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The potential synergistic effects of DCP-LA and resveratrol in mitigating cytotoxicity in an oxidative stress model of Alzheimer's disease

A Thesis in Neuroscience

By

Shady Barsoom

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Abstract

Alzheimer's disease (AD) is one of the most common neurodegenerative disorders whose prevalence is increasing each year. Alzheimer's is characterized by a progressive decline in memory, cognition, and motor activities. The two hallmark pathophysiological symptoms of AD are the formation of toxic amyloid beta (Aβ) plaques and neurofibrillary tangles (NFT's). As a result, several treatments and trials have been conducted to minimize or remove the presence of these harmful plaques and tangles, but success has been limited. A protein kinase C epsilon (PKCε) activator known as DCP-LA has been used to stimulate neurologically beneficial processes such as neurite outgrowth and synaptogenesis. In addition, through its interaction with PKCε, DCP-LA may help to reduce the amount of hyperphosphorylated tau that results in NFT's.

Resveratrol, a compound found in red wine and several foods, may also be beneficial in mitigating some of the neurodegenerative processes seen in AD by stimulating the activation of ADAM10, a metalloproteinase that may result in the decrease of harmful oligomeric Aβ plaques. While several studies have been conducted on the potential beneficial effects of DCP-LA and resveratrol independently, their effects when used in combination have not been fully characterized. Thus, I propose a hypothetical set of experiments that would seek to identify the possible synergistic or complementary effects of DCP-LA and resveratrol in mitigating the cellular symptoms of cytotoxicity in an FAB model of Alzheimer's disease. The use of DCP-LA and resveratrol in combination with each other may result in a more pronounced beneficial cellular response to cytotoxicity.

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PART I

Alzheimer's Disease: An Overview

Alzheimer's disease (AD) is a progressive neurodegenerative disease that affects millions of people each year. As of 2017, there are approximately 6 million people living with AD, with this number expected to increase to approximately 14 million by 2050 (Alzheimer's Association, 2017). This form of dementia is characterized by its multifaceted clinical symptoms and difficulty in subsequent treatment. As of the present, no definitive cure or effective treatment exist for Alzheimer's disease.

While AD is not an age-specific form of dementia, the risk of developing it increases over the age of 65 (Svob Strac et al., 2021). Patients with AD experience a slow progressive decline in their health that includes the eventual inability of the patient to carry out essential day-to-day activities, such as walking and eating, placing a heavy burden on family and caregivers. As the number of AD cases increases, the economic and social burdens of taking care of these patients is expected to increase as well. Financially, this includes costs of medications, long-term care facilities, and hospitalizations. The non-fiscal costs of AD may include increased familial strain, increased time off from work, and the emotional burden of living with this disease. Thus, the ever-increasing prevalence of AD, along with its burdens, warrant the development of an effective course of treatment for this major public health issue.

Clinical Symptoms and Risk Factors

Several clinical symptoms are associated with AD. Patients with this disease display increased forgetfulness, behavioral changes, and an overall decline in cognition. Behavioral

changes include apathy and depressive symptoms, which may progress to a decline in the performance of essential daily activities such as speaking and walking (Alzheimer's Association, 2017). However, clinical presentations of AD are not synonymous with the decline in cognition that sometimes accompanies normal aging (Atri, 2019). While normally aging individuals may have some memory decline and other overlapping symptoms with AD, they usually have little to no changes in their personality or affective behaviors; thus, changes in these behaviors in individuals are not indicative of normal aging and may point to AD or some other form of dementia. As the disease progresses, major cognitive impairments may emerge such as confusion, increased agitation, and delusions (Alzheimer's Association, 2017).

The risk of developing AD can vary from individual to individual, and several environmental and genetic risk factors have been identified. A meta-analysis of modifiable risk factors has shown that the presence of other comorbidities such as atherosclerosis and hypertension, along with lifestyle choices such as smoking status, diet, and exercise, have correlations with the development of this disease (Xu et al., 2015). Genetically, individuals who possess the apolipoprotein (APOE)-ε4 allele, which encodes proteins that help to transport cholesterol from astrocytes to neurons, have a much higher risk of developing this disease (Atri, 2019; Sen et al., 2012). While the exact role of the APOE-ε4 allele specifically in the progression of AD is still under active investigation, it remains a prominent area of interest for studying its progression. Moreover, mutations in the genes for amyloid precursor protein (APP), presenilin-1 (PS-1), and presenilin-2 (PS-2) have also been implicated in the development of early onset (familial) Alzheimer's (Bekris et al., 2010). For example, Wolfe et al. (1999) have shown that mutations in PS-1 in multiple cell models lead to the increased production of $A\beta_{1\text{-}42}$ (elaborated

on elsewhere) through the alteration of activity of γ secretases (Wolfe et al., 1999). Additionally, individuals who have family members with AD may be more likely to develop it as well.

Pathogenesis

AD is also characterized by a few classic molecular symptoms, namely an increase in amyloid-beta (Aβ) deposits and the hyperphosphorylation of tau, a microtubule-stabilizing protein. Aβ is a protein that is created by the cleavage of amyloid precursor protein (APP) by secretases in the brain. The major enzyme that cleaves APP is β-amyloid cleaving enzyme 1 (BACE-1), a β-secretase that is found in the central nervous system (CNS) (Sawda et al., 2017). As a result, inhibitors of this enzyme are being investigated in trials for individuals with AD. In the amyloidogenic pathway, APP is cleaved by β secretases to form β-C terminal fragments (CTFs) (Chen et al., 2017). After cleavage by β secretases, γ secretases may also cleave the β-CTFs to form the 40- and 42-amino acid-long form of Aβ ($A\beta_{1-40}$ and $A\beta_{1-42}$, respectively) (Chen et al., 2017; Zhou et al., 2005). Binding analyses of these two Aβ peptides indicated that $A\beta_{1-42}$ bound to metal ions with the highest affinity, which is important in studying the development of Aβ plaques because metal ions such as copper and zinc are dysregulated and have been indicated to build up in AD (Atwood et al., 2000); increased deposition of $A\beta_{1-42}$ specifically has been associated with the development of Alzheimer's disease. According to the amyloid cascade hypothesis, AD is primarily caused by the formation of Aβ either by its accumulation or cleavage from APP, causing neuronal death and neurofibrillary tangles (Hardy and Higgins, 1992; Sevigny et al., 2016). In patients with AD, Aβ accumulates without being adequately cleared by proteins such as apolipoprotein E (ApoE), creating neurologically harmful oligomeric protein

sheets (Agarwal et al., 2021). Specifically, individuals with two alleles coding for the ApoE4 protein are more at risk to develop Alzheimer's disease because this apolipoprotein variant has been hypothesized to stabilize Aβ deposits and lead to greater accumulation of Aβ in the brain (Sen et al., 2012). Aβ deposits may also lead to a decrease in the number of synapses present in AD brains through the reduction of the activity of certain enzymes such as protein kinase C, a process that will be elaborated on further elsewhere (Hongpasian et al., 2011). Terry et al. (1991) maintain that this loss of synapses is strongly correlated with the emergence of dementia in patients.

Another important contributor to the pathology of AD is the microtubule-associated protein, tau. Microtubules are important cytoskeletal components that aid in cellular trafficking and the structural stability of the cell. While the phosphorylation of tau is a normal neuronal mechanism that helps with microtubule function, namely in helping to stabilize and assemble microtubules (Wang and Mendelkow, 2016), the hyperphosphorylation of this protein leads to neurofibrillary tangles (NFT). These tangles are insoluble and disrupt normal microtubule function, causing a decay in vital neuronal processes such as transport and ultimately leading to degeneration (Agarwal et al., 2021). According to these same authors, the activation of glycogen synthase kinase 3β (GSK-3β) is required for the hyperphosphorylation of tau to occur, making regulation of this enzyme an important area of investigation. Tau may also be phosphorylated by several other kinases such as protein kinase A, Ca^{2+}-calmodulin-dependent kinase II (CaMKII), and cyclin dependent kinase, which can prime tau before it is phosphorylated by other kinases (Wang and Mendelkow, 2016; Hanger et al., 2009). Additionally, the hyperphosphorylation of tau may prevent its proper removal from the cell, which could occur by preventing its cleavage

by certain caspases (Guillozet-Bongaarts et al., 2006). Together, Aβ deposits and NFT's caused by hyperphosphorylated tau are hypothesized to lead to the neuronal damage that manifests as the pathological signs of AD.

AD is also characterized by oxidative stress. The formation of reactive oxygen species (ROS) facilitates this process through the Fenton reaction (Chen and Zhong, 2014). Normally, the neurotypical brain has mechanisms for alleviating the oxidative stresses that occur, namely through antioxidants like glutathione and manganese superoxide dismutase (MnSOD). However, in patients with AD, accumulating levels of Aβ and hyperphosphorylated tau create significant cytotoxicity. Aβ plaques interact with glutamatergic neurons, which can create excitotoxicity in the brain and lead to a loss of synapses (Choi, 1987; Svob Strac et al., 2021). Oxidative stress may promote the activation of β- and γ-secretases that can cleave APP into the harmful Aβ fragments that molecularly characterize AD (Anekonda, 2006; Chen and Zhong, 2014). Chen and Zhong (2014) also state that oxidative stress may increase the polymerization of tau and increase the activity of a kinase involved in phosphorylating it, which could result in the formation of the neurofibrillary tangles that characterize AD. Because the brain is quite active metabolically, it has the potential to create a vast quantity of ROS, highlighting the brain's vulnerability to the damaging effects of oxidative stress. Protecting the brain against excitotoxicity remains an important goal for many treatments that seek to alleviate the molecular stresses of AD.

AD patients also have compromised blood-brain barriers, which can contribute to chronic inflammation and the diminished ability of the barrier to protect against pathogens and other toxic molecules (Zenaro et al., 2017). The blood-brain barrier (BBB) is a highly effective layer

of cells that utilize tight junctions to regulate the transport of nutrients and waste into and out of the brain. In AD patients, the BBB's activities are diminished. Zenaro et al. (2017) also maintain that because the BBB is also responsible for the transport of nutrients to the brain, AD patients whose BBBs are affected may have compromised transport of nutrients, making their delivery to the brain less efficient.

Current Treatments for Alzheimer's Disease

Treating AD has proven difficult due to the complexity of the molecular mechanisms that lead to its emergence. Additionally, the difficulty in accurately diagnosing AD in living patients contributes to a delay in treatment. AD can only be fully diagnosed post-mortem, and several of its symptoms coincide with those of normal aging. Thus, it is possible for AD symptoms to be dismissed as simply evidence of normal aging, even though there are distinct differences between cognitive decline due to aging and decline due to AD. Also, the molecular symptoms of AD may begin up to one to two decades before any major clinical presentations arise (Sawda et al., 2017), contributing to the difficulty of beginning an effective course of treatment before this disease becomes significantly advanced.

As of today, there are four oral medications given to treat AD: donepezil, galantamine, rivastigmine, and memantine (the first three are acetylcholinesterase inhibitors, while memantine is an NMDA receptor antagonist) (Svob Strac et al., 2021). AD patients have some degeneration in cholinergic neurons, hence the use of acetylcholinesterase inhibitors, which prevent the breakdown of existing acetylcholine. The use of acetylcholinesterase inhibitors such as donepezil has been shown to help prevent the accumulation of Aβ proteins by shifting the cleavage of APP

away from their formation, presumably by decreasing BACE1 levels in the brain (Peron et al., 2018). However, these medications mostly treat the symptoms that manifest from AD, rather than prevent them from occurring.

Drugs that counter the effects of AD must cross the blood-brain barrier. As a result of this barrier, the ability of these drugs to reach the brain is quite minimal. While AD patients may have compromised BBBs as described above, it is still difficult to effectively transport these drugs in a way so that they are not degraded by the body's metabolic enzymes and actually exert their effects on the brain. Recently, researchers are exploring potential avenues for increasing the bioavailability of drugs using nanotechnology, in which drugs are packaged as nanoparticles or lipid membranes that can be delivered to the intended target and diffuse across the blood-brain barrier (Agarwal et al., 2021). The hope is that the combination of drugs that were hitherto low in availability can achieve their intended effect with a smaller concentration or alternate route of delivery. This is still a burgeoning field of study, but it offers some optimistic implications should the delivery methods of these drugs improve.

Another potential course of treatment is the use of vaccines, in which passive immunization of monoclonal antibodies that target Aβ fragments is used (Adolfsson et al., 2012). However, these immunizations are still under active development and undergoing trials because of safety concerns, especially since many of these immunization techniques can disrupt the blood-brain barrier. Recently, Biogen has conducted clinical trials on a monoclonal antibody known as aducanumab (brand name Aduhelm), which is a drug that can cross the blood-brain barrier and react with Aβ plaques, facilitating their clearance from the brain (Sevigny et al., 2016). However, it is important to note that aducanumab's approval and use for Alzheimer's

disease patients has been dubious at best due to its potentially severe side effects and questionable efficacy in reducing Aβ plaques in human patients.

Furthermore, because of AD's multifaceted pathophysiology and widespread effects in the brain, one potential avenue for investigation is the use of combination treatments. This would entail using compounds that either have similar effects and are used synergistically to make their combined effects more robust than either drug alone, or using drugs with differing mechanisms of action to have more widespread effects in the brain. Combination treatments are used for a variety of diseases, ranging from cardiovascular to neurological. They have also been used in some treatments for AD as well. One of the most common combination treatments involves memantine (NMDA receptor antagonist) with an acetylcholinesterase inhibitor such as donepezil, which has shown to be effective in slowing the progression of the disease, especially if this treatment was started early (Kabir et al., 2020; Cummings et al., 2019). NMDA receptors are responsive to glutamate, so inhibiting their action may help to prevent glutamate-mediated excitotoxicity. However, even combination treatments usually attempt to alleviate the symptoms of AD without actually preventing the underlying mechanisms behind them. Thus, finding a combination treatment that would be able to address not just one, but several aspects that cause the molecular mechanisms of AD could potentially be effective in significantly altering the course of this disease's progression. This could be established through the regulation of the activity of enzymes with multiple subcellular targets involved in the development of Alzheimer's disease.

Protein Kinase C Epsilon

Many molecular events that occur within organisms require phosphorylation, or the addition of a phosphoryl group to a target, to occur. The phosphorylation of target molecules can lead to their activation or inhibition, allowing for systemic modulation and specificity. Certain types of enzymes that carry out phosphorylation are called kinases. A well-known molecular class of kinases includes protein kinase C (PKC), a protein kinase that is the downstream target of certain metabotropic membrane receptors and second messenger cascades. Within the PKC family are several isoforms (or isozymes) that each have distinct structures and functions in the body. While there are approximately eleven different isoforms of PKC (Zeidman et al., 1999), one PKC isoform that is of particular interest in Alzheimer's disease pathology is protein kinase C-epsilon (PKCε). PKCε is the most abundant isoform of this family of kinases in the central nervous system (Akita, 2002).

PKCε is an enzyme that contains a conserved catalytic domain at the C-terminus and a variable regulatory domain at the N-terminus (Ochoa et al., 2001). PKCε is considered a novel protein kinase compared to the classical protein kinases due to its distinguishing characteristic of Ca^{2+} independence, but similar ability to bind activators such as diacylglycerol and phorbol esters (Zeidman et al., 1999). The regulatory domain of PKCε mediates the phosphorylation activity of this enzyme through two separate binding sites. The regulatory domain contains two cysteine-rich sites (C1 and C2) that bind to PKCε's activators such as diacylglycerol (DAG) and phorbol esters (Aksoy et al., 2004). The C1 domain specifically binds to the activators, while the C2 domain in classical kinases binds Ca^{2+} (Ochoa et al., 2001). Unlike the classical protein

kinases, however, PKCε does not bind Ca^{2+} in its regulatory domain and activates independently from Ca^{2+} binding.

When PKCε is inactive, it is normally bound to the Golgi apparatus and centrosome via anchoring proteins such as centrosome and Golgi localized PKN-associated protein (CG-NAP) (Takahashi et al., 2000). According to these same authors, when PKCε is phosphorylated sufficiently *in vitro*, it dissociates from the Golgi into the cytosol, where it is now mature and ready to bind to activators. Thus, in order for PKCε to activate and phosphorylate downstream targets, it itself must be phosphorylated as well.

Activation of PKCε occurs through a second-messenger signaling cascade (Figure 1). Ligand binding to a G-protein-coupled receptor (GPCR) embedded in the cell membrane of the neuron leads to the initiation of intracellular signaling events. The GPCR is composed of an α, β, and γ subunit. The α subunit is normally bound to inactive guanosine diphosphate (GDP), but upon ligand binding to the GPCR, it exchanges GDP for active guanosine triphosphate (GTP), thus allowing it to dissociate from the β/γ subunits and bind to a downstream enzyme called phospholipase C (PLC) or phospholipase A2 (Kanno et al., 2006). PLC catalyzes the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), an activator of PKCε (Falkenburger et al., 2013). Activated PKC is then translocated to sites within the cell through the interaction with anchoring proteins such as receptors for C-kinases (RACK's) (Ochoa et al., 2001). The specific RACK for PKCε has been identified as β'-COP. Additionally, where PKCε localizes within the cell depends on which second messenger is actually bound to it (Akita, 2002). PKCε can also be activated by exogenous substances such as DCP-LA, which will be elaborated further elsewhere.

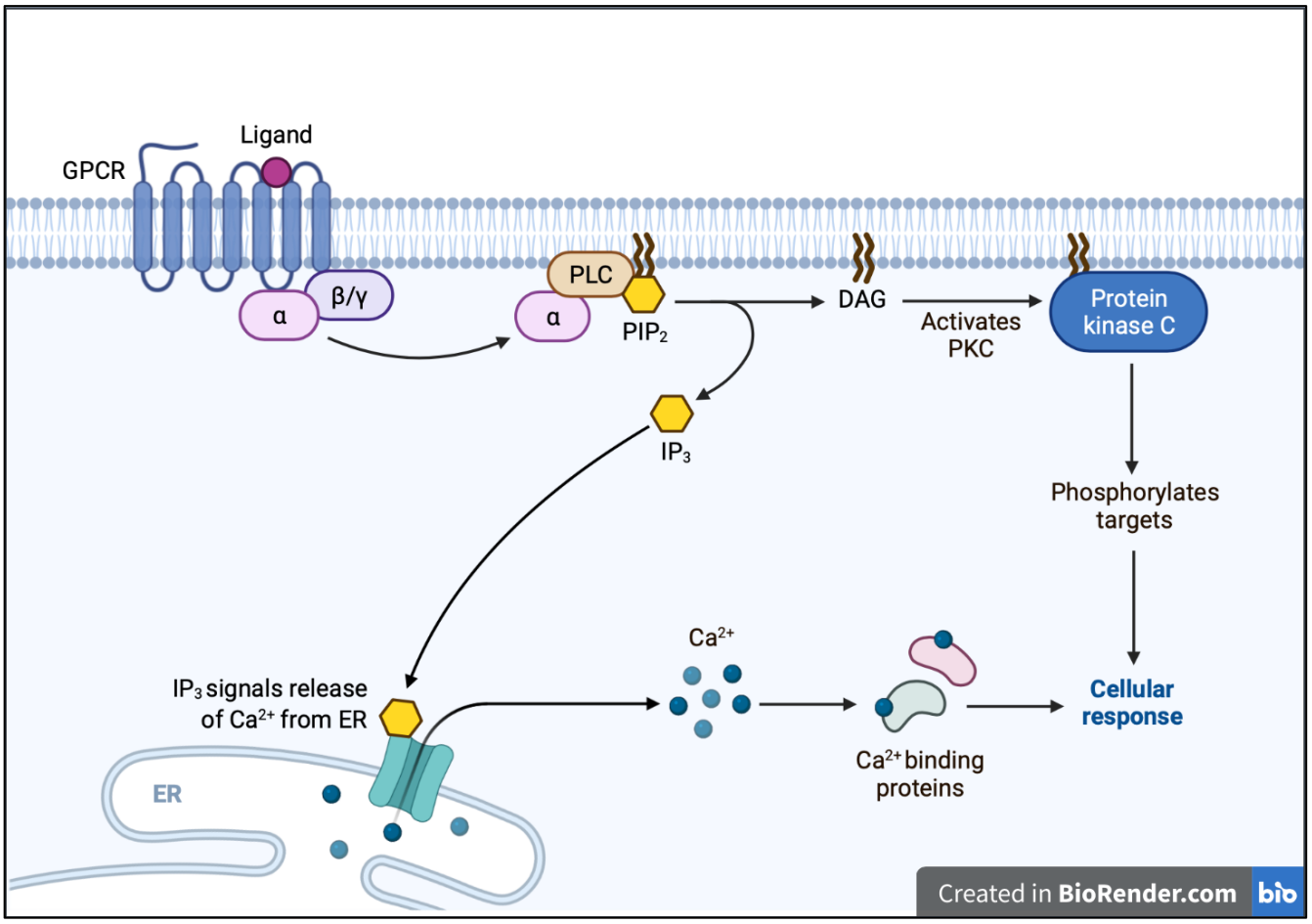

Figure 1: Signaling pathway for PKC – Activation of PKC occurs through intracellular cascades mediated by G protein-coupled receptors (GPCR's).

PKCε has previously been shown to be involved in synaptogenesis (Hongpaisan et al., 2011). As previously mentioned, the loss of synapses has a strong correlation with the symptoms of dementia in AD patients. Synapses allow for neuronal communication, and the brain's ability

to change its structure through the dynamic nature of its synapses is what allows it to adapt, learn, and remember (known as neuroplasticity). Thus, the generation of new synapses is vital for the brain to adapt to its environment, and the loss of synapses in a neurodegenerative disease such as AD compromises their dynamic nature and can be associated with the symptoms seen in AD patients.

Synaptogenesis, or the formation of new neuronal synapses, can partially be explained by long-term potentiation (LTP), a possible mechanism of how learning occurs in the brain. The brain has an increased ability to respond to excitatory postsynaptic potentials (EPSPs) through the increased number of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors on the postsynaptic membrane (Whitlock et al., 2006). AMPA receptors are activated by glutamate, an excitatory neurotransmitter that leads to excitatory post-synaptic potentials (EPSPs) in postsynaptic cells, so increasing the amount of AMPA receptors would allow the neurons to be more responsive to EPSPs. Whitlock et al. (2006) have shown that implementing a model of learning and memory in rats leads to an increase in the phosphorylation of AMPA receptor subunit GluR1, specifically at the serine-831 site. By measuring the level of phosphorylation of this subunit, they were able to use this as a proxy for indicating where LTP had occurred. From this technique, they found that trained rats had higher levels of GluR1 and GluR2 protein levels and phosphorylation, which indicated that rats who had undergone this training relative to controls had some neural molecular changes upon learning. These changes were explained as mediating LTP. Thus, increasing the number of AMPA receptors long-term on the postsynaptic membrane allows the neuron to be more easily excited by glutamate, creating

more EPSP's that are associated with LTP and indicating some level of learning (neuroplasticity) in the brain.

In Alzheimer's disease, the formation of Aβ plaques downregulates the activity of PKCε, leading to a decrease in AMPA receptors (Lucke-Wold et al., 2015). Liu et al. (2010) demonstrated that Aβ increases phosphorylation of a protein (S880) on the AMPA receptor that results in its removal from the cell surface; this process is mediated by PKC because inhibitors of PKC significantly decreased this effect (Liu et al., 2010). Transporting the increased numbers of AMPA receptor proteins is accomplished by postsynaptic density 95 (PSD-95), a scaffold protein that is one of the downstream targets of PKCε (Sen et al., 2016). Specifically, PKCε is a serine-threonine kinase that phosphorylates the serine-295 residue on PSD-95, allowing for its accumulation in the postsynaptic membrane and the eventual aggregation of the AMPA receptors that lead to LTP. Thus, PKCε may have the post-synaptic effect of mediating the increase in AMPA receptors to allow neurons to become more receptive to glutamate and contribute to more efficient neuronal communication in the brain.

Presynaptically, PKCε interacts with cytoskeletal proteins. Specifically, PKCε has an F-actin (filamentous actin) binding region that, when bound to F-actin, stabilizes the structural conformation of the kinase and may play a role in mediating the release of the excitatory neurotransmitter glutamate (Newton and Messing, 2010). This F-actin binding site is unique to the particular isoform of PKCε (Prekeris et al., 1996), making it a target of interest for neuroprotective drugs. Through the interaction with actin filaments presynaptically, it is possible that PKCε can lead to increased glutamate-mediated EPSPs via its interaction with F-actin.

Activating PKCε can also lead to decreased phosphorylation of tau. When certain isoforms of PKC are active, they can directly inhibit an enzyme called glycogen synthase-3β (GSK-3β), also known as tau protein kinase I (TPKI) (Ishiguro et al., 1993). GSK-3β was originally thought to be involved only in phosphorylating and inactivating glycogen synthase (for which the enzyme is named), but has now been shown to be an enzyme involved in several metabolic and signaling processes and can phosphorylate as many as forty substrates (Grimes and Jope, 2001; Jope and Johnson, 2004). As such, because it is a kinase involved in many processes, it must be tightly regulated and this regulation is crucial for proper cellular functioning. GSK-3β is particularly abundant in the brain (Grimes and Jope, 2001), meaning its dysfunction or dysregulation may potentially be involved in the development of psychiatric and neurodegenerative diseases like Alzheimer's. This regulation is particularly compelling as well, considering that GSK-3β can promote apoptosis, or programmed cell death (Jope and Johnson, 2004). Indeed, Pap and Cooper (1998) observed that overexpression of wild-type GSK-3 enzymes caused apoptosis in Rat-1 and PC12 cells, while using mutated GSK-3 resulted in cells with lower rates of apoptosis (Pap and Cooper, 1998). Jope and Johnson (2004) claim that GSK-3β is involved in neurite outgrowth and development, with inhibited GSK-3β correlating to extension of neuronal growth cones and vice versa. Since PKCε is involved in neurite outgrowth and development, it may be that this kinase regulates this phenomenon in a process mediated by GSK-3β.

Activating GSK-3β leads to its phosphorylation of proteins in the brain that can have several effects on neuronal structure and function. For example, when activated, GSK-3β can phosphorylate a microtubule-associated protein (MAP) like tau. Tau phosphorylation is normally

regulated by a balance between kinases and phosphatases (proteins that remove phosphoryl groups) (Plattner et al., 2006). However, when GSK-3β is overactivated, it can hyperphosphorylate tau, resulting in the insoluble neurofibrillary tangles that are characteristic of AD (Wang et al., 2007). However, as these authors demonstrate, tau is phosphorylated by a wealth of kinases, and their experiment involved using several of them in combination with each other to observe their effects on tau hyperphosphorylation, so GSK-3β's direct role in tau hyperphosphorylation is still under active investigation. Regardless, this hyperphosphorylation is an example of the dysregulation of the kinase/phosphatase balance that influences tau function such as microtubule stabilization and assembly. Additionally, according to Plattner et al. (2006), increased GSK-3β activity was directly correlated to increased tau phosphorylation and neurofibrillary tangles in transgenic mice. Moreover, the other classic molecular pathology of AD, Aβ deposits, is also involved in GSK-3β activity. Aβ is hypothesized to actually activate GSK-3β, which has been shown to be involved in tau hyperphosphorylation and leading to the impaired neuronal function that is observed in AD (Grimes and Jope, 2001). Takashima et al. (1998) have demonstrated that GSK-3β activity significantly increases when cells are exposed to $A\beta_{25\text{-}35}$ (another form of Aβ), which was correlated with the increased phosphorylation state of tau (Takashima et al., 1998). While GSK-3β has been shown to phosphorylate tau and the overactivation of this enzyme has been associated with apoptotic activities, it is important to note that there are several other cellular components at play in the formation of hyperphosphorylated tau and the cellular dysregulation that is observed in the AD brain.

Regulating GSK-3β activity can be accomplished by phosphorylation. Just as PKCε itself must be phosphorylated to activate other downstream targets, GSK-3β's level of activity is

influenced by its, and its substrates', phosphorylation activity (Cho and Johnson, 2003). This is undoubtedly part of the tightly regulated process of GSK-3β activity because of its direct involvement in several important cellular processes. GSK-3β activity is inhibited by the phosphorylation of Ser-9 on its N-terminal (Grimes and Jope, 2001; Jope and Johnson, 2004). According to Grimes and Jope (2001), one of the kinases that phosphorylates (and inactivates) GSK-3β is PKC. However, it is important to note that PKCε specifically does not directly phosphorylate GSK-3β, but activators of PKCε such as DCP-LA can indirectly inhibit GSK-3β while at the same time promoting synaptogenesis and increasing plasticity, which will be elaborated on elsewhere.

DCP-LA

PKCε has been shown to be activated by *cis*-unsaturated fatty acids such as arachidonic and linoleic acid (Kanno et al., 2006). Diacylglycerol (DAG), previously described as an activator of PKCε, is also composed of fatty acids attached to a glycerol molecule. This interaction is important because fatty acids such as these may play a role in improving synaptic transmission in regional areas of the brain such as the hippocampus through the proliferation of nicotinic acetylcholine receptors (nAChR) (Kanno et al., 2006; Nishizaki et al., 1999). Upregulating the number of nACh receptors has been suggested to improve synaptic plasticity and thus overall neurological function (Nishizaki et al., 1998). Conventional *cis*-unsaturated fatty acids have been challenging to use in treating cognitive disorders due to their rapid degradation in the brain; however, a more stable linoleic-acid derived fatty acid named 8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]- octanoic acid (DCP-LA) was created, and this compound has

been shown to also activate PKCε (Nagata et al., 2005). Tanaka and Nishizaki (2003) suggest that the double bonds in fatty acids like linoleic acid are important contributors to biological reactions *in vivo* but may also contribute to their rapid degradation in the brain (Tanaka and Nishizaki, 2003). Thus, this derivative of linoleic acid contains two cyclopropyl rings instead of double bonds, contributing to its increased chemical stability (Figure 2).

The creation of the two cyclopropyl rings means that DCP-LA can have four different spatial configurations, or diastereomers. For example, the two cyclopropyl rings in the structure may be in the same plane of space (α,α or β,β) or in opposing planes (α,β or β,α). The orientation of substituents around DCP-LA has a significant impact on its chemical reactivity due to its various binding availabilities and interactions with other molecules. The α,β form of DCP-LA (each cyclopropyl ring in a different plane of space) has been shown to display the most potent effect in activating PKCε of the four possible diastereomers (Shimizu et al., 2011). Using an *in situ* assay in PC-12 cells from rats, these authors found that not only did this particular α,β diastereomer of DCP-LA activate PKC the most, but that PKCε was the most highly activated isoform of PKC, thus highlighting DCP-LA's potential as a compound of interest in specifically activating a kinase involved in synaptic function.

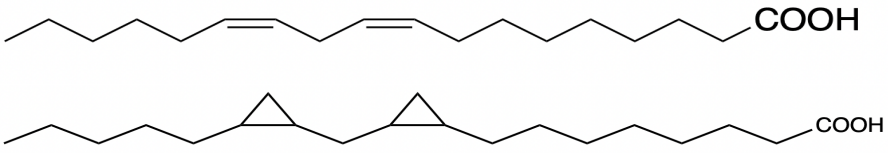

Figure 2: Structures of linoleic acid and DCP-LA – The double bonds of linoleic acid (top) have been replaced with the cyclopropyl rings in DCP-LA (bottom). Created with ChemDraw.

DCP-LA is an activator of PKCε. Kanno et al. (2006) demonstrated DCP-LA's ability to bind to PKCε through the inhibition of PKCε using a chemical inhibitor (GF109203X) and small interfering RNA (siRNA) and observing the subsequent decrease in DCP-LA. DCP-LA activity was measured by the amount of phosphorylated substrate that was formed as DCP-LA interacted with PKC. Because DCP-LA's activity was significantly reduced upon inactivation of PKCε, and it significantly activated this kinase more than the other isoforms of PKC that were studied, it was concluded that DCP-LA binds to PKCε selectively. DCP-LA specifically binds to the phosphatidylserine binding site on the C2 domain of PKCε, at the Arg-50 and Ile-89 amino acids; by selectively mutating these amino acids, it was shown that DCP-LA activity was dampened, indicating that these amino acids are its binding sites (Kanno et al., 2015).

Phosphatidylserine is an activator of PKCε (Nishizuka, 1992), meaning it has a specific binding site on the kinase. Experiments involving treating PKCε with both linoleic acid and its derivative DCP-LA simultaneously showed no significant additional increase in its activation, and neither of these linoleic acids increased PKC activation in the presence of dioleoyl-phosphatidylserine (a phosphatidylserine molecule that is a ligand for PKC) (Kanno et al., 2006). This suggests that linoleic acid and DCP-LA compete with phosphatidylserine for binding to PKCε, and phosphatidylserine binds with the highest affinity. More specifically, DCP-LA's carboxy terminal interacts with the Arg-50 portion of PKCε, while the two cyclopropyl rings interact with the Ile-89 portion (Kanno et al., 2015). Using fluorescent cytochemistry and PKCε binding assays, these same authors were able to deduce that DCP-LA binds to PKCε in the cytosol, but not the plasma membrane. Considering that PKCε is translocated to the cytosol upon its activation, it follows that DCP-LA can only bind to PKCε after the kinase has been

phosphorylated, activated, and finally moved to the cytosol. Thus, DCP-LA will not bind to unphosphorylated (inactive and membrane-bound) PKCε. However, experiments have shown that DCP-LA that is applied extracellularly can be taken up into the cell, after which it can bind to PKCε (Kanno et al., 2015).

In addition to binding to PKCε, DCP-LA can also inactivate GSK-3β through the inhibition of protein tyrosine phosphatase-1B (PTP1B) (Nishizaki, 2017). While GSK-3β is directly inactivated by PKC as explained previously, it can also be inactivated indirectly using a signaling cascade. In the absence of DCP-LA, a group of kinases known as receptor tyrosine kinases (RTK) proceeds to phosphorylate insulin receptor substrate-1 (IRS-1), which then activates phosphatidylinositol 3 kinase (P13K), which then phosphorylates and activates Akt1 that then inactivates GSK-3β (Tsuchiya et al., 2014; Figure 3). According to these same authors, PTP1B inhibits RTK's by de-phosphorylating them, thereby regulating their activity and allowing for GSK-3β activation. It is also important to note that PTP1B is involved in a multitude of important cellular functions such as signaling, the cell cycle, and mediating responses to stress (Haj et al., 2003). However, DCP-LA has been shown to inhibit PTP1B, allowing for RTK to be disinhibited and proceed to eventually inactivate GSK-3β (Nishizaki, 2017). Because PTP1B is expressed in several locations (Tsuchiya et al., 2014), inhibiting it using DCP-LA can have powerful effects in mediating GSK-3β inactivation and an eventual decrease in tau hyperphosphorylation. Thus, the decrease in tau hyperphosphorylation through inhibition of GSK-3β by DCP-LA-mediated inhibition of PTP1B may result in the mitigation of the neurofibrillary tangles that characterize Alzheimer's disease.

Additionally, DCP-LA facilitates long term synaptic transmission through two major mechanisms: upregulation of α7 nicotinic acetylcholine (nACh) receptors by trafficking them to the cell membrane and upregulation of the AMPA receptor subunits GluR1 and GluR2 (Kanno et al., 2013; Shimizu et al., 2011; Figure 3). Nicotinic acetylcholine receptors (nAChR) are an ionotropic subtype of acetylcholine receptors. Generally, activation of these kinds of receptors leads to transient excitatory postsynaptic potentials (EPSPs) in the postsynaptic membrane. The α7 nACh receptors specifically are localized in the presynaptic membrane and facilitate the release of glutamate in the hippocampus (Tanaka and Nishizaki, 2003). As cholinergic neurons are abundant in the brain, including the hippocampus, increasing the number of these receptors can facilitate an increase in the response of the postsynaptic neuron to glutamate (Haam and Yakel, 2017). Glutamate is the primary excitatory neurotransmitter in the brain, meaning its release via exocytosis creates EPSPs in the postsynaptic cell. α7 nACh receptors are tethered to the plasma membrane using a scaffolding protein named protein 4.1N that acts as a binding partner (Kanno et al., 2013). Protein 4.1N is expressed in the brain and co-localizes with other important downstream targets of PKCε like PSD-95, as well as postsynaptic AMPA receptor subunits GluR1 and GluR2, highlighting Protein 4.1N's important role not only in normal PKCε function, but as a potential protein of interest in studies involving treatment of AD with DCP-LA. Experiments *in vitro* have shown that DCP-LA increases the amount of these α7 nACh receptors in the brain (Tanaka and Nishizaki, 2003). The result of this upregulation in receptors is increased glutamate release, meaning the postsynaptic neuron is stimulated to an increased extent and facilitates LTP in the hippocampus. α7 nACh receptors are especially abundant in limbic regions of the brain such as the hippocampus, and thus upregulating them presynaptically

may help to improve long-term cognitive function; this phenomenon is even more compelling because patients with Alzheimer's disease have been shown to display impaired cholinergic systems and the number of cholinergic receptors in the hippocampus decreases as the brain ages (Tanaka and Nishizaki, 2003).

Thus, a potential long-term effect of DCP-LA entering the cell and increasing α7 nACh receptors is the cellular form of learning, LTP. Tanaka and Nishizaki (2003) maintain that this phenomenon occurs in a PKC-dependent manner because this increase in receptors does not occur upon inhibition of PKCε. However, PKCε is not indicated to phosphorylate the α7 nACh receptors because there is no binding site for phosphorylation from the specific isoform of this kinase, nor is protein 4.1N phosphorylated by PKCε (Kanno et al., 2012). While it is still unclear exactly what the phosphorylation target of PKCε specifically is in this process, through inhibition studies of this kinase, it is clear that it is crucial to the completion of this process.

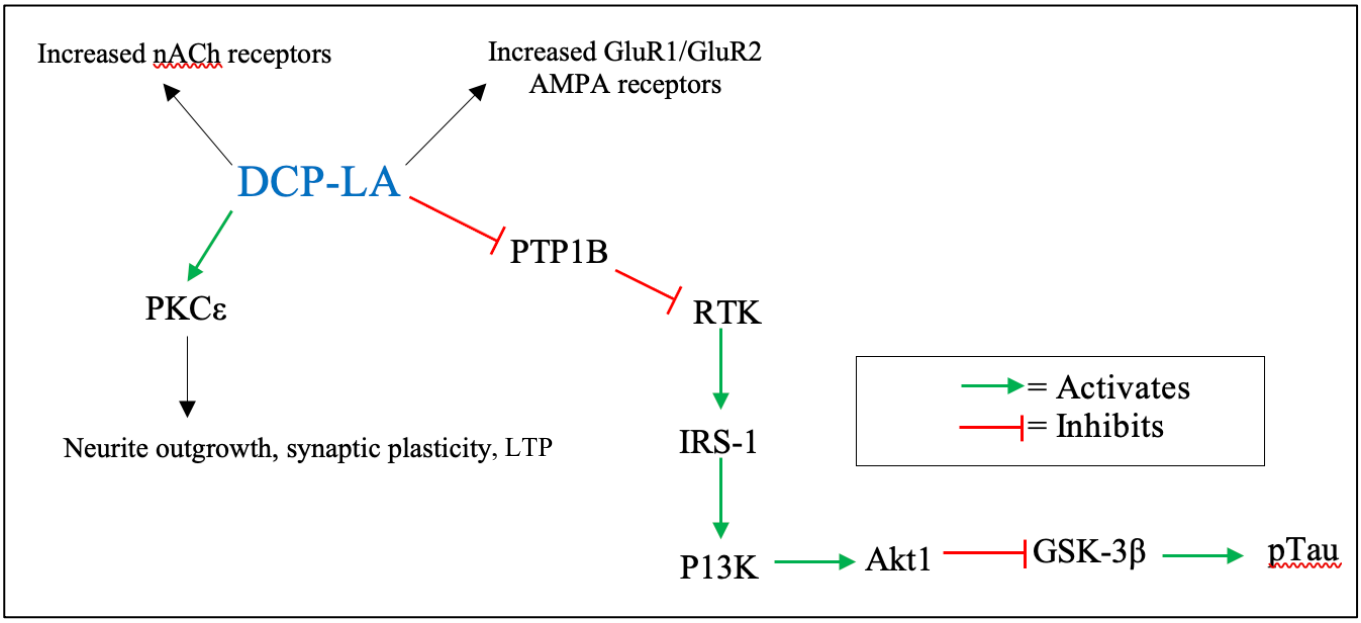

Figure 3: Major actions of DCP-LA (modified from Tsuchiya et al., 2014) – PKCε activation leads to neurite outgrowth and increased synaptic plasticity. DCP-LA upregulates nACh receptors and GluR1/GluR2 AMPA receptors. DCP-LA can indirectly inhibit GSK-3β through PTP1B inhibition. PKCε: protein kinase C-epsilon, RTK: receptor tyrosine kinase, PTP1B: protein tyrosine phosphatase 1B, IRS-1: insulin receptor substrate-1, PI3K: phosphoinositide-3-kinase, Akt1: AKT serine-threonine kinase-1, GSK-3β: glycogen synthase 3β, pTau: phosphorylated tau.

Resveratrol

It has been commonly noted that moderate consumption of wine can be beneficial in improving neurological health. Though the full effects of this intake in humans and the specific mechanisms involved are still under active investigation, the notion that red wine specifically has a positive impact on overall health has intrigued many in academic circles (Anekonda, 2006). Interestingly, multiple studies have shown that moderate red wine use has been associated with a lower incidence of Alzheimer's disease and other forms of dementia, although many of these incorporate other lifestyle factors that may have influenced their results. (Vingtdeux et al., 2008).

A population-wide analysis in Canada indicated that moderate red wine consumption was associated with a lower risk of developing AD (Lindsay et al., 2002). This study involved two evaluations of patients' (65 years and older) overall health spaced five years apart, but it is important to note that many of these patients died over the years, limiting the applicability of the study's results. Evaluating the effect of red wine on human patients' learning and memory can be challenging, and so, Wang et al. (2006) have shown that a specific red wine (with ethanol and water used as controls) slowed the decline of spatial memory (measured through latency times escaping a maze) and decreased $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ peptides in the Tg2576 transgenic mouse model of AD (Wang et al., 2006).

One active ingredient in red wine and many other foods such as peanuts, mulberries, and other plants is resveratrol. Resveratrol is a phytoalexin, meaning it is a compound produced by plants in response to external stressors or by parasites and fungi (Boue et al., 2009; Anekonda, 2006). It is also a polyphenol (Figure 4), displaying aromaticity and is chemically stable. There are stereoisomeric differences in resveratrol, with the *trans* configuration of resveratrol being biologically active (Walle, 2011; Howitz et al., 2003). Similar to the specificity for isomers seen in DCP-LA, where the α, β isomer was the most biologically active, resveratrol similarly displays most activity in activating a class of molecules called sirtuins while arranged in a specific conformation. Additionally, the structure of resveratrol is such that it is able to cross the blood-brain barrier and preserve its chemical structure, although many of resveratrol's metabolites are found in the blood (Sawda et al., 2017).

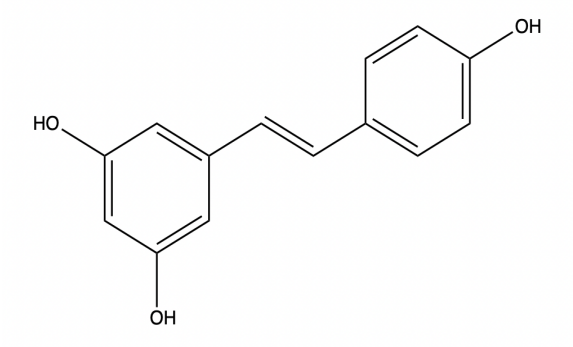

Figure 4: Structure of resveratrol. Created with ChemDraw.

Resveratrol has been shown to have many beneficial effects both *in vitro* and *in vivo*. Resveratrol may exert beneficial effects due to its ability to mimic caloric restriction (Gräff et al., 2013). Caloric restriction may help to reduce the occurrence of diseases that emerge with age. Specifically, these authors state that caloric restriction has been shown to dampen memory deficits and protect against Aβ aggregates as well as reduce the level of tau phosphorylation in mouse models of AD. However, since long-term calorie restriction may not be desirable to most people, a chemical imitation of it in the form of resveratrol seems to be more palatable. Thus, resveratrol may be able to simulate the effects of caloric restriction in way that does not require actual abstinence from certain foods. Another study suggests that resveratrol can reduce total cholesterol in hypercholesteremic rats (Peron et al., 2018).

Caloric restriction may help to reduce age-related diseases through the activation of a class of proteins called sirtuins. Sirtuins are conserved NAD-dependent deacetylases (Anekonda, 2006). NAD (nicotinamide adenine dinucleotide) is a coenzyme that is crucial for many important biological reactions such as cellular respiration. It is reduced to NADH in biochemical

reactions, and this ratio of NAD^+/NADH is carefully regulated to ensure that these reactions are occurring properly. Thus, with caloric restriction, this ratio is altered slightly because of the decrease in caloric energy. This alteration in the ratio allows for sirtuins to be activated. While sirtuins can be found in many organisms such as yeast (whose sirtuins are named SIR2), the homologs of sirtuins found in mammalian organisms specifically are known as SIRT1-7, with SIRT1 being extensively studied (Anekonda, 2006). While there are several small molecule activators of SIRT1, resveratrol is known to activate SIRT1 the most potently compared to other sirtuin activators, increasing its catalytic rate more than 13 times its control rate (Howitz et al., 2003).

Activation of SIRT1 can lead to lipolysis in white adipocytes (fat cells), which is an important consequence of caloric restriction (Picard et al., 2004). SIRT1 activation can also lead to the deacetylation of several targets involved in metabolism, which have a long-lasting impact on cellular function (Sawda et al., 2017). For example, SIRT1 has been shown to deacetylate p53, a major tumor-suppressing protein involved in the development of cancer because of its ability to promote apoptosis, or programmed cell death (Gräff et al., 2013). As such, this SIRT1-mediated deacetylation of p53 displayed increased cell survival in a neurodegenerative CK-p25 mouse model. Moreover, according to the amyloid cascade hypothesis, AD may be caused by the cleavage of amyloid precursor protein (APP), which results in the formation of the toxic Aβ deposits found in the brains of those with this disease. Sawda et al. (2017) hypothesize that resveratrol exerts its beneficial effects in the cell through two mechanisms: the cleavage of APP at a site different than the one that normally produces Aβ fragments and through the deacetylation of tau. One of resveratrol's hypothesized actions in preventing Aβ formation is

through the activation SIRT1, leading to the transcription of a gene named ADAM10 (Peron et al., 2018). ADAM10 is an α secretase that is part of a class of metalloproteinases that can cleave APP into a neuroprotective metabolite known as sAPPα. Therefore, by acting as an activator of ADAM10, resveratrol may help to mediate the cleavage of APP away from the site that normally produces the toxic Aβ fragments and decrease the amount of available unreacted APP. This would lower the chance of APP being cleaved by enzymes that could produce the Aβ fragments. According to Peron et al. (2018), however, ADAM10 is ubiquitously expressed in mammalian cells and is thus involved in several other cleavage processes; improper activation may lead to other undesired cellular events, a fact even more compelling considering that ADAM10 does have some tumor-promoting activities. Thus, the use of resveratrol to activate ADAM10 must be carefully regulated to ensure that cellular death or cancer-like proliferation are avoided.

Another beneficial effect of resveratrol is its ability to act as an antioxidant *in vitro* (Vingtdeux et al., 2008). This ability to attenuate the effects of reactive oxygen species (ROS) may be associated with the neuroprotective effects seen with moderate red wine consumption because it leads to decreased inflammation and prevents the peroxidation of membrane lipids, which reduces the load of ROS in the body. Since the brain uses significant supplies of oxygen and subsequently generates large amounts of ROS, it is vital that the formation of these ROS is regulated tightly, because these molecules can oxidize other cellular targets that eventually result in cellular damage or death. Resveratrol has been shown to upregulate several antioxidant enzymes in the brains of rats such as superoxide dismutase, catalase, and peroxidase (Mokni et al., 2007). In culture models of AD, resveratrol has also been shown to delay Aβ-induced toxicity, although the mechanism of how it accomplishes this is not fully understood (Vingtdeux

et al., 2008). Moreover, because of resveratrol's innate ability to cross the blood brain barrier, it serves as a promising agent to treat neurodegenerative diseases such as AD. Thus, resveratrol remains an important biological molecule for investigation because of its potential therapeutic effects, should they be more completely understood.

It is important to note that most of the beneficial effects of resveratrol have been observed *in vitro*. This is due to resveratrol's very low bioavailability, or the ability of an organism to absorb and use a given nutrient *in vivo* (Anekonda, 2006). Resveratrol given orally has low bioavailability because of the rapid breakdown of this compound into glucuronide and sulfate metabolites (Vingtdeux et al., 2008). It is possible that it is these metabolites that are responsible for the apparent decrease in memory deficits and improvements in those who moderately consume red wine. Even though resveratrol and these metabolites were not found in the brain of mice in a previous study, these researchers observed a decrease in Aβ in several regions of the brain (Karuppagounder et al., 2009). These metabolites are still under active research as to their possible role in ameliorating neural deficits, but resveratrol has been shown that it does not readily reach the brain intact when given orally because of its rapid degradation. Indeed, one of the most limiting factors to the therapeutic benefits of this drug is the difficulty in maintaining its original structure in biological systems. However, resveratrol has been shown to achieve its effects in a dose-dependent manner, and it has been shown to be relatively safe for use in humans, even at high concentrations (Sawda et al., 2017). There are a few studies that have investigated repeatedly dosing human participants with resveratrol, which may have some beneficial effects because a higher concentration of resveratrol may saturate the enzymes that break it down, allowing it to remain in the body for longer periods of time and reach the brain in

higher concentrations. Repeatedly dosing with resveratrol may also lead to its accumulation in tissues, which is another way it can be more highly detected in the blood (Walle, 2011). Additionally, most studies have used oral resveratrol, which has been clearly shown to break down readily. It is possible that administering resveratrol via other routes of delivery such as intravenously may improve its bioavailability because it would not be metabolized as extensively as if it had gone through the digestive system. Further research must be done to investigate the efficacy of these alternate routes of delivery in human subjects.

Resveratrol has been shown to interact with PKC and facilitates the phosphorylation of this kinase in a dose-dependent manner, meaning that co-treatment of cells with PKC activators and resveratrol may have dramatic benefits in rescuing against the neural deficits seen in AD models (Han et al., 2004). These researchers demonstrated that pre-treating primary hippocampal rat neurons with a PKC activator enhanced resveratrol's ameliorating effects of reducing Aβ-induced cell death, while applying a PKC inhibitor reduced these effects. However, it should be noted that this study showed that resveratrol was not likely to alter the expression of PKCε specifically in their model, and instead suggested that another isoform of PKC (PKCδ) was involved. Moreover, resveratrol has been suggested to bind to the phorbol ester site of one isoform of PKC (Pany et al., 2012), meaning that it may be possible for resveratrol to be chemically modified to bind specifically to PKCε and lead to its increased activation. With chemical modifications, resveratrol analogs may be used in conjunction with PKC activators to create a synergistic effect of improving cellular deficits in models of Alzheimer's disease.

Previous Work

My previous work sought to find a consistent concentration of DCP-LA that would significantly and consistently improve neuronal viability compared to control cells and that would adequately protect cells that were undergoing cytotoxic stress *in vitro*. Using E18 rat embryos, cortical rat neurons were cultured for two weeks prior to any experimentation. DCP-LA of various concentrations (25, 50, and 100 μM) were used in conjunction with agents of oxidative stress, namely NMDA and ferrous amyloid buthionine (FAB). Exogenously given NMDA acts on its receptors postsynaptically to simulate the increased excitotoxicity that is observed in AD (Wang and Reddy, 2017). While NMDA receptor function is critical for cell survival, overactivation of these receptors can lead to glutamate excitotoxicity and thus may function as a general inducer of oxidative stress in models of AD. FAB is a stressor more specific to AD models and comprises ferrous sulfate ($FeSO_4$), $A\beta_{1-42}$, and buthionine sulfoximine (BSO). $FeSO_4$ creates an environment of oxidative stress, $A\beta_{1-42}$ supplies the insoluble and harmful fragments, and BSO inhibits the cell's natural antioxidant mechanisms by inhibiting glutathione; thus, this FAB mixture can induce the AD phenotype *in vitro.* (Lecanu and Papadopoulos, 2013). Neuronal viability was measured using an MTS colorimetric assay, wherein the color of the wells containing the affected neurons was analyzed using computer software for significant changes, as more live cells give a darker color to the wells. MTS is a tetrazolium salt that is broken down into a colored dye known as formazan by dehydrogenases of the mitochondria of live cells (Ganguly et al., 2006). Only living cells would be able to catalyze this reaction, and so MTS serves as a measure of cell viability, with darker-colored wells indicative of more living neurons. Dendritic morphological changes and the level of acetylated

tubulin were measured using immunocytochemistry (ICC), which involved fixing the cells in paraformaldehyde, thus keeping them in place so their shapes can be observed. Staining was accomplished using anti-acetylated tubulin as a primary antibody and anti-mouse secondary antibodies conjugated to either FITC or Cy3 fluorescent proteins.

Achieving consistent results with FAB was challenging, mainly due to the difficulty in establishing a suitable concentration. During these experiments, a range of FAB concentrations were used ("low," "medium," or "high" FAB), yet each concentration showed inconsistent results both as negative controls and in the presence of a protective compound such as DCP-LA. Thus, NMDA was used as a stressor. This was done in order to measure DCP-LA's effectiveness in ameliorating cytotoxic effects in cultured cells until a suitable FAB concentration was developed. Using NMDA, the efficacy of DCP-LA was somewhat consistent, with unanticipated results due to contamination within the cell cultures and varying rates of metabolism of the neurons that meant feeding schedules were not always effective. However, for most trials, DCP-LA increased neuronal viability in stressed cells compared to control cells in NMDA alone, although these results were not statistically significant (Figure 5). 50 μM DCP-LA was also shown to have somewhat protected against cytotoxicity using 100 μM NMDA. Thus, 50 μM of DCP-LA were used for later experiments. However, later viability assays and ICC measuring acetylated tubulin also showed some inconsistency in bringing about the cellular changes expected from 50 μM, and so it was hypothesized that a greater concentration of DCP-LA was needed. 100 μM of DCP-LA were planned to be used, but with the global COVID-19 pandemic, these viability assays were unable to be carried out.

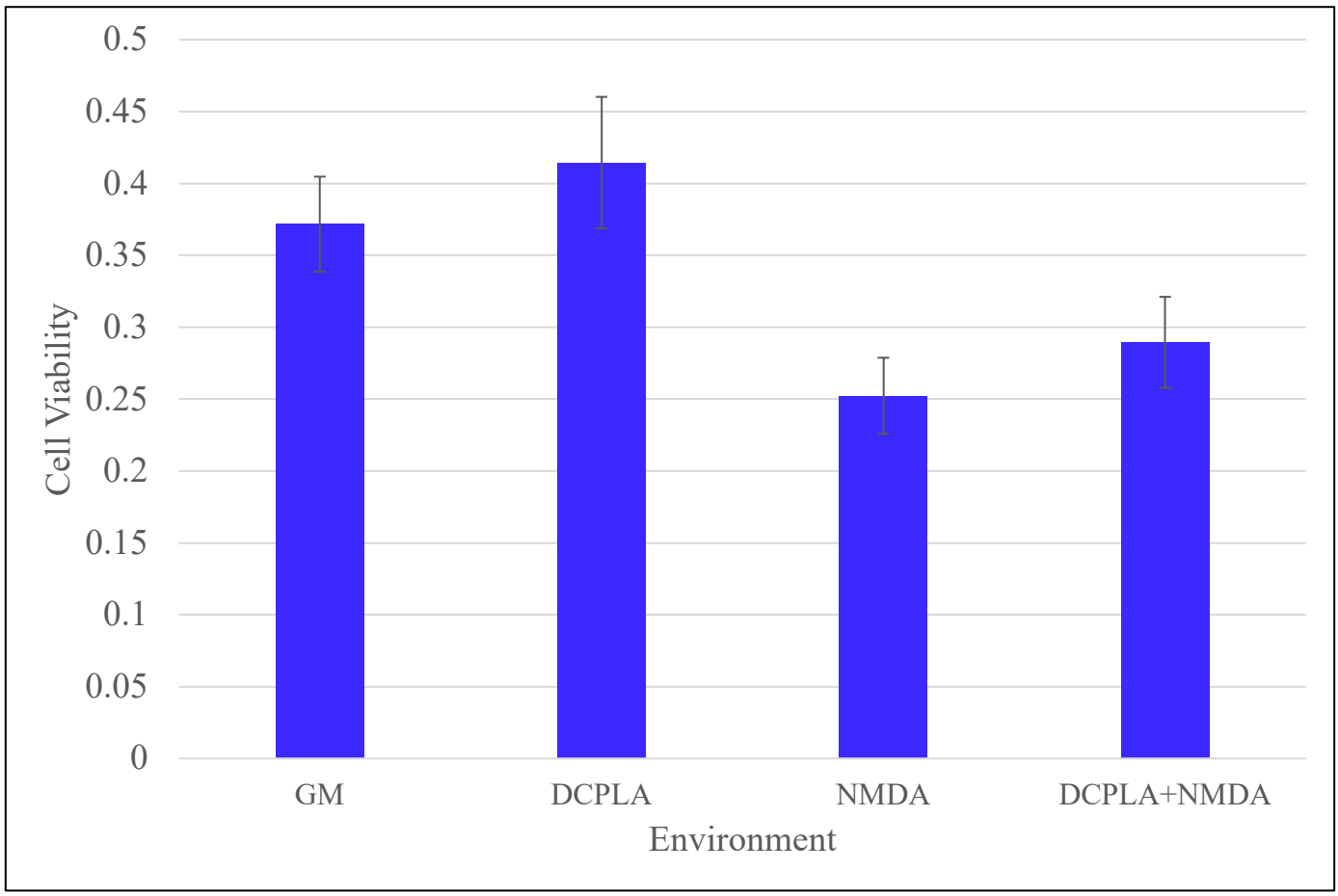

Figure 5: Survival of cortical rat neurons when treated with 50 µM DCP-LA in the presence of the cytotoxic stressor NMDA (100 µM) – Cortical rat neurons were cultured and treated with 50 µM DCP-LA dissolved in growth media (GM) in the presence and absence of 100 µM NMDA. Data are mean ± SEM with n = 15 for each condition. * $p<0.05$

Executive Summary

The development of Alzheimer's disease is one that has many aspects that are not all fully understood. The decline in neural integrity may begin years before clinical symptoms of AD arise, further adding to the complication of treating this disease effectively. According to the amyloid cascade hypothesis, AD is the result of the formation of harmful Aβ plaques and hyperphosphorylated tau that leads to neurofibrillary tangles (NFT's). Aβ plaques may have several detrimental effects in the brain, including decreasing the abundance of synapses, creating oxidative stress, and possibly upregulating enzymes like GSK-3β that can result in tau hyperphosphorylation. Aβ may also downregulate PKCε activity, which would decrease long term synaptic transmission and neurite outgrowth. Preserving or even upregulating PKCε activity is vital for maintaining neural integrity and protecting against Aβ-induced insults.

DCP-LA is an activator of PKCε that has the potential for upregulating both the amount of α7 nACh receptors and AMPA receptor subunits GluR1 and GluR2, facilitating long-term synaptic transmission and LTP. DCP-LA can also inactivate GSK-3β through PTP1B inhibition, which would limit the amount of tau hyperphosphorylation. Thus, the use of DCP-LA may prove beneficial in protecting against toxicity in Alzheimer's disease.

However, my previous work has shown that DCP-LA may not be as effective in protecting against cytotoxicity when used independently. Thus, a combination treatment of DCP-LA with another compound may provide more robust protection. Resveratrol is a compound found in red wine that mimic caloric restriction by activating a class of deacetylases known as sirtuins. Through the activation of sirtuins such as SIRT1, resveratrol can lead to several beneficial downstream effects such as a decrease in Aβ plaques through the upregulation of

ADAM10, a metalloproteinase that can cleave APP into the neuroprotective protein sAPPα. Resveratrol may also deacetylate tau, reducing its excessive phosphorylation.

The use of DCP-LA and resveratrol in combination has not been extensively studied. Thus, exploring how these two compounds may exert their effects synergistically may open several avenues in more effectively protecting against cytotoxic insults induced by AD. It is possible that using these two compounds in tandem can result in them mitigating Aβ plaque formation and tau hyperphosphorylation separately, or by one compound augmenting the other's effects. Both DCP-LA and resveratrol have been shown to provide some protective and rescuing properties in models of neurodegenerative diseases, and therefore using them in combination is a potential avenue for success in treating the neurological insults brought on by cytotoxicity in models of AD.

PART II

The results of using DCP-LA to preserve neuronal viability in excitotoxicity indicate that DCP-LA alone may not have been as effective in doing so in the presence of oxidative stress, and thus the possibility of using a combination treatment with another drug is explored here. Resveratrol is a plant-derived compound that has been correlated with the reduced incidence of AD, though this precise connection remains to be fully investigated. Resveratrol leads to the activation of SIRT1, which may have downstream effects such as reducing the accumulation of Aβ by the activation of secretases that cleave APP away from the site that produces the toxic Aβ fragments. Used in conjunction with DCP-LA, it is possible that resveratrol may reduce the amount of total Aβ produced, while DCP-LA facilitates the decrease in tau hyperphosphorylation and increases or preserves the number of synapses. Thus, these two mechanisms of action working in tandem may result in a pronounced improvement in neuronal viability, increased or preserved synapse number, decreased ROS, and decreased tau hyperphosphorylation *in vitro.*

Establishing a potential relationship between DCP-LA and resveratrol in mitigating damaging cytotoxic effects would require that each compound is independently evaluated for its potency in cell culture models before combining the two. In this way, the results of the combination treatment may be better explained by the results from the studies using each compound individually. As such, the suggested combination treatment explored here can best be understood in the context of how each individual treatment exerts its effect, and how these effects can possibly combine to produce an even more potent treatment for the cytotoxicity induced by Alzheimer's disease models. Thus, I present a hypothetical and systematic approach

to using a combination treatment of DCP-LA and resveratrol, first by evaluating each of their effects independently, then by combining the two compounds.

In vitro Models – DCP-LA and Resveratrol Alone

My previous work using DCP-LA yielded somewhat beneficial results when used at 50 µM concentrations in conjunction with 100 µM NMDA (Figure 5). Thus, the next steps would have been to repeat these experiments with NMDA to corroborate the results, as well as replace NMDA with FAB as a stressor due its more specific simulation of Alzheimer's disease *in vitro*. As previously mentioned, the global pandemic and finding a stable and effective concentration of FAB proved challenging, and consequently finding this concentration would have to be one of the first steps to accomplish before adding mitigating treatments such as DCP-LA and/or resveratrol. Maegerlein (2021) previously used 7.95 mg $FeSO_4$, 1µM Aβ, and 133.5 mg buthionine sulfoximine dissolved in 50 mL growth media (GM) to create her strongest FAB concentration, which she then used in addition to dilutions of this concentration to simulate the cytotoxic stresses observed in AD (Maegerlein, 2021); Witkowski (2021) also used these amounts of each component of FAB in her experiments and diluted them to a 1:4 concentration (Witkowski, 2021). The FAB concentration that would be used in these hypothetical combination experiments must be potent enough to induce cytotoxicity but not so powerful that it overwhelms the cell culture with ROS.

For use in an *in vitro* model, the maximal concentration of FAB used by both Maegerlein and Witkowski would be used to evaluate cytotoxicity (Maegerlein, 2021; Witkowski, 2021). This concentration would serve as an adequate starting point and would be diluted to create

100%, 50%, and 25% v/v concentrations. The purpose of this experiment would be to determine whether FAB can act as a sufficient oxidative stress-inducing agent, and the concentration that would do so. A sufficient level of oxidative stress would be characterized as a consistent 40-60% average reduction in neuronal viability across trials, as measured through an MTS assay. This would allow for better visualization of the possible effects of compounds like DCP-LA and resveratrol, when they would be used in future experiments. Having a 40-60% reduction in viability would allow room for observing any potential increases or decreases in viability with treatment of DCP-LA and resveratrol (or both).

The effect of FAB's excitotoxicity would be evaluated using four major measures: neuronal viability, superoxide levels, microtubule stability, and the relative number of synapses. A one-way ANOVA would be used to analyze the results of each of these four measures. Neuronal viability would be measured using an MTS viability assay. The amount of superoxide, which is a reactive oxygen species and indicator for oxidative stress, would be measured with a MitoSox assay, following the protocol outlined by Rinald (2020). Microtubule stability would be measured with immunocytochemistry (ICC). ICC involves fixing the cells, or preserving them in suspension, with paraformaldehyde so that their structures can be analyzed with immunofluorescence imaging (Pollice et al., 1992). Paraformaldehyde preserves these cells by creating intermolecular covalent cross-links (Kim et al., 2017), essentially halting all chemical reactions to allow for a clear image of the cellular structure to be taken (Figure 6). After fixing the cells in 4% paraformaldehyde, they would be washed with phosphate-buffered saline (PBS) and incubated with 5% Triton-100. The cells would be stained with a primary antibody, which is anti-acetylated tubulin raised in mice. A secondary antibody, anti-mouse IgG raised in goats that

is conjugated to either FITC (fluorescein isothiocyanate, anti-acetylated tubulin) (George et al., 2015), or Cy3. FITC and Cy3 glow blue-green and red under the appropriate wavelength of light, respectively, allowing for visualization acetylated microtubules. Acetylated tubulin is a marker for stable microtubules (Eshun-Wilson et al., 2019). Since tau functions in stabilizing microtubules, and hyperphosphorylation of tau results in the destabilization of these microtubules, using ICC with antibodies that indicate the amount of acetylated tubulin could be used as a proxy for the assessing the level of tau hyperphosphorylation. If tau was hyperphosphorylated, then it would destabilize the microtubules and result in a decrease in acetylated tubulin, measured by a decrease in fluorescent intensity of the fluorescent antibodies FITC and Cy3.

The number of synapses would be measured using markers for synaptophysin, a marker for presynaptic terminals and a way of measuring synaptogenesis (Gräff et al., 2013; Hongpaisan et al., 2011). A primary antibody such as SY38, which is an anti-synaptophysin IgG antibody raised in mice, could be used (abcam). A fluorescent secondary antibody such as H&L, which is an anti-mouse IgG fluorescent antibody raised in goats, could be used to visualize the synaptophysin. Measuring the abundance of synapses would seek to investigate whether oxidative stress and/or the presence of $A\beta_{1\text{-}42}$ negatively impacts synapse number and provide a more complete picture of what is occurring at the cellular level when neurons are exposed to oxidative stress.

It is hypothesized that FAB will act in a dose-dependent manner in all four measures. Neuronal viability and superoxide levels are expected to be the lowest in the 100% concentrated FAB medium, and highest in the GM control. The amounts of acetylated tubulin and

synaptophysin should be lowest in the 100% concentrated FAB solution, and highest in the control. If neuronal viability is consistently between 40-60% of the control at any one concentration of FAB (most likely the 25% or 50% concentration), this concentration would be used in the subsequent experiments involving DCP-LA, resveratrol, or both compounds.

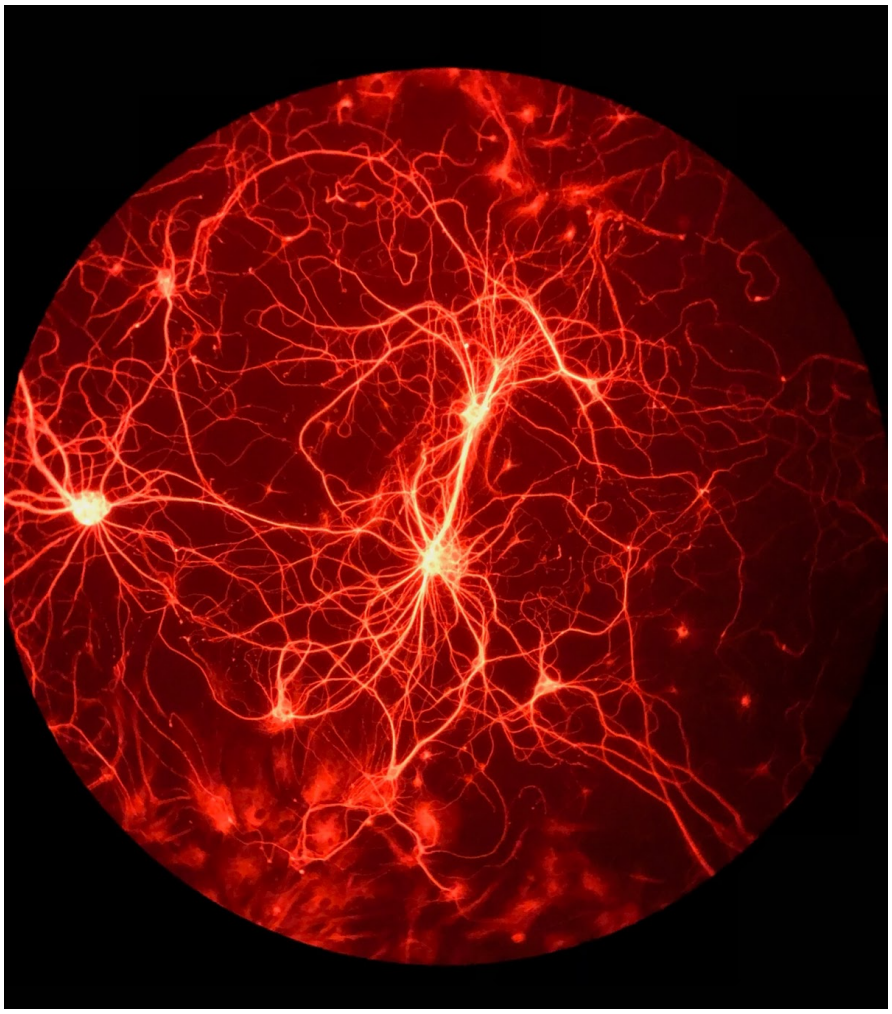

Figure 6: Example fluorescent microscope image of primary cortical rat neurons – Cortical rat neurons were fixed with 4% paraformaldehyde and treated with anti-acetylated tubulin and secondary antibodies conjugated to FITC and Cy3. The neurons depict fluorescent Cy3 antigen binding, which glows red when treated with the appropriate wavelength of light. The cell bodies of the neurons fluoresce the strongest, indicating more robust antibody-antigen interactions and greater protein levels. Image taken at Drew University, Madison NJ, by Shady Barsoom (2019).

A general procedure for this first proposed experiment is given below (Figure 7):

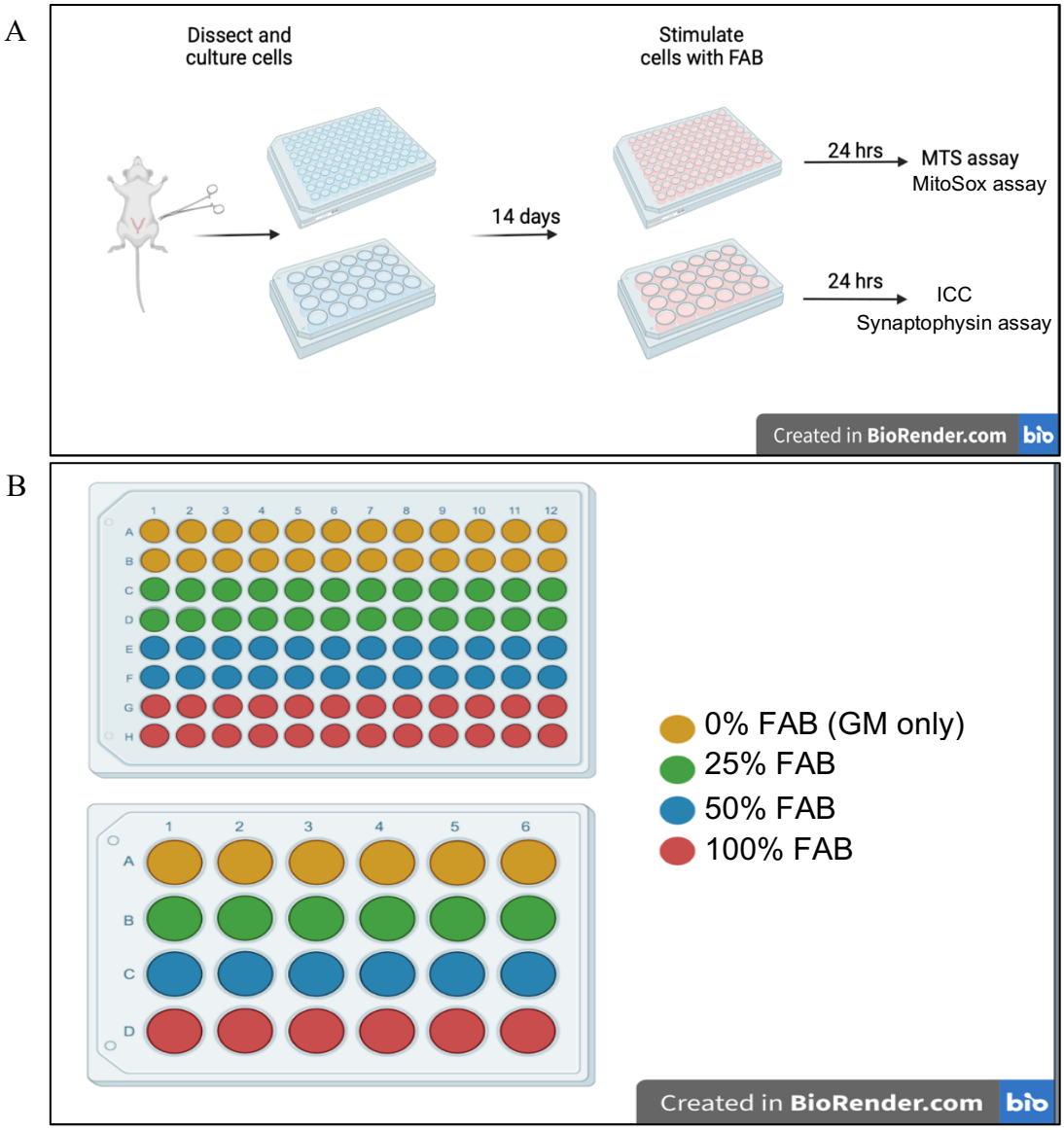

Figure 7: Procedure for determination of sufficient FAB concentration. A) General timeline of experiment, beginning with the culture of embryonic E18 cortical rat neurons for two weeks. 24 hours after stimulation of the plates with the respective FAB concentrations, an MTS assay and MitoSox assay would be conducted on the 96-well plate, while ICC and a synaptophysin assay to measure the relative number of synapses would be conducted on the 24-well plate. B) Plate map for both 96-well and 24-well plates. 100% FAB corresponds to the maximal concentration of FAB used (7.95 mg $FeSO_4$, 1μM Aβ, and 133.5 mg buthionine sulfoximine), while the 0% FAB is a positive control containing solely growth media (GM). Percentages represent dilutions v/v in GM.

To evaluate the possible neuroprotective effects of DCP-LA, a similar experiment to my previous work would be performed. The purpose of this particular experiment would be three-fold: 1) To assess whether DCP-LA has a neuroprotective ameliorating effect independently from FAB, 2) To assess whether DCP-LA protects or preserves neuronal function in the presence of FAB, and 3) To assess whether DCP-LA's effects are dose-dependent. E18 cortical rat neurons would be dissected and cultured for 14 days to allow them to grow in 96-well and 24-well plates, with replacement of the growth media occurring every other day. After 14 days, the cells would be assigned into four separate conditions: 1) growth media (GM), 2) DCP-LA (0, 10, 50, and 100 μM dissolved in DMSO), 3) FAB (using the predetermined concentration for inducing cytotoxicity at 40-60% viability), and 4) DCP-LA + FAB (DCP-LA would be dissolved in FAB and applied to the cells). Given that the results of my previous experiments with DCP-LA took place in NMDA as a stressor, a variety of dosages of DCP-LA would have to be required to re-establish the most effective concentration of DCP-LA in the FAB medium that would be previously ascertained. A two-way ANOVA would be conducted to evaluate for statistical significance. The overall process of this experiment can be seen in Figure 8:

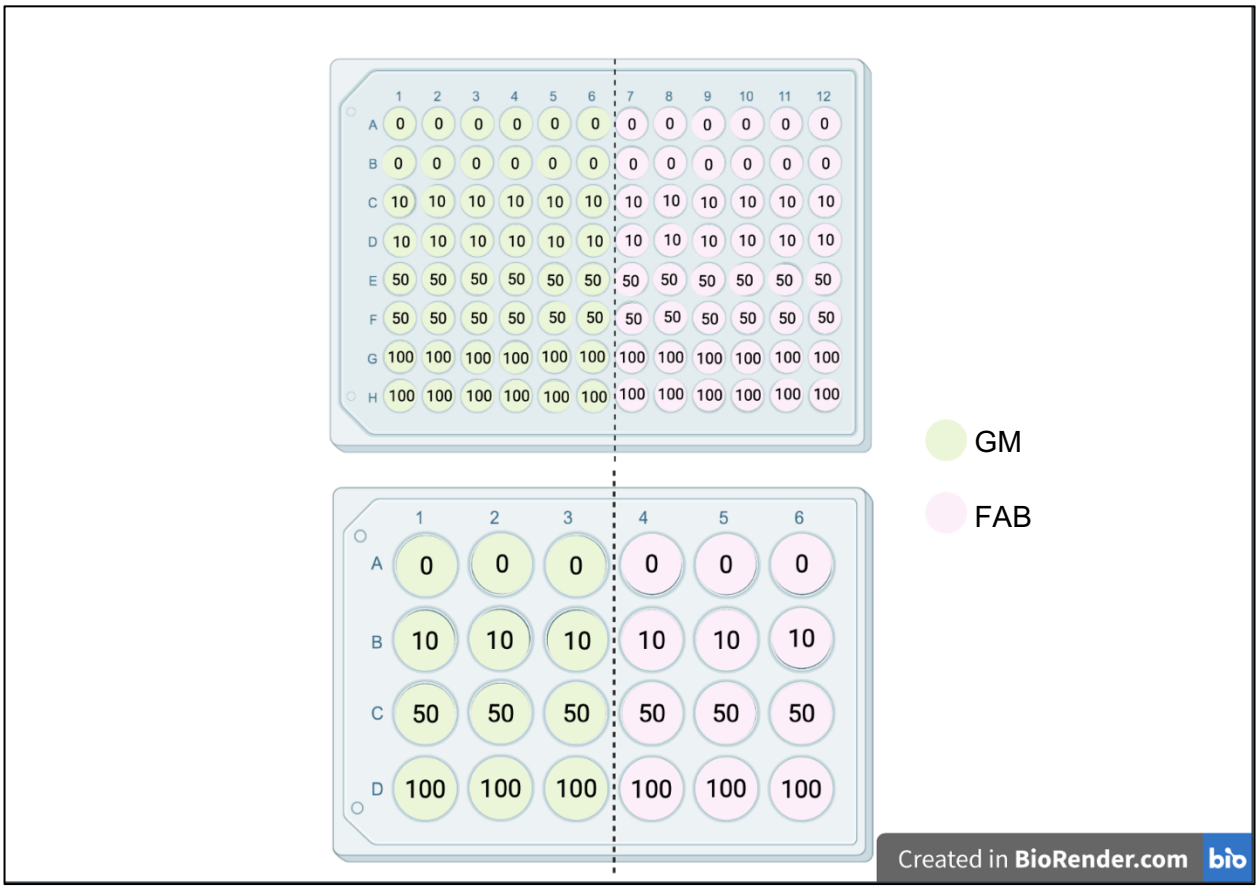

Figure 8: Plate map for DCP-LA-alone experiment – Both the 96-well and 24-well plates would be split into two halves, with one half containing no oxidative stress (GM) and the other half containing an oxidative stress environment (FAB). The numbers within the wells indicate the concentration of DCP-LA (in µM) that would be plated in its respective media (GM or FAB).

Following the treatment for each respective condition, the molecular and cellular effects would be evaluated. The same four measures as the FAB experiment would be evaluated (neuronal viability, superoxide levels, microtubule stability, and synapse number).

It is hypothesized that cells in the FAB alone condition would exhibit decreased overall viability than the cells treated with DCP-LA. It is also predicted that DCP-LA would dose-

dependently reduce the amount of superoxide levels and increase neuronal viability, acetylated tubulin, and synaptophysin. The highest concentration of DCP-LA would be predicted to have the most robust effects of these measures.

A similar experiment would be conducted using resveratrol. The goal of this experiment would be to evaluate 1) if resveratrol is neuroprotective alone without FAB, 2) if resveratrol would protect or preserve neuronal function in the presence of FAB, and 3) if resveratrol exerts these effects dose-dependently. The same four conditions as the DCP-LA alone experiment would be used, but with DCP-LA replaced with resveratrol: GM, FAB, resveratrol, and resveratrol + FAB. Similar to the DCP-LA experiment, a range of concentrations for resveratrol would be used. Modifying the range used by Han et al. (2004), the concentrations used would be 0, 10, 20, and 40 μM. Witkowski (2021) observed the most neuronal survival in cells treated with 20 μM of resveratrol, and so incorporating this concentration mid-range would also serve to corroborate her results and explore whether a higher concentration of resveratrol in these conditions may facilitate greater neuronal survival and function (Witkowski, 2021). The same four measures evaluated in the FAB-alone and DCP-LA-alone experiment would be used (viability, superoxide levels, acetylated tubulin, and synaptophysin). Resveratrol would also be dissolved in DMSO. Resveratrol has a wide range of solubility in both organic and polar solvents (Robinson et al., 2015). Statistical analysis would be conducted using a 2-way ANOVA. A plate map depicting the respective conditions that would be used is given in Figure 9:

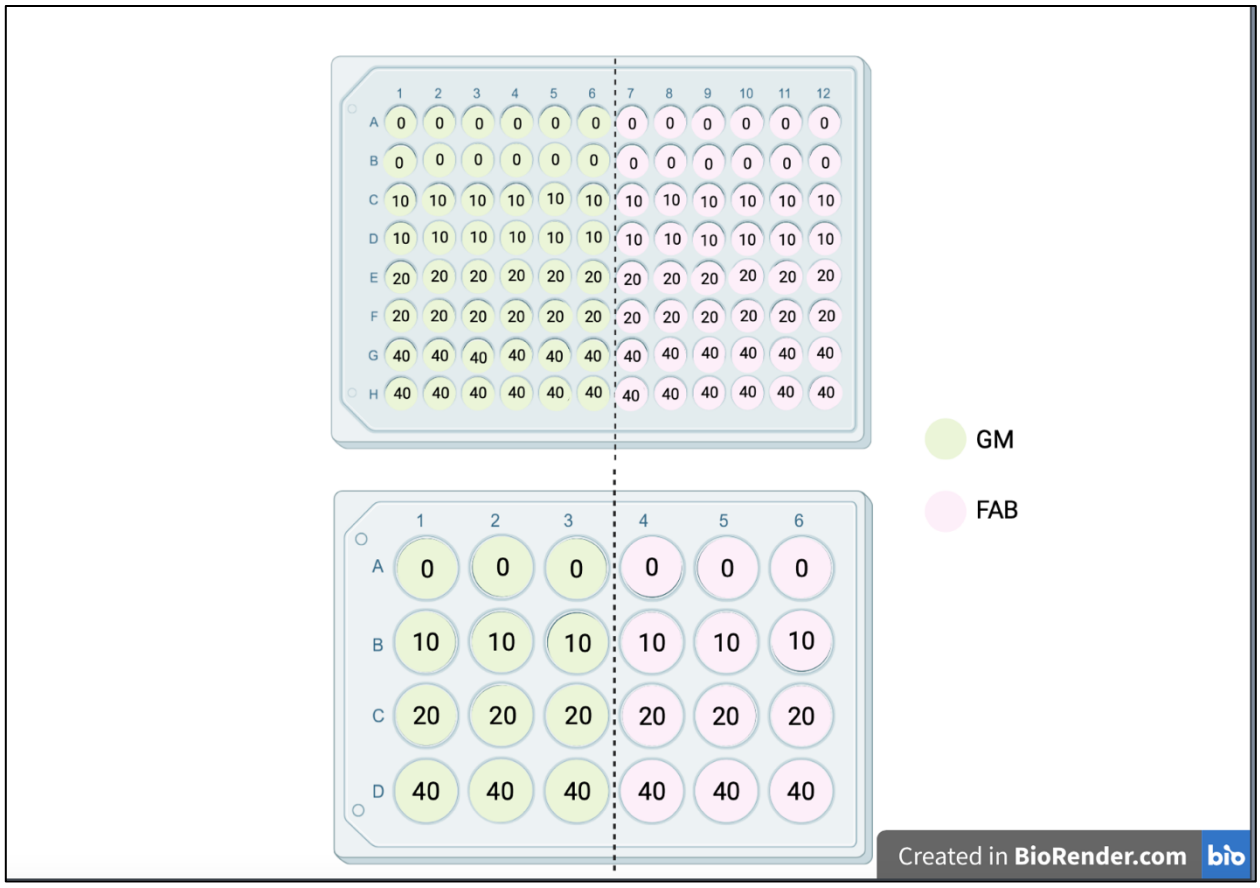

Figure 9: Plate map for resveratrol alone experiment – Both the 96-well and 24-well plates would be split into two halves, with one half containing no oxidative stress (GM) and the other half containing an oxidative stress environment (FAB). The numbers within the wells indicate the concentration of resveratrol (in µM) that would be plated in its respective media (GM or FAB).

In vitro Models – DCP-LA and Resveratrol Combined Treatment

Having gained further insight into how DCP-LA and resveratrol exert their effects independently in the excitotoxic FAB model of Alzheimer's disease, I would proceed to create a combination treatment. This combination approach would be established in two main ways: the

first is simply combining the two compounds into one solution and administering it to the cultured cells, whereas the second approach entails sequentially adding one compound to the other. As Han et al. (2004) have shown, administration of a PKC inhibitor prior to treatment with resveratrol significantly reduced the ameliorating effects of resveratrol against the damaging effects of Aβ and also reduced resveratrol's phosphorylation of certain PKC isoforms. Treatment with a strong PKC activator before adding resveratrol showed lower levels of Aβ toxicity. Thus, there is a possibility that DCP-LA and resveratrol may work synergistically by either a) independently mitigating classic hallmarks of AD to create an overall decline in toxicity or b) augmenting each other's effects, such as DCP-LA enhancing the effect of resveratrol so that the reduction of Aβ toxicity is even more pronounced. It would be important for this combination approach to experiment with the order in which these two compounds are added.

Essentially, three main goals must be achieved prior to using this proposed combination treatment of DCP-LA and resveratrol: a suitable concentration of FAB must be established such that there is sufficient excitotoxicity (40-60% reduction in neuronal viability) of the cells without overwhelming them, a sufficiently high concentration of DCP-LA must be created that consistently protects against FAB toxicity, and a sufficiently high concentration of resveratrol must be created that also consistently protects against FAB toxicity. Once the concentrations of these three compounds (FAB, DCP-LA, and resveratrol) are established, a combination treatment of DCP-LA and resveratrol could be explored in the FAB model of excitotoxicity. This combination treatment would utilize the same cell culture system and general procedures for dissection and growth. Both compounds would be dissolved in DMSO and would be at the

concentration that was deemed most effective in promoting cell survival in the previously discussed experiments where they were used independently.

Following incubation for 24 hours with the respective treatment, the cell cultures would be assessed for potential mitigating effects of this combination treatment by evaluating the same four major measures as the previous experiments: neuronal viability, superoxide levels, acetylated tubulin levels, and synaptophysin levels. These four categories would be measured using the same or similar laboratory techniques as previously described. It is hypothesized that cells treated with the combination treatment of DCP-LA and resveratrol would exhibit greater neuronal viability, decreased superoxide levels, and increased acetylated tubulin and synaptophysin than cells in FAB alone. These effects are predicted to be more robust in the combination treatment than either compound alone. The effect of various treatments would be measured using a two-way ANOVA.

PART III

Interpretation of Anticipated Results – DCP-LA Alone

In the DCP-LA alone treatment, DCP-LA of varying concentrations would be administered to cultured cortical rat neurons both in the presence and absence of FAB. After incubation for 24 hours, the aforementioned measures would be evaluated. The expected results would be an increase in neuronal viability, acetylated tubulin, and synaptophysin, with a decrease in superoxide levels in the DCP-LA-treated cells in the presence of FAB, as compared to FAB alone. To briefly summarize, there would be four conditions in this hypothetical experiment: GM alone, DCP-LA alone, FAB alone, and DCP-LA in FAB. The GM- and FAB-alone conditions would function as positive and negative controls, respectively, while the DCP-LA alone condition would assess whether this compound exerts any protective effects in the absence of a cytotoxic stressor. The DCP-LA in FAB experimental condition would evaluate whether DCP-LA provides a rescuing or preserving effect in the presence of a stressor.

In the DCP-LA-alone conditions, where there would be no FAB stressor, I would expect to see an increase, or at least a preservation of, neuronal viability as measured by an MTS assay. Should this occur, I would conclude that DCP-LA does have a beneficial impact on maintaining the health of the cell. Primary culture cells intrinsically do not last indefinitely, and so it would be expected that the number of live cells would naturally diminish over time. If DCP-LA significantly preserved the number of live cells compared to the GM positive control, then it would be a strong indicator of its beneficial effects. Moreover, if the levels of acetylated tubulin and synaptophysin were comparable or higher than the GM control as well, this would also strongly suggest that DCP-LA functions to preserve neuronal viability and synapse integrity by

limiting the amount of hyperphosphorylated tau and promoting neurite outgrowth. If neuronal viability decreased, then it is possible that the concentration of DCP-LA was too low for it to exert its effects or sufficiently protect against natural neuronal loss. It is also possible that the hypothesis that DCP-LA protects or rescues against neuronal loss in this particular model system is not supported. Other causes for decreased neuronal viability can be intrinsic, such as contaminated cells.

In the DCP-LA and FAB-treated conditions, I would expect to see a decrease in neuronal viability compared to the GM control, but there would be more live cells than the FAB-alone conditions. Similarly, I would expect to see higher levels of acetylated tubulin and synaptophysin in the DCP-LA treated conditions compared to the FAB-alone treatment. These anticipated results would strongly suggest that DCP-LA provides a protective and/or rescuing effect in the presence of a cytotoxic stressor such as FAB. If the neuronal viability assay showed no effects or even a decrease in viability in DCP-LA treated cells, it is likely that either the FAB concentration was still too potent and killed the cells by overwhelming them with ROS, or the concentration of DCP-LA was too low to exert a significant protective/rescuing effect from excitotoxicity. If the FAB concentration was too potent, but DCP-LA was exerting a protective effect, then I would expect to see superoxide levels in the DCP-LA-treated conditions that are comparable to FAB alone, and I would see some preservation of neuronal viability, acetylated tubulin, and synaptophysin. If the DCP-LA concentration was too low to exert its effects, then superoxide levels in all treated conditions would be comparable to the controls and neuronal viability, acetylated tubulin, and synaptophysin would not be significantly altered. Overall, the purpose of the DCP-LA-alone experiment would be to 1) evaluate whether DCP-LA has a surviving effect

in the absence of a stressor, 2) evaluate the potential protective or rescuing effect of DCP-LA in the presence of the stressor FAB, and 3) observe if these effects were dose-dependent.

The next steps for this particular experiment would be to repeat it with an even more dilute concentration of FAB and/or raising the concentration of DCP-LA. FAB would be diluted even further while keeping the concentration of DCP-LA the same and evaluating the same measures of neuronal integrity and excitotoxicity. Finding a more potent concentration of DCP-LA would likely entail using another agent of oxidative stress such as NMDA. While this compound does provide a more generalized excitotoxic model that is not specific to the molecular events characteristic of AD, it can be a useful tool in evaluating the potency of a compound such as DCP-LA without the influence of other disruptive factors such as the Aβ or antioxidant-suppressing mechanism of buthionine sulfoximine. NMDA may serve as a more reliable method of inducing excitotoxicity so that DCP-LA's concentration can be established. The intention with these repeated experiments is therefore to adjust the concentrations of FAB, DCP-LA, or both in order to more fully investigate whether there is indeed a neuroprotective effect of DCP-LA in an oxidative stress environment such as FAB.

Should the results of the DCP-LA-alone experiment be what was expected, some possible mechanisms for how DCP-LA exerts its effects could be predicted. If neuronal viability was maintained or even increased in the DCP-LA-treated conditions without FAB, then DCP-LA broadly helps to facilitate neuronal survival intrinsically. A more specific evaluation of DCP-LA's potential effects would be seen in the measurements of superoxide, acetylated tubulin, and synaptophysin. Should the levels of superoxide be reduced upon treatment with DCP-LA in the presence of FAB, this could be explained by DCP-LA's activation of PKCε, which may have led

to an increase in the cell's antioxidant mechanisms. Rinald (2020) suggested that PKCε activation may lead to activation of manganese superoxide dismutase (MnSOD), an enzyme that converts superoxide molecules to hydrogen peroxide, thereby neutralizing these ROS's effects. Additionally, Millien et al. (2022) have shown that PKCε resulted in decreased ROS and prevented MnSOD decreases from oxidative stress, highlighting an important potential interaction between this kinase and MnSOD (Millien et al., 2022). Whether PKCε activation via DCP-LA leads to an upregulation of MnSOD or prevents its downregulation that may result in the decreased superoxide levels anticipated in this proposed experiment is unclear, and would require more specific study into the interaction between PKCε and MnSOD.

If acetylated tubulin was increased or maintained in the DCP-LA-treated conditions, then it is possible that through the activation of PKCε, DCP-LA facilitated neuroprotective effects. For example, an increase in acetylated tubulin may suggest that DCP-LA inhibited GSK-3β activity, which would diminish the amount of tau hyperphosphorylation and result in increased microtubule stability. Should this be the case, then future experiments should more specifically investigate GSK-3β activity in the presence of DCP-LA and FAB. If acetylated tubulin decreased in the presence of DCP-LA alone, then it is possible that tau was phosphorylated by other kinases that would not be studied in these experiments. For example, the presence of hyperphosphorylated tau (as measured by a decrease in acetylated tubulin) in DCP-LA-treated conditions could mean that the concentration of DCP-LA used was not sufficient to lead to a decrease in GSK-3β activity, which could be corroborated by a decrease in neuronal viability as measured by the MTS colorimetric assay. Additionally, tau could have been phosphorylated by a host of other kinases that would not have been measured in this experiment, such as cyclin-

dependent kinase 5 (cdk5), 5' adenosine monophosphate-activated protein kinase (AMPK), protein kinase A, and tyrosine kinases (Noble et al., 2013). While GSK-3β plays a vital role in understanding how AD develops in certain models, this experiment would not detect its activity specifically, and so more detailed characterizations of GSK-3β activity and its impact on tau hyperphosphorylation would have to be conducted.

Should the levels of synaptophysin be increased or maintained in the DCP-LA-treated conditions, this would suggest that DCP-LA is acting as predicted by activating PKCε and leading to an increase in neurite outgrowth and synaptogenesis. As previously mentioned, DCP-LA can result in the trafficking of α7 nACh receptors and upregulating GluR1 and GluR2 AMPA receptor subunits, allowing the neuron to become more responsive to EPSPs and increasing the cell's ability to respond postsynaptically. One of the downstream targets of PKCε is PSD-95, which is responsible for the trafficking of these AMPA receptor proteins and may be involved in doing so in a process mediated by DCP-LA. While Rinald (2020) did not observe an increase in PSD-95 phosphorylation by PKCε, Sen et al. (2016) did, although Rinald acknowledged that the timing of his experiment varied from Sen et al.'s (Rinald, 2020; Sen et al., 2016). With this in mind, it is possible that synaptophysin levels would not be increased or maintained in this proposed experiment because of the potential short-lived nature of DCP-LA-mediated PKCε activation. Thus, to more fully characterize the effect of DCP-LA on PKCε activation and the subsequent change in synapse number, different time courses of treatment would have to be used.

Interpretation of Anticipated Results – Resveratrol Alone

The treatment of cells with resveratrol would follow a similar experimental procedure and measures as with DCP-LA. The goal of this particular proposed experiment would be similar to that of DCP-LA in determining any potential neural benefits of resveratrol both in the absence and presence of the cytotoxic stressor FAB. There would be four conditions: GM alone (positive control), resveratrol alone, FAB alone (negative control), and resveratrol + FAB. Resveratrol would be studied without the addition of FAB to determine whether it has potential neuroprotective effects on its own. Should neuronal viability, acetylated tubulin, and synaptophysin be increased or unchanged relative to the GM control, it would provide a strong indication that resveratrol is beneficial to neurons *in vitro* independently. Increased viability could be the result of increased tau-microtubule stability and decreased superoxide levels. Resveratrol has been hypothesized to deacetylate tau (Sawda et al., 2017). Cohen et al. (2011) have shown that acetylated tau displays impaired function because of its unstable interactions with microtubules, causing a decrease in neuronal survival (Cohen et al., 2011). Thus, should neuronal viability and acetylated tubulin increase in the resveratrol-treated conditions, it would strongly suggest that resveratrol facilitated this process through the deacetylation of tau and subsequent stabilization of this protein with microtubules.

Should there be a decrease in superoxide levels in resveratrol-treated conditions compared to FAB-alone conditions, this would most likely have occurred through the upregulation of the cell's natural antioxidant enzymes, which resveratrol has been suggested to do (Mokni et al., 2007). Additionally, if superoxide levels were significantly decreased in the resveratrol + FAB condition compared to the FAB-alone condition, then it is possible that

resveratrol alleviated oxidative stress through the same mechanism. Aβ has been suggested to induce lipid peroxidation and may generate ROS that can lead into the insoluble plaques that characterize AD (Huang et al., 2004). Therefore, it is possible that resveratrol may have activated SIRT1 and subsequently ADAM10, which would cleave APP into the neuroprotective peptide sAPPα instead of Aβ. While $A\beta_{1-42}$ would already be applied to the cell culture without the need for its formation from APP (because FAB contains ready-made Aβ), sAPPα may play a role in preserving the neurons under oxidative stress that is the result of Aβ, though the exact mechanism of how this protein functions in oxidative stress has yet to be identified.

An increase or maintenance of synaptophysin in resveratrol-treated conditions could be the result of the previously mentioned phenomena. Should resveratrol deacetylate tau and decrease superoxide levels via an upregulation of the cell's natural antioxidant mechanisms, it is likely that the neurons will be able to develop and form new synapses even in the presence of an oxidative stressor like FAB. If synaptophysin is not significantly altered in resveratrol-treated conditions, then it is possible that either the concentration of resveratrol was too low for it to exert its effects (a theory that would be supported by a decrease in neuronal viability and increase in superoxide levels compared to FAB alone), or that resveratrol has no indicated role in the formation or maintenance of synapses.

Interpretation of Anticipated Results – Combination Treatment

The specific goal of this experiment would be to assess whether an *in vitro* cytotoxic model of Alzheimer's disease would display improved neuronal viability, synapse integrity, and decreased oxidative stress and hyperphosphorylated tau through the synergistic effects of DCP-

LA and resveratrol. Neuronal viability would be measured with an MTS assay, superoxide levels with a MitoSox assay, tau hyperphosphorylation through ICC measuring acetylated tubulin, and synapse abundance through synaptophysin measurement. Since these four measures would have been evaluated in all of the preceding experiments, this would allow for DCP-LA and resveratrol's potential combined effect to be placed in context with their effects independently. All four of these measures have been indicated to be affected by oxidative stress, and so determining whether this proposed combination treatment would positively impact them would strongly suggest that these two compounds may alleviate oxidative stress.

Broadly, it is expected that the treated neurons would display a higher level of neuronal viability than cells in FAB alone or cells treated with either compound alone. Cell viability that indicates this robust effect would lend support to the synergistic or additive effects of these two compounds. Determining whether these effects are synergistic (ameliorating excitotoxicity through two independent mechanisms), or additive (one compound augmenting the other's effects), would be accomplished by comparing the results of this experiment with those that used DCP-LA and resveratrol independently. For example, if DCP-LA alone did not significantly alter superoxide levels, but resveratrol alone did, and the combination treatment showed a decrease in superoxide levels comparable to resveratrol alone, then it is likely that DCP-LA and resveratrol were working independently in mitigating superoxide levels because DCP-LA would not be indicated to have a role in doing so. If, however, there was a greater decrease in superoxide levels in the combination treatment than resveratrol alone, then it is possible that DCP-LA augmented resveratrol's effects in alleviating oxidative stress. If there is no significant change in any or all of these four measures with this combination treatment, it is entirely possible that the

hypothesis regarding DCP-LA and resveratrol's combined effects is not supported in this model system. However, this does not necessarily mean that DCP-LA and resveratrol do not have any potential combined effects; it just might not be in alleviating cytotoxicity. As such, investigating a combination treatment of these compounds should be applied to other models of AD such as transgenic mouse models or other neurodegenerative cell cultures.

Additionally, with this combination treatment, it is possible that the concentrations of resveratrol and DCP-LA would be too high. For example, while high concentrations of resveratrol have not been shown to cause adverse effects when given to human participants (Sawda et al., 2017), administering it *in vitro* may lead to an overactivation of a protein like ADAM10 more than what is desired, initiating apoptosis in treated cells. This overactivation could be experimentally determined by measuring ADAM10 mRNA levels with qPCR and fluorescently labeling markers of apoptosis, such as cytochrome c. Additionally, SIRT1 is involved in other cellular processes such as deacetylation of p53, meaning its excessive upregulation by the addition of an exogenous substance such as resveratrol can also create cellular dysfunction and even death if its concentration is too high. Additionally, DCP-LA at exceedingly high concentrations may lead to dephosphorylation of tau to a degree that it is not able to carry out its normal function by interacting with microtubules. Tau must be phosphorylated to some degree in order to stabilize microtubules, so very high concentrations of DCP-LA may interfere with this property. A destabilization in microtubules would likely result in neuronal dysfunction, since microtubules are found all throughout the axon and dendrites, the primary structures for neuronal communication.

Model Systems

While this hypothetical combination treatment of DCP-LA and resveratrol may help to shed some light on how neurons respond to oxidative stress in Alzheimer's disease, there are several factors to consider when evaluating this treatment. One example is the model system that is used. Alzheimer's disease incorporates several pathophysiological processes that occur over long periods of time, even years before symptoms manifest. The model system used in these proposed experiments focuses only on simulating the oxidative stress component of AD; however, oxidative stress is but one component of AD, part of several other important factors that play a role such as neuroinflammation and insulin resistance (Trujillo-Estrada et al., 2021). All of these factors initiate their own cellular events that are involved in the development of this disease. Only the effects of DCP-LA and resveratrol and protecting against oxidative stress specifically would be studied in these proposed experiments. Other contributing factors to the molecular events of AD are ignored in this system for simplicity, and so it is important that the results of this combination treatment are assessed within the broader context of this disease.

This model system also uses cultured embryonic cortical rat neurons. The rat nervous system is quite similar to that of humans, especially at the cellular level, which makes them useful simulations of human neurodegenerative disorders. However, it is important to note that this model studies neurons in isolation, away from the various complicated processes that occur in an intact brain. Thus, the results that would be anticipated in this model system should be taken in the broader context of the mammalian brain. Also, embryonic rat neurons were used as opposed to adult neurons, meaning younger neurons may have already higher levels of neuroplasticity than their adult counterparts, which would explain a lack of significant increases

in synapse abundance, for example, in these experiments. While there may be no significant increase in the number of synapses, this could stem from the already high levels of synaptogenesis because of the intrinsic nature of embryonic neurons.

Moreover, creating an applicable treatment using DCP-LA and resveratrol in a human model of AD would be crucial in assessing whether this combination is an effective one to use in the treatment of oxidative stress. One way that this can be accomplished is through the use of another *in vitro* model known as the SH-SY5Y cell line, which is derived from human neuroblastoma cells (Fontana et al., 2020). According to these same authors, this cell line is not as resistant to Aβ toxicity as something like the cortical rat neurons used in these proposed experiments. Should the combination treatment reveal that the FAB model was not as neurotoxic as previously anticipated, using the SH-SY5Y model may be a more prudent approach than attempting to adjust the concentrations of each individual component of FAB. Moreover, the SH-SY5Y cell line is a useful model system for investigating Aβ processing specifically. SH-SY5Y cells can be used to investigate how Aβ is cleared from the cells (Fontana et al., 2020). Using knockouts and transcriptional analysis in an SH-SY5Y cell line, Grimm et al. (2015) have shown that one enzyme involved in Aβ degradation is neprilysin (NEP), which interacts with APP and its cleavage product APP intracellular domain (AICD) to facilitate the degradation of Aβ (Grimm et al., 2015). While the proposed combination treatment would measure the rate of Aβ formation, using SH-SY5Y cells could be useful for evaluating the effect of oxidative stress on Aβ clearance.

Another *in vitro* cell line that may be of use in this proposed combination experiment are PC12 cells. PC12 cells are an immortalized cell line from a rat adrenal medulla tumor (a

pheochromocytoma) (Fontana et al., 2020), but can display neural characteristics when treated with certain growth factors (Gilson et al., 2015). Additionally, PC12 cells can be useful for more clearly understanding the processing events of APP to Aβ. Wencel et al. (2018) have shown that Aβ exposure in PC12 cells results in an increase γ secretase activity, which plays a role in how APP is cleaved (Wencel et al., 2018). Using PC12 cells may be useful in the proposed combination and resveratrol-alone experiments, where elucidating the mechanism by which APP is cleaved into sAPPα or harmful $A\beta_{1\text{-}42}$ proteins is vital to understanding how resveratrol may protect against excitotoxicity.

Moreover, an *in vivo* model of Alzheimer's disease should also be used to corroborate the results seen in these hypothetical combination experiments. Transgenic rat and mouse models would be useful for understanding the role that various proteins play in facilitating the development of the pathological characteristics of AD. For example, the transgenic mouse model Tg2576 can simulate the formation of Aβ because these transgenic mice overexpress APP that can be selectively mutated and they can develop Aβ proteins and plaques at a young age (Wang et al., 2006; Westermann et al., 2002). Manipulating the genetic makeup of transgenic models can also help to elucidate the role these various genes play in the development of Alzheimer's, and may explain certain results observed in the combination experiments. For example, overexpression of genes that express proteins such as SIRT1 and ADAM10 would be quite useful in understanding how these enzymes may interact with each other and work towards cleaving APP into its neuroprotective form sAPPα. However, great care must be taken when manipulating these kinds of genes because of their widespread distribution throughout the mammalian organism. These models could also clarify the broader behavioral effects stemming

from oxidative stress. The formation of Aβ plaques and neurofibrillary tangles in animal models would likely have an effect on their performance in certain tasks such as spatial memory and learning because of the role that these two pathologies play in synapse structure and integrity. Observing any changes in behavior following the treatment of these models with the combination treatment would be useful in evaluating whether this combination treatment is actually effective in reducing the severity of AD-like symptoms other than an oxidative stress model.

Suggestions for Future Research

Because of the multifaceted roles that several enzymes and cells play in the development and progression of Alzheimer's disease, it is challenging to analyze the entire process in one or a few experiments. Further, cytotoxicity is but one aspect of AD. The role of genetics and the degradation of cholinergic neurons are two examples of several that also have been shown to also be involved in the development of this disease, but would not be studied in these combination experiments.

The combination experiments can also be the source of much variation. For example, in both the DCP-LA- and resveratrol-alone experiments, only one time course would be used. It is possible that these two compounds exert their effects most optimally at different times. Thus, for future research, experimenting with different time courses using the same concentration of DCP-LA and resveratrol would be prudent in more fully understanding their possibly beneficial effects. The same impact of time may be seen in the FAB model. Again, the hypothetical FAB

experiment proposed here would only take place within one time frame. It is possible that FAB exerts its neurotoxic effects at a different time than what was predicted. Understanding the time course of when FAB, DCP-LA, and resveratrol exert their effects may help in elucidating the mechanisms by which cytoxicity and the drugs that mitigate it are interacting with each other.

In addition to considering other model systems as mentioned previously, it is possible that FAB may not be the most effective model for this combination experiment. For example, it may be that DCP-LA and resveratrol do in fact display synergistic effects, but it may not be in the context of FAB-induced cytotoxicity. Therefore, using different model systems such as PC12 and SH-SY5Y cells may provide a clearer picture in how DCP-LA and resveratrol could possibly work in tandem.

Moreover, the possibility of chemically altering the structures of DCP-LA, resveratrol, or both may allow for better integration with this combination treatment and with the respective models of AD. For example, Villalba and Alcaín mention several sirtuin activators that are not related to resveratrol specifically (Villalba and Alcaín, 2012). These activators may be more potent in upregulating SIRT1 activity and leading to a more robust change in tau hyperphosphorylation via ADAM10 expression. Modifying the structure of resveratrol itself so that it can interact with SIRT1 more effectively is also a possibility in increasing its effects. The same principle can be applied to DCP-LA, where modifying its structure chemically so that it is able to interact with PKCε more strongly and lead to more pronounced neurological benefits.

It is the hope that DCP-LA and resveratrol, while not having been studied in combination previously, together may provide a novel way of protecting against Aβ plaques and NFT's caused by tau hyperphosphorylation in a cytotoxic model of Alzheimer's disease.

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