SYNERGISTIC INDUCTION OF AD PATHOLOGY BY APOE AND DDT/DDE: ENVIRONMENTAL AND GENETIC COMPONENT TO NEURODEGENERATIVE DISEASE

By

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ABSTRACT OF DISSERTATION

Synergistic Induction of AD Pathology by APOE and DDT/DDE:

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Alzheimer's Disease is associated with numerous factors that have been found to increase an individual's risk for developing the disease. Some of these risks are avoidable, including exposure to environmental factors, or unavoidable including aging or genetic make-up. The importance of Apolipoprotein E (APOE) and its role in altering the risk of developing AD have been intensively investigated. The E4 allele of the APOE gene is the greatest single contributor, leading to a two-to-three-fold risk increase in AD. The E4 allele leads to induction of Amyloid Precursor Protein (APP) expression in neurons and in turn alters the amount of amyloid-beta (Aβ) that accumulates in the extracellular space. To study the individual effects of the E4 variant, independent of the other variants, CRISPR/Cas9 and lentiviral transduction were utilized to generate hemizygous E3/- and E4/- induced pluripotent stem cells (iPSCs) which could be differentiated into induced neurons (iNs) and induced astrocytes (iAs). The pesticide dichlorodiphenyltrichloroethane (DDT) is an environmental risk factor for AD, with persistent bioaccumulation and elevated serum levels of its metabolite, dichlorodiphenyldichloroethylene (DDE) in AD patients even after it was banned in the U.S. fifty years ago. DDT holds open the voltage gated sodium channels leading to hyperexcitability of the nervous system, but its actions, along with the effect of DDE, have not been well studied in humans. Isolation of the APOE variants, generated using the as described above, provided an efficient way to assess the combinatory effects of genetic and environmental risks for AD.

There have been few studies on whether DDT and DDE affects the human nervous system. We found that acute (≤ 6 hr) or chronic (≥ 24 hr) exposure to DDT and DDE resulted in alteration

of the electrophysiological properties of mammalian neurons. Membrane depolarization was induced following toxicant exposure and firing effects observed from short-term or immediate exposure could be reversed after 5 mins for DDT and 15 mins for DDE when compounds were washed out. However, long-term/chronic exposure resulted in long lasting alterations, with both toxicants resulting in a shift in the resting membrane potential (RMP) +10 mV. More importantly, DDE induced neuronal firing after prolonged exposure while DDT only produced shifts in the RMP. The ability for DDE to exert its toxicity after prolonged exposure as well as requiring additional time for reversing its effects after acute exposures, shows, for the first time, that the DDE metabolite can alter the physiology of human neurons. Although the toxicants were not found to directly induce APOE protein expression individually, these two factors have been shown to contribute to the development or potentiation of AD. Previous evidence showed that individuals with the E4 variant of APOE genotype and DDE accumulation in their blood serum exhibited more cognitive impairment when compared to AD patients who were non-carriers of the E4 variant. Therefore, we determined that exposure to these compounds along with APOE E4 potentiated hallmarks of AD pathology by increasing amyloid precursor protein (APP) and phosphorylated Tau (pTau) expression and amyloid beta 40 and 42 peptide (Aβ40/42) secretion. Interestingly, induction of APP, pTau, and Aβ40/42 were greater with DDE exposure compared to the vehicle group by approximately 2-fold for APP and pTau and 4-fold for amyloid beta; solidifying the dangers of what was once assumed the safer of the two compounds. Literature suggests that DDT, DDE and APOE can independently alter Ca^{2+} homeostasis in neurons. The modification of this vital cation may be one mechanism by which synergism of DDT or DDE exposure and a genotype of APOE E4 can induce the risk of developing and or progressing AD. Again, our observations revealed that exposing our APOE E3/E4 humans induced neurons to DDT or DDE increased AD markers. In addition, knowing that AD patients who are carriers for APOE E4 show signs of more cognitive impairment after exposure to DDT and/or DDE. This awareness has the potential to open doors for targeting different stages of AD as well as contribute to the regulatory efforts of DDT usage; as

being able to monitor or minimize exposure to this potent compound can reduce severe long-term effects.

DEDICATION

This dissertation is dedicated to my mother (Marva), father (Fitzroy), and sister (Marveann).

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CHAPTER 1: INTRODUCTION

1.1 Alzheimer's Disease and Therapeutic Strategies

Alzheimer's disease (AD) is the most prevalent neurodegenerative disease associated with neuronal damage, molecular accumulation of amyloid plaques and phosphorylated Tau protein (Powell et al., 2013; Rhinn et al., 2013). AD is an incurable, progressive disease that is the most predominant form of all dementia cases with an occurrence rate of 60-80 % (Imtiaz, Taipale, et al., 2017). In 1997 AD affected 2.32 million Americans, however, currently 6.2 million Americans are diagnosed with the disease (Brookmeyer et al., 1998; Rajan et al., 2021). In 1998, Brookmeyer predicted that the prevalence would quadruple over the course of 50 years, however, the occurrence has already increased by three-fold in half that time (Brookmeyer et al., 1998). As the rate for AD diagnosis is increasing, there have been many attempts at improving the patients' way of living. This is because an individual diagnosed with AD typically presents with varying symptoms ranging from memory decline, alterations in performance or mood, and even disorientation (Bature et al., 2017). Prior to the development of a conclusive method to diagnose AD in living individuals, diagnosis was confirmed by the discovery of neuritic plaques and neurofibrillary tangle (NFT) pathology in post-mortem patient brain examinations, however only after the confirmation of clinical signs antemortem (Hyman et al., 2012; Mirra et al., 1993; Montine et al., 2012). While a definitive diagnosis still requires an extensive evaluation of brain tissue post-mortem, AD diagnosis in living patients can be detected using β-amyloid position emission tomography (PET) imaging. Amyloid PET allowed for the selective detection of AD which was demonstrated in its first use reported in 16 patients diagnosed with mild AD and 9 control individuals (three aged 21 and six ranging from ages 58 to 80) (Klunk et al., 2004). The use of Pittsburgh Compound-B (PIB) showed that in AD patients, amyloid accumulation was significantly greater, in decreasing order, in the frontal cortex, the striatum, the parietal, occipital, and temporal cortex with 1.94-, 1.76-, 1.71-, 1.54-, and 1.52-fold increases respectively (Klunk et al., 2004). This new method created the

opportunity to approach and design clinical trials differently. While the method provided selective and quantifiable AD progression observations, there has been little success with numerous clinical trials, bringing on the need to assess various avenues for targeting AD. The contribution of safe and efficacious medicine have aided in managing the symptoms of AD in addition to cognitive stimulation therapy; however, efforts to manage disease prevention are lacking (Jha & Mukhopadhaya, 2021). In this review, we explore the wide variety of risks factors, both avoidable and non-avoidable, that contribute to the development of AD.

Over the course of studying AD, there have been multiple failed clinical trials (Jha & Mukhopadhaya, 2021), however, there are various medications that are approved to manage the associated symptoms. These drugs include various cholinesterase inhibitors, immunotherapies, and cognitive stimulation therapy (Jha & Mukhopadhaya, 2021). Aducanumab, marketed as Aduhelm, falls under the category of an immunotherapy, is a monoclonal IgG1 antibody that targets both soluble and insoluble amyloid beta; however two clinical phase III trials, EMERGE (N=1643) and ENGAGE (N=1653) show no improvement in cognitive decline (Jha & Mukhopadhaya, 2021; Salloway et al., 2022; Tolar et al., 2020). Some of the approved drugs such as Donepezil - Aricept®, Galantamine - Razadyne®, and Memantine - Namenda®, approach AD from a molecular standpoint managing the biochemical consequences that occur from neuronal cell death by targeting acetylcholinesterase enzyme and NMDA-receptor subtype of glutamate receptors to enhance cholinergic transmission and mitigate the abnormal transmission of glutamate respectively. While being able to manage the symptoms provides a tolerable quality of life for individuals diagnosed with AD, there is a need for determining contributing factors for susceptible populations. Whether these factors are individually or collectively influencing AD, they could be targeted to gain more information on a preventative approach or a treatment plan.

1.2 Disease Biology

Age is the primary risk for Alzheimer disease and most patients are diagnosed at 65 years or older. However, AD also affects individuals as young as 45 years of age although these cases are relatively rare (Zhu et al., 2015). Individuals diagnosed with AD under the age of 65 are classified as early-onset AD (EOAD). Individuals diagnosed at age 65 or older are considered to have late-onset AD (LOAD). EOAD and LOAD are two primary forms of AD, each having their own distinct clinical progression and cognitive function (Cho, Seo, Kim, et al., 2013; Cho, Seo, Ye, et al., 2013). EOAD, the minor form, is commonly familial, accounting for approximately 6.1% of AD cases but it tends to progress more aggressively and rapidly than LOAD (Chui et al., 1985; Zhu et al., 2015). Previous studies have also characterized EOAD as having a greater diversity in neurological and cognitive impairments compared to LOAD (Cho, Seo, Ye, et al., 2013).While not as aggressive as EOAD, LOAD's progression still involves drastic volume reductions in regions of the brain including the hippocampus and amygdala as well as clinical signs of cognitive decline (Cho, Seo, Ye, et al., 2013). Although AD can be categorized into either early or late-onset, the overall disease pathology has been widely assessed by two hallmarks; the abundance of amyloid deposition within the temporal lobe, anterior cingulate gyrus and parietal operculum and neurofibrillary tangles within the hippocampus, neocortex, and brain stem (Grothe et al., 2017; Henderson & Kerchner, 2018; Singh et al., 2012).

The human brain can be broken down into 3 parts, the brainstem, cerebrum, and cerebellum. Each of these parts play a vital role in making up the entirety of the brain with their unique function. The cerebellum, otherwise known as "little brain", only accounts for 10% of the brain's volume. Despite this low weight contribution, it's neural circuitry is highly dense containing ~80% of the roughly 86 billion neurons in the brain (David et al., 2018; Roostaei et al., 2014). Due to the dense circuitry of tiny tightly packed granule neurons, the cerebellum plays a vital role in motor learning and control as well as the increasing understanding for its contribution to non-motor

cognitive function (Roostaei et al., 2014). On the other hand, the cerebrum accounts for ~80% of the brain's volume but is only comprised of roughly 20% neurons and this is because of its abundance in dendrites, synapses, glial cells, numerous axonal projections and larger neurons (David et al., 2018). The cerebrum is comprised of two hemispheres, an outer layer (gray matter) called the cortex and an inner layer (white matter) both of which work together to support a variety of cognitive function related processes (Jawabri & Sharma, 2022). The cortex can be further sectioned into four lobes, the frontal, parietal, temporal, and occipital lobe. The final part of the brain is the brainstem that structurally connects the cerebrum to the spinal cord and cerebellum and is also comprised of both gray and white matter. The gray matter houses nerve cell bodies and form brainstem nuclei while the white matter contains the axons of nerves navigating to other structures which their corresponding cell bodies originated from different parts of the central nervous system (Basinger & Hogg, 2022). The intricate connections that the brain makes, and its profoundly divided structure have not yet been fully understood or even explored. Its complexity makes it a vital organ in the body to protect because any disruptions can result in a cascade of detrimental effects. For example, disruptions in any of the four lobes of the cerebrum can lead to personality disorders, loss of sensation, memory loss, visual hallucinations and even deafness (Helmstaedter et al., 2018; Huff et al., 2022; Klingner & Witte, 2018; Malloy & Richardson, 1994). Most importantly, disruptions in regions including the thalamus, hippocampus and amygdala have been associated with AD (Son et al., 2017; Zhao et al., 2015). Those regions are a part of the cerebrum, more specifically medial temporal lobes which has been speculated to be involved in semantic memory or conscious long-term memory that can be retrieved after a delay in time (Knierim, 2015; Son et al., 2017). Therefore, it is clear how dysfunctions in these temporal lobes are associated with disorders such as AD that demonstrate clinical signs including memory loss.

AD is typically associated with the accumulation of amyloid beta (Aβ), a proteolytic product of Amyloid Precursor Protein (APP). APP functionally supports the brain by stimulating

synapse formation and neural plasticity (Sabo et al., 2003; Tominaga-Yoshino et al., 2001). The role of APP in AD is thought to be due to altered proteolytic cleavage into amyloid peptides. These smaller peptides, known collectively as amyloid beta (Aβ), are produced through two pathways, the canonical pathway or the amyloidogenic pathway (Figure 1.1). The production of Aβ from APP occurs where α-secretase (composed of TACE and ADAM-10) cleaves APP on residue 16 resulting in a disruption of the Aβ sequence (Lammich et al., 1999; Sisodia, 1992). These smaller peptides can be broken down even further by γ-secretase, where Presenilin 1 and 2 are components of the complex, allowing for clearance from the cell (Nishimura et al., 1999). Through the amyloidogenic pathway, the dominant pathway in neuronal cells, APP cleavage is carried out by β-secretase (BACE) and subsequentially γ-secretase (Laferla et al., 1995; Simons et al., 1996; Tambini et al., 2016). Unlike α-secretase, β-secretase cleaves APP leaving the Aβ sequence intact allowing γ-secretase to cleave at the C-terminal end of the Aβ peptide resulting in multiple isoforms ranging in lengths of 36-43 amino acid (Mawuenyega et al., 2013). These Aβ peptides can accumulate, forming oligomers and ultimately plaques which can disrupt synaptic plasticity and impede communication across the neural network (Walsh et al., 2002). The major isoforms associated with AD that have been detected in human cerebrospinal fluid are Aβ-38, -40, and -42; Aβ-40 is produced in greatest quantities (Portelius et al., 2009; Portelius et al., 2012). While not the major isomer, Aβ-42 has a greater affinity to form aggregates based on measuring turbidity at various time point; this contributes to its neurotoxicity (Iwatsubo et al., 1994; Jarrett et al., 1993). During AD progression, the Aβ-42 isoform becomes more prevalent, destroying the dynamic equilibrium that exists between the intracellular and extracellular accumulation of Aβ (Laferla et al., 1995; Oddo et al., 2006; Wirths et al., 2002). Both intracellular and extracellular accumulation of these peptides contribute to AD pathology as well as accelerate other dysfunctions in neurovascular system such as cerebral β-amyloidosis (Zlokovic, 2008). Evidence has supported that extracellular Aβ has the potential to activate caspases through the cell surface death receptors activation

(RajaRajeswari et al., 2016). Intracellular Aβ is a dominant feature of mitochondrial dysfunction which can, in turn, affect calcium levels, as well as participating in the hyper-phosphorylation of Tau (RajaRajeswari et al., 2016).

The second hallmark of AD is the development of neurofibrillary tangles (NFTs) that are caused by the hyperphosphorylation of neuronal microtubule associated protein (MAP) Tau. Microtubules form the cytoskeleton of cells contributing to their structural morphology. Neuronal microtubules support axons and are primarily responsible for the transportation of supplies such as nutrients and neurotransmitters to different parts of the cell. These microtubules are rich in Tau, a

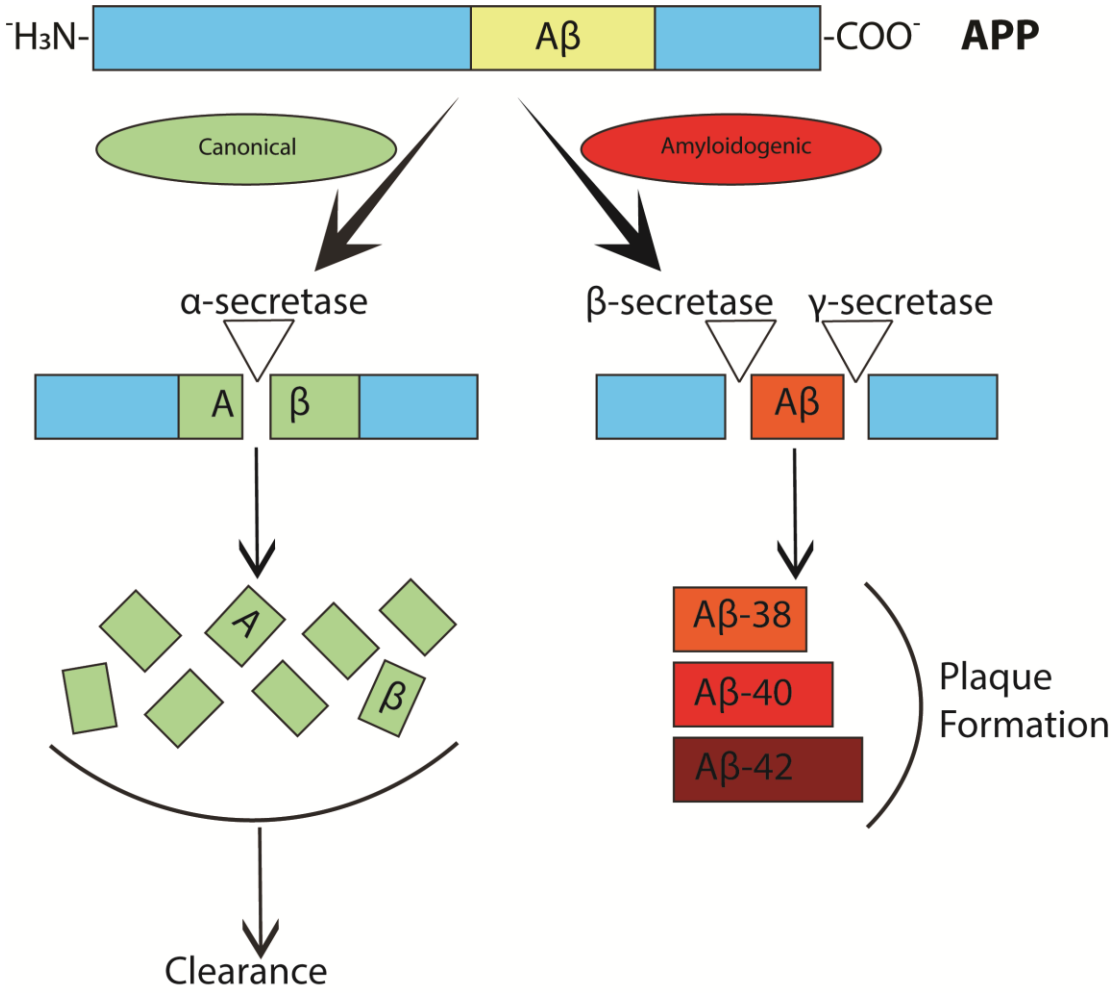


Figure 1.1 Processing of APP via the canonical and the amyloidogenic pathway. Diagram describes the Canonical pathway (left panel) where APP is cleaved by α-secretase into smaller peptides which can be further processed by γ-secretase for clearance by astrocytes and microglia. The amyloidogenic pathway (right panel) describes APP being cleaved by both β- and γ- secretase leaving the amyloid beta sequence intact. In AD, the intact Aβ can be found in 3 isoform residue lengths (38, 40, and 42) and can accumulate to form plaques due to the difficulty of astrocytes and microglia to further break them down.

protein serving as a non-tubulin component essential in the structural assembly (Lindwall & Cole, 1984). Under normal circumstances, Tau contains two or three phosphate groups aiding in its 'glue-like' functionality, however, during AD pathology, hyperphosphorylation can result in up to 9 moles of phosphates for every mole of Tau protein (Alonso et al., 1994). This hyperphosphorylation encourages inhibition of tubulin polymerization and disrupts the interaction of the microtubules with actin filaments (Lindwall & Cole, 1984; Selden & Pollard, 1983). Evidence supports that this abnormal phosphorylation promotes the generation of paired-helical filaments (PHF) through the hyperphosphorylated Tau interacting with itself or other neuronal components (Grundke-Iqbal et al., 1986). PHF are insoluble and therefore tend to accumulate as neurofibrillary tangles within the cell body of neurons. Tau pathology has also been implicated in other neurodegenerative disorders including progressive supranuclear palsy, post-encephalitic parkinsonism, and amyotrophic lateral sclerosis-parkinsonism-dementia complex of Guam (Do Carmo et al., 2021; Feany & Dickson, 1996).

1.3 Risks of Developing AD

While most research has been focused on resolving the rapid degeneration of neurons caused by amyloid plaque accumulation and neurofibrillary tangles, there is a need for research that concentrates on addressing the risks of developing AD. Some epidemiological studies have proposed that the progression from mild cognitive impairment to full onset AD is influenced by several factors including (but not limited to) age, genetics, sex, prior health complications, diet, and the environment (Xue et al., 2017). These risks can be categorized as either controllable, such as diet or environmental contributions, or uncontrollable factors: age and sex. Of the several factors involved, aging presents itself as the leading risk due to its unavoidable nature. Through the extensive exploration of aging, evidence supports its role in causing disruptions in the structural and signaling environment that is necessary for survival, differentiation and proliferation of immature neurons from neural stem cells; this reduction in neurogenesis is the main contributor to

the observed cognitive decline (Lazarov & Tesco, 2016). Impairment of cognitive function can also be affected by reactive oxygen species (ROS) which targets pathways responsible for maintaining neuronal and mitochondrial function, apoptosis as well as inflammation (Delbarba et al., 2016; Leuner et al., 2007; Xu et al., 2015). Although age is the leading cause of the development and progression of AD, more specifically LOAD, this review will focus on the need for more studies that can identify any connectivity these risk factors play in increasing the risk of AD.

1.4 Genetic Risks

Researchers have been interested in finding genetic connections with many well-known diseases and AD is no exception. Literature suggests that genetics play a pivotal role in only the development but also the progression of AD. A large two-stage meta-analysis study of genome-wide association studies, Powell (2013), mentions some of the additional genes found to have high associations with AD based on the genome-wide significance level: *CR1*, *BIN1*, *CD2AP*, *EPHA1*, *CLU, MS4A6A, PICALM, APOE, ABCA7,* and *CD33*. This study discussed the negative and positive impacts of these genes. A reduction in *CD33* and *CLU* expression has been correlated with decreasing the risk of AD by interfering with Aβ peptide accumulation as these genes participate in mediating inflammation (Powell et al., 2013; Stevens et al., 2014). In both mouse primary neurons and human induced pluripotent stem cell derived neurons the presence of Aβ increases intracellular levels of CLU while decreasing its secretion (Killick et al., 2014; Robbins et al., 2018). This effect may likely be the cause of *CLU* contribution to Aβ accumulation as CLU protein secretion has been linked to macrophage infiltration and enhanced phagocytic activity (Weng et al., 2021). Powell's meta-analysis also identified several new gene associations with AD including: *SORL1*, *CASS4,* and *FERMT2, HLA-DRB5-DRB1, INPP5D, MEF2C, PTK2B, CELF1*, and *NME8*. The two hallmarks of AD, Aβ plaque accumulation and neurofibrillary tangles, are reinforced by *SORL1* and *FERMT2* expression, respectively, while suggesting new pathways for AD, including disruption in axonal transport regulated by CELF1 and NME8 (Powell et al., 2013). *INPP5D* also

participates in microglia and myeloid cellular function in addition to gene expression regulation and post-translational modification of proteins (Powell et al., 2013). Mutations in the presenilin 1 and 2 (*PSEN1*, *PSEN2*) genes have also been observed in some AD patients; these genes encode the active components of γ-secretase thus mutations in these genes as well as point mutations in the *APP* gene can alter APP processing, boosting Aβ-42 production and oligomerization (Barucker et al., 2015; Laferla et al., 1995; Tambini et al., 2016; Walsh et al., 2002; Wirths et al., 2002).

Although there are several important genes associated with AD, the strongest, most consistent, and earliest genetic risk factor is Apolipoprotein E (*APOE*) (Powell et al., 2013; Stevens et al., 2014). ApoE is a plasma protein, derived from the *APOE* gene, that participates in cholesterol transportation by binding to ApoE receptors to mediate the uptake of lipoproteins including very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) (Huang et al., 2017; Strittmatter et al., 1993). However, in the presence of injury, APOE production increases proportionally to the increase in APOE mRNA in the brain. Observations show that in AD, ApoE binds to intracellular neurofibrillary tangles and extracellular senile plaques (Strittmatter et al., 1993). There are three genetic variants of *APOE*: *APOE2* (E2), *APOE3* (E3), and *APOE4* (E4) which can be differentiated based on a cysteine-arginine substitution or the quantity of cysteine at positions 112 and 158 (Utermann et al., 1977; Utermann et al., 1980; Weisgraber et al., 1981). Amino acid analysis of the human APOE variants reveal that at these sites the amount of cysteine residues are as follows: the E2 variant has zero, E3 has one, while the E4 variant has 2 residues (Weisgraber et al., 1981). These substitutions alter the charge of APOE which in turn affects how each protein variant of APOE folds (Weisgraber et al., 1981). In fact, this substitution may be linked to the E4 variant increased susceptibility to proteolysis (Rohn, 2013). The differences among the APOE variants suggest their differential contribution to AD. Evidence even supports that the E4 variant has the highest genetic risk for developing AD, E3 is relatively neutral while the E2 is the

protective variant, reducing the risk of developing the disease (Huang et al., 2017; Strittmatter et al., 1993; Zhao et al., 2014).

Worldwide, the E3 variant is the most prevalent with a 77.9% frequency while the prevalence of the E2 and E4 variants are 8.4% and 13.7% of the population respectively (Farrer, 1997). However, the frequency of the E4 variant increases to approximately 40% among individuals diagnosed with AD (Farrer, 1997). Although AD is influenced by numerous different genes, this review will focus on the impact of *APOE*. A recent study explored the mechanisms supporting differences among the three variants. Huang (2017) not only provided evidence of a pathway by which ApoE potentially exacerbates AD, but it also establishes significant differences in the rate at which the ApoE isoforms secrete Aβ-40 and Aβ-42, enhance APP mRNA and protein levels, and activate the mitogen-activated protein (MAP) kinases that are expressed in the brain. As previous literature suggests, E4 is the most potent variant, disrupting the delicate balance of intracellular Aβ accumulation from glia to neurons (Zhao et al., 2014). The E4 variant is not only associated with greater Aβ accumulation, but it is also a factor in Tau-mediated pathology. Depressed neuronal viability and increased tumor-necrosis factor-α (TNF-α) have been observed in Tau-expressing neurons co-cultured with E4-expressing glia (Shi et al., 2017). Shi (2017) also confirmed that the effects observed in the presence of the E4 variant, as it pertains to Tau-mediated neurodegeneration, is independent from observations when assessing Aβ pathology. Both characterizations of AD pathology, Aβ accumulation and neurofibrillary tangles, are enhanced by the presence of the E4 isoform compared to the other isoforms in both in vivo and in vitro models.

Mechanistically, the way in which ApoE participates in enhancing or reducing the risk for developing AD has not been extensively explored. Huang (2017) reported that ApoE is secreted from glia, the supporting cells essential for neuronal survival, and causes an increase in APP and Aβ production through a non-canonical pathway. ApoE firsts binds to surface ApoE receptors which then leads to the activation a series of MAP kinases commencing with DLK/MAP3K12,

MKK7/MAP2K7, and ending with ERK/MAPK. The activation pathway was categorized as a non-canonical since observations show activation of ERK by MKK7/MAP2K7 rather than its typical downstream activation of JNK. ApoE increases the half-life of DLK instead of increasing DLK protein production causing it to persist longer. The activation of MAP kinases leads to phosphorylation of the transcription factor, c-Fos, allowing it to bind to an AP-1 site in the promoter region on the APP gene, resulting in the increased transcription of APP and ultimately Aβ production (Huang et al., 2017). However, this is not the earliest evidence that MAP kinases potentially contribute to AD. In 2001 Ohkubo demonstrated that in primary rat hippocampal neurons, ApoE4 activates the ERK cascade, which in turn stimulates cAMP-response element-binding protein (CREB) transcriptional activity, whereas the E3 variant was unable to do so (Ohkubo et al., 2001). The increased transcriptional activity of genes dependent on CREB include

Bcl2 and c-Fos, and c-Fos plays a key role in upregulating APP transcription (Huang et al., 2017; Ohkubo et al., 2001). In 2004, ApoE receptors, in conjunction with other factors, were shown to modify neurite outgrowth through calcium-dependent MAP kinase pathways that could possibly promote neurodegeneration (Qiu et al., 2004). Multiple studies have pointed to the potential of ApoE to target pathways known to be involved in the progression of AD.

There is evidence showing that ApoE contributes to AD by causing mitochondrial dysfunction (Tambini et al., 2016). This dysfunction as well as the ability for ApoE to target several signaling pathways including MAPK/JNK, ERK/MAPK and Reelin is a result of the lipoprotein bound ApoE binding to cell surface receptors. In primary mouse embryonic cortical neurons, the ApoE variants have been identified to differentially bind to ApoE receptors to inhibit c-Jun N-

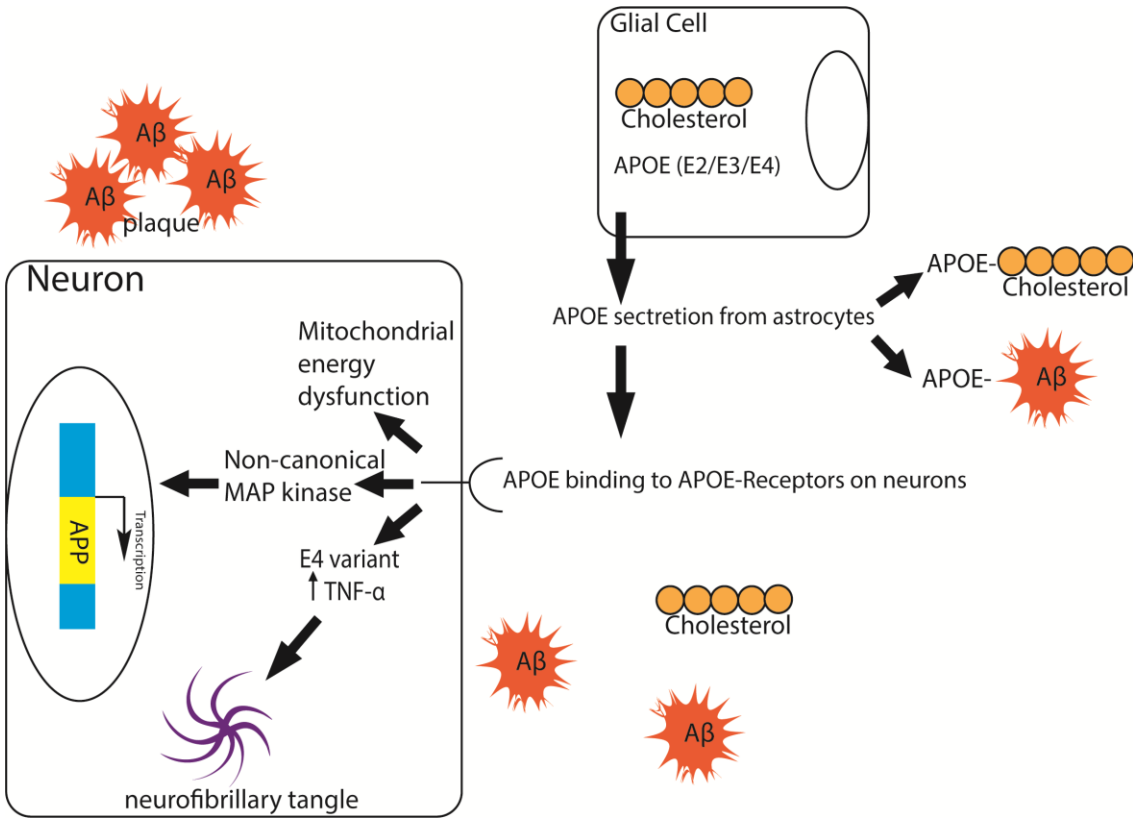


Figure 1.2 Apolipoprotein E Role in AD. Diagram describes the role that APOE plays in AD pathology. APOE secreted from astrocytes can target lipoproteins and aid in their transport and uptake. APOE can also aid in the clearance of amyloid beta to prevent its accumulation and plaque formation. APOE can bind to the APOE receptors located on neurons to initiate various pathways including the non-canonical MAP kinase that induces APP transcription. APOE binding can also lead to mitochondrial dysfunction. Finally, independent of the amyloidogenic pathway, the E4 variant can induce inflammation and lead to neurofibrillary tangles.

terminal kinase 1/2 (JNK1/2), activate the extracellular signal-regulated kinase 1/2 (ERK1/2), and increase the phosphorylation of disabled 1 (Dab1) associated with MAPK/JNK, ERK/MAPK, and Reelin signaling pathways (Hoe et al., 2005). Chang (2005) determined that the lipid-binding region also interacts with the mitochondria, this interaction as well as activation and suppression of key signaling pathways may work together to cause neurotoxicity and mitochondrial dysfunction (Chang et al., 2005; Hoe et al., 2005). Similar to the effects of the variants of APOE as it pertains to Aβ secretion and Tau hyperphosphorylation, the level of mitochondrial dysfunction can also vary depending on the APOE variant encoded, that is, E4 results in more mitochondrial injury (Chang et al., 2005). Subsequent studies showed that presenilin-deficient cells and fibroblasts removed from patients with AD have enhanced mitochondria-associated endoplasmic reticulum membranes (MAM) (Tambini et al., 2016). MAMs are involved in lipid metabolism which includes the catabolism of fatty acids and the production of cholesterol, fatty acids, steroids, and phospholipids by acting as an intracellular signaling hub to allow communication between the endoplasmic reticulum (ER) and the mitochondria (Sala-Vila et al., 2016; Tambini et al., 2016). The Tambini (2016) study also demonstrated variant differences to the risk of AD when a correlation was found that carriers of the E4 variant had higher functioning MAM compared with E3 carriers. These studies imply why APOE genotype has such a significant influence on AD; it not only contributes to the well-studied pathways explored in the disease, but it is also involved in the less explored avenues.

1.5 Sex Differences

AD affects both sexes, but with a higher incidence in females. This is most apparent when males and females above the age of 80 were compared within the US, European and Asian populations (Fratiglioni et al., 1997; Roberts et al., 2014; Ruitenberg et al., 2001; Yoshitake et al., 1995). Additional evidence supports these findings, where before the age of 80, the incidence of AD can be variable, with either sex having a higher incidence or no significant differences exist,

depending on study population's ethnicity, and therefore likely reflects that sub-population's genetic background (Agüero-Torres et al., 1998; Gao et al., 1998; Matthews et al., 2016; Tschanz et al., 2011). There have been multiple potential explanations as to why older females tend to have a higher incidence of developing the disease. However, the increased proportion of females diagnosed with AD may be explained by their increased life expectancy compared to males (Chêne et al., 2015; Hebert et al., 2001; Plassman et al., 2007; Seshadri et al., 1997). However, when other risk factors are considered in conjunction with sex, we can make educated inferences as to why females tend to have higher incidences of AD. Another explanation for this may be the fact that men have an increased risk of developing vascular dementia as opposed to AD when compared to women (Gao et al., 1998). Women also have unavoidable factors that contribute to AD generation such as menopause due to the rapid depletion of estrogen and progesterone, which may be protective earlier in life (Imtiaz, Taipale, et al., 2017). In fact, studies of over 200,000 Finnish women physician prescribed estrogen hormone therapy for more than 10 years revealed a reduction in AD risk (Imtiaz, Taipale, et al., 2017; Imtiaz, Tuppurainen, et al., 2017). Interestingly, of both sexes genotyped for the E4 variant of APOE, women are at an even greater risk. However, independent of sex, the AD risk associated with the E4 variant is dose-dependent; one allele increases the risk by 2-fold and two alleles increases it by 8-10-fold with a 95% confidence interval (CI) of 2.03-3.96 for each additional E4 allele (Corder et al., 1993). As opposed to men who are carriers of one allele of the E4 variant, females having one allele of E4 pose a greater risk of developing AD (Gao et al., 1998; Payami et al., 1996). In fact, evidence suggests that age, genetics, and sex collaborate to alter AD risk; the age of AD onset is approximately 4 years earlier for women who carry just one APOE E4 allele as opposed to males carrying one APOE E4 allele (Bickeböller et al., 1997; Duara et al., 1996). The information gathered indicates several explanations as to why women have an increased risk of AD and supporting evidence suggests that a women's risk of AD, compared to men, can be independent of other contributing factors. Evidence supports a variety of

risks involved in the generation and progression of AD; however, other studies also support the possibility of different synergistic relationships involved in contributing to the disease.

1.6 Environmental Risks

Risk factors of age and sex are unavoidable, however, there are avoidable factors that affect the risk of AD such as an individual's dietary choice and their environment. Epidemiological studies provide evidence suggesting that exposure to metals, medications, solvents, and pesticides pose a risk for AD (Li, Zhou, et al., 2015; Tyas et al., 2001). The heavy metals lead and cadmium have been associated with neurodegeneration (Bakulski et al., 2020). Lead readily crosses the blood brain barrier (BBB) by replacing calcium. Exposure can result in a disruption of metal signaling in the brain leading to oxidative stress and ultimately neuroinflammation (Zhu et al., 2013). Cadmium, on the other hand, does not readily cross the BBB, but it can alter its permeability and promote Aβ accumulation by promoting the amyloidogenic pathway (Li et al., 2012; Notarachille et al., 2014). Studies have also confirmed that middle-aged to older adults regularly taking medications including anticholinergic antidepressants have an increased risk of developing dementia (Coupland et al., 2019). In addition, solvents such as toluene, benzene and alcohols have an increased odds ratio associated with the onset of AD (Kukull et al., 1995). However, this review will focus on the correlation between AD and environmental contaminants such as pesticides.

Pesticides have been used extensively in agriculture leading to direct and indirect exposure to humans. This has revealed some of their adverse effects to humans such as inducing injury to the brain and reproductive organs, or even leading to various types of cancers (Baldi et al., 2011; Parron et al., 2011). Some classes of pesticides of concern include carbamates, organophosphates (OPs), and organochlorides (OCPs). Acute exposure to these pesticides produces short-term effects such as conformational changes to voltage-gated sodium channel isoforms $Na_V1.1$-1.6 and 1.7 by OCPs in the brain and inhibition of cholinesterase, an enzyme involved in breaking down acetylcholine, by both OPs and carbamates (Baldi et al., 2011; Rush et al., 2007). In a study

assessing different concentrations of certain types of OPs (Phorate) and carbamates (Mexacarbate), the mean inhibition potency to cholinesterase was determined to be $1.00 \pm 0.16 \times 10^{8}$ and $1.46 \pm 0.056 \times 10^{7}$ $M^{-1}min^{-1}$ respectively (Singh & Agarwal, 1983). While there are studies that have explored the neurological effects after long-term exposures, Baldi sought to fill this gap by completing a 4–5-year follow-up study with 614 elderly French vineyard workers with occupational exposure to pesticides. Observations revealed that altered cognitive function and increased risk for the progression to dementia was associated with chronic exposure to pesticides (Baldi et al., 2011; Baldi et al., 2003). This observation was in line with an ecological study based in Andalusia (South Spain) with a population of 17,429 patients recorded between 1998 and 2005. Averaged prevalence rates of neurological conditions coincided with the selected area's environmental exposure to pesticides were used to showed that areas with greater pesticide, use had a higher prevalence of AD when compared to areas with lower pesticide use (Parron et al., 2011). Although these studies covered the general category of pesticides, the longevity of OCPs, more specifically dichlorodiphenyltrichloroethane (DDT) and its metabolites, has been linked to

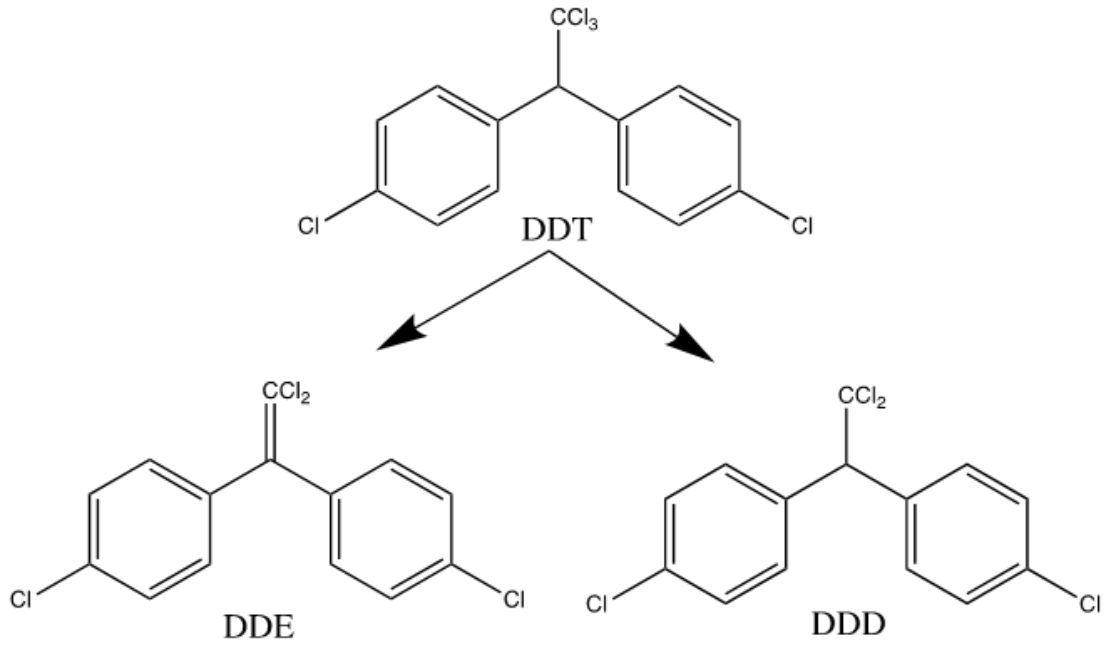


Figure 1.3 Structure of DDT, DDE, and DDD. Visual representation of the structure of DDT (Top) and its metabolites DDE (Bottom Left) and DDD (Bottom Right).

AD and Parkinson's disease (PD) (Fleming et al., 1994; Richardson et al., 2014; Richardson et al., 2009). Other organochlorines β-HCH and dieldrin also have been shown to increase the risk of AD in the north Indian population (Singh et al., 2013). As these compounds are still persistent in the environment and/or continue to be used, their contribution to AD remains that much more relevant.

DDT and its metabolites dichlorodiphenyldichloroethylene (DDE) and dichlorodiphenyldichloroethane (DDD) belong to one of the three classes of OCPs (Hatcher et al., 2008). The discovery of DDT was a substantial milestone in the control of disease-carrying insects (Davies et al., 2007; Läuger et al., 1944; Mellanby & British Crop Protection, 1992; Müller, 1946). It was widely used for many years as it was inexpensive to produce and was effective due to its long half-life between 2-15 years in soil and up to 150 years in an aquatic environment (ATSDR, 2002; Davies et al., 2007). However, due to negative effects on wildlife as well as the unknown adverse effects on humans, a ban on the use of DDT in the U.S. by the EPA began in the early 1970s (Jürgens et al., 2016; Rehwagen, 2006). Negative adverse effects were observed in multiple species of birds and marine animals in the wild as well as in experimental animals during controlled

studies. In the environment, DDE was found to reduce the population size of bald eagles and brown pelican as a result of eggshell thinning while oral dosing of 30 mg/kg/day of DDT revealed tremors in 90 % of the female Osborne-Mendel rats (ATSDR, 2002). However, the potential molecular effects of DDT and its metabolites on brain function have never been studied in humans.

The metabolism of DDT to DDE is favored under aerobic conditions while anaerobic conditions promote the formation of DDD (Jürgens et al., 2016). Fifty years after the EPA ban, DDT and its metabolites can still be found in deep sediments of soil, water, and even in the serum of individuals where it has been associated with increased risk for onset of AD (Jürgens et al., 2016; Richardson et al., 2014). In 2014, DDE levels were determined in existing serum samples from 86 AD patients and 79 control patients from the Emory University Alzheimer's Disease Research Center and the University of Texas Southwestern Medical School's Alzheimer's Disease Center (Richardson et al., 2014). There was a 3.8-fold increase in DDE levels observed in AD patients compared to control individuals revealing DDE association to increased AD risk with an odds ratio of 4.18 (Richardson et al., 2014). DDT remains the most effective deterrent for malaria-carrying mosquitoes, reducing transmission by 90% in ongoing spraying programs in India, making its reintroduction an added source of contamination (WHO, 2006). Therefore, accumulation of these compounds is both persistent and ongoing, partly due to past contamination of soils and other environmental sources, as well as from continued usage of DDT, through bioaccumulation, biomagnification, and transportation across long distances in the upper atmosphere.

DDT was originally thought to selectively target the nervous system of insects until extensive use revealed it could also be toxic to other species. Clinical signs of DDT poisoning can be identified by the presence of tremors, seizures, headaches and nausea (ATSDR, 2002; Davies et al., 2007), all of which could be attributed to effects on the nervous system. The toxic mechanism of DDT is thought to require direct interaction with the voltage-gated sodium channels (Na_v) (Stuart et al., 1987; van der Bercken, 1972). These channels are embedded in the neuronal membrane and

function to allow the influx of sodium ions in response to action potential initiation (Lenaeus et al., 2017). DDT prolongs Na_v opening by decreasing the channel's ability to be closed or inactivated and thereby induces a rapid bursting activity, resulting in reduced ability to repolarize after an action potential, as well as increasing the negative afterpotential, requiring more time to recover before generating another action potential (Chen et al., 2019; Lucas & Renou, 1992; Shrager et al., 1969; van der Bercken, 1972). When studying the electrical property of a neuron exposed to DDT, the rapid bursting phenotype can be identified as the continuous attempt to generate an action potential before the neuron has fully returned back to its resting membrane potential (RMP). This foundational information on where DDT targets and its toxic behavior was heavily based primarily on the studies conducted in insects.

In insects, these properties are what makes DDT an effective pesticide. Moths exposed to 1 mg in 10 µL acetone of DDT onto their abdomen immediately began displaying more active behaviors whereas after three hours tremors and uncoordinated movements were observed (Lucas & Renou, 1992). This coincided with recordings of a single sensillum, a simple sensory receptor, revealed the replacement of a single action potential spike with short intervals of bursts (multiple spikes) in moths treated with DDT and this pattern was more evident when exposure time increased (Lucas & Renou, 1992). A single perfused giant axon dissected from crayfish exposed to 7 µM DDT internally for 7 minutes and externally for 45 minutes (exposure applied to either side of its membrane) resulted in irreversible increases in the negative afterpotential and decreases in impedance; increasing the internal dose caused these effects to occur 3 times faster (Shrager et al., 1969). However, a similar perfusion of DDT onto squid giant axon did not respond as it did in crayfish, where the negative afterpotential was only amplified by a small extent (Narahashi & Haas, 1967; A. M. Shanes, 1949). In crab leg nerves, the height of spikes as well as repetitive activity was enhanced after exposure to 1 part in a million to 10 million parts DDT in sea water or artificial sea water for 30 minutes, however the negative afterpotential was unchanged (Abraham M. Shanes,

1949). In lobster giant axons, exposure to $5x10^{-4}$ M DDT per liter artificial seawater resulted in delayed inhibition of sodium conductance, yielding an increase in potassium conductance, increasing the negative afterpotential (Narahashi & Haas, 1967). Rainbow trout brain synaptosomes treated with 100 µM DDT for 20 minutes confirmed observations seen in squid giant axons; the compound was unable to enhance sodium flux (Stuart et al., 1987). Recordings from myelinated nerve fibers of *Xenopus* clawed toads or *Rana pipiens* frog nerves treated with DDT showed rapid repetitive responses which were more abundant with prolonged exposure time (van der Bercken, 1972) and concentration ranging from 20-195 minutes and 0.1 to 10 mg respectively (Shanes, 1951). Although repetitive responses were observed only in the myelinated sensory fibers when compared to the motor fibers of *Xenopus* laevis, the repetitive firing was consistent and stronger in nerve fibers of *Rana pipiens* (ÅRhem & Fraxkexhaeuser, 1974; ÅRhem et al., 1974; van der Bercken, 1972). These results, although inconsistent, provide evidence that DDT can affect species other than the insects for which it was designed to be selective. Knowing this, we can make a prediction that DDT may also unselectively target human neurons in a similar manner.

Unfortunately, there are limited studies on a direct relationship between DDT and DDE on neuronal function in mammals and none in humans. In rats, it was reported that clinical signs such as tremors directly correlated with the concentration of DDT found in the brain, whether the rats were exposed to small doses over time or to one large dose (ÅRhem & Fraxkexhaeuser, 1974; ÅRhem et al., 1974; Dale et al., 1962). Accumulation of DDT occurs in the brain, kidney, liver, fat and serum, however, only traces of DDE were detected in the serum as the concentrations were well below the limit of experimental detection for the Schechter-Haller method (Dale et al., 1962; Dale et al., 1963). Electrochemically, DDT-exposed sciatic nerve from Purdue-Wister rats show the same repetitive burst as seen in insects, lobster and frogs (Shankland, 1964). DDT suppressed activity-induced gene expression changes in mouse cerebellar granule cells (Imamura et al., 2005). In addition, concentrations of 10 and 100 µM DDE was found to induce a caspase-3-dependent

apoptosis in embryonic neural cells (Wnuk, Rzemieniec, Litwa, Lasoń, et al., 2016). Studies suggest that this toxicity is likely independent of any potential action of Na_v channels, but instead the caspase-3-dependent apoptosis observed post DDE exposure is likely a result of aryl hydrocarbon receptor (AHR), constitutive androgen receptor (CAR) and retinoid X receptor β induced signaling (Wnuk, Rzemieniec, Litwa, Lasoń, et al., 2016; Wnuk et al., 2020). This may be a potential mechanism of developmental neurotoxicity, but it does not explain neurological symptoms in chronic adult exposure.

Some studies have provided evidence that DDT and its metabolites increase the development and progression of AD, but the mechanism by which this potentiation occurs is unclear (Hatcher et al., 2008; Li, Kim, et al., 2015; Richardson et al., 2014). Exposing human neuroglioma (H4) cells to DDT revealed increases in APP and BACE1, but impairs the extracellular degradation and intracellular clearance of Aβ (Li, Kim, et al., 2015). The downstream target genes ATP-binding cassette transporter A1 and insulin-degrading enzyme were also affected, corroborating the variety of biochemical alterations that have been observed in AD patients (Li, Kim, et al., 2015). Furthermore, while chronic bioaccumulation of DDE is likely to be found in exposed individuals, there has been no direct study of DDE action on physiological response in mammalian or human neurons. It is crucial to determine whether these compounds act on human Na_v to determine if current WHO regulatory efforts should be adjusted to minimize exposure and diminish the risk of disease development. This is especially crucial since levels of it and its metabolites are still found in 75-80 % of serum samples (Richardson et al., 2014) which is most likely due to the lipophilic, bioaccumulation, and biomagnification qualities in the environment (Hatcher et al., 2008).

1.7 Combined effect of environmental and genetic risks.

Individually, environmental, and genetic factors have been shown to influence the development of AD. However, there is a lack of research that focuses on how multiple factors can

influence the disease. EOAD has a more distinct genetic trigger, however the most common LOAD is likely due to a combination of several risk factors from all three categories: age, genetics, the environment, and sex (Li, Kim, et al., 2015). Singh (2014) observed that cytochrome P450 2D6*4 (CYP2D6*4) and glutathione *S*-transferase pi1 *B and *C (GSTP1*B and GSTP1*C) polymorphisms in 100 AD patients and 100 control patients between the ages of 50 and 85 from North India, may interact with OCPs, β-HCH, dieldrin, and copper to impact AD risk. Observations revealed that the CYP2D6*4 allele did not show to increase AD risk (Furuno et al., 2001; Singh et al., 2014), however there is evidence that the observation is controversial (Tanaka et al., 1998). On the other hand, GSTP1*B and GSTP1*C allelic frequencies were significantly increased in AD patients with p values 0.009 and 0.011 respectively which is interpreted as both variants having an influence of the AD risk (Singh et al., 2014). In addition, AD patients who also has significantly higher levels of OCPs, β-HCH and dieldrin in their serum were also genotyped to have higher allele frequencies of CYP2D6*4, GSTP1*B, and GSTP1*C (Singh et al., 2014). Using the same study population, Singh also investigated associations between APOE E4 and OCPs and risk of AD diagnosis. Again, by collecting blood samples from these individuals, they were able to observe that AD prevalence was exacerbated in carriers of the E4 variant who also had high levels of β-HCH, dieldrin, and cholesterol in their serum (Singh et al., 2012). The negative association the E4 variant has to AD is further confirmed in a study that was previously discussed. It was a novel discovery to associate high DDE levels in the serum with AD diagnosis, however, a gene-environmental risk relationship was also observed when more cognitive impairment was prevalent in AD patients who were also carrying APOE E4 (Richardson et al., 2014). There is evidence supporting that the risk for LOAD is associated with genetic and environmental factors, although further studies are necessary.

1.8 Model systems

The development of animal models for AD has led to an increase in learning about its pathophysiology. When developing a disease model system, the organism of choice is a non-human species that has previously been well studied in order to understand its biology and can provide insight to the disease in more complex organism. These model systems can pave the way for discovering mechanistic pathways involved in the disease pathology as well as explore underlying causes and potential treatments that cannot be tested in humans for ethical reasons. However, despite some genetic similarities, some species including rats and mice need genomic manipulation in order to recapitulate phenotypes and symptoms similar to human genetic diseases but this effort can be expensive and time intensive. To address this limitation, some invertebrate models including *Drosophila melanogaster* and *Danio rerio* have been developed. The *Drosophila melanogaster* also known as the fruit fly, has been used to evaluate the metabolism of APP by assessing the involvement of Tau, localization of Aβ, and the modulation of BACE1 by using the GAL4-UAS system to generate transgenic flies with the human Tau or APP and BACE1 (Finelli et al., 2004; Greeve et al., 2004; Shulman & Feany, 2003). Evidence show that these transgenic flies are advantageous at providing new information on the two hallmarks of AD. Overexpression of Tau and induction of glycogen synthase kinase (GSK3β) induce intracellular inclusions that resemble neurofibrillary tangles while transgenic APP and BACE1 flies show Aβ accumulation is localized in the endoplasmic reticulum (ER), lysosomes and Golgi apparatus (Iijima et al., 2008; Jackson et al., 2002). Zebrafish (*Danio rerio*) has also been used to evaluate neurofibrillary tangles and Aβ plaque accumulation due to the presence of the orthologous human genes in its genome (Chen et al., 2009; Groth et al., 2002; Leimer et al., 1999; Musa et al., 2001). In fact, the primary structure of PSEN1 revealed conservation in zebrafish when compared to the human form (Leimer et al., 1999). The wild-type zebrafish PSEN1 promotes the generation of toxic Aβ in a similar manner to human familial AD (FAD) PSEN1 mutations (Leimer et al., 1999). These invertebrate models

provide a more cost-effective and timely approach to exploring AD in preparation for using a more complex system more closely related to humans.

The discovery of genes that are associated with AD have impacted the generation of many of these animal models, this holds true when considering more complex animal systems. Mice have been utilized to address potential mechanisms involved in AD pathogenesis as they have an even higher degree of genetic conservation with humans as it relates to the structure and function of the medial temporal lobe including the hippocampus (Waterston et al., 2002). The most common AD mouse model is the 5XFAD. This mouse line possesses 5 genetic mutations associated with AD three of which are the Swedish, London (V7171), and Florida (I716V) in the APP gene and two mutations in the PSEN1 gene, notably, M146L and L286V (Oakley et al., 2006). These mice have been shown to have cognitive impairment, high level expression of Aβ-42 and experience neuronal loss around four months (Oakley et al., 2006). When considering an AD model designed around a polymorphic gene such as APOE, it becomes a bit more challenging. While the mouse apoE4 has the corresponding arginine at the 112 position, the protein is not structurally or functionally conservative to humans due to the differences in the rest of the sequence (Raffai et al., 2001). As a result, human apoE targeted-replacement (TR) mice were utilized where arginine at position 61 was incorporated into the genome of the mouse by microinjecting the gene-targeting vector; this would result in an equivalent APOE E4 domain (Grootendorst et al., 2005; Raffai et al., 2001). Observations reveal that the apoE4-mice show more synaptic and cognitive impairment compared to the apoE3-mice (Grootendorst et al., 2005; Raffai et al., 2001; Trommer et al., 2004). These model systems have been developed based on mutations in an autosomal dominant gene or based on the presence of a polymorphism that increases AD risk. However more recently, these components were combined to generate FAD/APOE-Tg models. In these models, the E4 variant has been shown to increase Aβ associated dysfunctions within insulin signaling (Chan et al., 2016). Researchers have also explored using *in vitro* model systems in 2D and 3D based on induced

pluripotent stem cells (iPSCs) with similar genetic mutations to address the inability for the mouse to elicit some of the important pathological components such as high generation of toxic amyloid beta and neurofibrillary tangles (Choi et al., 2014; Paquet et al., 2016). These models systems have allowed for a broad scope and extensive exploration of AD pathology.

1.9 Conclusion

Age, genetics, sex, and environmental factors all play pivotal, independent, and synergistic roles in the development and progression of AD. Age, the strongest risk factor for the disease, defines the major difference between the two forms of AD: EOAD and LOAD. EOAD is normally thought to be caused by genetic variation, while the major form, LOAD, is associated with more of a combination of the general risk factors associated with AD. Although there is extensive research on genetic variations that contribute to the disease there needs to be more work done on environmental factors because, unlike genetics, our exposure to toxicants can be limited. In addition to studies on environmental factors, supplementary mechanistic data needs to be provided on the interaction of the environmental and genetic contributions. More specifically, there is a need for studies that focus on the association between APOE, the main genetic contributor, and DDT, a prevalent environmental concern.

CHAPTER 2: GENERATION AND VALIDATION OF APOE VARIANTS IN INDUCED PLURIPOTENT STEM CELLS AND NEURAL LINEAGE CELLS USING CRISPR CAS9 AND LENTIVIRAL TRANSDUCTION

2.1 Abstract

Studies have revealed the importance of Apolipoprotein E (APOE) and its role in altering the risk of developing pathologies such as Alzheimer Disease (AD). The variants of the gene, E2, E3, and E4, all have been shown to independently alter its coded proteins' role in various disease states. The most common variant, E3, is considered to be neutral in developing AD whereas E4 has been deemed the strongest genetic risk factor for AD; E2 functions as a protector. While an APOE genotype in an individual is defined by the variant(s) identified in both alleles of the gene, evidence revealed that one allele of the E4 variant increases the risk of AD by two- to three-fold (Corder et al., 1993). To properly study these variants individually and collectively, the genome editing tool CRISPR/Cas9 and lentiviral transduction were utilized. Using CRISPR/Cas9, we generated hemizygous E3 and E4 induced pluripotent stem cells (iPSCs) from a heterozygous E3/E4 line via non-homologous end joining (NHEJ) of frameshift deletions. We constructed multiple lentiviruses expressing E3 or E4 APOE using inducible or tissue-specific promoters and fluorescent markers or drug resistance. Lentiviral transduction allowed us to reintroduce any of the individual variants of APOE to the knockout APOE null (APOE -/-) iPSC line, induced neurons (iNs), and induced astrocytes (iAs) lines. Relevant protein expression of the gene was apparent in transduced APOE E2, E3, or E4 HEK 293 cells when they revealed both intracellular and extracellular differences in APOE quantities when compared to untransfected HEK cells. The stable cell types that are carriers of the major variants of APOE give us the ability to distinguish and study their individual effects. Being able to identify the individual role of specific variants in disease development will aid in how to establish populations that are more susceptible to these disorders as well as how to approach mechanistic interventions.

2.2 Introduction

Apolipoprotein E (APOE) plays a vital role in transporting and mediating the uptake of lipoproteins including very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) in the blood (Mendivil et al., 2013). In more general terms, ApoE maintains the homeostasis of cholesterol and other lipids within the brain. With the discovery of the E4 variant of APOE being the strongest risk factor of the development of AD, it became crucial for researchers to explore the involvement of cholesterol in the disease (Corder et al., 1993; Strittmatter et al., 1993). In fact, human brain tissue samples (the middle frontal gyrus and cerebellum) collected from confirmed AD patients via autopsy as well as cerebral cortical tissue from C57BL/6 mice revealed accumulation of cholesterol and long-chain ceramides (Cutler et al., 2004). Another function of ApoE is its ability to clear amyloid beta from the central nervous system(Mendivil et al., 2013; Verghese et al., 2013). In AD, ApoE has been shown to bind to extracellular senile plaques as well as the intracellular neurofibrillary tangles (Strittmatter et al., 1993). As a result of its wide functionality, APOE partakes in the mechanism of many different disorders such as Alzheimer Disease (AD). An APOE genotype is defined by the variant identified in both alleles of the gene. There are three common variants of APOE, each independently altering the proteins' major functional role as it pertains to disease states (Verghese et al., 2013). Considering the relationship between APOE and AD, the E2 variant has been observed to reduce both the amyloid pathology and cognitive impairment while having a limited effect on Tau pathology (Goldberg et al., 2020; Morris et al., 2010; Shinohara et al., 2016). The E3 variant is the most common form, appearing in over 70 % of the population and is considered to be neutral in the risk for developing AD (Corder et al., 1994; Goldberg et al., 2020). The E4 variant of APOE has been deemed the strongest genetic factor for developing AD by driving the transcription of amyloid precursor protein (APP) (Huang et al., 2017; Strittmatter et al., 1993). The risk associated with developing AD is not only affected by the presence of the E4 variant, but it is also dose-dependent; two alleles of E4 increase AD risk

by 8-10-fold (Corder et al., 1993). Due to the individual contributions of specific variants to the risk of developing AD, it is important that there are different methods and model systems for studying underlying mechanisms responsible for altered susceptibility to pathogenesis.

Genome editing, independent of the technique used, is a novel method developed to enhance the designs and approach of preclinical studies. As observations show that genetics play a crucial role in disease development and/or progression, the technologies developed to target and regulate the expression of individual genes have provided understanding of disease pathogenesis. There are multiple ways to effectively target and regulate the expression of individual genes. For the purposes of this study, the focus will be on Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) - Cas9 and Lentiviral Transduction.

CRISPR-Cas9 is a RNA-guided nuclease system that is more specific, efficient and has a more user-friendly design when compared with other editing technologies, one being Zinc-finger nucleases (ZFNs) (Nelson et al., 2016; Ran et al., 2013). Mechanistically, a small twenty nucleotide long guide RNA (gRNA) is designed to target a specific site on the DNA corresponding to the gene of interest (Mali et al., 2013; Sapranauskas et al., 2011). The Cas9 protein is guided by the gRNA by forming a complex which targets the region of DNA associated with the gene of interest (Garneau et al., 2010; Gasiunas et al., 2012; Liang et al., 2015). Once at the recognition site, the Cas9 nuclease catalyzes a double stranded break (DSB) on the DNA to initiate genomic DNA repair mechanisms (Gasiunas et al., 2012). Homologous Recombination (HR) and Non-Homologous End Joining (NHEJ) are two major DSB repair pathways; both of which can be used to achieve the desired outcome. HR is considered the alternative DNA repair mechanism due to its occurrence rate and its activity being primarily in dividing cells when compared to NHEJ (Saleh-Gohari & Helleday, 2004). This mechanism requires a template from an exogenous source where it can be a single-stranded DNA oligonucleotide (ssODNs) or a double-stranded DNA (dsDNA) with homology donor of the insertion sequence (Jinek et al., 2013; Kanca et al., 2019). Unfortunately,

its efficiency is dependent on the template as well as the state and type of cell the repair is required (Saleh-Gohari & Helleday, 2004). On the other hand, NHEJ is an error-prone DNA repair mechanism that occurs majority of the time. This is because NHEJ takes precedence in the absence of a template (Jinek et al., 2013). The errors are developed from the insertions/deletions (in/dels) that form during the ligation process. However, this can be beneficial when trying to knockdown a gene as these in/dels can result in frameshift mutations and premature stop codons (Perez et al., 2008). The multicomponent system allows for the efficiency in targeting and editing genes of interest.

The capacity to alter the expression of individual genes using lentiviral transduction has been extensively explored (Durand & Cimarelli, 2011). Lentiviral transduction uses a viral vector to deliver viral RNA which is then reverse-transcribed into DNA and incorporated into the genome, altering the expression of the targeted genes within a cell (Kafri et al., 2000; Li et al., 2010). This system works in both dividing and non-dividing cells as well as having the added benefit of maintaining said altered gene expression (Kafri et al., 2000). The system allows us to not only to target different genes of interests but to also change multiple components involved with altering gene expression such as the transcriptional activators/promoters and co-expressors, for example a drug-resistant gene. This quickly allows the system to become a powerful tool. We sought to develop stable variations of the APOE genotype in multiple cell lines using these effective genetic editing technologies. The ability to distinguish each variant of APOE in different cell types offers the opportunity to study their individual effects on the diseases the gene plays a major role in such as AD.

2.3 Material and Methods

Materials

Induced Pluripotent Stem Cells (iPSC) were maintained in mTeSR™ 1 medium (STEMCELL Technologies; 05850). Before use, 6-well sterile culture dishes were coated with Matrigel

(Corning® Matrigel® hESC-Qualified Matrix; 354277) and incubated (5 % O_2, 5 % CO_2, 37 °C) for at least 45 minutes or overnight. Cells were maintained to until they reached approximately 80 % confluency prior to weekly passage or single cell retrieval. Cells were split once a week using ReLeSR™ (STEMCELL Technologies; 05872) or Dispase (BD Biosciences; 354235) to preserve healthy colony formation by minimizing spontaneous differentiation. To obtain single cells, iPSCs were rinsed with phosphate-buffered saline (PBS) before incubating with 1 mL ACCUTASE™ (STEMCELL Technologies; 07920) for 5 minutes. PBS was used to inactivate ACCUTASE activity by diluting the cell mixture. Single cells were then collected, counted and prepared for the appropriate application. In order to prevent apoptosis after single cell collection, 5 µM Y27632 (y-compound, Tocris; 1254) was added to mTeSR™ 1 medium.

Generation, Maintenance and Validation of Induced Pluripotent Stem Cell (iPSC) Lines

The APOE positive (heterozygous E3/E4) and the APOE negative (homozygous deleted: APOE -/-) induced pluripotent stem cell (iPSC) lines were obtained from the Dr. Xianmin Zeng (XCell Science, Inc.). During initial Western blot assessment of the APOE -/- cells, there was evidence of ApoE production. We intended to distinguish APOE -/- cells from contaminated positive expressing cells to create an efficient negative control.

To purify the APOE -/- line, approximately $1x10^6$ cells were collected. Fluorescence-activated cell sorting (FACS) was used to distribute one cell per well into a 96 well plate containing mouse embryonic fibroblast (MEFs) and maintained in KSR (20% Knockout Serum Replacement, 1X NEAA, 1X GlutaMAX™ Supplement, 1X beta mercaptoethanol, 10 ng/mL bFGF, in DMEM/F12) medium for 1 week, refreshing the media after 3 days. Once colonies had formed in each well from the single cell (approximately one week), colonies were picked using a plunger connected to a modified glass capillary tube. A glass capillary was heated in the center, stretched and cut using sharp glass cutters; both ends were then heated to round out the edges to prevent the plate from

being scratched. Twenty colonies were selected and plated on a Matrigel-coated 96 well plate and maintained in mTeSR™ 1 medium supplemented with y-compound until all cells were 70-80 percent confluent. Once confluent, cells were treated with 30 µL ACCUTASE™ for 5 mins followed by 100 µL of mTeSR to stop the proteolytic activity of ACCUTASE and transferred to a 96 well round bottom plate and spun to collect the cell pellet. 100 µL of the supernatant was removed and cells were resuspended in 100 µL mTeSR supplemented with y-compound and plated onto two Matrigel-coated plates at different densities (plate A = 100 µL and plate B = 20 µL). Due to the size of the wells and to reduce the risk of losing material, cells were not counted; cell estimation was based on cell suspension volume added. Once confluent, plate A was lysed in Quickextract (Lucigen, catalog number QE09050), heated to 68°C for 6 minutes followed by 98°C for 2 minutes. Lysates were then PCR amplified using primers DET3-F [5'-GACGAGACCATGAAGGAGTTGA -3'] and DET3-R [5'- CGAACCAGCTCTTGAGGCG -3'], treated with exoSAPIT (Thermo Fisher, catalog number 75001.4X.1.ML) for purification and sent for sequencing with the F primer. APOE -/- candidates were validated further with immunofluorescence via IN Cell Analyzer 6000 and Western Blotting was used. Of the 20 colonies expanded, 7 were selected as clonal APOE -/- lines.

The hemizygous lines (E3/- and E4/-) were a result of Non-Homologous End Joining (NHEJ) from the CRISPR Cas9 system to target either the E3 or E4 allele of APOE in the heterozygous E3/E4 iPSC line. Three million E3/E4 iPSC were electroporated (amaxa Nucleofector) in suspension with a mixture of 2 µg Cas9 protein, 200 ng of gRNA (to target the E3 allele C9 major gRNA was used and the E4 allele was targeted using C9 minor gRNA), and 0.4 µg of GFP plasmid. Cells were added to Matrigel-coated 24 well plates and incubated overnight (5 % O_2, 5 % CO_2, 37 °C). On the following day, GFP expression was checked and confirmed before collecting cells for FACS sorting. GFP-positive cells, per condition, were co-cultured with MEFs on 6 well plates at 3 different densities (1k, 2k and 5k) and maintained in KSR medium. Culture maintenance, procedure

for picking and screening colonies were carried out similarly to purifying the APOE null cell line. For both conditions 72 colonies were selected, the best hemizygous line was then chosen and expanded after sequence verification. All FACS sorting were carried out by the EOHSI Core Facility at Rutgers University.

Differentiation of Induced Neurons from iPSC

To collect single cells for glutamatergic neuronal differentiation, iPSCs were treated with ACCUTASE™ (STEMCELL Technologies; 07920) and infected in suspension with lentivirses containing: the doxycycline-inducible neurogenin 2 (Ngn2) co-expressed with the drug resistance gene puromycin, the reverse tetracycline-controlled transactivator (rtTA), 5 µM Y27632 (y-compound, Tocris; 1254) and 2 ng/µL of doxycycline (Pang et al., 2011; Vierbuchen et al., 2010; Zhang et al., 2013). Transduced cells were selected by addition of 1 µg/ml puromycin from days 2 to 4 following infection. At day-five post infection, the induced neurons (iNs) were ACCUTASE™-treated, plated on the mouse glia and treated with a final concentration of 4 µM cytosine arabinoside (AraC; Sigma-Aldrich; C1768) on day 8 to inhibit cell division in the glial cells. Co-cultures were then maintained with half-media changes of Neurobasal Plus Medium plus B27+ (NB2) (Thermo Fisher Scientific: catalog number A3653401) every 4-5 days until neuronal maturity; approximately 36 days post lentiviral infection. Electrophysiology was used to assess maturity by measuring the Resting Membrane Potential (RMP) and spontaneous glutamate release (sEPSCs).

Differentiation of Induced Astrocytes from iPSC

To collect single cells for astrocyte differentiation, iPSCs were treated with ACCUTASE™ (STEMCELL Technologies; 07920) and infected in suspension with lentiviruses containing: the doxycycline inducible SRY-Box Transcription Factor 9 (Sox9) co-expressed with the drug

resistance gene puromycin, the doxycycline-inducible Nuclear Factor I B (Nfib) co-expressed with the drug resistance gene hygromycin, the reverse tetracycline-controlled transactivator (rtTA), 5 µM Y27632 (y-compound, Tocris; 1254) and 2.5 ng/µL of doxycycline. The early stages of differentiation was adopted until days 12-16 when the cells and media were harvested (Canals et al., 2018).

Western Blot

To screen APOE null candidates, cells were lysed using RIPA lysis buffer containing 50 mM Tris HCl, 150 mM NaCl, 1.0% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 1.0 mM EDTA, 0.1% (w/v) SDS, 0.01% (w/v) sodium azide at a pH of 7.4 and 1X HALT protease inhibitor (Thermo Fisher; 78430). To collect extracellular proteins, induced astrocytes were maintained in Optimem 1X (Gibco; 31985-070) for 24 hrs prior to media collection and concentrated using a Amicon® Ultra-15 Centrifugal Filter Unit (MilliporeSigma; UFC901024). The Pierce™ BCA Protein Assay Kit (Thermo Fisher; 23225) and a spectrophotometer set to 562 nm were utilized to quantify protein concentrations. Protein (30 µg intracellular and 2.5 µg extracellular) were loaded onto a SDS-polyacrylamide 4-12% Bis-Tris gel (Life Technologies) and resolved by electrophoresis. Protein bands were transferred for 8 minutes onto polyvinylidene fluoride membranes using the Invitrogen iBlot Dry Blotting System. Once transferred, membranes were stained using the Revert™ 700 Total Protein stain and wash solution kit (LI-COR Biosciences; 926-11010) to reveal each lane's total protein content; staining was then stripped using the kit's reversal solution prior to blocking. Membranes were blocked in 5% non-fat milk in 0.5% Tween 20-PBS for 1 hr. before incubating them with primary antibodies overnight at 4°C. All primary antibodies were diluted in 5% non-fat milk in 0.5% Tween 20-PBS. Species-appropriate secondary antibodies (LI-COR Biosciences; 926-68071 & 926-32210) were used to probe the primary antibodies. Li-Cor Odyssey FC was used to detect and quantify protein signals.

Immunocytochemistry

Cells maintained on Matrigel-coated glass coverslips were fixed with 4% Paraformaldehyde (PFA) for 15 mins at room temperature and washed three times with phosphate-buffered saline (PBS) for 10 mins. Fixed cells were then permeabilized using the blocking buffer (0.1 % Triton X-100 and 4 % normal goat serum in PBS) for 1 hr at room temperature. Blocking buffer was then replaced by the primary antibodies diluted in the blocking buffer and incubted bind overnight at 4 °C. Prior to incubating the coverslips for 2 hrs with the appropriate Alexa Fluor secondary antibodies and Hoechst 33342 (Thermo Fisher; H3570) diluted in the blocking buffer, they were washed three times with immune-wash buffer (0.05 % Triton X-100 in PBS) for 10 mins. Coverslips were mounted onto glass slides using mounting medium in preparation for confocal imaging using a Zeiss LSM700.

t-SNE Analysis in R

Data from our single-cell RNA sequencing (scRNAseq) experiment (alcohol reverses effects of KCNJ6 variants on neurons) was reanalyzed as they contain information using the same types of cultures. RStudio (RStudio Software Company, Boston, MA) with the installed Rtsne and ggplot2 packages were used to generated t-distributed stochastic neighbor embedding (tSNE) plots to visualize patterns and trends in the data of cells mapped by distinct clusters of cell types coordinately expressing gene expression. Separate plots were generated to visual APP and APOE within the cell type clusters; presence of color indicate positive expression.

2.4 Results

To generate stable hemizygous APOE E3/- and E4/- iPSC lines, the CRISPR/Cas9 genome editing tool was used to excise either the E4 or E3 allele, respectively, from the heterozygous E3/E4 genotype. Candidate cell cultures exhibiting evidence of a deletion were isolated and confirmed by inserting the PCR amplified region of APOE ((5'-CGGACATGGAGGACGTGCG-3') targeted by the Cas9 protein into StrataClone blunt PCR cloning vector and then picking individual bacterial colonies to separate the two alleles (Figure 2.1). The amplified APOE region overlaps the rs429358 region that distinguishes E3 from E4 by the base change from thymine (T) to cytosine (C) respectively. Sequencing of these colonies identified iPSC clones having one unedited allele and

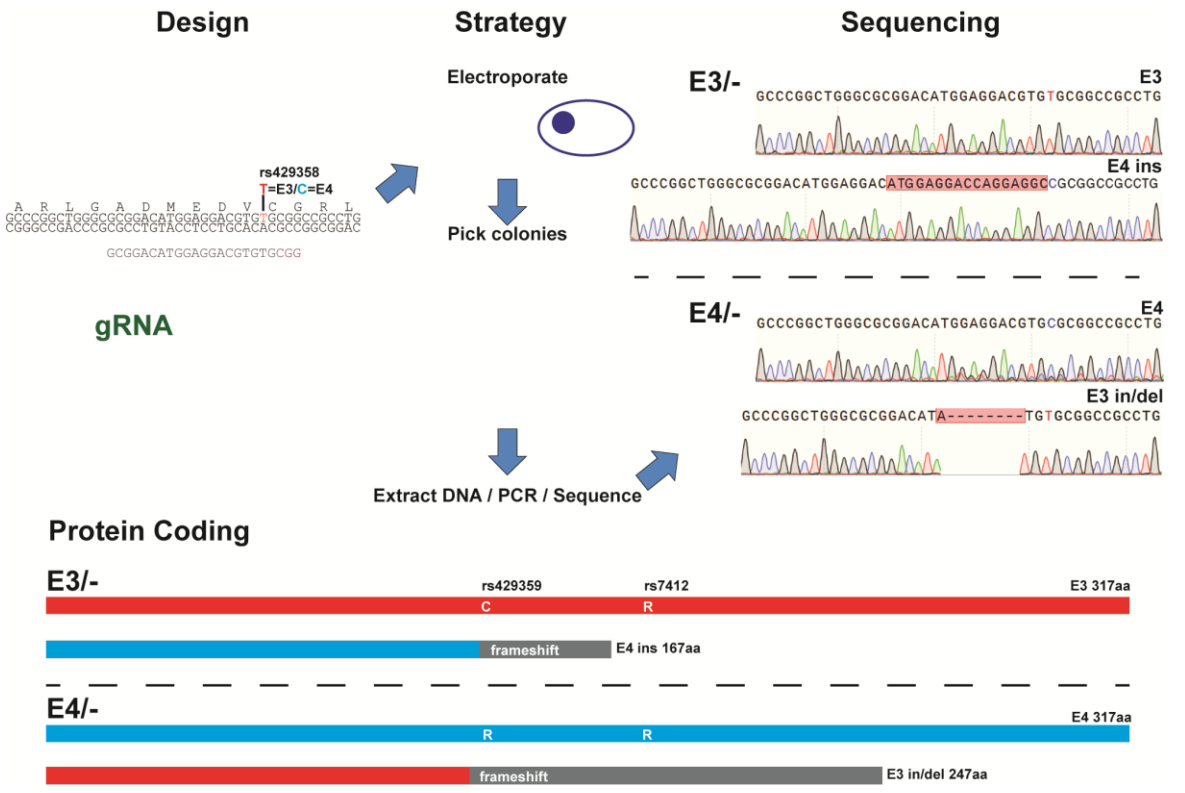


Figure 2.1 Strategy for CRISPR/Cas9 editing of APOE. A gRNA targeting sequence was identified overlapping rs429358, which distinguishes E3 (T) from E4 (C). Two versions were prepared, named C9 or C9min (5'-CGGACATGGAGGACGTGCG-3'; not shown) to target E3 or E4, respectively. In vitro transcribed C9maj or C9min were mixed with recombinant Cas9 protein and a GFP-expressing plasmid before electroporating into iPSC in suspension. One day later, GFP+ iPSC cultures were dissociated and plated at low density onto irradiated mouse embryonic fibroblasts. After one week, colonies were visualized and picked by hand into 96 well plates. After these cultures expanded, they were split and one replicate plate was harvested for genomic DNA. PCR amplified fragments containing the chosen mutation site were screened by sequencing and confirmed by cloning into plasmids for sequencing isolated alleles. For the two selected iPSC lines, one is E3/- (containing an unedited E3 allele and an E4 allele with a 17 bp insertion replacing a 4 bp deletion) and the other is E4/- (containing unedited E4 and an E3 allele with a single A residue replacing a 8 bp deletion). The inserted/deleted alleles predict the coding of a frameshifted protein sequence (shown in grey) leading to premature termination.

one with a frameshift insertion or deletion that would produce frameshifted and truncated protein, yielding either E3/- or E4/- iPSC clones. These iPSC lines provided the basis for determining the effect of each variant of APOE in neurons and astrocytes.

We obtained an APOE-/- knockout iPSC line from Dr. Zeng and we aimed to confirm that it did not synthesize APOE protein. Experiments carried out in the APOE -/- iNs differentiated from the iPSC line received were assessed using Western blot and revealed positive expression for ApoE (Figure 2.2A). This observation led us to believe that our APOE -/- line was contaminated with APOE positive cells. Fluorescence-activated cell sorting (FACS) to purify the null cells was conducted after suspecting APOE positive cells contamination. Isolated cells were then expanded, and a portion was lysed to detect APOE protein by Western blotting. The various APOE null candidates were compared to the heterozygous E3/E4 iPSC line revealing that all candidates produced no ApoE (Figure 2.2B). However, some candidate lines, such as APOE -/- B and F, produced less APOE when compared

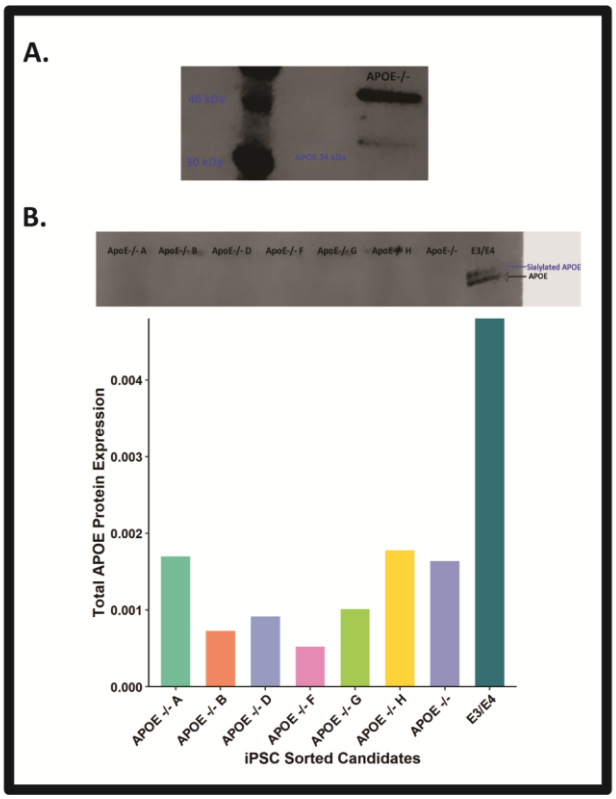


Figure 2.2 Isolation of APOE null iPSC candidates. APOE null candidates were identified from the APOE positive contaminated cells FACS sorted. Western blotting was used to assess the APOE protein quantity in each potential candidate and compared to the heterozygous APOE E3/E4 iPSC line. All candidates lack APOE, shown as the absent APOE band. Total APOE protein for each sample was quantified and normalized to its total protein detected in the lane (not shown). Western blot presented as a representative of two technical replicates.

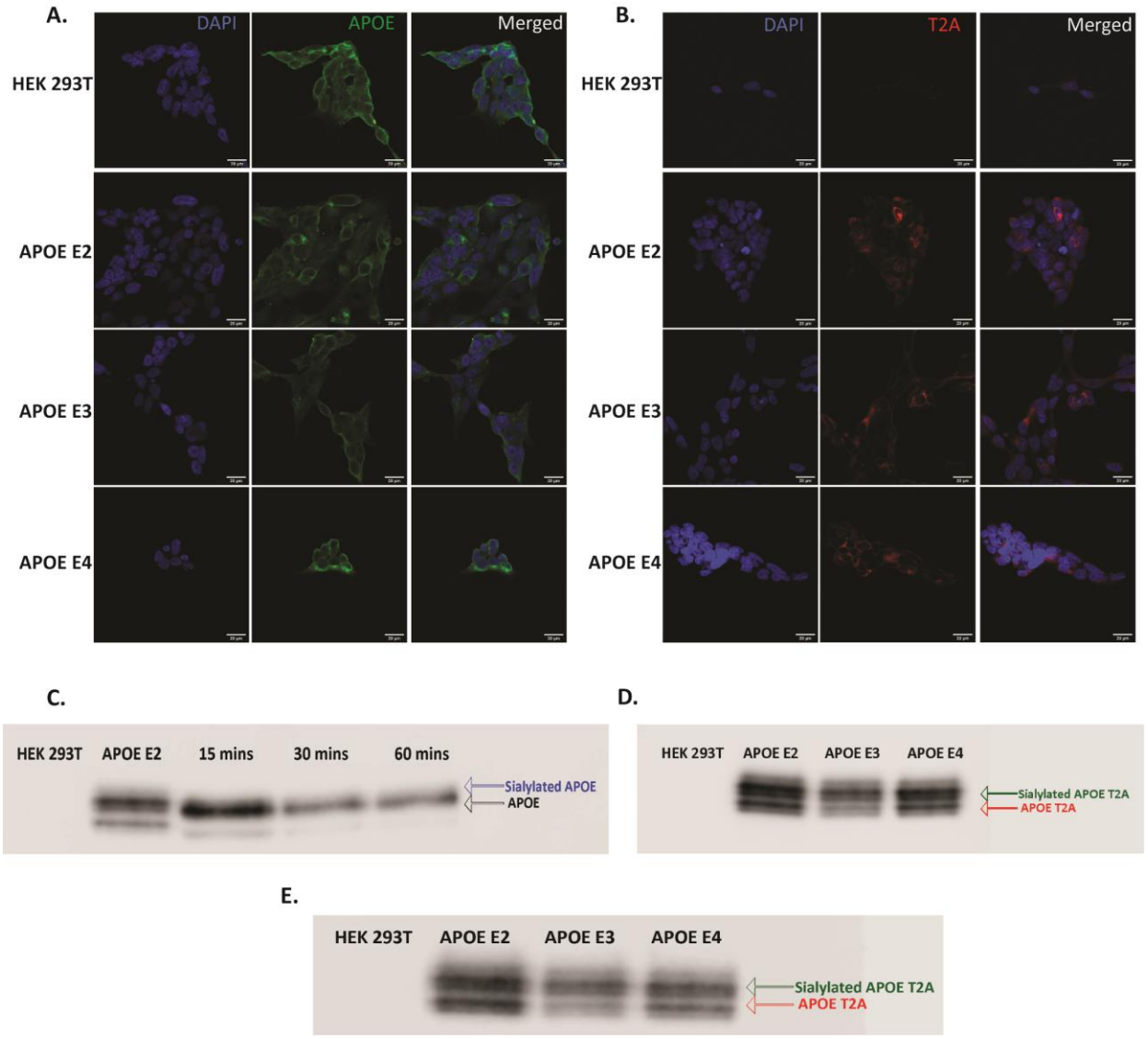


Figure 2.3 Intracellular and Extracellular APOE expression from HEK293 Cells Overexpressing E2, E3, or E4. (A-B) Representative images of intracellular APOE expression in APOE-transduced HEK 293T cells: HEK 293T, APOE E2 Transfected HEK 293T, APOE E3 Transfected HEK 293T, and APOE E4 Transfected HEK 293T expressing APOE (green), or T2A (red) in addition to the positive nuclei detected by Hoechst (blue). (C-E) Media secreted from non-Transfected and Transfected FUGW-APOE-Puro^{R} (APOE E2, E3, or E4) HEK 293T cells were collected and concentrated using 10 kDa molecular weight cut-off Centricon filters. APOE protein was then detected by APOE Recombinant Rabbit Monoclonal Antibody (D) and T2A (E) via Western Blot analysis. Uninfected HEK293 cells served as negative control. Images presented as a representative of five field views. Western blot presented as a representative of three technical replicates.

to the unsorted. The variation of APOE being produced in the selected candidates compared to APOE -/- could be a result of alternative splicing or translational reinitiation. Previous studies have observed that knockout lines generated using the CRISPR-Cas9 system tend to start re-expressing the targeted gene defined as the identification of residual protein expression (Makino et al., 2016; Smits et al., 2019). Cells that continue to divide starts to compensate for the lack of protein by

inducing exon skipping and translational reinitiation (Smits et al., 2019). As these cells are still actively replicating, this is a strong possibility contributing to the variability.

To assess extracellular APOE, we utilized human embryonic kidney cells (HEK 293T) transfected with the constitutive lentivirus containing one of the APOE variants. The synthesis of ApoE begins in the endoplasmic reticulum from where it is transported to and post-translationally modified in the Golgi apparatus; the mechanism does not deviate from the canonical secretory pathway (Lanfranco et al., 2021). Multiple *O*-linked oligosaccharide ApoE modifications have been found in cerebrospinal fluid (CSF) with as little as zero and no more than two sialic acid residues bound (Flowers et al., 2019; Lanfranco et al., 2021; Lee et al., 2010). Intracellular and extracellular APOE was detected from transfected FUGW-APOE-PuroR (APOE E2, E3, or E4) HEK 293T cells. As a result of the presence of the fused, self-cleaving peptide T2A, detection of APOE was carried out by both APOE (Figure 2.3A) and T2A (Figure 2.3B) antibodies. Extracellular APOE was detected as either unmodified or modified with the sialic acid group, each tagged with T2A. Transfected HEK APOE E2 cells treated with neuraminidase resulted in the removal of the sialic acid group converting the APOE detected to the unmodified T2A tagged form (Figure 2.3C). This supports the conclusion that APOE is sialylated as reported previously (Lee et al., 2010), since the observed molecular weight was reduced after being treated with neuraminidase. This allows us to synthesize and utilize biologically relevant extracellular APOE for future experiments.

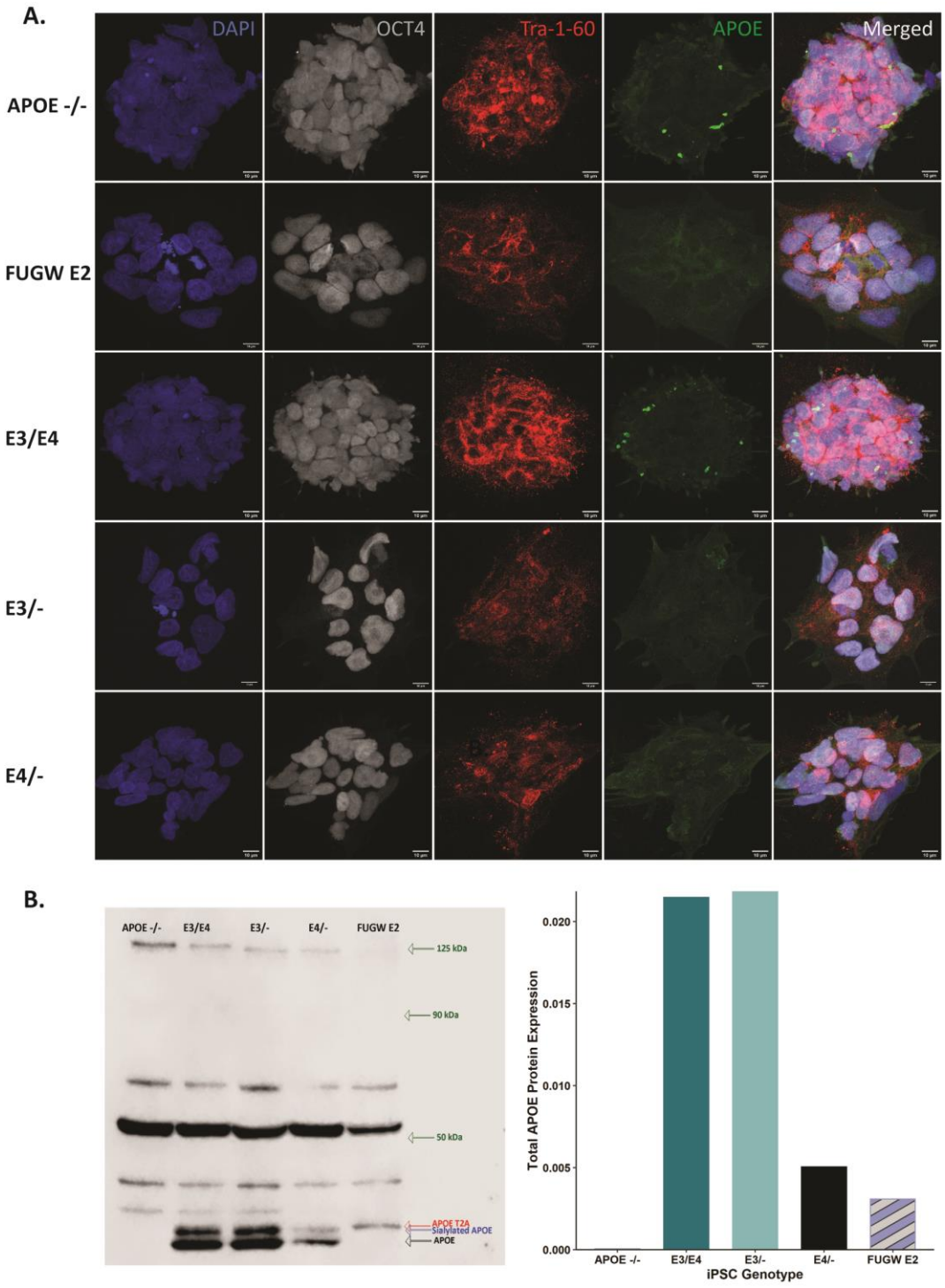


Figure 2.4 Pluripotency validation and APOE expression in iPSCs. (A) Representative images of the: sorted APOE -/-, APOE -/- infected FUGW APOE E2, heterozygous APOE E3/E4, hemizygous E3/-, and hemizygous E4/- iPSCs expressing OCT4 (grey), Tra-1-60 (red), and APOE (green) in addition to the positive nuclei detected by Hoechst (blue). (B) Western Blot representation explains the presence of APOE expression in the sorted APOE -/- line via immunocytochemistry by the non APOE bands detected above the molecular weight of interest. APOE bands were detected in 3 forms; unmodified APOE, sialylated APOE, and APOE tagged with T2A. Total APOE protein for each sample was quantified and normalized to its total protein detected in the lane. Images presented as a representative of five field views. Western blot presented as a representative of two technical replicates.

Pluripotency of APOE null, heterozygous E3/E4, hemizygous E3/-, E4/- and APOE E2 transduced iPSCs were determined with positive OCT4 and Tra-1-60 immunocytochemistry (Figure 2.4A). Although FACS sorting confirmed isolation of APOE null cells, there is detectable signal in all lines when probing for APOE. To confirm that the positive signal is a result of cross reactivity, cells were lysed and immunoreactive protein was assessed by Western blots (Figure 2.4B). Detection of bands with multiple apparent molecular weights revealed bands matching predicted molecular weights specific for native APOE or sialylated APOE as well as alternate bands, presumably the source of cross reactivity. Normally, APOE is detected at 34 kDa (Lee et al., 2010). A higher molecular weight of APOE is detected (~37 kDa) in the viral-transduced line as a result of fusing APOE with the self-cleaving peptide T2A engineered to allow co-expression of drug resistance or fluorescent protein. APOE detected in the transduced line was only detected with one molecular weight without clear evidence of sialylation. Based on the Western, the APOE -/- cells do not produce any detectable APOE protein and using lentiviral constructs, we are able to constitutively incorporate APOE into its genome, thereby allowing the cells to produce APOE-T2A.

Although there was no evidence that the CRISPR-Cas9 hemizygous cell lines produced lower amounts of APOE compared to the heterozygous E3/E4 (Figures 2.4 and 2.5), we sought to ectopically regulate APOE expression in the APOE knockout background. To do this, we constructed viruses driven by a dox-inducible promoter and performed a doxycycline (dox) dose-response in the E2 transduced induced neurons (iNs) to determine if APOE expression could be artificially manipulated. Neurons differentiated from the APOE -/- iPSCs and infected with the pTet-O APOE E2 lentivirus, show a proportional change in APOE protein expression with varying concentrations of dox; the higher the concentration of dox, the greater the expression of APOE (Figure 2.5). This is in line with other studies that show increasing the concentrations of the tetracycline up-regulates the expression of the gene of interest (Gossen & Bujard, 1992; Gossen et al., 1995). However, even at the lowest effective concentration of dox (0.2 mg/ml), the APOE expression was several folds higher than the expression observed in the endogenous heterozygous E3/E4 iNs, reducing the biological utility of this approach. However,

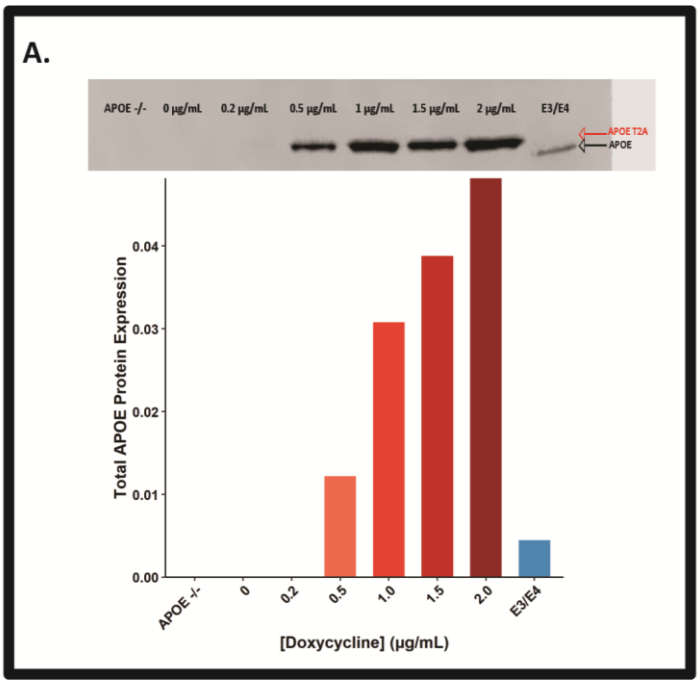


Figure 2.5 Doxycycline concentration proportionally affects the APOE E2 protein expression in iNs. Western Blot representation displays increasing APOE protein expression in p-TetO APOE E2 infected APOE -/- iNs incubated with increasing concentrations of doxycycline for 24 hr. APOE bands were detected in two forms; unmodified APOE and APOE tagged with T2A. There was no APOE detected in the untransduced APOE -/- iNs, therefore the APOE detected is purely from the lentivirus. Total APOE protein for each sample was quantified and normalized to its total protein detected in the lane. Western blot presented as a representative of two technical replicates.

these results confirm our ability to alter the expression of APOE in a lentiviral-transduced line by altering the concentration of dox.

While APOE is generally expressed in many cell types, its expression in neurons has caused some controversy. Neuronal APOE is dependent on its response to neuronal injury and its location in the brain (Xu et al., 1999; Xu, 2006). Studies have confirmed that APOE is primarily expressed and secreted by astrocytes; therefore studying APOE expression in this cell type may be more relevant to its biology (Boyles et al., 1985). In 2019, investigators conducting the Religious Order Study and the Memory and Aging Project (ROSMAP) prepared single-cell RNA sequencing data to identify transcriptionally distinct subpopulations involved in AD pathology (Bennett et al., 2018). ROSMAP was a joint aging study that collected information from older individuals that consisted of their cognitive assessment and neuro-imaging ante mortem as well as an autopsy postmortem. By re-analyzing the data, we were able to assess the gene expression of APP and

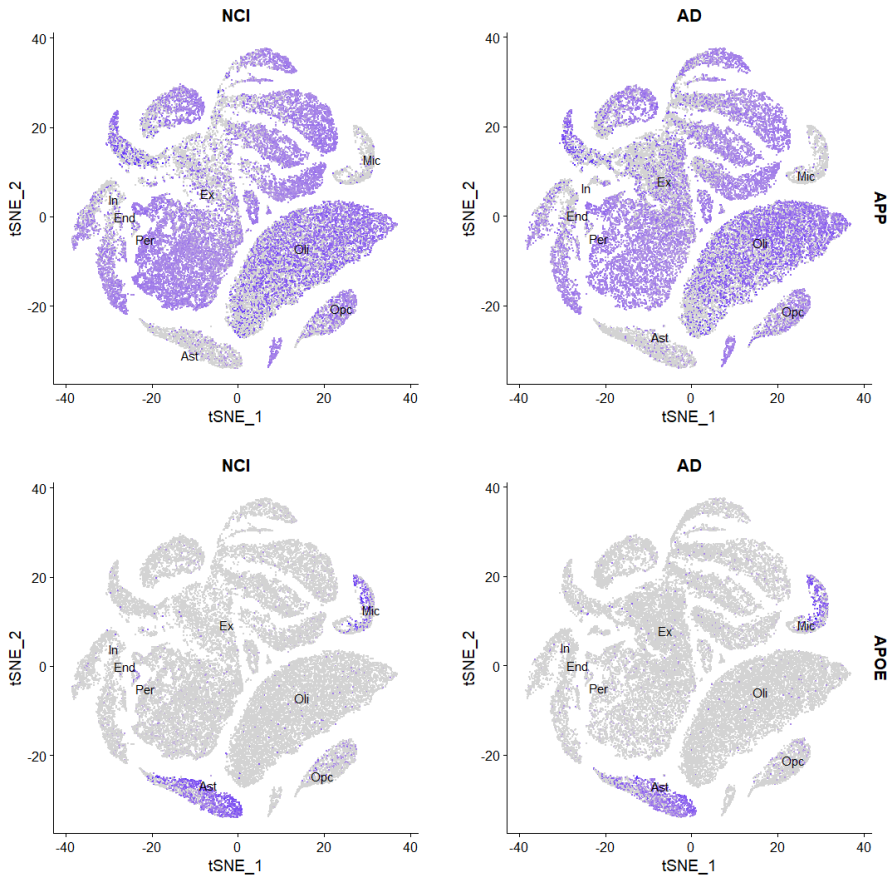


Figure 2.6 APOE expression is abundant in non-neuronal cells. (A) tSNE plot displaying cell type clustering. Neuronal cells are one of the main primary sources of APP, however, APOE is primarily expressed in other cell types like astrocytes and oligodendrocytes. Cell types: Ex: Excitatory neurons, In: Inhibitory neurons, Ast: Astrocytes, Oli: Oligo-dendrocytes, Opc: Oligodendrocyte precursors, Mic: Microglia, Per: Perivascular cells, End: Endothelia. Diagnosis: NCI: no cognitive impairment, AD, Alzheimer's disease.

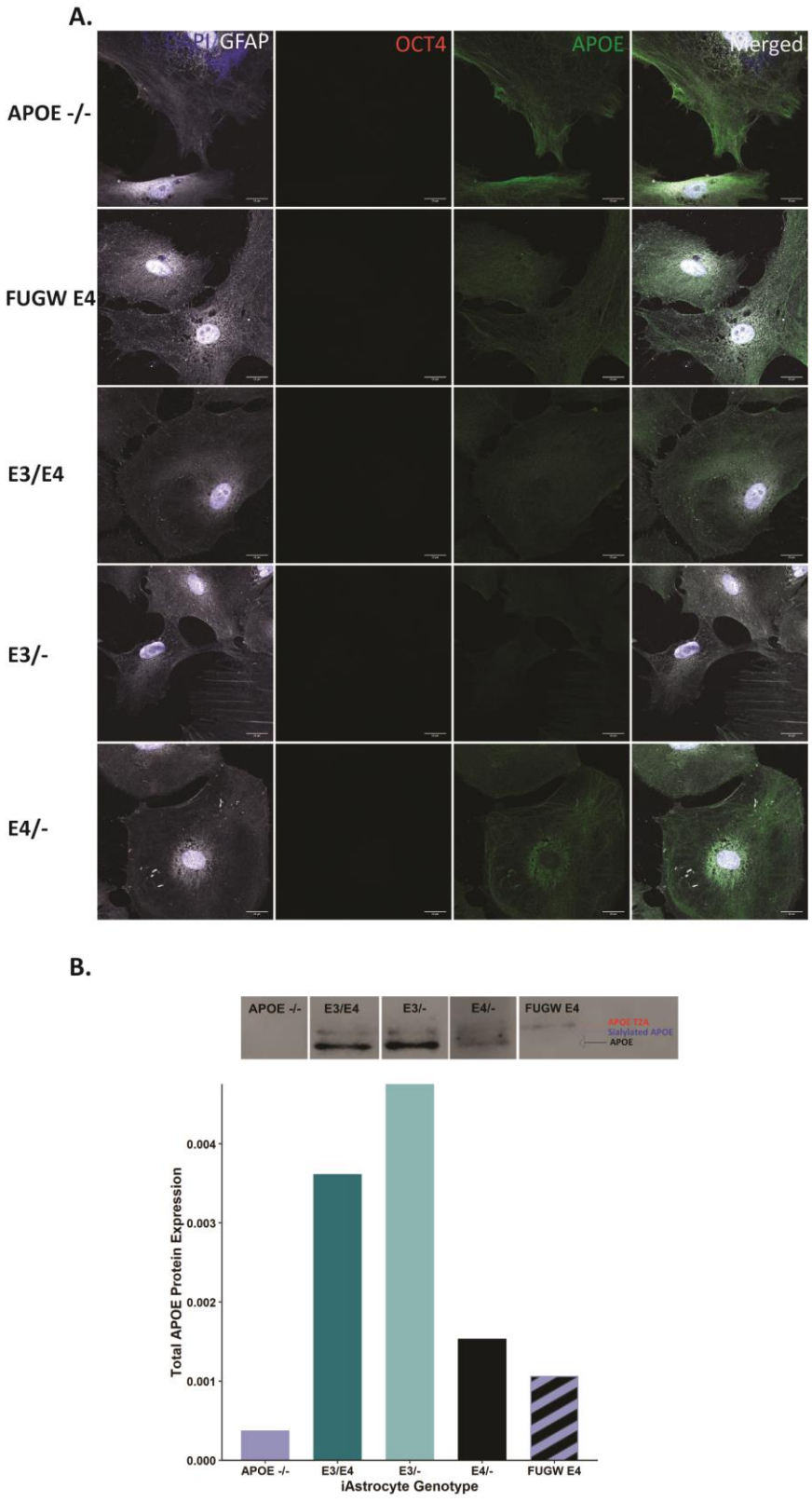


Figure 2.7 Astrocytic validation and APOE expression in iPSCs. (A) Representative images of the: sorted APOE -/-, APOE -/- infected constitutive FUGW APOE E4 lentivirus, heterozygous APOE E3/E4, hemizygous E3/-, and hemizygous E4/- iAs expressing GFAP (grey), APOE (green) and the absence of OCT4 (red) in addition to the positive nuclei detected by Hoechst (blue). (B) Western Blot representation shows APOE bands detected in 3 forms; unmodified APOE, sialylated APOE, and APOE tagged with T2A. Total APOE protein for each sample was quantified and normalized to its total protein detected in the lane. Images presented as a representative of five field views. Western blot presented as a representative of two technical replicates.

Visually, there was no difference between the types of cells that express the APP gene when comparing the NCI and AD groups. We were also able to confirm that neurons have little or no expression of APOE while astrocytes and microglia are the primary sources in human brain (Figure 2.7).

Being able to confirm that APOE is predominantly expressed in astrocytes, we wanted to differentiate the various APOE iPSC lines to astrocytes (iAs). The astrocytic identity of APOE null, heterozygous E3/E4, hemizygous E3/-, E4/- and APOE E4 transduced iAs was confirmed with staining for glial fibrillary acidic protein (GFAP) as well as the absence of the pluripotency marker Octamer-binding transcription factor 4 (OCT4) via ICC (Figure 2.7A). As observed in the iPSCs (Figure 2.4), all APOE iAS cell lines exhibited similar cross reactivity when assessing APOE immunofluorescence (Figure 2.7A), however, the absence of APOE in the APOE-null iAS was determined by Western blotting (Figure 2.7B). APOE rescue is also observed in the APOE E4 transduced line. Total APOE was defined as both sialylated and unmodified APOE. Detecting sialylated APOE provides additional evidence that astrocytes robustly express and secrete ApoE protein. Finally, similar to what was seen in the iPSCs, these cells were differentiated from, a higher molecular weight of APOE is detected in the transduced line as a result of the presence of self-cleaving peptide T2A.

2.5 Discussion

We were able to develop a set of iPSC lines expressing each of the three variants of APOE using both CRISPR-Cas9 technology and lentiviral transductions. CRISPR-Cas9 cleavage near rs429358 resulted in error-prone NHEJ repair and short frameshift deletions, giving us isogenic hemizygous genotypes of APOE (E3/- and E4/-). If hemizygosity leads to haploinsufficiency, or the inability to maintain normal function when one copy of a gene is deleted or inactivated, this would have been a limitation associated with using these hemizygous lines. However, we observed that the expression levels were not different from a diploid cell isogenic line APOE E3/E4. When

it comes to disorders, in particular AD, the variant quantity of APOE appears to influence the risk of developing and worsening the condition (Bickeböller et al., 1997; Corder et al., 1993; Duara et al., 1996). An advantage is that with only one functional allele per cell, we could easily manipulate or add to it with viral transduction. Our generation of hemizygous lines are in line with the variable 2- 20 % success rate of HR DNA repair rates seen in CRISPR/Cas9 edited iPSCs (Simkin et al., 2022). Literature also suggests that poor genotype screening often leads to the effective creation of "homozygous" iPSCs, which misses various genomic defects namely insertions and deletions (Simkin et al., 2022; Weisheit et al., 2020). Our results indicate that through the thorough genotype screening of the hemizygous lines, there was no difference in ApoE protein expression compared to the heterozygous E3/E4 cell line.

The tools designed and developed were intended to be used to generate genetically appropriate AD neuronal model systems. To do this, our aim was to differentiate human iPSCs into neurons and astrocytes and determine how the APOE variants influence the hallmarks of AD. Based on the data presented in the iPSCs and the iAs, having only one allele of APOE did not affect the amount of protein being synthesized, providing a great model to assess the APOE variants separately. This hemizygous genotype also did not affect the differentiation state of the iPSCs and iAs indicative of their positive pluripotency (Figure 2.4A) and astrocytic markers (Figure 2.7A) presented. Developing a way to separately study the most common APOE variant, E3, and the variant associated with the highest risk, E4, can make a significant contribution to exploring APOE associated disorders. We also displayed evidence of successfully isolating the APOE -/- cells from those that may have potentially undergone alternative splicing or translational reinitiation; these provide an excellent negative control to add to our model system.

As astrocytes are the major cells expressing APOE, we provide evidence that we can edit this gene in iPSCs and carry out its expression through astrocyte differentiation. Lentiviral transfection provided another way to assess the variants of APOE individually. Cells infected with the drug

inducible lentiviral constructs allow us to change the expression of APOE by altering the concentration of doxycycline the cells are exposed to. However, in some cases, researchers may not have the capability of designing their model systems based on having that necessary cell-to-cell contact between neurons and astrocytes. Therefore, we presented an alternative by designing lentiviruses and transfecting efficient cells for the sole purpose of collecting and extracellular nutrients that can be used to mirror a healthy environment. The constitutive lentiviruses used to create stable HEK 293T APOE variations allowed us to collect extracellular APOE that mimicked the free and sialylated APOE detected from the iAs. This suggests that studies looking to assess the behavior of extracellular APOE *in vitro* can utilize the FUGW-APOE-PuroR (APOE E2, E3, or E4) lentivirus to infect transfect competent cells that are very efficient at producing protein such as HEK 293T cells and reintroducing the collected media to another model system.

We provide evidence on how genetic editing tools can be employed to generate biologically relevant model systems. The ability to manipulate the individual variants of APOE in our pluripotent cell lines as well as maintain said genotype through differentiation allows us to design a system that encompasses different genotypes. When it is difficult to differentiate and maintain cells that secrete nutrients and small molecules important for the normal physiology of the targeted cells, lentivirus transductions in other cell types for the sole purpose of collecting the secreted factors in order to reintroduce them to the targeted cells in a system may be preferred. Both CRISPR Cas9 and lentiviral transduction systems supply alternative approaches for future studies assessing the hallmarks of AD, as well as provide other means to study the role each variant of APOE has on other disorders including cardiovascular diseases and many more.

CHAPTER 3: DDT AND DDE EXPOSURE ALTERS RESPONSIVENESS OF MOUSE AND HUMAN NEURONS

3.1 Abstract

Developed in the 1930s, dichlorodiphenyltrichloroethane (DDT) quickly became the most effective pesticide against insects carrying Malaria and other insect borne human diseases. While it is effective due to its long half-life, the excessive use of DDT revealed the negative effects it has on the environment and wildlife as well its potential adverse effect on humans. This revelation caused the EPA to ban the use of it in 1972 within the US, however, remnants of it as well as DDE, one of its metabolites, can be found in water and soils, in addition to the blood of exposed individuals. There have been several studies in species including crayfish, frog and rats to further confirm DDT's toxic mechanism of action. However, the same level of analysis has not been carried out for its metabolite DDE, which has always been assumed to be less effective or deactivated compared to its parent compound. We determined the ability of DDT to interact with the voltage-gated sodium channels expressed on human induced neurons (iNs). We have also demonstrated that the metabolite DDE at concentrations of 1μM is also capable of eliciting generation of continuous rapid action potentials, presumably by holding open the voltage-gated sodium channel as has been demonstrated for DDT. Acute and chronic exposure to DDT and DDE at a concentration of 1 μM elicited an induction of +10 mV shift in resting membrane potential (RMP). Interestingly, DDE provoked stronger affinity to the neurons shown by the longer wash-out period of 15 minutes before its toxicity was reversed when compared to the 5 minutes required for DDT. Results indicate that both DDT and DDE inhibit the closing of mammalian voltage gated sodium channels, without resulting in significant cell death, at concentrations below 10 μM. These results provide further reasons to: (1) regulate the current usage of DDT in countries still being affected by malaria, (2) argue for additional research that explores the adverse effects in human to

the once-classified “safer” metabolite DDE, and (3) reveal potential pathways that can be targeted for therapy after chronic pesticide exposure.

3.2 Introduction

The discovery of the selective organochloride pesticide (OCP), dichlorodiphenyltrichloroethane (DDT) was a substantial milestone in the control of disease-carrying insects (Davies et al., 2007; Läuger et al., 1944; Mellanby & British Crop Protection, 1992; Müller, 1946). DDT use was widespread for many years as it was inexpensive to produce and was effective due to its long half-life (ATSDR, 2002; Davies et al., 2007). However, due to negative effects on wildlife as well as the unknown adverse effects on humans, a ban on the use of DDT in the U.S. by the EPA began in 1972 (Jürgens et al., 2016; Rehwagen, 2006). However, the potential effects of DDT and its metabolites on brain function have never been studied in humans.

Depending on conditions, DDT decays in the environment into two different metabolites: dichlorodiphenyldichloroethylene (DDE) and dichlorodiphenyldichloroethane (DDD). The formation of DDE is favored under aerobic conditions while anaerobic conditions promote the formation of DDD (Jürgens et al., 2016). Fifty years after the EPA ban, this compound and its metabolites can still be found in deep sediments of soil, water (Jürgens et al., 2016), and even in the serum of individuals, where it has been associated with increased risk for onset of Alzheimer Disease (Richardson et al., 2014). With ongoing spraying programs in India, DDT continues to be the most efficient repellent for malaria-carrying mosquitoes, reducing transmission by 90%, making its reintroduction a new source of contamination. (WHO, 2006). Therefore, accumulation of these compounds is ongoing, partly due to past contamination of soils and other environmental sources, as well as from continued usage of DDT, through bioaccumulation, biomagnification, and transportation across long distances in the upper atmosphere.

DDT was designed to selectively target the nervous system of insects until extensive use revealed it could also be toxic to other species. The toxic mechanism of DDT is thought to require

direct interaction with the voltage gated sodium channels (Na_v) (Stuart et al., 1987; van der Bercken, 1972). These channels are embedded in neuronal membrane and function to moderate the influx of sodium ions in response to action potential initiation (Lenaeus et al., 2017). DDT increases the negative afterpotential, requiring more time to recover before generating another action potential because it decreases the channel's ability to be closed or inactivated (Chen et al., 2019; Lucas & Renou, 1992; Shrager et al., 1969; van der Bercken, 1972). These characteristics are what make DDT an efficient pesticide in insects. In fact, moths exposed to 1 µg of DDT onto their abdomen immediately became more active, with tremors and disorganized movements seen after three hours (Lucas & Renou, 1992). This was corroborated by recordings of a single sensillum, a basic sensory receptor, which demonstrated that in moths treated with DDT a single action potential spike was replaced by short intervals of bursts (multiple spikes), and that this pattern became more apparent as exposure progressed (Lucas & Renou, 1992).

Evidence supports the ability of DDT to also affect the nervous systems of non-target species. Researchers sought to determine if this toxicant could harm aquatic life because DDT is known to persist up to 150 years in aquatic environments (ATSDR, 2002; Davies et al., 2007). DDT exposure in artificial seawater to lobster giant axons resulted in a decrease sodium conductance, resulting in an increase in potassium conductance, raising the negative afterpotential (Narahashi & Haas, 1967). Internal exposure induced irreversible increases in the negative afterpotential and decreases in impedance in a single perfused giant axon dissected from crayfish exposed to DDT from either side of its membrane; increasing the dose increased these effects (Shrager et al., 1969). Rainbow trout brain synaptosomes treated with DDT confirmed observations seen in squid giant axons; the compound was unable to enhance sodium flux (Stuart et al., 1987). These studies conducted in various aquatic species showed that the accumulation of DDT in aquatic environments behave in a similar manner to what was observed in insects, this indicates that DDT is not very selective. The lack of selectivity is further confirmed when recordings from DDT-treated

myelinated nerve fibers in *Xenopus* clawed toads or *Rana pipiens* frog nerves revealed rapid repeating responses that became more numerous with increased exposure time (van der Bercken, 1972) and concentration (Shanes, 1951). Although repetitive responses were observed only in the myelinated sensory fibers when compared to the motor fibers of *Xenopus* laevis, the repetitive firing was consistent and stronger in nerve fibers of *Rana pipiens* (ÅRhem & Fraxkexhaeuser, 1974; ÅRhem et al., 1974; van der Bercken, 1972). These additional findings show that DDT impacts multiple species, which encourages our speculation that it may have a similar interaction with human neurons.

There have been no direct studies of whether or how DDT and DDE interact with human neurons. Clinical signs such as tremors were found to be directly correlated with the concentration of DDT found in the brain in rats, regardless of whether the rats were given small doses over time or one large dose (ÅRhem & Fraxkexhaeuser, 1974; ÅRhem et al., 1974; Dale et al., 1962). DDT-exposed sciatic nerves in Purdue-Wister rats display the same repeating burst seen in insects, lobsters, and frogs (Shankland, 1964). These findings show that both chemicals cause similar behaviors in the mammalian nervous system, but there is still no evidence showing any direct negative impacts on the human neurological system.

Furthermore, while chronic bioaccumulation of DDE is likely to be found in exposed individuals, there has been no direct study of DDE action on mammalian or human neurons. It is crucial to determine whether these compounds act on human Na_v to determine if current regulatory efforts should be adjusted to minimize exposure which could ultimately diminish the risk. Here we use primary cultures of mouse cortical neurons and excitatory human neurons derived from induced pluripotential stem cells (iPSC) to identify the immediate and chronic effects of both DDT and DDE.

3.3 Materials and Methods

Stem cell culture and rapid neuronal induction

Induced pluripotent stem cells (iPSCs) were maintained on Matrigel (Corning® Matrigel® hESC-Qualified Matrix; 354277) coated 6-well plates with mTeSR™ 1 medium (STEMCELL Technologies; 05850). Cells were split weekly using ReLeSR™ (STEMCELL Technologies; 05872) to preserve colony formation. To collect single cells for glutamatergic neuronal differentiation (iNs), iPSCs were treated with ACCUTASE™ (STEMCELL Technologies; 07920) and infected in suspension with multiple lentiviruses containing the doxycycline inducible neurogenin 2 (Ngn2) co-expressed with the drug resistance gene puromycin, or the reverse tetracycline-controlled transactivator (rtTA), plus addition of 5 µM Y27632 (Y-compound, Tocris; 1254) and 2 ng/µL of doxycycline. Transduced cells were selected by addition of 1 µg/ml puromycin from days 2 to 4 following infection. At day 5 post infection, the iNs were plated on primary mouse glia and treated with 4 µM cytosine arabinoside (Ara C, Sigma-Aldrich; C1768) on day 8 to inhibit the growth of the dividing glial cells. Co-cultures were then maintained with half medium changes of Neurobasal Plus Medium plus B27+ (NB2) (Thermo Fisher Scientific: catalog number A3653401) every 4-5 days until neuronal maturity; approximately 36 days post lentiviral infection. Electrophysiology was used to assess maturity by measuring the Resting Membrane Potential (RMP) and spontaneous glutamate release (sEPSCs).

Primary mouse neuron culture

Primary mouse cortical neurons were dissected from postnatal C57BL/6J pups aged 0-1 days. Cells were seeded on 0.1 mg/mL Poly-L-Lysine (Sciencell, catalog number 0413, PLL) and 5 µg/mL natural mouse laminin protein (Fisher Scientific, catalog number 23-017-015) coated plates and coverslips. Cells were shipped with ice-cold HE media, filling the wells completely. Parafilm was used to wrap the plates after they were sealed with an adhesive plate sealer. Plates were shipped overnight from the laboratory of Dr. Jason Richardson (Florida International University) to Rutgers University in New Jersey in a Styrofoam shipping container with pre-chilled

4°C ice packs. Upon arrival, shipping media was immediately replaced with warm (37°C) culture media consisting of: Neurobasal medium (Thermo Fisher Scientific, catalog number 21103049), 2% B-27 (Thermo Fisher Scientific, catalog number 17504044), 1% penicillin-streptomycin (Thermo Fisher Scientific, catalog number 15140122), and 1% GlutaMAX™ Supplement (Thermo Fisher Scientific, catalog number 35050061). The morphology and density of the cells were checked 2 days post shipment. On day 4, a half media change with culture media with a final concentration of 5 µM cytosine arabinoside (AraC; Sigma-Aldrich; C1768). Every 3-4 days post, half media changes were carried out until neuronal maturation on day 14.

Treatments

Dichlorodiphenyltrichloroethane (DDT) (Chem Service, Inc; N-10876-100MG) and dichlorodiphenyldichloroethylene (DDE) (Chem Service, Inc; N-10875-100MG) were dissolved in sterile DMSO (Sigma Aldrich; D2650) to a final concentration of 100 mM. Compounds are stored at -20°C in small volumes in amber glass vials (Sigma Aldrich; 29663-U) to avoid frequent freeze thaw cycles. The purity and concentration of DDT and DDE were validated using GC-MS by the Chemical Analysis Facility Core at EOHSI, Rutgers University. Mature mammalian neurons were exposed to vehicle control DMSO, 1 µM or 10 µM of DDT and DDE for 6 or 24 hours prior to whole-cell patch-clamp recordings. Propidium iodide experiments were carried out on neurons exposed to vehicle control DMSO, 1 µM, 10 µM, 20 µM, and 50 µM of DDT and DDE for 24 hours. All DDT and DDE dilutions were made in amber glass vials using sterile DMSO, which did not exceed 0.2 % (v/v) in treatment media.

Electrophysiology

Functional analyses of mammalian cultures were conducted using whole-cell patch-clamp electrophysiology (Halikere et al., 2020; Popova et al., 2019; Scarnati et al., 2020) which were

performed by Dr. Dina Popova. Pipettes used for patching were pulled from borsilicate glass capillary tubes (Warner Instruments; 64-0793) using a PP830 pipette puller (Narishige). All recording were done in HEPES buffer consisting of (in mM): 140 NaCl, 5 KCl, 2 CaCl2, 2 MgCl2, 10 HEPES, and 10 glucose. The pH was adjusted to 7.4 with NaOH. For current-clamp recordings, a K-gluconate internal solution was used, which consisted of (in mM): 126 K-gluconate, 4 KCl, 10 HEPES, 0.05 EGTA, 4 ATP-magnesium, 0.3 GTP-sodium, and 10 phosphocreatine. The pH was adjusted to 7.2 with KOH and osmolality was kept between 270–290 mOsm. For all the experiments intrinsically active neurons (pacemaker-like) were pre-selected since both chemical compounds exert their function through open channel mechanisms. Resting membrane potential, intrinsic and induced action potential firing parameters were recorded in current clamp mode with acute treatments performed via bath application of compounds. Electrophysiological data were analyzed using Clampfit 10.5 (Molecular Devices).

Immunocytochemistry and imaging

Neurons were fixed with ice cold 100% methanol for 30 min at -20°C and permeabilized using 0.02% Triton X-100 in PBS for 10 min at room temperature. Coverslips were then incubated in blocking buffer (3% bovine serum albumin and 5% normal goat serum in PBS) for 30 min at room temperature and then incubated with primary antibodies diluted in blocking buffer overnight at +4°C, washed with PBS three times, and subsequently incubated in secondary antibodies diluted in PBS for 1 h at room temperature. Confocal imaging analysis was performed using a Zeiss LSM700.

Statistical Analysis

Data were expressed as mean ± standard error of the mean (SEM) from at least three independent experiments with each treatment in duplicate. One-way repeated measures ANOVA

was used as appropriate. Statistical differences were adjusted using the Bonferroni multiple testing correction method. RStudio (RStudio Software Company, Boston, MA) was used for statistical analysis with *p <0.05, **p <0.01, ***p <0.001 considered as statistically significant.

3.4 Results

To identify the interaction of DDT and DDE with mammalian neuronal physiology, we tested the initial effects upon introduction of each compound, or a longer-term effect based on a 24 hr exposure prior to testing. Both human and mouse cultured neurons were tested. For mouse, primary cortical neurons were prepared from newborn pups, and for human, excitatory neurons resembling mid-level cortical neurons were prepared by Ngn2 induction from induced pluripotent stem cells (iPSC) (Pang et al., 2011; Vierbuchen et al., 2010; Zhang et al., 2013).

1. DDT and DDE induce immediate physiological responses in mammalian neurons

It is well accepted that DDT and DDE affect the nervous systems of insects and other non-mammalian organisms through modulation of voltage gated sodium channels (Na_v channels) which can lead to cytotoxic cell death through excessive depolarization (Stuart et al., 1987; van der Bercken, 1972). Limited studies suggest that DDT and its metabolites can similarly target the

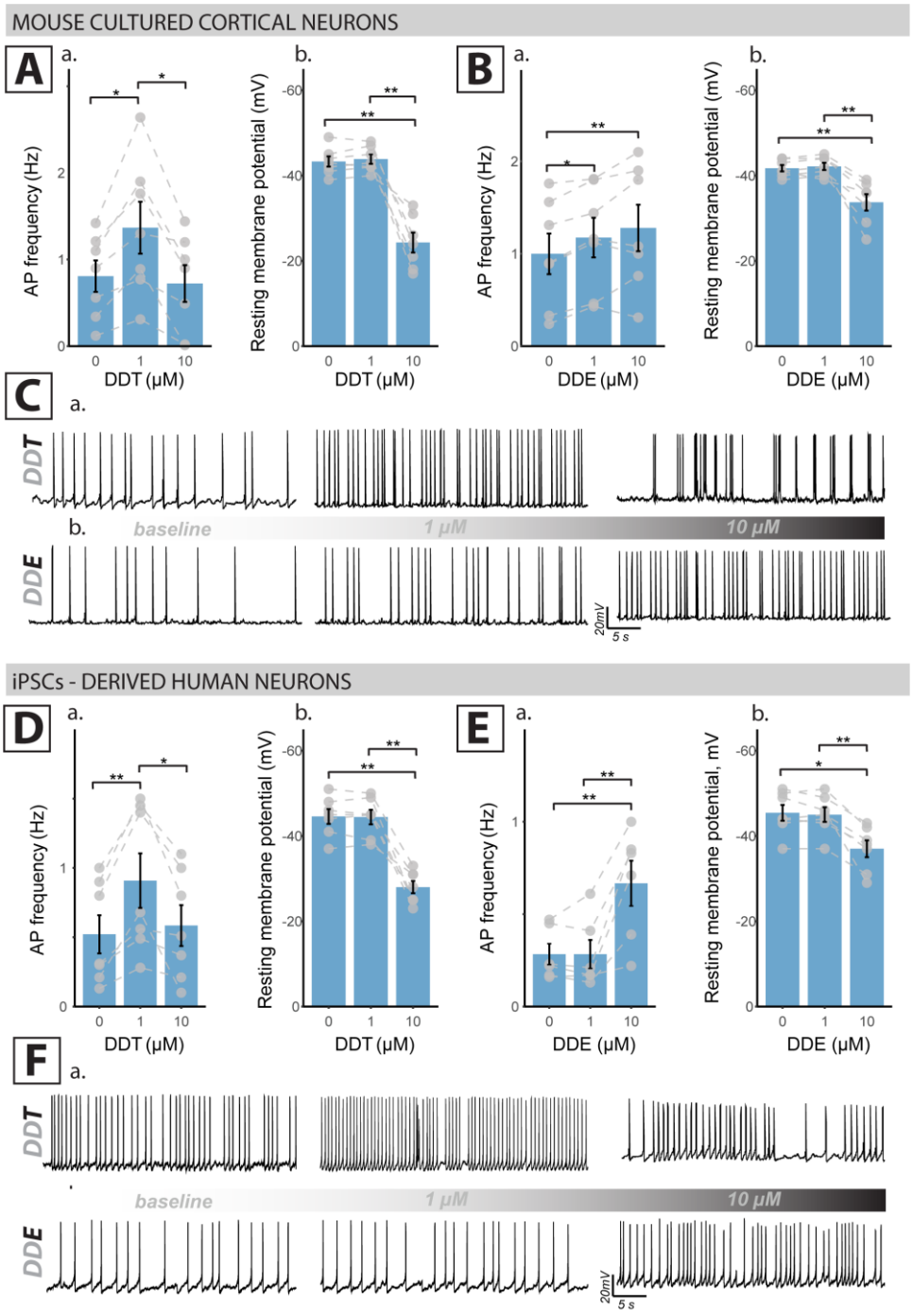


Figure 3.1 DDT and DDE potentiate action potentials and reduce resting membrane potential in a dose-dependent manner in both mouse and human neurons. Panels depict results from (A-C) primary mouse cortical neurons and (D-F) iPSC-derived induced human neurons. (A, D) Quantification of responses to acute application of 1 or 10 μM DDT treatment. Plots summarize changes in (left) AP firing of neurons and (right) resting membrane potential. (B, E) Quantification of responses to acute application of 1 or 10 μM DDE treatment. Results show changes in (left) AP firing of neurons and (right) resting membrane potential. (C, F) Representative traces of spontaneous AP firing of neurons treated with increasing concentrations of (top) DDT and (bottom) DDE (0, 1 and 10 μM respectively). Plots show individual cells as dots, with mean as bar ± SEM. Results were analyzed using repeated measures ANOVA to evaluate statistical differences in the same cells over multiple treatments. Bonferroni multiple testing correction method was used to assess comparisons (*p <0.05, **p <0.01, ***p <0.001). Electrophysiology experiments performed by Dr. Dina Popova from the Hart lab.

mammalian nervous system (Imamura et al., 2005; Shankland, 1964), potentially including human neurons. Our goal was to investigate whether if DDT and DDE induce comparable physiological responses in iPSC-derived, induced human neurons (iNs) or in primary mouse cortical neuron cultures. This approach allowed us to investigate details of DDT and DDE action in parallel on two mammalian nervous system paradigms.

Glutamate-releasing excitatory iPSC-induced human neurons used in the study were produced using Ngn2 reprogramming (Pang et al., 2011; Vierbuchen et al., 2010; Zhang et al., 2013) and quality of the cultures was evaluated prior to each experiment. Using immunocytochemistry (ICC), we probed neurons for expression of the neuron-specific microtubule-associated protein 2 (MAP2) and a marker of synaptic vesicles, synapsin 1 (SYN1). Using electrophysiology, we assessed physiological maturity of neurons by probing: (i) for ability to release glutamate (spontaneous excitatory postsynaptic currents, sEPSCs) (Figure 3.2), (ii) resting membrane potential (RMP) and (iii) the ability to fire action potentials. All results are consistent with the presence of neuronal features, physiologically activity, and spontaneous firing, prerequisites for studying a potential sodium channel modifier.

Since DDT and its metabolite DDE are known to primarily affect Na_v channels (Chen et al., 2019; Lucas & Renou, 1992; Shrager et al., 1969; van der Bercken, 1972) we first tested if spontaneous activity of both glutamatergic human iNs and primary mouse cortical neurons would respond similarly to initial treatment. Changes in RMP and AP firing were measured through bath application of increasing concentrations of compounds (Figure 3.1).

Figure 3.2 Representative traces of spontaneous excitatory post-synaptic currents (sEPSCs). Traces depicts iPSC-derived induced human neurons exposure to DMSO (left trace) and DDT or DDE (right trace). Exposure to either DDT or DDE results in increased glutamate release from the cells.

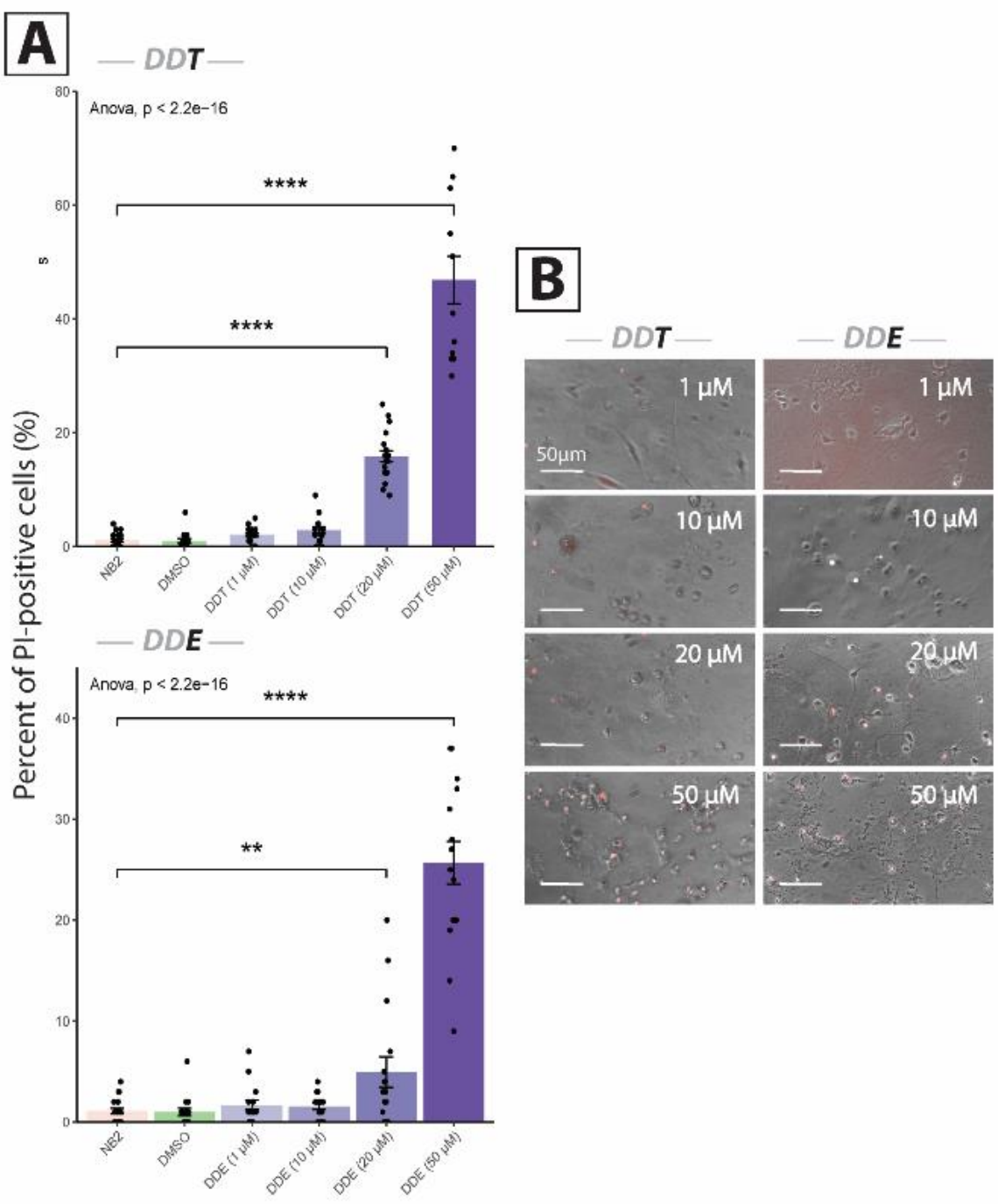


Figure 3.3 Low concentration exposure of DDT and DDE does not result in significant cell death. Panels A-B depicts results for iPSC-derived induced human neurons and C-D for primary mouse cortical neurons. Panels A and C display the percentage of PI-positive cells after a 24-hour exposure to DDT and DDE with concentrations ranging from 0-50 µM. Representative images from the dose response are summarized in panel B for human induced neurons and panel D for primary mouse cortical neurons. Bar plots show individual cells as dots, with the bar showing the mean and error bars ± SEM. Results were analyzed using repeated measures ANOVA to evaluate statistical differences. Bonferroni multiple testing correction method was used to assess comparisons (*p <0.05, **p <0.01, ***p <0.001, ****p<0.0001).

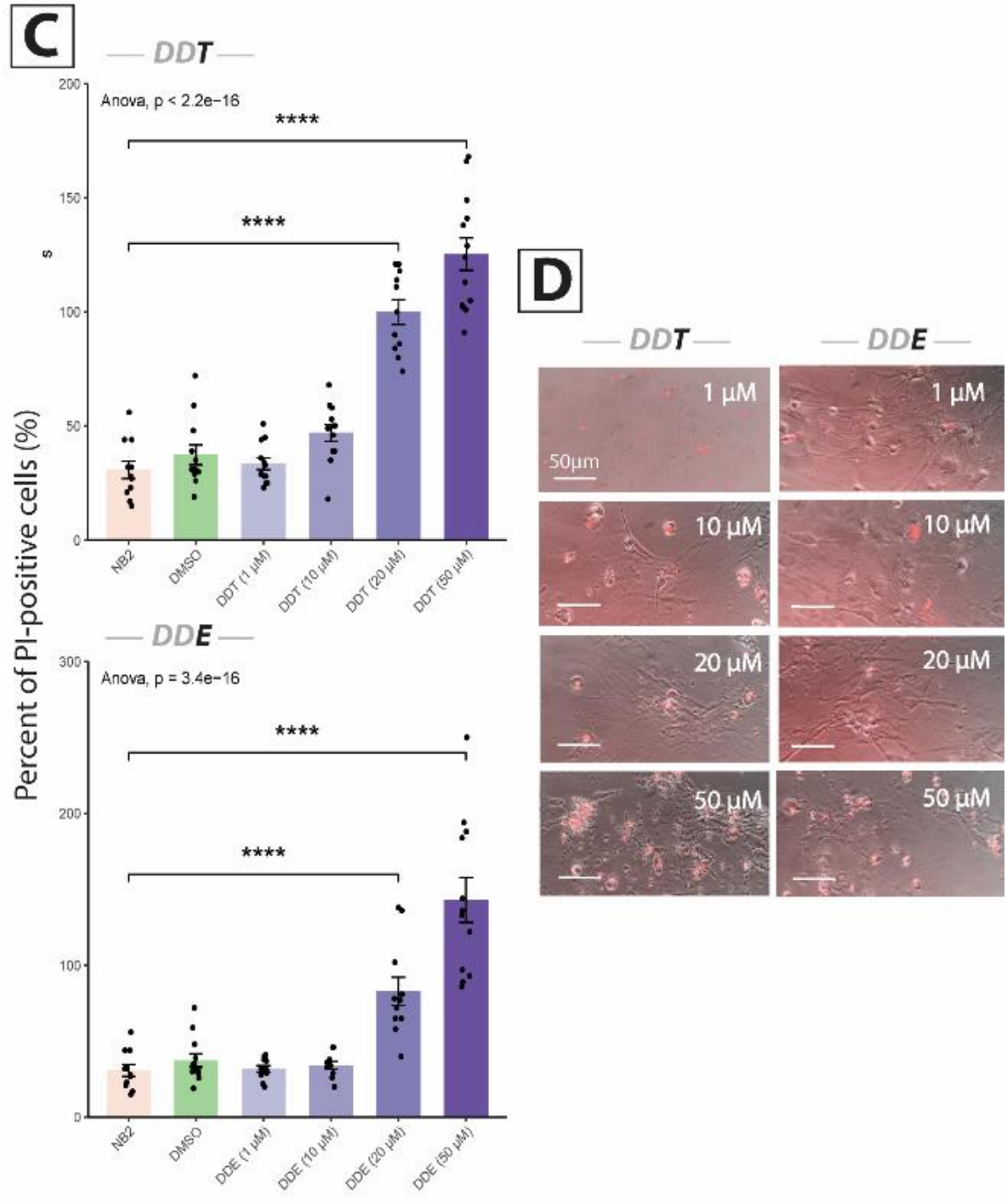


Figure 3.3 Cont. Low concentration exposure of DDT and DDE does not result in significant cell death. Panels A-B depicts results for iPSC-derived induced human neurons and C-D for primary mouse cortical neurons. Panels A and C display the percentage of PI-positive cells after a 24-hour exposure to DDT and DDE with concentrations ranging from 0-50 µM. Representative images from the dose response are summarized in panel B for human induced neurons and panel D for primary mouse cortical neurons. Bar plots show individual cells as dots, with the bar showing the mean and error bars ± SEM. Results were analyzed using repeated measures ANOVA to evaluate statistical differences. Bonferroni multiple testing correction method was used to assess comparisons (*p <0.05, **p <0.01, ***p <0.001, ****p<0.0001).

We found that both compounds induced immediate changes in RMP and AP firing. However, we observed discrepancies in dose responses of human and mouse neurons to DDT and DDE. Interestingly, 1 µM DDT infusion induced rapid changes in AP firing while DDE administration induced similar effects only at 10 µM. In parallel to firing, we also observed that DDT and DDE at a 10 µM concentration induced a significant shift in RMP toward greater depolarization, with DDT effects being more pronounced than DDE, which would suggest robust involvement of Na_v channels into responses. No substantial differences were observed to DDT and DDE administration between human and mouse neurons.

Although these results might suggest differences in potency of the two compounds, with DDT presumably being more potent, there is also the possibility that they might have different mechanisms of action or other properties such as lipophilicity.

2. DDT or DDE induces cell death in a concentration-dependent manner

The toxic mechanism of DDT and DDE has been well studied in other systems (Wnuk, Rzemieniec, Litwa, Lason, et al., 2016). High concentrations of compound initially induce hyperactivity, which is transformed into paralysis and subsequently excitotoxic cell death. We determined the concentrations of DDT and DDE that induce cell death in mammalian neurons. Mouse cortical neurons were exposed to 1, 10, 20, or 50 µM DDT or DDE for 24 hr, and then toxicity was measured using propidium iodide (PI) live cells imaging (Figure 3.3). Appearance of red PI signal in neuronal nuclei indicated DNA binding due the loss of membrane integrity as result of cell death. Our results demonstrate that only the highest concentrations that we tested of both drugs (20 and 50 µM) induce significant cell death (more than 50%), whereas lower concentrations (1 and 10 µM) did not show significant cell death over vehicle control (DMSO). Evidence suggests that the human brain is rarely if ever exposed to neurotoxic concentrations of either DDT or DDE (Richardson et al., 2014).

3. Initial effects of DDT and DDE on human and mouse neurons are reversible

We found that both compounds induce an immediate response associated with membrane depolarization and/or induction of AP firing. We then wished to determine if the observed physiological differences could be reversed or if they remain stable when the toxicants are no longer present. To answer this question, we measured physiological properties during a 5 min bath application of DDT and DDE through a perfusion system followed by a period of washout.

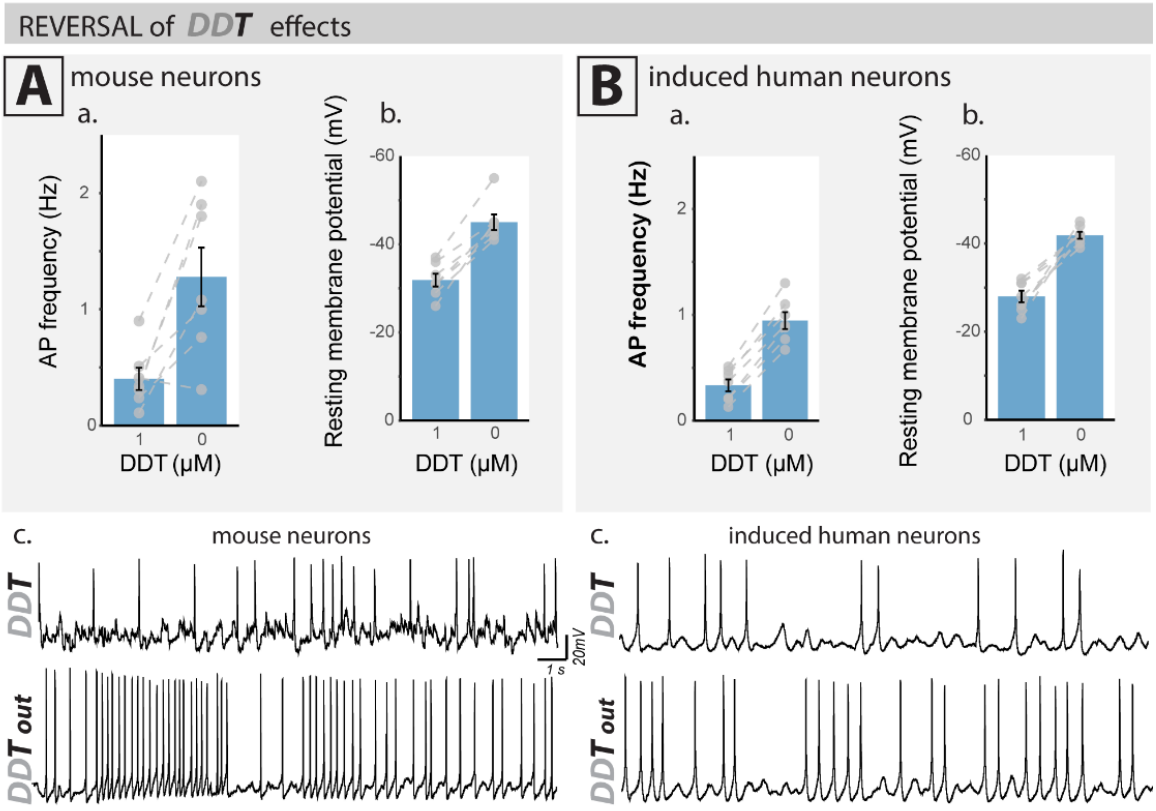


Figure 3.4 Reversal of acute DDT effects. (A) Quantification of the reversal of DDT effects by washout in primary mouse neurons, showing (left) increased AP firing, (right) reduced resting membrane potential, and (bottom) representative trace showing firing patterns of neurons with (DDT) 1 μM DDT present in the recording solution or (DDT out) representative trace showing "normalization" of neuronal firing pattern after 5 min of washout. (B) A similar reversal of effects was observed in human neurons. Repeated measures ANOVA was used to evaluate statistical differences (*p <0.05, **p <0.01, ***p <0.001). Electrophysiology experiments performed by Dr. Dina Popova from the Hart lab.

Both mouse and human neurons exhibited reversibility following acute DDT and DDE administration. However, the time courses of the response were different: DDT-induced membrane

depolarization and firing were reversed by 5 min of washout (Figure 3.4), while at least 15 min of wash out was required to reverse the effects of DDE (Figure 3.5). These findings are consistent with previously-reported results on the potentially different mechanisms of DDT and DDE on neurons where DDT induced c-*fos* and BDNF mRNA expression while DDE had no effect (Imamura et al., 2005). There is also evidence of both DDT and DDE exhibiting different lipophilic properties where DDE tends to accumulated more in human adipose tissue when compared to DDT

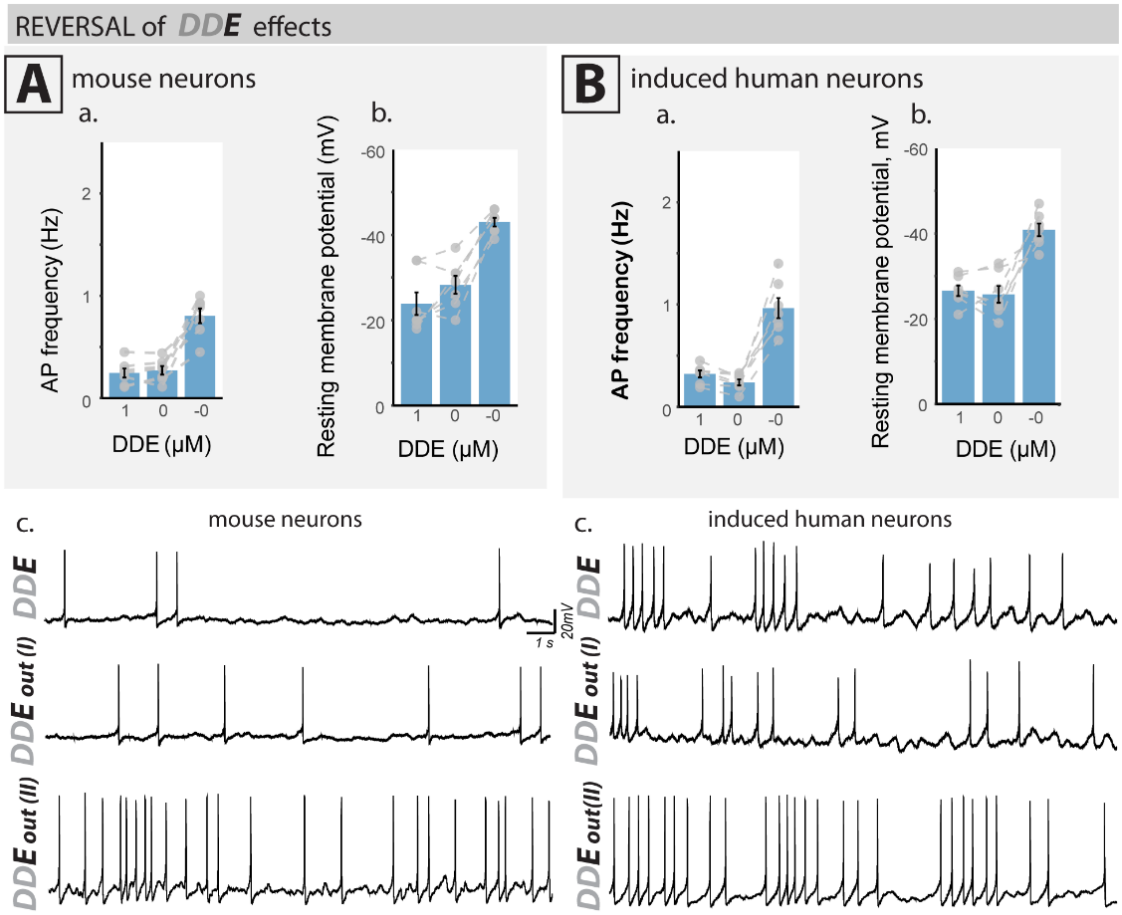


Figure 3.5 Reversal of acute DDE effects. (A) Quantification the reversal of DDE effects by washout in primary mouse neurons, showing (*left*) changes in APs firing, (*right*) changes in resting membrane potential, and (*bottom*) representative trace showing firing pattern of neurons with (*DDE*) 1 μM DDE present in recording solution, (*DDE out(I)*) representative trace showing mild "normalization" of neuronal firing pattern after 5 min washout and (*DDE out(II)*) representative trace showing complete "normalization" of neuronal firing pattern after 15 min washout (B) A similar reversal of effects was observed in human neurons. Repeated measures ANOVA was used to evaluate statistical differences (*p <0.05, **p <0.01, ***p <0.001). Electrophysiology experiments performed by Dr. Dina Popova from the Hart lab.

(Morgan & Roan, 1971). These would explain the observed discrepancies in the kinetics of both the wash in and wash out experiments.

4. Chronic DDT and DDE exposure induces specific burst-like responses in human and mouse neurons.

While both compounds induce rapid and immediate physiological responses in mouse and human neurons, dose-dependently shifting RMP and inducing AP firing, we hypothesized that a longer-term exposure that mimicked effects of a more chronic environmental exposure would induce compensatory responses in neurons. Since concentrations ranging from 0.28-76 μg/l of both compounds have been found in the serum of humans (Imamura et al., 2005), it is important to determine how prolonged exposure to DDT or its metabolite may affect neuronal activity.

We found that either a 6 hr or 24 hr pre-treatment with DDT or DDE induced specific patterns of activity in mammalian neurons (Figure 3.7). Overall, a phenotype characterized by the presence of burst-like events was observed in more than a half of the recorded neurons at a time when compounds were no longer present in a recording medium. These results suggest that prolonged exposure of neurons to DDT or DDE can lead to longitudinal changes in neurons affecting their default physiological properties.

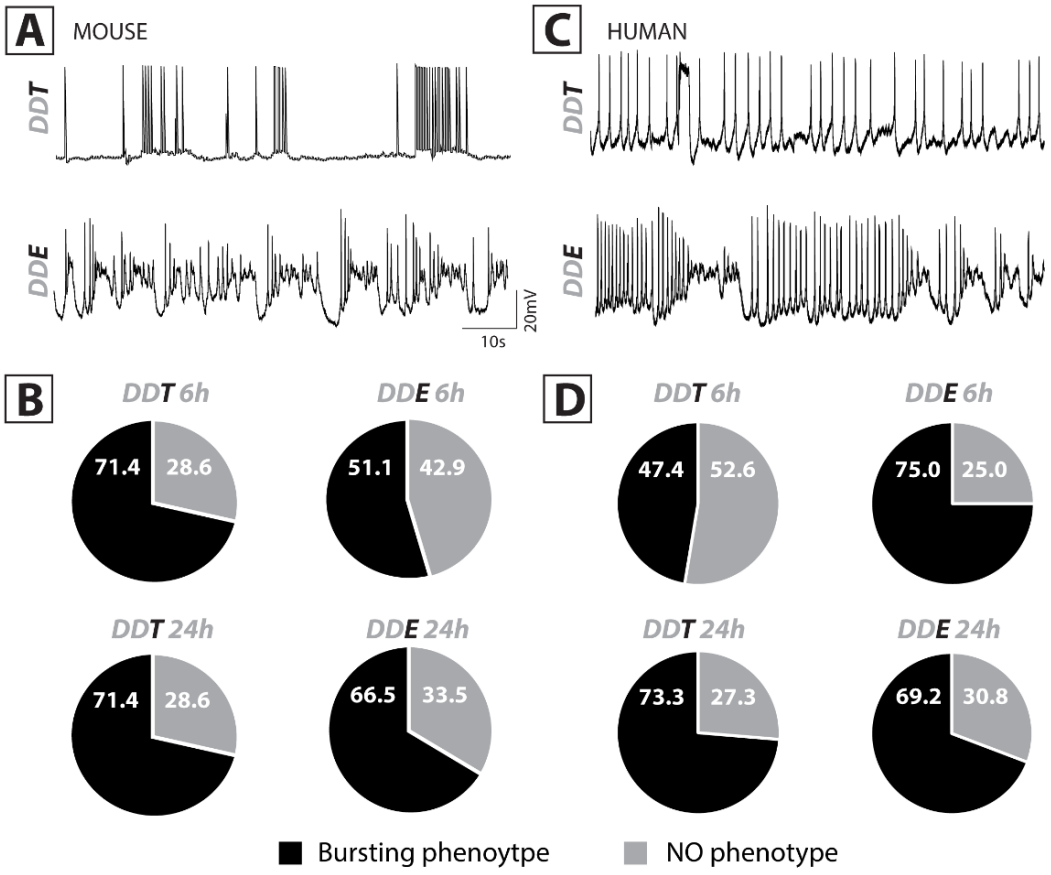


Figure 3.6 Prolonged treatment with DDT or DDE induces bursting phenotypes. (A & C) Representative traces showing examples of burst-like events following prolonged (6 or 24 hr) DDT or DDE exposure in (A) primary mouse cortical neurons or (C) induced human neurons. (B & D) The observed frequencies of the burst-like phenotype are shown in pie charts for (B) mouse or (D) human neurons. The black colored portion shows the proportion with bursting phenotypes and the grey portion indicates neurons without bursting phenotype. Bursting phenotype was not apparent in untreated neurons (not shown). Electrophysiology experiments performed by Dr. Dina Popova from the Hart lab.

5. Chronic DDT and DDE exposure induces changes to the resting state of mammalian neurons.

If prolonged exposure of mammalian neurons to DDT and DDE induced specific burst-like activity, then how is it manifested at the physiological level? To investigate this, after 6 or 24 hr treatments with DDT and DDE in human and mouse neurons, we measured several electrophysiological parameters such as RMP, basal AP firing as well as induced excitability. We found no differences between untreated (control) neurons and neurons exposed to DMSO vehicle control, whereas at both 6 hr and 24 hr treatment with DDT and its metabolite DDE introduced substantial changes to the physiological properties of mammalian neurons. We found that among all tested variables, RMP was affected most drastically, while spontaneous action potentials and action potentials generated after probing the neurons remained constant. We found that both toxicants at both time points on average induced approximately a +10 mV resting membrane potential shift. This result confirms our observation that prolonged DDT and DDE exposure induces long term differences in mammalian neurons which were distinct from acute administration.

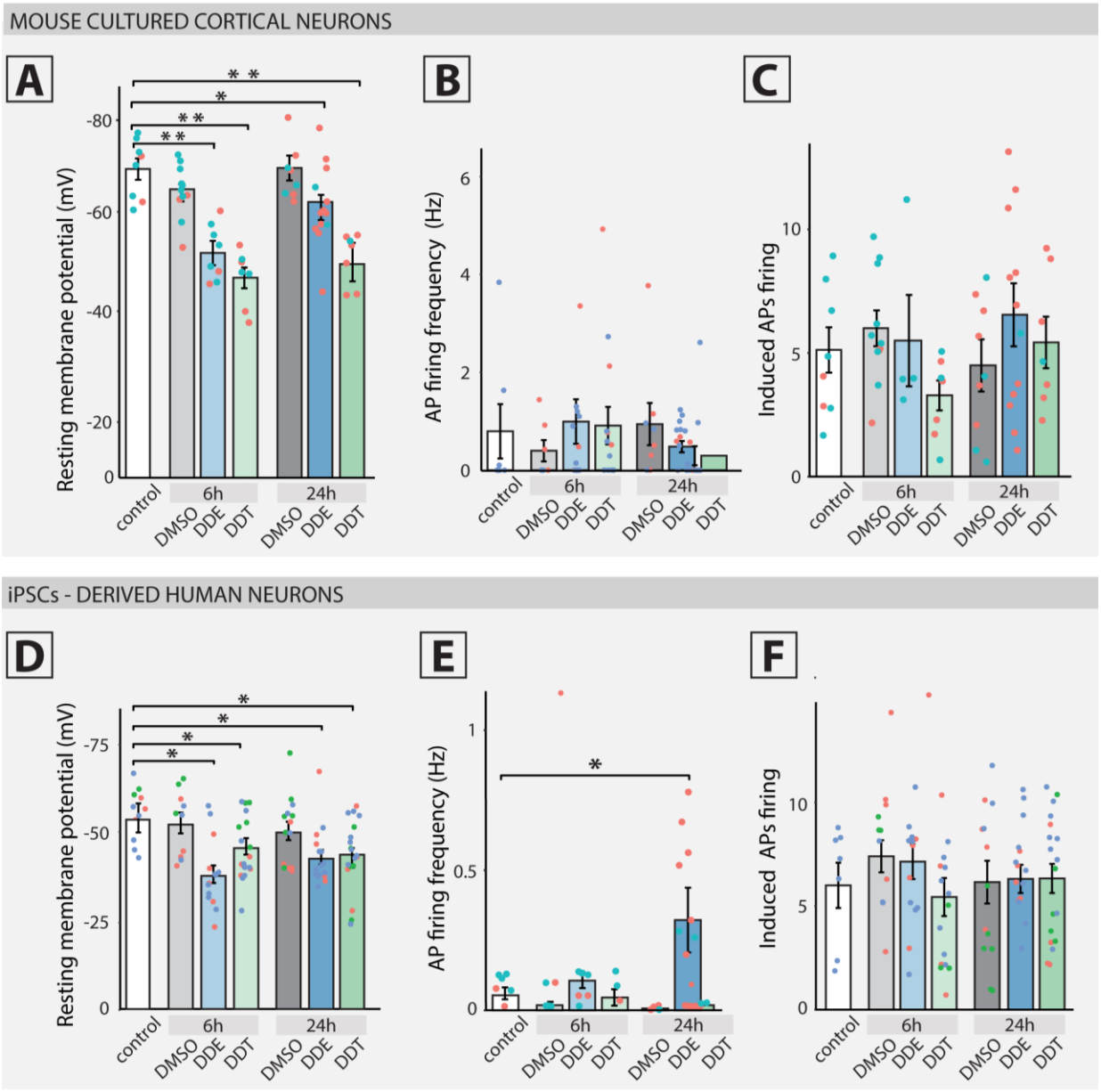


Figure 3.7 Prolonged treatment with DDT or DDE alters the resting state of the neurons. (A) Resting membrane potential (RMP) measured in primary mouse cortical neurons reveals DDT and DDE induces a +10 mV significant shift at 6 and 24 hr exposures. (B) Basal AP firing frequency measured in primary mouse cortical neurons showed no significant changes after exposures. (C) Induced AP firing frequency induced by step current injections in primary mouse cortical neurons remained unchanged when comparing the treated and untreated. (D) Resting membrane potential (RMP) measured in iPSC-induced human neurons revealed trends similar to observations seen in primary mouse cortical neurons. (E) Basal AP firing frequency measured in iPSC-induced human neurons was only induced at 24 hr with DDE exposure. (F) Induced AP firing frequency induced by step current injections in iPSC-induced human neurons showed no changes among groups. All exposures were carried out for 6 or 24 hr. with 1 µM DDT or DDE; DMSO treatment was used as vehicle control. Number of batches of independently generated cultures are depicted in colors of dots. One-way ANOVA was used to evaluate statistical differences (*p <0.05, **p <0.01). Electrophysiology experiments performed by Dr. Dina Popova from the Hart lab.

3.5 Discussion

This study demonstrates that the insecticide DDT and its metabolite DDE exert direct functional effects on mammalian neurons. Using both mouse cortical primary cultures as well as

iPSC-derived excitatory human neurons, we demonstrated that both compounds induced immediate and long-lasting changes in activity of the neurons affecting excitability. We found that only the highest doses tested (20 and 50 µM) induced neuronal lethality in dose-dependent manner, and these doses are well above 1.89 ng/mg cholesterol concentrations found in human brain (Richardson et al., 2014). Although the directions of the activity changes mediated by DDT and DDE were similar, we found that DDE caused delayed initial responses in neurons with pronounced accumulation effects, requiring longer washout periods to reverse its effects. Also, by comparing acute and longitudinal treatment strategies, we have found that prolonged exposure induces appearance of specific burst-like events in neurons which was paralleled by changes in resting membrane potential. To our knowledge, this is the first study to assess specific physiological effects of DDT and DDE action on the nervous system, specifically in mammalian neurons, which is of high relevance to human health since epidemiological studies report an association of neurodegenerative disorders with environmental exposure to organochlorine pesticides (Baldi et al., 2003; Li, Kim, et al., 2015; Richardson et al., 2009; Singh et al., 2013).

Previous studies suggest that in non-mammalian neurons DDT and its metabolite DDE affect sodium channels (Na_v) through stabilization of the open state, causing prolonged channel opening which led to persistent depolarization and hyperactivity of the nervous system (Chen et al., 2019; Lucas & Renou, 1992; Shrager et al., 1969; van der Bercken, 1972). Prolonged exposure to either DDT or DDE induces paralysis and in high concentrations it can lead to cell death through apoptosis (Wnuk, Rzemieniec, Litwa, Lason, et al., 2016).

To confirm that these compounds have similar effects on mammalian cells, we performed parallel electrophysiological analysis of cultured mouse and human neurons where DDT or DDE were introduced acutely throughout the perfusion system and physiological properties of the neurons were evaluated. We found that at a relatively low dose (1 µM) DDT induced immediate effects, causing firing of the neurons and a modest shift in resting membrane potential, while a

higher dose (10 µM) induced loss of firing and a significant shift in membrane potential. Importantly, washout of DDT showed a fast reversal of the effects on firing and membrane potential. These data suggest that, as in non-mammalian neurons, DDT at low doses potentiates sodium channel activity resulting in firing of neurons, but in high doses induces channel inactivation causing strong depolarization of the neurons and imbalance in K-Na conductance (Dong et al., 2014).

Similar to DDT, DDE also induced firing and depolarization in neurons but less dramatically, compared with DDT. However, the washout patterns were different, where in the reversal of DDE required 15 minutes compared to the 5 minutes required for DDT. Such discrepancies could be explained by different mechanism of action of investigated compounds. According to the literature, DDE exhibits higher lipophilicity compared to DDT, meaning it will partition into the lipid component of the membrane, prolonging its interaction with sodium channels (Imamura et al., 2005). This is supported when DDE levels in adipose tissue increased 13 times faster in individuals who ingested 5 mg of DDE when compared to adipose levels of DDT after DDT ingestion (Morgan & Roan, 1971). Levels of DDE in the adipose tissue of wild and laboratory animals as well as its levels in breast milk support this interpretation (ATSDR, 2002; Van Den Berg et al., 2017).

Although the synthetic pyrethroids that have been investigated exert their insecticidal activity through disruption of the nervous system, none of them is particularly toxic. However *in vitro* administration of high doses of DDT and DDE was shown to induce cell death (Wnuk, Rzemieniec, Litwa, Lason, et al., 2016). To investigate neurotoxicity of the compounds, we also determined if DDT and DDE exposure can induce cell death. We treated neurons with increasing concentration of the compounds at doses ranging between 1- 50 µM. Significant cell death was found only with highest concentrations of 20 and 50 µM, which correlates well with previously

published data using 10 and 100 μM concentrations (Wnuk, Rzemieniec, Litwa, Lason, et al., 2016).

We found that acute administration of the compounds induces clear physiological responses in mammalian neurons. But to begin to predict effects of chronic environmental exposure, prolonged treatment strategies are also important to access. We used 6 and 24 hr treatments with low dose of the compounds (1 μM) to evaluate if any physiological differences will be observed. First, we have detected immediate appearance of a distinct phenotype characterized by specific burst-like events in both mouse and human neurons after treatment which persist even at a time when compounds were no longer present in a recording medium. Events are consistent with alterations after potentiation – the hyperpolarization stage of the AP -- which could be explained by several mechanisms: (i) long-term modulation of sodium channels through secondary signaling mechanism (e.g., removal from the membrane or inactivation), (ii) alteration in Na^+-K^+ homeostasis and decrease in a K^+ conductance leading to accumulation of K^+ inside the cell, and/or (iii) bioaccumulation in a neuronal membranes, which would cause extended action on the nervous system. Prolonged exposure to these compounds alters the natural physiology of neurons that persists even after the toxicants have been removed.

The phenotypic observations were paralleled by significant overall depolarization of neurons exposed to 6 and 24 hr DDT and DDE treatments compared to vehicle control (DMSO) and untreated controls. Interestingly, we found that in comparison to acute effects, prolonged exposure only induced a shift in RMP but not in basal or induced firing of the neurons, except in the 24 hr DDE group. These findings may be a result of the overstimulation and ultimately lead to the exhaustion of the neurons. The greater effects of DDE could also be another result of its lipophilic properties. This interpretation is based on our observations of the prolonged wash-out time needed to reverse the effects of DDE as well as previous assessments revealing more accumulation of DDE vs. DDT in human adipose tissue after daily ingestion for up to 183 days

(Morgan & Roan, 1971). In the long term, these changes could lead to either diminished responsiveness of the neurons, since it is close to depolarization block of the Na channels (-20mV), or in another way – increased excitability, where minimal stimulation will cause physiological response in a neuron.

In conclusion, our findings align well with previous physiological and behavioral observation from non-mammal organisms where DDT and DDE target sodium channels (Na_v) through stabilization of the open state, causing prolonged channel opening, hyperactivity at the level of the behavior, which leads to persistent depolarization of neurons. However, our results uniquely demonstrate that even a relatively short-prolonged exposure can have consequences on human brain neurons, affecting the ability to function in circuits. This further expresses some concerns DDT and DDE where even in a controlled and minimized setting, exposure may lead to long term health effects in humans. Since evidence points to ongoing chronic environmental exposure in humans, more work is required to identify strategies to wash out bioaccumulated DDT or especially DDE, or to ameliorate effects on neuronal function.

CHAPTER 4: THE E4 VARIANT OF APOE, DDT AND DDE SYNERGIZE TO INDUCE APP, PTAU AND Aβ40/42 IN HUMAN STEM CELL DERIVED NEURONS

4.1 Abstract

The E4 variant of the Apolipoprotein E (APOE) gene contributes the greatest single genetic risk for Alzheimer Disease (AD), but it is also affected by factors including age and environmental exposure. The pesticide dichlorodiphenyltrichloroethane (DDT) has been found to be an environmental contaminant associated with an increased risk for developing AD. The risk is related to the capability of DDT to persist in the environment as well as its ability to bioaccumulate. Elevated serum levels of its metabolite, dichlorodiphenyldichloroethylene (DDE), have been found in AD patients despite the parent compound having been banned in the U.S. over fifty years ago. Previous results found that there were significant interactions between serum DDE levels, APOE genotype and cognitive dysfunction, with APOE E4 genotype and higher DDE levels being associated with worsened cognitive function. To examine the mechanisms underlying this relationship, we prepared induced excitatory neurons (iNs) from human iPSCs carrying either an APOE knockout (APOE -/-), both E3 and E4 variant alleles (E3/E4), or hemizygous lines with either the E3 (E3/-) or E4 (E4/-) variant and exposed them to environmentally relevant concentrations (250 nM, 500 nM and 1 μM) of DDT and DDE. Results show that both DDT and DDE increased levels of APOE, amyloid precursor protein (APP) and phosphorylated Tau (pTau) as well as enhanced secretion of amyloid beta 40/42 (Aβ40/42). Potentiation of Aβ40/42 secretion, APP and pTau expression was more prominent when neurons carrying the E4 variant were exposed to low 250 and 500 nM concentrations of DDE. The greater lipophilicity of DDE may result in enhanced physical interaction of DDE with the E4 variant of APOE. These results provide a potential mechanistic framework to understand the synergistic relationship observed epidemiologically.

4.2 Introduction

Alzheimer's disease (AD) accounts for 60-80 % of all dementia cases; characterizing it as the most prevalent neurodegenerative disease (Imtiaz, Taipale, et al., 2017). The disease pathology is associated with neuronal damage due to the molecular accumulation of amyloid plaques and phosphorylated Tau (pTau) protein (Powell et al., 2013; Rhinn et al., 2013). However, while its hallmarks have been identified and studied, AD remains incurable (Imtiaz, Taipale, et al., 2017). However to improve the quality of life, AD patients are typically prescribed cholinesterase inhibitors Donepezil - Aricept® and Galantamine - Razadyne® that may help in managing the cognitive and behavioral symptoms, but there is a need for assessing the contributing factors of AD (Jha & Mukhopadhaya, 2021). The incidence of the disease can be affected by age, genetics, and environmental factors such as pesticides. While many individual risk factors have been well established, combinatory factors have not been extensively explored. A 2014 study revealed a 3.8-fold higher level of dichlorodiphenyldichloroethane (DDE), a metabolite of the pesticide dichlorodiphenyltrichloroethane (DDT), found in the serum of Alzheimer's disease (AD) patients (Richardson et al., 2014). This study also observed that SY5Y human neuroblastoma cells exposed to 1 µM DDT and DDE for 24 hr induced APP protein expression. Moreover, DDE levels were higher in the serum of AD patients which was associated with more cognitive impairment in individuals carrying the E4 variant of APOE (Richardson et al., 2014). With that in mind, we wanted to explore the synergistic relationship between genetics and environmental contributions on the potentiation of AD pathogenesis in an *in vitro* model.

ApoE is a plasma protein, encoded by the APOE gene, which participates in cholesterol transport and uptake by binding to ApoE receptors (Huang et al., 2017; Strittmatter et al., 1993). There is substantial evidence that the strongest genetic risk factor for developing AD is the E4 variant of APOE (Huang et al., 2017; Saeed et al., 2018; Strittmatter et al., 1993; Zhao et al., 2014) and when considering sex differences, is even stronger in women (Altmann et al., 2014). Evidence

also supports the relatively lower risk of the E3 variant and the protective feature of the E2 variant reducing the risk of developing the disease (Goldberg et al., 2020; Huang et al., 2017). The variants differ based on the quantity of cysteine residues at positions 112 and 158, where the E4 variant has two, E3 has 1 and E2 has zero (Weisgraber et al., 1981). These differences alter the charge of APOE which in turn alters how the protein is folded. As a result of this altered protein folding, the E4 variant is may be more susceptible to proteolysis, compared to the E3 and E2 variants, which would result in less APOE to transport cholesterol and clear Aβ (Rohn, 2013). Although studies have explored the behavior of APOE as it relates to lipid transportation, there are still gaps in how it influences the progression of AD.

Several studies have explored how the gut microbiome and gut-brain interaction influence the development of AD (Jiang et al., 2017). The gut-brain axis is responsible for monitoring and integrating gut functions. It serves to connect the cognitive regions of the brain to the functions of the peripheral intestine (Carabotti et al., 2015). Neuro-immuno-endocrine mediators are used to maintain the bidirectional communication between the gut's autonomic (ANS) and enteric (ENS) nervous systems with the central nervous systems (CNS) and the hypothalamic pituitary adrenal (HPA) axis (Carabotti et al., 2015). Afferent signals from the ANS travels through the vagal, enteric, and spinal pathways before reaching the CNS where efferent signals from the HPA are relayed to the intestinal wall for an adaptive response (Tsigos & Chrousos, 2002). The gut microbiome has influences on the gut-brain axis to initiate anxiety and depression when a study revealed Alistipe, a genus in the phylum Bacteroidota, has been linked to inducing stress in mice (Naseribafrouei et al., 2014). There are multiple factors that alter the gut microbiome such as diet, age, and even APOE. The variant differences of APOE observed with the risk of AD, E2 lowering the risk and E4 increasing the risk, are consistent when looking at the gut microbiome composition. Bacterial taxa and metabolomic analysis of human fecal samples and APOE–targeted replacement (TR) mice revealed that the abundance of certain "good" bacteria in particular Prevotellaceae and

Ruminococcaceae was higher in the presence of E3 and E2 when compared to E4 (Tran et al., 2019). The reduction of these bacteria typically associated with a healthy individual could contribute to the increased risk the E4 variant has on AD development. Interestingly, Prevotellaceae and Ruminococcaceae abundance has been observed to be drastically lowered in AD patients (Vogt et al., 2017). In addition, the APOE variants have also been shown to differentially alter the rate at which the Aβ-40 and Aβ-42 peptides are secreted, enhance APP mRNA and protein levels, and activate the mitogen-activated protein (MAP) kinases that are expressed in the brain (Huang et al., 2017). Another study observed that the presence of the E4 variant impacts the ability of human microglia-like cells to control their cytokine secretion, perform metabolic function, and carry-out phagocytosis (Konttinen et al., 2019). The influence APOE has on the development and/or progression of AD appears to be targeted through direct and indirect pathways.

Pesticides in the OCP class include DDT and its metabolite DDE (Hatcher et al., 2008). In order to combat malaria and other vector-borne diseases, DDT was the primary pesticide used in agriculture during the era of World War II (Hatcher et al., 2008; Richardson et al., 2014). Due to the long half-life of DDT ranging from 2-15 years in the soil and up to 20 years for its metabolite DDE, it poses as a persistent threat to the environment (ATSDR, 2002; Hatcher et al., 2008). Their other properties include their ability to bioaccumulate and biomagnify (ATSDR, 2002). Biologically, these compounds are highly lipophilic and have been known to accumulate in human adipose tissue with the capability to transport back and forth to the blood (Morgan & Roan, 1971). DDT is known to target the embedded voltage gated sodium channels (Na_v) of neurons preventing its normal function of closing and controlling the influx of sodium ions (Lenaeus et al., 2017; Stuart et al., 1987; van der Bercken, 1972). This chemical modification induces a rapid burst of activity resulting in delayed repolarization after an action potential and increased negative afterpotential, requiring more time to recover before generating another action potential (Chen et al., 2019; Lucas & Renou, 1992; Shrager et al., 1969; van der Bercken, 1972). Biochemically, evidence supports

DDT's ability to target downstream genes ATP-binding cassette transporter A1 as well as a reduce the efficiency in oxidative phosphorylation showing to be more prominent in brain mitochondrial fraction than the liver (Byczkowski, 1976; Li, Kim, et al., 2015; Wu et al., 2021). These observations corroborate some of the variety of biochemical alterations that have been observed in AD patients. More direct associations namely increases in APP, BACE1, and impairment of the extracellular degradation and intracellular clearance of Aβ have been observed with DDT exposure to human neuroglioma (H4) cells (Li, Kim, et al., 2015; Wu et al., 2021).

Individually, these factors, the E4 variant of APOE and exposure to DDT, have been observed to induce AD risk directly and indirectly. However, there are limited studies observing the combinatory phenomenon because of the difficulty of studying this human disease in animal models. To bridge this gap, human stem cell-derived neurons, the only ethical way of preparing human neuron cultures, were utilized to explore the mechanistic interactions between APOE and DDT and how they can synergistically enhance the risk of developing AD by increasing hallmarks of AD. A better understanding of this can provide insight to the pathogenesis of Alzheimer's disease which can ultimately lead to the identification of novel mediators relating to the disease. This awareness also has the potential in opening doors for targeting different stages of AD.

4.3 Material and Methods

Materials

Induced Pluripotent Stem Cells (iPSC) were maintained in mTeSR™ 1 medium (STEMCELL Technologies; 05850). Before use, 6-well sterile culture dishes were coated with Matrigel (Corning® Matrigel® hESC-Qualified Matrix; 354277) and incubated (5 % O_2, 5 % CO_2, 37°C) for at least 45 minutes or overnight. Cells were maintained to until they reached approximately 80% confluency prior to weekly passage or single cell retrieval. Cells were split once a week using ReLeSR™ (STEMCELL Technologies; 05872) or Dispase (BD Biosciences; 354235) to preserve healthy colony formation by minimizing spontaneous differentiation. To obtain single cells, iPSCs

were rinsed with phosphate-buffered saline (PBS) before incubating with 1 mL ACCUTASE™ (STEMCELL Technologies; 07920) for 5 minutes. PBS was used to inactivate ACCUTASE activity by diluting the cell mixture. Single cells were then collected, counted and prepared for the appropriate application. In order to prevent apoptosis after single cell collection, 5 µM Y27632 (y-compound, Tocris; 1254) was added to mTeSR™ 1 medium.

Differentiation of Induced Neurons from iPSC

To collect single cells for glutamatergic neuronal differentiation, iPSCs were treated with ACCUTASE™ (STEMCELL Technologies; 07920) and infected in suspension with lentiviruses containing doxycycline inducible neurogenin 2 (Ngn2) co-expressed with the drug resistance gene puromycin, the reverse tetracycline-controlled transactivator (rtTA), 5 µM Y27632 (y-compound, Tocris; 1254) and 2 ng/µL of doxycycline (Pang et al., 2011; Vierbuchen et al., 2010; Zhang et al., 2013). Transduced cells were selected by addition of 1 µg/ml puromycin from days 2 to 4 following infection. At day 5 post infection, the induced neurons (iNs) were plated on the mouse glia and treated with a final concentration of 4 µM cytosine arabinoside (AraC; Sigma-Aldrich; C1768) on day 8 to inhibit the growth of the dividing glial cells. Co-cultures were then maintained with half media changes of Neurobasal Plus Medium plus B27+ (NB2) (Thermo Fisher Scientific: catalog number A3653401) every 4-5 days until neuronal maturity; approximately 36 days post lentiviral infection. Electrophysiology was used to assess maturity by measuring the Resting Membrane Potential (RMP) and spontaneous glutamate release (sEPSCs).

Treatments

Dichlorodiphenyltrichloroethane (DDT) (Chem Service, Inc; N-10876-100MG) and dichlorodiphenyldichloroethylene (DDE) (Chem Service, Inc; N-10875-100MG) were dissolved in sterile DMSO (Sigma Aldrich; D2650) to a final concentration of 100 mM. Compounds are stored

at -20°C in small volumes in amber glass vials (Sigma Aldrich; 29663-U) to avoid frequent freeze thaw cycles. The purity and concentration of DDT and DDE were validated using GC-MS by the Chemical Analysis Facility Core at EOHSI, Rutgers University (spectra shown in Appendix). Mature mammalian neurons were exposed to vehicle control (DMSO), 1 µM, 250 nM, or 500 nM of DDT and DDE for 24 or 48 hours prior to PFA fixation or cell lysis. All DDT and DDE dilutions were made in amber glass vials using sterile DMSO, which did not exceed 0.2 % (v/v) in treatment media.

Western Blot

Cells were lysed using RIPA lysis buffer containing 50 mM Tris HCl, 150 mM NaCl, 1.0% (v/v) NP-40, 0.5% (w/v) Sodium Deoxycholate, 1.0 mM EDTA, 0.1% (w/v) SDS, 0.01% (w/v) sodium azide at a pH of 7.4 and 1X HALT protease inhibitor (Thermo Fisher; 78430). The Pierce™ BCA Protein Assay Kit (Thermo Fisher; 23225) and a spectrophotometer set to 562 nm was utilized to quantify protein concentrations. Protein (30 µg intracellular and 2.5 µg extracellular) were loaded onto a SDS-polyacrylamide 4-12% Bis-Tris gel (Life Technologies) and resolved by electrophoresis. Protein bands were transferred onto polyvinylidene fluoride membranes using the Invitrogen iBlot Dry Blotting System for 8 minutes. Once transferred, membranes were stained using the Revert™ 700 Total Protein stain and wash solution kit (LI-COR Biosciences; 926-11010) to reveal total protein content; staining was then stripped prior to blocking. Membranes were blocked in 5% non-fat milk in 0.5% Tween 20-PBS for 1 hr. before incubating them with primary antibodies overnight at 4°C. All primary antibodies were diluted in 5% non-fat milk in 0.5% Tween 20-PBS. Species-appropriate secondary antibodies (LI-COR Biosciences; 926-68071 & 926-32210) were used to probe the primary antibodies. Li-Cor Odyssey FC was used to detect protein signals.

Immunocytochemistry - Confocal

A two-to-one ratio of glia (50,000 cells) to neurons (100,000 cells) were maintained on Matrigel-coated glass coverslips were fixed with 4% PFA for 15 mins at room temperature and washed three times with phosphate-buffered saline (PBS) for 10 mins. Fixed cells were then permeabilized using the blocking buffer (0.1 % Triton X-100 and 4 % normal goat serum in PBS) for 1 hr. at room temperature. Blocking buffer was then replaced with the primary antibodies diluted in the blocking buffer and allowed to bind overnight at 4 °C. Prior to incubating the coverslips for 2 hr with the appropriate Alexa Fluor secondary antibodies and Hoechst 33342 (Thermo Fisher; H3570) diluted in the blocking buffer, they were washed three times with immune-wash buffer (0.05 % Triton X-100 in PBS) for 10 mins. Coverslips were mounted onto glass slides using mounting medium in preparation for confocal imaging using a Zeiss LSM700. Neurons chosen for analysis were selected based on healthy MAP2 staining indicative of cohesive neurites.

Immunocytochemistry - BZ-X800

Cells maintained on Matrigel-coated Greiner black 96-well plates (Greiner bio-one, 655090) were fixed with 4% PFA for 15 mins at room temperature and washed three times with phosphate-buffered saline (PBS) for 10 mins. Fixed cells were then permeabilized using the blocking buffer (0.1 % Triton X-100 and 1 % BSA in PBS) for 1 hr. at room temperature. Blocking buffer was then replaced with the primary antibodies diluted in the blocking buffer and allowed to bind overnight at 4 °C. Secondary antibody binding was performed at room temperature in the dark for 1 hr. with the appropriate Alexa Fluor secondary antibodies diluted in the blocking buffer. Cells were washed three times with 1X PBS for 5 mins before adding 1:1000 DAPI solution made in 1x PBS for 10 mins. BZ-X800 analyzer Macro Cell Count software was used to analyze images. Parameters such as setting thresholds for integrated density were set to control images; parameters were then applied automatically to the other images.

Multi-Spot Assay System (MSD)

Extracellular Aβ40 and Aβ42 was assessed post DDT and DDE neuronal exposure using the V-PLEX Plus Aβ Peptide Panel 1 (4G8) Kit. MSD detection antibody, wash buffer, read buffer T and plate were prepared following the kits recommendations. Samples were stored at -80 °C and thawed on ice prior to introducing it neat (25 µL total) to prepared plates for an overnight incubation at 4°C. Plate was analyzed using MESO QuickPlex SQ 120.

Statistical Analysis

Data were expressed as mean ± standard error of the mean (SEM). One-way repeated measures ANOVA was used as appropriate. Statistical differences for Figures 4.2-4.5 were adjusted using Bonferroni multiple testing correction method. RStudio (RStudio Software Company, Boston, MA) was used for statistical analysis with *p <0.05, **p <0.01, ***p <0.001 considered as statistically significant. Figures 4.6 and 4.7, statistical differences were determined with Tukey's multiple comparisons test. GraphPad Prism 7.03 (GraphPad Software, San Diego, CA) was used for statistical analysis with *p <0.05 considered as statistically significant.

4.4 Results

The overarching goal was to determine the potential synergistic relationship between APOE E4 and DDT or DDE on inducing AD pathology. However, we first sought to determine if these toxicants could alter the expression of APOE.

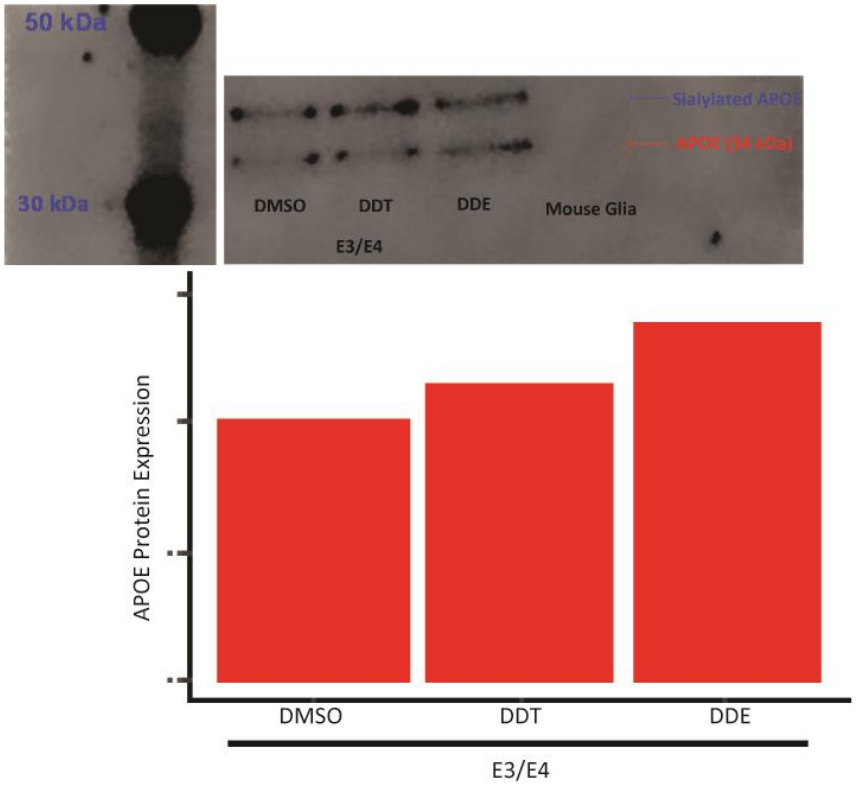


Figure 4.1 DDT and DDE induce APOE protein expression. (A) Western Blot represents the effects of APOE in E3/E4 human differentiated iNs post exposure to DDT or DDE. APOE bands were detected in 2 forms, unmodified APOE and sialylated APOE (top). Unmodified APOE protein for each sample was quantified and normalized to its total protein detected in the lane (bottom). E3/E4 iNs were co-cultured on mouse glia, which did not express human APOE, as demonstrated by the "Mouse Glia" lane presented above. The concentration of DDT and DDE was 1 µM diluted in DMSO. Exposure time was limited to 48 hours. Quantification is presented as one independent study.

We wished to determine whether DDT or DDE is capable of altering the expression of APOE in neurons, so we exposed differentiated neurons to DDT and DDE and monitored APOE protein expression. Co-cultures of heterozygous E3/E4 iNs grown on primary mouse glia were exposed to DDT or DDE for 48 hours. As revealed in our electrophysiology studies and other studies DDE lipophilicity is greater than DDT (Imamura et al., 2005; Morgan & Roan, 1971). This was observed when individuals who ingested 20 mg/day for 183 days revealed a mean regression

rate of DDT stored in adipose tissue of 2.95 mg/day and a rate of 0.35 mg/day for individuals who ingested 10 mg/day (Morgan & Roan, 1971). However, there were no significant loss of DDE stored in adipose tissues within the 8 months after stopping exposure of individuals who ingested 5 mg/day for 92 days (Morgan & Roan, 1971). Knowing the difference in lipophilicity and APOE's participation in lipid transportation, we proposed that APOE expression may trending higher with DDE. As APOE is heavily involved in transportation of lipoproteins, the interaction between DDE and APOE may be greater when compared to APOE and DDT. However, we showed that APOE expression in heterozygous E3/E4 iNs following DDT and DDE exposure is virtually the same. (Figure 4.1). While DDT and DDE did not reveal that there is a direct relationship with ApoE, the pathways affected by either DDT or APOE may overlap, revealing a potential gateway to how both components can work together to increase the risk of developing AD..

To validate whether the presence or absence of APOE alongside DDT and DDE affects the initiation or progression of AD, we exposed APOE-/- and E3/E4 iNs to both toxicants and assessed the expression of amyloid precursor protein (APP) and phosphorylated Tau (pTau) by

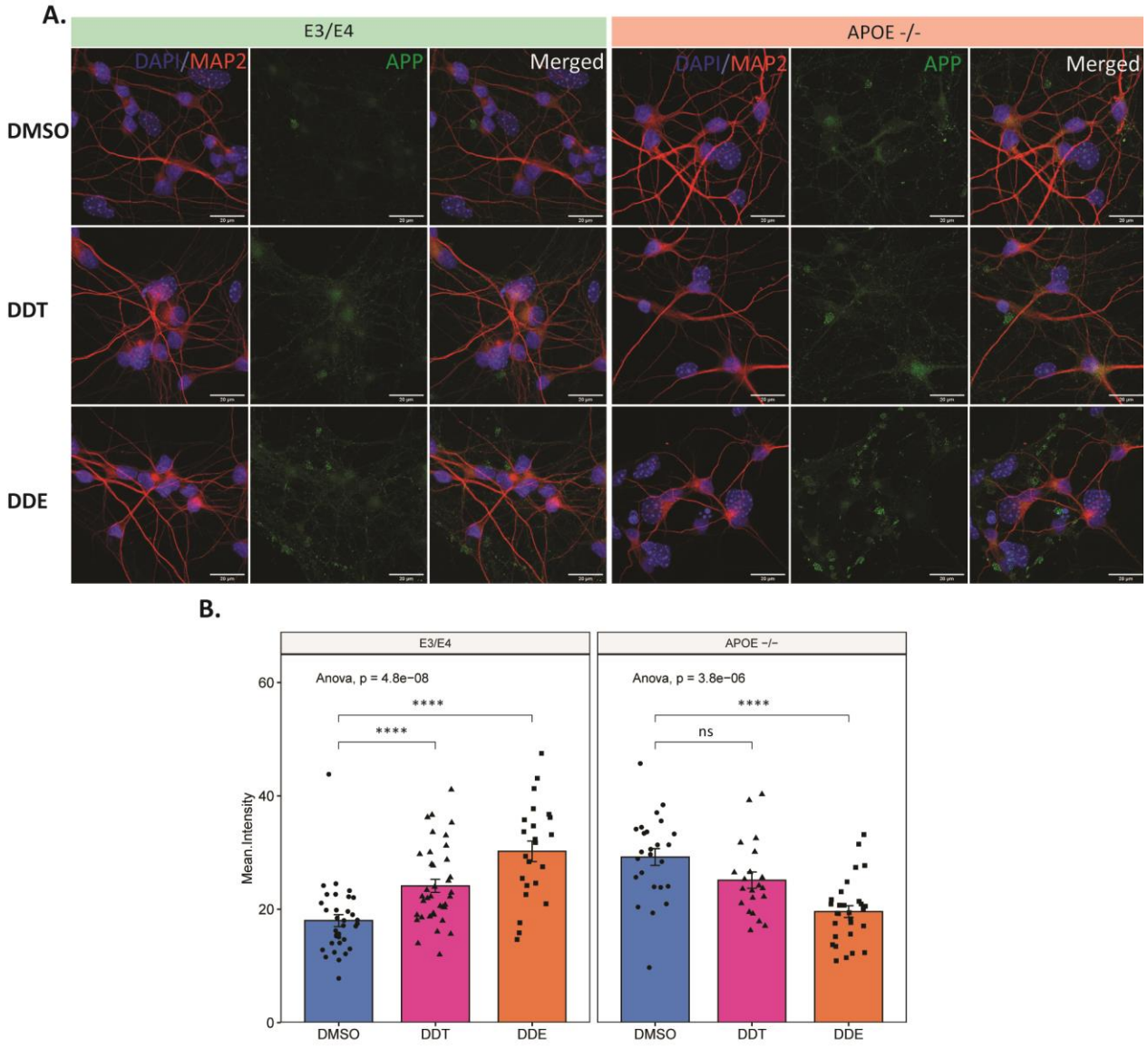


Figure 4.2 DDT and DDE increase APP expression in human iNs after 24 hours. Representative images (A) of APOE E3/E4 (Left) and APOE -/- iNs (Right) expressing Hoechst (blue), MAP2 (red), and APP (green). APP expression was assessed only in MAP2 positive neurons. (B) APP intensity was measured per cell, quantified, and compared across all groups. APP expression was significantly increased in the heterozygous E3/E4 iNs after 24 hr exposure to DDT and DDE (left panel B). However, APOE -/- iNs revealed no change in APP expression with DDT exposure and a significantly decrease in APP expression post DDE (right panel B). One-way ANOVA was used to evaluate statistical differences followed by pairwise t-test Bonferroni correction for multiple measurements (ns p>0.05, *p <0.05, **p <0.01, ***p <0.001, ****p <0.001). Each data point represents measurements from one cell.**p <0.001). Each data point represents measurements from one cell.

immunocytochemistry. Prior to using our more sensitive data collection method, we intended to

determine if there are any detectable changes when looking at the cultures has a whole. After exposing the heterozygous E3/E4 iNs to DDT and DDE for 24 hr, we were able to observe significant increases in APP expression (Figure 4.2A). APP expression was not changed when APOE -/- iNs were exposed to DDT for 24 hr but was significantly lowered when the cells were exposed to DDE. This data provides the first evidence that APOE is required for DDT and DDE to induce APP. However, to determine if a longer exposure to DDT and DDE mimics or worsens the induction of APP, we exposed the iNs for 48 hr APP expression is significantly increased after a 48 hr exposure to DDT and DDE in the heterozygous E3/E4 iNs. APP expression was not changed when APOE -/- iNs were exposed to DDT or DDE for 48 hr. This further supports the requirement for APOE in observing any inductions in APP expression after DDT and DDE exposure.

Next, we looked at pTau, which in the hyperphosphorylated state can lead to the destabilization of neuronal microtubules (Alonso et al., 1994; Gong et al., 2005). While Tau is used

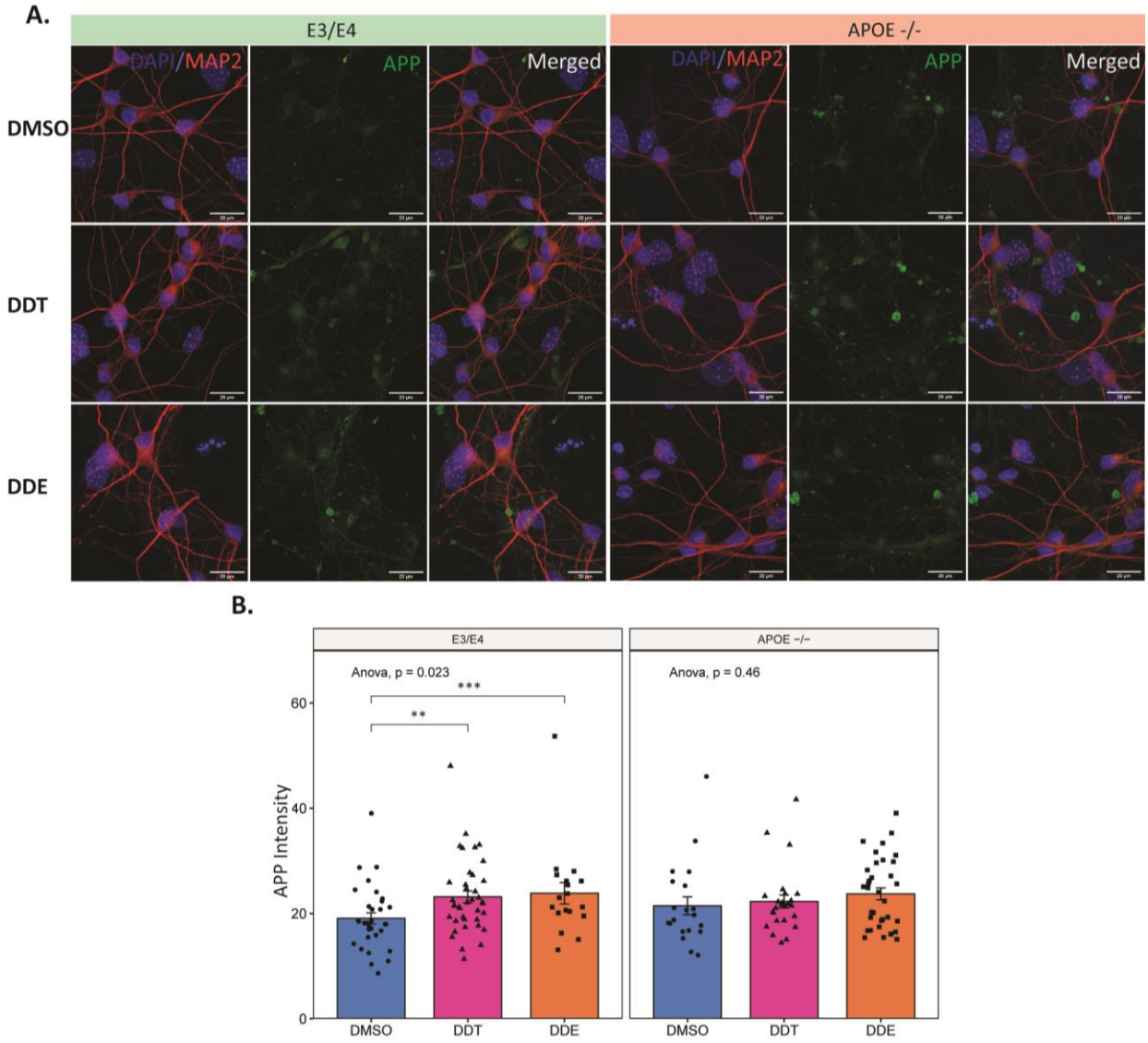


Figure 4.3 DDT and DDE increase APP expression in human iNs after 48 hours. Representative images (A) of APOE E3/E4 (Left) and APOE -/- (Right) iNs expressing Hoechst (blue), MAP2 (red), and APP (green). APP expression was assessed only in MAP2 positive neurons. (B) APP intensity was measured per cell, quantified, and compared across all groups. APP expression was significantly increased in the heterozygous E3/E4 iNs after 48 hr exposure to DDT and DDE (left panel B). However, APOE -/- iNs revealed no change in APP expression with DDT and DDE exposure (right panel B). One-way ANOVA was used to evaluate statistical differences followed by pairwise t-test Bonferroni correction for multiple measurements (ns p>0.05, *p <0.05, **p <0.01, ***p <0.001, ****p <0.001). Each data point represents measurements from one cell.

clinically to detect general neurodegeneration, pTau is a marker specifically upregulated during AD pathology as it is associated with the formation of neurofibrillary tangles (NFT) (Wattmo et al., 2020). We wanted to gain further confirmation that the type of distinct molecular feature observed in our cultures was specific to AD, so we observed if there were any changes in pTau,

after exposing human iNs for 24 hr to DDT and DDE. The expression of pTau was increased in E3/E4 neurons exposed to DDE but revealed no significant changes when exposed to DDT (Figure 4.4A). There were no changes in expression of pTau when the APOE -/- neurons were exposed to

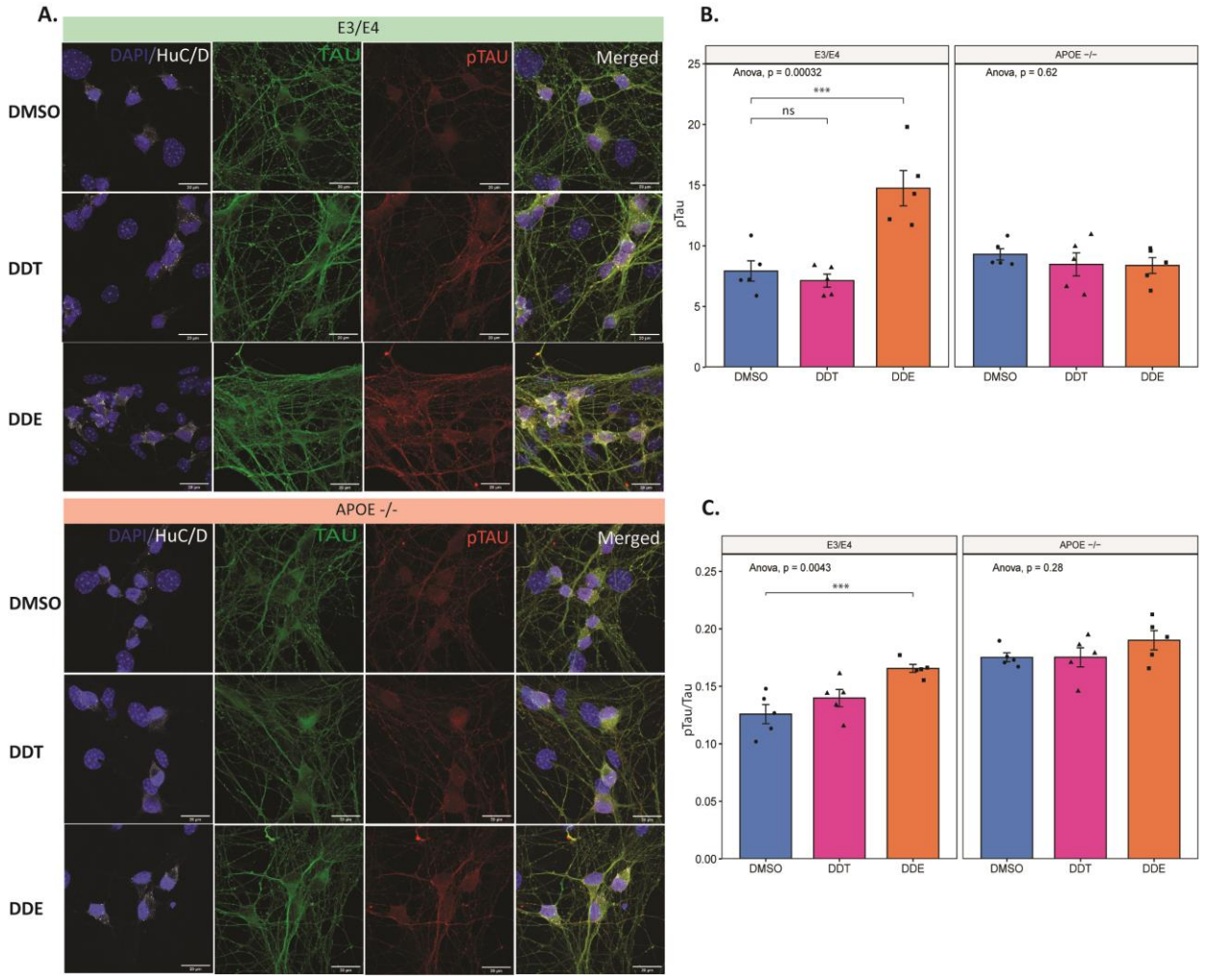


Figure 4.4 DDT and DDE increase pTau expression in human iNs after 24 hours. Representative images (A) of heterozygous APOE E3/E4 (Top) and APOE -/- (Bottom) iNs expressing Hoechst (blue), HuC/D (gray), Tau (green), and pTau (red). Phosphorylated Tau (pTau) was assessed only in HuC/D positive neurons. (B) pTau intensity was measured per field, quantified, and compared across all groups. The expression of pTau was significantly increased in the heterozygous E3/E4 iNs after 24 hr exposure to DDE while no significant changes when exposed to DDT (left panel B). APOE -/- iNs revealed no change in pTau expression after DDT and/or DDE exposure (right panel B). The ratio of pTau to Tau was quantified showing same significant increases in the E3/E4 iNs exposed to DDE (left panel C) and lack thereof in the null line exposed to both compounds. APOE -/- iNs revealed no change in pTau expression after DDT and/or DDE exposure (right panel C). One-way ANOVA was used to evaluate statistical differences followed by pairwise t-test Bonferroni correction for multiple measurements (ns $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.001$). Each data point represents averaged measurements from multiple cells in one field view.

either DDT or DDE (Figure 4.4B). No alteration in pTau expression when considering the APOE -/- neurons emphasize the importance of the genotype where these toxicants are concerned.

However, considering that DDT increases APP expression, the absence of pTau induction in the heterozygous neurons that were exposed to DDT was unexpected.

Cerebrospinal fluid (CSF) Aβ40/42 and pTau biomarkers have been used to clinically detect AD due to their high sensitivity and specificity (Shi et al., 2011). However, the collection of CSF for the purpose of analyzing these biomarkers can be invasive and expensive. To minimize the sampling side effects, research has been conducted to detect AD biomarkers in the saliva. The first study assessed saliva from AD patients and control patients using Mass spectrometry (Shi et al., 2011), while the other utilized western blotting (Pekeles et al., 2018). Despite the assessment method used, both investigations found significant increases in pTau/Tau ratios in AD patients compared to controls. This demonstrates that salivary Tau may be shifted toward the phosphorylated form which is important for disease onset and progression in AD patients. As a result, we wanted to determine if we would see a similar induction when assessing pTau/Tau after exposing human iNs for 24 hr to DDT and DDE. The same pattern observed with pTau was maintained when the ratio between pTau and Tau were assessed.

To determine if longer exposure of DDT and DDE leads to greater inductions in Tau pathology markers, we exposed the iNs for 48 hr. Contrary to the results observed with APP expression during both time points, 24 hr and 48 hr exposures revealed opposite effects. After 48 hr exposure to DDT and DDE, there was a significant increase in pTau, however, there was a

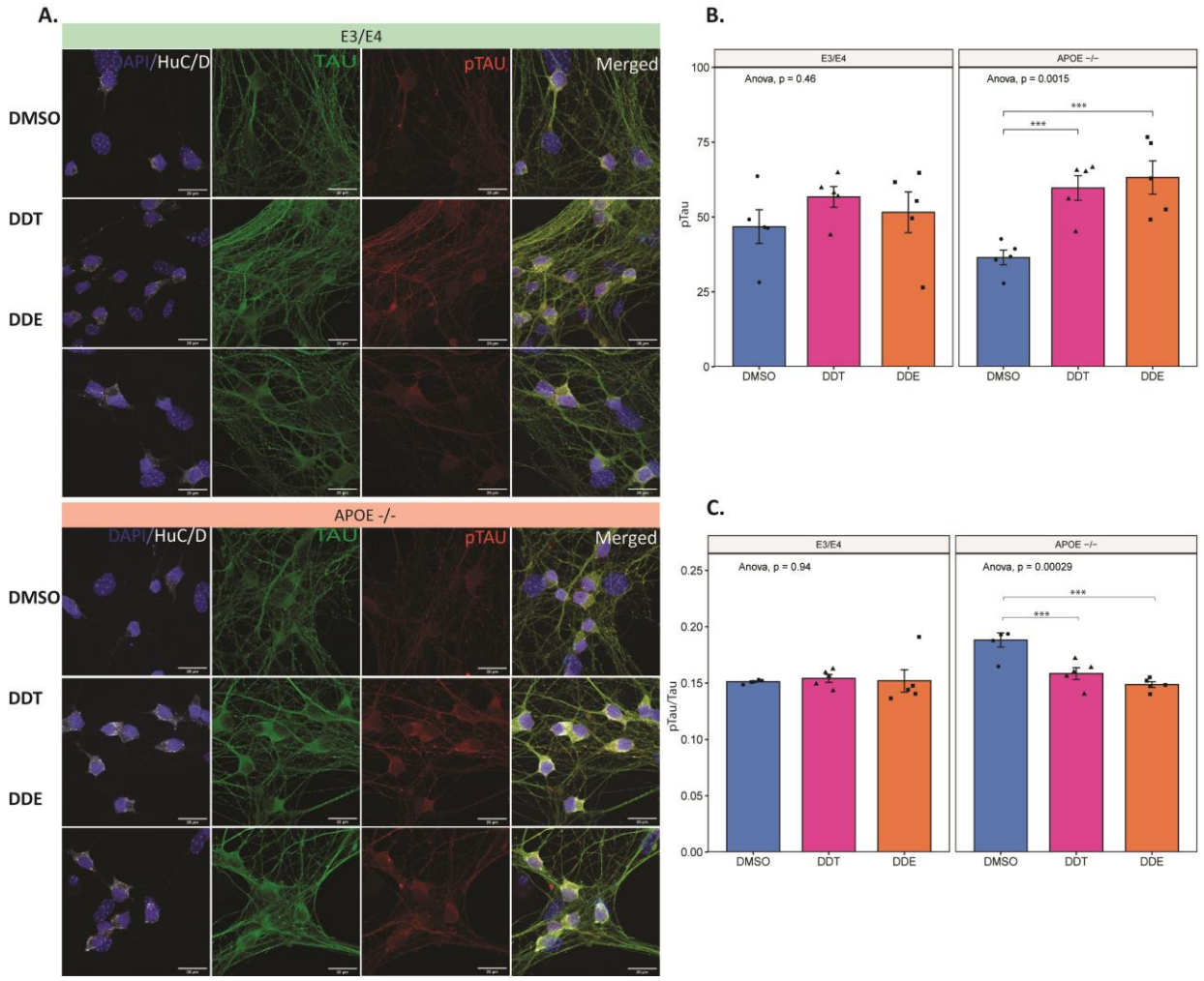


Figure 4.5 DDT and DDE increase pTau expression in human iNs after 48 hours. Representative images of (A) APOE E3/E4 and (B) APOE -/- iNs expressing Hoechst (blue), HuC/D (gray), Tau (green), and pTau (red). Phosphorylated Tau (pTau) was assessed only in HuC/D positive neurons. (C) pTau intensity was measured per field, quantified, and compared across all groups. The expression of pTau was significantly increased in the APOE -/- iNs after 48 hr exposure to DDT and DDE (right panel C). E3/E4 iNs revealed no change in pTau expression after DDT and/or DDE exposure (left panel C). The ratio of pTau to Tau was quantified showing a downward significance in the null cells exposed to DDT and DDE (right panel D). There was still no change when the E3/E4 iNs exposed to DDT and DDE were assessed (left panel D). One-way ANOVA was used to evaluate statistical differences followed by pairwise t-test Bonferroni correction for multiple measurements (ns $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.001$). Each data point represents averaged measurements from multiple cells in one field view.

significant decrease in pTau/Tau ratio in the APOE -/- iNs (Figure 4.5). However, neither compound induced any changes in the E3/E4 iNs. These confounding results suggest that the

presence of the APOE genotype, in this case the E3 and E4 variants, may change the rate of

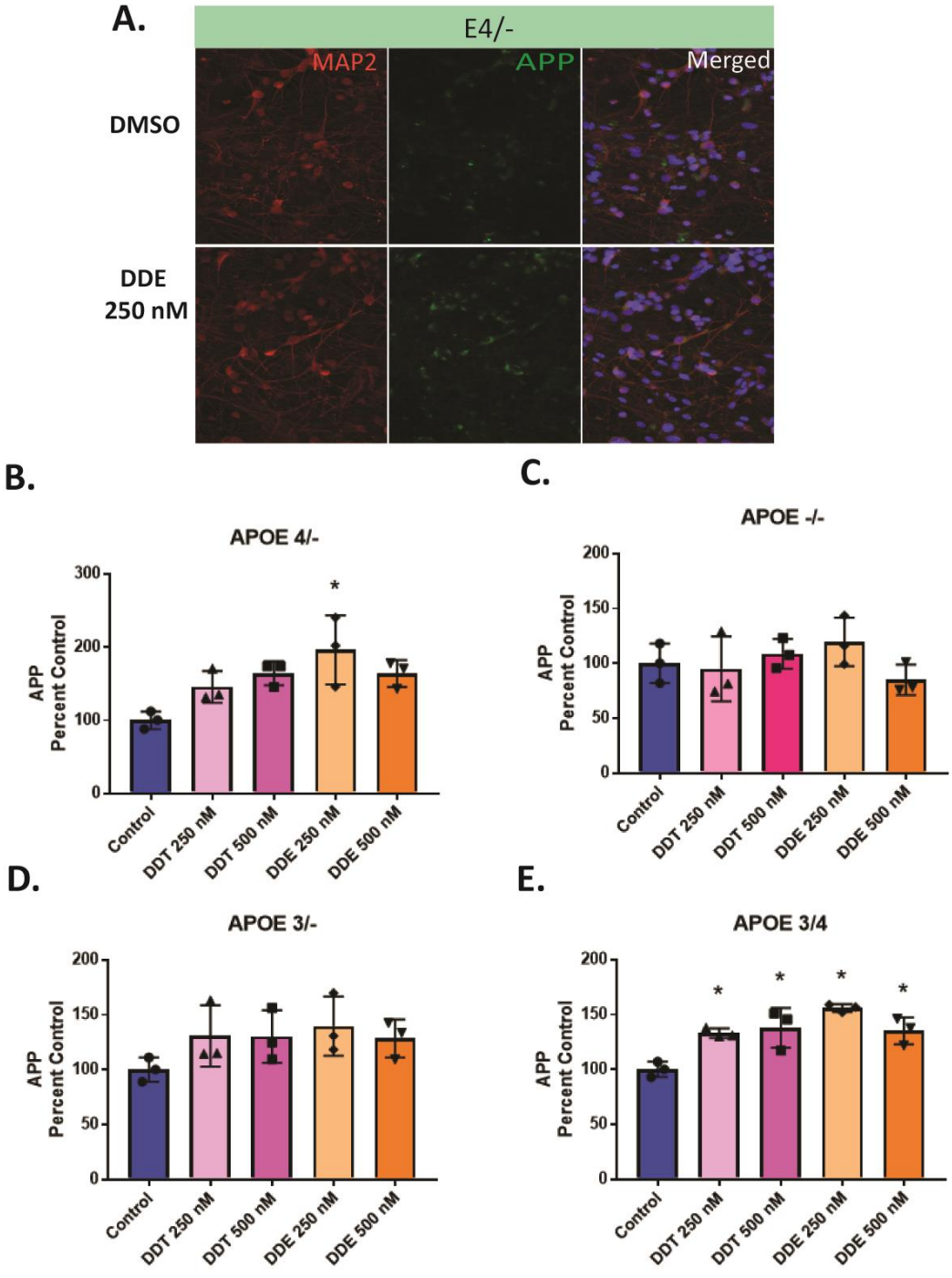


Figure 4.6 DDT and DDE potentiate expression of APP in human iNs carrying the E4 variant. (A) Representative images of APOE E4/- treated with DMSO or DDE 250 nM expressing DAPI (blue), MAP2 (red), and APP (green). Quantified integrated density of APP measured exclusively within MAP2 stained neurons and normalized to number of neurons genotyped as: (B) APOE E4/-, (C) APOE -/-, (D) APOE E4/-, and (E) APOE E3/E4. APP expression was significantly increased in the E4/- iNs after DDE 250 nM exposure (B) and is increased with DDT and DDE at both concentrations in the heterozygous E3/E4 iNs (E). Neuronal exposures were limited to 24 hr while concentrations of DDT and DDE were limited to 250 nM and 500 nM. Integrated density was threshold to control and analyzed using the BZ-X800 analyzer Macro Cell Count software. One-Way ANOVA with Tukey's multiple comparisons test (*p <0.05). Data provided by Ferass Sammoura in the Richardson lab.

response in Tau phosphorylation therefore suggesting its role in pTau regulation. However, the variable expression seen in the APOE -/- iNs, suggest that APOE is not essential in Tau pathology regulation.

To (1) increase the sensitivity of detectable APP, (2) collect data comparable to the data collected from primary mouse neurons, and (3) minimize the variability observed, APP expression assessed using a high throughput imaging system. APOE -/-, E3/-, E4/-, and E3/E4 iNs were exposed to DDT and DDE for 24 hr. Following exposure, APP expression is significantly increased only in the presence of the E4 variant (Figure 4.6 B & E). E4/- iNs exposure to DDT and DDE resulted in significant APP induction only to 250 nM of

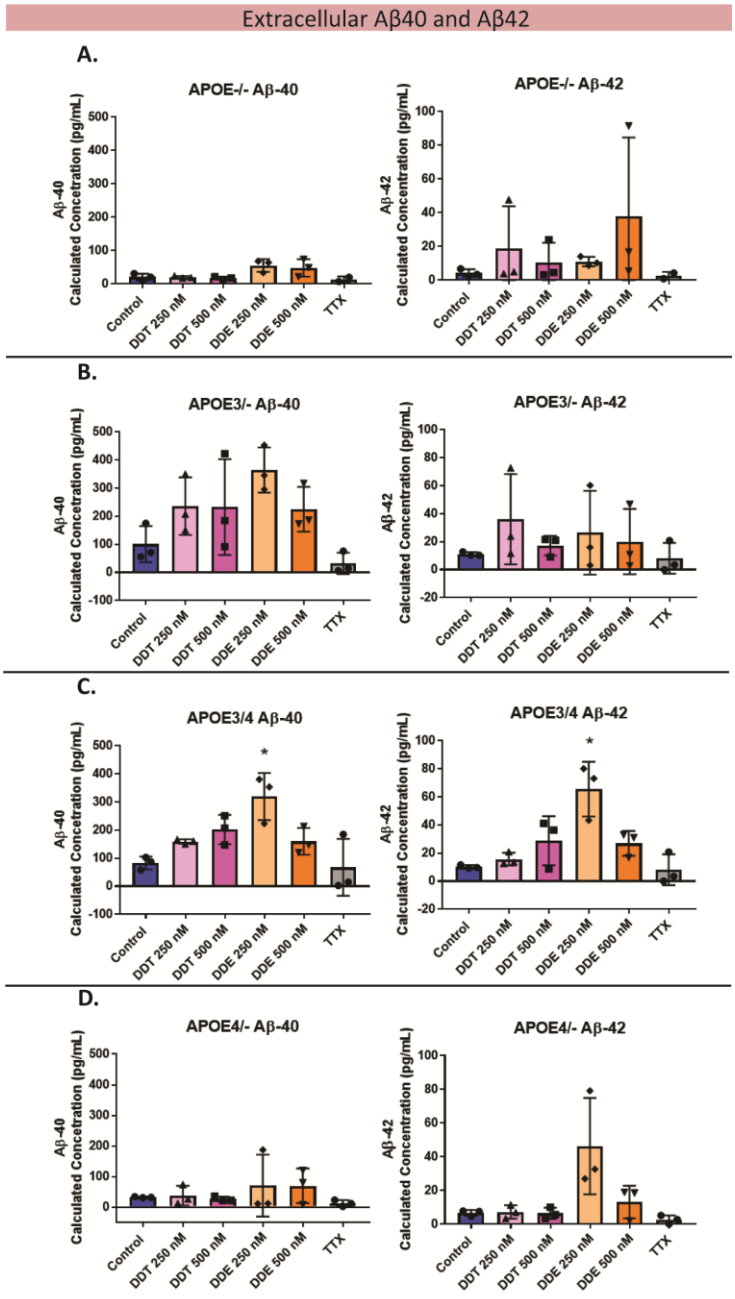


Figure 4.7 DDT and DDE increase Aβ40 and Aβ42 in the presence of the E4 variant. Plots show the extracellular concentration of Aβ40 (left) and Aβ42 (right) in (A) APOE -/-, (B) APOE E3/-, (C) APOE E3/E4, and (D) APOE E4/- after cultures were exposed to DDT and DDE. /- treated with DMSO or DDE 250 nM expressing DAPI (blue), MAP2 (red), and APP (green). Significant increases in Aβ40 (left panel C) and Aβ42 (right panel C) observed after 250 nM DDE exposure to E3/E4 neurons. Aβ40 and Aβ42 concentration trends upward, although variable, in the APOE E4/- neurons. Tetrodotoxin was used as a negative control, blocking the accumulation of the peptides via the inhibition of APP endocytosis (Sullivan 2014). Media collections carried out post neuronal exposures limited to 24 hr and concentrations of DDT and DDE were limited to 250 nM and 500 nM. Data represented as means (pg/mL) +/- SD. N = 3, each N represents a separate batch. One-Way ANOVA with Tukey's multiple comparisons test (*p <0.05). Data provided by Ferass Sammoura in the Richardson lab.

DDE. However, induction was significant when the E3/E4 iNs were exposed to DDT and DDE at both 250 and 500 nM concentrations. Exposing APOE -/- and E3/- iNs to DDT and DDE showed no significant increases in APP expression. The high throughput BZ-X800 analyzer Macro Cell Count software allowed miniscule changes in APP expression to be detected. APOE appears to be crucial in observing any upward trends in APP expression, but significant increases are potentiated when the E4 variant is present.

Finally, we aimed to evaluate whether the induction of APP would be reflected in the extracellular accumulation of Aβ40 and Aβ42, so we assayed the media after a 24 hr exposure to DDT and DDE. DDE exposure resulted in an increased secretion of Aβ40 and Aβ42 in the E3/E4 neurons (Figure 4.7C). However, there were no significant increases in the E4/- iNs exposed to DDE. As with the APP expression observations, there were no significant changes in Aβ40 and Aβ42 secretion in cell lines lacking the E4 variant. Using the Multi-Spot Assay System (MSD) gave us the ability to detect pg/mL levels and observe minute fluctuations of Aβ40 and Aβ42. The Aβ40 levels detected in the APOE -/- are much lower in magnitude than what was detected in the other APOE genotypes. This supports a role for APOE in the non-canonical pathway to induce APP, the precursor protein to these peptides (Huang et al., 2017). Indirectly, the induced APP expression observed in Figure 4.6 is consistent with the increased levels of Aβ40 and Aβ42 accumulation, as expected. The concentration range of Aβ40 and Aβ42 detected in the media are consistent with well-known fold differences reported in the literature (Gu & Guo, 2013). However, in the absence of APOE, the Aβ concentrations detected of both forms fall within the same limits.

Toxicant	Concentration	Genotype				Change in Markers		
		APOE -/-	E3/E4	E3/-	E4/-	APP	pTau	Aβ
DDT	250 nM	■				↔	/	↔
			■			↑	/	↔
				■		↔	/	↔
					■	↔	/	↔
DDT	500 nM	■				↔	/	↔
			■			↑	/	↔
				■		↔	/	↔
					■	↔	/	↔
DDT	1 µM	■				24 hr ↔	24 hr ↔	/
						48 hr ↔	48 hr ↑	
			■			24 hr ↑	24 hr ↔	/
						48 hr ↑	48 hr ↔	
DDE	250 nM	■				↔	/	↔
			■			↑	/	↑
				■		↔	/	↔
					■	↑	/	↔
DDE	500 nM	■				↔	/	↔
			■			↑	/	↔
				■		↔	/	↔
					■	↔	/	↔
DDE	1 µM	■				24 hr ↓	24 hr ↔	/
						48 hr ↔	48 hr ↔	
			■			24 hr ↑	24 hr ↑	/
						48 hr ↑	48 hr ↔	

Table 4.1 Data Summary: Changes are denoted by increase (↑), no change (↔), decrease (↓), and measurement not performed (/).

4.5 Discussion

The results of this dissertation revealed that DDT and DDE did not directly induce ApoE protein expression, however we were able to detect a synergistic relationship between the E4 variant and the environmental toxicants on the potentiation of AD. Our goal to first verify that DDT and DDE had an effect on human neurons, revealed that APP expression was significantly increased in the E3/E4 iNs with both toxicants after exposing for 24 and 48 hr. However, in the absence of APOE, DDT and DDE did not alter APP expression. This may be a result of APOE being involved in multiple AD pathways, both direct and indirect such as the ability for APOE to modulate cholesterol which is another known risk for developing AD (Huang et al., 2017; Tran et al., 2019). We observed significant increases in pTau expression only when the E3/E4 iNs were exposed to DDE for 24 hr and not (1) 48 hr exposure nor by (2) DDT at either time points. These findings imply that APOE may influence the rate of response in pTau regulation, but it does not appear to be required. Additionally, they also suggest that the chemical properties of DDT and DDE differ enough to produce such variable outcomes in pTau expression. Individually, DDT, DDE and APOE alter Ca^{2+} homeostasis which may be one of the mechanisms in which their synergism potentiates AD. The variants of APOE also differentially alter the intracellular concentrations of Ca^{2+} where the highest levels were associated with the E4 variant (Jiang et al., 2015). In fact, forcing cell depolarization and initiating Ca^{2+} influx was shown to reduce the neurotoxic effects associated with APOE fragmentation; E3 fragments less toxic than E4 (Marques et al., 1996; Tolar et al., 1999).

The conflicting results could also be contributed by other factors and in order to understand the unexpected outcome, several components were considered. The first may be related to the length of time the cells were exposed to the vehicle control DMSO. DMSO has the potential to alter the membrane permeability affecting the cells normal function and could allow larger molecules to readily travel across the membrane (De Ménorval et al., 2012). However, literature suggests that DC-3F cells, (Chinese hamster lung fibroblast cells) exposed to concentrations of 10

% vol DMSO for short periods did not result in the membrane permeabilizing to calcium (De Ménorval et al., 2012). The final concentration of DMSO used in the treatment was 0.2% vol, much lower than the 10% vol tested. Another factor related to what we observed during the electrophysiology experiments was exposure duration. We revealed how the normal physiology of the neurons became compromised the longer they interact with the compounds. Longer exposure resulted in a permanent +10 mV shift in the resting membrane potential (RMP) suggesting that the permeability of the membrane allows for more unmonitored inward diffusion of sodium and calcium ions (Popova *et. al*, 2022; manuscript in preparation). Next, the variability observed for APP may be a result of the lack of sensitivity in how the AD markers are assessed. While in theory differentiating our induced pluripotent stem cells to neurons by introducing transcriptional factor NGN2 should yield only glutamatergic neurons. However, single-cell RNA sequencing data from similar iN cultures demonstrate that they contain multiple classes of neurons that are at different stages of maturation (Popova *et. al*, 2022; manuscript in preparation). As neurons selected to assess APP and pTau expression were based on the quality of the processes labeled by microtubule-associated protein 2 (MAP2), we are most likely omitting a pool of neurons that may not be fully mature or categorized as glutamatergic. Lastly, the concentrations of DDT and DDE may have been too high to isolate modest changes in APP and pTau expression. Originally, 1 µM concentration of DDT and DDE used was based on the serum levels detected in humans who ingested the compounds for 2 to 6 months (Morgan & Roan, 1971). However, mouse neurons exposed to nanomolar (nM) concentrations of DDT and DDE produced more robust APP inductions, approximately 1.5-fold increases (Sammoura *et. al*., unpublished data). Higher concentrations of the toxicants may be initiating other pathways related and unrelated to AD masking subtle changes in AD markers.

We next sought to improve the sensitivity of analyzing the expression of APP. Our sensitive assaying systems revealed human iNs exposed to 250 nM of DDE resulted in significantly

greater expression of APP only in the presence of the E4 variant. Interestingly, iNs selected randomly by the BZX software or objectively under the confocal revealed that between the two toxicants, DDE has the greater effect. These effects are likely due to DDE being more lipophilic than DDT where binding is tighter and the effects are allotted a longer time to persist (Morgan & Roan, 1971). This pattern was also confirmed during our washout experiments where persistent excitability from DDE exposure was prolonged compared to DDT due to its delayed clearance. To determine the binding affinities for DDT and DDE, we could utilize membrane potential sensitive dyes and Fluorescence resonance energy transfer (FRET) technology (Felix et al., 2004). Using these two components we could measure the occupancy/activation of Na_V channels at multiple timepoints over the course of 24 hr. Neurons would be previously exposed to one concentration of DDT or DDE for approximately 24 hr which should be followed by rinsing the excess compounds away. We could computationally compare DDT and DDE binding by analyzing Na_V channel activation as a function of FRET signal versus time. Finally, we were able to detect extracellular levels of Aβ40 and Aβ42. Secretion of these peptides signified the amyloidogenic endocytosis of APP known to occur during AD pathogenesis (Barucker et al., 2015; Gu & Guo, 2013; Mawuenyega et al., 2013; Sisodia, 1992). The secretion of Aβ40 and Aβ42 was enhanced in E3/E4 neurons after exposure to DDE and not DDT. The concentration of Aβ40 was found to be several folds higher than what was detected of the Aβ42 peptide. This finding is in line with what has been reported the literature. In AD, amyloid beta plaques are more abundant with Aβ42 although concentrations of Aβ40 peptide can be found up to several folds higher (Gu & Guo, 2013).

Bioaccumulation post DDT exposure is likely to be detected in individuals as DDE which has also resulted in the occurrence of more cognitive impairment when compared to its parent compound (Medehouenou et al., 2019). Exposure is predominantly from DDE contamination as DDE is the most prevalent environmental metabolite of DDT (ATSDR, 2002). In fact, a study that observed absorption, storage, and the metabolic conversion of ingested DDT and it's metabolites

noted that conversion of ingested DDT lead to a slow conversion to DDE (Morgan & Roan, 1971). As previous clinical observations revealed that DDE accumulation was associated with more cognitive impairment, finding the ability for DDE to cause a greater induction in APOE, APP and pTau expression as well as extracellular Aβ40/42 concentrations was anticipated (Richardson et al., 2014). These markers, APP, pTau, and Aβ40/42 in an individual genotyped as a carrier of the E4 variant shows significant increases after exposure to the more lipophilic toxicant, DDE. This evidence supports the clinical symptoms that has been seen in individuals exposed to the compounds and therefore can be used as a basis to further investigate combinatory risk factors on neurodegenerative diseases.

CHAPTER 5: Conclusion/Future Directions

Alzheimer's disease (AD) is the most common form of dementia, accounting for 6-8 out of every 10 cases. It is linked to increased cognitive impairment, amyloid beta plaque accumulation, and phosphorylated Tau protein. The incidence of the disease can be affected by increasing age, genetics, and environmental factors such as an introduction of pesticides. There is substantial evidence that the strongest genetic risk factor for developing AD is the E4 variant of APOE (Powell et al., 2013; Stevens et al., 2014). There were also environmental contributions observed in another study exposing a positive correlation between AD and increasing levels in serum DDE, a metabolite of the organophosphate pesticide DDT (Richardson et al., 2014). Together, these factors, the E4 variant of APOE and exposure to DDT, have been observed to produce more cognitive impairment in AD patients (Richardson et al., 2014). However, there are limited studies observing this phenomenon and the mechanism(s) behind it due to difficulties in studying this human disease in animal models. My work sought to bridge this gap by utilizing stem cell-derived neurons, the only way of studying live human neurons in the laboratory, to explore the mechanistic interactions between APOE and DDT/DDE. This is in addition to determining how they can synergistically enhance the risk of developing AD by increasing the disease hallmarks.

To assess the contribution of each APOE variant, both CRISPR-Cas9 technology and lentiviral transduction were used to develop stable cell lines. Generation of the hemizygous, E3/- and E4/-, iPSCs using CRISPR-Cas9 showed no reduction in ApoE protein expression despite there only being one allele. Upon initial assessment of the APOE -/- cells received from the Buck Institute, we observed basal APOE protein synthesis. To generate an appropriate negative control, we purified our APOE -/- iPSCs and showed APOE protein expression much lower in our potential candidates compared to E3/E4. We were able to alter the expression of APOE in APOE -/- iNs, induced with dox-inducible lentivirus (pTet-O-APOE E2-PuroR), by changing the concentration of doxycycline, similar to previous research that looked at other genes (Gossen & Bujard, 1992;

Gossen et al., 1995). The levels detected were always greater compared to the normal endogenous levels in the E3/E4 line. We developed and used our constitutive FUGW-APOE-PuroR (APOE E2, E3, or E4) lentivirus to transduce APOE -/- iPSCs, iNs, and iAs and reintroduce the individual variants of APOE. Expression was detected by probing for APOE and the tagged self-cleaving peptide T2A. We provided evidence that the functionality of the cells was unaffected after genetic editing and transduction because we were able to differentiate iPSCs into neurons and astrocytes, while maintaining their respective genotypes. We were also able to show that the genetic contributions were not primarily based in the differentiated neurons but that astrocytes produced biologically relevant ApoE. This was further confirmed when our re-analysis of the 2019 Religious Order Study and the Memory and Aging Project (ROSMAP) single-cell RNA sequencing data (Figure 2.6) identified astrocytes as the cell types being highly expressive of APOE (Bennet, 2019; Mathys et al., 2019). This allowed us to create stable HEK 293T APOE variations for the collection of extracellular APOE mimicking the free and sialylated forms detected in the iAs. The foundation for developing dependable and functionally stable model systems was established by using CRISPR-Cas9 technology and lentiviral transduction.

Prior to assessing the synergistic effects of APOE and DDT and DDE, we first sought to confirm the ability of DDT and DDE to modify the voltage gated sodium channels in human neurons. Although limited, the toxic mechanism of DDT has been well established in various species including a few studies conducted in mammals (ÅRhem et al., 1974; Chen et al., 2019; Dale et al., 1962; Stuart et al., 1987). However, the same level of research has not been carried out for its metabolite DDE which has always been assumed to be ineffective or deactivated compared to its parent compound. Although DDT was designed to target insect sodium channel, we were able to confirm that both DDT and its metabolite DDE are able to interact and prevent the closing of the voltage gated sodium channels on human iNs. This resulted in continuous rapid action potential generation, however depending on the exposure time, we showed that these adverse effects are

reversible once the toxicants are removed. Interestingly, DDE provoked a stronger effect when a longer time course was required to reverse its toxicity when compared to DDT. The longer time course needed to stop the rapid action potential generation and repolarize the resting membrane potential is most likely due to the high lipophilicity of DDE versus DDT (Imamura et al., 2005).

The chemical property differences of DDE to DDT were also reflected when we assessed the synergistic relationship between the E4 variant of APOE and the environmental toxicants. We were able to show that both DDT and DDE induced AD pathology by initiating persistent generation of action potentials, increases the expression of APP, pTau as well as Aβ40/42 secreted concentrations in human neurons. However, DDE exposure resulted in more robust changes, which could be due to its strong lipophilic characteristics as the quantity of both toxicants used was the same. The lipophilic nature of DDT and DDE have been explored in middle-aged adult men who ingested the compounds for 2-6 months; findings described DDE as having a greater propensity to be stored in adipose tissues compared to the significant rate reduction of DDT at 2.95 mg/day in individuals ingesting 20 mg/day for 183 days (Morgan & Roan, 1971). The concentrations of DDT and DDE used in the current studies were similar to the levels detected in the serum of humans who have been previously exposed (Morgan & Roan, 1971; Richardson et al., 2014). Nevertheless, in the current studies, lower concentrations of DDE resulted in significantly greater APP expression and Aβ40/42 concentrations which was accentuated in the presence of the E4 variant. Therefore, higher concentrations of the compounds may result in off-target effects or non-canonical pathway activations that can mask or divert from the minute detectable changes in APP, pTau and Aβ40/42 .

There is always room to develop, progress, and explore new routes in research. An interesting future path would be to see how the dose or concentration of haploid copy/genome of the E4 variant affects the risk of Alzheimer's disease in our system. According to the literature, the E4 variant is dose-dependent, with one allele increasing the risk of developing AD by 2-fold and

two alleles increasing the risk by 8-10-fold (Corder et al., 1993). Our main goal could be to determine if increasing the expression of APOE E4 would result in a higher expression of APP, pTau and Aβ40/42 secretion. A suitable model system would need to be established before these markers could be assessed. Co-culturing E4/- iNs with E4/- iAs would be utilized to assess the risk of one E4 variant. We would infect APOE -/- iPSCs with our dox inducible lentivirus pTet-O-APOE E4-PuroR, drug select the transduced cells from the non-transduced and differentiate them to iNs and iAs. The iNs and iAs would be co-cultured in different groups subjected to increasing concentrations (0.2 -0.5 µg/mL) of doxycycline to increase APOE E4 expression. Concentrations of doxycycline would be selected based on the dose response carried out in neuronal expression of APOE (Figure 2.5). It is also known that inducing gene expression with lower amounts of doxycycline can lessen the harmful impacts of long-term exposure, which promote mitochondrial proteotoxic stress (Moullan et al., 2015; T. Das et al., 2016). Due to the time required to reach neuronal maturity and the risks associated with long-term doxycycline exposure, exposure to varying concentrations of the antibiotic would be limited to the final week of neuronal development. Negative controls would include APOE -/- iNs co-cultured with APOE -/- iAs, as well as transduced APOE -/- iNs co-cultured with transduced APOE -/- iAs treated with 0 µg/mL of doxycycline. At the end of the treatment, media would be collected to assess Aβ40/42 secretion, as well as APP and pTau expression via ICC and Western blot. We hypothesize that increasing the expression of APOE E4 would produce higher expression of APP and Aβ40/42, however, based on our own observations, we may not see a proportional induction of pTau with APOE expression.

Our observations revealed that the induction of pTau by DDT and DDE were independent of APOE genotype. Studies conducted showed that hyperphosphorylation of Tau has been associated with intracellular Aβ and mitochondrial dysfunction (RajaRajeswari et al., 2016). These factors contribute to reasons why our induction of pTau remained independent. However, an important avenue to explore would be to determine how DDT and DDE enhances pTau expression.

In the simplest form, could DDT and DDE induce the formation of reactive oxygen species (ROS) which in turn alters the mitochondria and ultimately results in hyperphosphorylation of Tau? Reviewing the literature shows that DDT and DDE have been known to induce the generation of ROS, hydrogen peroxide and other oxidative stress mediators as demonstrated in human blood mononuclear cells collected from healthy unexposed individuals DDE was shown to induce ROS by 25-fold and DDT by 19-fold (Pérez-Maldonado et al., 2005). DDE specifically was also shown to increase ROS as well as reduce cellular protein enzymes Phospholipid hydroperoxide glutathione peroxidase (PHGPx, GPx4) and Superoxide dismutase (SOD), which are all important enzymes in reducing excessive amounts of ROS, as evidenced by detecting enzyme (i.e. PHGPx,) protein and mRNA expression in testicular tissue using western blotting and real-time quantitative PCR post exposure (Mota et al., 2011; Quan et al., 2016; Song et al., 2008). One of the most prominent targets for toxicity is mitochondrial malfunction which is commonly a result of excessive ROS disrupting the electron transport chain, targeting mitochondrial DNA leading to mutations and disturbance of Ca^{2+} homeostasis and membrane permeability (Mota et al., 2011). The previous studies conducted provides connections for how DDT and DDE could, independent of APOE, lead to inducing pTau expression. This could be an innovative avenue to pursue and confirm our suspicions about the phenomenon.

To explore if induced pTau expression caused by DDT and DDE exposure in our human iNs are a result of mitochondrial dysfunction, we would first expose APOE -/- iNs to both compounds for 48 hr. Only the APOE -/- cells showed significant pTau expression after 48 hr of exposure, according to our findings. We would first need to assess the ability of DDT and DDE to induce the generation of ROS in human neurons. This could be confirmed by utilizing flow cytometry to detect ROS using 2',7'-dichlorofluorescein diacetate (DCFH-DA). DCFH diffuses into the cell becoming trapped where it is oxidized by hydrogen peroxide or low-molecular-weight peroxides forming a fluorescent compound 20,70-dichlorofluorescein (Pérez-Maldonado et al.,

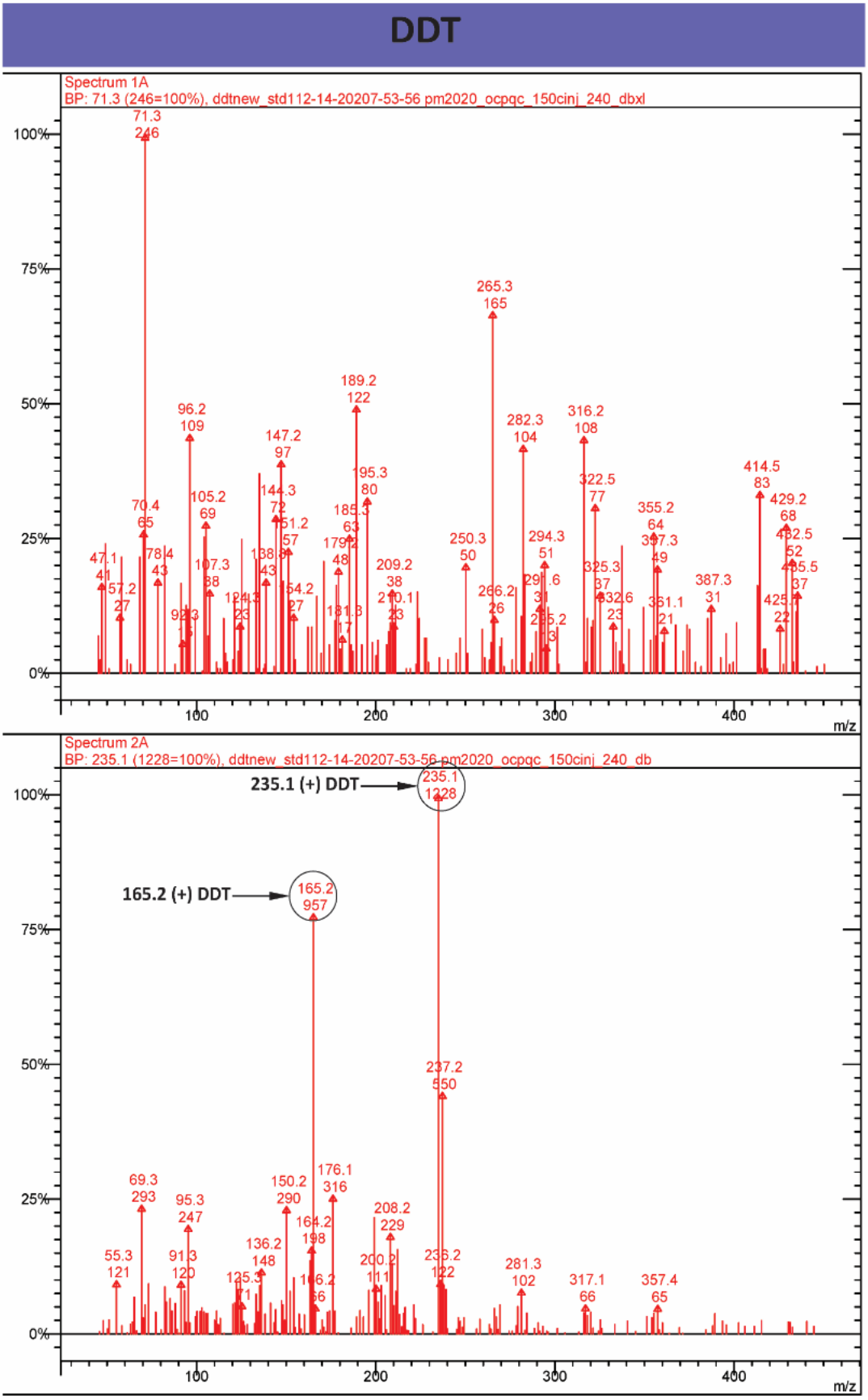
2005). We could also explore a potential reduction of SOD by immunofluorescent staining of the cells for mitochondria SOD using MnSOD and cytoplasmic using CuZnSOD. Both toxicants are expected to produce ROS, but DDE may produce more, based on our own observations and the literature (Pérez-Maldonado et al., 2005). After confirming the ability of DDT and DDE to increase ROS and reduce antioxidant enzymes in human neurons, we would then confirm that exposure results in mitochondrial dysfunction by ratiometric Ca^{2+} indicator. As these experiments would be carried out in our co-culture system, cells should be infected with a neuronal mitochondrial reporter hSyn.2mtYC3.6, as well as, the calcium indicator calbryte 590 to measure cytosolic and mitochondrial Ca^{2+} changes via multiphoton laser-scanning microscopy (Calvo-Rodriguez et al., 2020). We would expect to see mitochondria of exposed neurons with higher concentrations of mitochondrial Ca^{2+} compared to the unexposed. Finally, we would assess the expression of pTau in DDT and DDE exposed APOE -/- iNs compared to control via Western blot and immunocytochemistry probing for pTau. This series of potential experiments create a foundation for determining that Tau pathology is enhanced by DDT and DDE independently from APOE.

My dissertation focused on the association between APOE, the main genetic contributor, DDT, and DDE prevalent environmental concerns on AD. Being able to better understand the pathogenesis of Alzheimer's disease could ultimately lead to the identification of novel mediators relating to the disease. These novel mediators can include personalized medications that target multiple pathways depending on the patients APOE genotype. For instance, as the APOE variants function differently in clearing Aβ40/42, knowing the APOE genotype of the AD patient could allow us to create a unique regimen. An individual who is a carrier of the E4 variant may require a higher dose of aducanumab, as the build-up of Aβ40/42 has been described as being higher compared to patients without the E4 variant; alternatively, a different drug could be prescribed. It is also known that human elimination of DDT/DDE may be enhanced during exercise due to the increased perspiration therefore prescribing the right medication along with increasing physical

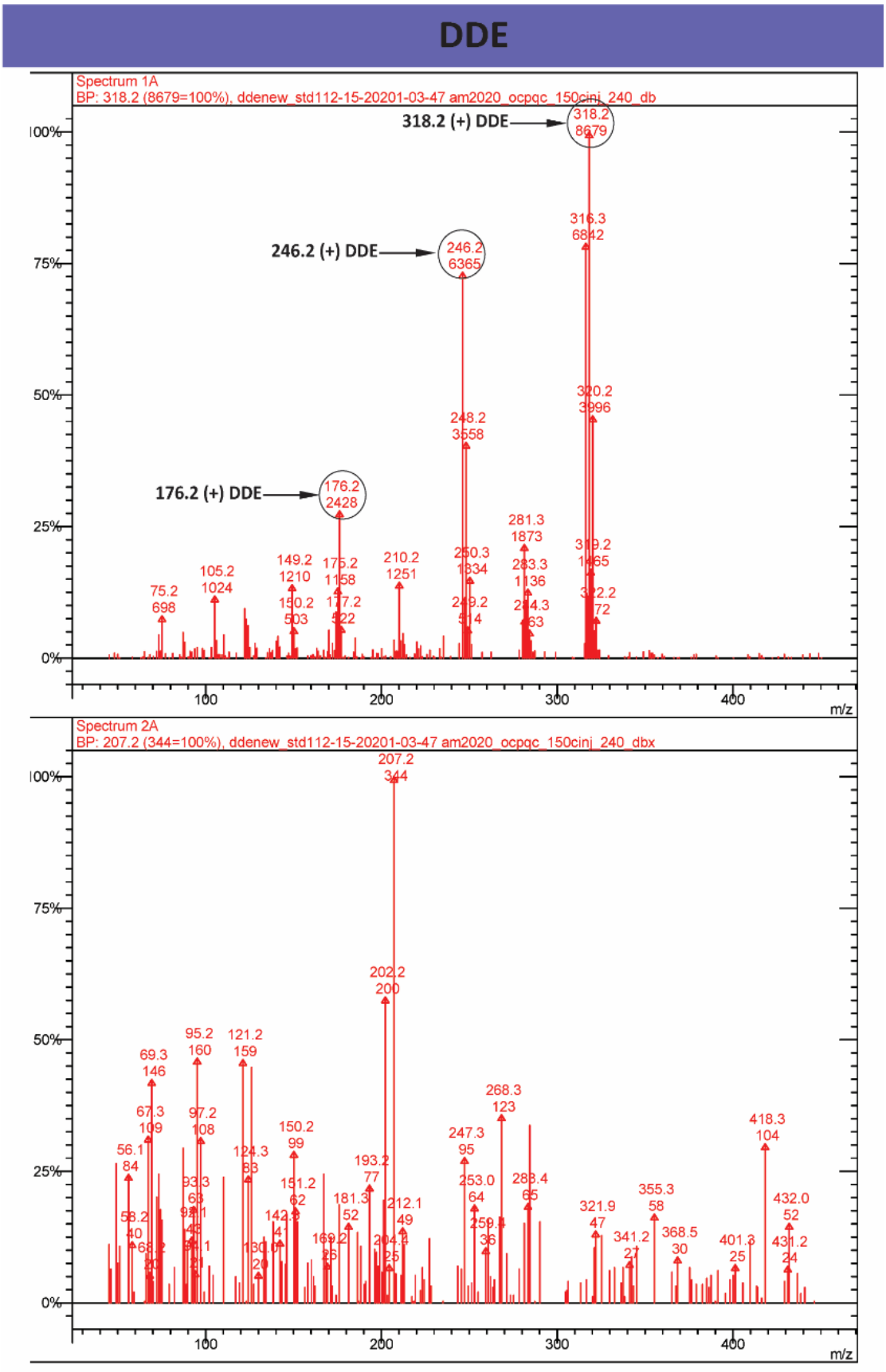
activity may further aid in mitigating the progression of the disease (Genuis et al., 2016). This may be worth exploring because based on my observations, AD pathology in enhanced in the presence of the strongest genetic contributor and an environmental insult. This awareness also has the potential in opening doors for targeting different stages of AD as well as contribute to the regulatory efforts of DDT usage.

APPENDIX

GC-MS verifies the purity of DDT (Spectra 1) and DDE (Spectra 2). In both spectrum, plots 2A represents the expanded retention time for DDT and 1A for DDE. DDT purity was determined by the presence of ion patterns associated with DDT, peaks 235.1 and 165.2, in 2A and lack of DDE ion patterns in 1A. While DDE purity was determined by the positive identification of ion patterns 318.2, 246.2, and 176.2, in 1A and the absence of DDT ion patterns in 2A.



Spectra 1: GC-MS verifies DDT purity



Spectra 2: GC-MS verifies DDE purity

REFERENCES

Agüero-Torres, H., Fratiglioni, L., Guo, Z., Viitanen, M., & Winblad, B. (1998). Prognostic Factors in Very Old Demented Adults: A Seven-Year Follow-Up From a Population-Based Survey in Stockholm. *Journal of the American Geriatrics Society*, *46*(4), 444-452. https://doi.org/10.1111/j.1532-5415.1998.tb02464.x

Alonso, A. C., Zaidi, T., Grundke-Iqbal, I., & Iqbal, K. (1994). Role of Abnormally Phosphorylated Tau in the Breakdown of Microtubules in Alzheimer Disease. *Proceedings of the National Academy of Sciences - PNAS*, *91*(12), 5562-5566. https://doi.org/10.1073/pnas.91.12.5562

Altmann, A., Tian, L., Henderson, V. W., & Greicius, M. D. (2014). Sex modifies the APOE-related risk of developing Alzheimer disease. *Annals of Neurology*, *75*(4), 563-573. https://doi.org/10.1002/ana.24135

ÅRhem, B. P., & Fraxkexhaeuser, B. (1974). DDT and Related Substances: Effects on Permeability Properties of Myelinated Xenopus Nerve Fibre. Potential Clamp Analysis [https://doi.org/10.1111/j.1748-1716.1974.tb05706.x]. *Acta Physiologica Scandinavica*, *91*(4), 502-511. https://doi.org/https://doi.org/10.1111/j.1748-1716.1974.tb05706.x

ÅRhem, P., Frankenhaeuser, B., Göthe, R., & O'Bryan, P. (1974). DDT and Related Substances on Myelinated Nerve: Effects on Permeability Properties [https://doi.org/10.1111/j.1748-1716.1974.tb05665.x]. *Acta Physiologica Scandinavica*, *91*(1), 130-132. https://doi.org/https://doi.org/10.1111/j.1748-1716.1974.tb05665.x

ATSDR. (2002). *Toxicological profile for DDT, DDE, and DDD*. Agency for Toxic Substances and Disease Registry (ATSDR)

Bakulski, K. M., Seo, Y. A., Hickman, R. C., Brandt, D., Vadari, H. S., Hu, H., & Park, S. K. (2020). Heavy Metals Exposure and Alzheimer's Disease and Related Dementias. *J Alzheimers Dis*, *76*(4), 1215-1242. https://doi.org/10.3233/JAD-200282

Baldi, I., Gruber, A., Rondeau, V., Lebailly, P., Brochard, P., & Fabrigoule, C. (2011). Neurobehavioral effects of long-term exposure to pesticides: results from the 4-year follow-up of the PHYTONER study. *Occup Environ Med*, *68*(2), 108-115. https://doi.org/10.1136/oem.2009.047811

Baldi, I., Lebailly, P., Mohammed-Brahim, B., Letenneur, L., Dartigues, J., & Brochard, P. (2003). Neurodegenerative diseases and exposure to pesticides in the elderly. *American Journal of Epidemiology*, *157*(5), 409-414. https://login.proxy.libraries.rutgers.edu/login?url=https://search.ebscohost.com/login.aspx?direct=true&db=c8h&AN=106677934&site=eds-live

Barucker, C., Chang, P. K. Y., Hancock, M. A., Liebsch, F., Hossain, S., Charron, F. M., McKinney, R. A., Multhaup, G., Bittner, H. J., Hildebrand, P. W., Cameron, S., Shaw, H., Rao, Y., Harmeier, A., Dembny, P., Gensler, M., Zhuang, W., Rabe, J. P., Schmitz, D., & Lurz, R. (2015). Aβ42-oligomer Interacting Peptide (AIP) neutralizes toxic amyloid-β42 species and protects synaptic structure and function [Article]. *Scientific Reports*, *5*. https://doi.org/10.1038/srep15410

Basinger, H., & Hogg, J. P. (2022). Neuroanatomy, Brainstem. In *StatPearls*. StatPearls Publishing Copyright © 2022, StatPearls Publishing LLC.

Bature, F., Guinn, B. A., Pang, D., & Pappas, Y. (2017). Signs and symptoms preceding the diagnosis of Alzheimer's disease: a systematic scoping review of literature from 1937 to 2016. *BMJ Open*, *7*(8), e015746. https://doi.org/10.1136/bmjopen-2016-015746

Bennet, D. A. e. a. (2019). Religious Orders Study and Rush Memory and Aging Project. *Journal of Alzheimer's Disease*, *64*.

Bennett, D. A., Buchman, A. S., Boyle, P. A., Barnes, L. L., Wilson, R. S., & Schneider, J. A. (2018). Religious Orders Study and Rush Memory and Aging Project. *J Alzheimers Dis*, *64*(s1), S161-s189. https://doi.org/10.3233/jad-179939

Bickeböller, H., Campion, D., Brice, A., Amouyel, P., Hannequin, D., Didierjean, O., Penet, C., Martin, C., Pérez-Tur, J., Michon, A., Dubois, B., Ledoze, F., Thomas-Anterion, C., Pasquier, F., Puel, M., Demonet, J. F., Moreaud, O., Babron, M. C., Meulien, D., . . . Clerget-Darpoux, F. (1997). Apolipoprotein E and Alzheimer disease: genotype-specific risks by age and sex. *American journal of human genetics*, *60*(2), 439-446.

Boyles, J. K., Pitas, R. E., Wilson, E., Mahley, R. W., & Taylor, J. M. (1985). Apolipoprotein E associated with astrocytic glia of the central nervous system and with nonmyelinating glia of the peripheral nervous system. *Journal of Clinical Investigation*, *76*(4), 1501-1513. https://doi.org/10.1172/jci112130

Brookmeyer, R., Gray, S., & Kawas, C. (1998). Projections of Alzheimer's disease in the United States and the public health impact of delaying disease onset [Article]. *American Journal of Public Health*, *88*(9), 1337-1342. https://login.proxy.libraries.rutgers.edu/login?url=https://search.ebscohost.com/login.aspx?direct=true&db=edselc&AN=edselc.2-52.0-0031695197&site=eds-live

Byczkowski, J. Z. (1976). The mode of action of p,p'=DDT on mammalian mitochondria. *Toxicology*, *6*(3), 309-314. https://www.ncbi.nlm.nih.gov/pubmed/136771

Calvo-Rodriguez, M., Hou, S. S., Snyder, A. C., Kharitonova, E. K., Russ, A. N., Das, S., Fan, Z., Muzikansky, A., Garcia-Alloza, M., Serrano-Pozo, A., Hudry, E., & Bacskai, B. J. (2020). Increased mitochondrial calcium levels associated with neuronal death in a mouse model of Alzheimer's disease. *Nature Communications*, *11*(1). https://doi.org/10.1038/s41467-020-16074-2

Canals, I., Ginisty, A., Quist, E., Timmerman, R., Fritze, J., Miskinyte, G., Monni, E., Hansen, M. G., Hidalgo, I., Bryder, D., Bengzon, J., & Ahlenius, H. (2018). Rapid and efficient induction of functional astrocytes from human pluripotent stem cells. *Nat Methods*, *15*(9), 693-696. https://doi.org/10.1038/s41592-018-0103-2

Carabotti, M., Scirocco, A., Maselli, M. A., & Severi, C. (2015). The gut-brain axis: interactions between enteric microbiota, central and enteric nervous systems. *Annals of gastroenterology*, *28*(2), 203-209. https://pubmed.ncbi.nlm.nih.gov/25830558

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4367209/

Chan, E. S., Shetty, M. S., Sajikumar, S., Chen, C., Soong, T. W., & Wong, B. S. (2016). ApoE4 expression accelerates hippocampus-dependent cognitive deficits by enhancing Aβ impairment of insulin signaling in an Alzheimer's disease mouse model. *Sci Rep*, *6*, 26119. https://doi.org/10.1038/srep26119

Chang, S., Ma, T. r., Miranda, R. D., Balestra, M. E., Mahley, R. W., & Huang, Y. (2005). Lipid- and receptor-binding regions of apolipoprotein E4 fragments act in concert to cause mitochondrial dysfunction and neurotoxicity. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(51), 18694-18699. https://doi.org/10.1073/pnas.0508254102

Chen, M., Du, Y., Wu, S., Nomura, Y., Zhu, G., Zhorov, B. S., & Dong, K. (2019). Molecular evidence of sequential evolution of DDT- and pyrethroid-resistant sodium channel in Aedes aegypti. *PLOS Neglected Tropical Diseases*, *13*(6), e0007432. https://doi.org/10.1371/journal.pntd.0007432

Chen, M., Martins, R. N., & Lardelli, M. (2009). Complex splicing and neural expression of duplicated tau genes in zebrafish embryos. *J Alzheimers Dis*, *18*(2), 305-317. https://doi.org/10.3233/jad-2009-1145

Chêne, G., Beiser, A., Au, R., Preis, S. R., Wolf, P. A., Dufouil, C., & Seshadri, S. (2015). Gender and incidence of dementia in the Framingham Heart Study from mid-adult life. *Alzheimer's & Dementia*, *11*(3), 310-320. https://doi.org/10.1016/j.jalz.2013.10.005

Cho, H., Seo, S. W., Kim, J. H., Suh, M. K., Kim, G. H., Noh, Y., Ye, B. S., Kim, H. J., Yoon, C. W., Chin, J., Na, D. L., Choe, Y. S., Lee, K. H., Lee, J. H., & Kim, J. S. (2013). Amyloid

deposition in early onset versus late onset alzheimer's disease [Article]. *Journal of Alzheimer's Disease*, *35*(4), 813-821. https://doi.org/10.3233/JAD-121927

Cho, H., Seo, S. W., Ye, B. S., Kim, G. H., Noh, Y., Kim, H. J., Yoon, C. W., Kang, S. J., Chin, J., Na, D. L., Kim, J. H., Kim, C., Seong, J. K., Kim, C. H., Kim, S. T., & Lee, K. H. (2013). Changes in subcortical structures in early- versus late-onset Alzheimer's disease [Article]. *Neurobiology of Aging*, *34*(7), 1740-1747. https://doi.org/10.1016/j.neurobiolaging.2013.01.001

Choi, S. H., Kim, Y. H., Hebisch, M., Sliwinski, C., Lee, S., D'Avanzo, C., Chen, H., Hooli, B., Asselin, C., Muffat, J., Klee, J. B., Zhang, C., Wainger, B. J., Peitz, M., Kovacs, D. M., Woolf, C. J., Wagner, S. L., Tanzi, R. E., & Kim, D. Y. (2014). A three-dimensional human neural cell culture model of Alzheimer's disease. *Nature*, *515*(7526), 274-278. https://doi.org/10.1038/nature13800

Chui, H. C., Teng, E. L., Henderson, V. W., & Moy, A. C. (1985). Clinical subtypes of dementia of the Alzheimer type. *Neurology*, *35*(11), 1544. https://doi.org/10.1212/WNL.35.11.1544

Corder, E. H., Saunders, A. M., Risch, N. J., Strittmatter, W. J., Schmechel, D. E., Gaskell, P. C., Rimmler, J. B., Locke, P. A., Conneally, P. M., Schmader, K. E., Small, G. W., Roses, A. D., Haines, J. L., & Pericak-Vance, M. A. (1994). Protective effect of apolipoprotein E type 2 allele for late onset Alzheimer disease. *Nature Genetics*, *7*(2), 180-184. https://doi.org/10.1038/ng0694-180

Corder, E. H., Saunders, A. M., Strittmatter, W. J., Schmechel, D. E., Gaskell, P. C., Small, G. W., Roses, A. D., Haines, J. L., & Pericak-Vance, M. A. (1993). Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. *Science*, *261*(5123), 921-923. https://doi.org/10.1126/science.8346443

Coupland, C. A. C., Hill, T., Dening, T., Morriss, R., Moore, M., & Hippisley-Cox, J. (2019). Anticholinergic Drug Exposure and the Risk of Dementia. *JAMA Internal Medicine*, *179*(8), 1084. https://doi.org/10.1001/jamainternmed.2019.0677

Cutler, R. G., Kelly, J., Storie, K., Pedersen, W. A., Tammara, A., Hatanpaa, K., Troncoso, J. C., & Mattson, M. P. (2004). Involvement of oxidative stress-induced abnormalities in ceramide and cholesterol metabolism in brain aging and Alzheimer's disease. *Proceedings of the National Academy of Sciences*, *101*(7), 2070-2075. https://doi.org/doi:10.1073/pnas.0305799101

Dale, W. E., Gaines, T. B., & Hayes, W. J. (1962). Storage and excretion of DDT in starved rats. *Toxicol Appl Pharmacol*, *4*(1), 89-106. https://doi.org/https://doi.org/10.1016/0041-008X(62)90078-9

Dale, W. E., Gaines, T. B., Hayes, W. J., & Pearce, G. W. (1963). Poisoning by DDT: Relation between Clinical Signs and Concentration in Rat Brain. *Science*, *142*(3598), 1474-1476. http://www.jstor.org.proxy.libraries.rutgers.edu/stable/1712006

David, Chad, & Matthew. (2018). Development and Evolution of Cerebral and Cerebellar Cortex. *Brain, Behavior and Evolution*, *91*(3), 158-169. https://doi.org/10.1159/000489943

Davies, T. G. E., Field, L. M., Usherwood, P. N. R., & Williamson, M. S. (2007). DDT, pyrethrins, pyrethroids and insect sodium channels. *IUBMB Life*, *59*(3), 151-162. https://doi.org/10.1080/15216540701352042

De Ménorval, M.-A., Mir, L. M., Fernández, M. L., & Reigada, R. (2012). Effects of Dimethyl Sulfoxide in Cholesterol-Containing Lipid Membranes: A Comparative Study of Experiments In Silico and with Cells. *PLoS ONE*, *7*(7), e41733. https://doi.org/10.1371/journal.pone.0041733

Delbarba, A., Abate, G., Prandelli, C., Marziano, M., Buizza, L., Arce Varas, N., Novelli, A., Cuetos, F., Martinez, C., Lanni, C., Memo, M., & Uberti, D. (2016). Mitochondrial Alterations in Peripheral Mononuclear Blood Cells from Alzheimer's Disease and Mild

Cognitive Impairment Patients. *Oxid Med Cell Longev*, *2016*, 5923938. https://doi.org/10.1155/2016/5923938

Do Carmo, S., Spillantini, M. G., & Cuello, A. C. (2021). Editorial: Tau Pathology in Neurological Disorders [Editorial]. *Frontiers in Neurology*, *12*. https://doi.org/10.3389/fneur.2021.754669

Dong, K., Du, Y., Rinkevich, F., Nomura, Y., Xu, P., Wang, L., Silver, K., & Zhorov, B. S. (2014). Molecular biology of insect sodium channels and pyrethroid resistance. *Insect Biochem Mol Biol*, *50*, 1-17. https://doi.org/10.1016/j.ibmb.2014.03.012

Duara, R., Barker, W. W., Lopez-Alberola, R., Loewenstein, D. A., Grau, L. B., Gilchrist, D., Sevush, S., & St George-Hyslop, S. (1996). Alzheimer's disease: interaction of apolipoprotein E genotype, family history of dementia, gender, education, ethnicity, and age of onset. *Neurology*, *46*(6), 1575-1579. https://doi.org/10.1212/wnl.46.6.1575

Durand, S., & Cimarelli, A. (2011). The inside out of lentiviral vectors. *Viruses*, *3*(2), 132-159. https://doi.org/10.3390/v3020132

Farrer, L. A. (1997). Effects of Age, Sex, and Ethnicity on the Association Between Apolipoprotein E Genotype and Alzheimer Disease. *JAMA*, *278*(16), 1349. https://doi.org/10.1001/jama.1997.03550160069041

Feany, M. B., & Dickson, D. W. (1996). Neurodegenerative disorders with extensive tau pathology: A comparative study and review. *Annals of Neurology*, *40*(2), 139-148. https://doi.org/10.1002/ana.410400204

Felix, J. P., Williams, B. S., Priest, B. T., Brochu, R. M., Dick, I. E., Warren, V. A., Yan, L., Slaughter, R. S., Kaczorowski, G. J., Smith, M. M., & Garcia, M. L. (2004). Functional assay of voltage-gated sodium channels using membrane potential-sensitive dyes. *Assay Drug Dev Technol*, *2*(3), 260-268. https://doi.org/10.1089/1540658041410696

Finelli, A., Kelkar, A., Song, H. J., Yang, H., & Konsolaki, M. (2004). A model for studying Alzheimer's Abeta42-induced toxicity in Drosophila melanogaster. *Mol Cell Neurosci*, *26*(3), 365-375. https://doi.org/10.1016/j.mcn.2004.03.001

Fleming, L., Mann, J. B., Bean, J., Briggle, T., & Sanchez-Ramos, J. R. (1994). Parkinson's disease and brain levels of organochlorine pesticides [Article]. *Annals of Neurology*, *36*(1), 100-103. https://doi.org/10.1002/ana.410360119

Flowers, S. A., Grant, O. C., Woods, R. J., & Rebeck, G. W. (2019). O-glycosylation on cerebrospinal fluid and plasma apolipoprotein E differs in the lipid-binding domain. *Glycobiology*, *30*(2), 74-85. https://doi.org/10.1093/glycob/cwz084

Fratiglioni, L., Viitanen, M., von Strauss, E., Tontodonati, V., Herlitz, A., & Winblad, B. (1997). Very Old Women at Highest Risk of Dementia and Alzheimer's Disease. *Neurology*, *48*(1), 132. https://doi.org/10.1212/WNL.48.1.132

Furuno, T., Kawanishi, C., Iseki, E., Onishi, H., Sugiyama, N., Suzuki, K., & Kosaka, K. (2001). No evidence of an association between CYP2D6 polymorphisms among Japanese and dementia with Lewy bodies. *Psychiatry Clin Neurosci*, *55*(2), 89-92. https://doi.org/10.1046/j.1440-1819.2001.00793.x

Gao, S., Hendrie, H. C., Hall, K. S., & Hui, S. (1998). The Relationships Between Age, Sex, and the Incidence of Dementia and Alzheimer Disease. *Archives of General Psychiatry*, *55*(9), 809. https://doi.org/10.1001/archpsyc.55.9.809

Garneau, J. E., Dupuis, M.-È., Villion, M., Romero, D. A., Barrangou, R., Boyaval, P., Fremaux, C., Horvath, P., Magadán, A. H., & Moineau, S. (2010). The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature*, *468*(7320), 67-71. https://doi.org/10.1038/nature09523

Gasiunas, G., Barrangou, R., Horvath, P., & Siksnys, V. (2012). Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proceedings*

of the National Academy of Sciences, *109*(39), E2579-E2586. https://doi.org/10.1073/pnas.1208507109

Genuis, S. J., Lane, K., & Birkholz, D. (2016). Human Elimination of Organochlorine Pesticides: Blood, Urine, and Sweat Study. *Biomed Res Int*, *2016*, 1624643. https://doi.org/10.1155/2016/1624643

Goldberg, T. E., Huey, E. D., & Devanand, D. P. (2020). Association of APOE e2 genotype with Alzheimer's and non-Alzheimer's neurodegenerative pathologies. *Nature Communications*, *11*(1). https://doi.org/10.1038/s41467-020-18198-x

Gong, C. X., Liu, F., Grundke-Iqbal, I., & Iqbal, K. (2005). Post-translational modifications of tau protein in Alzheimer's disease. *J Neural Transm (Vienna)*, *112*(6), 813-838. https://doi.org/10.1007/s00702-004-0221-0

Gossen, M., & Bujard, H. (1992). Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proceedings of the National Academy of Sciences*, *89*(12), 5547-5551. https://doi.org/10.1073/pnas.89.12.5547

Gossen, M., Freundlieb, S., Bender, G., MÜLler, G., Hillen, W., & Bujard, H. (1995). Transcriptional Activation by Tetracyclines in Mammalian Cells. *Science (American Association for the Advancement of Science)*, *268*(5218), 1766-1769. https://doi.org/10.1126/science.7792603

Greeve, I., Kretzschmar, D., Tschäpe, J. A., Beyn, A., Brellinger, C., Schweizer, M., Nitsch, R. M., & Reifegerste, R. (2004). Age-dependent neurodegeneration and Alzheimer-amyloid plaque formation in transgenic Drosophila. *J Neurosci*, *24*(16), 3899-3906. https://doi.org/10.1523/jneurosci.0283-04.2004

Grootendorst, J., Bour, A., Vogel, E., Kelche, C., Sullivan, P. M., Dodart, J. C., Bales, K., & Mathis, C. (2005). Human apoE targeted replacement mouse lines: h-apoE4 and h-apoE3 mice differ on spatial memory performance and avoidance behavior. *Behav Brain Res*, *159*(1), 1-14. https://doi.org/10.1016/j.bbr.2004.09.019

Groth, C., Nornes, S., McCarty, R., Tamme, R., & Lardelli, M. (2002). Identification of a second presenilin gene in zebrafish with similarity to the human Alzheimer's disease gene presenilin2. *Dev Genes Evol*, *212*(10), 486-490. https://doi.org/10.1007/s00427-002-0269-5

Grothe, M. J., Barthel, H., Sepulcre, J., Dyrba, M., Sabri, O., & Teipel, S. J. (2017). In vivo staging of regional amyloid deposition. *Neurology*, *89*(20), 2031-2038. https://doi.org/10.1212/wnl.0000000000004643

Grundke-Iqbal, I., Iqbal, K., Tung, Y. C., Quinlan, M., Wisniewski, H. M., & Binder, L. I. (1986). Abnormal phosphorylation of the microtubule-associated protein tau (tau) in Alzheimer cytoskeletal pathology. *Proceedings of the National Academy of Sciences*, *83*(13), 4913-4917. https://doi.org/10.1073/pnas.83.13.4913

Gu, L., & Guo, Z. (2013). Alzheimer's Aβ42 and Aβ40 peptides form interlaced amyloid fibrils. *Journal of Neurochemistry*, *126*(3), 305-311. https://doi.org/10.1111/jnc.12202

Halikere, A., Popova, D., Scarnati, M. S., Hamod, A., Swerdel, M. R., Moore, J. C., Tischfield, J. A., Hart, R. P., & Pang, Z. P. (2020). Addiction associated N40D mu-opioid receptor variant modulates synaptic function in human neurons. *Molecular Psychiatry*, *25*(7), 1406-1419. https://doi.org/10.1038/s41380-019-0507-0

Hatcher, J. M., Delea, K. C., Richardson, J. R., Pennell, K. D., & Miller, G. W. (2008). Disruption of dopamine transport by DDT and its metabolites. *Neurotoxicology*, *29*(4), 682-690. https://doi.org/10.1016/j.neuro.2008.04.010

Hebert, L. E., Scherr, P. A., McCann, J. J., Beckett, L. A., & Evans, D. A. (2001). Is the Risk of Developing Alzheimer's Disease Greater for Women than for Men? *American Journal of Epidemiology*, *153*(2), 132-136. https://doi.org/10.1093/aje/153.2.132

Helmstaedter, C., Elger, C. E., & Vogt, V. L. (2018). Cognitive outcomes more than 5 years after temporal lobe epilepsy surgery: Remarkable functional recovery when seizures are controlled. *Seizure*, *62*, 116-123. https://doi.org/10.1016/j.seizure.2018.09.023

Henderson, V. W., & Kerchner, G. A. (2018). Alzheimer's Disease☆. In *Reference Module in Biomedical Sciences*. Elsevier. https://doi.org/https://doi.org/10.1016/B978-0-12-801238-3.00017-9

Hoe, H.-S., Harris, D. C., & Rebeck, G. W. (2005). Multiple pathways of apolipoprotein E signaling in primary neurons. *Journal of Neurochemistry*, *93*(1), 145-155. https://doi.org/10.1111/j.1471-4159.2004.03007.x

Huang, Y.-W. A., Zhou, B., Wernig, M., & Südhof, T. C. (2017). ApoE2, ApoE3, and ApoE4 Differentially Stimulate APP Transcription and Aβ Secretion. *Cell*, *168*(3), 427-441.e421. https://doi.org/https://doi.org/10.1016/j.cell.2016.12.044

Huff, T., Mahabadi, N., & Tadi, P. (2022). Neuroanatomy, Visual Cortex. In *StatPearls*. StatPearls Publishing

Copyright © 2022, StatPearls Publishing LLC.

Hyman, B. T., Phelps, C. H., Beach, T. G., Bigio, E. H., Cairns, N. J., Carrillo, M. C., Dickson, D. W., Duyckaerts, C., Frosch, M. P., Masliah, E., Mirra, S. S., Nelson, P. T., Schneider, J. A., Thal, D. R., Thies, B., Trojanowski, J. Q., Vinters, H. V., & Montine, T. J. (2012). National Institute on Aging-Alzheimer's Association guidelines for the neuropathologic assessment of Alzheimer's disease. *Alzheimers Dement*, *8*(1), 1-13. https://doi.org/10.1016/j.jalz.2011.10.007

Iijima, K., Chiang, H. C., Hearn, S. A., Hakker, I., Gatt, A., Shenton, C., Granger, L., Leung, A., Iijima-Ando, K., & Zhong, Y. (2008). Abeta42 mutants with different aggregation profiles induce distinct pathologies in Drosophila. *PLoS ONE*, *3*(2), e1703. https://doi.org/10.1371/journal.pone.0001703

Imamura, L., Kurashina, K., Kawahira, T., Omoteno, M., & Tsuda, M. (2005). Additional repression of activity-dependent c-fos and BDNF mRNA expression by lipophilic compounds accompanying a decrease in Ca2+ influx into neurons. *Neurotoxicology*, *26*(1), 17-25. https://doi.org/10.1016/j.neuro.2004.07.008

Imtiaz, B., Taipale, H., Tanskanen, A., Tiihonen, M., Kivipelto, M., Heikkinen, A.-M., Tiihonen, J., Soininen, H., Hartikainen, S., & Tolppanen, A.-M. (2017). Risk of Alzheimer's disease among users of postmenopausal hormone therapy: A nationwide case-control study. *Maturitas*, *98*, 7-13. https://doi.org/https://doi.org/10.1016/j.maturitas.2017.01.002

Imtiaz, B., Tuppurainen, M., Rikkonen, T., Kivipelto, M., Soininen, H., Kröger, H., & Tolppanen, A.-M. (2017). Postmenopausal hormone therapy and Alzheimer disease. *Neurology*, *88*(11), 1062. https://doi.org/10.1212/WNL.0000000000003696

Iwatsubo, T., Odaka, A., Suzuki, N., Mizusawa, H., Nukina, N., & Ihara, Y. (1994). Visualization of Aβ42(43) and Aβ40 in senile plaques with end-specific Aβ monoclonals: Evidence that an initially deposited species is Aβ42(43). *Neuron*, *13*(1), 45-53. https://doi.org/https://doi.org/10.1016/0896-6273(94)90458-8

Jackson, G. R., Wiedau-Pazos, M., Sang, T. K., Wagle, N., Brown, C. A., Massachi, S., & Geschwind, D. H. (2002). Human wild-type tau interacts with wingless pathway components and produces neurofibrillary pathology in Drosophila. *Neuron*, *34*(4), 509-519. https://doi.org/10.1016/s0896-6273(02)00706-7

Jarrett, J. T., Berger, E. P., & Lansbury, P. T. (1993). The carboxy terminus of the .beta. amyloid protein is critical for the seeding of amyloid formation: Implications for the pathogenesis of Alzheimer's disease. *Biochemistry*, *32*(18), 4693-4697. https://doi.org/10.1021/bi00069a001

Jawabri, K. H., & Sharma, S. (2022). Physiology, Cerebral Cortex Functions. In *StatPearls*. StatPearls Publishing

Jha, A., & Mukhopadhaya, K. (2021). *Alzheimer's disease : diagnosis and treatment guide* (1st ed. 2021. ed.). Springer. https://doi.org/10.1007/978-3-030-56739-2

Jiang, C., Li, G., Huang, P., Liu, Z., & Zhao, B. (2017). The Gut Microbiota and Alzheimer's Disease. *J Alzheimers Dis*, *58*(1), 1-15. https://doi.org/10.3233/jad-161141

Jiang, L., Zhong, J., Dou, X., Cheng, C., Huang, Z., & Sun, X. (2015). Effects of ApoE on intracellular calcium levels and apoptosis of neurons after mechanical injury. *Neuroscience*, *301*, 375-383. https://doi.org/10.1016/j.neuroscience.2015.06.005

Jinek, M., East, A., Cheng, A., Lin, S., Ma, E., & Doudna, J. (2013). RNA-programmed genome editing in human cells. *eLife*, *2*. https://doi.org/10.7554/elife.00471

Jürgens, M. D., Crosse, J., Hamilton, P. B., Johnson, A. C., & Jones, K. C. (2016). The long shadow of our chemical past – High DDT concentrations in fish near a former agrochemicals factory in England. *Chemosphere*, *162*, 333-344. https://doi.org/https://doi.org/10.1016/j.chemosphere.2016.07.078

Kafri, T., Van Praag, H., Gage, F. H., & Verma, I. M. (2000). Lentiviral Vectors: Regulated Gene Expression. *Molecular Therapy*, *1*(6), 516-521. https://doi.org/10.1006/mthe.2000.0083

Kanca, O., Zirin, J., Garcia-Marques, J., Knight, S. M., Yang-Zhou, D., Amador, G., Chung, H., Zuo, Z., Ma, L., He, Y., Lin, W.-W., Fang, Y., Ge, M., Yamamoto, S., Schulze, K. L., Hu, Y., Spradling, A. C., Mohr, S. E., Perrimon, N., & Bellen, H. J. (2019). An efficient CRISPR-based strategy to insert small and large fragments of DNA using short homology arms. *eLife*, *8*, e51539. https://doi.org/10.7554/eLife.51539

Killick, R., Ribe, E. M., Al-Shawi, R., Malik, B., Hooper, C., Fernandes, C., Dobson, R., Nolan, P. M., Lourdusamy, A., Furney, S., Lin, K., Breen, G., Wroe, R., To, A. W. M., Leroy, K., Causevic, M., Usardi, A., Robinson, M., Noble, W., . . . Lovestone, S. (2014). Clusterin regulates β-amyloid toxicity via Dickkopf-1-driven induction of the wnt–PCP–JNK pathway. *Molecular Psychiatry*, *19*(1), 88-98. https://doi.org/10.1038/mp.2012.163

Klingner, C. M., & Witte, O. W. (2018). Somatosensory deficits. *Handb Clin Neurol*, *151*, 185-206. https://doi.org/10.1016/b978-0-444-63622-5.00009-7

Klunk, W. E., Engler, H., Nordberg, A., Wang, Y., Blomqvist, G., Holt, D. P., Bergström, M., Savitcheva, I., Huang, G. F., Estrada, S., Ausén, B., Debnath, M. L., Barletta, J., Price, J. C., Sandell, J., Lopresti, B. J., Wall, A., Koivisto, P., Antoni, G., . . . Långström, B. (2004). Imaging brain amyloid in Alzheimer's disease with Pittsburgh Compound-B. *Ann Neurol*, *55*(3), 306-319. https://doi.org/10.1002/ana.20009

Knierim, J. J. (2015). The hippocampus. *Current Biology*, *25*(23), R1116-R1121. https://doi.org/10.1016/j.cub.2015.10.049

Konttinen, H., Cabral-da-Silva, M. e. C., Ohtonen, S., Wojciechowski, S., Shakirzyanova, A., Caligola, S., Giugno, R., Ishchenko, Y., Hernández, D., Fazaludeen, M. F., Eamen, S., Budia, M. G., Fagerlund, I., Scoyni, F., Korhonen, P., Huber, N., Haapasalo, A., Hewitt, A. W., Vickers, J., . . . Malm, T. (2019). PSEN1DE9, APPswe, and APOE4 Confer Disparate Phenotypes in Human

iPSC-Derived Microglia. *Stem Cell Reports*, *13*(4), 669-683. https://doi.org/10.1016/j.stemcr.2019.08.004

Kukull, W. A., Larson, E. B., Bowen, J. D., McCormick, W. C., Teri, L., Pfanschmidt, M. L., Thompson, J. D., O'Meara, E. S., Brenner, D. E., & van Belle, G. (1995). Solvent exposure as a risk factor for Alzheimer's disease: a case-control study. *Am J Epidemiol*, *141*(11), 1059-1071; discussion 1072-1059. https://doi.org/10.1093/oxfordjournals.aje.a117370

Laferla, F. M., Tinkle, B. T., Bieberich, C. J., Jay, G., & Haudenschild, C. C. (1995). The Alzheimer's Aβ peptide induces neurodegeneration and apoptotic cell death in transgenic mice [Article]. *NATURE GENETICS*, *9*(1), 21-30. https://doi.org/10.1038/ng0195-21

Lammich, S., Kojro, E., Postina, R., Gilbert, S., Pfeiffer, R., Jasionowski, M., Haass, C., & Fahrenholz, F. (1999). Constitutive and regulated -secretase cleavage of Alzheimer's amyloid precursor protein by a disintegrin metalloprotease. *Proceedings of the National Academy of Sciences*, *96*(7), 3922-3927. https://doi.org/10.1073/pnas.96.7.3922

Lanfranco, M. F., Sepulveda, J., Kopetsky, G., & Rebeck, G. W. (2021). Expression and secretion of apoE isoforms in astrocytes and microglia during inflammation. *Glia*, *69*(6), 1478-1493. https://doi.org/https://doi.org/10.1002/glia.23974

Läuger, P., Martin, H., & Müller, P. (1944). Über Konstitution und toxische Wirkung von natürlichen und neuen synthetischen insektentötenden Stoffen [https://doi.org/10.1002/hlca.194402701115]. *Helvetica Chimica Acta*, *27*(1), 892-928. https://doi.org/https://doi.org/10.1002/hlca.194402701115

Lazarov, O., & Tesco, G. (2016). *Genes, Environment and Alzheimer's Disease* [Book]. Academic Press. https://login.proxy.libraries.rutgers.edu/login?url=https://search.ebscohost.com/login.aspx?direct=true&db=nlebk&AN=1144264&site=eds-live

Lee, Y., Kockx, M., Raftery, M. J., Jessup, W., Griffith, R., & Kritharides, L. (2010). Glycosylation and Sialylation of Macrophage-derived Human Apolipoprotein E Analyzed by SDS-PAGE and Mass Spectrometry. *Molecular & Cellular Proteomics*, *9*(9), 1968-1981. https://doi.org/10.1074/mcp.m900430-mcp200

Leimer, U., Lun, K., Romig, H., Walter, J., Grünberg, J., Brand, M., & Haass, C. (1999). Zebrafish (Danio rerio) presenilin promotes aberrant amyloid beta-peptide production and requires a critical aspartate residue for its function in amyloidogenesis. *Biochemistry*, *38*(41), 13602-13609. https://doi.org/10.1021/bi991453n

Lenaeus, M. J., Gamal El-Din, T. M., Ing, C., Ramanadane, K., Pomès, R., Zheng, N., & Catterall, W. A. (2017). Structures of closed and open states of a voltage-gated sodium channel. *Proceedings of the National Academy of Sciences*, *114*(15), E3051. https://doi.org/10.1073/pnas.1700761114

Leuner, K., Pantel, J., Frey, C., Schindowski, K., Schulz, K., Wegat, T., Maurer, K., Eckert, A., & Müller, W. E. (2007). Enhanced apoptosis, oxidative stress and mitochondrial dysfunction in lymphocytes as potential biomarkers for Alzheimer's disease. *J Neural Transm Suppl*(72), 207-215. https://doi.org/10.1007/978-3-211-73574-9_27

Li, G., Kim, C., Kim, J., Yoon, H., Zhou, H., & Kim, J. (2015). Common Pesticide, Dichlorodiphenyltrichloroethane (DDT), Increases Amyloid-beta Levels by Impairing the Function of ABCA1 and IDE: Implication for Alzheimer's Disease [Article]. *J Alzheimers Dis*, *46*(1), 109-122. https://doi.org/10.3233/JAD-150024

Li, G., Zhou, H., Kim, C., Kim, J., Yoon, H., & Kim, J. (2015). Common pesticide, dichlorodiphenyltrichloroethane (DDT), increases amyloid-β levels by impairing the function of ABCA1 and IDE: Implication for Alzheimer's disease [Article]. *Journal of Alzheimer's Disease*, *46*(1), 109-122. https://doi.org/10.3233/JAD-150024

Li, M., Husic, N., Lin, Y., Christensen, H., Malik, I., McIver, S., Daniels, C. M. L., Harris, D. A., Kotzbauer, P. T., Goldberg, M. P., & Snider, B. J. (2010). Optimal promoter usage for lentiviral vector-mediated transduction of cultured central nervous system cells. *Journal of neuroscience methods*, *189*(1), 56-64. https://doi.org/10.1016/j.jneumeth.2010.03.019

Li, X., Lv, Y., Yu, S., Zhao, H., & Yao, L. (2012). The effect of cadmium on Aβ levels in APP/PS1 transgenic mice. *Experimental and Therapeutic Medicine*, *4*(1), 125-130. https://doi.org/10.3892/etm.2012.562

Liang, P., Xu, Y., Zhang, X., Ding, C., Huang, R., Zhang, Z., Lv, J., Xie, X., Chen, Y., Li, Y., Sun, Y., Bai, Y., Songyang, Z., Ma, W., Zhou, C., & Huang, J. (2015). CRISPR/Cas9-mediated gene editing in human tripronuclear zygotes. *Protein & Cell*, *6*(5), 363-372. https://doi.org/10.1007/s13238-015-0153-5

Lindwall, G., & Cole, R. D. (1984). Phosphorylation affects the ability of tau protein to promote microtubule assembly. *Journal of Biological Chemistry*, *259*(8), 5301-5305. https://doi.org/10.1016/s0021-9258(17)42989-9

Lucas, P., & Renou, M. (1992). Electrophysiological study of the effects of deltamethrin, bioresmethrin, and DDT on the activity of pheromone receptor neurones in two moth species. *Pesticide Biochemistry and Physiology*, *43*(2), 103-115. https://doi.org/https://doi.org/10.1016/0048-3575(92)90024-T

Makino, S., Fukumura, R., & Gondo, Y. (2016). Illegitimate translation causes unexpected gene expression from on-target out-of-frame alleles created by CRISPR-Cas9. *Scientific Reports*, *6*(1), 39608. https://doi.org/10.1038/srep39608

Mali, P., Yang, L., Esvelt, K. M., Aach, J., Guell, M., DiCarlo, J. E., Norville, J. E., & Church, G. M. (2013). RNA-Guided Human Genome Engineering via Cas9. *Science*, *339*(6121), 823-826. https://doi.org/doi:10.1126/science.1232033

Malloy, P. F., & Richardson, E. D. (1994). Assessment of frontal lobe functions. *J Neuropsychiatry Clin Neurosci*, *6*(4), 399-410. https://doi.org/10.1176/jnp.6.4.399

Marques, M. A., Tolar, M., Harmony, J. A., & Crutcher, K. A. (1996). A thrombin cleavage fragment of apolipoprotein E exhibits isoform-specific neurotoxicity. *Neuroreport*, *7*(15-17), 2529-2532. https://doi.org/10.1097/00001756-199611040-00025

Mathys, H., Davila-Velderrain, J., Peng, Z., Gao, F., Mohammadi, S., Young, J. Z., Menon, M., He, L., Abdurrob, F., Jiang, X., Martorell, A. J., Ransohoff, R. M., Hafler, B. P., Bennett, D. A., Kellis, M., & Tsai, L.-H. (2019). Author Correction: Single-cell transcriptomic analysis of Alzheimer's disease. *Nature*, *571*(7763). https://doi.org/10.1038/s41586-019-1329-6

Matthews, F. E., Stephan, B. C. M., Robinson, L., Jagger, C., Barnes, L. E., Arthur, A., & Brayne, C. (2016). A two decade dementia incidence comparison from the Cognitive Function and Ageing Studies I and II. *Nature Communications*, *7*(1), 11398. https://doi.org/10.1038/ncomms11398

Mawuenyega, K. G., Kasten, T., Sigurdson, W., & Bateman, R. J. (2013). Amyloid-beta isoform metabolism quantitation by stable isotope-labeled kinetics. *Analytical Biochemistry*, *440*(1), 56-62. https://doi.org/10.1016/j.ab.2013.04.031

Medehouenou, T. C. M., Ayotte, P., Carmichael, P.-H., Kröger, E., Verreault, R., Lindsay, J., Dewailly, É., Tyas, S. L., Bureau, A., & Laurin, D. (2019). Exposure to polychlorinated biphenyls and organochlorine pesticides and risk of dementia, Alzheimer's disease and cognitive decline in an older population: a prospective analysis from the Canadian Study of Health and Aging. *Environmental Health*, *18*(1). https://doi.org/10.1186/s12940-019-0494-2

Mellanby, K., & British Crop Protection, C. (1992). *The DDT story*. British Crop Protection Council. //catalog.hathitrust.org/Record/002615237
http://hdl.handle.net/2027/mdp.39015029255109

Mendivil, C. O., Rimm, E. B., Furtado, J., & Sacks, F. M. (2013). Apolipoprotein E in VLDL and LDL With Apolipoprotein C-III is Associated With a Lower Risk of Coronary Heart Disease. *Journal of the American Heart Association*, *2*(3), e000130-e000130. https://doi.org/10.1161/jaha.113.000130

Mirra, S. S., Hart, M. N., & Terry, R. D. (1993). Making the diagnosis of Alzheimer's disease. A primer for practicing pathologists. *Arch Pathol Lab Med*, *117*(2), 132-144.

Montine, T. J., Phelps, C. H., Beach, T. G., Bigio, E. H., Cairns, N. J., Dickson, D. W., Duyckaerts, C., Frosch, M. P., Masliah, E., Mirra, S. S., Nelson, P. T., Schneider, J. A., Thal, D. R., Trojanowski, J. Q., Vinters, H. V., & Hyman, B. T. (2012). National Institute on Aging-Alzheimer's Association guidelines for the neuropathologic assessment of Alzheimer's

disease: a practical approach. *Acta Neuropathol*, *123*(1), 1-11. https://doi.org/10.1007/s00401-011-0910-3

Morgan, D. P., & Roan, C. C. (1971). Absorption, Storage, and Metabolic Conversion of Ingested DDT and DDT Metabolites in Man. *Archives of Environmental Health: An International Journal*, *22*(3), 301-308. https://doi.org/10.1080/00039896.1971.10665848

Morris, J. C., Roe, C. M., Xiong, C., Fagan, A. M., Goate, A. M., Holtzman, D. M., & Mintun, M. A. (2010). APOEpredicts amyloid-beta but not tau Alzheimer pathology in cognitively normal aging. *Annals of Neurology*, *67*(1), 122-131. https://doi.org/10.1002/ana.21843

Mota, P. C., Cordeiro, M., Pereira, S. P., Oliveira, P. J., Moreno, A. J., & Ramalho-Santos, J. (2011). Differential effects of p,p′-DDE on testis and liver mitochondria:Implications for reproductive toxicology. *Reproductive Toxicology*, *31*(1), 80-85. https://doi.org/10.1016/j.reprotox.2010.09.010

Moullan, N., Mouchiroud, L., Wang, X., Ryu, D., Williams, E. G., Mottis, A., Jovaisaite, V., Frochaux, M. V., Quiros, P. M., Deplancke, B., Houtkooper, R. H., & Auwerx, J. (2015). Tetracyclines Disturb Mitochondrial Function across Eukaryotic Models: A Call for Caution in Biomedical Research. *Cell Rep*, *10*(10), 1681-1691. https://doi.org/10.1016/j.celrep.2015.02.034

Müller, P. (1946). 204. Über Zusammenhänge zwischen Konstitution und insektizider Wirkung I (Dichlordiphenyl-trichlor-äthan-Derivate und verwandte Verbindungen) [https://doi.org/10.1002/hlca.19460290626]. *Helvetica Chimica Acta*, *29*(6), 1560-1580. https://doi.org/https://doi.org/10.1002/hlca.19460290626

Musa, A., Lehrach, H., & Russo, V. A. (2001). Distinct expression patterns of two zebrafish homologues of the human APP gene during embryonic development. *Dev Genes Evol*, *211*(11), 563-567. https://doi.org/10.1007/s00427-001-0189-9

Narahashi, T., & Haas, H. G. (1967). DDT: Interaction with Nerve Membrane Conductance Changes. *Science*, *157*(3795), 1438. https://doi.org/10.1126/science.157.3795.1438

Naseribafrouei, A., Hestad, K., Avershina, E., Sekelja, M., Linløkken, A., Wilson, R., & Rudi, K. (2014). Correlation between the human fecal microbiota and depression. *Neurogastroenterol Motil*, *26*(8), 1155-1162. https://doi.org/10.1111/nmo.12378

Nelson, C. E., Hakim, C. H., Ousterout, D. G., Thakore, P. I., Moreb, E. A., Rivera, R. M. C., Madhavan, S., Pan, X., Ran, F. A., Yan, W. X., Asokan, A., Zhang, F., Duan, D., & Gersbach, C. A. (2016). In vivo genome editing improves muscle function in a mouse model of Duchenne muscular dystrophy. *Science*, *351*(6271), 403-407. https://doi.org/doi:10.1126/science.aad5143

Nishimura, M., Yu, G., Levesque, G., Zhang, D. M., Ruel, L., Chen, F., Milman, P., Holmes, E., Liang, Y., Kawarai, T., Jo, E., Supala, A., Rogaeva, E., Xu, D. M., Janus, C., Levesque, L., Bi, Q., Duthie, M., Rozmahel, R., . . . St George–Hyslop, P. (1999). Presenilin mutations associated with Alzheimer disease cause defective intracellular trafficking of β-catenin,a component of the presenilin protein complex. *Nature Medicine*, *5*(2), 164-169. https://doi.org/10.1038/5526

Notarachille, G., Arnesano, F., Calò, V., & Meleleo, D. (2014). Heavy metals toxicity: effect of cadmium ions on amyloid beta protein 1-42. Possible implications for Alzheimer's disease. *Biometals*, *27*(2), 371-388. https://doi.org/10.1007/s10534-014-9719-6

Oakley, H., Cole, S. L., Logan, S., Maus, E., Shao, P., Craft, J., Guillozet-Bongaarts, A., Ohno, M., Disterhoft, J., Van Eldik, L., Berry, R., & Vassar, R. (2006). Intraneuronal beta-amyloid aggregates, neurodegeneration, and neuron loss in transgenic mice with five familial Alzheimer's disease mutations: potential factors in amyloid plaque formation. *J Neurosci*, *26*(40), 10129-10140. https://doi.org/10.1523/jneurosci.1202-06.2006

Oddo, S., Caccamo, A., Smith, I. F., Green, K. N., & LaFerla, F. M. (2006). A dynamic relationship between intracellular and extracellular pools of Aβ [Article]. *American Journal of Pathology*, *168*(1), 184-194. https://doi.org/10.2353/ajpath.2006.050593

Ohkubo, N., Mitsuda, N., Tamatani, M., Yamaguchi, A., Lee, Y.-D., Ogihara, T., Vitek, M. P., & Tohyama, M. (2001). Apolipoprotein E4 Stimulates cAMP Response Element-binding Protein Transcriptional Activity through the Extracellular Signal-regulated Kinase Pathway. *Journal of Biological Chemistry*, *276*(5), 3046-3053. https://doi.org/10.1074/jbc.M005070200

Pang, Z. P., Yang, N., Vierbuchen, T., Ostermeier, A., Fuentes, D. R., Yang, T. Q., Citri, A., Sebastiano, V., Marro, S., Südhof, T. C., & Wernig, M. (2011). Induction of human neuronal cells by defined transcription factors. *Nature*, *476*(7359), 220-223. https://doi.org/10.1038/nature10202

Paquet, D., Kwart, D., Chen, A., Sproul, A., Jacob, S., Teo, S., Olsen, K. M., Gregg, A., Noggle, S., & Tessier-Lavigne, M. (2016). Efficient introduction of specific homozygous and heterozygous mutations using CRISPR/Cas9. *Nature*, *533*(7601), 125-129. https://doi.org/10.1038/nature17664

Parron, T., Requena, M., Hernandez, A. F., & Alarcon, R. (2011). Association between environmental exposure to pesticides and neurodegenerative diseases. *Toxicol Appl Pharmacol*, *256*(3), 379-385. https://doi.org/10.1016/j.taap.2011.05.006

Payami, H., Zareparsi, S., Montee, K. R., Sexton, G. J., Kaye, J. A., Bird, T. D., Yu, C. E., Wijsman, E. M., Heston, L. L., Litt, M., & Schellenberg, G. D. (1996). Gender difference in apolipoprotein E-associated risk for familial Alzheimer disease: a possible clue to the higher incidence of Alzheimer disease in women. *American journal of human genetics*, *58*(4), 803-811. https://pubmed.ncbi.nlm.nih.gov/8644745

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1914663/

Pekeles, H., Qureshi, H. Y., Paudel, H. K., Schipper, H. M., Gornistky, M., & Chertkow, H. (2018). Development and validation of a salivary tau biomarker in Alzheimer's disease. *Alzheimer's & Dementia: Diagnosis, Assessment & Disease Monitoring*, *10*(1), 53-60. https://doi.org/10.1016/j.dadm.2018.03.003

Pérez-Maldonado, I. N., Herrera, C., Batres, L. E., González-Amaro, R., Díaz-Barriga, F., & Yáñez, L. (2005). DDT-induced oxidative damage in human blood mononuclear cells. *Environmental Research*, *98*(2), 177-184. https://doi.org/10.1016/j.envres.2004.11.001

Perez, E. E., Wang, J., Miller, J. C., Jouvenot, Y., Kim, K. A., Liu, O., Wang, N., Lee, G., Bartsevich, V. V., Lee, Y.-L., Guschin, D. Y., Rupniewski, I., Waite, A. J., Carpenito, C., Carroll, R. G., S Orange, J., Urnov, F. D., Rebar, E. J., Ando, D., . . . June, C. H. (2008). Establishment of HIV-1 resistance in CD4+ T cells by genome editing using zinc-finger nucleases. *Nature Biotechnology*, *26*(7), 808-816. https://doi.org/10.1038/nbt1410

Plassman, B. L., Langa, K. M., Fisher, G. G., Heeringa, S. G., Weir, D. R., Ofstedal, M. B., Burke, J. R., Hurd, M. D., Potter, G. G., Rodgers, W. L., Steffens, D. C., Willis, R. J., & Wallace, R. B. (2007). Prevalence of Dementia in the United States: The Aging, Demographics, and Memory Study. *Neuroepidemiology*, *29*(1-2), 125-132. https://doi.org/10.1159/000109998

Popova, D., Desai, N., Blendy, J. A., & Pang, Z. P. (2019). Synaptic Regulation by OPRM1 Variants in Reward Neurocircuitry. *The Journal of Neuroscience*, *39*(29), 5685-5696. https://doi.org/10.1523/jneurosci.2317-18.2019

Portelius, E., Brinkmalm, G., Tran, A. J., Zetterberg, H., Westman-Brinkmalm, A., & Blennow, K. (2009). Identification of novel APP/Abeta isoforms in human cerebrospinal fluid. *Neurodegener Dis*, *6*(3), 87-94. https://doi.org/10.1159/000203774

Portelius, E., Zetterberg, H., Dean, R. A., Marcil, A., Bourgeois, P., Nutu, M., Andreasson, U., Siemers, E., Mawuenyega, K. G., Sigurdson, W. C., May, P. C., Paul, S. M., Holtzman, D. M., Blennow, K., & Bateman, R. J. (2012). Amyloid-β(1-15/16) as a marker for γ-secretase

inhibition in Alzheimer's disease. *J Alzheimers Dis*, *31*(2), 335-341. https://doi.org/10.3233/JAD-2012-120508

Powell, J., Lambert, j.-c., Gu, W., Crane, P., Hardy, J., berr, C., Bosso, P., Schott, J., Kowall, N., Reposo, R.-L., Aspelund, T., Singleton, A., Seshadri, S., Pickering-Brown, S., Escott-Price, V., Reisberg, B., duron, e., Buxbaum, J., Smith, A. V., . . . Launer, L. J. (2013). Meta-analysis of 74,046 individuals identifies 11 new susceptibility loci for Alzheimer's disease. *NATURE GENETICS*, *45*(12), 1452-U1206. https://login.proxy.libraries.rutgers.edu/login?url=https://search.ebscohost.com/login.aspx?direct=true&db=edswsc&AN=000327715800010&site=eds-live

Qiu, Z., Hyman, B. T., & Rebeck, G. W. (2004). Apolipoprotein E Receptors Mediate Neurite Outgrowth through Activation of p44/42 Mitogen-activated Protein Kinase in Primary Neurons. *Journal of Biological Chemistry*, *279*(33), 34948-34956. https://doi.org/10.1074/jbc.M401055200

Quan, C., Shi, Y., Wang, C., Wang, C., & Yang, K. (2016). p,p'-DDE damages spermatogenesis via phospholipid hydroperoxide glutathione peroxidase depletion and mitochondria apoptosis pathway. *Environmental Toxicology*, *31*(5), 593-600. https://doi.org/https://doi.org/10.1002/tox.22072

Raffai, R. L., Dong, L. M., Farese, R. V., Jr., & Weisgraber, K. H. (2001). Introduction of human apolipoprotein E4 "domain interaction" into mouse apolipoprotein E. *Proc Natl Acad Sci U S A*, *98*(20), 11587-11591. https://doi.org/10.1073/pnas.201279298

Rajan, K. B., Weuve, J., Barnes, L. L., McAninch, E. A., Wilson, R. S., & Evans, D. A. (2021). Population estimate of people with clinical Alzheimer's disease and mild cognitive impairment in the United States (2020–2060). *Alzheimer's & Dementia*. https://doi.org/10.1002/alz.12362

RajaRajeswari, P., Viswanadha Raju, S., & Ashour, A. S. (2016). Active site cavities identification of amyloid beta precursor protein: Alzheimer disease study. In (pp. 319): IEEE.

Ran, F. A., Hsu, P. D., Wright, J., Agarwala, V., Scott, D. A., & Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. *Nature Protocols*, *8*(11), 2281-2308. https://doi.org/10.1038/nprot.2013.143

Rehwagen, C. (2006). WHO recommends DDT to control malaria. *Bmj*, *333*(7569), 622. https://doi.org/10.1136/bmj.333.7569.622-b

Rhinn, H., Fujita, R., Qiang, L., Cheng, R., Lee, J. H., & Abeliovich, A. (2013). Integrative genomics identifies APOE ε4 effectors in Alzheimer's disease. *Nature*, *500*(7460), 45. https://login.proxy.libraries.rutgers.edu/login?url=http://search.ebscohost.com/login.aspx?direct=true&db=edb&AN=89496489&site=eds-live

Richardson, J. R., Roy, A., Shalat, S. L., von Stein, R. T., Hossain, M. M., Buckley, B., Gearing, M., Levey, A. I., & German, D. C. (2014). Elevated serum pesticide levels and risk for Alzheimer disease. *JAMA Neurol*, *71*(3), 284-290. https://doi.org/10.1001/jamaneurol.2013.6030

Richardson, J. R., Shalat, S. L., Buckley, B., Winnik, B., O'Suilleabhain, P., Diaz-Arrastia, R., Reisch, J., & German, D. C. (2009). Elevated serum pesticide levels and risk of Parkinson disease [Article]. *Archives of Neurology*, *66*(7), 870-875. https://doi.org/10.1001/archneurol.2009.89

Robbins, J. P., Perfect, L., Ribe, E. M., Maresca, M., Dangla-Valls, A., Foster, E. M., Killick, R., Nowosiad, P., Reid, M. J., Polit, L. D., Nevado, A. J., Ebner, D., Bohlooly-Y, M., Buckley, N., Pangalos, M. N., Price, J., & Lovestone, S. (2018). Clusterin Is Required for β-Amyloid Toxicity in Human iPSC-Derived Neurons [Original Research]. *Frontiers in Neuroscience*, *12*. https://doi.org/10.3389/fnins.2018.00504

Roberts, R. O., Knopman, D. S., Mielke, M. M., Cha, R. H., Pankratz, V. S., Christianson, T. J. H., Geda, Y. E., Boeve, B. F., Ivnik, R. J., Tangalos, E. G., Rocca, W. A., & Petersen, R. C.

(2014). Higher risk of progression to dementia in mild cognitive impairment cases who revert to normal. *Neurology*, *82*(4), 317-325. https://doi.org/10.1212/wnl.0000000000000055

Rohn, T. T. (2013). Proteolytic cleavage of apolipoprotein E4 as the keystone for the heightened risk associated with Alzheimer's disease. *Int J Mol Sci*, *14*(7), 14908-14922. https://doi.org/10.3390/ijms140714908

Roostaei, T., Nazeri, A., Sahraian, M. A., & Minagar, A. (2014). The human cerebellum: a review of physiologic neuroanatomy. *Neurol Clin*, *32*(4), 859-869. https://doi.org/10.1016/j.ncl.2014.07.013

Ruitenberg, A., Ott, A., van Swieten, J. C., Hofman, A., & Breteler, M. M. B. (2001). Incidence of dementia: does gender make a difference? *Neurobiology of Aging*, *22*(4), 575-580. https://doi.org/https://doi.org/10.1016/S0197-4580(01)00231-7

Rush, A. M., Cummins, T. R., & Waxman, S. G. (2007). Multiple sodium channels and their roles in electrogenesis within dorsal root ganglion neurons. *The Journal of Physiology*, *579*(1), 1-14. https://doi.org/10.1113/jphysiol.2006.121483

Sabo, S. L., Ikin, A. F., Buxbaum, J. D., & Greengard, P. (2003). The Amyloid Precursor Protein and Its Regulatory Protein, FE65, in Growth Cones and SynapsesIn VitroandIn Vivo. *The Journal of Neuroscience*, *23*(13), 5407-5415. https://doi.org/10.1523/jneurosci.23-13-05407.2003

Saeed, U., Mirza, S. S., Macintosh, B. J., Herrmann, N., Keith, J., Ramirez, J., Nestor, S. M., Yu, Q., Knight, J., Swardfager, W., Potkin, S. G., Rogaeva, E., St. George-Hyslop, P., Black, S. E., & Masellis, M. (2018). APOE -ε4 associates with hippocampal volume, learning, and memory across the spectrum of Alzheimer's disease and dementia with Lewy bodies. *Alzheimer's & Dementia*, *14*(9), 1137-1147. https://doi.org/10.1016/j.jalz.2018.04.005

Sala-Vila, A., Navarro-Lérida, I., Sánchez-Alvarez, M., Bosch, M., Calvo, C., López, J. A., Calvo, E., Ferguson, C., Giacomello, M., Serafini, A., Scorrano, L., Enriquez, J. A., Balsinde, J., Parton, R. G., Vázquez, J., Pol, A., & Del Pozo, M. A. (2016). Interplay between hepatic mitochondria-associated membranes, lipid metabolism and caveolin-1 in mice. *Scientific Reports*, *6*(1), 27351. https://doi.org/10.1038/srep27351

Saleh-Gohari, N., & Helleday, T. (2004). Conservative homologous recombination preferentially repairs DNA double-strand breaks in the S phase of the cell cycle in human cells. *Nucleic Acids Research*, *32*(12), 3683-3688. https://doi.org/10.1093/nar/gkh703

Salloway, S., Chalkias, S., Barkhof, F., Burkett, P., Barakos, J., Purcell, D., Suhy, J., Forrestal, F., Tian, Y., Umans, K., Wang, G., Singhal, P., Budd Haeberlein, S., & Smirnakis, K. (2022). Amyloid-Related Imaging Abnormalities in 2 Phase 3 Studies Evaluating Aducanumab in Patients With Early Alzheimer Disease. *JAMA Neurol*, *79*(1), 13. https://doi.org/10.1001/jamaneurol.2021.4161

Sapranauskas, R., Gasiunas, G., Fremaux, C., Barrangou, R., Horvath, P., & Siksnys, V. (2011). The Streptococcus thermophilus CRISPR/Cas system provides immunity in Escherichia coli. *Nucleic Acids Research*, *39*(21), 9275-9282. https://doi.org/10.1093/nar/gkr606

Scarnati, M. S., Boreland, A. J., Joel, M., Hart, R. P., & Pang, Z. P. (2020). Differential sensitivity of human neurons carrying μ opioid receptor (MOR) N40D variants in response to ethanol. *Alcohol*, *87*, 97-109. https://doi.org/10.1016/j.alcohol.2020.05.004

Selden, S. C., & Pollard, T. D. (1983). Phosphorylation of microtubule-associated proteins regulates their interaction with actin filaments. *The Journal of biological chemistry*, *258*(11), 7064-7071. https://doi.org/10.1016/S0021-9258(18)32333-0

Seshadri, S., Wolf, P. A., Beiser, A., Au, R., McNulty, K., White, R., & D'Agostino, R. B. (1997). Lifetime risk of dementia and Alzheimer's disease : The impact of mortality on risk estimates in the Framingham study. *Neurology*, *49*(6), 1498-1504. https://doi.org/10.1212/WNL.49.6.1498

Shanes, A. M. (1949). ELECTRICAL PHENOMENA IN NERVE : II. CRAB NERVE. *Journal of General Physiology*, *33*(1), 75-102. https://doi.org/10.1085/jgp.33.1.75

Shanes, A. M. (1949). Electrical phenomena in nerve; squid giant axon. *The Journal of general physiology*, *33*(1), 57-73. https://doi.org/10.1085/jgp.33.1.57

Shanes, A. M. (1951). Electrical phenomena in nerve. III. Frog sciatic nerve [https://doi.org/10.1002/jcp.1030380103]. *Journal of Cellular and Comparative Physiology*, *38*(1), 17-40. https://doi.org/https://doi.org/10.1002/jcp.1030380103

Shankland, D. L. (1964). Involvement of spinal cord and peripheral nerves in DDT-poisoning syndrome in albino rats. *Toxicol Appl Pharmacol*, *6*(2), 197-213. https://doi.org/https://doi.org/10.1016/0041-008X(64)90105-X

Shi, M., Sui, Y. T., Peskind, E. R., Li, G., Hwang, H., Devic, I., Ginghina, C., Edgar, J. S., Pan, C., Goodlett, D. R., Furay, A. R., Gonzalez-Cuyar, L. F., & Zhang, J. (2011). Salivary tau species are potential biomarkers of Alzheimer's disease. *J Alzheimers Dis*, *27*(2), 299-305. https://doi.org/10.3233/jad-2011-110731

Shi, Y., Yamada, K., Liddelow, S. A., Smith, S. T., Zhao, L., Luo, W., Tsai, R. M., Spina, S., Grinberg, L. T., Rojas, J. C., Gallardo, G., Wang, K., Roh, J., Robinson, G., Finn, M. B., Jiang, H., Sullivan, P. M., Baufeld, C., Wood, M. W., . . . Holtzman, D. M. (2017). ApoE4 markedly exacerbates tau-mediated neurodegeneration in a mouse model of tauopathy. *Nature*, *549*(7673), 523-527. https://doi.org/10.1038/nature24016

Shinohara, M., Kanekiyo, T., Yang, L., Linthicum, D., Shinohara, M., Fu, Y., Price, L., Frisch-Daiello, J. L., Han, X., Fryer, J. D., & Bu, G. (2016). APOE2eases cognitive decline during Aging: Clinical and preclinical evaluations. *Annals of Neurology*, *79*(5), 758-774. https://doi.org/10.1002/ana.24628

Shrager, P. G., Macey, R. I., & Strickholm, A. (1969). Internal perfusion of crayfish, giant axons: action of tannic acid, DDT, and TEA. *J Cell Physiol*, *74*(1), 77-90. https://doi.org/10.1002/jcp.1040740111

Shulman, J. M., & Feany, M. B. (2003). Genetic modifiers of tauopathy in Drosophila. *Genetics*, *165*(3), 1233-1242. https://doi.org/10.1093/genetics/165.3.1233

Simkin, D., Papakis, V., Bustos, B. I., Ambrosi, C. M., Ryan, S. J., Baru, V., Williams, L. A., Dempsey, G. T., McManus, O. B., Landers, J. E., Lubbe, S. J., George, A. L., & Kiskinis, E. (2022). Homozygous might be hemizygous: CRISPR/Cas9 editing in iPSCs results in detrimental on-target defects that escape standard quality controls. *Stem Cell Reports*, *17*(4), 993-1008. https://doi.org/10.1016/j.stemcr.2022.02.008

Simons, M., de Strooper, B., Multhaup, G., Tienari, P. J., Dotti, C. G., & Beyreuther, K. (1996). Amyloidogenic processing of the human amyloid precursor protein in primary cultures of rat hippocampal neurons. *J Neurosci*, *16*(3), 899-908.

Singh, D. K., & Agarwal, R. A. (1983). Inhibition kinetics of certain organophosphorus and carbamate pesticides on acetylcholinesterase from the snail Lymnaea acuminata. *Toxicology Letters*, *19*(3), 313-319. https://doi.org/https://doi.org/10.1016/0378-4274(83)90136-4

Singh, N. K., Banerjee, B. D., Bala, K., Basu, M., & Chhillar, N. (2014). Polymorphism in Cytochrome P450 2D6, Glutathione S-Transferases Pi 1 Genes, and Organochlorine Pesticides in Alzheimer Disease: A Case-Control Study in North Indian Population. *J Geriatr Psychiatry Neurol*, *27*(2), 119-127. https://doi.org/10.1177/0891988714522698

Singh, N. K., Chhillar, N., Banerjee, B. D., Bala, K., Basu, M., & Mustafa, M. (2013). Organochlorine pesticide levels and risk of Alzheimer's disease in north Indian population [Article]. *Human & Experimental Toxicology*, *32*(1), 24-30. https://doi.org/10.1177/0960327112456315

Singh, N. K., Chhillar, N., Banerjee, B. D., Bala, K., Mukherjee, A. K., Mustafa, M. D., & Mitrabasu. (2012). Gene-environment interaction in Alzheimer's disease. *Am J Alzheimers Dis Other Demen*, *27*(7), 496-503. https://doi.org/10.1177/1533317512456067

Sisodia, S. S. (1992). Beta-amyloid precursor protein cleavage by a membrane-bound protease. *Proceedings of the National Academy of Sciences*, *89*(13), 6075-6079. https://doi.org/10.1073/pnas.89.13.6075

Smits, A. H., Ziebell, F., Joberty, G., Zinn, N., Mueller, W. F., Clauder-Münster, S., Eberhard, D., Fälth Savitski, M., Grandi, P., Jakob, P., Michon, A.-M., Sun, H., Tessmer, K., Bürckstümmer, T., Bantscheff, M., Steinmetz, L. M., Drewes, G., & Huber, W. (2019). Biological plasticity rescues target activity in CRISPR knock outs. *Nature Methods*, *16*(11), 1087-1093. https://doi.org/10.1038/s41592-019-0614-5

Son, S.-J., Kim, J., & Park, H. (2017). Structural and functional connectional fingerprints in mild cognitive impairment and Alzheimer's disease patients. *PLoS ONE*, *12*(3), e0173426. https://doi.org/10.1371/journal.pone.0173426

Song, Y., Liang, X., Hu, Y., Wang, Y., Yu, H., & Yang, K. (2008). p,p′-DDE induces mitochondria-mediated apoptosis of cultured rat Sertoli cells. *Toxicology*, *253*(1-3), 53-61. https://doi.org/10.1016/j.tox.2008.08.013

Stevens, B. W., DiBattista, A. M., William Rebeck, G., & Green, A. E. (2014). A gene−brain−cognition pathway for the effect of an Alzheimer's risk gene on working memory in young adults [Article]. *Neuropsychologia*, *61*, 143-149. https://doi.org/10.1016/j.neuropsychologia.2014.06.021

Strittmatter, W. J., Saunders, A. M., Schmechel, D., Pericak-Vance, M., Enghild, J., Salvesen, G. S., & Roses, A. D. (1993). Apolipoprotein E: high-avidity binding to beta-amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer disease. *Proceedings of the National Academy of Sciences*, *90*(5), 1977-1981. https://doi.org/10.1073/pnas.90.5.1977

Stuart, A. M., Bloomquist, J. R., & Soderlund, D. M. (1987). Pharmacological characterization of the voltage-dependent sodium channels of rainbow trout brain synaptosomes. *Brain research*, *437*(1), 77-82. https://doi.org/10.1016/0006-8993(87)91528-9

T. Das, A., Tenenbaum, L., & Berkhout, B. (2016). Tet-On Systems For Doxycycline-inducible Gene Expression. *Current Gene Therapy*, *16*(3), 156-167. https://doi.org/10.2174/1566523216666160524144041

Tambini, M. D., Pera, M., Kanter, E., Yang, H., Guardia-Laguarta, C., Holtzman, D., Sulzer, D., Area-Gomez, E., & Schon, E. A. (2016). ApoE4 upregulates the activity of mitochondria-associated ER membranes. *EMBO reports*, *17*(1), 27-36. https://doi.org/10.15252/embr.201540614

Tanaka, S., Chen, X., Xia, Y., Kang, D. E., Matoh, N., Sundsmo, M., Thomas, R. G., Katzman, R., Thal, L. J., Trojanowski, J. Q., Saitoh, T., Ueda, K., & Masliah, E. (1998). Association of CYP2D microsatellite polymorphism with Lewy body variant of Alzheimer's disease. *Neurology*, *50*(6), 1556. https://doi.org/10.1212/WNL.50.6.1556

Tolar, M., Abushakra, S., Hey, J. A., Porsteinsson, A., & Sabbagh, M. (2020). Aducanumab, gantenerumab, BAN2401, and ALZ-801—the first wave of amyloid-targeting drugs for Alzheimer's disease with potential for near term approval. *Alzheimer's Research & Therapy*, *12*(1). https://doi.org/10.1186/s13195-020-00663-w

Tolar, M., Keller, J. N., Chan, S., Mattson, M. P., Marques, M. A., & Crutcher, K. A. (1999). Truncated Apolipoprotein E (ApoE) Causes Increased Intracellular Calcium and May Mediate ApoE Neurotoxicity. *The Journal of Neuroscience*, *19*(16), 7100-7110. https://doi.org/10.1523/jneurosci.19-16-07100.1999

Tominaga-Yoshino, K., Uetsuki, T., Yoshikawa, K., & Ogura, A. (2001). Neurotoxic and neuroprotective effects of glutamate are enhanced by introduction of amyloid precursor

protein cDNA. *Brain research*, *918*(1-2), 121-130. https://doi.org/10.1016/s0006-8993(01)02983-3

Tran, T. T. T., Corsini, S., Kellingray, L., Hegarty, C., Le Gall, G., Narbad, A., Müller, M., Tejera, N., O'Toole, P. W., Minihane, A.-M., & Vauzour, D. (2019). APOE genotype influences the gut microbiome structure and function in humans and mice: relevance for Alzheimer's disease pathophysiology. *The FASEB Journal*, *33*(7), 8221-8231. https://doi.org/10.1096/fj.201900071r

Trommer, B. L., Shah, C., Yun, S. H., Gamkrelidze, G., Pasternak, E. S., Ye, G. L., Sotak, M., Sullivan, P. M., Pasternak, J. F., & LaDu, M. J. (2004). ApoE isoform affects LTP in human targeted replacement mice. *Neuroreport*, *15*(17), 2655-2658. https://doi.org/10.1097/00001756-200412030-00020

Tschanz, J. T., Corcoran, C. D., Schwartz, S., Treiber, K., Green, R. C., Norton, M. C., Mielke, M. M., Piercy, K., Steinberg, M., Rabins, P. V., Leoutsakos, J.-M., Welsh-Bohmer, K. A., Breitner, J. C. S., & Lyketsos, C. G. (2011). Progression of cognitive, functional, and neuropsychiatric symptom domains in a population cohort with Alzheimer dementia: the Cache County Dementia Progression study. *The American journal of geriatric psychiatry : official journal of the American Association for Geriatric Psychiatry*, *19*(6), 532-542. https://doi.org/10.1097/JGP.0b013e3181faec23

Tsigos, C., & Chrousos, G. P. (2002). Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress. *J Psychosom Res*, *53*(4), 865-871. https://doi.org/10.1016/s0022-3999(02)00429-4

Tyas, S. L., Manfreda, J., Strain, L. A., & Montgomery, P. R. (2001). Risk factors for Alzheimer's disease: a population-based, longitudinal study in Manitoba, Canada. *International Journal of Epidemiology*, *30*(3), 590-597. https://doi.org/10.1093/ije/30.3.590

Utermann, G., Hees, M., & Steinmetz, A. (1977). Polymorphism of apolipoprotein E and occurrence of dysbetalipoproteinaemia in man. *Nature*, *269*(5629), 604-607. https://doi.org/10.1038/269604a0

Utermann, G., Langenbeck, U., Beisiegel, U., & Weber, W. (1980). Genetics of the apolipoprotein E system in man. *American journal of human genetics*, *32*(3), 339-347. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1686062/pdf/ajhg00189-0045.pdf

Van Den Berg, M., Kypke, K., Kotz, A., Tritscher, A., Lee, S. Y., Magulova, K., Fiedler, H., & Malisch, R. (2017). WHO/UNEP global surveys of PCDDs, PCDFs, PCBs and DDTs in human milk and benefit–risk evaluation of breastfeeding. *Archives of Toxicology*, *91*(1), 83-96. https://doi.org/10.1007/s00204-016-1802-z

van der Bercken, J. (1972). The effect of DDT and dieldrin on myelinated nerve fibres. *European Journal of Pharmacology*, *20*(2), 205-214. https://doi.org/https://doi.org/10.1016/0014-2999(72)90150-1

Verghese, P. B., Castellano, J. M., Garai, K., Wang, Y., Jiang, H., Shah, A., Bu, G., Frieden, C., & Holtzman, D. M. (2013). ApoE influences amyloid- (A) clearance despite minimal apoE/A association in physiological conditions. *Proceedings of the National Academy of Sciences*, *110*(19), E1807-E1816. https://doi.org/10.1073/pnas.1220484110

Vierbuchen, T., Ostermeier, A., Pang, Z. P., Kokubu, Y., Südhof, T. C., & Wernig, M. (2010). Direct conversion of fibroblasts to functional neurons by defined factors. *Nature*, *463*(7284), 1035-1041. https://doi.org/10.1038/nature08797

Vogt, N. M., Kerby, R. L., Dill-Mcfarland, K. A., Harding, S. J., Merluzzi, A. P., Johnson, S. C., Carlsson, C. M., Asthana, S., Zetterberg, H., Blennow, K., Bendlin, B. B., & Rey, F. E. (2017). Gut microbiome alterations in Alzheimer's disease. *Scientific Reports*, *7*(1). https://doi.org/10.1038/s41598-017-13601-y

Walsh, D. M., Klyubin, I., Fadeeva, J. V., Cullen, W. K., Anwyl, R., Wolfe, M. S., Rowan, M. J., & Selkoe, D. J. (2002). Naturally secreted oligomers of amyloid B protein potently inhibit

hippocampal long-term potentiation in vivo [Article]. *Nature*, *416*(6880), 535. https://login.proxy.libraries.rutgers.edu/login?url=https://search.ebscohost.com/login.aspx?direct=true&db=aph&AN=6770978&site=eds-live

Waterston, R. H., Lindblad-Toh, K., Birney, E., Rogers, J., Abril, J. F., Agarwal, P., Agarwala, R., Ainscough, R., Alexandersson, M., An, P., Antonarakis, S. E., Attwood, J., Baertsch, R., Bailey, J., Barlow, K., Beck, S., Berry, E., Birren, B., Bloom, T., . . . Lander, E. S. (2002). Initial sequencing and comparative analysis of the mouse genome. *Nature*, *420*(6915), 520-562. https://doi.org/10.1038/nature01262

Wattmo, C., Blennow, K., & Hansson, O. (2020). Cerebro-spinal fluid biomarker levels: phosphorylated tau (T) and total tau (N) as markers for rate of progression in Alzheimer's disease. *BMC Neurology*, *20*(1). https://doi.org/10.1186/s12883-019-1591-0

Weisgraber, K. H., Rall, S. C., & Mahley, R. W. (1981). Human E apoprotein heterogeneity. Cysteine-arginine interchanges in the amino acid sequence of the apo-E isoforms. *Journal of Biological Chemistry*, *256*(17), 9077-9083. https://doi.org/10.1016/s0021-9258(19)52510-8

Weisheit, I., Kroeger, J. A., Malik, R., Klimmt, J., Crusius, D., Dannert, A., Dichgans, M., & Paquet, D. (2020). Detection of Deleterious On-Target Effects after HDR-Mediated CRISPR Editing. *Cell Reports*, *31*(8), 107689. https://doi.org/10.1016/j.celrep.2020.107689

Weng, X., Zhao, H., Guan, Q., Shi, G., Feng, S., Gleave, M. E., Nguan, C. C., & Du, C. (2021). Clusterin regulates macrophage expansion, polarization and phagocytic activity in response to inflammation in the kidneys. *Immunol Cell Biol*, *99*(3), 274-287. https://doi.org/10.1111/imcb.12405

WHO. (2006). WHO gives indoor use of DDT a clean bill of health for controlling malaria. *Indian J Med Sci*, *60*(10), 439-441.

Wirths, O., Multhaup, G., Czech, C., Feldmann, N., Blanchard, V., Tremp, G., Beyreuther, K., Pradier, L., & Bayer, T. A. (2002). Intraneuronal APP/Aβ Trafficking and Plaque Formation in β-Amyloid Precursor Protein and Presenilin-1 Transgenic Mice. *Brain Pathology*, *12*(3), 275. https://login.proxy.libraries.rutgers.edu/login?url=https://search.ebscohost.com/login.aspx?direct=true&db=edb&AN=64866810&site=eds-live

Wnuk, A., Rzemieniec, J., Litwa, E., Lasoń, W., Krzeptowski, W., Wójtowicz, A., & Kajta, M. (2016). The Crucial Involvement of Retinoid X Receptors in DDE Neurotoxicity. *Neurotoxicity Research*, *29*(1), 155-172. https://doi.org/10.1007/s12640-015-9572-6

Wnuk, A., Rzemieniec, J., Litwa, E., Lason, W., Krzeptowski, W., Wojtowicz, A. K., & Kajta, M. (2016). The Crucial Involvement of Retinoid X Receptors in DDE Neurotoxicity. *Neurotox Res*, *29*(1), 155-172. https://doi.org/10.1007/s12640-015-9572-6

Wnuk, A., Rzemieniec, J., Przepiórska, K., Wesołowska, J., Wójtowicz, A. K., & Kajta, M. (2020). Autophagy-related neurotoxicity is mediated via AHR and CAR in mouse neurons exposed to DDE. *Science of The Total Environment*, *742*, 140599. https://doi.org/10.1016/j.scitotenv.2020.140599

Wu, D., Hu, Y., Song, M., & Li, G. (2021). Dichlorodiphenyltrichloroethane Impairs Amyloid Beta Clearance by Decreasing Liver X Receptor α Expression [Original Research]. *Frontiers in Aging Neuroscience*, *13*. https://doi.org/10.3389/fnagi.2021.634948

Xu, L. H., Xie, H., Shi, Z. H., Du, L. D., Wing, Y. K., Li, A. M., Ke, Y., & Yung, W. H. (2015). Critical Role of Endoplasmic Reticulum Stress in Chronic Intermittent Hypoxia-Induced Deficits in Synaptic Plasticity and Long-Term Memory. *Antioxid Redox Signal*, *23*(9), 695-710. https://doi.org/10.1089/ars.2014.6122

Xu, P.-T., Gilbert, J. R., Qiu, H.-L., Ervin, J., Rothrock-Christian, T. R., Hulette, C., & Schmechel, D. E. (1999). Specific Regional Transcription of Apolipoprotein E in Human Brain

Neurons. *The American Journal of Pathology*, *154*(2), 601-611. https://doi.org/10.1016/s0002-9440(10)65305-9

Xu, Q. (2006). Profile and Regulation of Apolipoprotein E (ApoE) Expression in the CNS in Mice with Targeting of Green Fluorescent Protein Gene to the ApoE Locus. *Journal of Neuroscience*, *26*(19), 4985-4994. https://doi.org/10.1523/jneurosci.5476-05.2006

Xue, H., Sun, Q., Liu, L., Zhou, L., Liang, R., He, R., & Yu, H. (2017). Risk factors of transition from mild cognitive impairment to Alzheimer's disease and death: A cohort study. *Comprehensive Psychiatry*, *78*, 91-97. https://doi.org/10.1016/j.comppsych.2017.07.003

Yoshitake, T., Kiyohara, Y., Kato, I., Ohmura, T., Iwamoto, H., Nakayama, K., Ohmori, S., Nomiyama, K., Kawano, H., Ueda, K., Sueishi, K., Tsuneyoshi, M., & Fujishima, M. (1995). Incidence and risk factors of vascular dementia and Alzheimer's disease in a defined elderly Japanese population. *Neurology*, *45*(6), 1161. https://doi.org/10.1212/WNL.45.6.1161

Zhang, Y., Pak, C., Han, Y., Ahlenius, H., Zhang, Z., Chanda, S., Marro, S., Patzke, C., Acuna, C., Covy, J., Xu, W., Yang, N., Danko, T., Chen, L., Wernig, M., & Südhof, T. C. (2013). Rapid single-step induction of functional neurons from human pluripotent stem cells. *Neuron*, *78*(5), 785-798. https://doi.org/10.1016/j.neuron.2013.05.029

Zhao, H., Li, X., Wu, W., Li, Z., Qian, L., Li, S., Zhang, B., & Xu, Y. (2015). Atrophic Patterns of the Frontal-Subcortical Circuits in Patients with Mild Cognitive Impairment and Alzheimer's Disease. *PLoS ONE*, *10*(6), e0130017. https://doi.org/10.1371/journal.pone.0130017

Zhao, W., Dumanis, S. B., Tamboli, I. Y., Rodriguez, G. A., Moussa, C. E., William rebeck, G., & Jo ladu, M. (2014). Human APOE genotype affects intraneuronal Aβ1-42 accumulation in a lentiviral gene transfer model [Article]. *Human Molecular Genetics*, *23*(5), 1365-1375. https://doi.org/10.1093/hmg/ddt525

Zhu, G., Fan, G., Feng, C., Li, Y., Chen, Y., Zhou, F., Du, G., Jiao, H., Liu, Z., Xiao, X., Lin, F., & Yan, J. (2013). The effect of lead exposure on brain iron homeostasis and the expression of DMT1/FP1 in the brain in developing and aged rats. *Toxicol Lett*, *216*(2-3), 108-123. https://doi.org/10.1016/j.toxlet.2012.11.024

Zhu, X.-C., Tan, L., Wang, H.-F., Jiang, T., Cao, L., Wang, C., Wang, J., Tan, C.-C., Meng, X.-F., & Yu, J.-T. (2015). Rate of early onset Alzheimer's disease: a systematic review and meta-analysis. *Annals of translational medicine*, *3*(3), 38-38. https://doi.org/10.3978/j.issn.2305-5839.2015.01.19

Zlokovic, B. V. (2008). The Blood-Brain Barrier in Health and Chronic Neurodegenerative Disorders. *Neuron*, *57*(2), 178-201. https://doi.org/10.1016/j.neuron.2008.01.003