CHARACTERIZATION OF THE 5XFAD TRANSGENIC MOUSE AND THE EFFECTIVENESS OF HYDROXYLPYCLEN IN THE DISAGGREGATION OF Aβ PLAQUES AND RESTORATION OF COGNITIVE FUNCTION IN ALZHEIMER'S DISEASE

by

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CHARACTERIZATION OF THE 5XFAD TRANSGENIC MOUSE AND THE EFFECTIVENESS OF HYDROXYLATED PYCLEN IN THE DISAGGREGATION OF Aβ PLAQUES AND RESTORATION OF COGNITIVE FUNCTION IN ALZHEIMER'S DISEASE

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ABSTRACT

Alzheimer's disease (AD), a neurodegenerative disorder with no known cure or treatment, is the most prevalent form of age-related dementia. As both the rate and total number of those diagnosed continues to increase, AD has become a global concern. The transgenic 5xFAD mouse model expresses familial AD due to mutations in both amyloid-beta precursor protein (APP) and presenilin-1 (PS1). The AD hallmarks exhibited include amyloid-beta (Aβ) plaque deposition around 2 months of age and hippocampus synaptic dysfunction around 9 months of age, which result in severe accelerated cognitive impairment. Through contextual fear conditioning (CFC) and radial arm water maze paradigms, as well as Thioflavin-S Aβ plaque staining, this study characterized in our own lab the AD hallmarks that have been previously demonstrated in the 5xFAD mouse model. Copper-chelation, antioxidants, and reactive oxygen species (ROS) reduction have shown promise individually as potential treatment options for AD. The novel hydroxylpyclen from Green Research Laboratory has been previously shown to exhibit all of these capabilities, while not detrimentally interfering with cell processes or viability. Therefore, after characterization, this study explored hydroxylpyclen as a potential treatment in 9 month old, transgenic positive 5xFAD mice. By injecting mice daily for 28 days with 222mg/kg hydroxylpyclen, our lab has shown promise for an AD treatment option by producing improvement in context-dependent cognitive behavior as compared to controls in CFC, as well as reductions in Aβ hippocampal plaque and ROS species.

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INTRODUCTION

In 1901, Dr. Aloysius Alzheimer, working in the insane asylum of Frankfurt am Main, observed a 51-year old female patient, Auguste Deter. Mrs. Deter presented with no motor deficits, but numerous behavioral anomalies, including disorientation, deliriousness, auditory hallucinations, paraphasia, screaming, and a "rapid loss of memory." After suffering for four and half years, Mrs. Deter died. In his 1907 report of the case, Alzheimer found in the place of neurons, "a tangle of fibrils," and in the cortex, "miliary foci caused by the deposition of a special substance." He determined these to be the pathological hallmarks of pre-senile dementia, an undocumented "special illness." Today, the "special substance" is referred to as amyloid beta (Aβ) plaques and the illness, named after its founder, as Alzheimer's disease (AD) (Alzheimer, 1995).

Since its original description, AD, the neurodegenerative disease of aging, has become the leading cause of dementia and a rising global concern (Cai, 2011). Over 5 million total Americans currently suffer from AD, and, of this, 1 out of every 9 Americans aged 65 and older has the disease. Every 67 seconds another American develops AD, making this disease the sixth leading cause of death in the US. In 2014, the direct healthcare cost for AD patients was an estimated $214 billion, predicted to increase to $1.2 trillion in 2050. The prevalence of the disease is increasing. Between 2000 and 2010, the number of deaths from AD increased 68%, compared to an overall decrease in the number of deaths from other major diseases including breast and prostate cancer, heart disease, stroke, and HIV (Alzheimer Association). As AD primarily affects those aged 65+, it becomes daunting to recognize that the population of this age demographic is expected to increase 79.2% from the year 2010 to 2030, and 120.1% by 2050 (Admin on

Aging). AD will surely continue to be a highly relevant issue for generations in the future, as there is currently no approved treatment for its onset or progression.

The initiating mechanism of AD pathology is recognized to be Aβ formation and plaque deposition (Finder, 2010). Aβ is derived from amyloid precursor protein (APP), a transmembrane protein with unknown biological function. However, APP has been implicated to be involved in several cellular processes including cell adhesion, neurite growth, synaptogenesis, neuronal protein transport, transmembrane signal transduction, and calcium metabolism (Zheng, 2011). The actual production of Aβ is the result of two sequential cleavages within APP by β-secretase (BACE) and γ-secretase (Cai, 2011). Cleavage by γ-secretase results in the production of predominately $A\beta_{40}$ and $A\beta_{42}$, the latter isoform being more associated with AD plaques due to its hydrophobic and amyloidogenic nature (Iwatsubo, 1996). Presenilin 1 (PS1), one of four components within the γ-secretase complex, is a regulatory protein, which modulates the activity level of γ-secretase. Alternatively, if APP is initially cleaved by α-secretase, then this results is a non-amyloidogenic pathway, as the cleavage occurs within the Aβ forming domain of the peptide. Once formed, the Aβ protein can exist in several aggregation states ranging from monomer, soluble oligomer, and insoluble fibril. The conversion into insoluble neuritic plaques and fibrils is a result of self-association due to β-sheet domain stacking (Jawhar, 2011).

After the initial formation of Aβ, neural degeneration and dysfunction results from a number of consequent causes, including free radical formation, oxidative stress, inflammatory processes, neurofibrillary tangles (NFT), microgliosis, astrocytosis, and cell apoptosis (Cai, 2011). The neural degeneration associated with AD is also marked by

elevated synaptic loss. As synapses are important sites for processes necessary to memory formation, including long-term potentiation and neuron plasticity, their loss is highly correlated to the state of dementia experienced by AD patients (Jawhar, 2011).

Oxidative stress is defined as a steady state level of oxidative damage, caused by reactive oxygen species (ROS) and an imbalance between the formation of cellular oxidants and antioxidative processes (Bernhard, 2007). Implicated as an initiator and a consequence of Aβ formation, oxidative stress production, sensitivity, and damage increases with age, as does the progression of AD (Cai, 2011). Oxidative stress, which is increased by the production and deposition of Aβ, results in inflammation, blood-brain barrier (BBB) dysfunction, apoptosis, and even increased activity of β- and γ-secretases. In a self-perpetuating cycle, each of these consequences of oxidative stress in turn results in further production and deposition of Aβ (Cai, 2011). Once this relentless cycle begins, it is clear to see how AD, and its associated neurodegeneration, progresses so rapidly.

Aging, especially after ages 65 years and older, is the most significant risk factor for developing sporadic AD. Genetics is another major risk factor, predisposing individuals expressing the inherited mutations towards development of AD. Because individuals with an AD genetic background can develop the disease as early as 16-65 years of age, this form of early onset AD has been termed familial Alzheimer's disease (FAD). Genetic studies of AD prone families have revealed that mutations in the APP, PS1, and PS2 genes are responsible for most forms of FAD. Although FAD represents only 5-10% of all AD cases, it is relevant because of its ability to be replicated and studied through the use of transgenic mouse models (Jawhar, 2011).

Currently, our laboratory is utilizing the 5xFAD (Tg6799) transgenic mouse in its study of AD pathology. The 5xFAD double transgenic mouse expresses five AD mutations, disposing them to early-onset cognitive and memory impairments that result from accelerated Aβ plaque accumulation and increased $A\beta_{42}$ formation. Through site-directed mutagenesis, the Swedish (KM670/671NL), Florida (I716V), and London (V717I) mutations were introduced into the APP gene and the M146L and L286V mutations were introduced into the PS1 gene. This model is maintained on a C57/Bl6SJL background in a hemizygous state and passes on to offspring both the APP and PS1 transgenes as a single transgene. As a result, transgenic positive 5xFAD (5xFAD+) mice inherit and express all 5 mutations, while transgenic negative 5xFAD (5xFAD-) mice neither inherit nor express any of the 5 mutations. The Swedish mutation results in a greater total production of Aβ, while the other four mutations result in an increase in the production of the specific $A\beta_{42}$ isoform. As a result of these mutations, Aβ plaque deposition within the brain is significantly accelerated, evidenced as early as 2 months of age in layer 5 of the cortex and throughout the hippocampus and cortex by 6 months of age. The 5xFAD mouse, lacking NFT formation, represents a predominantly amyloid AD model (Oakley, 2006).

Additional AD pathologies exhibited by the 5xFAD model include age-dependent synaptic degeneration, neuron loss, and cognitive dysfunction. Synaptic degeneration was demonstrated by a reduction in synaptic markers. Neuron loss, a feature uncommon in AD mouse models, was evidenced by 9 months of age (Oakley, 2006). 5xFAD mice also display synapse disruption, specifically in the area of the hippocampus. This results in context-dependent memory loss, demonstrated by as early as 6 months of age through

impairments in the contextual fear conditioning (CFC) and radial arm water maze (RAWM) paradigms (Kimura, 2009). The first objective of the present study was to characterize the 5xFAD model by validating the previously described age-dependent hallmarks of this AD model in our own laboratory. Being able to replicate the existing data on accelerated plaque deposition and contextual memory impairment in this model provides a control for comparison purposes and allows our laboratory to use the 5xFAD model in future studies, such as AD therapeutic interventions.

The level of extracellular Aβ deposition in the brain, histologically measured through plaque counts, is not highly correlated with the level of cognitive deficiency exhibited in Alzheimer's patients. Patients, found to have Aβ plaques throughout the brain, sometimes exhibit no dementia characteristic of AD symptomology. Instead, the degree of cognitive deficiency is much more highly correlated with the concentration of soluble oligomeric Aβ, undetectable by immunohistochemical techniques (Lue, 1999). Studies have also shown that *in vivo* microinjection of human Aβ oligomers into rats were sufficient in inhibiting synaptic plasticity and hippocampal long-term potentiation. This provides additional evidence for the correlation between soluble Aβ and cognitive deficiency, rather than monomeric Aβ or amyloid fibrils (Walsh, 2002). The insoluble Aβ aggregates may in fact serve as a neuroprotective equilibrium with soluble Aβ, providing a reservoir to lower the concentrations of the soluble neurotoxic form (Hardy, 2002).

In addition, the accumulation of intraneuronal Aβ has also been implicated as a predominating factor in synapse and neuron dysfunction. Finding deposition of intraneuronal Aβ in AD vulnerable brain regions suggests that this accumulation precedes extracellular deposition (Gouras, 2000). Intracellular Aβ is believed to arise from two

pathways, intracellular APP processing and reuptake of extracellular Aβ (Jawhar, 2011). Within neurons, Aβ accumulation leads to increased lysosomal permeability and content leakage, resulting in cell necrosis and apoptosis (Ditaranto, 2001). Additionally, Aβ localization to mitochondria produces decreased oxygen consumption, increased H_2O_2, and decreased cellular respiration enzyme activity (Caspersen, 2005). All of these effects result in a high correlation between intraneuronal Aβ accumulation and the pathological cognitive deficiencies (Wirths, 2010). Therefore, it is of high interest and relevance to devise a targeting strategy towards the more symptomatic Aβ peptide.

Presently, the clinically approved drug treatments for AD consist of symptom alleviation, mainly through control of behavioral instability (Hardy, 2002). However, six predominant categories have been proposed and are being investigated, both clinically and non-clinically, for the treatment of AD. These treatment strategies include: 1) inhibition of the APP cleaving proteases, β- and γ-secretase; 2) active or passive immunization of Aβ to enhance clearance; 3) reduction of inflammation, which is augmented as Aβ accumulates; 4) reduction of cholesterol levels; 5) prevention of Cu^{2+} and Zn^{2+}-dependent Aβ aggregation through chelation; 6) prevention of neurodegeneration through antioxidant, neuroprotective, and/or neurotrophic compounds (Hardy, 2002). After characterization of the 5xFAD mouse in our laboratory, future studies on this model can begin to investigate the effectiveness of these treatment strategies. Employing several of the aforementioned strategies, specifically strategies 3, 5, and 6, the hydroxylpyclen compound, 1,4,7,10-Tetraazacylododecane (Figure 1),

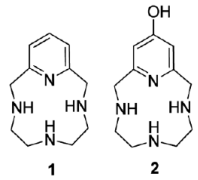

Figure 1. Pyclen (1); Hydroxylpyclen (2)

provided by the Chemistry Research Laboratory of Dr. Kayla Green, at Texas Christian University, is one such potential treatment for AD currently under investigation.

Copper (Cu) is an essential trace element crucial for the proper functioning of several physiological processes in living systems. Elevated levels of Aβ have been shown to disrupt Cu homeostasis through a histidine rich, high-affinity Cu^{2+} binding site within $A\beta_{42}$ (Singh, 2013). In addition, Cu^{2+}-binding mediates Aβ precipitation and aggregation into insoluble plaques (Bush, 2002). Therefore, Cu^{2+} contamination within brain capillaries is associated with an increase in Aβ levels and a reduction in Aβ transport and clearance (Singh, 2013). Once bound to Cu^{2+}, Aβ becomes electrochemically active. Via Fenton chemical reactions, Aβ becomes capable of converting molecular oxygen into hydrogen peroxide (H_2O_2), a pro-oxidant, through reduction of Cu^{2+} (Lynch, 2000). The generation of ROS in the brain by these reactions results in exacerbated oxidative stress, leading to elevated inflammation and neuronal death (Lincoln, 2012).

As metal ion dysregulation and resulting oxidative stress have been implicated in the development of neurological diseases such as AD, therapies aimed at restoring this disrupted pathway are under current investigation (Lincoln, 2013). Pyclen (Figure 1) is the backbone of PCTA, a potential MRI contrast agent. Studies have demonstrated that pyclen is not only a Cu/Zn ion-specific chelating agent, but also exhibits antioxidant capabilities. Through spectroscopic and TEM/SEM imaging studies, pyclen was shown to disaggregate Aβ plaques, as well as to protect from metal-induced Aβ aggregation. Additionally, antioxidant assays performed in cell culture studies have demonstrated pyclen as providing protective antioxidant capabilities (Lincoln, 2012).

In an attempt to enhance the antioxidant potency of pyclen, the Green Research Laboratory hydroxylated the pyridine group to a pyridol. The resulting compound, hydroxylplycen, was then tested to evaluate its chelation and antioxidant capabilities. Hydroxylpyclen maintained the ability to disaggregate metal-induced Aβ plaque formation through Cu/Zn chelation. Studies also showed that hydroxylpyclen displayed considerably more potential as an antioxidant than pyclen. In addition, hydroxylpyclen was shown to cease Cu ion redox reactions implicated in the formation of ROS. Cell culture studies revealed that hydroxylpyclen significantly reduced oxidative stress-induced cellular death when compared to pyclen. Further, hydroxylpyclen was capable of entering cells, while not disrupting vital cytosolic metalloenzyme functioning (Lincoln 2013). The results of these studies illustrate the promise of hydroxylpyclen as treatment for AD pathologies. However, evaluation of the effectiveness of this compound within an organism is still required.

Recently, several studies have tested additional chelating compounds in order to investigate the efficacy of this particular treatment strategy. Utilizing clioquinol (CQ), a hydrophobic antibiotic and Cu/Zn chelator able to cross the BBB, chelation of metal ions has been shown to reduce the levels of Aβ plaque deposition in vivo by as much as 49% in the APP2576 transgenic mouse model. However, treatment also resulted in a slight increase in soluble Aβ levels (Cherny, 2001). In other studies, CQ markedly reduced the levels of soluble extracellular Aβ within hours and within days restored cognitive deficiencies to exceed that of control littermates, the amyloid-bearing transgenic AD mouse model, APPswe/PS1dE9 (Adlard, 2008). Despite its effectiveness in mouse models and phase II clinical trials, further evaluation of CQ was abandoned due to

possible side effects including subacute myelo-optic neuropathy (Lincoln 2012). Currently, PBT2, a second-generation analogue of CQ lacking the cross-reactive iodine, is undergoing testing in phase II clinical trials with promising results, including a reduction in frontal lobe cognitive deficits and cerebrospinal fluid $A\beta_{42}$ levels (Adlard 2008).

Continuing with the promise shown in this treatment strategy, the second objective of the present study is to investigate the effectiveness of a Cu^{2+}-chelator in reducing the levels of Aβ plaque deposition and associated cognitive impairments. However, this is to be achieved instead by treating the 5xFAD transgenic mouse model with the hydroxylpyclen compound of Green Research Laboratory. This compound, because of its antioxidant capabilities, may also reduce the generation of oxidative stress and radical species, further alleviating the pathological hallmarks of AD, namely plaque deposition and progressive cognitive decline.

MATERIALS & METHODS

Experimental Subjects

Experimentally naïve 3, 6, 9, and 12 month-old male 5xFAD transgenic mice, both transgenic positive (5FAD+) and negative (5xFAD-), bred in the Texas Christian University vivarium from a breeding stock obtained from Jackson Laboratory (Bar Harbor, ME), were utilized in characterization experiments. Experimentally naïve 9-month old male 5xFAD transgenic positive mice were utilized in the hydroxylpyclen studies, as well as the Thioflavin-S staining study designed for plaque counts. All subjects were housed in groups of one to four in standard polycarbonate mouse cages. All subjects were on the same 12-h light/dark schedule, and both food and water were

available *ad libitum*. All animals were housed and cared for in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 2010), and in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Texas Christian University.

Treatment Conditions (Characterization)

Transgenic positive 5xFAD mice have been shown to develop Aβ plaques and associated cognitive deficits without experimental intervention in an age-dependent manner (Oakley, 2006). Therefore, experimentally naïve transgenic positive and negative 5xFAD mice (n = 44) were allowed to age independently until reaching 3, 6, 9, or 12 months of age. Upon reaching the desired age, groups of mice underwent behavioral testing and biological assessments.

Treatment Conditions (Hydroxylpyclen study)

Test subjects, all experimentally naïve 9-month transgenic positive 5xFAD males, were intraperitoneal (i.p.) injected once daily for a span of 28 days. Injections consisted of either hydroxylpyclen (n = 12) or saline (n= 12). Hydroxylpyclen was prepared in aqueous saline to a stock concentration of 33.3mg/mL. Test subjects were weighed each day and injected with either hydroxylpyclen at a concentration of 222mg/kg or with 200μL of saline. At the conclusion of the 28 days, subjects underwent behavioral testing, followed by tissue collection.

Contextual Fear Conditioning

Contextual fear conditioning (CFC) is a standard behavioral paradigm used to evaluate cognition in mice. CFC is a hippocampal-dependent task that uses context cues, in order to measure whether cognitive deficits are present (Phillips & LeDoux, 1992).

There are a variety of approaches, utilizing different timing strategies, as well as several types and combinations of contextual stimuli. One common approach consists of a training day, which includes an acclimation time and a consolidation time following an adverse stimulus (usually a mild shock). The second day, testing day, consists of an established resting time in the unit. The amount of freezing time recorded on testing day indicates whether the mouse was able to remember the context cues in conjunction with the negative stimulus (shock). Prolonged freezing is correlated with better learning and memory, whereas a lack of freezing correlates to cognitive deficits. The units (Coulbourn Instruments, Whitehall, PA, 7Wx7Dx12H) utilize visual cues, e.g., black polka dot walls, as well as olfactory cues, in this case peppermint oil diluted with water at a ratio of 1:10. Mice experienced the shock stimulus through an electrified grid floor while their behavior was monitored and analyzed using FreezeFrame™ (ActiMetrics Software, Wilmette, IL).

The protocol used for the contextual fear conditioning is in accordance with Kranjac et al. (2011). Visual and olfactory cues were shown to enhance freezing time (Kranjac et al., 2011) and an olfactory cue such as this is as an indicator of hippocampal integrity (Yoon, 2007).

For our study, on training day, each mouse was given 180 seconds to acclimate to the unit, followed by a 2-second 0.7mA shock. The mouse remained in the unit for a 1-minute interval period, until another 2-second 0.7mA shock was given. The mouse then remained in the unit another 30 seconds with no shock before removal, representing a period of consolidation or memory imprinting. Testing day merely involved monitoring the freezing behavior of the mice in the unit over a 180s time interval (in the absence of

the shock). After testing of all mice was completed, percent freezing was analyzed. Freezing time of the mice was analyzed using a one-way analysis of variance (ANOVA) procedure (Statview 5.0, SAS, Cary, NC) to assess significant differences, employing genotype (transgenic +/transgenic-) between each age group (3, 6, and 9 months) in the characterization study and treatment (hydroxylpyclen/saline) in the hydroxylpyclen study as independent variables. All statistical analyses used an alpha level of 0.05.

Radial Arm Water Maze

Radial arm water maze (RAWM) is a learning and memory task combining the spatial complexity and performance measurement of the dry radial arm water maze with the rapid learning and strong motivation observed in the Morris water maze. This protocol allows for assessment and quantification of working memory deficits exhibited in Alzheimer's-like transgenic mouse species. Specifically, the task utilizes context-dependent learning, which requires proper hippocampal functioning. Failure to learn the task effectively is evidence for cognitive deficits.

RAWM protocol was followed in accordance with Alamed et al., 2006. Animals were placed in the metal RAWM apparatus, which consists of 8 evenly spaced swim paths (arms) extending from a central circular pool. For each mouse, a goal arm is randomly assigned containing an escape platform. The goal arm location remains constant for a given mouse throughout the experiment. Test subjects were given 60s to locate their specific goal arm containing the escape platform. On training day (recorded in blocks 1-5), the platform alternated between visible (above water) and hidden (below water), for each trial (15 trials). The following day, testing day (recorded in blocks 6-10), the platform was hidden throughout all 15 trials. During each trial, the number of errors,

which included entry into a wrong arm (any arm not the goal arm) or pausing >15s, were recorded. Time to reach the goal arm was recorded as well. Triangulation to the goal arm was guided by multiple extra-maze context cues, such as constant researcher placement and various wall images. Number of errors was analyzed using ANOVA procedures (Statview 5.0, SAS, Cary, NC) applying Genotype (transgenic +/transgenic -) as independent variables. All statistical analyses used alpha level of 0.05.

Tissue Extraction and Preparation

Following behavioral testing, test subjects were humanely euthanized in accordance with approved procedures and brains removed. Brains were dissected down the interhemispheric fissure. The hippocampus of one half of the brain was removed and snap-frozen in a dry ice and ethanol slurry (stored at -80^0C for future analyses). The other half of the brain was fixed in 4% paraformaldehyde for 48 hours. This fixed tissue was then washed in phosphate-buffered saline (PBS), embedded in 3% agarose, and 30μm sagittal sections were cut using an etched sapphire blade on a Leica VT1000 S vibrating microtome. Tissue sections were stored in wells containing a 1% paraformaldehyde (PFA) + 0.03% azide solution.

Thioflavin-S Staining Procedure

Four random sections from 8 test subject brains, four hydroxylpyclen and four saline (32 sections total), were chosen for hippocampal tissue staining with Thioflavin-S. Sections were washed three times with distilled water (DiH_2O) for 10 minutes. Sections were then placed in 500μl Thioflavin-S (Sigma Aldrich). Thioflavin-S concentration was prepared as 0.5gm in 50.0mL DiH_2O. Sections were then washed in 50% ethanol twice for 2 min. Sections were then washed again three times in DiH_2O for 10 minutes.

Sections were placed in 500μl To-Pro3 for 15 minutes. To-Pro3 concentration was prepared in a 1:1000 ratio with DiH_2O. Sections were washed with DiH_2O three times for 5 minutes. Finally, stained sections were adhered to a slide and a coverslip was applied for microscopic counting of Aβ plaques.

Plaque Counts

Aβ plaque fluorescence was visualized on a Zeiss LSM 710 confocal microscope by exciting bound Thioflavin-S with a HeNe laser at a wavelength of 488nm. Total plaque count for each hippocampal section (n = 32) was obtained by two independent researchers using ImageJ software. The mean plaque count of the four hippocampal sections was calculated for each test subject (n = 8). The mean scores from the two researchers were then averaged. Statistical analysis between groups (compound/saline) was performed to determine if significant differences in Aβ plaque counts existed between treatment groups.

RESULTS

During the characterization study, the Contextual Fear Conditioning (CFC) paradigm revealed significant behavioral and cognitive differences between transgenic positive (5xFAD+) and non-transgenic (5xFAD-) 5xFAD mice in an age-dependent manner (Fig. 2). As expected, during training when subjects first learned to associate the established context cues with the adverse shock stimulus, there were no significant differences in percent freezing time between positive or negative transgenic status, regardless of age (3, 6, and 9 months) ($F(1,26)$: 0.07; $F(1, 14)$: 1.132; $F(1,16)$: 0.472; respectively, *ns*). During testing, which assessed the following day whether the test subjects successfully learned to associate the context cues with the negative stimulus, the

3-month 5xFAD+ mice demonstrated no significant differences in percent freezing time compared with the 3-month 5xFAD- mice (F(1,26): 0.531, *ns*). The 6 and 9-month old 5xFAD+ subjects exhibited significantly less mean percent freezing time than non-transgenic 5xFAD mice (5xFAD-) in the corresponding age group (F(1,14): 10.174; F(1,16): 5.128 , respectively, $p < 0.05$).

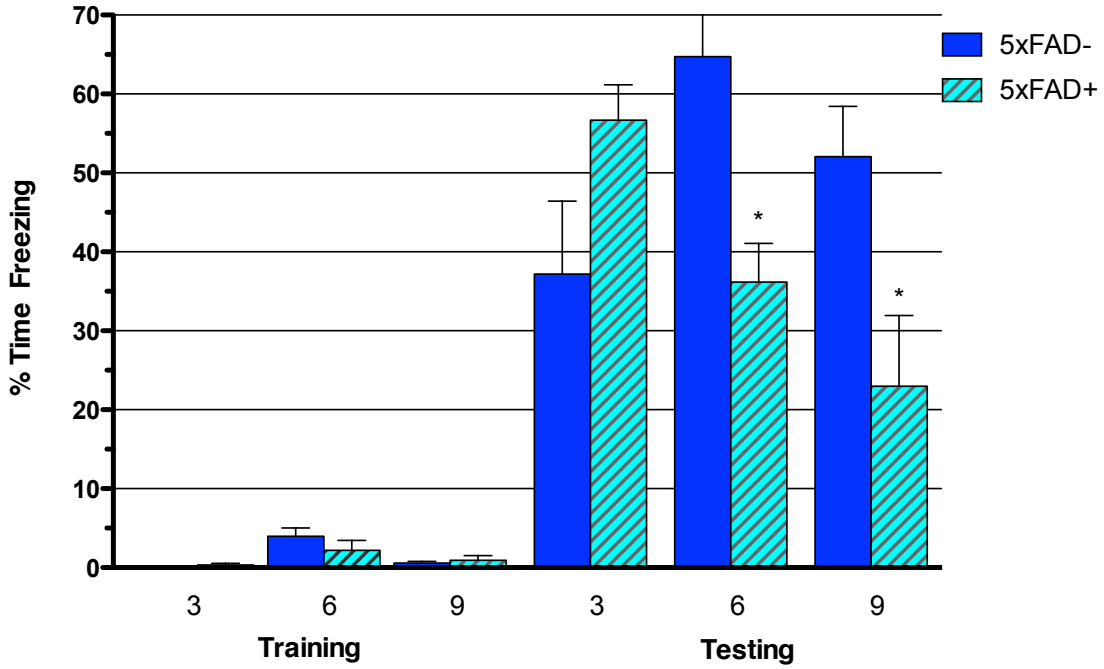

Figure 2. Results of Characterization Study. Contextual Fear Conditioning (CFC) behavioral testing data demonstrating the age-dependent cognitive deficits characteristic of the 5xFAD model. 3, 6, and 9-month age groups, both transgenic positive and negative 5xFAD male subjects, were evaluated (n = 44). 5xFAD+ represents transgenic positive 5xFAD mice, while 5xFAD- represents non-transgenic control mice. Less % time freezing in training is indicative of cognitive deficits. *= $p < 0.05$; bars represent ±SEM.

The Radial Arm Water Maze (RAWM) is another behavioral paradigm utilized in the characterization study to further evaluate the cognitive deficits exhibited by the 5xFAD+ mouse (Fig. 3). During training (Blocks 1-5), subjects learn to triangulate the position of their specific goal arm (location of platform) through context cues. In testing (Blocks 6-10), subjects were evaluated the following day as to whether they learned the behavior, evidenced by fewer errors in finding their specific goal arm through context cue

association. Our results showed a main effect of Transgenic Status (F(1,5) 33.83, $p < 0.05$) and a main effect of Block (F(9,45): 5.44, $p < 0.05$). In addition, results demonstrated a significant interaction effect between Block by Transgenic Status (F(9,45) 2.57, $p < 0.05$) showing that 9 month 5xFAD+ mice commit significantly more errors than 9 month 5xFAD- mice. Pairwise comparisons revealed that 5xFAD+ mice exhibited significant increases in the number of errors made compared to 5xFAD- mice at Blocks 2, 4, 5, 6, 9, and 10.

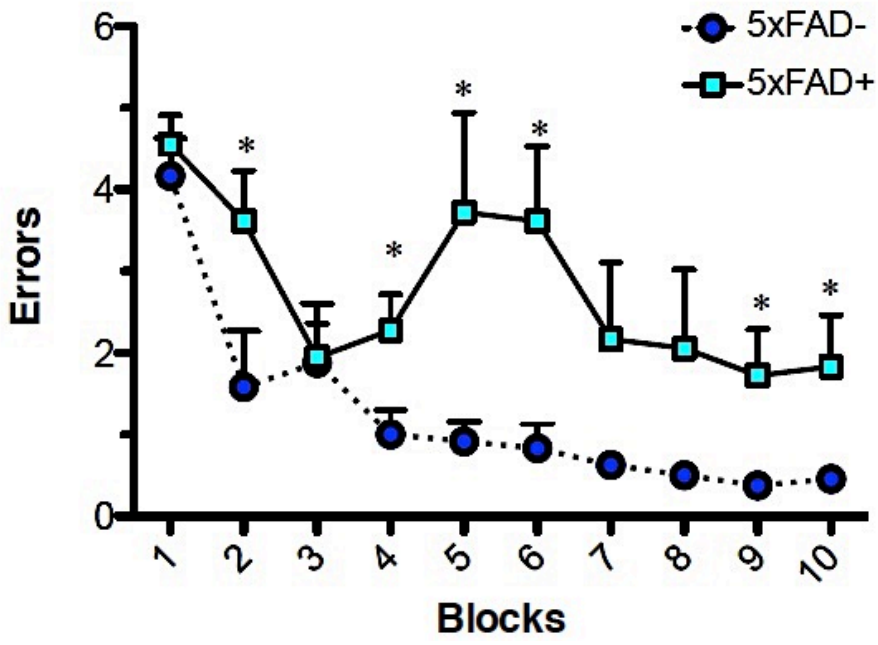

Figure 3. Results of Characterization Study. Radial Arm Water Maze (RAWM) behavioral testing data demonstrating the cognitive deficits characteristic of the 5xFAD model. Each Block consists of the number of errors averaged over 3 trials, therefore, 30 trials total per mouse. Blocks 1-5 represent training (alternating visible and hidden platform), while Blocks 6-10 represent testing of memory consolidation (hidden platform throughout). 5xFAD+ represents transgenic positive 5xFAD mice, while 5xFAD- represents non-transgenic control mice. Errors represent the number of times subjects entered the wrong arm or >15s delay. Trial ended after subject found their specific goal arm (platform location) or after 60s passed. Errors are indicative of cognitive deficits. *= $p < 0.05$; bars represent ±SEM.

Stained hippocampal tissue sections revealed an age-dependent increase in the accumulation of extracellular Aβ plaques characteristic of the 5xFAD+ mice (Fig. 4). No

Aβ plaques were detectable in Thioflavin-S stained 5xFAD- hippocampi, regardless of age group. Thioflavin-S staining (green fluorescence) revealed Aβ plaque deposition distributed in the area of the cortex (inset – green arrow) as early as 3 months of age in the 5xFAD+ mouse. In 5xFAD+ mice aged 6, 9, and 12 months, Aβ plaque accumulation was detected in both the cortex and hippocampus. Further, as 5xFAD+ mice aged from 3 to 12 months, there is an increased extracellular Aβ plaque burden, evidenced by a greater distribution of Thioflavin-S green fluorescence.

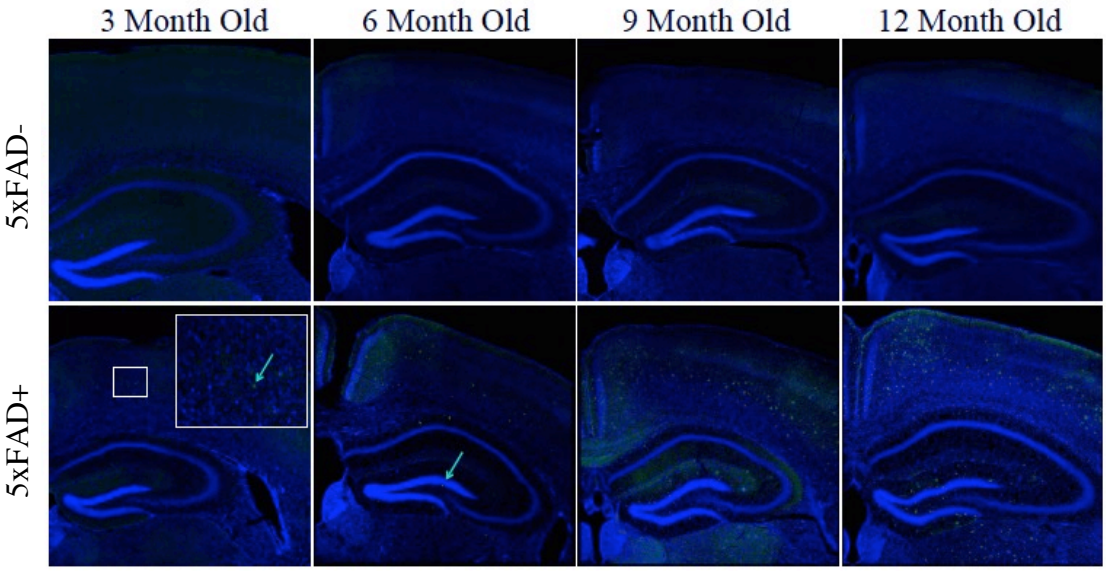

Figure 4. Results of Characterization Study. Representative stained tissue sections demonstrating the age-dependent Aβ plaque accumulation characteristic of the 5xFAD model. 5xFAD+ represents transgenic positive 5xFAD mice, while 5xFAD- represents non-transgenic control mice. In the 3-month-old 5xFAD+ mice, Aβ plaque formation (inset - green arrow) can be detected in the cortex. In 6-12 month old 5xFAD+ mice, Aβ plaque formation can be detected in both the cortex and the hippocampus. Thioflavin S (green) detects Aβ aggregates. TO-PRO 3 (blue) labels nuclei.

In the hydroxylpyclen study, 9-month 5xFAD+ injected with either hydroxylpyclen or saline were evaluated in the CFC paradigm to test for cognitive differences and improvement (Fig. 5). In training, as expected, there were no significant differences between 5xFAD+ treatment groups ($F(1, 22)$: 0.547, *ns*). During testing, there

were no significant differences between saline and hydroxylpyclen-injected subjects (F(1,22): 0.081, *ns*). Hydroxylpyclen-injected FAD+ subjects exhibited a slight increase in percent time freezing compared to saline-injected subjects.

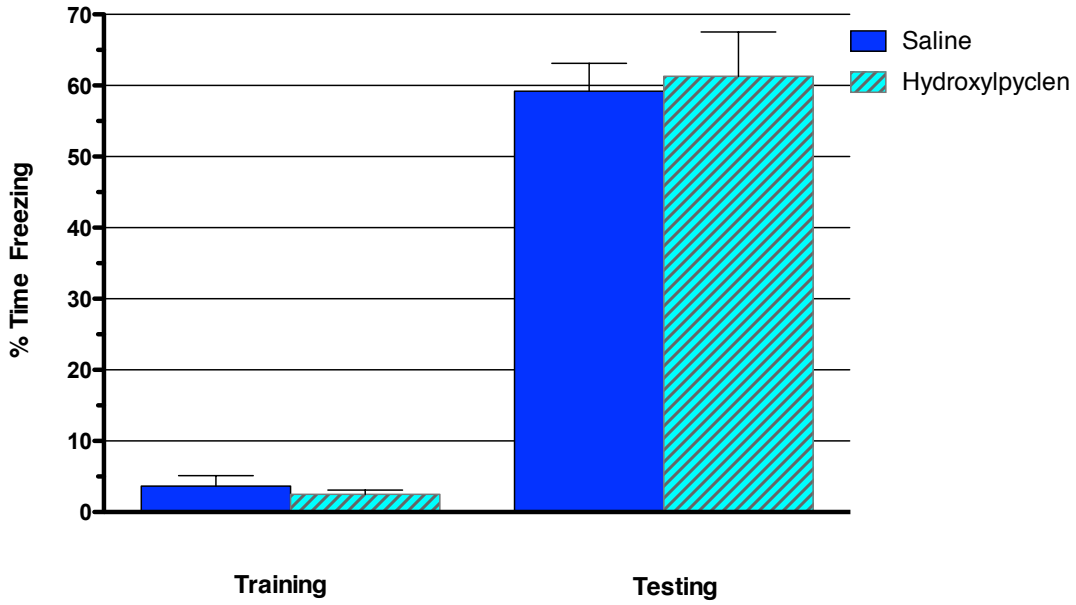

Figure 5. Results of Hydroxylpyclen Study. Percent mean freezing time (bars represent ±SEM) during Contextual Fear Conditioning (CFC) for 9-month-old transgenic positive 5xFAD male mice (n = 24) treated with either Saline or Hydroxylpyclen.

In tissue staining, 9-month 5xFAD+ hippocampal sections were stained with Thioflavin-S to detect for any reduction in extracellular Aβ plaque accumulation (Fig. 6). Representative images revealed that 5xFAD+ subjects injected with hydroxylpyclen exhibited a marked reduction in Aβ plaque burden and density compared with saline-injected subjects (Fig. 6 Left). Further, 60x magnification images of Aβ plaques within these representative hippocampal sections suggested a decrease in both plaque diameter and size in hydroxylpyclen-injected 5xFAD+ subjects (Fig. 6 Right).

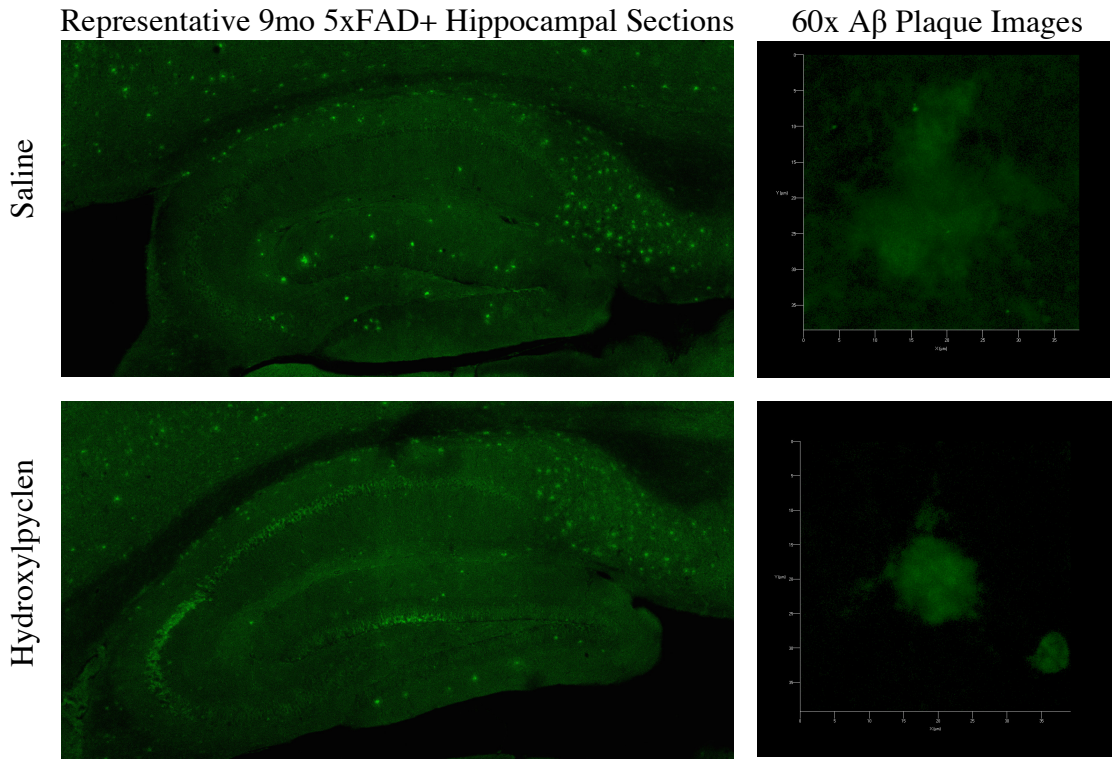

Figure 6. Results of Hydroxylpyclen Study. Representative hippocampal tissue sections of 9-month-old 5xFAD positive male mice. Stained for visualization of Aβ plaques using Thioflavin-S (green fluorescence) to show relative Aβ plaque accumulation comparing hydroxylpyclen to saline-injected test subjects (Left). 60x magnified images of Thioflavin-S stained hippocampal tissue sections demonstrating relative Aβ plaque sizes between treatment groups (Right).

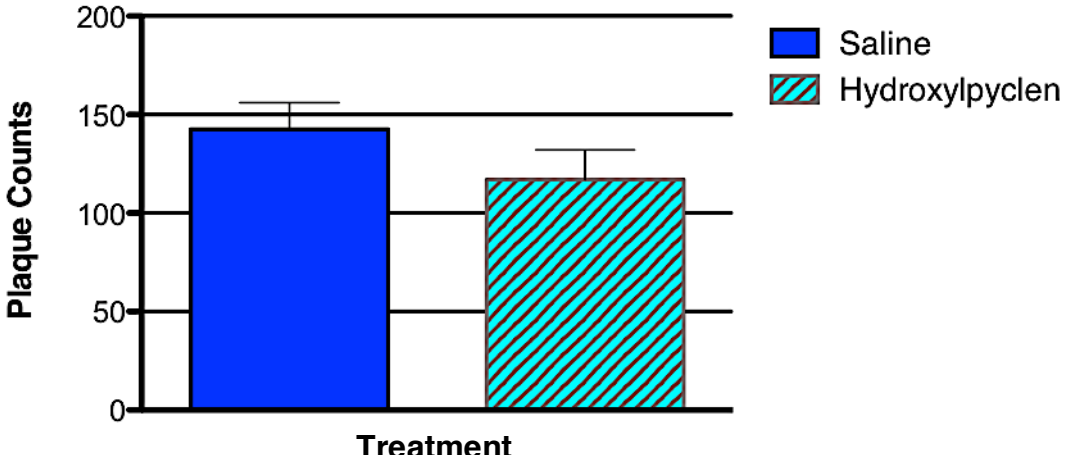

Figure 7. Results of Hydroxylpyclen Study. Mean Aβ plaque counts (bars represent ±SEM) for 9-month-old transgenic positive 5xFAD male mice (n = 8) treated with either Saline or Hydroxylpyclen. Mean Aβ plaque counts represent averages obtained by two independent researchers taken from Thioflavin-S stained hippocampal sections (n = 8; 4 hydroxylpyclen, 4 saline).

Plaque counts in the Thioflavin-S stained hippocampal sections revealed a trending, non significant reduction in Aβ plaque number in hydroxylpyclen-injected 5xFAD+ subjects compared to saline-injected subjects (F(1,6): 1.582, *ns*; Fig. 7).

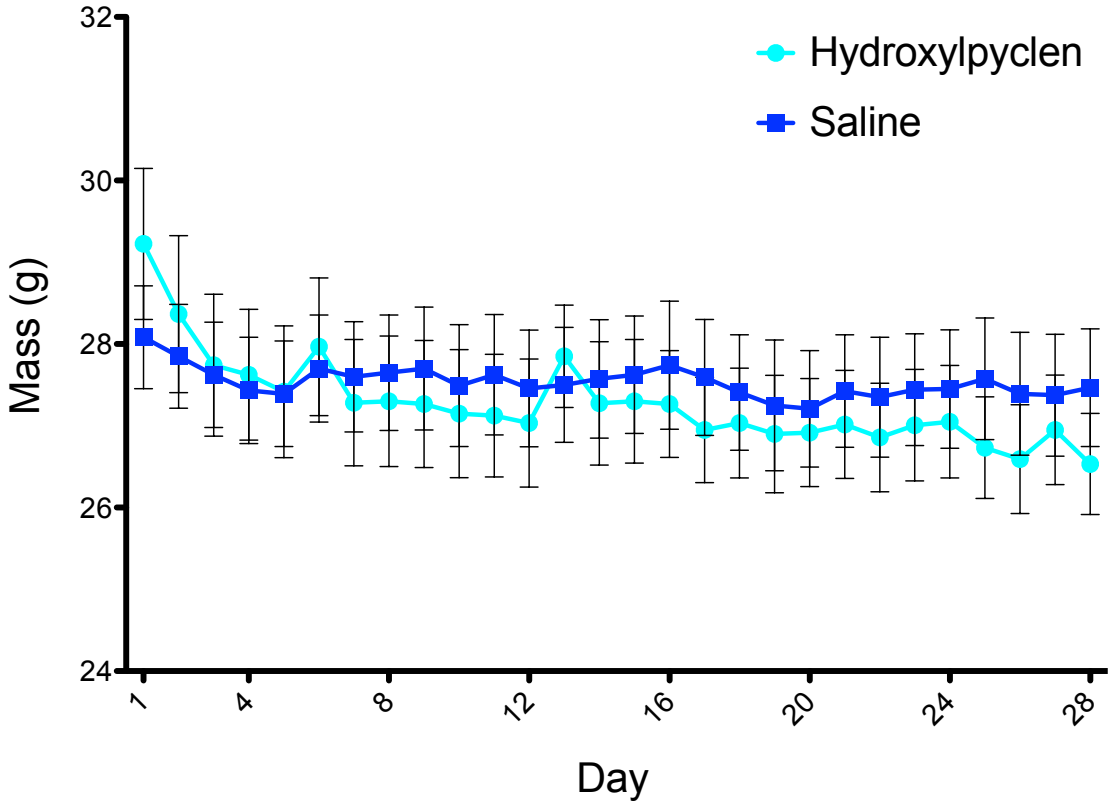

Figure 8. Results of Hydroxylpyclen Study. Mean (bars represent ±SEM) mass (in grams) for 9-month-old transgenic positive 5xFAD male mice (n = 24) treated with either Saline or Hydroxylpyclen over the course of 28 daily injections.

Mass data (Fig 8.) revealed no significant differences (F(1,22): 0.069, *ns*) between hydroxylpyclen and saline-injected 9-month 5xFAD+ mice over the course of the study. Further, the experimental protocol and dose of hyrdoxylpyclen revealed to be non-fatal as no subjects were lost over the course of the study.

DISCUSSION

The results of the characterization study demonstrated the age-dependent AD pathologies known to be exhibited by the 5xFAD mouse model. As mentioned previously, the 5xFAD+ exhibits cognitive deficiencies as early as early as 6 months of age (Kimura, 2009). Using the behavioral paradigm, contextual fear conditioning (CFC), this study was able to successfully show the detrimental age-dependent decline in cognition associated with the 5xFAD (Fig. 2). CFC results revealed that 5xFAD+ mice demonstrated no significant differences in percent freezing time, thus cognition, during training day. This shows that both transgenic positive and negative mice were novel to the protocol, expressing little difference in their initial exposure to the contextual chamber and adverse stimulus. On training day, test subjects, correctly learning to associate the context cues with the adverse shock stimulus, as a natural inclination to fear, should freeze more. Subjects, unsuccessfully associating the context cues with the adverse stimulus, freeze significantly less, thus indicating the presence of cognitive deficiencies. As expected from previous studies on this model, during training day, 3-month-old 5xFAD+ mice showed no significant differences from 5xFAD- mice in cognition. However, 6 and 9-month-old 5xFAD+ mouse froze significantly less during training day, indicative of impairments in contextual memory association and consolidation. In addition, 9-month 5xFAD+ mice exhibited less percent freezing time than the 6-month 5xFAD+ mice, further evidence for an age-dependent effect.

To further characterize the impairments in contextual learning and memory formation, this study also utilized the behavioral paradigm, radial arm water maze (RAWM), in 9-month-old 5xFAD (Fig. 3). Blocks 1-5 represent training day. During

training, 5xFAD+ demonstrated significant increases in the number of errors required to find the goal arm during Blocks 2, 4, and 5. This shows that 5xFAD- mice were able to learn to associate the context cues with the goal arm more rapidly even upon initial exposure. Further, on testing day (Blocks 6-10), 5xFAD+ showed increases in the number of errors during each Block and significant increases during Blocks 6, 9, and 10. As contextual learning is hippocampus dependent (Maren & Holt, 2000), these findings in the contextual behavioral paradigms, CFC and RAWM, suggest that transgenic positive 5xFAD exhibit deteriorations in hippocampal synaptic function over time. Therefore, this study was able to characterize the age-dependent cognitive deficiencies exhibited by the 5xFAD+ by successfully replicating the results of previous studies.

This study then set out to characterize another hallmark of AD exhibited by the 5xFAD mouse, the accelerated deposition and accumulation of extracellular Aβ plaques. Previous studies have demonstrated that Aβ plaques are visibly expressed by the 5xFAD as early as 2 months of age (Oakley, 2006). As revealed by Thioflavin-S representative hippocampal tissue stains in Fig. 4, green fluorescent dots, indicative of Aβ plaques being present, are detectable within the cortex of 3-month-old 5xFAD+ mice and are detectable within the both the cortex and hippocampus of 6, 9, and 12-month-old 5xFAD+ mice. However, there are no green fluorescent dots, i.e., no Aβ plaques, present within any of the 5xFAD- stained hippocampal tissue stains, regardless of age. Therefore, this study was able to demonstrate the age-dependent manner in which 5xFAD+ mice exhibit accelerated extracellular Aβ plaque accumulation. It is interesting to note that 5xFAD+ display hippocampal Aβ at 6 months of age, the same age at which they initially demonstrate hippocampal-dependent learning deficits. This provides support to the theory

that Aβ accumulation disrupts neural synaptic function, resulting in inhibited cell-to-cell communication and impairments in memory formation (Palop & Mucke, 2010).

Studies characterizing additional AD hallmarks exhibited by the 5xFAD mouse would allow for increased methods of evaluation when using the 5xFAD mouse in future studies. For example, previously demonstrated 5xFAD hallmarks not characterized in this study include intraneuronal $A\beta_{42}$ accumulation at 1.5 months of age, neuron loss exhibited at 9 months of age (Eimer & Vassar, 2013), gliosis at 2 months of age, and synapse degeneration at 4 months of age (Oakley, 2006). Further, there exist several additional behavioral paradigms for evaluating both learning and memory in transgenic mouse models, including Morris water task, radial maze, Y-maze, T-maze, passive and active avoidance, delayed nonmatching to position, and eye blink conditioning (Crawley & Paylor, 1997). Characterizing the cognitive deficiencies of the 5xFAD mouse with these additional tasks would allow for an increased array of evaluators when utilizing the 5xFAD in future studies.

Through this study, our lab was able to successfully characterize the AD hallmarks exhibited by the 5xFAD model, specifically, the age-dependent cognitive deficits and accumulation of extracellular Aβ plaques. This allowed our lab to use the 5xFAD model in subsequent experimental and interventional studies, for example, testing the effectiveness of hydroxylpyclen as a potential treatment for AD.

Results from the hydroxylpyclen study evaluated the effectiveness of the compound in reversing the AD hallmarks exhibited by the 5xFAD, specifically improving cognition in CFC and reducing extracellular Aβ plaque accumulation through plaque imaging and counts. CFC results (Fig. 5) indicated a slight increase in percent

freezing time, although not significant. This is indicative of slight cognitive improvement; however, more trials to increase sample size may reveal more significant results. Interestingly, all 9-month-old 5xFAD, both hydroxylpyclen and saline-injected, exhibited an elevated percent freezing time during testing day, a finding that was not expected. As the subjects in the hydroxylpyclen study were transgenic positive, known to exhibit cognitive impairments, if hydroxylpyclen were ineffective, it would be expected for all mice to have exhibited a decreased percent freezing time on testing day. Further, the percent freezing time should be comparable to the 9-month-old 5xFAD+ mice from the characterization study. However, comparisons between the two studies revealed that injected 9-month-old 5xFAD+ mice had a greatly increased percent freezing time compared with identical un-injected 9-month-old 5xFAD+ mice. It is theorized that the 28 daily injections sensitized the 5xFAD in the hydroxylpyclen study to exhibiting a fear response, thus, causing them to naturally freeze more. In turn, this injection-induced freezing may be shadowing the true effects of hydroxylpyclen administration on cognition.

Next, this study evaluated the effects of hydroxylpyclen administration on Aβ plaque deposition and accumulation in the hippocampus. In representative Thioflavin-S stained hippocampal tissue sections (Fig. 6), hydroxylpyclen-injected subjects revealed a greatly reduced Aβ plaque burden in the hippocampus compared to saline-injected animals. Further, even if Aβ plaques were not completely eliminated, Aβ plaque size was greatly reduced, as suggested by representative 60x magnified images. Plaque counts (Fig. 7) quantified these findings by showing that Aβ plaque load was greatly reduced in hydroxylpyclen-injected animals; however, not significantly reduced. There proved to be

great variability within the number of Aβ plaques exhibited by each section, which produced a high standard deviation during statistical analysis. Increasing the number of stained sections analyzed may reveal statistical significance.

Further testing of brain tissue and hippocampal sections may reveal that hydroxylpyclen is able to replicate *in vivo* the properties previously demonstrated in cell culture studies. Hydroxylpyclen has been shown previously to exhibit antioxidant capabilities, able to reduce ROS within cells, thus, reducing overall oxidative stress. As mentioned before, one of half of the hippocampus from each hydroxylpyclen-injected mouse was flash frozen for future analysis of reactive oxygen species (ROS) levels. Although not evaluated in the current study, this analysis will reveal the antioxidant capabilities of hydroxylpyclen *in vivo*. In addition, examining hippocampal tissue sections for the presence of gliosis, namely microglial activation, through additional staining protocols may reveal the potential of hydroxylpyclen in reducing neuroinflammation. Lastly, examinations into overall copper levels and intracellular copper levels within the neural tissue and neurons, respectively, of hydroxylpyclen-injected subjects will evaluate its ability to chelate copper *in vivo*.

This study alone does not fully evaluate the potential of hydroxylpyclen as a treatment option for AD. A multitude of possible directions still exist for future studies with hydroxylpyclen. For example, hydroxylpyclen administration could be tested in a younger age group, such as mice 6 months of age. At this age in the 5xFAD, cognitive impairment remains demonstrable, but perhaps not as far-gone as 9-month 5xFAD to still allow for improvements. In 9-month-old 5xFAD, severe synaptic disruption and neuronal death may have already taken place, leading to irreversible cognitive deficiencies

(Serrano-Pozo, 2011). In addition to the 6-month group, hydroxylpyclen administration can begin in an even earlier age group, such as 3-month-old subjects. At this age, the drug may prove to be effective as a preventative treatment option, maintaining and protecting cognitive function before deficiencies are expressed. As mentioned earlier, there exist numerous more behavioral paradigms, which evaluate learning and memory. Utilizing one of these may prove to be more effective at revealing the effects on cognition due to hydroxylpyclen administration.

Dosage studies would also be of great interest in future studies with hydroxylpyclen. The concentration of injections in the current study (222mg/kg) resulted in no obvious detrimental side effects to the subjects, as evidenced by no significant differences in mass compared to saline-injected subjects, no significant effects to internal viscera examined during postmortem autopsy, and no mortalities. A more concentrated dose may result in increased treatment effectiveness, while still retaining biocompatibility. Another direction would be to administer hydroxylpyclen for a longer duration. There are currently implantable administration devices, including osmotic pumps (AZLET pumps), controlled-release pellets, and cannulas, that allow for prolonged drug exposure by progressively delivering compound into the system at a steady rate for a period of days, weeks, or months. These administration methods bypass the use of injections. Further, they can be implanted subcutaneously and intraperitoneally or attached to catheters for intravenous, intracerebral, and intra-arterial administration (Shumizu, 2004). If the number of injections did, in fact, sensitive the mice and, thus, shadow the effects of hydroxylpyclen on cognition, then limiting this number may also reveal its true effectiveness. Thus, a shorter exposure time to hydroxylpyclen may reveal

to be more effective. Exposure durations would further influence the dosage requirements.

Future studies may also examine how effectively the compound is functioning *in vivo*. Evaluating, either experimentally or through a computer model analysis, the efficiency of hydroxylpyclen in crossing the blood-brain barrier (BBB) and its effectiveness in reaching target neural tissues would be of great interest (Crivori, 2000; Seelig, 1994). Chemical modifications, such as lipidation or conjugation to carriers, and biological modifications, such as carrier-mediated transporters and codrugs, to hydroxylpyclen may allow for more facilitated transport or diffusion into the desired target neural tissues (Pardridge, 2003). In addition, brain targeting may also be accomplished by fusing hydroxylpyclen to "molecular Trojan horses," compounds that cross the BBB through binding to specific endogenous receptor-mediated transporters (Pardridge, 2007). The compound could also be administered directly to the circulatory system, cerebrospinal fluid (CSF), or target neural tissues via intravenous, intraspinal or intracerebral injections, respectively, to possibly increase potency (Shumizu, 2004).

Clearly, the potential of hydroxylpyclen as a viable treatment option for AD has not been exhausted by this study. Many more studies are possible, which may pinpoint the administration factors and dosage characteristics necessary to render the compound most effective in the potential treatment of Alzheimer's disease.

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