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IMMUNOTHERAPY IN COMBINATION WITH BEHAVIORAL ENRICHMENT IN A CANINE MODEL OF AGING

Paulina R. Davis
University of Kentucky, pau.davis@uky.edu

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Paulina R. Davis, Student

Dr. Elizabeth Head, Major Professor

Dr. Robert Hadley, Director of Graduate Studies

IMMUNOTHERAPY IN COMBINATION WITH BEHAVIORAL ENRICHMENT IN A CANINE MODEL OF AGING

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By
Paulina Reneé Davis

Lexington, Kentucky

Director: Dr. Elizabeth Head, Associate Professor of Pharmacology & Nutritional Sciences
Lexington, Kentucky

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ABSTRACT OF DISSERTATION

IMMUNOTHERAPY IN COMBINATION WITH BEHAVIORAL ENRICHMENT IN A CANINE MODEL OF AGING

Alzheimer's disease (AD) is characterized by cognitive decline and hallmark neuropathology, including β-amyloid (Aβ). Therapeutic strategies for AD are focusing on reducing Aβ. Canines develop Aβ neuropathology and cognitive decline with age similar to AD patients. In previous studies, immunization with Aβ1-42 (VAC) in aged canines decreased brain Aβ but did not improve cognition. Behavioral enrichment (ENR) improved cognition without reducing brain Aβ. We hypothesized that VAC combined with ENR would provide cognitive benefits and reduce Aβ neuropathology, as compared individual VAC and ENR treatments. Aged beagles were placed into groups: control, VAC with fibrillar Aβ1-42, ENR, and combination treatment (VAC+ENR) for 18 months. Learning and memory was evaluated throughout the study. Serum IgG antibody titers, cerebral spinal fluid (CSF) and brain Aβ were measured. Serum anti-Aβ1-42 IgG increased significantly in VAC animals. ENR but not VAC significantly increased CSF Aβ1-40. No cognitive improvements were observed in any group. VAC significantly reduced brain Aβ1-40 and 1-42, as well as reduced plaque load. An overall slowing of plaque accumulation was seen in the ENR group. VAC and ENR were able to modify pathology when used as separate treatments; however, the combination treatment did not succeed in further reducing Aβ or improving cognition. Previous AD clinical trials using immunotherapy yielded similar outcomes to our study showing reduced Aβ pathology but little to no cognitive improvements. In combination these results suggest that future studies should focus on prevention approaches both in the canine model and in human clinical trials.

KEYWORDS: Alzheimer Disease, Beta-Amyloid, Dog, Vaccine, Enrichment

Paulina Davis

Student's Signature

September 22, 2014

Date

IMMUNOTHERAPY IN COMBINATION WITH BEHAVIORAL ENRICHMENT IN A CANINE MODEL OF AGING

By

Paulina Reneé Davis

Elizabeth Head
Director of Dissertation

Robert Hadley
Director of Graduate Studies

September 22, 2014

I would like to dedicate this work to my grandparents, Robert E. Davis, Blanche Davis, Humberto Rodriguez, and Yolanda Rodriguez.

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CHAPTER ONE: Alzheimer's disease

Frequency of Alzheimer's disease in the US and Internationally

In the United States, 5.4 million people are living with Alzheimer disease (AD), (178, 495, 496). The number of people living with the disease is expected to triple by 2050. This increase in prevalence of AD is thought to be due to the general population living longer as well as the "baby boom" generation reaching the age of 75, a time at which the most common late onset of the disease is seen (22, 323). AD occurrence increases exponentially between the ages of 75 and 85 with about 50% of the population over the age of 85 being affected (113, 121, 178). In 2010, the Center for Disease Control and Prevention reported 83,494 deaths due to AD. However, a recent study by James et al. indicated the death toll may be closer to 500,000 placing AD as the 3rd top killer in the US as opposed to its previous place at 6th. Only heart disease and cancer are higher than AD in number of deaths per year (197).

Economic Impact

Not only is the prevalence and number of deaths caused by AD increasing, but the global economy is significantly impacted. The estimated annual global economic burden due to dementia is between $315 and $604 billion (189, 486). These vast costs come as little surprise with 12% of the population being elderly, of whom make up 26% of physician visits, one third of hospital stays and prescriptions, 40% of emergency responses, and 90% of nursing home residents (22). These numbers will only grow as noted earlier due to the aging "baby boom"

population. By 2029, all “baby boomers” will be at least 65 years old totaling an estimated 70 million people in the US aged 65 years and older.

In 2009, the Alzheimer’s Association totaled direct costs to Medicare and Medicare as well as indirect costs to businesses for employees who are caregivers of individuals with AD and other dementias to be estimated at more than $203 billion in 2013 and expected to increase to $1.2 trillion in 2050 (23, 150, 224). In 2008, the average individual out of pocket costs for Medicare beneficiaries over the age of 65 with AD and other dementias was $9,754 per person per year for healthcare and long-term care services, with the payments being highest for those living in nursing homes and assisted living facilities (22, 23). The total costs in 2004 from all sources of hospice care for these beneficiaries totaled $2.8 billion (22). In addition to these expenses, are those that were not paid to individuals who voluntarily cared for those with dementia. Whether it was family, a friend, or neighbor, over 15 million people provided unpaid care for an individual with AD or other dementia in 2008 (9, 22, 23). In 2012, these caregivers totaled 17.5 billion (22, 448). The time provided by these caregivers is valued at $216 billion dollars (22, 448). For perspective, the value of caregiver time totaled in 2011 equaled half the net sales from Walmart and eight times the sales of McDonald’s (448). While AD is quickly becoming an epidemic among the elderly, so has the cost of care for these individuals on the US economy.

History

AD was first described in1906, by Dr. Alois Alzheimer after treating a patient who exhibited progressive memory decline (10). Augusta Deter, a 51 year-old

woman, died 5 years later after she was first seen by Dr. Alzheimer (10, 431). It was noted at autopsy that the patient's cerebral cortex was atrophied (10). At this time, Alzheimer also identified histopathological changes that would later come to be known as the hallmark lesions of AD, neuritic plaques and neurofibrillary tangles (NFTs). In 1910, Emil Kraepelin named presenile dementia "Alzheimer's disease" to honor Alois Alzheimer (395, 431). A clinical diagnosis of AD is made when a patient exhibits dementia with progressive decline, although a final diagnosis of AD cannot be confirmed until post mortem examination and requires the presence of AD pathological findings (96).

Cognitive and Behavioral Changes

A diagnosis of dementia is established when an individual exhibits the loss of 2 or more of the following cognitive domains: memory, language, calculation, orientation, or judgment (1, 2). However, for the individual to have a "probable AD" diagnosis, they must have dementia that is clinically documented along with deficits in at least 2 cognitive domains, absence of other systemic disorders, and progressive worsening of memory (269, 270, 336, 338). Individuals with AD show difficulty remembering new information and exhibit confusion, disorganized thinking, impaired judgment, and disorientation with time, space and location. In addition, those with AD experience frequent changes in mood, are easily agitated, and often experience anxiety. These psychological changes often lead to restlessness and sleep deprivation (12, 208, 285). Most AD patients require assistance with bathing, dressing, using the bathroom, eating, and other daily

activities. The gradual decline in memory eventually increases in severity until the symptoms become debilitating (35).

If not noted by patient report or by a family member, friend, or caregiver, the first signs of cognitive impairment can be detected through direct observation by a clinician during an Annual Wellness Visit (15). If symptoms of cognitive impairment are present, a brief structured patient assessment of cognition is performed using one of several available tools including the Mini-Mental State Examination (MMSE), the Memory Impairment Screen (MIS), the General Practitioner Assessment of Cognition (GPCOG), or the Mini-Cog (59, 183, 192, 249, 281). In addition, informant assessments of the patient can be conducted using the GPCOG, AD8, or short Informant Questionnaire on Cognitive Decline in the Elderly (IQCODE). If any of these assessments indicate possible cognitive impairment, a full dementia evaluation can be conducted and may include an assessment of multiple cognitive domains, a neurologic exam, standard laboratory tests, and structural brain imaging. Results of a full dementia evaluation will help determine an appropriate diagnosis such as mild cognitive impairment or AD, or determine other possible causes (75).

Familial and Sporadic Alzheimer's disease

There are two types of AD, familial and sporadic. When at least two generations of a family has been reported to have AD with a mutation that is inherited, it is considered of the familial type (41, 303). Sporadic AD occurs with some genetic or outside contributing factors increasing one's risk. AD is further defined as being early (EOAD) or late onset (LOAD) based on the age of onset of

the disease. EOAD occurs before the age of 65 and LOAD occurs after (37, 497). Almost all cases of sporadic AD are LOAD. EOAD makes 1-6% of AD cases of which 60% are familial AD (57, 63). While familial AD must have a genetic component, sporadic forms of AD may also have a genetic component (76, 77, 333, 350-355). However, a majority of the cases are unpredictable with various factors including past medical history, environment, and lifestyle factors that may increase or decrease the risk of AD and the age of onset.

Genetic Contributions to AD

Of the 60% familial cases that are EOAD, 13% are due to autosomal dominant inheritance of specific genes (57, 63). Researchers have found several genes to be associated with AD, but the three most commonly linked to familial EOAD include mutations on the amyloid precursor protein (APP), presenilin 1 (PSEN1), and presenilin 2 (PSEN2) genes (141, 243, 244, 348, 349, 397). Over 200 mutations are reported in these three genes alone (35). A consistent feature of all these mutations is elevated Aβ peptide levels (discussed in more detail later), enhanced aggregation of Aβ, early onset of AD neuropathology, and cognitive decline (25). The APP gene encodes an integral type 1 membrane glycoprotein and when cleaved by beta and gamma secretases results in different Aβ peptide isoforms. Mutations in APP result in altered ratios of Aβ isoforms in the brain and can account for 10-15% of familial autosomal dominant EOAD cases (157, 339). The PSEN1 and PSEN2 genes encode two proteins that are components of the gamma-secretase complex, which are expressed in many different tissues including the brain and are involved in cleaving APP to Aβ (242-244, 348). APP

processing will be discussed in further detail in Chapter 2. The mutations reported in PSEN1 and PSEN2 may result in a modified gamma secretase cleavage of APP. This altered cleavage activity leads to altered Aβ ratios with an increase in AB_{1-42} (107). PSEN1 accounts for a majority of the reported mutations and is the most common cause of familial EOAD making up 18-50% of the autosomal dominant cases (382, 432, 447).

Only one gene has been widely studied as the strongest risk factor for sporadic LOAD, apolipoprotein E (APOE). APOE is a three-allele polymorphism (ε2, ε3, ε4) encoding for a glycoprotein that carries cholesterol in the blood stream and maintains lipid metabolism and transport (22, 76, 77, 349, 402). Additionally it can influence the clearance of cerebral Aβ in AD individuals (76, 77). The effect APOE has on AD depends on which alleles are expressed. ApoE2 has exhibited protective properties and can act on longevity, ApoE4 increases the risk of AD, and ApoE3 is considered neutral (76, 77). Humans express two alleles of APOE. The effects of the two alleles a person expresses act in a dose dependent manner (76, 77). For example, having at least one ApoE4 allele leads to a higher risk of developing LOAD compared to individuals with no ApoE4 allele. However, the risk of AD is 15 fold higher in homozygous carriers of ApoE4 (21). Individuals with ApoE4 show more Aβ and NFT pathology when compared to individuals with other ApoE allele expression (35). The risk of developing AD by ApoE genotype can be modified further by non-genetic factors such as head trauma or stroke (184, 185, 300). The frequencies and distribution of the ApoE alleles vary widely among populations (504, 505) however the ApoE3 allele is the most commonly expressed

(35). APOE, along with APP, PSEN1, and PSEN2 make up less than 30% of the genetic variance in EOAD and LOAD, however, many other genes are currently being studied and reported (89, 431).

Non-Genetic Contributions

While APOE can influence risk of LOAD, other non-genetic variables can increase, or decrease risk as well. Risk of AD increases with age, female gender, and lower education (121, 236, 430). Females have a risk of AD three times higher than that of males (125). This may be due to the fact that women on average live longer than men, allowing more time for the development of AD (22). Education is an important factor influencing risk of AD (210-212, 421, 422, 506). The supporting idea for this risk is that individuals with higher education develop a greater "cognitive reserve capacity" than those with lower education (422). By having a greater reserve, a maintenance of cognition or delay of dementia onset may occur as a consequence of compensating for any functional deficits caused by AD pathology (11).

Comorbidities can also influence AD risk. Individuals with systemic hypertension, diabetes mellitus, cardiovascular disease, and cerebrovascular disease all experience a greater risk for developing AD (191, 229, 251, 265, 306, 412). Those with type II diabetes have a two-fold increased risk for AD (117, 250, 251, 317). Middle-aged individuals with high serum cholesterol levels are also at a higher risk of AD later in age (220, 481). Observational studies have shown mid-life hypertension to increases likelihood of AD (267). However, low blood pressure later in life can also increase chances of developing AD (334, 356). History of

traumatic brain injury or stroke dramatically increases risk of AD (122, 318), especially in APOE4 carriers. Some environmental factors that also appear to contribute to risk of AD include lack of social engagement, smoking, and heavy alcohol drinking. Individuals who are ApoE4 carriers are especially affected by smoking and heavy drinking (4, 16, 273, 311).

Protective Factors

While there are several factors that increase the risk of developing AD, there are also several that are associated with reduced risk of disease including social and cognitive enrichment, physical activity, and diet (112, 372, 373, 466). Exercise activates neural plasticity, remodels neuronal circuitry, and promotes vascularization (38, 80, 462). Cognitive enrichment such as reading, social activities, knitting, tabletop games, information processing activities, and playing musical instruments have all shown to be protective and reduce risk of dementia and AD (36, 84). Diet has been widely studied for protective factors against the development of sporadic AD. Omega-3, vitamin D, and folic acid have all been shown to reduce risk of AD (110, 289-292, 374, 472). In addition, several studies suggest that a Mediterranean diet of vegetables, legumes, fruits, and cereals, unsaturated fats, fish, some dairy, meat and poultry, and wine is also associated with decreased risk (415). While genetic factors and medical history can influence the onset of AD, there are many lifestyle practices an individual can engage in to reduce their risk.

Neuropathology of AD

In parallel, or prior to, the development of cognitive and behavioral changes, individuals with AD accumulate several types of neuropathology that become progressively worse as the disease evolves. In the 1960's, the 2 classic lesions of AD, NFTs and senile plaques, were confirmed as hallmark pathology for the disease (215, 431). During the 1980s, various neurotransmitter deficits were identified in AD (146, 147, 298, 329). Additional forms of neuropathology that contribute to AD have also been described including changes in brain volume, synaptic and neuronal loss, as well as decreased neurogenesis. A brief description of each of these key pathologies is described next.

Brain Volume and Neuronal Loss

The medial temporal lobe, including the hippocampus and entorhinal cortex, is the first area in the brain to show atrophy in individuals with AD and occurs early in the disease as shown with structural MRI (26, 48, 52, 106, 216, 337, 414, 449, 450). Atrophy, including loss of myelination, synapses, and neurons, begins in the temporal lobe and develops into the neocortex, notably the frontal and parietal regions, at later stages of the disease (26, 48, 52, 73, 90, 106, 188, 195, 284, 380, 414, 449). Several longitudinal studies have shown that increased rates of ventricular widening as well as regional and whole brain atrophy in otherwise healthy individuals increases risk of developing cognitive decline due to AD later in life (64, 196, 357). Several studies have indicated a strong correlation between cognitive ability and synaptic numbers (93, 247, 369, 375, 377-379, 441). With the loss of synaptic contacts and neurons, a significant reduction of grey matter in the

hippocampus and entorhinal cortex is seen in later stages of AD (308). This grey matter loss correlates with decreased cognitive performance, especially so with memory (227, 439). However, synaptic and neuronal loss is experienced in multiple regions of the brain in AD, possibly explaining the diverse cognitive changes seen with the progression of the disease (376).

Neurogenesis

Neurogenesis takes place primarily in two regions of the adult brain, the subgranular zone of the dentate gyrus of the hippocampus and subventricular zone of the lateral ventricles (111). Fully functioning neurons can be generated from progenitor cells. These cells proliferate into immature neurons and migrate to the granule cell layer where they mature and integrate into preexisting circuitry (502). Interestingly, several genetic contributors to familial AD modulate neurogenesis, including PS1 and APP (237). While PS1 positively regulates neural progenitor cell differentiation, soluble APP positively regulates proliferation (62, 126, 127, 238). However, mutations in these two genes, as seen in familial AD, alter alpha and gamma secretase activity and reduce soluble APP, respectively, which may suppress neurogenesis (as reviewed in (237)). These deficiencies in neurogenesis may occur early in AD, prior to plaques, NFT, or neuronal loss, thus further supporting the idea that familial AD involved proteins directly affect neurogenesis which may contribute to the development of AD (238).

The link between changes in learning and memory with age and losses in neurogenesis is complex (237). The hippocampus is one of the first regions of the brain affected in AD, and is also one of only two brain regions where neurogenesis

occurs. Thus supporting the hypothesis that pathological processes of AD impact neurogenesis, and vice versa (161, 205, 206, 248, 386, 467, 468, 508). It has been proposed that when these disease processes begin to take place, rates of neurogenesis will increase in attempt to compensate before a significant loss in neurogenesis is observed (5, 222, 228, 248, 399, 400). Decreases in neurogenesis may underlie cognitive impairments associated with dementia (69, 161, 205, 206, 237, 248, 386, 467, 468, 508). Suppressing neurogenesis causes deficits in hippocampal dependent learning; while other cognitive domains appear unaffected (238, 507). Furthermore, additional studies that increase neurogenesis in mice lead to improved performance in pattern separation and spatial memory (363, 423).

Neurofibrillary Tangles

In 1963, neurofibrillary tangles (NFTs) were first identified as one of the hallmark lesions of AD and found to be made up of paired helical filaments (PHFs) (190, 215, 431). Then in 1986, researchers found that NFTs were comprised of microtubule associated protein tau (58, 151, 225, 304, 494). Tau protein has a normal function of stabilizing microtubule formation and disassembly in neurons. In its non-phosphorylated state, tau protein binds microtubules and binds less tightly once phosphorylated (56). However, increased phosphorylation, or hyper-phosphorylation, of tau can lead to aggregation of the protein within the cell and formation of paired helical filaments (PHF) (151). Ultimately these PHFs lead to the formation of neurofibrillary tangles (NFT) (142, 226, 240, 264). There are 19 specific amino acid sequences that are frequently phosphorylated in the formation

of NFTs in AD (29, 142, 226, 240, 264). Tau protein can be phosphorylated by several kinases, but the three commonly acting on phosphorylation sites of interest in AD include MAP kinase, GSK-3 and cdk5 (104, 254).

The extent and distribution of NFTs correlates with the severity of dementia in AD (20, 49). There are different morphological stages of NFTs including pre-, intra-, and extra- neuronal (219). Pre-neuronal NFTs include intracellular punctate aggregates of hyperphosphorylated tau inside an otherwise healthy neuron. Once these aggregates start to form filamentous structures, paired helical filaments (PHFs), an NFT is then considered intra-neuronal. NFTs enter the final morphological stage, extra-neuronal, when the neuron dies and only an extra cellular NFT, or “ghost tangle”, remains (219).

In addition to morphological changes, NFTs have defined stages for progression in terms of severity and location within the brain known as Braak stages I-VI (19, 52). A 900 autopsy case study spanning the ages 25 to 95 of demented and non-demented brains showed that younger cases showed a pattern of NFT deposition that spread with more advanced ages (307). NFTs are first observed in layer II of the entorhinal cortex (transentorhinal cortex) following a predictable sequence spreading onward to other regions of the brain (19, 20, 50, 53, 187, 326, 460, 461). Braak stages I and II are defined as having NFTs present in the transentorhinal region of the brain with the absence of cognitive impairments (55, 200). At these stages, this area is generally void of any Aβ deposits of plaques (54). Progression into Braak stages III and IV show more extensive NFT pathology into the hippocampus, however, most of the neocortex is unaffected. When NFTs

reach the hippocampus, limbic circuits become disrupted that is associated with declines in cognition seen at these stages. This disruption is further exacerbated in Braak stages V and VI when NFTs are prevalent throughout the cerebral cortex. These stages of neuropathology are used for confirmation of clinical AD diagnosis (283, 451). NFTs have been seen in other neurodegenerative diseases in the absence of Aβ pathology, and it can be assumed that NFTs can occur independently of plaques in the progression of AD (389). This is seen in Braak stages I and II of NFT progression. However, for a final diagnosis of AD, the presence of senile plaques is also required.

β-Amyloid

In addition to NFTs, a final diagnosis of AD at post mortem examination also requires the presence of Aβ plaques (33, 96, 201). As with most of the neuropathology observed in AD, Aβ pathology occurs early in the disease before cognitive impairments are observed (389). Genetic mutations of APP, PSEN1, or PSEN2 seen in familial AD can influence the age at which Aβ pathology is first seen. According to the amyloid cascade hypothesis, excess accumulation of Aβ peptide induces a series of events including the formation of insoluble and soluble oligomers followed by aggregate stress, the formation of NFTs, and ultimately leading to neuronal death and AD (159). At the start of the cascade, APP is cleaved by β- and γ- secretase and the resultant cleavage product is the Aβ peptide (476). Depending on the location at which γ- secretase cleaves APP, various species of Aβ peptide can be produced. The two most common Aβ peptide species are $A\beta_{1-40}$ and $A\beta_{1-42}$ (194, 389). These Aβ peptides can accumulate into

oligomers, then fibrils, which then aggregate further to make up Aβ plaques (97, 138, 261). The spatial pattern of Aβ deposition begins in the frontal cortex spreading to the lateral and parietal regions early in the disease (18, 50-52, 445). Later, Aβ deposition spreads further into the occipital lobe and motor cortices with the entorhinal cortex and hippocampal region being affected last (18, 51). Whether Aβ deposition directly causes neuronal loss is unknown, but Aβ is toxic and can alter neuron function (294). For example, many studies have shown Aβ deposition impairs long term potentiation, a physiological substrate for memory, and neuronal plasticity in the hippocampus (245, 389).

The extent of Aβ pathology including peptide production and isoforms, the various types of plaques that form, and effects on cognition will be described in greater detail in the following chapter.

Vascular Aβ and Cerebral Amyloid Angiopathy

In addition to Aβ plaques, Aβ peptides can deposit in association with the vasculature (138). Aβ was originally isolated from meningeal blood vessels of individuals with Down syndrome and AD (138, 139). Aβ deposition in vascular walls is characteristic of cerebral amyloid angiopathy (CAA) (138, 389). CAA is defined as amyloid protein aggregated within the blood vessels of brain tissue that can be stained by Congo red, an immunohistochemical stain that labels compacted amyloid protein aggregates (342). While Aβ protein makes up Aβ plaques and CAA, there is a low correlation between the two events and each can occur in absence of the other (389, 465). Population studies show that 55-59% of patients with dementia show CAA and in up to 98% of the AD cases (199, 214). Though

the majority of the AD population shows CAA in the brain, the degree of CAA burden among individuals with AD can vary widely (389, 465). Genetic mutations associated with AD, including APOE and PSEN1, are also risk factors for CAA deposition (149, 299, 325, 499). While CAA can be found throughout the brain, the distribution pattern is much like that of Aβ plaques beginning in the frontal, parietal, temporal, and occipital cortices and later in the hippocampus and entorhinal cortex (443, 444). Interestingly, a study documenting and assessing various forms of neurodegenerative pathology in relation to cognitive function indicated that CAA may correlate more strongly with the presence of dementia than with other forms of amyloid pathology (266). However, the contribution of CAA to dementia is not well understood (465).

Accumulation of Aβ in the vasculature may be due to efforts of clearing the peptide from the brain or microglial uptake and deposition into the vascular lumen. Vascular Aβ deposition prompts smooth muscle cells of these vessels to produce vascular Aβ, allowing further Aβ deposition. Affected vessels show thickened walls with amyloid deposits and degeneration of smooth muscle cells (260, 301). On rare occasion, advanced CAA damage of the vessel walls can cause them to rupture resulting in microhemorrhages or hemorrhagic stroke (389, 489). When hemorrhaging does occur, it is predominantly located in the frontoparietal, temporal, and occipital regions of the brain. A definitive clinical diagnosis of CAA-related hemorrhages cannot be made until postmortem examination, but a probable diagnosis can be made by MRI or CT imaging (223). In comparison, a probable diagnosis of CAA can be made with the observation of multiple

hemorrhages confined to lobar brain areas with no other explanation for the pathology (148). Cerebral microhemorrhages are seen in 16.7 to 32% of AD patients as seen using MRI (78, 79, 155, 156, 297). When using positron emission tomography (PET) with amyloid labeling ^{11}C-Pittsburgh Compound B (PiB), microhemorrhages usually occur in regions that have concentrated amyloid deposits of individuals with and without AD (99).

CHAPTER TWO: β-Amyloid

APP and APP Processing

The Aβ peptide is a cleavage product of the amyloid precursor protein (APP) (152, 153). The Aβ peptide was first sequenced from amyloid deposits in meningio-cerebral blood vessels of a patient with Down's syndrome (DS) and AD (137, 140). Subsequently, APP was localized to chromosome 21 (209, 343, 433). APP is a single transmembrane polypeptide glycoprotein whose function is unknown (476). APP is commonly cleaved between residues 16 and 17 of the Aβ region by α-secretase resulting in a soluble ectodomain region (APPs-α) and its release into vesicle lumens. Alternatively, APP can be cleaved by β-secretase cutting APP to release a truncated form of soluble APP (APPs-β), which is also released into the vesicle lumen (393). Cleavage by either α-secretase or β-secretase also results in a carboxy terminal fragment (CTF) still within the membrane that will ultimately be cleaved by γ-secretase resulting in either the peptide p3 (if first cleaved by α-secretases) or Aβ peptide (if first cleaved by β-secretase) (464, 500). The majority of APP undergoes processing by α- and γ-secretases while less undergoes the amyloidogenic processing by β-secretase and γ-secretase (387). Various mutations in APP or PSEN1 or PSEN2 genes, as described in the genetic risk factors of AD section of Chapter 1, can lead to increased amyloidogenic processing of APP. Genetic mutations identified in the APP or PS gene causing AD in addition to the observation that older individuals with trisomy 21 DS having an earlier onset of AD and faster progression of the disease led to the amyloid hypothesis (141, 158, 179, 295, 309). The amyloid

hypothesis proposes that production of Aβ and its intracellular deposition in neurons along with extracellular formation of diffuse and neuritic plaques is the initiating factor resulting in tau hyperphosphorylation and activation of microglia, ultimately leading to neurodegeneration (119, 159).

Aβ Peptide

During the amyloidogenic processing of APP, γ-secretase can cleave the CTF at various sites leading to multiple isoforms of Aβ peptide ranging from 36 to 43 amino acids long (74, 136, 194). The two most common isoforms of Aβ are $A\beta_{1-40}$ and $A\beta_{1-42}$ which are 40 or 42 amino acids in length, respectively (74, 136, 194). While the biological function of Aβ peptide is not well known, it does have a hydrophobic structure that self-aggregates into dimers, trimers, tetramers, oligomers, and fibrils (262). In general $A\beta_{1-40}$ is more soluble, less toxic, and found in plaques, but more commonly associated with deposition in the blood vessels (324, 389, 498). $A\beta_{1-42}$ on the other hand is more hydrophobic making it more readily aggregated to form fibrils and plaques representing the majority of parenchymal Aβ (324, 389, 477). Up to 90% of Aβ in the brains of individuals of AD can be of the $A\beta_{42}$ species (144). Individuals with familial AD due to APP mutations will have increased levels of extracellular $A\beta_{1-42}$ or both $A\beta_{1-40}$ and $A\beta_{1-42}$ depending on the mutation (61, 68, 427), while PSEN1 or PSEN2 mutations selectively increase $A\beta_{1-42}$ levels (382).

Soluble and Insoluble Aβ

The various forms of Aβ can be either soluble or insoluble. The soluble form of Aβ can be found in CSF, plasma, and serum as well as in brain tissue (262,

390). Soluble CSF $A\beta_{1-40}$ and $A\beta_{1-42}$ can be used as biomarkers to predict AD pathology progression in the brain (153, 394, 398). CSF $A\beta_{1-42}$ is reduced in individuals with AD and inversely proportional to the level of cognitive impairment and Aβ pathology in the brain of that individual (13, 128). Generally, those without AD have higher levels of CSF Aβ and lower levels of brain Aβ. Lower CSF Aβ would indicate the movement of Aβ from the periphery into the brain (13, 128). While CSF Aβ levels inversely correlate with AD pathology in the brain and cognitive decline, Plasma $A\beta_{1-40}$ and $A\beta_{1-42}$ levels are more variable and less reliable as a biomarker of AD (268, 272, 463). Additionally, the levels of $A\beta_{1-40}$ and $A\beta_{1-42}$ are much higher and more easily measured in CSF than plasma $A\beta_{1-40}$ and $A\beta_{1-42}$ levels (268, 272, 463). The insoluble form of Aβ can only be found in tissue (294, 473). Insoluble Aβ is generally fibrous in nature and makes up a large proportion of Aβ plaques (262). The Aβ that forms plaque cores is generally more insoluble than that from vascular deposits (262). While both soluble and insoluble Aβ correlate with cognitive impairment, soluble Aβ levels measured by biochemical assays appear to better correlate with cognition than insoluble dense plaque deposition measured immunohistochemically (252, 271, 396).

Aβ Oligomers

Aβ is also very toxic in its soluble oligomeric form (252, 271). When Aβ monomers assemble with one another they can form soluble oligomers which can exist in multiple forms such as dimers, trimers, or Aβ*56 (a dodecameric Aβ formation) (235). Two studies in the early 1990's demonstrated a poor correlation between fibrillar Aβ and cognitive decline in patients with AD (98, 442). Later

soluble non-fibrillar Aβ levels were shown to have a strong correlation with AD (252, 271). With these studies it was suggested that soluble Aβ may be a greater contributor to progression of AD than the previously thought deposited fibrillar Aβ (252, 271). Since Aβ oligomers provide more surface area for interaction with neural synapses than plaques, they are thought to be more synaptotoxic. Oligomers modulate both pre- and post-synaptic structure and functions in a dose dependent manner (313, 388). This synaptotoxicity by oligomers causes inhibition of long term potentiation in the hippocampus contributing to cognitive decline (470).

Aβ Plaques

Aβ is deposited extracellularly and aggregates into plaques (389). As mentioned, Aβ is self-aggregating and first forms polymers that then create beta pleated sheet formations making up Aβ fibrils (389). The insoluble Aβ fibrils may be inactive but are reservoirs of smaller Aβ assemblies (153, 294, 394, 398). These fibrils can then become cytotoxic when misfolded leading to amyloidosis and aggregating to form Aβ plaques (389). Both $A\beta_{1-40}$ and $A\beta_{1-42}$ can be found in plaques, but since $A\beta_{1-42}$ is more fibrillogenic of the two it is observed in Aβ plaques earlier in the disease (145, 193). Two types of plaques can form, diffuse and dense plaques. Diffuse plaques are primarily made of $A\beta_{1-42}$, while dense plaques contain both $A\beta_{1-40}$ and $A\beta_{1-42}$ (263). When stained by immunohistochemistry, diffuse plaques have a cloud like structure while dense plaques are more globular.

Post-translationally modified Aβ

Aside from the multiple Aβ isoforms that can result in the c-terminal cleavage by γ-secretase, there can also be N-terminal heterogeneity (429). This heterogeneity leads to various shorter peptides including $A\beta_{5\text{-}40}$, $A\beta_{5\text{-}42}$, $A\beta_{3\text{-}40}$, and $A\beta_{3\text{-}42}$ (65, 429). Additional post-translational modifications can occur with these N-truncated Aβ peptides. For example, an amino terminal modification can occur in which there is a proteolytic removal of residues 1 and 2 (Asp and Ala) (287). Another type of post-translational modification that can occur is the cyclizing of residue 3 or 11 of Aβ (Glu) to a pyroglutamate (pE) by glutaminyl cyclase (QC) (287, 384). The most prominent forms of the pEAβ species are $A\beta_{3(pE)\text{-}40}$, $A\beta_{3(pE)\text{-}42}$, $A\beta_{11(pE)\text{-}40}$, and $A\beta_{11(pE)\text{-}42}$ (365). As stated earlier, up to 90% of Aβ in the brain ends in $A\beta_{42}$ (144). Truncated and modified Aβ make up most of the $A\beta_{42}$ in the brain with $A\beta_{3(pE)\text{-}42}$ being the most prevalent form (144, 428). The prevalence of N-truncated and modified Aβ peptides is even greater in the brains of patients with familial AD compared to those with sporadic AD suggesting that post-translationally modified Aβ has a decisive role in the development of AD (282, 361). pEAβ is more readily aggregated, more toxic, and is resistant to degradation (8, 162, 362, 383, 385, 487). Schilling et al. studied the seeding and oligomerization capacity of the pEAβ peptide species and found that formation of seeds required for forming fibrils was very rapid compared to unmodified Aβ peptide (383). This suggests the pEAβ peptide species is more toxic and could initiate Aβ aggregation and plaque formation by unmodified Aβ (383). Since pEAβ peptide species promotes the advancement of Aβ pathology early in AD, then this

species could be a marker of older Aβ deposits (383). With the N-terminal pyroglutamyl present, pEAβ is resistant to N-terminal targeted degradation, adding to the toxicity of this Aβ peptide species (364). These attributes suggest that the pEAβ species plays a prominent role in the overall progression of AD (162, 186, 362, 383, 440) and may determine the severity of disease state in an individual (360).

Not only is pEAβ involved with Aβ pathology in AD, but two recent studies indicate an involvement in hyperphosphorylated tau pathology (256, 305). In 2012, Nussbaum et al. examined the connections between pEAβ and tau in AD, finding that the toxicity of pEAβ and tau were dependent on one another (305). Later, Mandler et al in 2014 measured and compared $pEA\beta_3$, full-length Aβ, and hyperphosphorylated tau loads in the frontal cortex and entorhinal cortex of 41 post mortem brains of both individuals with AD and controls (256). As expected, all loads were higher in AD. Interestingly, when looking at $pEA\beta_{3-x}$ independently of full-length Aβ, $pEA\beta_3$ predicted AD and hyperphosphorylated tau while full-length Aβ only predicted AD (256). High levels of hyperphosphorylated tau came with greater loads of $pEA\beta_3$, but were not affected by the absence of full-length Aβ (256). The greater toxicity, resistance to degradation, correlation with hyperphosphorylated tau and the progression of AD make pEAβ a critical peptide to evaluate in future AD studies and therapeutic development.

CHAPTER THREE: Dog Model

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The Canine Model of Human Aging and Alzheimer's disease

Some of the most commonly studied animal models of human brain aging are rodents and nonhuman primates (129). Other animals, including wolves, bears, cats, and dogs, naturally develop human-like neuropathology (172). Of these animals, cats and dogs tend to have similar living environments to humans (172). Canines, however, show cognitive decline with age and develop most aspects of neuropathology seen in aged human brain including AD patients (81, 87). Such neuropathology includes Aβ pathology, reduced brain volume, neuronal loss, and impaired neurogenesis (81, 163). In addition to the similar cognitive decline and accumulation of neuropathological hallmarks to humans with AD, drugs exhibit similar pharmacokinetics when administered to dogs or humans (for example statins - (7, 132)), making them an appropriate model for translational studies on therapeutic drugs. Not only are dogs easy to handle due to their long history of domestication, but pet dogs also share similar living conditions and diets to humans (30, 87, 316). Canines are highly motivated by food reward when conducting cognitive tests, which makes them cooperative research subjects by reducing or eliminating deprivation protocols for motivation. Thus, this cooperativeness eliminates many physiological stressors that can affect cognitive testing results present in other animal models such as rodents that require food deprivation or cold water for motivation (40). The similar cognitive decline and

accumulation of neuropathology to humans makes the canine model of aging useful for translational research on neurodegenerative diseases, especially AD.

Cognitive Outcome Measures

There are several measures of cognition that are age-sensitive and treatment-sensitive in dogs that can be used as intervention outcome measures to assess different cognitive abilities with analogous tasks in nonhuman primates and in humans. Much like humans, the aging canine shows cognitive decline with various cognitive domains and cortical pathways being differentially affected (277). Dogs show cognitive deficits due to age in tests measuring complex learning, executive function, spatial learning and attention, and memory (67, 82, 166, 277, 280, 410, 424, 434, 435, 437). In addition to cognitive domain variability, individual dogs also show variability in cognitive function as seen in humans (3). This variability becomes most apparent in old canines, and using spatial learning and memory tasks, three groups of animals can be identified: (1) successful agers, (2) impaired dogs whose scores fell 2 standard deviations above the mean of the young animals, and (3) severely impaired dogs who failed to learn the task (172). The availability of age-matched animals with and without cognitive deficits allowed researchers to determine which types of neuropathology contribute to individual cognitive impairments in these animals (e.g. (166)).

Several tasks, similar to those used for testing cognition in non-human primates, have been developed to measure cognitive decline in the aging canine (274, 276, 277). Such tasks include landmark discrimination, oddity discrimination, object, size and black / white discrimination and reversal tasks, and a spatial

memory task. Cognitive testing occur in a modified Wisconsin General Testing Apparatus such that the motor and sensory demands are consistent across tasks (277). For each task, 10-12 trials are given per day and dogs are tested daily until a predetermined criterion level of performance is reached; total error scores are added up across days to provide a measure of learning and/or memory for each animal. These tasks are described in more detail below to illustrate how a test battery can be developed to measure the function of several brain circuits that may be differentially affected by age and/or treatment in aging dogs.

Landmark Discrimination Task

The landmark discrimination task, which measures visuospatial function and allocentric learning, involves presenting dogs with two identical objects, one of which is adjacent to a third object that serves as a landmark (274). Animals are required to recognize that the landmark is an indicator of which object covers the food reward, and selection of the object closest to this landmark by the animal is considered a correct response. The task is made successively more difficult by placing the landmark further away from the object covering the reward. Previous work shows that aged dogs are impaired on the landmark task and show age decrements in their ability to determine how close the landmark is to the correct object (274, 276).

Oddity Discrimination Task

The oddity discrimination task measures complex learning, as well as prefrontal cortex function (82). Aged dogs show deficits in oddity discrimination learning (82, 280). In this task, dogs are presented with three objects

simultaneously, two of which are identical and a third that is unique. A correct response is indicated when the dog chooses the unique object, resulting in a reward. To prevent a floor effect and detect progressive age decline, the oddity aspect of this task is made successively more difficult. Animals progress through four sets of three objects and each subsequent set contains a unique object, which is more difficult to distinguish from others than the previous set (280). Interestingly, young dogs can solve this problem by using the strategy of selecting the novel object for each successive set of objects such that error scores plateau; in contrast, aged dogs do not learn a strategy but re-learn each set of objects as a new problem (82, 280).

Object, Size and Black/White Discrimination

Tests of object, size and black/white discrimination are administered to measure associative learning ability. Object discrimination involves presenting dogs with two different objects simultaneously with one of the two objects consistently rewarded. Dogs must learn to select the same object each presentation with the left/right position being randomly determined. Similarly, the size discrimination objects differ in size (small/large) and the black/white discrimination task objects differ only in color (black/white) (278). Object, size and black/white discrimination are also progressively more difficult for animals to solve given the similarities in the objects increasing. Thus, these 3 tasks in combination can serve as different test versions (much like in clinical studies in people) to assess longitudinal changes in learning while reducing practice effects (278).

Object, Size or Black/White Reversal

Executive function can be evaluated immediately after discrimination learning has been completed by using the object, size or black/white reversal tasks. The reversal tasks differ from the original discrimination task in that the positive and negative objects for reward contingencies are reversed after animals have learned the initial discrimination (278, 279). Reversing the reward contingencies can show perseverative behaviors (persistent choice of previously correct object), which are frontal cortex dependent (474). A subset of the discrimination learning tasks and all reversal learning tasks are age dependent, with reversal learning being consistently more impaired with age (277-279, 410, 437).

Spatial Memory Task

Memory also declines with age in dogs. The most useful age-sensitive task we have used is a spatial memory task, in which dogs are required to recognize the location of a sample stimulus and then respond to a different location during the test trial. We refer to this as a delayed non-match to position task (DNMP) and it involves showing animals a single object covering a food reward either on the left or right food well. After animals move the object and obtain the reward, the object is withdrawn from sight for a predetermined delay period (e.g. 10s). Subsequently animals are given two identical objects to choose from; one is the same object in the same position as before and one is in a novel position. The correct response is to select the object covering the novel location. Results published in 1995 (170) suggested that the task was age-sensitive. We

subsequently developed a 3-choice visuospatial working memory task that allows determination of the differential age-dependent strategies (e.g. cognitive or stimulus-dependent strategies) dogs use in solving the problem (66). In this task, rather than just the left and right food wells, a center well is also included to make the task more difficult. Further, this task shows minimal practice effects in longitudinal studies (177). The time course of the development of cognitive decline was identified and deterioration in spatial ability occurs early in the aging process, between 6 and 7 years of age in dogs (424).

Behavioral/Functional Outcome Measures

In addition to cognitive outcome measures, researchers and veterinarians are interested in measuring functional outcomes. Further, laboratory-based cognitive testing as described above is labor intensive and requires many months to years to obtain data. An open field test can be used to observe the behavioral patterns of animals in an empty room for 10 minutes. During this task, movement, sniffing, urinating, grooming, rearing, jumping, vocalization, and inactivity are noted (171, 409, 411). Self-recognition can be evaluated through the mirror test, originally developed for primates (91, 130), by observing the reaction of each animal with a mirror and their reflection. Exploratory behavior of canines can be assessed through a curiosity test in which animals are presented with various novel play objects. During their time with the objects, the amount of time the dogs spend in physical contact with or sitting next to the objects is recorded as well as their frequency of sniffing the objects (411). Social responsiveness of dogs can be gauged through a few different tasks: a human interaction test, silhouette test,

and the model dog test. A human interaction test is performed by the presence of a person in the middle of the room and recording the reaction of the dog to that person by measuring the time the dog is in physical contact with the person, time sitting or standing beside the person, and frequency sniffing the person (167). The silhouette test records the animals frequency of sniffing the front and rear regions of a cardboard silhouette of a dog posted onto a wall (123). The model dog test also records the sniffing frequency of the dogs, but this time in response to the presence of a life size model dog in the center of a room (411).

Behavioral patterns in these functional tasks show age effects as well as differential effects based on the presence of intact/impaired cognition. In 2001, Siwak et al. characterized the behavioral profiles of young (2 to 4 years), aged (9-15 years) cognitively impaired, and aged non-impaired beagles (411). Young dogs tend to show greater responsiveness to changes in environments such as the addition of novel objects and a person. They also showed greater social responsiveness spending the most time next to or sniffing a person, silhouette, and model dog. Aged unimpaired dogs were still responsive to alterations in environment, but to a lesser degree than the young animals. Additionally, aged unimpaired dogs spent the least amount of time reacting to the mirror during the self-recognition task. Unlike either the young or aged unimpaired canines, the aged impaired canines were unresponsive to all stimuli presented to the environment and randomly moved about the room in pacing/aimless behavior. However, the aged impaired dogs did spend the most time interacting with the mirror in the self-recognition test (411).

Measures of canine function can also be assessed in a clinical setting (231-233). Clinical measures have been developed consisting of pet dog owner based evaluation of dog behavioral changes (46, 47, 72, 233, 330, 331) similar to those used in human clinical evaluations, such as the Mini Mental State Exam (MMSE). Although there are different versions of these questionnaires, all appear to be sensitive to the presence of canine cognitive dysfunction (233). The evaluation consists of items such as walking, posture/emotion of expression, elimination behavior, life rhythm, play behavior, exploratory behavior, learned specific behavior, adaptive capabilities, and interactions with other animals or with owners. The items of individual questionnaires can be used to derive scores that distinguish between normally and pathologically aging dogs. Adult and older dogs generally score worse with these types of evaluation tools, and old dogs show individual variability in terms of the amount of cognitive dysfunction reported (47).

Dog Neuropathology and Outcome Measures

Just as canines can exhibit cognitive decline with age similar to aging humans and patients with AD, several human-type neuropathologies have been reported in dogs (81). In particular, the canine model has long been suggested as an excellent model of Aβ pathogenesis (490). Several changes observed in the aged canine brain are associated with cognition and are discussed below.

Brain Volume

Individuals with AD show significant cortical and hippocampal atrophy and ventricular enlargement relative to non-demented age matched controls (6, 340) and losses in brain volume correlate with cognitive decline (105, 114). Similar

events are seen in aged canines. On cross sectional MR imaging, aging canines show increased cortical atrophy and ventricular widening (143, 218, 425). Ventricular widening over time was observed by MRI in a 3-year longitudinal study (426). Canine cortical atrophy occurs earliest in the prefrontal cortex and later with age in the hippocampus (436). As with humans, the more extensive the cortical/hippocampal atrophy seen in aged canines the more pronounced the cognitive deficits (347, 436).

Neuronal Loss

There is some evidence for neuronal loss in AD that could account for brain volume losses seen in brain imaging (404, 478). With normal brain aging, neuronal loss is only seen in the hilus (478, 479), while neuronal loss is much more widespread in individuals with AD (42, 480). Individuals with AD experience neuronal loss in the CA1, CA2, CA4, dentate gyrus and subiculum of the hippocampus (42, 327, 480). In aged beagles, the hilus of the dentate gyrus showed fewer neurons compared to younger dogs (408). Beagles with fewer neurons in the hilus made significantly more errors when performing the size discrimination task (408). Similarly, Pugliese et al. found that a loss of Purkinje cells of the cerebellum in canines correlated with data acquired by questionnaires quantifying behavioral deficits (330). However, neuronal loss may not account for all of the brain atrophy observed by MR as the loss of neuronal dendritic spines occurs with AD (221, 312) but to our knowledge, there are currently no studies published evaluating similar changes with age in dogs.

Neurogenesis

While selective neuronal loss may occur with aging, the brain is also able to produce new neurons. The hippocampus, for example, grows new neurons in the subgranular layer (111), as described in Chapter 1. Neurogenesis has been explored in aged beagles using BrdU and doublecortin staining methods. Siwak-Tapp et al. measured neurogenesis in aged beagles using BrdU and found that animals over the age of 13 showed a significant loss of neurogenesis (407). Fewer newer BrdU positive neurons was associated with poorer cognitive function in learning and memory (407).

B-Amyloid

Beta-amyloid (Aβ) is derived from a longer precursor protein, the amyloid precursor protein (APP). The APP sequence of Canis familiaris has 98% homology with human APP (http://www.ensembl.org/Canis_familiaris/) and an identical amino acid sequence (207, 391). Additionally, dog Aβ peptides may undergo the same posttranslational modifications as in humans (31, 371). These similarities make canines a viable aging model without the need for genetic modification or overexpression of mutant human proteins (391).

The Aβ present in canines is ultrastructurally fibrillar and, though more compact deposits may form, it generally aggregates into diffuse plaques (88, 133, 293, 359, 454, 455, 458). This type of Aβ deposition most resembles early AD pathology (81, 257, 288) (Figure 3.1A). Since most AD therapeutics studied today are likely to have a greater affect if applied earlier in the disease progression, the early AD-like pathology canines produce makes them an attractive model for

preclinical prevention studies (258). As with cognitive decline, AD-like neuropathology has a region specific progression in both humans and canines (52, 133, 168, 391, 445, 488). Though this progression in dogs is similar to that reported in humans, it is not identical. In canines, the accumulation of Aβ begins in the prefrontal cortex (approximately 8 years at age of onset) and continues to develop with increasing age to include other regions such as the temporal and occipital cortex (81, 168, 358). The severity of neuropathology can vary between individual animals but can be linked to the extent of cognitive decline (72, 86, 169, 347). For instance, animals who perform worse in reversal learning tasks have greater Aβ pathology in the prefrontal cortex, while those deficient in size discrimination learning show higher amounts of Aβ in the entorhinal cortex (86, 166, 322).

Aβ peptide can also be measured in the cerebrospinal fluid (CSF) of dogs (370). Measuring CSF Aβ as a ratio of $A\beta_{42}$/ $A\beta_{40}$ is a good predictor of Aβ in the brain in dogs (176). While brain Aβ increases with age, CSF Aβ decreases with age reflecting the hypothesis that Aβ migrates from the periphery and deposits in the brain with age and AD.

Aside from the fibrillar Aβ found in diffuse plaques in AD, a smaller, more soluble form of Aβ, oligomeric Aβ, - is also seen in the aged dog brain. This more toxic form of Aβ affects synaptic function and can be found in plaques (213, 390, 471). Higher levels of oligomers are present in canines and humans with increasing age and cognitive decline. The greater the cognitive deficit, the more prevalent oligomers are in the brain (321, 453). Similar to fibrillar Aβ, oligomeric

Aβ can be measured in CSF, where levels are inversely related to levels in the brain (176).

Cerebrovascular Pathology

Aβ can also aggregate in the cerebral blood vessel walls and cause cerebrovascular pathology (27, 180, 328). This type of deposition is referred to as cerebral amyloid angiopathy (CAA) (Figure 3.1B, C, D). Typically CAA is composed of the shorter Aβ 1-40 peptide (27, 180, 491). Both humans and canines exhibit CAA pathology, with a particular vulnerability in the occipital cortex (28). CAA impairs the blood brain barrier, vascular function, and can cause microhemorrhages and occasionally hemorrhagic strokes (92, 328, 456). Because of these complications, CAA may contribute to cognitive decline in both humans (27, 109, 302, 342) and canines (133, 164, 456, 457). Much like humans, canines develop microhemorrhages with age (457) (Figure 3.1E). These cerebral hemorrhages are present in both animals with and without CAA, but are more common in those with the blood vessel pathology (457). Given the significant overlap of cerebrovascular pathology with AD, the spontaneous accumulation of CAA in dogs also offers as yet, an underappreciated model system to test the effects of cerebrovascular pathology on cognition and AD neuropathology.

Neurofibrillary Tangles

One hallmark AD pathology canines do not produce is NFTs (359, 391). While no research to date has observed NFTs in the canine brain, the increased phosphorylation seen at some sites of tau in AD cases also occurs in cognitively impaired canines (173, 230, 315, 332, 475). This lack of NFT pathology could

possibly be due to significant differences in the tau protein sequence between canines and humans (http://www.ensembl.org/Canis_familiaris/). However, an advantage to dogs not accumulating NFTs is that they serve as a model that is selective for Aβ pathology and ideally suited for testing interventions that target this toxic protein.

Treatment Studies in Aged Dogs

Several studies have investigated therapeutic strategies using the canine model of aging and AD with both cognitive and neuropathological outcome measures (Table 3.1).

Antioxidant-rich Diet in Combination with Behavioral Enrichment

One of the earliest studies to develop a treatment for cognitive dysfunction in aged dogs tested an antioxidant-rich diet in combination with behavioral enrichment in aged dogs. The rationale for this study was observations of increased oxidative damage in the canine brain (346, 347, 413) and studies in mouse models of AD showing environmental enrichment benefited cognition and reduced Aβ pathology (17, 202, 239). The diet included vitamins E and C, fruits and vegetables, lipoic acid and carnitine. The behavioral enrichment included increased exercise, interaction with other dogs, and cognitive enrichment (82, 276, 278, 279). Compared to control animals, those receiving an antioxidant-rich diet committed fewer errors during landmark acquisition and retention tasks (276) as well as oddity discrimination tasks (82). Treatment with an antioxidant diet and behavioral enrichment resulted in improved performance during black and white object discrimination and reversal (278). Pop and colleagues found dogs provided

with both behavioral enrichment and an antioxidant diet have an overall reduction in Aβ pathology across multiple regions of the brain (322) However, when looking at group treatment effects, only the antioxidant-treated animals had a significant reduction in Aβ plaque pathology. Additionally, the combination treatment approach of behavioral enrichment and an antioxidant-rich diet in aged canines was unable to reduce existing brain Aβ, but was able to slow the accumulation of Aβ (322). While plaque load was affected by the combined intervention, soluble and insoluble $A\beta_{1-40}$ was not reduced, and only soluble levels of $A\beta_{1-42}$ were lowered specifically in the prefrontal cortex. A trend towards a significant decrease in oligomers, specifically in the parietal cortex, was observed in canines receiving the combined treatment (322). Interestingly, the combination group also showed reduced oxidative damage (310) with the antioxidant diet group alone showing reduced mitochondrial dysfunction (175). Further, the behavioral enrichment group, independent of the antioxidant diet treatment showed less neuron loss in the hippocampus (408) as well as improved levels of brain derived neurotrophic factor (115).

Supplemental Medium-Chain TAG

Supplemental medium-chain TAG (MCT) increases ketone levels in the brain, and these ketones can in turn be used as an alternative energy source. In 2010, Pan and colleagues measured cognitive effects of this supplement on the landmark discrimination, oddity discrimination, and 2 choice egocentric spatial learning tasks. Results indicated aged dogs given a diet with MCT

supplementation performed better than those receiving a control diet in all tasks (314).

Medical Food Cocktail

In contrast, fewer benefits on cognition were observed in a study using a medical food cocktail (174). Dogs receiving a combination cocktail containing an extract of turmeric containing 95% curcuminoids, an extract of green tea containing 50% epigallocatechingallate, N-acetyl cysteine, R-alpha lipoic acid and an extract of black pepper containing 95% piperine exhibited fewer errors compared to control animals during the landmark task indicating improved spatial attention. However, other areas of cognition were unaffected and brain Aβ remained unchanged (174).

Cholesterol-lowering Drugs

Several studies in the aged dog have tested the effects of drugs already approved for use in humans, with novel applications to brain aging. For example, several cross-sectional or case-control epidemiological studies revealed a striking link between cholesterol-lowering drugs (e.g. statins and others) and a 20-70% reduction in risk of developing AD (108, 154, 204, 344, 345, 492, 493, 503). Modest cognitive benefits have been reported in preliminary AD clinical trials with simvastatin (406) and atorvastatin (416-419). In particular, AD patients with mild to moderate dementia who were treated with 80 mg/day atorvastatin had significantly improved scores on one measure of cognition (ADAS-Cog) at 6 months of treatment, with smaller non-significant benefits at 12 months (419).

Statins may reduce the risk of incident AD through the prevention of Aβ production (160, 405). In rodent models, treatment with inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) or statins reduces Aβ (319). However, rodents respond to statin treatment by massively upregulating HMG-CoA reductase levels (7, 118, 446, 452). To compensate, long-term studies in rodent often employ physiologically excessive doses, making it difficult to translate the results of these studies into human trials.

The dog model is particularly useful to study chronic statin treatment, given similarities with humans in terms of dose requirements, responsiveness, drug handling, and metabolism (7, 132). For example, 12 dogs were treated with 80 mg/day of atorvastatin for 14.5 months (296). Peripheral levels of cholesterol, low density lipoproteins, triglycerides and high density lipoproteins were reduced in treated dogs. Surprisingly, a transient impairment in reversal learning was observed, suggesting prefrontal dysfunction. Spatial memory remained unchanged up to over a year of treatment. The lack of cognitive benefits of treatment was also reflected by a lack of reduction in plasma, CSF, and brain Aβ. Interestingly, BACE1 protein level was decreased in the brains of atorvastatin-treated dogs. This intriguing outcome may suggest that statins might be more useful to prevent the production of Aβ through lowering BACE1 if started in animals in middle age, consistent with human studies indicating that middle-aged individuals using statins are protected from AD.

Immunotherapy

In 2008, a therapeutic approach that directly targeted Aβ reduction was explored in which aged beagles were actively immunized with fibrillar $A\beta_{1-42}$ for 2 years (VAC) based upon previous work in transgenic mouse models of AD (381). Schenk and colleagues were one of the first groups to explore the immunotherapy approach and found that active immunization with fibrillar $A\beta_{1-42}$ reduced Aβ pathology in aged mice while preventing accumulation in young mice (381). Additionally, behavioral outcomes improved in treated mice (198, 286). When testing this immunotherapy approach in a larger animal model, the aging caning, results showed no improvement in cognitive function, but interestingly a long term maintenance of executive function was noted based on error scores from the size reversal learning task (177). However, significant benefits to brain pathology were observed in the VAC dogs who showed significantly decreased Aβ plaque load in prefrontal, entorhinal, and occipital cortical regions, as well as reduced CAA (177). While soluble and insoluble brain $A\beta_{1-40}$ and $A\beta_{1-42}$ significantly decreased in treated canines, there was no significant reduction in soluble oligomers. This study suggests that reducing or eliminating pre-existing Aβ in aging dogs is not sufficient to improve cognition.

Outcomes from the longitudinal dog active vaccination study are similar to reports of Aβ immunotherapy clinical trials in patients with AD where no differences between antibody responders and placebo groups on several cognitive and disability scales was observed. A small number of patients enrolled in the AN1792 study have come to autopsy and show Aβ plaque reduction without any effect on

the extent of neurofibrillary tangles or CAA (120, 259, 301). Further, the frontal cortex showed the largest response to immunotherapy (259), which is similar to our observations in the dog. The most recent autopsy study of 8 patients that were in the AN1792 study further confirm reduced Aβ pathology in response to treatment, 5 years after the last injection (182). However, reduction of brain Aβ did not slow disease progression and 7 of 8 patients had severe end stage dementia prior to death. (134). Interestingly, a composite score of a neuropsychological test battery indicated “less worsening” of decline in antibody responders after 12 months and an improvement in the memory domain (134).

In contrast, Bosch et al. recently (2013) showed benefits of an active fibrillar $A\beta_{40}$ and $A\beta_{x\text{-}40}$ combination vaccine on cognition in aged companion beagles and pet dogs treated for 51 days (46). Over the course of treatment, cognitive evaluations by questionnaire were given at 31 days post treatment and at the end of treatment. Immunized animals showed a significant improvement in cognitive evaluation scores at both 31 and 51 days post treatment compared to pre-immunized scores (46). Differences in the formulation, the outcome measures or the source of animals may explain the positive effects in the Bosch study compared with the previous beagle immunotherapy studies.

Table 3.1. Treatment Studies in Aging Dogs

Treatment	Sample size and Age	Landmark Discrim.	Oddity Discrim.	Size Discrim.	Size Reversal	Black White Discrim.	Black/white Reversal	Spatial Memory	Question -naire	Publication
Antioxidant diet	28 old (8-13 yrs)	Improved	Improved	Improved	Improved	Improved	Improved	Improved	N/A	(82, 278, 279)
Behavioral Enrichment		N/A	N/A	Improved	Improved	Improved	Improved	Improved	N/A	
Antioxidant Diet + Behavioral Enrichment		Improved	Improved	Improved	Improved	Improved	Improved	Improved	N/A	
MCT Dietary supplement	24 old (9-10 yrs)	Not Improv.	Impaired	N/A	N/A	N/A	N/A	Impaired	N/A	(314)
Medical Food Cocktail	18 old (8-9 yrs)	Improved	Not Improv.	Not Improv.	Not Improv.	Not Improv.	Not Improv.	Not Improv.	N/A	(174)
Atorvastatin	10 old (9-13 yrs)	N/A	N/A	Not Improv.	Impaired	Not Improv.	Not Improv.	Not Improv.	N/A	(296)
Fibrillar Aβ1-42 Immunotherapy	20 old (8-13 yrs)	Not Improv.	Not Improv.	Not Improv.	Maintained	Not Improv.	Maintained	Not Improv.	N/A	(177)
Fibrillar Aβ 1-40 & x-40 Immunotherapy	12 old (11-18 yrs)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Improved	(46)

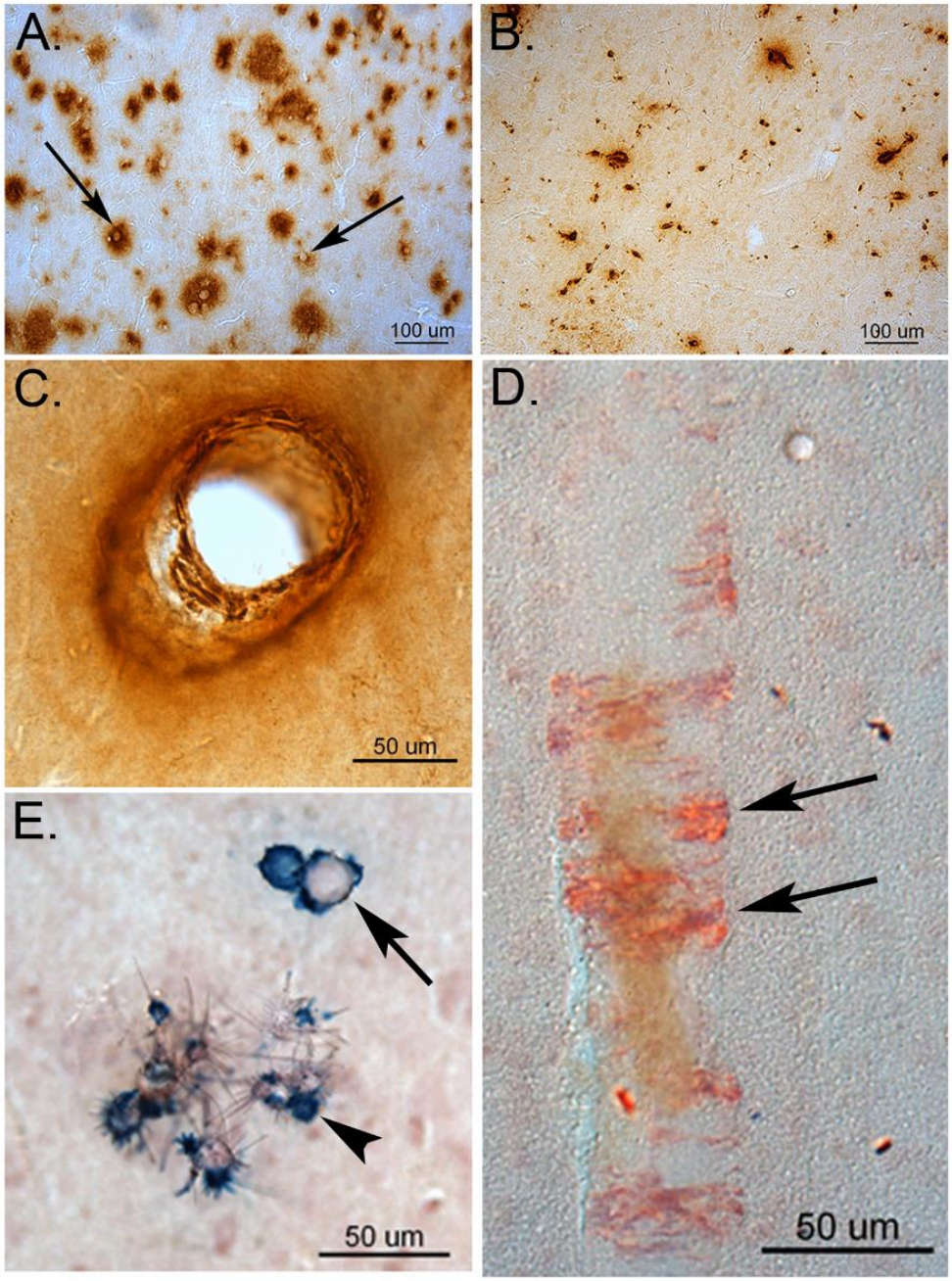
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Figure 3.1. Aβ Pathology in an Aged Beagle.

The prefrontal cortex of a 13.8 year old beagle immunostained with Aβ 1-16 (6E10) showing Aβ deposition. Arrows indicate intact neurons within diffuse plaques (A). CAA clustering in the prefrontal cortex of a 12.7 year old beagle immunostained with Aβ 1-16 (6E10) (B). Cross section of a blood vessel with CAA in the prefrontal cortex of a 14.5 year old beagle immunostained with Aβ 1-16 (6E10) (C). CAA shown in a longitudinal blood vessel of a 13.7 year old beagle (occipital cortex) stained with Congo red (C). Note the striations of CAA along the blood vessel wall indicated by arrows (C). Microhemorrhages are seen by Prussian blue staining in the prefrontal cortex of a 13.8 year old beagle (E). The arrow points to a cross section of a blood vessel with a microhemorrhage and the arrowhead indicates a hemosiderin laden perivascular microglia (E).

CHAPTER FOUR: Significance and Rationale

Introduction

5.1 million people in the United States are affected by Alzheimer's disease (AD), the most common form of dementia, with no current treatment available (495). As described in Chapter 1 (Alzheimer's Disease), the hallmark lesions of AD include neurofibrillary tangles (NFTs), and plaques made up of the β-amyloid protein (Aβ) that result from cleavage of the amyloid precursor protein (APP) (255, 389). While there is no cure for AD, there are various approved drugs for use as symptomatic treatments of AD. Three of these drugs are acetylcholinesterase inhibitors, donepezil, rivastigmine, and galantamine, and a noncompetitive NMDA-receptor antagonist, memantine (124, 131). These drugs only act to manage the symptoms of AD for a limited period of time until the symptoms are too great and the drugs become ineffective. In addition, no current biomarker can determine when AD pathology will occur or how it will progress in an individual, resulting in a variable age of onset of disease. An individual may be clinically normal while their brain may have sufficient pathology for an AD diagnosis (85). For those reasons, researchers have spent the past several years developing numerous therapeutic strategies to specifically reduce neuropathology and improve cognition in AD patients (392)

An exciting therapeutic strategy being evaluated is immunotherapy (immunization or vaccination). Specifically, several immunotherapies being explored target the reduction of Aβ. This approach aims to reduce Aβ accumulation and increase its clearance in AD patients with the goal of reversing

cognitive decline due to the AD neuropathology. Both active and passive Aβ immunotherapies have been explored by researchers.

Aβ Immunotherapy as a Therapeutic for AD

Active vaccination involves the administration of a vaccine containing an antigen to induce the recipient's immune response that produces antibodies against that antigen. The benefit of this type of vaccine is that only a small number of vaccinations are required to promote an immune response to produce antibodies and maintain that response. However, the disadvantage is the variability in immune response between patients. Passive immunotherapy involves the delivery of antibodies against the antigen of interest derived from a source other than the recipient. The benefit to this type of immunization is the ability to administer the desired amount of therapeutic antibodies. The disadvantage is that passive immunization requires repeated injections or infusions in order to maintain the desired antibody concentration in the recipient over time (43, 44, 234).

The mechanism by which immunotherapy works is still unclear, however there are several hypotheses. Only about 0.1% of antibodies in the periphery are able to pass into the brain (43, 44, 203, 234). Although a majority of antibodies do not pass into the brain, the volume of anti-amyloid antibodies in the periphery can cause a "peripheral sink" effect driving the movement of Aβ out of the brain and into the periphery (43, 44, 203, 234). This "peripheral sink" hypothesis has been demonstrated in multiple animal models (94). Of the antibodies that do reach the brain, several possible mechanisms could contribute to the reduction of Aβ levels

and pathology. For one, the anti-Aβ antibodies could bind to soluble forms of Aβ increasing their clearance or causing a shift of equilibria leading to insoluble Aβ breaking down into a more soluble form (234). Antibodies could be binding to Aβ plaques promoting microglial activation to aid in clearing out plaques (34, 43, 45, 301). Additionally, bound Aβ to antibodies may disrupt its ability to aggregate into plaques (234).

Active vaccination with the Aβ peptide was first described by Schenk and colleagues (381). A study of transgenic mice vaccinated with the Aβ peptide demonstrated that not only was Aβ accumulation reduced in older animals with pre-existing Aβ pathology, but it was prevented in younger mice (prior to Aβ pathology) as well. In addition, behavioral outcomes were improved (198, 286). As a result, the study progressed to a clinical trial in which mild to moderate AD patients were immunized with fibrillar $A\beta_{1-42}$ with QS-2 in polysorbate 80 as an adjuvant (181). Promising initial data showed 20% of the AD patients developed antibodies to fibrillar Aβ and had improved brain function (181). However, in a second larger clinical trial in 2005, while some patients developed antibodies and had reduced Aβ plaques, no cognitive improvement was seen, and the trial was ultimately halted when a subset of patients developed aseptic meningoencephalitis (135). Some of the patients who had developed meningoencephalitis possessed an elevated t-cell response in the brain. This T-cell response is thought to be associated with the adjuvant used in the vaccine, QS-2 in polysorbate 80 (135).

Subsequently, a second generation of immunotherapy, passive immunotherapy, has been developed. In 2010, there were 15 passive Aβ

immunotherapies in clinical trials including bapineuzumab and solanezumab (241). These two passive immunotherapies, like active immunotherapy, aim to remove preexisting Aβ pathology and reduce cognitive decline.

Bapineuzumab is a humanized monoclonal antibody to $A\beta_{1-5}$. In mice, this antibody reduced Aβ pathology (94, 438). In Phase III clinical trials, bapineuzumab may have reduced Aβ accumulation and phosphorylated tau levels as seen in cases that reached autopsy. However, the treatment failed to improve cognition in patients with and without the ApoE4 allele, a gene that associated with an increased risk of developing AD (341, 366, 367, 403). It was suggested that the dosage of bapineuzumab used in the Phase III trials was too low to reach the desired primary outcomes (366, 367), however Phase II trials using greater doses resulted in more cases of edema and microhemorrhages in treated patients (368, 420). The doses used in Phase III trials showed no significant adverse effects due to bapineuzumab treatment (366, 367).

Solanezumab, another humanized monoclonal antibody, targets $A\beta_{16-24}$, and reverses memory impairment in the PDAPP mouse model of AD (32, 94, 100, 438). However, solanezumab immunotherapy leads to variable effects on Aβ burden including both reduction (94) or no change (100). Solanezumab subsequently was tested in two Phase III clinical trials, but both trials failed to meet prespecified primary outcomes of improving cognition and function (101, 102). While primary outcome measures were not met, a reduced rate of cognitive decline seen in patients with mild AD was observed in one study (101, 102). Additionally, no significant adverse effects are seen due to the solanezumab treatment (101, 102,

401). Further studies will continue to explore solanezumab in patients with mild AD or those who lack the clinical symptoms of AD but show brain Aβ accumulation through biomarker measures (101, 102).

Although the passive immunotherapy approach was thought to be safer and more promising than active vaccination since no adjuvant is needed, bapineuzumab and solanezumab both failed to meet efficacy expectations and fulfil primary outcomes of reducing or slowing down cognitive decline in AD patients (101, 102, 341, 366, 367, 401, 403). Additionally, there are still concerns with adverse effects such as edema and intracerebral microhemorrhages (60, 320, 368, 420, 483). Thus, there is a critical need to continue to refine and develop novel therapeutics for AD. Two possible reasons for negative clinical trials outcomes are (1) the preclinical animal model was not a predictor of human clinical trial outcomes and (2) the serious adverse events were harmful to the patients due to the immunotherapies themselves.

Aβ Immunotherapy in the Canine Model of AD

In Chapter 3 we describe a unique animal model, the canine, which shows similar neuropathology and cognitive decline to humans with AD. Canines naturally produce APP that has 98% homology with human APP, develop Aβ neuropathology, and show cognitive decline with age, similar to AD patients (253, 277, 475, 501). The similar neuropathology and cognitive decline coupled with their common living conditions with humans make dogs useful for translational studies on neurodegenerative diseases such as AD.

The active vaccine used in this study differs from past clinically tested active vaccines in that it uses Aluminum hydroxide (Alum) as an adjuvant in place of the QS-2 in polysorbate 80. This Alum adjuvant is commonly used in other active vaccines that can be safely administered in humans and causes minimal, if any, T-cell responses in the brain. As discussed in Chapter 3,in a study reported in 2008, aged beagles were actively immunized with fibrillar Aβ1-42 using Alum as an adjuvant (VAC) for 2 years (177). In addition, VAC dogs had significantly decreased Aβ plaque load (177). No serious adverse events such as those reported in the previously described human clinical trials were reported. However, Aβ immunotherapy in aging dogs with preexisting pathology led to no improvement in cognitive function, but interestingly a long term maintenance of executive function that was noted based on error scores from the size reversal learning task (177). These results suggest that reducing Aβ alone was insufficient to improve cognition but that over time, lower levels of brain Aβ can support cognitive maintenance.

Behavioral Enrichment in the Canine Model of AD

Another therapeutic strategy being explored is behavioral enrichment. Behavioral enrichment (ENR) includes environmental enrichment, exercise, social engagement, and cognitive enrichment. Though a combined ENR approach has not been explored in other animal models, individual aspects of ENR have been studied. In rodents, exercise leads to increased brain derived neurotropic factor and improved learning (80). However, the effects of ENR on transgenic mouse models of AD have shown significant variability between studies (17, 202, 239). In

people, individuals who live behaviorally enriched lifestyles, which includes exercising or participation in activities that involve information processing, tend to show less brain atrophy with age and exhibit reduced risk of dementia (70, 71, 485).

As discussed in Chapter 3, behavioral enrichment (ENR) in the canine model of aging has been evaluated. In a study of aged canines, ENR included increased exercise, interaction with other dogs, and cognitive enrichment. In 2004 and 2005, Milgram and colleagues found ENR of aged canines to decrease the rate of cognitive decline and improve cognitive function (278, 279).

A Combination Approach to Improve Cognition and Reduce Pathology in the Canine Model of AD

Immunotherapy decreases Aβ pathology in humans and AD animal models, while ENR improves overall cognition, growth factor levels and supporting neuron number with varying Aβ outcomes.(116, 278, 279, 407). These results suggest the exploration of a combination treatment approach with the potential for additive effects to improve brain function and health with age. This study evaluates the combination approach of active Aβ vaccination and behavioral enrichment by examining their effects on cognition and neuropathology. We hypothesize that additive benefits of improved cognition with reduced AD like pathology will be exhibited in treated aged canines receiving a combination treatment approach of active immunization with fibrillar $A\beta_{1-42}$ using aluminum hydroxide (ALUM) as an adjuvant (VAC) with ENR.

CHAPTER FIVE: Methodology

Canines

The study was started with 40 beagles with 37 animals from the Lovelace Respiratory Research Institute (LRRI) (Albuquerque, NM) and 3 from Harlan (Riglan Farms, Inc., Mount Horeb, Wisconsin) (Table 5.1). All animals were reproductively intact. At the start of baseline, the ages of the dogs ranged from 10.5 to 13.6 years. At this age range, all study animals should have significant prefrontal Aβ pathology (168). Animals were housed singly in kennel buildings with indoor/outdoor runs measuring 91cm x 600cm, unless otherwise noted as part of the ENR treatment. Animals were fed Harlan Teklad Global Diet (25% protein – Teklad Pioneer Lab Diets, Madison, WI) once daily. Water was available for the animals at all times. All animals were given a thorough veterinary examination to assure they were in good health before inclusion in the study. Examinations included physical examination, neurological examination, and analysis of blood biochemistry. All procedures done with the animals were conducted in accordance with LRRI-approved animal protocols and the National Institutes of Health Policy on Humane Care and Use of Laboratory Animals.

Testing Apparatus

As described previously (277), the testing apparatus was a 0.609 X 1.15 X 1.08 m wooden box constructed from press board coated with melamine. The box contained a sliding black Plexiglas tray containing three food wells. Adjustable vertical stainless steel bars provided openings appropriate for individual dog sized and made up the front of the box. The bottom of the barrier opened up so that a

sliding tray would be able to be pushed either toward or out-of-view of the dog. A 60W light was placed above the presentation tray to light the objects. Data acquisition was controlled by a customized program, DOGMA (MetaCog Testing Systems, New Westminster, BC). This program controlled randomization procedures and timing, indicated the placement of the reward, and stored all of data. Each trial began when an experimenter pressed a key and the program would provide an audio cue to present the tray to the dog. The dog's response would be recorded by identifying the location (left, right, or center) on the keyboard or by a mouse. This also indicated the end of the trial and began an intertrial interval. One teaspoon of wet dog food was formed into a ball and served as the food reward. Each dog was given either 10 or 12 trials a day (depending on the task). The dogs were tested 5 day a week.

Baseline Cognitive Testing

All dogs underwent a series of baseline tests and error scores during this testing were used to counterbalance placement into treatment groups such that each group contained both good and poor performers. All animals were given a reward and object approach learning task and then a simple object discrimination and reversal learning task. After discrimination learning, dogs were given a spatial non-matching-to-position memory task. All tasks were performed as described in Chapter 3, Dog Model. After placement into treatment groups, VAC and ENR protocols were started, and cognitive testing was conducted for 19 months while treatment was ongoing.

Treatment Groups

The total errors made by each dog during baseline testing were summed and used to rank animals according to total error scores. These cognitive test scored were used to place animals into one of four treatment groups, making sure that groups were balanced by baseline performance and age. These treatment groups included (1) immunization with Alum only (n = 8) (C/C), (2) ENR with immunization with Alum only (n = 8) (E/C), (3) immunization with fibrillar $A\beta_{1-42}$ and Alum (n = 8) (C/V), or (4) ENR with immunization with fibrillar $A\beta_{1-42}$ and Alum (n = 10) (E/V) (Table 5.2).

Behavioral Enrichment Procedure

Dogs receiving ENR (groups E/C and B/V) were given a 20 min walk outdoors in groups of 3-4 animals three times a week. Play toys were rotated through their kennels on a weekly basis. ENR animals received cognitive enrichment involving additional testing procedures including: landmark discrimination learning, variable distance landmark discrimination, oddity discrimination learning and a second re-test on landmark discrimination after ~16 months of treatment. For these cognitive tasks, only dogs receiving ENR were included however we were able to compare VAC and non-VAC dogs on each measure (Table 5.3).

Immunization Procedure

Fibrillar Aβ (provided by Dr. Charles Glabe, University of California at Irvine) was prepared by adding 500 µl of phosphate buffer solution (PBS), pH 7.5, to 0.5 mg of peptide, and the sample was vortexed and incubated overnight at 37°C in a water bath before formulation with the adjuvant. To prepare Aβ for immunization,

0.5 mg of fibrillar Aβ (500 µl) was added to 50 µl of 2% aluminum hydroxide suspension (Accurate Chemical, Westbury, NY) and 450 µl of PBS and vortexed. Animals in the C/C and E/C groups received Alum only. Animals were immunized subcutaneously in the back of the neck and monitored for adverse reaction. Animals were boosted every month with an additional single injection for 18 months.

Treatment Cognitive Testing

At predetermined time points during the study, animals were given tests to measure spatial attention (landmark discrimination learning), spatial memory (three-choice spatial testing), oddity learning, discrimination learning, and reversal learning (black/white discrimination and size discrimination) (Table 5.3). All tasks were performed as described in Chapter 1, Dog Model.

Serum and CSF Collection

Blood samples were obtained at baseline, taken immediately before the first immunization to obtain a pre-immune sample, monthly for six months, and then every six months thereafter. Blood was collected in 10 cc collection tubes and centrifuged, and the supernatant (serum) used to assay anti-Aβ antibodies. Serum samples were thawed, aliquoted, and frozen again at -80°C for later use. Cerebral spinal fluid (CSF) was collect from each animal at the start of the study before the first immunization (baseline), 12 months after start of treatment, and at the time of euthanasia. CSF was drawn from the lateral ventricles, aliquoted, frozen, and were stored at -80°C for later use.

Euthanasia and Tissue Collection

At the end of the study, animals were anesthetized with sodium pentobarbital (Nembutal). Blood was collected in 10 cc red top tubes and centrifuged, and the supernatant (serum) used to assay anti-Aβ antibodies. When animals were in deep surgical stage, the brains were rapidly removed. Procedures were performed in accordance with LRRI Institutional Animal Care and Use Committee protocols. The left hemisphere was placed in 4% paraformaldehyde at 4°C for48 hours before transfer to PBS, pH 7.4 with 0.02% sodium azide and stored at 4°C. The right hemisphere was coronally sectioned and stored at -80°C.

Serum IgG ELISA

$A\beta_{1-42}$ antibody response was measured over nine time points of the study by enzyme-linked immunosorbent assay (ELISA). 96 well flat bottom plates (Microtiter Immunlon 2 HB, Fisher, cat# 14-245-61) were coated with 5µg/ml fibrillar $A\beta_{1-42}$ in 0.1M phosphate buffered saline (PBS) (pH 7.5) and incubated overnight at 4°C. Blank wells received PBS only. After incubation, plates were washed three times in Tris buffered saline with 0.05% Tween-20 (TBST) (pH 7.5). Plates were then blocked with blocking buffer (TBST with 3% bovine serum albumin (BSA)) and incubated for two hours at 37° on a plate rocker. Plates that were not used immediately after blocking were stored at 4°C until needed. Once blocked, plates were washed three times in TBST. Serum samples being used were serially diluted in 1:10 dilution of blocking buffer (0.3% BSA in 2mM TBST) to 1:100, 1:400. 1:800, and 1:1600. Antibody 6E10 ($A\beta_{1-16}$, Covance, Dedham, MA; cat# SIG-39320) was used for standards and serially diluted to 1:10,000,

1:20,000, 1:40,000, 1:80,000, 1:160,000, and 1:320,000. Wells received 100µl of each sample, 6E10 antibody, or PBS. For a positive control and serum only control wells, 100 µl of 6E10 at 1:10,000 dilution and 1:200 dilution of sample was added, respectively. Plates were incubated for one hour at 37°C on a plate rocker. After three washes in TBST, 100 µl of horseradish peroxidase (HRP) conjugated secondary antibody diluted in blocking buffer was added to each well. Canine sample wells received anti-dog IgG-HRP (Bethyl Laboratories, Montgomery, TX; cat# A40-116P) as secondary, while standard and control wells received anti-mouse IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA; cat# SC-2005). Plates were incubated with secondary antibody for one hour at 37°C on a plate rocker. Following three washes in TBST, 100µl of 1 Step Ultra 3, 3',5,5'-tetramethylbenzidine solution (TMB) (Thermo Scientific, cat# 34028) (room temperature) was added to each well to start the reaction. TMB reaction was held for 3 minutes and then stopped by adding 100µl of 1N sulfuric acid. After 5 minutes when reaction has completely stopped, plates were read at 450 nm using a Multiscan FC plate reader (Thermo Scientific).

Frozen Tissue Extractions

Frozen tissue underwent a basic three step serial extraction before being used to measure Aβ content. Tissue first went through a phosphate buffered saline (PBS) extraction. 200mg of tissue was homogenized in 1 mL of 4° 1x PBS with complete protease inhibitor cocktail (PIC; with EDTA; Amresco, Solon, OH, cat# M222-1mL) (pH 7.4). Homogenization was done using a polytron at maximum speed. Raw homogenate was added into 1.5 mL centrifuge tubes and centrifuged

at 20,000 x g for 30 minutes at 4°C. Supernatant was collected and stored at -80°C while the pellet was saved and used for the following extraction by sodium dodecyl sulfate (SDS). Here, room temperature 2% SDS with PIC (in water) was added to the PBS pellets. The total volume of 2% SDS added to the PBS pellet was determined by calculating 70% the volume of PBS raw homogenate. Samples were sonicated with 10x 0.5 second pulses with an amplitude of 20% (Fisher sonic Dismembrator, Model 500). Samples were centrifuged at the same conditions as stated earlier. Supernatant was collected and stored while the pellet was used for the following Formic Acid extraction. For the last extraction by formic acid, samples were diluted 1:40. First samples were diluted 1:20 in neutralization buffer (Tris Phosphate Buffer) followed by a 1:1 dilution in antigen capture (AC) buffer (0.02M sodium phosphate buffer (pH=7), 0.4M NaCl, 0.02 M EDTA, 0.4% Block Ace (Serotec, Raleigh, NC), 0.2% BSA, 0.05% CHAPS, and 0.05% NaN3). Add 4° equal volume of 70% formic acid as that of SDS added to the PBS pellets to the SDS pellets. Samples were sonicated with 10x 0.5 second pulses with an amplitude of 20% and centrifuged at 20,000 x g for 30 minutes at 4°C. The underlying aqueous layer was collected and stored at -80°C for later use.

CSF and Brain Aβ ELISAs

Beta-amyloid (1-40, 1-42, and total) was measured in CSF and tissue by sandwich ELISA. Capture antibodies Ab42.5 (human sequence $A\beta_{1-16}$) for $A\beta_{1-40}$ capture, and 2.1.3 (end specific for $A\beta_{1-42}$) were diluted to 10 µg/mL in PBS and added (50µL) to each well of a 384 well plate (Immulon, cat# 4HBX). Any unused wells were filled with 50 µL of PBS. Once loaded, plates were sealed with sealing tape

and incubated overnight at 4°C. Plates were then emptied and rinsed with 1X PBS. To block the plates, 100µL of Synblock (Serotec, cat# BUFO34C) was added to each well, sealed and incubated for 2 hours at room temperature on a plate rocker. Once blocked, plates were emptied and dried for two hours at room temperature. Plates not used the same day could be sealed and stored at 4° in a dessicator for later use. Before antigen capture, plates were washed twice in 1X PBS. Samples were then added (100µL) in triplicate and then plates were sealed and incubated overnight at 4°C. Fluid from wells was discarded and washed twice with 1X PBS and 1X PBST. Next, 100µL of biotinylated detection antibodies biotinylated 13.1.1 (end specific for $A\beta_{1-40}$, and biotinylated 4G8 (human sequence $A\beta_{17-24}$, Covance) diluted in detection buffer (DB) (0.02M sodium phosphate buffer (pH=7), 0.002% Thimerosal, 0.002M EDTA, 0.4M NaCl, and 1% BSA) was added to the plates, sealed, and incubated at room temperature for four to six hours. Following an incubation in detection antibody, plates were emptied, washed, and filled with 100µL of NeutrAvidin-horse radish peroxidase (Pierce Biotechnologies, Rockford, IL) diluted in DB. After incubating for two hours at room temperature, solution was discarded and plates were washed several times. For developing, a 1:1 mixture of TMB developing solution (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was made and added (100µL) to each well and allow to incubate for about five minutes. Developing reaction was stopped by adding 100µL of stop buffer (5.6% O-Phosphoric Acid) to the wells and then plates were read with a BioTek multiwell plate reader at λ450nm. Total levels of $A\beta_{1-40}$ and $A\beta_{1-}$

$_{42}$ were determined by calculating the sum of levels in each fraction (PBS, SDS, and FA).

Plaque Load IHC

Tissue was stained for Aβ plaques by using anti-Aβ_{1-42} (Invitrogen, Carlsbad, CA; cat# 44-344; 1:500), 6E10 (Covance, Dedham, MA; cat# SIG-39320; 1:1000), and PyroGlu3 (Novus Biological, Littleton, CO; cat# NBP1-44048; 1:500) (Table 5.4) antibodies. Tissue was pre-treated in 90% formic acid for 4 min and washed in Tris-buffered saline (TBS) (pH 7.5). Next, a 30 min treatment in 3% hydrogen peroxide and 10% methanol was done to block endogenous peroxidase activity. After two washes in TBS, sections were then washed in TBS with 0.1% Triton X-100 (Sigma X-100) and blocked in TBS with 0.1% Triton X-100 and 2% Bovine serum albumin for 30 min to block non-specific sites. Sections were then incubated with primary antibody overnight at room temperature. Following the primary antibody the tissue was incubated in biotinylated secondary antibody for rabbit or mouse (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA; cat# PK-6101 (rabbit), PK-6102 (mouse)). After several washes sections were incubated for one hour in an avidin-biotin complex (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA; cat# PK6101). Detection was visualized with 3'-diaminobenzidine and hydrogen peroxide (DAB, Vector Labs, Burlington, CA; cat# SK4100). Sections were mounted on Superfrost/Plus slides (Labsco Scientific America, cat# LSA4951), left to dry, dehydrated, and coverslipped with 66m glass coverslips (Labsco Scientific America, cat# LS529J) using Depex mounting media

(Electron Microscopy Sciences, cat# 13515). Appropriate controls included sections eliminating primary or secondary antibodies, and all were negative.

Prussian Blue Staining

Prussian blue staining was used to identify microhemorrhages in PFCTX, OCTX, and Hippo tissue for all study cases. With this stain, only iron from hemosiderin containing microglia in the extracellular matrix is colorized (indicative of microhemorrhages). Prior to staining, tissue was mounted onto Labsco Superfrost/Plus slides and air dried overnight. Slides were rehydrated in distilled water for 30 seconds and then incubated in 2% potassium ferrocyanide with 2% 6N concentrated HCl (made in distilled water) for 30 minutes. After incubation, slides were rinsed twice in distilled water for five minutes each and then once in tap water for another five minutes. Once rinsed, slides were incubated in filtered 1% neutral red solution (J.T. Baker, cat# R746-03) (mixed overnight) for two minutes. Tissue was rinsed three times in tap water for 1 minute each, and dehydrated by dipping slides four times in 95% ethanol and four times in 100% ethanol. Slides were cleared twice in xylene for five minutes each and then coverslipped with Depex mounting media.

Baseline Comparison for Plaque Load and Prussian Blue

We were interested in estimating the extent of Aβ pathology and microhemorrhages in our dogs prior to the start of treatment to characterize changes in pathology over time and with immunotherapy/behavioral enrichment. We selected 10 archive cases (Table 5.5) that ranged in age from 10.8 to 13.5 years to compare changes in plaque loads and number of microhemorrhages

before treatment to after 19 months of treatment, particularly with ENR study animals. These archive cases had no previous treatment or cognitive testing done. Tissue for archive cases were collected using the same methods as the study cases.

Immunohistochemistry was used to measure total Aβ, $A\beta_{1-42}$, and PyroGlu3 plaque loads in the PFCTX and PCTX and was conducted in the same manner as with study case tissue. Prussian Blue staining was used to count the number of microhemorrhages in the PFCT and OCTX regions of the brain and was done in the same manner as with study case tissue.

Image Analysis for Plaque Load and Prussian Blue

To quantify the extent of Aβ plaque labeling, images were captured using ImagePro 6.3 with an Olympus Q-Color 5 camera on an Olympus BX51 microscope at 20x objective uniformly, five of the superficial layers and five of the deep layer. Quantification was done by image analysis using ImageJ to yield load values, the percent area occupied by positive labeling (Figure 5.1). A threshold was identified for each antibody/marker and applied to calculate a total average load of each image. The loads of all 10 images were averaged for each subject. These subject averages were then used to find a treatment group average for treatment group comparisons.

Quantification of Prussian blue staining entailed manually counting microhemorrhages in each tissue sample at 20X objective. Prussian blue labeling must have been within 2 cell diameters of a blood vessel to be considered a microhemorrhage (Figure 5.2). Counts were totaled for each subject in each brain

region. For each brain region subject averages were used to calculate a treatment group average to be used for treatment group comparisons.

Table 5.1. Dogs used in the Study

Dog ID	Sex	Date of Birth	Baseline Date	Age at Baseline (mo)	Date of Death	Age at Death (mo)
1625A	M	9/16/1997	4/18/2010	151.1	9/20/2010	156.2
1625C	M	9/16/1997	4/18/2010	151.1	7/23/2010	154.3
1628A	M	1/3/1998	4/18/2010	147.6	6/20/2012	173.7
1628C	M	1/3/1998	4/18/2010	147.6	6/18/2012	173.6
1633D	M	4/27/1998	4/18/2010	143.8	4/26/2012	168.1
1633S	F	4/27/1998	4/18/2010	143.8	6/20/2012	169.9
1635A	M	6/9/1998	4/18/2010	142.4	7/23/2010	145.5
1635S	F	6/9/1998	4/18/2010	142.4	2/26/2012	164.7
1635T	F	9/8/1998	4/18/2010	142.4	4/11/2012	166.2
1636V	F	6/28/1998	4/18/2010	141.8	6/7/2011	155.4
1637B	M	8/21/1998	4/18/2010	140.7	6/18/2012	166.8
1637T	F	7/29/1998	4/18/2010	140.7	6/19/2012	166.8
1638U	F	8/20/1998	4/18/2010	140	9/2/2010	144.5
1639B	M	8/21/1998	4/18/2010	140	6/19/2012	166.1
1639C	M	8/21/1998	4/18/2010	140	6/18/2012	166
1639T	F	8/21/1998	4/18/2010	140	6/18/2012	166
1639W	F	8/20/1998	4/18/2010	140	6/19/2012	166.1
1640B	M	9/8/1998	4/18/2010	139.4	6/19/2012	165.5
1640C	M	9/8/1998	4/18/2010	139.4	6/19/2012	165.5
1640S	F	9/8/1998	4/18/2010	139.4	5/17/2012	164.4
1640U	F	9/8/1998	4/18/2010	139.4	8/26/2011	155.7
1640V	F	9/8/1998	4/18/2010	139.4	4/18/2011	151.4
1640W	F	9/12/1998	4/18/2010	139.4	5/11/2012	164.2
1641A	M	9/12/1998	4/18/2010	139.3	6/20/2012	165.4
1641B	M	9/12/1998	4/18/2010	139.3	6/18/2012	165.3
1641T	F	9/12/1998	4/18/2010	139.3	8/22/2010	143.4
1641U	F	9/8/1998	4/18/2010	139.3	4/15/2011	151.2
1641V	F	9/12/1998	4/18/2010	139.3	6/18/2012	165.3
1642A	M	7/24/1999	4/18/2010	128.9	6/20/2012	155
1642B	M	7/24/1999	4/18/2010	128.9	6/20/2012	155
1642S	F	7/24/1999	4/18/2010	128.9	12/29/2011	149.3
1643C	M	7/24/1999	4/18/2010	128.9	7/2/2010	131.4
1643T	F	7/26/1999	4/18/2010	128.8	6/20/2012	154.9
1645A	M	8/10/1999	4/18/2010	128.4	6/20/2012	154.5
1645T	F	8/10/1999	4/18/2010	128.4	6/19/2012	154.4
1646T	F	10/16/1999	4/18/2010	126.1	6/18/2012	152.2
1646U	F	10/16/1999	4/18/2010	126.1	6/18/2012	152.2
D009	M	12/8/1996	4/18/2010	160.4	7/9/2011	175.1
D012	F	11/12/1996	4/18/2010	161.3	6/19/2012	187.3
D045	F	9/15/1996	4/18/2010	163.2	6/19/2012	189.2

Table 5.2. Treatment Group Assignments

Dog ID	Treatment Group	Time on Treatment (mo)
1625A	--	0
1625C	--	0
1628A	C/C	19.6
1628C	C/C	19.5
1633D	E/V	17.8
1633S	E/C	19.6
1635A	--	0
1635S	E/V	15.8
1635T	C/V	17.3
1636V	E/V	7.1
1637B	C/V	19.5
1637T	E/C	19.6
1638U	--	0
1639B	E/V	19.6
1639C	C/V	19.5
1639T	C/C	19.5
1639W	C/C	19.6
1640B	E/V	19.6
1640C	C/C	19.6
1640S	C/V	18.5
1640U	E/C	9.8
1640V	E/C	5.5
1640W	C/C	18.3
1641A	E/C	19.6
1641B	E/C	19.5
1641T	--	0
1641U	E/C	5.4
1641V	C/C	19.5
1642A	C/V	19.6
1642B	E/V	19.6
1642S	C/V	13.9
1643C	--	0
1643T	C/V	19.6
1645A	E/V	19.6
1645T	E/V	19.6
1646T	E/V	19.5
1646U	C/C	19.5
D009	E/C	8.2
D012	C/V	19.6
D045	E/V	19.6

Table 5.3. Cognitive Testing Timeline

Study Event	Time Between Boosts	Study Month
Baseline		
Serum and Plasma Time B1		-6.0
Blood Biochemistry - Time B1		-6.0
Physical and Neurological Examinations Time B1		-6.0
Pretraining - Phase 1		-6.0
Reward Approach Learning		-6.2
Pretraining - Phase 3		-6.2
Object Approach learning		-6.0
Baseline CSF sample		
Baseline - Object Discrimination Learning		-4.8
Baseline - Object Reversal Learning		-4.6
Baseline - 2 choice spatial learning		-4.0
Baseline - 3 choice spatial learning		-3.7
Baseline - 3 choice spatial memory		-1.1
Blood Biochemistry - Time B2		-0.7
Treatment		
Serum and Plasma Imm 0		0.7
Immunization-1	0	0.7
Begin behavioral enrichment protoocol		0.7
Serum and Plasma Imm 2w		1.2
Immunization-2	14	1.2
Serum and Plasma Imm 1m		1.6
Immunization 3	14	1.7
Landmark Testing - Land0-Land4		1.7
Serum and Plasma Imm 2m		2.6
Immunization 4	28	2.6
Serum and Plasma Imm 3m		3.5
Immunization 5	28	3.5
Oddity Discrimination Learning		4.2
Serum and Plasma Imm 4m		4.5
Immunization 6	28	4.5
Serum and Plasma Imm 5m		5.4
Immunization 7	28	5.4
Physical and Neurological Examinations		6.1
Serum and Plasma Imm 6m		6.3
Immunization 8	28	6.3

Table 5.3, continued

Blood Biochemistry 6 m		6.3
Immunization 9	28	7.3
Immunization 10	28	8.2
Time 1 - Size Discrimination Learning		8.4
Time 1 - Size Reversal Learning		8.9
Immunization 11	28	9.1
Time 1 - Spatial Acquisition		10.0
Immunization 12	28	10.1
Immunization 13	28	11.0
Immunization 14	28	11.9
Blood Biochemistry 12m		11.9
Physical and Neurological Examinations		11.9
Serum and Plasma Imm 12m		11.9
CSF sample		12.1
Time 1 - Spatial Memory		12.4
Immunization 15	28	12.9
Immunization 16	28	13.8
Immunization 17	28	14.7
Immunization 18	28	15.7
Time 2 - Landmark Variable Distance Retest		16.1
Immunization 19	28	16.6
Time 2 - Black/White Discrimination Learning		17.0
Immunization 20	28	17.5
Blood Biochemistry 18m		17.5
Serum and Plasma Imm 18m		17.5
Time 2 - Black/White Reversal Learning		17.7
Physical and Neurological Examinations		18.2
Immunization 21	28	18.5
Time 2 - Spatial Memory		19.1
Immunization 22	28	19.4
BrdU injections once daily for 5 days		20.1
Immunization 23	28	20.3
Serum and Plasma Imm 23m		20.5
Blood Biochemistry 24m		20.5
CSF sample immediately prior to euthanasia		20.5
Euthanasia		20.5

Table 5.4. Antibodies used for Immunohistochemistry

Antibody Name	Target	Dilution	Secondary	Manufacturer
Aβ 1-42	Aβ (42)	1:500	Rabbit	Invitrogen
6E10	Aβ (1-16)	1:1000	Mouse	Covance
PyroGlu3	Aβ (3 pE)	1:500	Mouse	Novus Biological

Table 5.5. Pre-Treatment Dogs used in the study

Dog ID	Sex	Date of Birth	Date of Death	Age at Death (mo)
1425S	F	11/26/1983	6/3/1997	162.4
1470S	F	1/22/1986	6/11/1997	136.7
1485V	F	12/1/1986	4/1/1999	148.1
1509S	F	3/2/1988	12/18/1998	129.6
1580S	F	5/15/1991	12/11/2002	139.0
1634U	F	6/7/1998	1/29/2010	139.9
1634V	F	6/7/1998	4/7/2009	130.1
1639U	F	8/21/1998	4/6/2010	139.6
1639V	F	8/21/1998	2/17/2010	138.0
D304	F	7/30/1991	12/23/2002	136.9

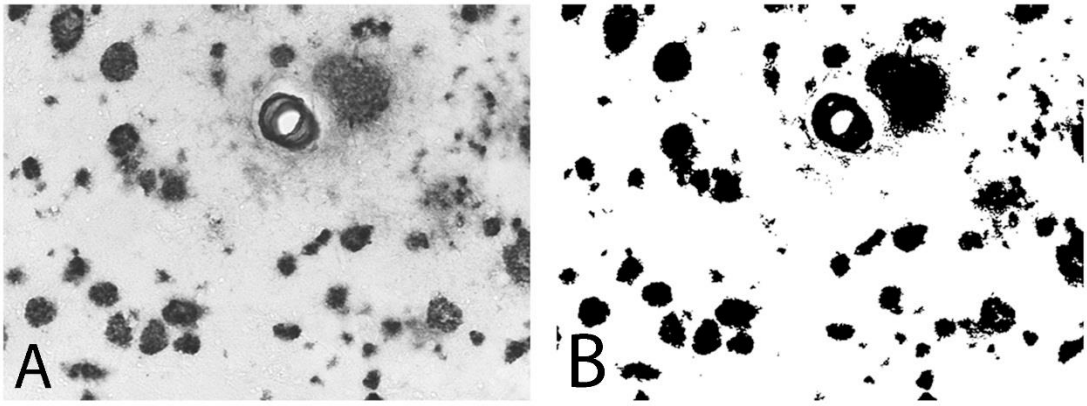


Figure 5.1. Quantification of Aβ Plaque loads.

Black and white image taken of 6E10 labeled tissue (A) and the same capture that has been thresholded so that only positive labeling is seen in black (B). This threshold is used to obtain a measure of total area occupied by positive labeling for 6E10.

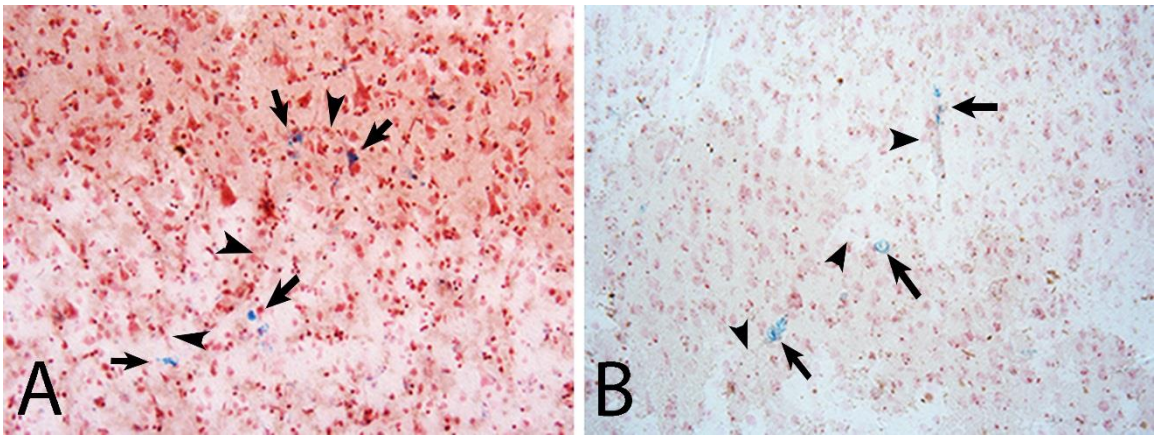


Figure 5.2. MHs in the FCTX of AD and aged canine.

Aged canines show MHs just as seen in aged humans. Arrows point to MHs in FCTX tissue stained with Prussian Blue of a human with AD (A) and of an aged canine PFCTX (B). Arrowheads point to blood vessel associated with each neighboring MH. Bleed labeling must have been within 2 cell diameters of a blood vessel to be considered a microhemorrhage.

CHAPTER SIX: Results

Cognitive Outcomes

Landmark Discrimination Learning

All dogs receiving ENR were given the landmark discrimination task. Thus, a comparison between dogs receiving the vaccine could be compared to those not receiving the vaccine, with all dogs being behaviorally enriched. Testing began 1 month after the first vaccination and 2 weeks after the first boost.

In the 18 (n=10 E/V, n=8 C/V) dogs that completed landmark 0, a significantly higher error score was observed in the dogs provided with the vaccine relative to the dogs receiving behavioral enrichment alone ($t(16)=2.7$ $p=0.016$)(Figure 6.1). However, based on our previous research, rather than the vaccinated dogs doing more poorly, this effect is due to significantly lower error scores in all dogs that was pronounced in the E/C group when compared to previous studies of similarly aged dogs (276). In landmark 1 discrimination learning, no significant group effects were observed ($t(16)=1.45$ $p=0.17$). Interestingly, in landmark 1 learning, two of the vaccinated dogs made over 100 errors to learn the task whereas the average error scores for the E/C condition was 9.88+/-4.74 and for the remaining dogs on E/V condition averaging 5.0 +/- 1.46 errors. Scores of over 100 errors are typical of untreated aged animals as previously reported (275, 276). A differential vaccine effect was also not observed for landmark 2 (2 cm distance) ($t(14)<1$ $p=0.77$) or landmark 4 (4 cm distance) ($t(14)<1$ $p=0.48$). A similar outcome was noted if a repeated measures general linear models approach is used to detect differential effects of the vaccine across all landmark tasks although the effect of distance on

error scores overall was significant ($F(3,42)=2.8$ $p=0.05$) suggesting the further distances resulted in higher error scores reflecting increasing difficulty of the task (Figure 6.1).

The variable distance landmark test was conducted for a period of 20 days with either 1, 2 or 4 cm distances appearing each day, four times/day for a total of 12 trials/day. The total number of errors made during the 20 days of testing was not different between the E/V nor E/C groups ($t(13)<1$ $p=0.53$). Next, the accuracy was calculated for individual distances for each dog as further distances are more difficult for animals to detect proximity to the correct response. All dogs performed between 62-72% correct with little variability across distances. A repeated measures general linear models analysis confirmed a lack of main effect of distance ($F(2,24)=2.2$ $p=0.133$) and of treatment group ($F(1,12)<1$ $p=0.53$). The interaction, was also not significant ($F1,12)< 1$ $p=0.78$) (Figure 6.2). This is contrast to previous reports of the variable distance landmark task varying as a function of distance (177).

After 15.2 months of treatment, 4 E/C and 8 E/V dogs were given a second assessment of the landmark variable distance task for 240 trials. There was a significant effect of distance of the landmark and error scores such that longer distances led to greater numbers of errors ($F(2,20)=12.90$ $p=<.0005$). There were no differences between the group receiving the vaccine (E/V) and the control group (E/C) or an interaction between distance and treatment condition (data not shown).

Oddity Discrimination Learning

A comparison was made between the 8 vaccinated (E/V) vs 10 non-vaccinated (E/C) dogs on the oddity task after 4.3 months of treatment with all dogs having behavioral enrichment. When individual oddity problems were analyzed using t-tests to compare the two groups we did not see any significant differences. For oddity 1, 7 E/C dogs and 9 E/V dogs reached criterion levels of responding ($t(14)<1$ p=n.s.). For oddity 2, 7 E/C dogs and 9 E/V dogs learned the task ($t(14)=1.40$ $p=0.18$). For oddity 3, 6 E/C dogs and 9 E/V dogs learned but no significant differences were observed ($t(13)<1$ p=n.s.). Last, for oddity 4, 6 E/C dogs and 9 E/V dogs learned but error scores were similar ($t(13)=1.37$ $p=0.20$). In a second repeated measures analysis (4 oddity tasks) using only dogs that were able to reach criterion levels of responding on all tasks, the two groups (E/C vs E/V) were compared. There was a significant main effect of the oddity task ($3,39)=4.75$ $p=0.006$) suggesting increasing difficulty, but no treatment group by oddity task interaction ($F(3,39)=0.39$ $p=0.76$). Overall there were no treatment group differences ($F(1,13)=3.41$ $p=0.088$)(data not shown).

Discrimination and Reversal Learning

After 7.6 months of treatment, all dogs were given a size discrimination and reversal learning problem. This task allowed us to compare all 4 treatment groups and test the hypothesis that the combined treatment led to greater cognitive benefits than either treatment alone and as compared to controls. All dogs still on study learned the size discrimination problem. Using a univariate analysis of variance (behavioral enrichment, vaccine), there was no significant effect of the

vaccine alone ($F(1,30)<1$ p=n.s.), the behavioral enrichment alone ($F(1,30)=0.36$ $p=0.55$) nor the interaction of the two treatments ($F(1,30)<1$ p=n.s.). For size reversal learning, 28 dogs in total learned the task. No significant improvements in the vaccine group alone ($F(1,28)=0.33$ $p=0.57$), the behavioral enrichment group alone ($F(1,28)=0.98$ $p=0.33$) nor in the combination group ($F(1,30)=0.029$ $p=0.87$) was observed (data not shown).

We next compared baseline levels of discrimination and reversal learning to size discrimination learning to determine if there was a maintenance of function in treated animals. In this repeated measures analysis, we observed a significant effect of time (or of task difficulty) between baseline discrimination learning and size discrimination learning ($F(1,26)=10.74$ $p=0.003$) with error scores being higher on the size task. No group differences nor a group by time interaction was observed suggesting no treatment effects on the maintenance of discrimination learning (data not shown). Similarly for reversal learning, there was a significant effect of time (or of task difficulty) overall ($F(1,24)=17.1$ $p<.0005$) with size reversal leading to higher error scores but no main effects of each treatment nor a time by group interaction (data not shown).

After 16.1 months of treatment, all dogs on the study were given the final discrimination learning and reversal tasks, black/white discrimination and black/white reversal learning. On the black/white discrimination learning task, 7 C/C, 4 E/C, 3 C/V and 7 E/V animals were able to reach criterion. There was no main effect of the vaccine ($F(1,21) = 1.49$ $p=0.24$), the behavioral enrichment ($F(1,21)=0.07$ $p=0.80$) nor a significant combination treatment effect ($F(1,21)=0.12$

p=0.73) (data not shown). Similarly, of the 6 C/C, 4 E/C, 3 C/V and 6 E/V dogs able to reach criterion on the black/white reversal learning task, no main effect of the vaccine ($F(1,19)=0.32$ $p=0.58$), of the behavioral enrichment ($F(1,19)=0.79$ $p=0.40$) nor of the combination treatment ($F(1,19)=3.26$ $p=0.09$) was observed (data not shown).

To detect any treatment effects over time (i.e. a possible maintenance of function) we compared baseline object discrimination to size discrimination and to black/white discrimination only in animals able to reach criterion for all 3 tasks. Overall there was a significant increase in error scores over time in all groups ($F(2,32)=3.4$ $p=0.05$) suggesting both an aging effect and an increase in task difficulty but no effect of the vaccine ($F(2,32)=0.11$ $p=0.90$) or of the behavioral enrichment alone ($F(2, 32) =0.56$ $p=0.58$). Interestingly, there was a significant effect of the combination treatment group ($F(2, 32)=4.0$ $p=0.03$) and as can be seen in Figure 6.3A, the combination group had the lowest average error scores. Reversal learning also showed a significant main effect of time in dogs that could learn the problem ($F(2,30)=10.7$ $p<.0005$) but there were no main effects of the vaccine alone ($F(2,30)=2.8$ $p=0.08$) or the behavioral enrichment alone ($F(2,30)=0.09$ $p=0.91$) nor of the combination treatment ($F(2,30)=1.12$ $p=0.34$) on change in error scores over time (Figure 6.3B). Interestingly, as can be seen in Figure 6.3B, the E/V group had the highest average error scores on reversal learning, whereas the two single treatment groups showed lower error scores.

Spatial Acquisition and Memory

Once dogs had been treated for 9.4 months, they were retested on the 3 choice spatial memory problems. At this time, dogs were given 50 days maximum to sequentially re-acquire the task at progressively increasing delays beginning with a 5 second delay. A multivariate analysis of variance was used to determine whether group differences were present with the initial re-learning at a 5 second delay. In this analysis, 7 C/C dogs, 4 E/C dogs, 7 C/V dogs and 7 E/V dogs reached criterion levels of responding. No significant main effects of the vaccine ($F(1,25)=0.577$ $p=0.46$), of behavioral enrichment ($F(1,25)=1.83$ $p=0.19$) nor of the combined treatment ($F(12,25)=0.039$ $p=0.85$) was observed (data not shown).

At the end of spatial acquisition testing, dogs were given a variable spatial memory task where the delays of 20, 70 or 110 seconds could occur on a single day. On this phase of the test, 8 C/C, 4 E/C, 8 C/V and 8 E/V dogs completed all 240 trials. The main effect of the delay interval on accuracy was marginally significant ($F(2,48)=2.91$ $p=0.06$) but no delay by vaccine group ($F(2,48)=0.71$ $p=0.50$), no delay by behavioral enrichment group ($F(2,48)=1.11$ $p=0.34$) nor a delay by vaccine group by behavioral group interaction ($F(2,48)=1.26$ $p=0.29$) was found. Thus, neither treatment alone or in combination resulted in improved spatial memory (Figure 6.4A and B).

A third and final test of spatial memory alone (without the acquisition phase) was initiated after 18.1 months of treatment. For this test, 8 C/C, 4 E/C, 5 C/V and 6 E/V dogs remained on study. The overall effect of delay on accuracy was blunted in this last test ($F(2,38)=2.85$ $p=.07$) most likely due to the smaller sample size.

There was no delay by vaccine group effect ($F(2,38)=0.72$ $p=0.49$) or delay by behavioral enrichment group effect ($F(2,38)=0.31$ $p=0.74$). Interestingly, there was an interaction between the delay interval accuracy in the combination treatment group ($F(2,38)=3.62$ $p=0.04$). Figure 6.4C shows that this was primarily due to the poorer performance of the E/C and C/V groups at the short 20 second delay whereas the C/C and E/V groups showed a progressive decline in accuracy from 20 to 110 seconds delays.

To detect any changes in spatial memory as a function of treatment over the 18 months of the study, a repeated measures analysis was used for each delay interval separately (20,70,110s). At the 20 second delay ($F(2,36)=16.12$ $p<.0005$), the 70 second delay ($F(2,36)=5.51$ $p=0.008$) and the 110 second delay ($F(2,36)=17.32$ $p<.0005$) there was an overall decrease in accuracy over time suggesting an aging effect. There was no apparent maintenance of spatial memory over time as a function of treatment (Figure 6.4 D, E, F).

IgG Anti-fibrillar Aβ Antibody Response in Serum

To determine if the anti-fibrillar Aβ given to VAC treated animals induced an immune response, we measured fibrillar Aβ1-42 antibody titers (Figure 6.5). Past active vaccine studies showed an increase in Aβ antibody titers in treated animals (177). Therefore, we hypothesized the C/V and E/V groups would have an increase in Aβ antibody titers over time. At baseline, there were low and variable levels of anti-Aβ titers in serum across dogs. Thus, to reduce individual variability of measurements due to baseline titers and to allow comparisons across groups over time, the difference between each time point measure of anti-Aβ titers from

baseline was calculated. Similar to previous studies, fibrillar Aβ antibody titers significantly increased over time in VAC animals ($F(1,19)=12.5$ $p=0.002$) (Figure 6.5A). No main effect of behavioral enrichment was seen on antibody titers ($F(1,19)=1.16$ $p=0.30$). Further, there was no interaction between the two treatments ($F(1,19)=1.32$ $p=0.26$). While antibody titers in the combination treatment group did increase over time, as can be seen in Figure 6.5B, the maximum response did not reach that of the vaccine only group (Figure 6.5A). Post hoc tests show that at time 23 months the combination group had lower titers ($p=0.018$, LSD). Additionally, the antibody titer response in the combination treatment group was delayed about 2 months (Figure 6.5A).

CSF Aβ

CSF Aβ levels are lower in AD compared with non-demented elderly controls as Aβ from the periphery deposits in the brain decreasing CSF Aβ and increasing brain Aβ and cognitive impairment (for review, see (14)). In animal studies, mice receiving passive immunization with antibodies against soluble Aβ experience an increase in CSF Aβ (100). Additionally, dogs receiving active vaccination experienced a non-significant increase in CSF $Aβ_{1-40}$ and decrease in $Aβ_{1-42}$ (165). We hypothesized that CSF Aβ levels would be higher in the C/V and E/V treatment groups compared to the C/C and E/C groups as a result of the VAC. CSF $Aβ_{1-40}$ and $Aβ_{1-42}$ were measured by sandwich ELISA. A significant increase in CSF $Aβ_{1-40}$ levels was seen in response to ENR ($F(1,22)=5.76$ $p=0.03$) (Figure 6.6A), while there was no effect of the vaccine ($F(1,22)=0.41$ $p=0.53$) (Figure 6.6B) or an interaction between the two treatments ($F(1,22)=1.16$ $p=0.29$) (Figure 6.7).

Though not significant, the E/V treatment group trended towards having the largest impact in increasing CSF $A\beta_{1-40}$ compared to all other treatment groups (Figure 6.7). No treatment effect was seen for ENR ($F(1,22)=0.38$ $p=0.54$) alone for $A\beta_{1-42}$ (Figure 6.8A). VAC had no treatment effect in lowering or raising CSF $A\beta_{1-42}$ over time ($F(1,22)=0.40$ $p=0.40$), but VAC animals did have significantly higher levels than non-VAC animals at 12 months ($F(1,22)=8.089$ $p=0.008$) (Figure 6.8B). Last, there was also no interaction treatment effect on CSF $A\beta_{1-42}$ for the two treatments over time ($F(1,22)=0.06$ $p=0.81$) (Figure 6.9).

Aβ Plaque Load

Based on the previous canine vaccine study indicating that Aβ plaque loads were decreased due to VAC, we hypothesized that animals from the C/V and E/V treatment groups would have decreased Aβ plaque loads compared to non VAC animals (177). We also hypothesized that the E/V group would have a greater reduction in plaque load than the C/V group as an effect of the combination treatment approach. To test this hypothesis we measured the extent of plaques containing $A\beta_{1-42}$, total Aβ, and pyroglutamate Aβ in the PFCTX, OCTX, PCTX, and ECTX by immunohistochemistry.

$A\beta_{1-42}$

$A\beta_{1-42}$ plaque load was measured in the PFCTX, OCTX, PCTX, and ECTX in all animals and compared between treatments. Plaque load was significantly decreased as a result of VAC (both C/V and E/V groups) in the PFCTX ($F(1, 34)= 33.04$ $p= <0.001$), OCTX ($F(1, 34)= 14.92$ $p= 0.001$), and PCTX ($F(1, 33)= 14.06$ $p= 0.001$), while no reduction was seen in the entorhinal cortex (ECTX) ($F(1, 34)=$

1.89 p= 0.39) (Figure 6.10). Compared to the C/C group, the C/V group showed a significantly lower $A\beta_{1-42}$ plaque load in the PFCTX (Bonferroni, p=<0.001), OCTX (Bonferroni, p=0.04), and PCTX (Bonferroni, p=0.02) (Figure 6.10). However there was no significant difference between the C/C and C/V groups when comparing plaque loads in the ECTX (Bonferroni, p=1.00) (Figure 6.10). No significant reduction in $A\beta_{1-42}$ plaque load was seen as an effect of ENR in the OCTX (F(1,34)=0.19 p=0.67), PCTX (F(1,33)=2.21 p=0.15), or ECTX (F(1,34)=0.754 p=0.39) (Figure 6.10). However, a reduction in the PFCTX due to ENR trended towards significance (F(1,34)=3.87 p=0.06) (Figure 6.10). Similarly, when looking at individual treatment groups, the E/C group had a significantly lower plaque load compared to the C/C group in the PFCTX (Bonferroni, p=0.02), while no difference was seen in the OCTX (Bonferroni, p=1.00), PCTX (Bonferroni, p=0.55), or ECTX (Bonferroni, p=1.00) (Figure 6.10). No additive effects were seen between the VAC and ENR in reducing $A\beta_{1-42}$ plaque load in the OCTX (F(1,34)=0.14 p=0.71), PCTX (F(1,33)=1.18 p=0.29), or ECTX (F(1,34)=0.18 p=0.68) (Figure 6.10). Statistically by two way ANOVA, there was a significant additive effect of VAC and ENR in decreasing $A\beta_{1-42}$ plaque load in the PFCTX (F(1,34)=6.54 p=0.02), however by post hoc the E/V treatment group did not have a lower plaque load than the C/V group, (p=1.000). In the presence of active vaccine, the ENR group provides no additional benefit. But, where there was no vaccine, the ENR treatment makes a difference such that $A\beta_{1-42}$ plaque load is lower. In other words, there is no additional effect of behavioral enrichment on the

vaccine group, but in the absence of vaccine, ENR resulted in lower $A\beta_{1-42}$ plaques compared to the C/C group.

Total Aβ

Total Aβ (6E10) plaque load was measured in the PFCTS, OCTX, PCTX, and ECTX in all animals. A significant decrease in total Aβ plaques was seen due to VAC (both C/V and E/V groups) in all brain regions (PFCTX, $F(1,34)= 52.91$ $p= <0.001$; OCTX, $F(1,34)= 13.65$ $p= 0.001$; PCTX, $F(1,33)= 10.70$ $p= 0.003$; ECTX, ($F(1, 34)= 6.60$ $p= 0.02$) (Figure 6.11). When comparing the C/V group to C/C animals, C/V dogs had lower plaque loads in the PFCTX, OCTX, and PCTX (Bonferroni: $p=<0.005$, $p=0.05$, $p=0.01$ respectively) (Figure 6.11). ENR did not have an effect on the reduction of total Aβ plaque load in any of the examined regions (PFCTX, $F(1,34)=2.95$ $p=0.10$; OCTX, $F(1,34)=0.34$ $p=0.57$; PCTX, $F(1,33)=2.82$ $p=0.10$; ECTX, $F(1,34)=.23$ $p=0.64$) (Figure 6.11). Though there was no overall effect due to ENR, the E/V group did have lower total Aβ plaque loads compared to the C/C group in the PFCTX, OCTX, and PCTX (Bonferroni: $p=<0.001$; $p=0.05$; $p=0.01$ respectively) (Figure 6.11). No additive effects were seen between VAC and ENR in decreasing total Aβ plaque loads from any of the PFCTX ($F(1,34)=1.78$ $p=0.19$), OCTX ($F(1,34)=0.13$ $p=0.73$), PCTX ($F(1,33)=3.26$ $p=0.08$), or ECTX ($F(1,34)=0.03$ $p=0.86$) (Figure 6.11). The combination group, E/V, did however have decreased total Aβ plaque loads compared to controls, C/C (Figure 6.11). Decreased total Aβ plaque loads was seen in the PFCTX ($p=<0.001$), OCTX ($p=0.03$), and PCTX ($p=0.01$) (Bonferroni) (Figure 6.11).

Pyroglutamate Modified Aβ

Previous immunotherapy studies did not look into the effects of the treatment on post-translationally modified Aβ. Since post translationally modified Aβ, more specifically AβpE3, is considered to be a more toxic and chronobiologically older form of Aβ, we tested our vaccine on its ability to reduce this form of Aβ. Here we measured AβpE3 plaques loads in the PFCTX, OCTX, PCTX, and ECTX in all study animals. We hypothesized that VAC would significantly reduce AβpE3 plaque loads in all regions examined and more so in the E/V treatment group dogs. We found that VAC had a significant effect in decreasing AβpE3 plaques loads (Figure 6.12). AβpE3 plaque loads were decreased in PFCTX ($F(1, 30)= 10.00$ $p= 0.004$) and PCTX ($F(1, 29)= 6.50$ $p= 0.02$), while no change was seen in the OCTX ($F(1, 30)= 2.32$ $p= 0.14$) or ECTX ($F(1, 30)= 3.13$ $p= 0.09$) (Figure 6.12). The C/V group trended towards a significantly lower AβpE3 plaque load in the PCTX compared to that of the C/C group (Bonferroni, $p=0.06$) (Figure 6.12). No reduction in plaque load was seen in any regions examined due to ENR (PFCXT, $F(1,30)=0.09$ $p=0.77$; OCTX, $F(1,30)=0.84$ $p=0.37$; PCTX, $F(1,29)=1.30$ $p=0.26$; ECTX, $F(1,30)=1.59$ $p=0.22$) and or the combination therapy approach (PFCXT, $F(1,30)=0.05$ $p=0.83$; OCTX, $F(1,30)=1.32$ $p=0.26$; PCTX, $F(1,29)=2.33$ $p=0.14$; ECTX, $F(1,30)=2.65$ $p=0.11$) (Figure 6.12).

Comparison of Plaque Load Over Time Due to Treatments

As mentioned, PFCTX had lower $Aβ_{1-42}$ plaque loads as an effect of ENR that trended towards significance. In addition, though no ENR effect was

statistically seen in lowering total Aβ plaque loads in the PFCTX and PCTX, the E/C treatment group did have significantly lower loads than the C/C group. What was unclear was if these lower plaque loads were due to a clearance of Aβ or maintenance of pre-existing pathology by the ENR. In order to investigate this further, we used PFCTX and PCTX tissue of archive cases that were age matched to study cases at their baseline age and stained them for $A\beta_{1-42}$ and total Aβ. The results would provide measurements that would represent the $A\beta_{1-42}$ and total Aβ plaque loads of the study cases at baseline before treatment began.

In the PFCTX, a significant group effect was seen when comparing $A\beta_{1-42}$ ($F(4, 44)= 9.447$ $p= <0.001$) (Figure 6.13), total Aβ ($F(4, 44)= 10.923$ $p= <0.001$) (Figure 6.14), and AβpE3 ($F(4, 44)= 9.752$ $p= 0.009$) (Figure 6.15) plaque loads between the pre-treatment group to the study treatment groups. The pre-treatment group had significantly lower $A\beta_{1-42}$ (Bonferroni, p=0.050) (Figure 6.13) and total Aβ (Bonferroni, p=0.014) (Figure 6.14) plaque loads than the C/C group indicating that an increase in these plaque loads was seen with age. The plaque loads of the pre-treatment group did not statistically differ from the E/C group, suggesting that a maintenance effect due to the ENR was likely in keeping Aβ $A\beta_{1-42}$ (Bonferroni, p=1.000) (Figure 6.13) and total Aβ (Bonferroni, p=1.000) (Figure 6.14) plaque loads maintained compared to the C/C group (Figure 6.13). Similar results were seen in the PCTX. A significant group effect was seen when comparing $A\beta_{1-42}$ ($F(4, 44)=4.780$ $p=0.003$) (Figure 6.13), total Aβ ($F(4, 44)= 3.297$ $p= 0.021$) (Figure 6.14), and AβpE3 ($F(4, 44)=3.321$ $p= 0.020$) (Figure 6.15) plaque loads between the pre-treatment group to the study treatment groups in the PCTX.

Compared to the C/C group, the pre-treatment group had significantly lower $A\beta_{1-42}$ (Bonferroni, p=035) (Figure 6.13) indicating that an increase in plaque load was seen with age. As seen in the PFCTX, the pre-treatment group $A\beta_{1-42}$ plaque load did not differ significantly from the E/C group in the PCTX either, again suggesting a maintenance effect due to ENR was likely keeping $A\beta_{1-42}$ plaque loads lower than the C/C group (Bonferroni, p=1.000) (Figure 6.13).

Soluble and Insoluble Brain Aβ

We hypothesized that the levels of soluble and insoluble Aβ in the brain would be reduced due to VAC and that the E/V treatment group would show an even greater reduction. To test this hypothesis we measured PBS, SDS, and FA extracted $A\beta_{1-40}$ and $A\beta_{1-42}$ from the PFCTX, OCTX, PCTX and HIPPO regions of the brain by sandwich ELISA.

$A\beta_{1-42}$

VAC significantly decreased $A\beta_{1-42}$ in the PBS, SDS and FA extracts of the PFCTX (PBS, $F(1, 34)= 2.518$ $p= 0.016$; SDS, $F(1, 34)=31.244$ $p= <0.005$; FA, $F(1, 34)=5.610$ $p= 0.024$) and OCTX (PBS, $F(1, 34)= 5.782$ $p= 0.023$; SDS, $F(1, 34)= 14.451$ $p= 0.001$; FA, $F(1, 34)= 3.914$ $p= 0.057$), while the PCTX and ECTX remained unchanged (Figure 6.13 A, C, E). No significant reduction was seen due to ENR in PBS, SDS or FA extracted $A\beta_{1-42}$ levels from any of the examined brain regions. However, ENR increased SDS extracted $A\beta_{1-42}$ in the HIPPO that trended towards significance ($F(1,34)=3.514$ $p=0.071$) (Figure 6.16 B, D,F). No combination treatment effect was seen with VAC and ENR, except for a decrease in SDS extractable $A\beta_{1-42}$ in the PFCTX that trended towards significance

(F(1,34)=3.461 p=0.073) and a significant increase in PBS extractable $A\beta_{1-42}$ in the HIPPO (F(1,34)=4.529 p=0.042). These results suggest that the VAC reduced soluble and insoluble levels of $A\beta_{1-42}$ in the PFCTX and OCTX. Additionally, the combination treatment may have been effective in breaking down SDS extractable $A\beta_{1-42}$ into a more soluble state leading to increased PBS extractable $A\beta_{1-42}$.

$A\beta_{1-40}$

In addition to Aβ $A\beta_{1-42}$, levels of soluble and insoluble forms of $A\beta_{1-40}$ were also measured. No significant decrease was seen due to VAC on $A\beta_{1-40}$ in any fraction from the PCTX. There was a significant decrease in $A\beta_{1-40}$ extractable by FA in the PFCTX (F(1, 34)=8.790 p= 0.006) and OCTX (F(1, 34)= 3.914 p= 0.057) (Figure 6.17 E). However, no significant reduction due to VAC was seen in PBS or SDS extractable $A\beta_{1-40}$ in either of these regions (Figure 6.17 A and C). An increase was seen in PBS extracted $A\beta_{1-40}$ due to VAC in the HIPPO (F(1, 34)= 5.433 p= 0.027) (Figure 6.17 A). ENR had no effect on increasing or decreasing PBS, SDS, or FA extractable $A\beta_{1-40}$ in any of the brain regions of interest (Figure 6.17 B, D, F). Additive effects of VAC and ENR were only seen with increasing SDS extractable $A\beta_{1-40}$ in the PCTX (F(1,34)=6.150 p=0.019) and HIPPO (F(1,34)=12.465 p=.001). These results suggest that the VAC as well as combination treatment may have been effective in breaking down FA extractable $A\beta_{1-42}$ into a more soluble state leading to increased levels of PBS (VAC) or SDS (combo) extractable $A\beta_{1-40}$.

$A\beta_{42/40}$ Ratio

The ratio of $A\beta_{42/40}$ was calculated as an indicator of AD pathology and onset of the disease (95, 217). A high $A\beta_{42/40}$ ratio would indicate a greater abundance of $A\beta 1\text{-}_{42}$ than $A\beta_{1\text{-}40}$, while a low $A\beta_{42/40}$ ratio would indicate greater Aβ 1-40 levels in the brain. We hypothesized a lower $A\beta_{42/40}$ ratio in the VAC animals since animals were vaccinated with fibrillar $A\beta 1\text{-}_{42}$ and a reduction of $A\beta 1\text{-}_{42}$ was expected. No significant changes were seen in the ratio of $A\beta_{42/40}$ in the PBS, SDS, and FA extracted samples from the PCTX or OCTX (Figure 6.18 A, C, E). There was a significant decrease due to VAC seen in PBS ($F(1, 34)= 15.732$ $p= <0.005$) and SDS ($F(1, 34)=29.668$ $p= <0.005$) extractable $A\beta_{42/40}$ ratio in the PFCTX (Figure 6.18 A and C). No increase or decrease due to ENR alone was observed (Figure 6.18 B, D, F). However, a decrease in the ratio is found in combination treatment of VAC and ENR in SDS extractable $A\beta_{42/40}$ ratio ($F(1,34)=5.994$ $p=0.020$). Interestingly, in the HIPPO, no increase or decrease due to VAC was found, but a significant increase due to ENR was detected in PBS extracted $A\beta_{42/40}$ ratio ($F(1,34)=5.101$ $p=0.031$) (Figure 6.18 B). There is also an additive effect due to VAC and ENR in increasing PBS extractable $A\beta_{42/40}$ ratio ($F(1,34)=5.066$ $p=0.032$) and increasing SDS extractable $A\beta_{42/40}$ ratio ($F(1,34)=7.668$ $p=.010$). These results suggest that the ratio of $A\beta_{42/40}$ was overall reduced in soluble extracts of the PFCXT due to VAC and the combination of VAC ENR. In the HIPPO, an increase in PBS extracted $A\beta_{42/40}$ is seen which could suggest the breakdown of less soluble forms seen in the SDS or FA extracts into

more soluble Aβ seen in the PBS extract. Collectively these results support the decrease in plaque loads seen in the PFCTX and HIPPO.

Microhemorrhages

Based upon previous studies in transgenic mice (320, 335, 482-484) and human clinical trials (459) we hypothesized that C/V and E/V groups may have more microhemorrhages than the C/C and E/C groups due to the vaccine. To detect increased microhemorrhages, we stained sections from the PFCTX, OCTX, and HIPPO brain regions using Prussian blue, which detects iron in the extracellular matrix. Counts of the number of microhemorrhages were used to detect treatment associated differences.

The total number of bleeds across all brain regions ranged between 0 and 23. The most bleeds was seen in the PFCTX having a range from 1 to 10 bleeds, with the exception of one dog having 17 bleeds. This canine in particular was a female, started the study at the age of 11.6 years, and was in the E/C treatment group. The OCTX had similar bleed counts ranging from 0 to 7. Fewer bleeds were seen in the HIPPO with a range of 0 to 3, with the exception of two dogs that had 7 and 23 bleeds. The animal that experienced 7 bleeds was a male, started the study at the age of 12.3 years, and was in the C/C group. The other dog that experienced 23 bleeds in the HIPPO was a female, started the study at the age of 10.7 years, and was part of the E/V treatment group. Further, if a dog did show a bleed in the HIPPO, it usually occurred in the CA3 region compared to area CA1 or dentate gyrus. Neither ENR ($\chi^2(1)=0.025$ $p=0.876$) (data not show) nor VAC ($\chi^2(1)=0.350$ $p=0.554$) (Figure 6.19) significantly increased microhemorrhage

frequency in the PFCTX. Additionally no individual group effect was seen on increasing the number of microhemorrhage frequency ($\chi^2(3)=0.984$ $p=0.805$) (Figure 6.20). There was also no change in the microhemorrhages in the HIPPO due to ENR ($\chi^2(1)=0.355$ $p=0.551$) (data not shown), VAC ($\chi^2(1)=0.078$ $p=0.780$) (Figure 6.19), or individual group ($\chi^2(3)=0.853$ $p=0.837$) (Figure 6.20). In the OCTX, however, there was a statistically significant increase in microhemorrhages due to VAC ($\chi^2(1)=6.501$ $p=0.011$) (Figure 6.19) and group effect ($\chi^2(3)=8.372$ $p=0.039$) (Figure 6.20). Though an increase in bleeds was statistically seen due to VAC, neither the C/V nor E/V treatment groups show more microhemorrhages than the C/C animals (Figure 6.20). However fewer microhemorrhages were seen in the E/C animals in the occipital cortex compared to C/V animals (Figure 6.20). It is possible that this decrease in bleeds of the E/C group (non VAC animals) could have led to the statistically apparent higher frequency of bleeds due to VAC. No increase in the number of microhemorrhages was observed due to a combination treatment effect in the occipital cortex.

Comparison of Microhemorrhages Over Time Due to Treatments

With the finding that E/C group had fewer bleeds in the OCTX compared to the other treatment groups and C/C group, we wanted to determine whether ENR was reducing the number of bleeds seen or having a maintenance effect as observed with plaque loads To do this we used the same 10 archive cases as used for plaque load analysis that were age matched to study dogs at the start of the study (10.5-13.6 years) (Pre-Treatment). We stained PFCTX and OCTX tissue sections from these pre-treatment dogs with Prussian Blue.

The total number of bleeds in the PFCTX from this pre-treatment group of dogs ranged from 1 to 8, similar to numbers seen in the treatment groups and control group. Statistically, there was no group difference in microhemorrhage counts in the PFCTX between the pre-treatment group and the four study groups ($\chi^2(4)=1.103$ $p=0.894$) (Figure 6.21). In the OCTX, pre-treatment dogs only showed 0 to 3 microhemorrhages compared to the 0 to 7 range seen in the study dogs. There was the exception of one pre-treatment dog that had 5 bleeds. There was a group difference in microhemorrhage frequency between the groups including the Pre-Treatment group ($\chi^2(4)=15.400$ $p=0.004$) (Figure 6.21). Additionally, the pre-treatment group showed approximately the same number of microhemorrhages as the E/C treatment group (Figure 6.21). While the C/C, C/V, and E/V treatment groups do not look to differ in number of bleeds, they do appear significantly higher than the pre-treatment and E/C groups. This would suggest that the E/C treatment is having a maintenance or protective effect against microhemorrhages in the occipital cortex while all other treatment groups experience more bleeds with age independently of the VAC.

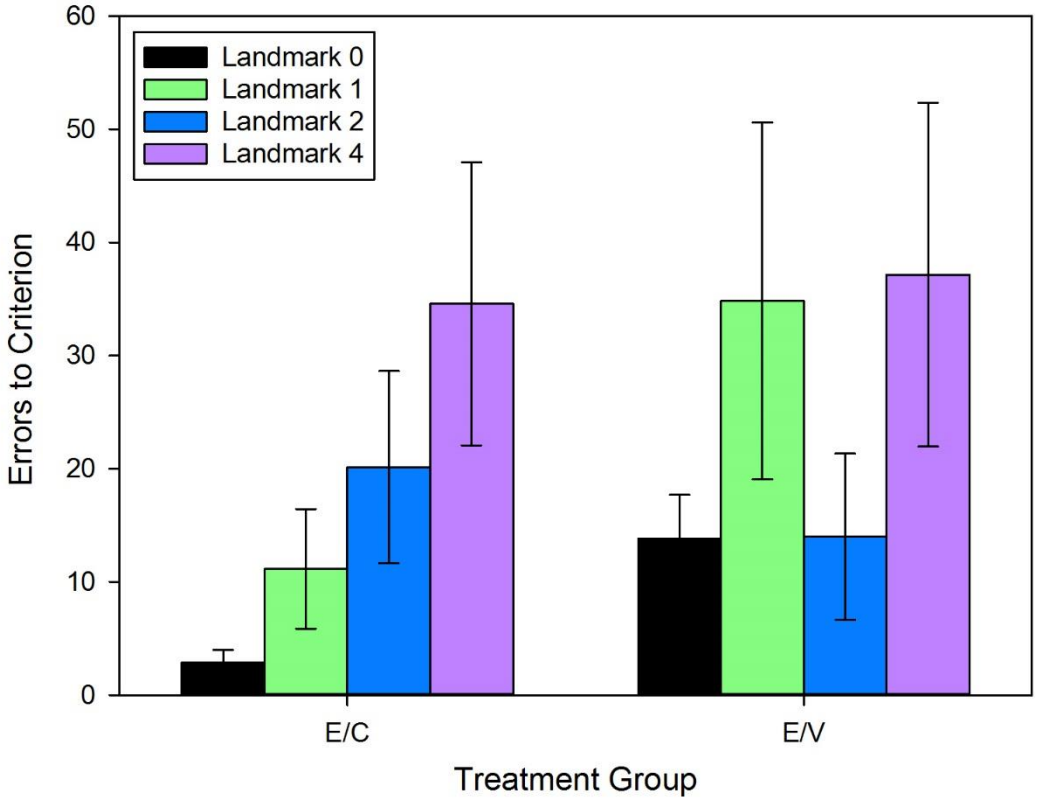


Figure 6.1. Landmark Discrimination Learning.

At Landmark 0, among, dogs that received ENR, dogs that received ENR with VAC (E/V) had higher error scores than dogs receiving ENR only (E/C) ($t(16)=2.7$ $p=0.016$). No group effect was seen for Landmarks 1,2, and 4. Overall, higher error scores occurring as the landmark distance (and thus task difficulty) increased ($F(3, 42)=2.8$ $p=0.05$).

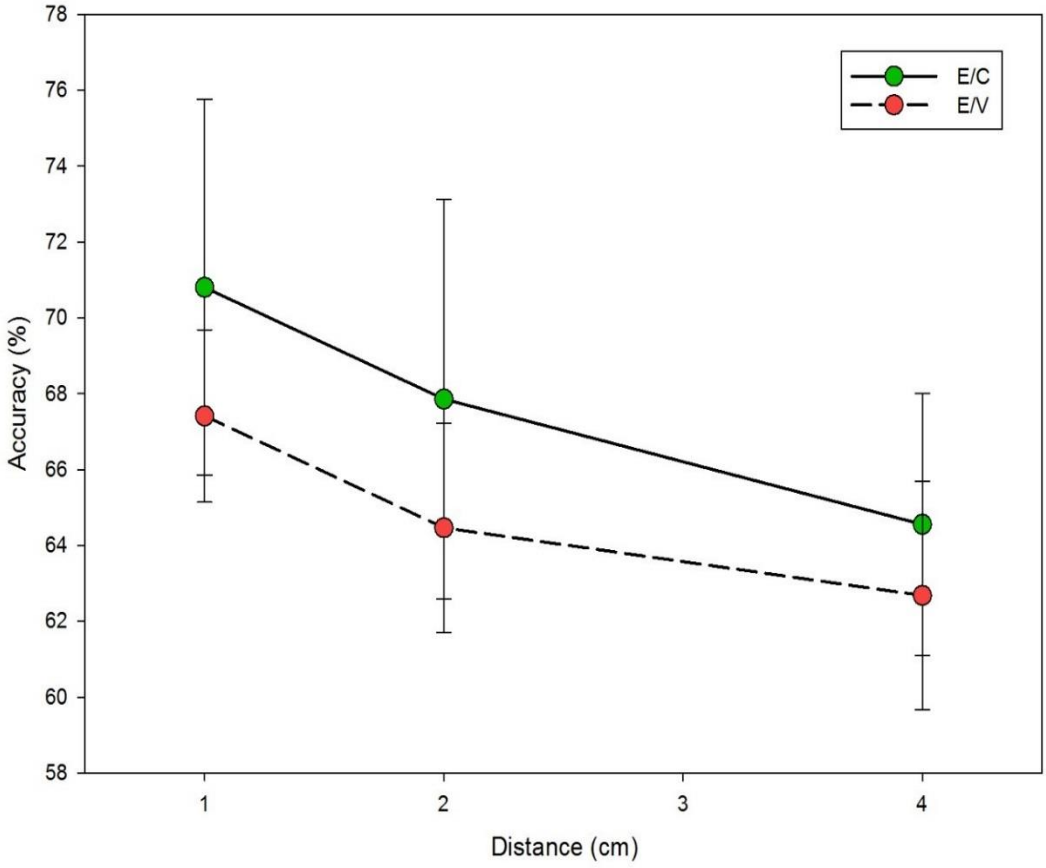
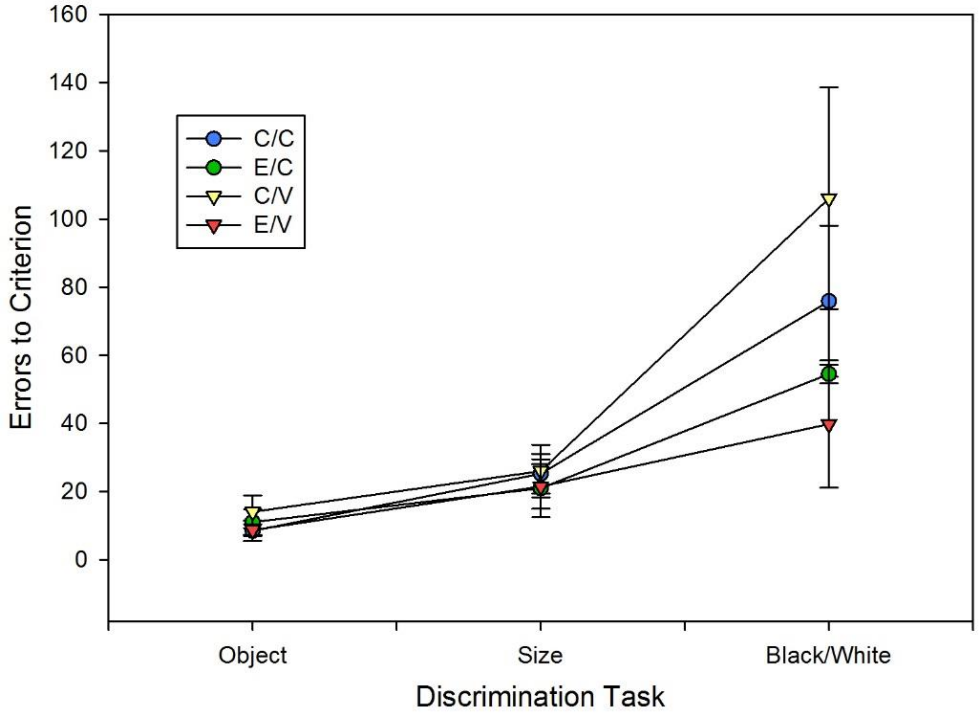


Figure 6.2. Variable distance landmark.

Dogs that received both VAC and ENR (E/V) did not differ in error scores for the variable distance landmark task compared to those receiving only ENR (E/C). Distance also had no effect on error scores during this task.

A


160
140
120
100
80
60
40
20
0
Errors to Criterion
C/C
E/C
C/V
E/V
Object
Size
Black/White
Discrimination Task

B

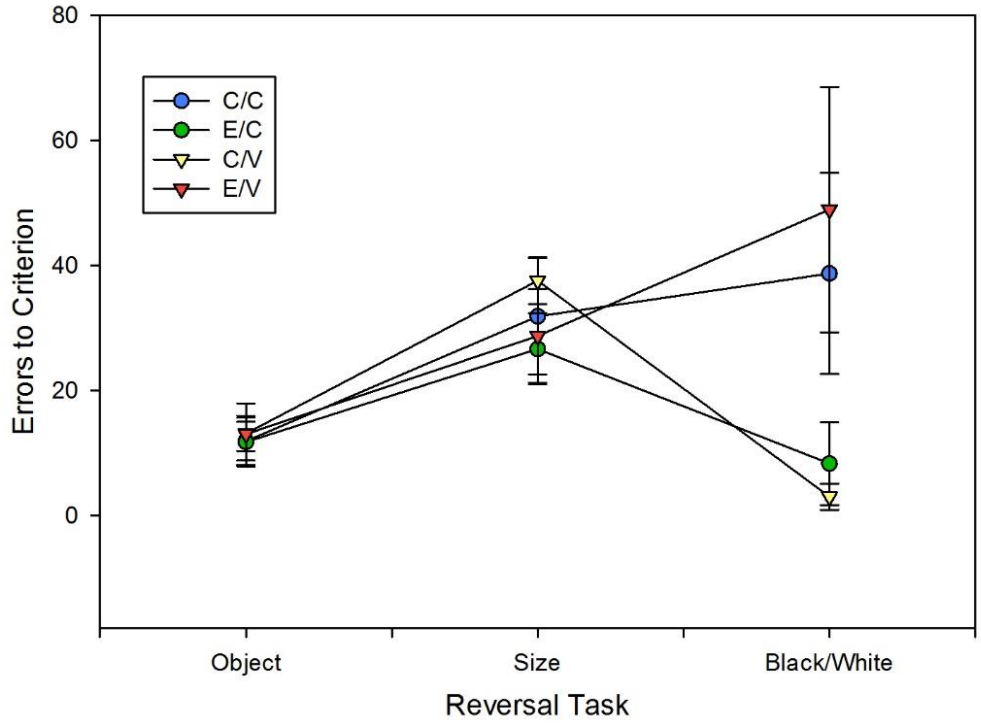

80
60
40
20
0
Errors to Criterion
C/C
E/C
C/V
E/V
Object
Size
Black/White
Reversal Task

Figure 6.3. Discrimination and reversal learning over time.

(A) In discrimination learning, error scores increased over time in all groups ($F(2,32)=3.4$ $p=0.05$). No effect of the vaccine or of the behavioral enrichment alone was seen, however there was a significant effect of the combination treatment group ($F(2, 32)=4.0$ $p=0.03$). The combination treatment group had the lowest error scores. (B) Reversal learning also indicated an increase in error scores over time in all groups ($F(2,30)=10.7$ $p<0.0005$) 2 $p=0.34$). No treatment effects were seen on error scores over time for VAC, ENR, or the combination treatment. E/V treatment group had the highest error scores on reversal learning.

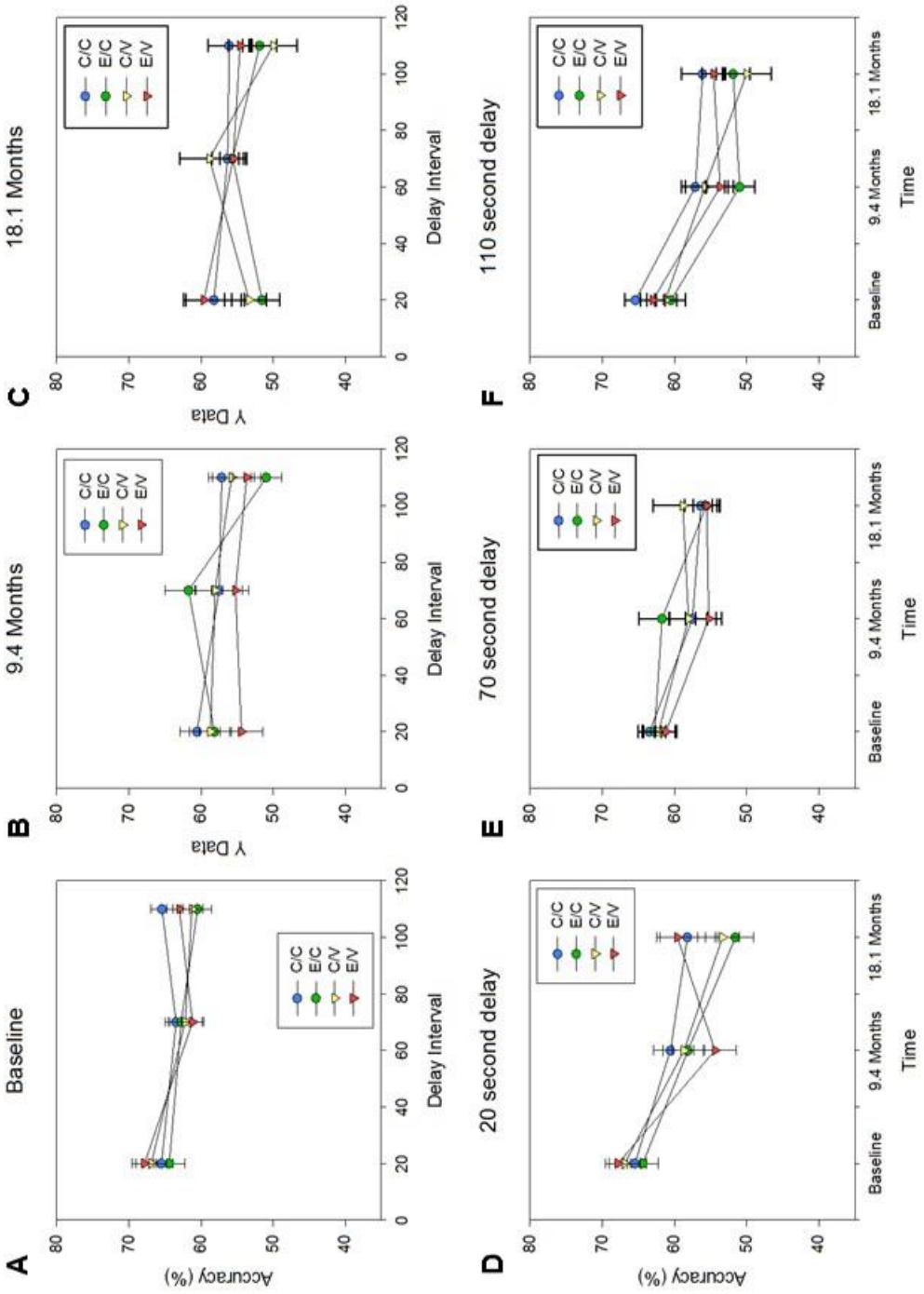
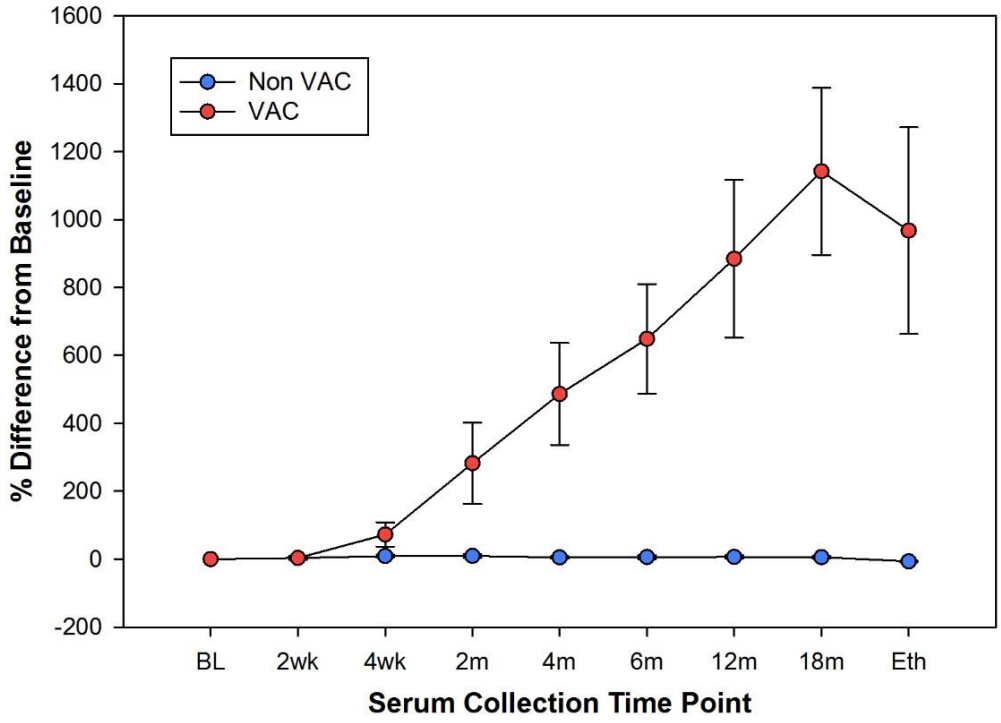

A
Baseline
B
9.4 Months
C
18.1 Months
D
20 second delay
E
70 second delay
F
110 second delay
C/C
E/C
C/V
E/V
Accuracy (%)
Y Data
Delay Interval
Time
Baseline
9.4 Months
18.1 Months
0
20
40
60
80
100
120
40
50
60
70
80

Figure 6.4. Variable delay spatial memory task.

(A) No difference between treatment groups was seen in the accuracy of performance across increasing delay intervals on the spatial memory task when tested at baseline. (B) When re-tested 9.4 months into treatment, there was no improvement observed due to VAC or ENR treatment alone or in combination. (C) Testing after 18.1 months into treatment indicated an interaction between delay interval and accuracy on the spatial memory task in the combination treatment group ($F(2,38)=3.62$ $p=0.04$). However, this may be due to the poor accuracy of the E/C and C/V groups during the 20s delay. (D,E,F) A decrease in accuracy over time was seen for each delay interval (20 second delay, $F(2,36)=16.12$ $p<.0005$; 70 second delay, $F(2,36)=5.51$ $p=0.008$; 110 second delay, $F(2,36)=17.32$ $p<.0005$).

A


Non VAC
VAC
% Difference from Baseline
1600
1400
1200
1000
800
600
400
200
0
-200
BL
2wk
4wk
2m
4m
6m
12m
18m
Eth
Serum Collection Time Point

B

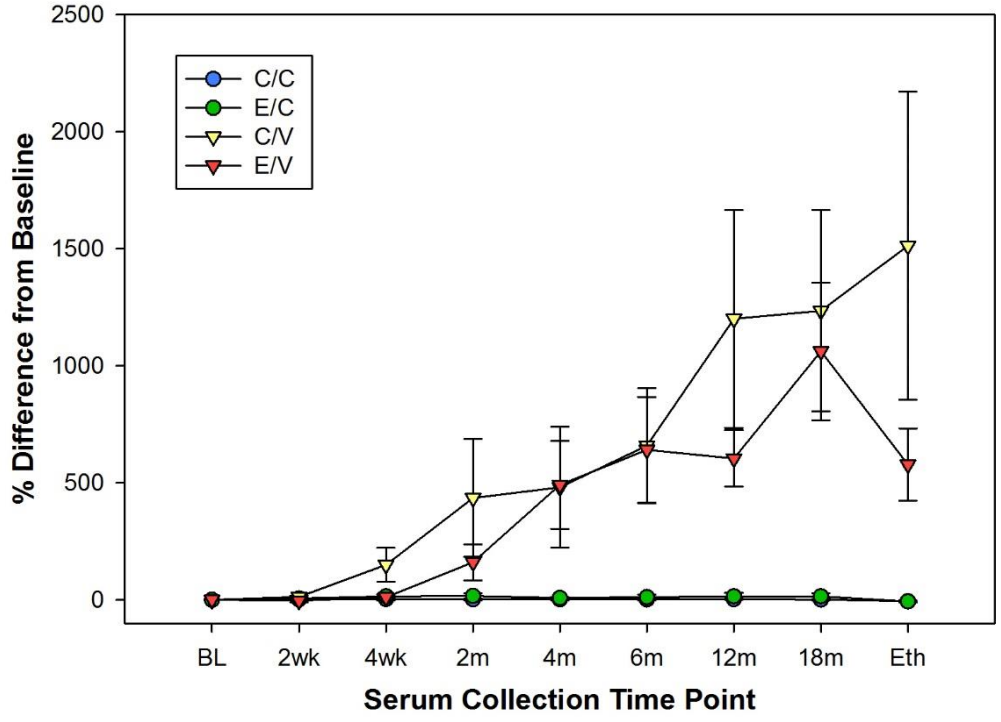
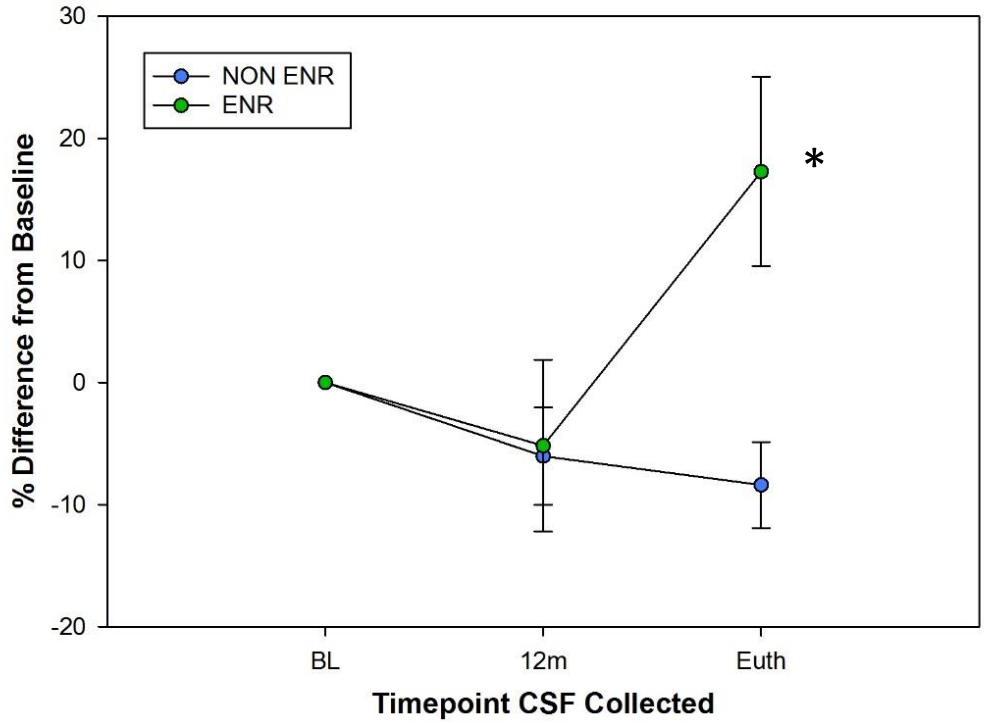

C/C
E/C
C/V
E/V
% Difference from Baseline
2500
2000
1500
1000
500
0
BL
2wk
4wk
2m
4m
6m
12m
18m
Eth
Serum Collection Time Point

Figure 6.5. Anti-Aβ antibody titers over time as a function of treatment.

IgG response was measured in the serum of all animals over multiple timepoints from baseline to euthanasia. VAC animals developed an anti- Aβ IgG response over time ($F(1,19)=12.5$ $p=0.002$) and was maintained as a result of the active vaccine (A). When observing all treatment groups, both VAC groups (C/V and E/V) developed an antibody response and maintained it (B). However the E/V response to the vaccine was delayed and did not reach the same maximum titers as observed in dogs receiving the vaccine alone C/V.

A


NON ENR
ENR
30
20
10
0
-10
-20
% Difference from Baseline
*
BL
12m
Euth
Timepoint CSF Collected

B

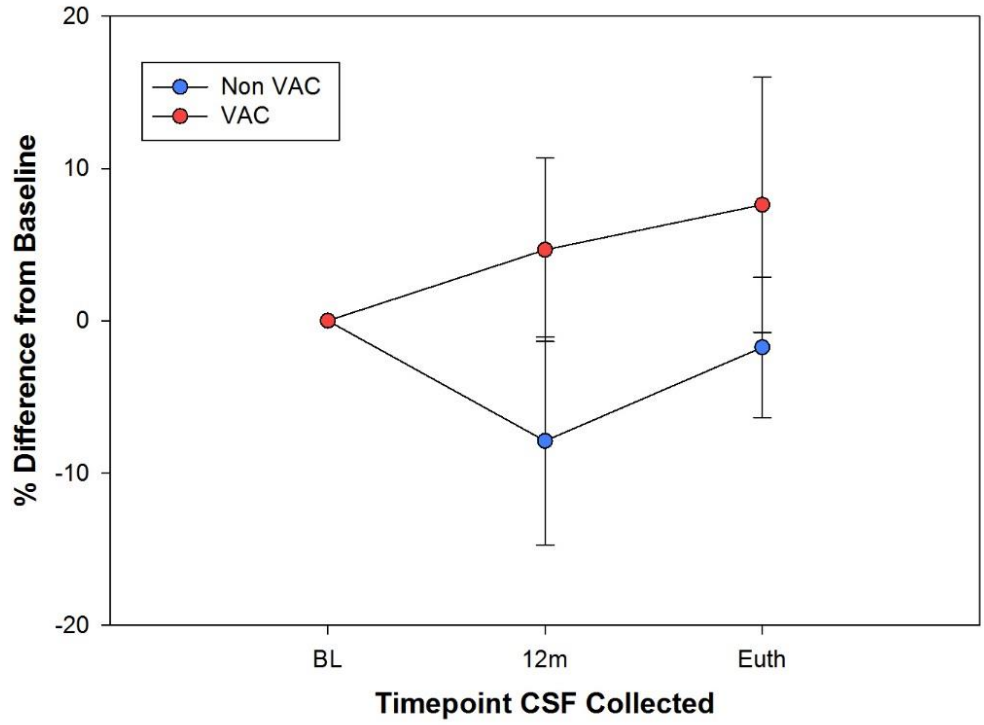

Non VAC
VAC
20
10
0
-10
-20
% Difference from Baseline
BL
12m
Euth
Timepoint CSF Collected

Figure 6.6. Change in average CSF $A\beta_{1-40}$ over the course of treatment.

CSF $A\beta_{1-40}$ was measured in all dogs across three time points through the duration of treatment. ENR led to a significant increase in CSF $A\beta_{1-40}$ ($F(1,22)=5.76$ $p=0.03$) (*) (A). VAC neither increased nor decreased the levels of $A\beta_{1-40}$ in CSF with the time points that were collected (B).

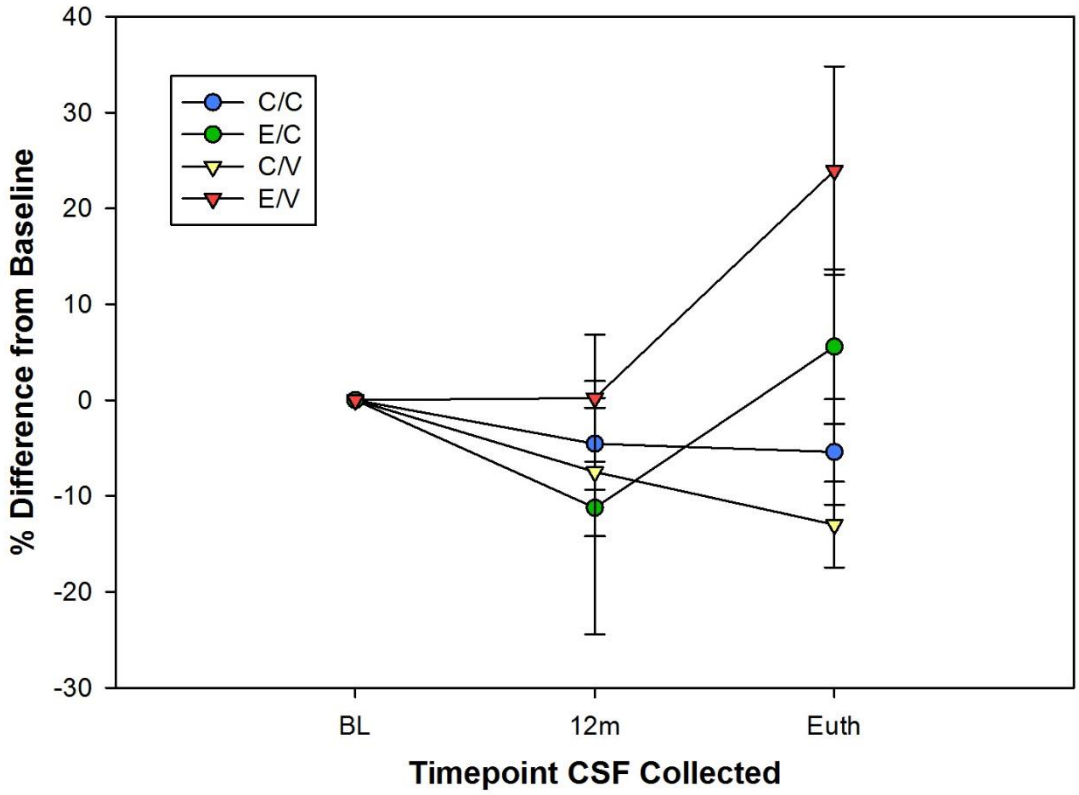
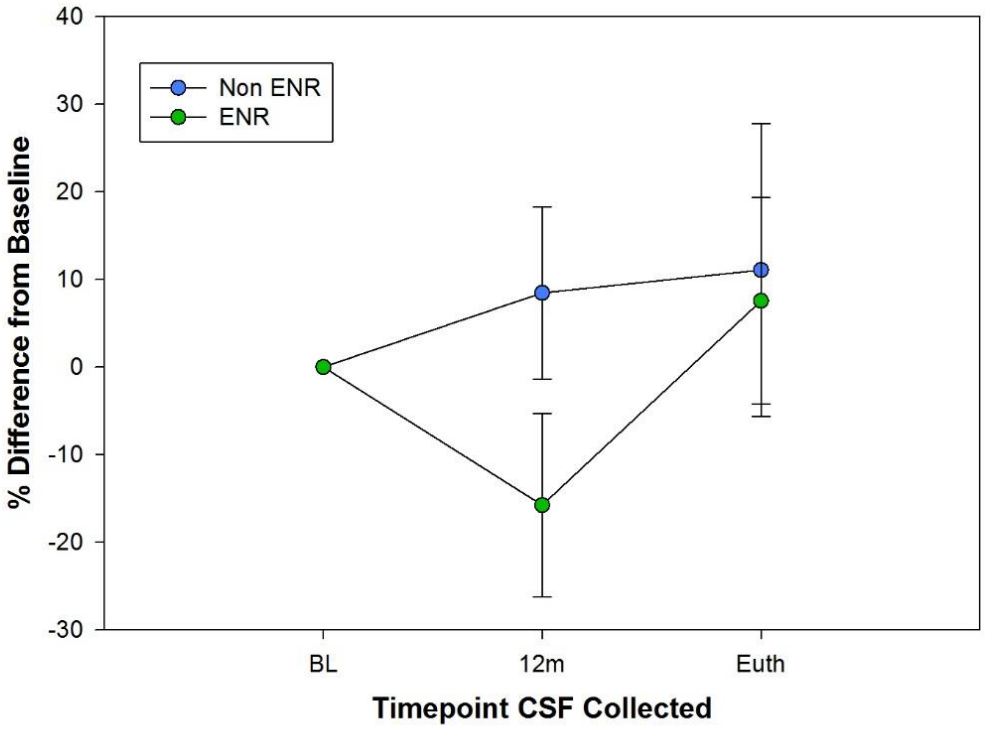


Figure 6.7. Change in average CSF $A\beta_{1-40}$ over course of study in all four treatment groups.

No change in CSF $A\beta_{1-40}$ was seen over time in any treatment group. However, the combination treatment group E/V did have the greatest increase in CSF $A\beta_{1-40}$ by the end of the study.

A


Non ENR
ENR
% Difference from Baseline
40
30
20
10
0
-10
-20
-30
BL
12m
Euth
Timepoint CSF Collected

B

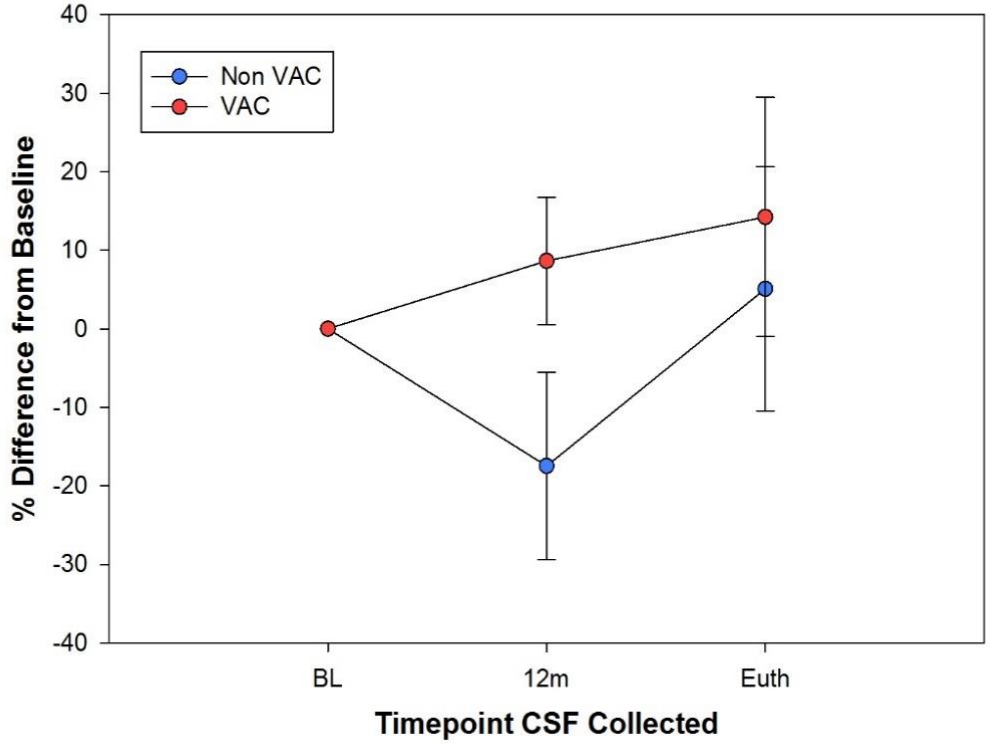

Non VAC
VAC
% Difference from Baseline
40
30
20
10
0
-10
-20
-30
-40
BL
12m
Euth
Timepoint CSF Collected

Figure 6.8. Change in average CSF Aβ1-42 over the course of treatment.

CSF $A\beta_{1\text{-}42}$ was measured in all dogs across three time points through the duration of treatment. No systematic effects were seen due to any treatment (A, B).

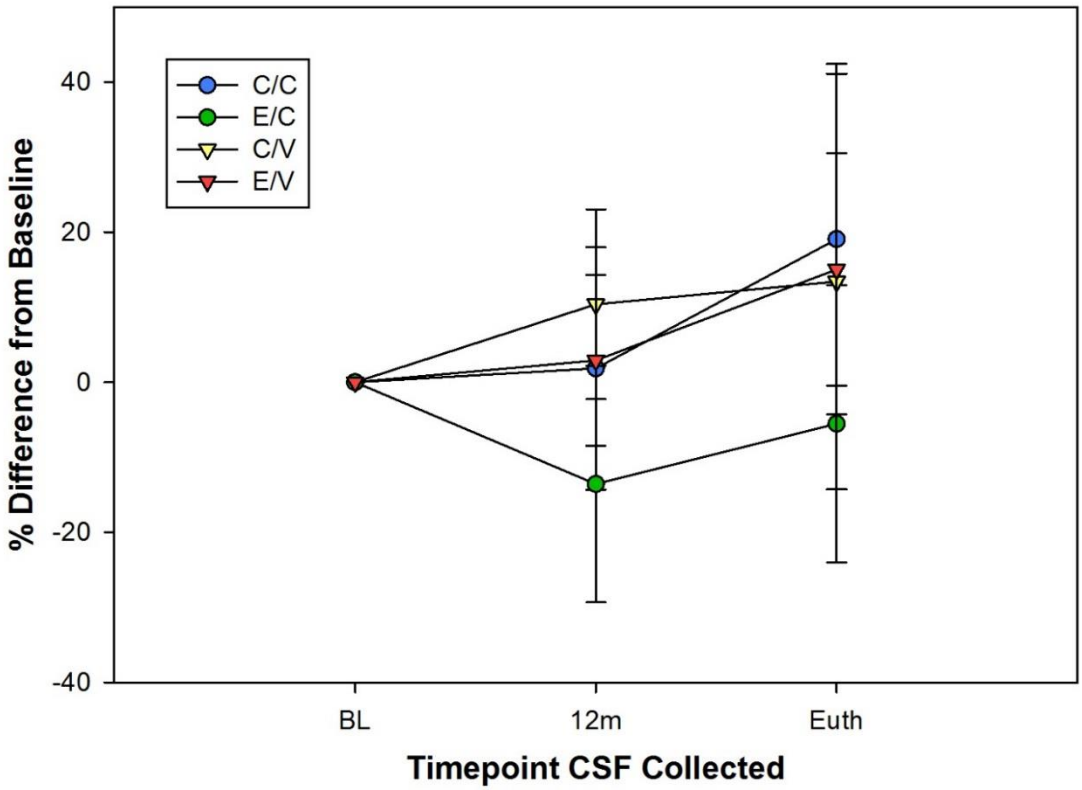


Figure 6.9. Change in average CSF Aβ1-42 over course of study in all four treatment groups.

None of the treatments significantly changed CSF Aβ1-42 over time.

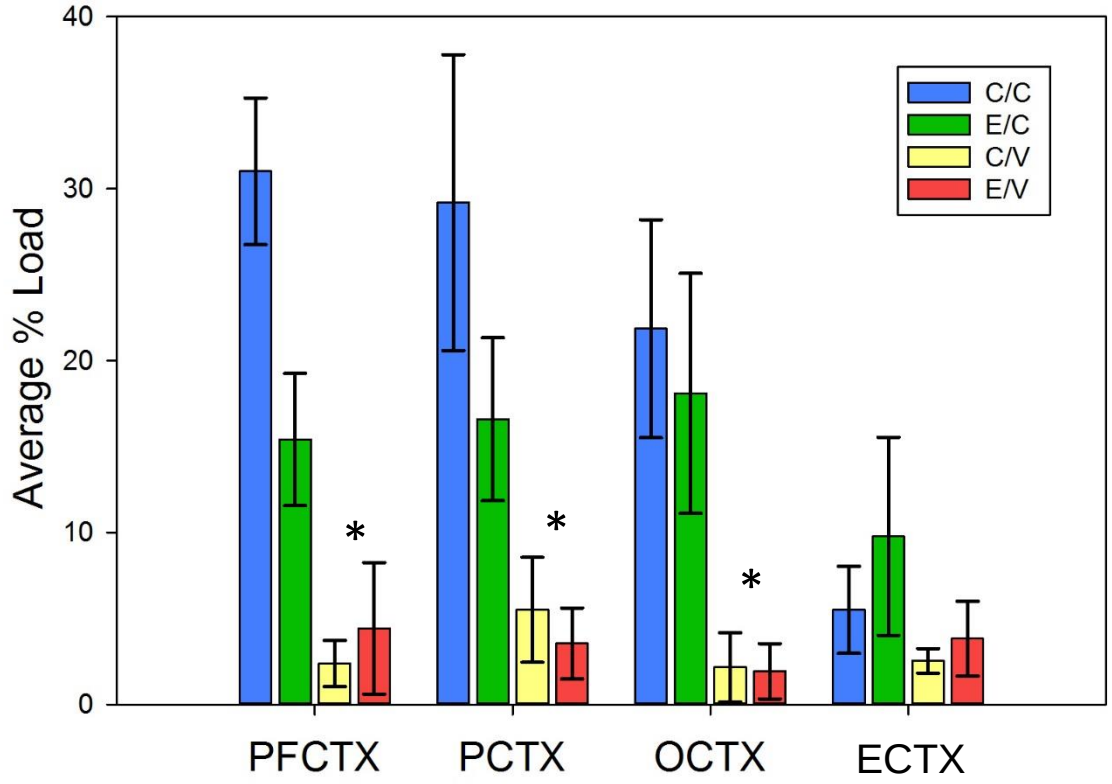


Figure 6.10. Average $A\beta_{1-42}$ plaque loads in PFCTX, PCTX, OCTX, and ECTX regions of the brain.

$A\beta_{1-42}$ plaque loads were reduced in most brain regions of VAC animals (C/V and E/V) (PFCTX ($F(1, 34)= 33.04$ $p= <0.001$); OCTX ($F(1, 34)= 14.92$ $p= 0.001$); PCTX ($F(1, 33)= 14.06$ $p= 0.001$) (*). No additive effect was seen due to the combination of VAC and ENR (E/V). Though not as low as the C/V or E/V groups, E/C animals did have lower loads of $A\beta_{1-42}$ plaques than the C/C group in the PFCTX (Bonferroni, $p=0.02$).

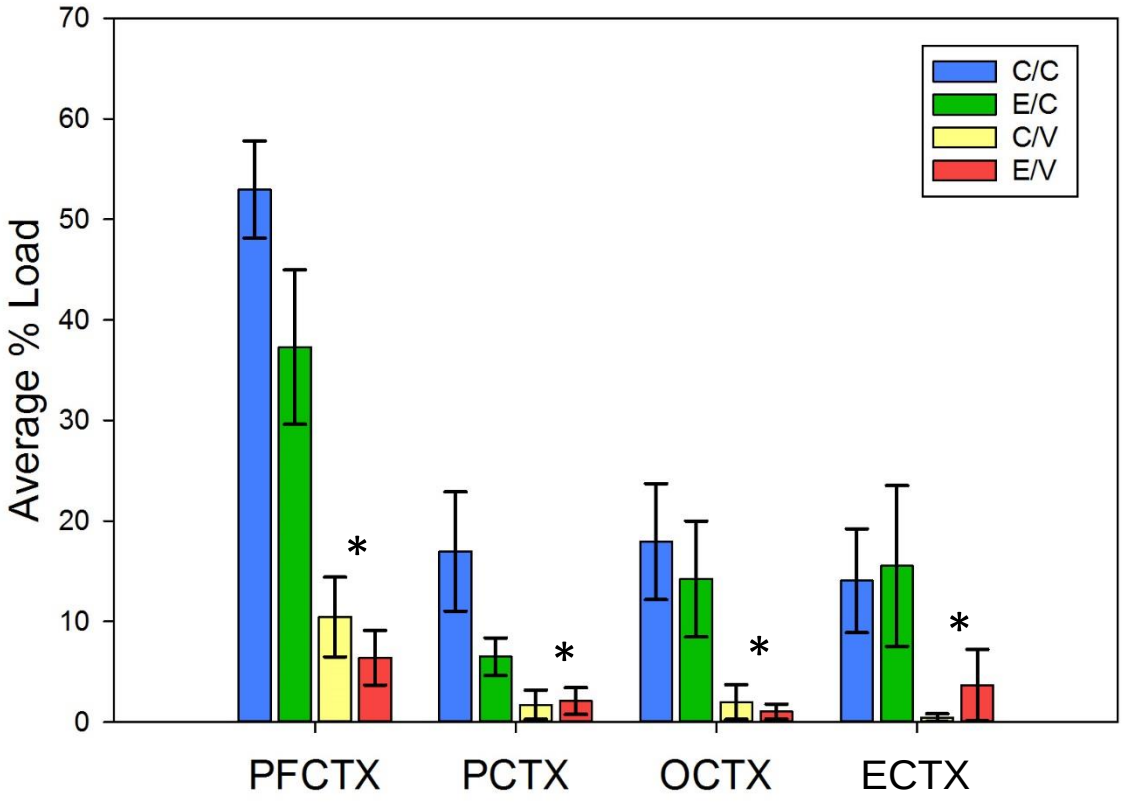


Figure 6.11. Average total Aβ plaque loads in PFCTX, PCTX, OCTX, and ECTX regions of the brain.

Total Aβ plaque load were reduced in the brains of VAC animals (C/V and E/V) (PFCTX, $F(1,34)= 52.91$ $p= <0.001$; OCTX, $F(1,34)= 13.65$ $p= 0.001$; PCTX, $F(1,33)= 10.70$ $p= 0.003$; ECTX, ($F(1, 34)= 6.60$ $p= 0.02$). No additive effect was seen due to the combination of VAC and ENR (E/V). Lower levels of total Aβ plaque loads were observed in the E/C group compared to the C/C group in the PCTX (Bonferroni, $p=0.06$).

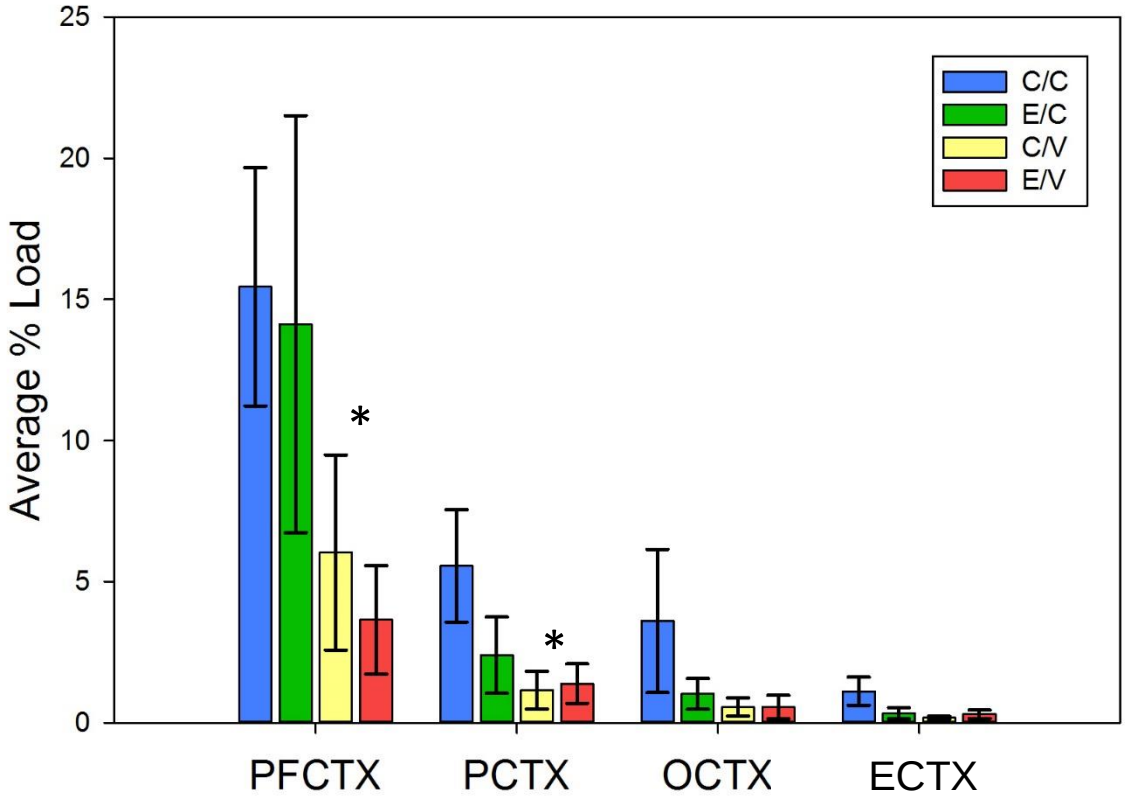


Figure 6.12. Average pyro glutamate modified Aβ (AβpE3) plaque loads in PFCTX, PCTX, OCTX, and ECTX regions of the brain.

Plaque levels of AβpE3 were overall lower than $A\beta_{1\text{-}42}$ and total Aβ plaque levels in all regions of the brain. Though post-translationally modified, VAC was able to reduce plaque loads containing this more toxic form of Aβ in the PFCTX ($F(1, 30)= 10.00$ $p= 0.004$) and PCTX ($F(1, 29)= 6.50$ $p= 0.02$). E/C animals did not differ in AβpE3 plaque loads from those of the C/C treatment group in any brain region.

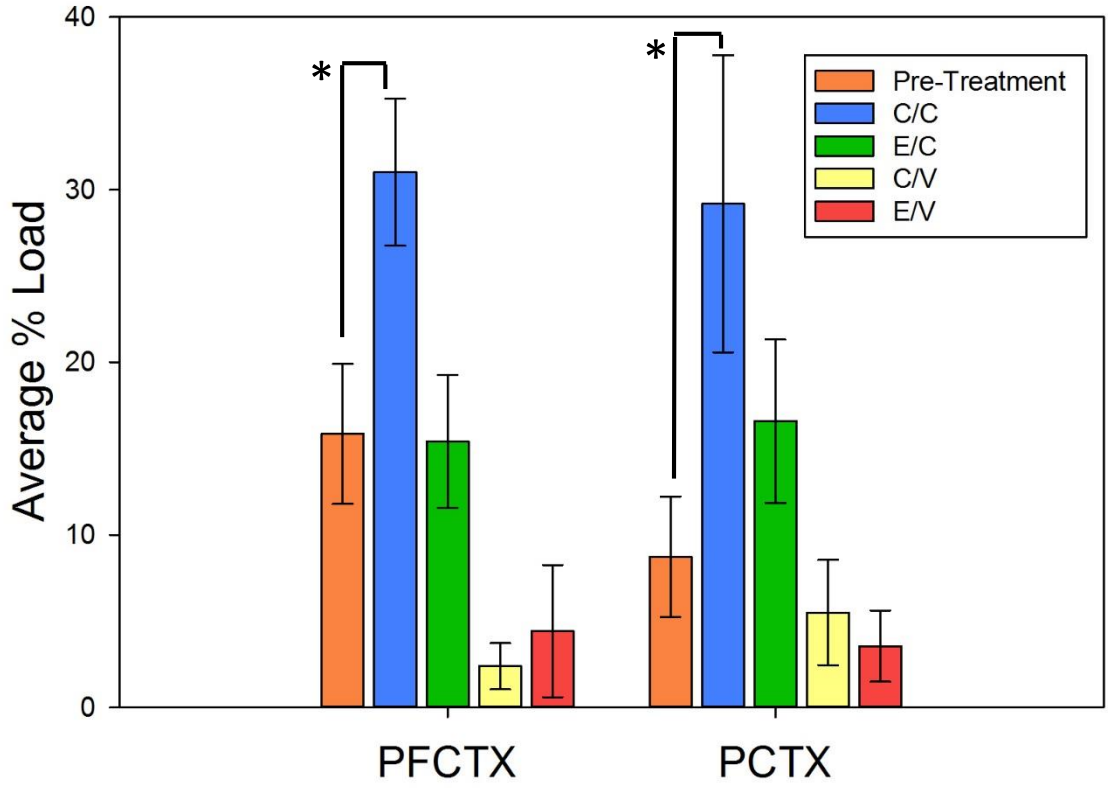


Figure 6.13. Changes in average Aβ1-42 plaque loads between Pre-Treatment animals and treated study animals in PFCTX and PCTX regions of the brain.

When comparing pretreatment animals to C/C, there is a significant increase in $A\beta_{1\text{-}42}$ plaque load with age (PFCTX, Bonferroni, p=0.050; PCTX Bonferroni, p=035). Pre-treatment animals did not differ from E/V animals suggesting a possible maintenance of plaque loads over time due to ENR (PFCTX, Bonferroni p=1.000; PCTX, Bonferroni, p=1.000).

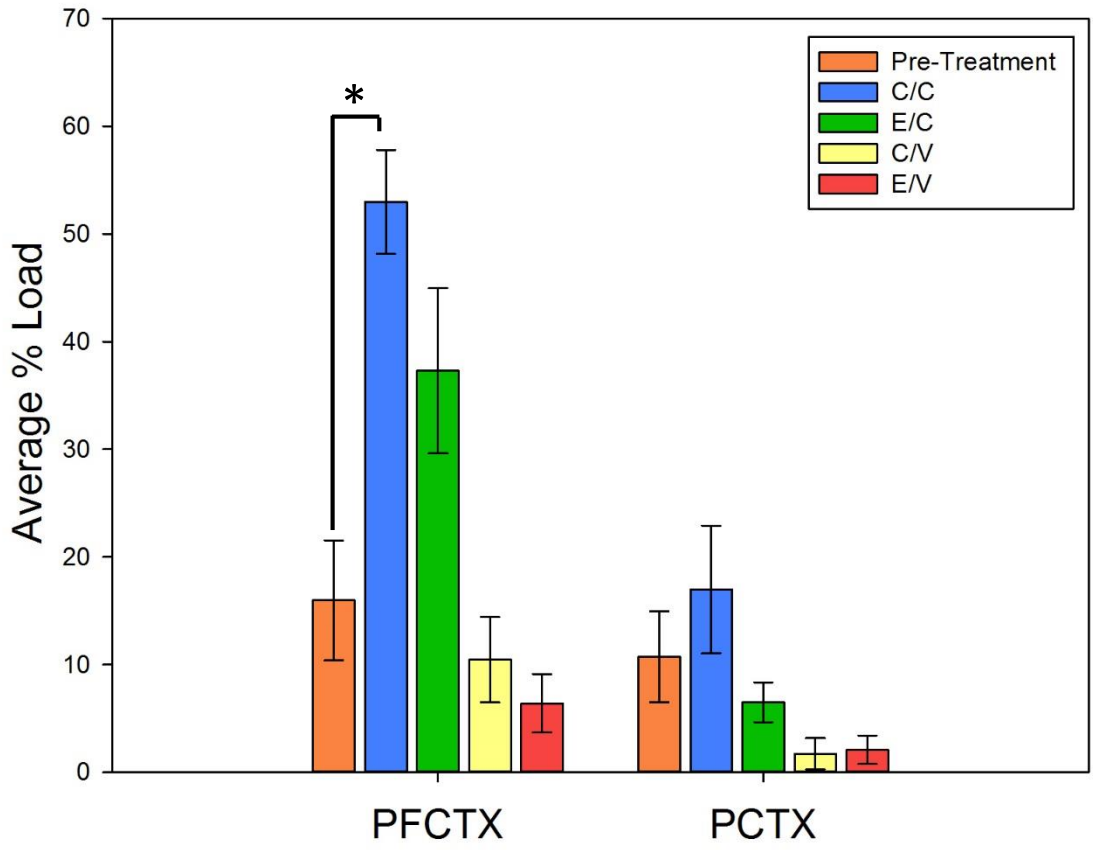


Figure 6.14. Changes in average total Aβ plaque loads between pre-treatment animals and study animals in PFCTX and PCTX regions of the brain.

Pre-treatment animals had lower levels of total Aβ plaque loads compared to control (C/C) animals in the PFCTX (Bonferroni, p=0.014), while there was no difference compared to E/C animals (Bonferroni, p=1.000). This lack of change in the E/C group suggests that ENR could be counteracting the natural age dependent increase in total Aβ plaque loads as seen in C/C animals. No systematic changes were seen in the PCTX for total Aβ plaque loads.

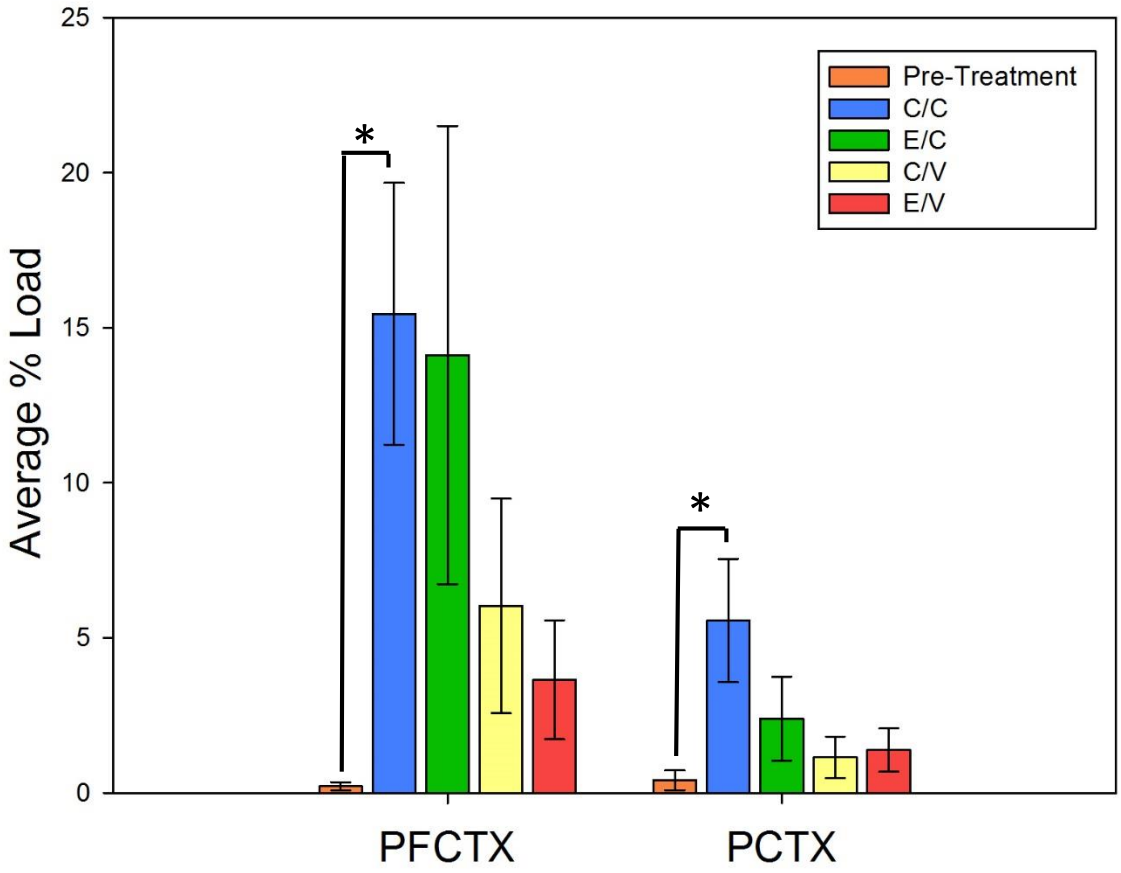
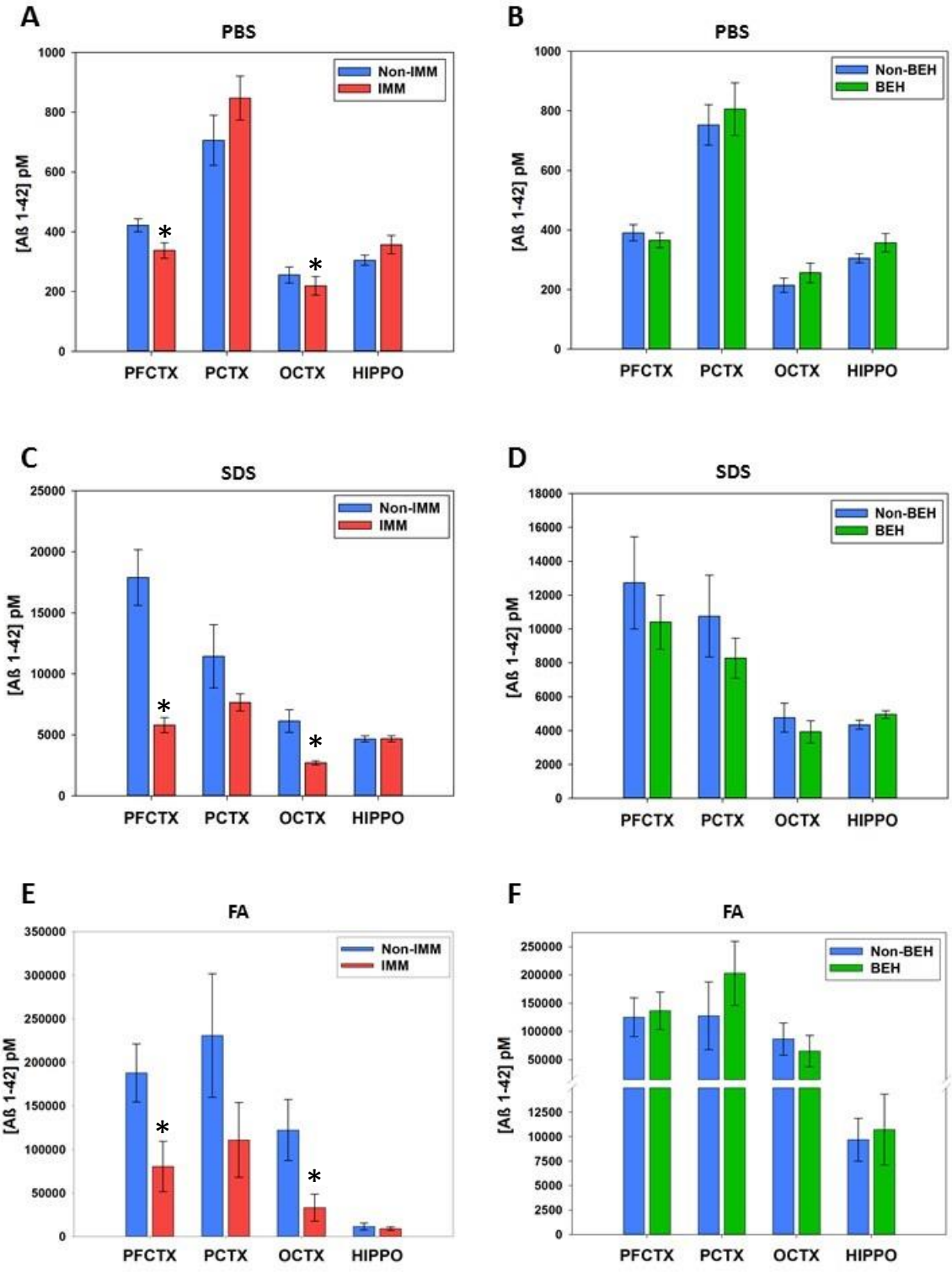


Figure 6.15. Changes in average AβpE plaque loads between pre-treatment animals and study animals in PFCTX and PCTX regions of the brain.

Significant group effects are seen in AβpE plaque loads between pre-treatment animals and treated study animals in both the PFCTX ($F(4, 44)= 9.752$ $p= 0.009$) and PCTX ($F(4, 44)=3.321$ $p= 0.020$). There was very little AβpE in pre-treatment dogs. While VAC reduced AβpE in treated animals compared to C/C or E/C groups, levels do not return back to those seen before the start of the study as represented by the pre-treatment group.


A
PBS
Non-IMM
IMM
[Aß 1-42] pM
1000
800
600
400
200
0
*
*
PFCTX
PCTX
OCTX
HIPPO
B
PBS
Non-BEH
BEH
[Aß 1-42] pM
1000
800
600
400
200
0
PFCTX
PCTX
OCTX
HIPPO
C
SDS
Non-IMM
IMM
[Aß 1-42] pM
25000
20000
15000
10000
5000
0
*
*
PFCTX
PCTX
OCTX
HIPPO
D
SDS
Non-BEH
BEH
[Aß 1-42] pM
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
PFCTX
PCTX
OCTX
HIPPO
E
FA
Non-IMM
IMM
[Aß 1-42] pM
350000
300000
250000
200000
150000
100000
50000
0
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PFCTX
PCTX
OCTX
HIPPO
F
FA
Non-BEH
BEH
[Aß 1-42] pM
250000
200000
150000
100000
50000
12500
10000
7500
5000
2500
0
PFCTX
PCTX
OCTX
HIPPO

Figure 6.16. Soluble and insoluble brain $A\beta_{1-42}$ as a function of treatment.

Higher levels of FA extractable brain Aβ 1-42 were seen compared to PBS and SDS across all groups. VAC reduced all forms of extractable $A\beta_{1-42}$ in the PFCTX (PBS, $F(1, 34)= 2.518$ $p= 0.016$; SDS, $F(1, 34)=31.244$ $p= <0.005$; FA, $F(1, 34)=5.610$ $p= 0.024$)and OCTX (PBS, $F(1, 34)= 5.782$ $p= 0.023$; SDS, $F(1, 34)= 14.451$ $p= 0.001$; FA, $F(1, 34)= 3.914$ $p= 0.057$) (A, C, E). No change in soluble or insoluble Aβ was seen due to ENR (B, D, F), except for an increase in SDS extractable $A\beta_{1-42}$ in the HIPPO ($F(1,34)=3.514$ $p=0.071$) that trended towards significance (D).

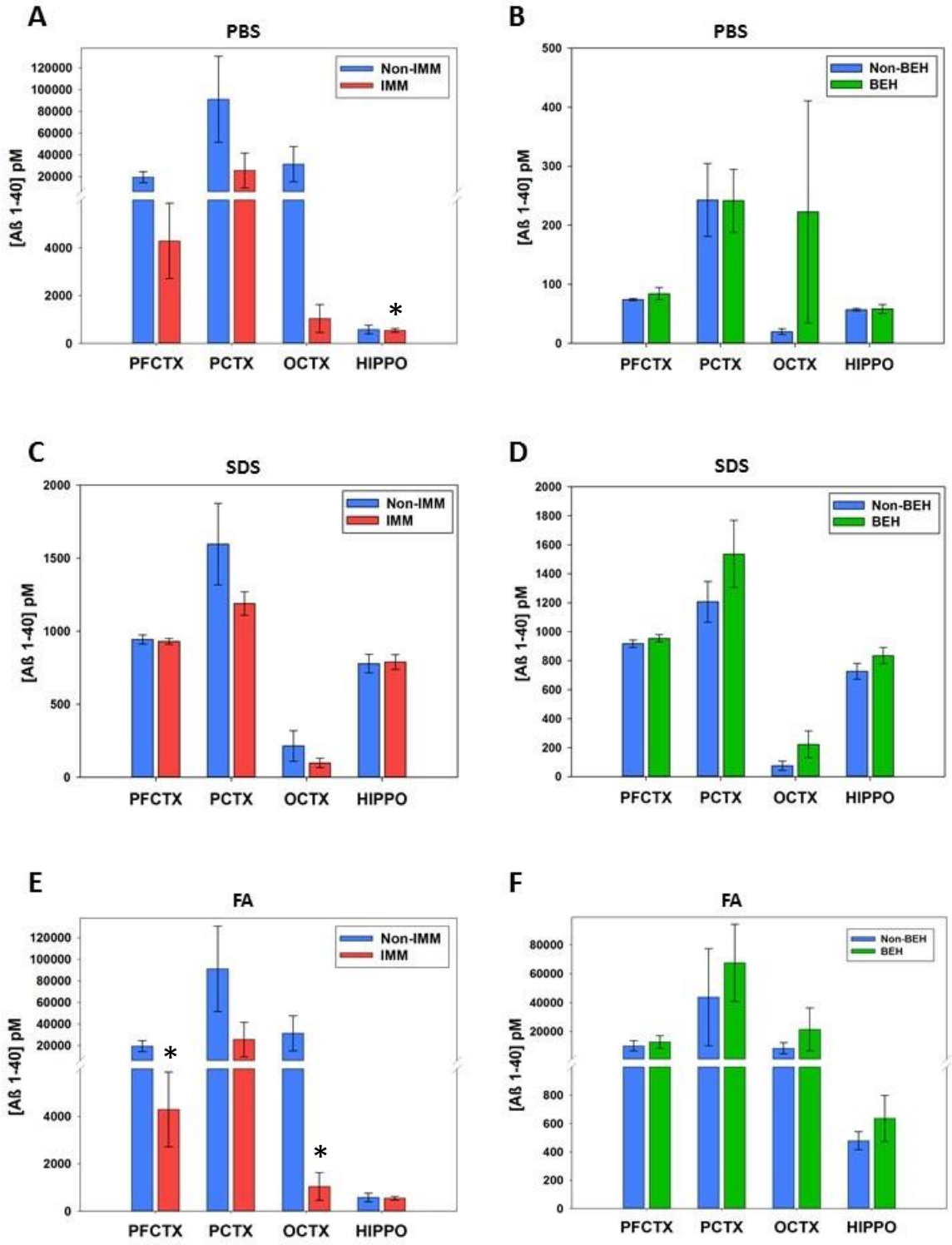
A
PBS
Non-IMM
IMM
[Aß 1-40] pM
120000
100000
80000
60000
40000
20000
4000
2000
0
*
PFCTX
PCTX
OCTX
HIPPO
B
PBS
Non-BEH
BEH
[Aß 1-40] pM
500
400
300
200
100
0
PFCTX
PCTX
OCTX
HIPPO
C
SDS
Non-IMM
IMM
[Aß 1-40] pM
2000
1500
1000
500
0
PFCTX
PCTX
OCTX
HIPPO
D
SDS
Non-BEH
BEH
[Aß 1-40] pM
2000
1800
1600
1400
1200
1000
800
600
400
200
0
PFCTX
PCTX
OCTX
HIPPO
E
FA
Non-IMM
IMM
[Aß 1-40] pM
120000
100000
80000
60000
40000
20000
4000
2000
0
*
*
PFCTX
PCTX
OCTX
HIPPO
F
FA
Non-BEH
BEH
[Aß 1-40] pM
80000
60000
40000
20000
800
600
400
200
0
PFCTX
PCTX
OCTX
HIPPO

Figure 6.17. Soluble and insoluble brain $A\beta_{1-40}$.

VAC increased PBS extracted $A\beta_{1-40}$ in the HIPPO ($F(1, 34)= 5.433$ $p= 0.027$) (A), while it reduced insoluble FA extractable $A\beta_{1-40}$ in the PFCTX ($F(1, 34)=8.790$ $p= 0.006$) and OCTX ($F(1, 34)= 3.914$ $p= 0.057$) (E). No change in soluble or insoluble Aβ was seen due to ENR (B, D, F).

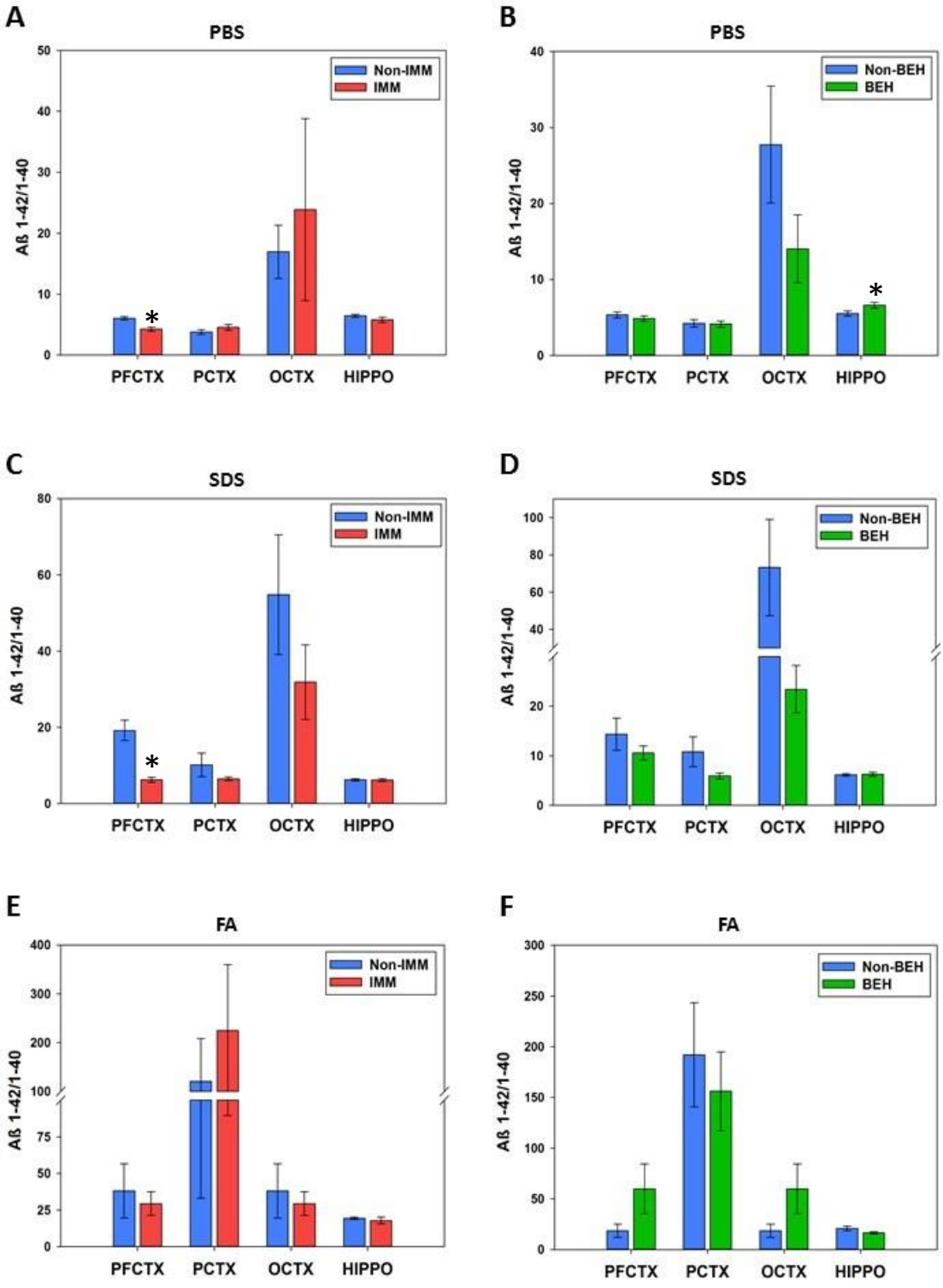
A
PBS
Non-IMM
IMM
Aß 1-42/1-40
PFCTX
PCTX
OCTX
HIPPO
B
PBS
Non-BEH
BEH
Aß 1-42/1-40
PFCTX
PCTX
OCTX
HIPPO
C
SDS
Non-IMM
IMM
Aß 1-42/1-40
PFCTX
PCTX
OCTX
HIPPO
D
SDS
Non-BEH
BEH
Aß 1-42/1-40
PFCTX
PCTX
OCTX
HIPPO
E
FA
Non-IMM
IMM
Aß 1-42/1-40
PFCTX
PCTX
OCTX
HIPPO
F
FA
Non-BEH
BEH
Aß 1-42/1-40
PFCTX
PCTX
OCTX
HIPPO

Figure 6.18. Soluble and insoluble brain $A\beta_{1-42}$/ $A\beta_{1-40}$ Ratios

VAC decreases PBS ($F(1, 34)= 15.732$ $p= <0.005$) extractable Aβ 42/40 ratio in the PFCTX(A), ENR increased PBS extracted Aβ 42/40 ratio ($F(1,34)=5.101$ $p=0.031$) in the HIPPO (B). SDS extractable Aβ 42/40 ratio in the PFCTX was reduced by VAC ($F(1, 34)=29.668$ $p= <0.005$) (C). No increase or decrease due to ENR was observed in SDS extractable Aβ 42/40 ratio (D). FA extractable Aβ 42/40 ratio was neither increased nor decreased by VAC or ENR (E, F).

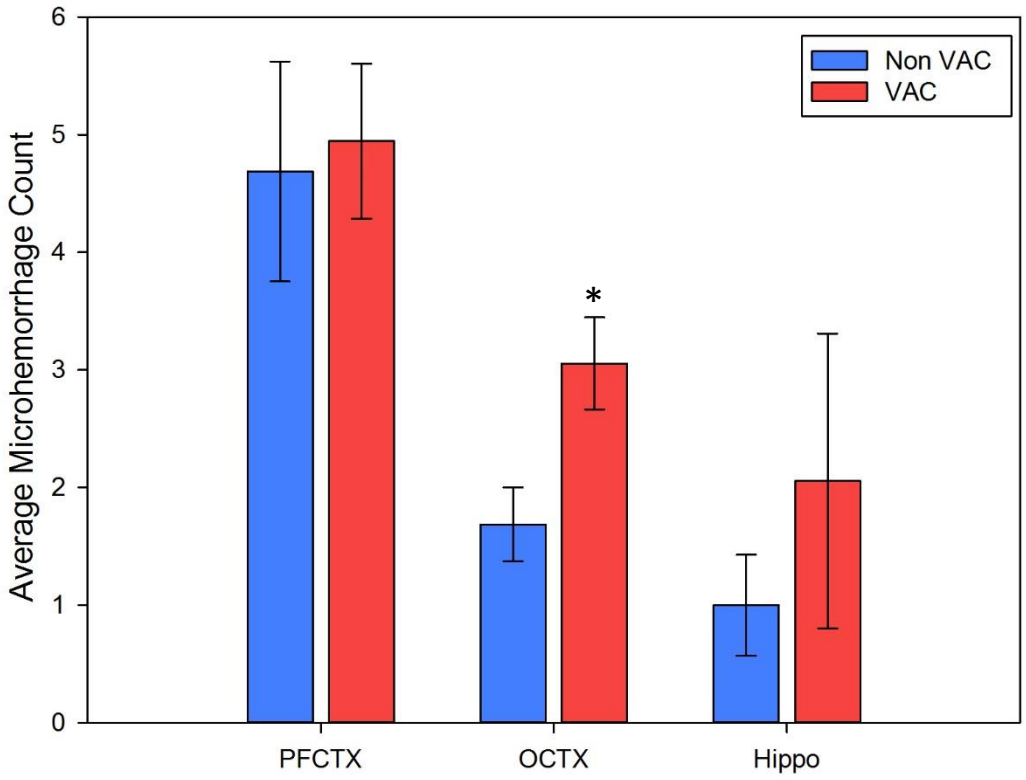

Figure 6.19. Microhemorrhages frequency in the PFCTX, OCTX, and Hippo regions of the brain in VAC treated animals compared to non VAC treated animals.

VAC does not cause an increase in microhemorrhages in the PFCTX or Hippo regions of the brain. However, VAC appears to increase the number of microhemorrhages in the OCTX but there was significant individual variability ($\chi^2(1)=6.501$ $p=0.011$).

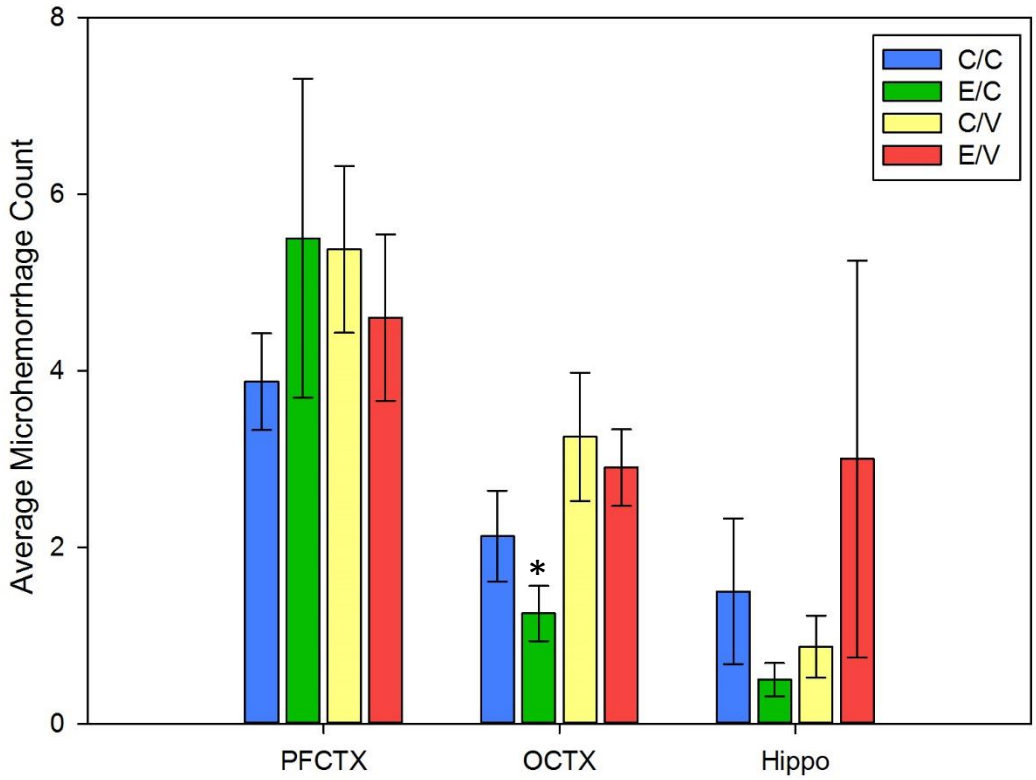

Figure 6.20. Microhemorrhage occurrence in the PFCTX, OCTX, and Hippo regions of the brain as a function of treatment group.

No increases in microhemorrhage frequency in the PFCTX or Hippo were seen as a consequence of treatment. While VAC increases bleed events in the OCTX ($\chi^2(1)=6.501$ $p=0.011$), the number of bleeds observed in VAC animals (C/V and E/V groups) does not differ significantly from the control animals (C/C) (Bonferroni- C/V, $p=0.852$; E/V, $p=1.000$). However, the E/C treatment group showed fewer microhemorrhages in the OCTX than the C/C group (Bonferroni, $p=0.071$) suggesting that ENR may be protective in that region of the brain.

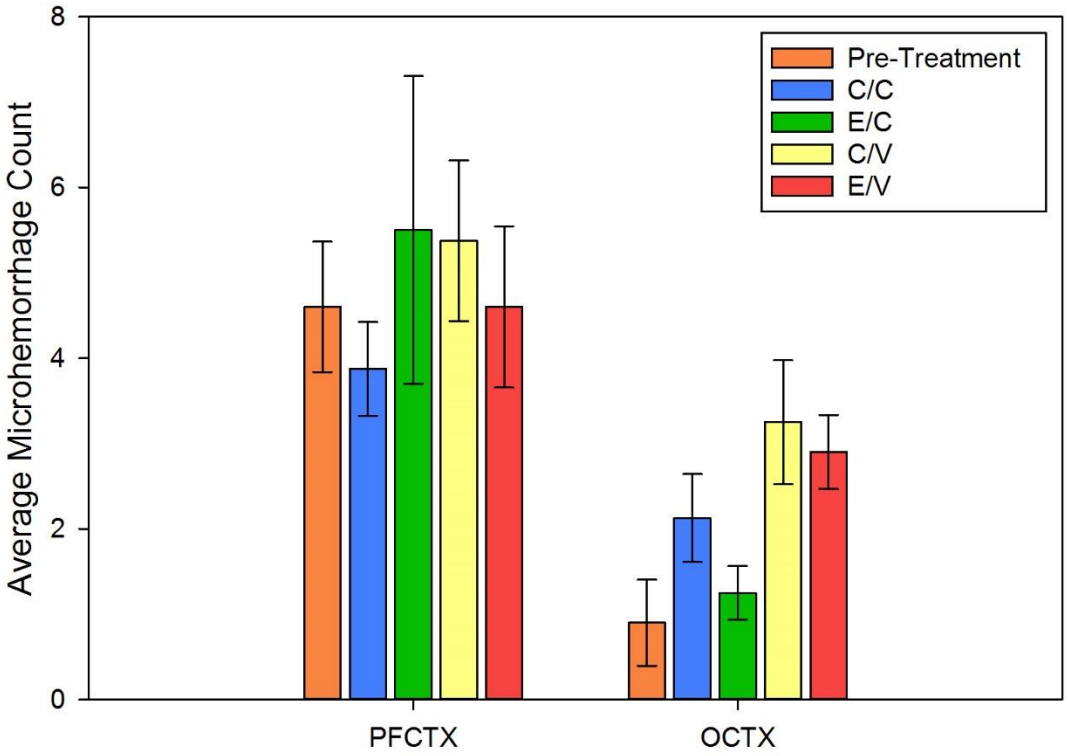

Figure 6.21. Microhemorrhage occurrence in the PFCTX and OCTX regions of the brain in pre-treatment and study animals.

In the PFCTX there were no differences in microhemorrhages between any of the treatment groups. However, in the OCTX, pre-treatment and E/C animals trends towards having fewer microbleeds than C/C and VAC dogs. Since E/C animals appear to show the same frequency of microbleeds as the pre-treatment animals, we suggest that the ENR is maintaining brain health and preventing microhemorrhages that may naturally occur with age.

CHAPTER SEVEN: Discussion

Previous human clinical trials using active vaccination with fibrillar $A\beta_{1-42}$, though discontinued due to cases of meningoencephalitis, showed evidence of reduced Aβ pathology within the brain (135, 181). Consequently, passive immunization approaches that eliminate the possibility of the previous adverse events have been and are currently being investigated in patients with mild to moderate AD; these immunotherapies reduce Aβ pathology and show modest reductions in rates of cognitive decline (101, 103, 366, 367). While these passive immunotherapies do not lead to meningoencephalitis, they have caused microhemorrhaging in several patients as seen by magnetic resonance imaging (368, 420). Our lab has investigated active immunization in aged canines using an adjuvant that is safe for use in mice and humans having few adverse effects (24, 83, 177, 246). In a previous study in aged canines, active vaccination with fibrillar $A\beta_{1-42}$ using Alum as an adjuvant reduced Aβ pathology in the brain and helped maintain executive function without causing adverse effects such as meningoencephalitis as seen with past active vaccinations (177). However, improved cognition in response to the vaccine was not observed in the canine study as reported in transgenic mouse studies (198, 286). Thus, it is possible that the vaccine alone and reduction of Aβ was insufficient to improve cognition in aged dogs with neuropathology. We hypothesized that adding a second intervention that would lead to neuronal repair may be beneficial when combined with the vaccine. We focused on behavioral enrichment as a second “arm” to this study.

Behavioral enrichment, consisting of exercise, cognitive enrichment, social engagement, and environmental enrichment, is being evaluated in clinical trials for AD in humans and animal models. Exercise and environmental enrichment improve learning in rodents but shows variability between studies in AD mouse models (17, 80, 239). In humans, behaviorally enriched lifestyles, involving exercise, cognitive enrichment, and social engagement, helps reduce brain atrophy with age and reduces risk of dementia (165, 274). Furthermore, in canines, ENR improves cognitive function without affecting Aβ levels in the brain (71, 485). While immunotherapies reduce Aβ pathology in humans and animals as well as aid in cognitive maintenance, ENR improves cognition and reduces risk of dementia but has variable outcomes on levels of brain Aβ. This study sought to combine active VAC with ENR predicting they would build upon one another and lead to greater cognitive improvement as well as decreased Aβ pathology compared to each individual treatment alone.

One aspect of the combination therapy we wanted to test was its effects on cognition. Based on our previous studies, ENR improves overall cognition in aged canines (71, 485). However, in a previous canine active vaccine study, no improvements in cognition were observed (177). We had hypothesized that our study animals receiving ENR only or VAC only would show similar results to past studies but animals receiving both ENR and VAC would exhibit greater cognitive improvements. Our results however, indicated no systematic effects by any treatment on improving cognition. The lack of cognitive improvement in the E/C group came as a surprise. It was noted however, that all tested animals actually

performed better with lower error scores at baseline than dogs in past studies did during their baseline testing. Also, housing protocols and the diet now provided for research canines has improved substantially since our last ENR study. It is possible that the housing conditions have improved enough that an ENR threshold has been met between all treatment groups just through these conditions where no additional cognitive benefits can occur even with added social engagement or cognitive enrichment. Results from the current study strongly suggest that reversing age-associated and Aβ-dependent cognitive decline is challenging and prevention may be more beneficial. Results from our neurobiological studies support this conclusion as will be discussed next.

After 19 months of treatment with the active vaccine of fibrillar $A\beta_{1-42}$, VAC animals had increased antibody titers against fibrillar $A\beta_{1-42}$. In C/V animals, this response was first seen at 4 weeks after treatment, while the E/V treated dogs experienced a delayed response at 2 months. While both groups receiving VAC maintained antibody levels through the rest of the study, the maximum response of the E/V treatment group never reached that of the C/V treatment group. It is possible that ENR in the combination treatment suppressed the immune response initiated by the vaccine causing a lower maximum antibody response but was still sufficient to reduce brain Aβ to the same extent as the C/V group. Overall, the elevated levels of anti- $A\beta_{1-42}$ antibodies indicate that the active vaccination was successful in initiating the production of antibodies against fibrillar $A\beta_{1-42}$. This result mirrors that of the human trials using active vaccination, where patients showed an antibody response between one and two months after treatment (181).

CSF Aβ levels correlate with disease in patients with AD who show lower CSF Aβ levels than non-demented individuals (for review, see (14)). To monitor Aβ changes throughout treatment, CSF samples were collected before treatment, 12 months into treatment, and at euthanasia. We hypothesized that VAC animals would have higher Aβ levels than non-VAC animals. These expected results would suggest the vaccine was succeeding in removing the Aβ from the brain and moving it into the periphery. However, VAC did not lower or raise CSF $A\beta_{1-42}$ or $A\beta_{1-40}$ compared to dogs that did not receive VAC. Several contributing factors may exist for these unexpected results. Samples were only able to be drawn at two time points before euthanasia. It is possible that levels of CSF Aβ may have become elevated at some time between the baseline and 12 months collection time points as Aβ was cleared from the brain. This elevation would then be followed by a reduction once a majority of Aβ was removed from the brain and periphery. Essentially, once Aβ was cleared from the brain and into the periphery, Aβ would also be removed from the CSF. This possible mechanism could have been captured had additional time point collections of CSF been made. Unfortunately, in order to collect CSF, the animals must be sedated, which can be physiologically stressful for aged animals. For this reason, CSF draws were limited to three spaced out collection time points.

While VAC did not reduce CSF $A\beta_{1-42}$ or $A\beta_{1-40}$, ENR did increase CSF $A\beta_{1-40}$. As discussed earlier in Chapter 2, $A\beta_{1-40}$ is the prominent isoform of Aβ peptide involved in amyloidosis in the vasculature of the brain (324, 389, 498). One possible explanation for the increased CSF $A\beta_{1-40}$ is that the exercise component

of the ENR improved blood perfusion and cerebrovascular health and aided in the clearance of Aβ deposited in the vasculature to the periphery. Additionally, this increase in CSF $A\beta_{1-40}$ was selective and not seen with $A\beta_{1-42}$. Since $A\beta_{1-42}$ tends to form plaques in the parenchyma of the brain rather than within blood vessels, this lack of effect by ENR on CSF $A\beta_{1-42}$ further supports the idea of ENR acting specifically on the cerebrovasculature. (324, 389, 477).

Several active and passive immunotherapies studied as a therapeutic for AD have shown positive results in reducing Aβ plaque pathology in both animal models and patients with AD (94, 177, 341, 367, 368, 381, 403, 438). In the present study, use of active immunization with fibrillar $A\beta_{1-42}$ in combination with ENR to treat aging canines also reduces Aβ pathology in several regions of the brain. VAC treated dogs, including both the C/V and E/V treatment groups, had decreased Aβ plaque loads compared to non-immunized animals in the PFCTX ($A\beta_{1-42}$ and total Aβ), OCTX ($A\beta_{1-42}$ and total Aβ), PCTX ($A\beta_{1-42}$ and total Aβ), and ECTX (total Aβ) regions of the brain. In addition, AβpE3, post-translationally modified Aβ, was reduced in the PFCTX and PCTX regions of the brain in VAC animals. Previous vaccine studies using the canine model have not explored the vaccine's potential in reducing post-translationally modified Aβ. Post-translationally modified Aβ, including AβpE3, has shown to be more toxic and involved in the initial stages of the disease thereby making it a crucial therapeutic target for clearance in a clinical setting (8, 162, 362, 383, 385, 487).

The previous ENR study in aged canines did not exhibit any kind of reduction in Aβ pathology in response to treatment (322). Interestingly, in the current study

ENR showed a trend towards reducing $A\beta_{1-42}$ in the PFCTX. ENR led to reduced total Aβ plaque loads in the PFCTX and PCTX. However, ENR did not show any treatment effects in reducing AβpE3. With this finding we became curious if the lower Aβ pathology was due to a clearance effect or maintenance effect due to the ENR. Since this reduction due to ENR seen in our study was not as great as that seen by VAC, we hypothesized that the ENR had a maintenance effect on Aβ plaque loads rather than a clearance effect.

To test if ENR was having a maintenance or clearance effect on plaque loads, we used PFCTX and PCTX brain tissue (regions that appeared to have the greatest treatment effect by ENR) from 10 canine cases from our archive tissue inventory. Dogs were selected at matched ages to the baseline ages of the treatment study animals. These pre-treatment dogs represented the average plaque loads of the treatment study dogs prior to the start of treatment, providing a means of comparing change in plaque loads with age (with C/C group dogs) and with treatment (with E/C, C/V, and E/V group dogs).

Results showed that pre-treatment dogs exhibited significantly lower plaque loads than C/C dogs for all types of Aβ examined illustrating the increase of plaque loads in the canine with age over time. $A\beta_{1-42}$ plaque loads of the PFCTX in pre-treatment dogs most resembled that of the E/C treatment group suggesting that the effects seen by ENR were likely maintenance of plaque loads in treated dogs rather than a clearance of Aβ plaques. This maintenance could have either been a decreased rate of Aβ accumulation or slowing of Aβ accumulation. $A\beta_{1-42}$ plaque loads in the PCTX of the pre-treatment animals fell between the E/C treatment

group and VAC animals. However, the E/C group loads were still lower than that of the C/C group. It is likely that the ENR was able to slow the rate of $A\beta_{1-42}$ plaque formation in the PCTX while VAC cleared plaque loads. A similar effect is suggested with total Aβ plaque loads in the PFCTX and PCTX. Unlike the $A\beta_{1-42}$ and total Aβ plaque loads, AβpE3 average plaque load for the pre-treatment group was significantly lower than all treatment groups on the present study. ENR appears to have no effect on AβpE3 plaque loads, while the VAC reduced/cleared this post-translationally modified form of Aβ (VAC animals). The lack of change in AβpE3 plaque loads suggests that ENR was not affecting preexisting Aβ, but rather the further accumulation of Aβ plaques. These results support ENR having a maintenance effect rather than enhancing clearance.

Aβ found in the periphery is of soluble form, however, both soluble and insoluble Aβ are found in the brain (262, 294, 390, 473). Plaque loads do not provide us with information regarding the changes in soluble as compared to insoluble Aβ. Though insoluble Aβ primarily makes up plaque pathology, this form of Aβ may not all be aggregated into plaque and can also be found in blood vessels. Additionally, plaque loads do not provide insight on soluble Aβ levels since this form of Aβ is not associated with plaques (262, 294, 390, 473). To further investigate the combination treatment effects of the present study serial extracted soluble and insoluble Aβ was measured in all treatment groups. We found that VAC reduced both soluble (PBS and SDS extracts) and insoluble (FA extract) $A\beta_{1-42}$ from the PFCTX and OCTX compared to non VAC dogs. Our findings confirmed the earlier mentioned clearance of $A\beta_{1-42}$ plaques seen by VAC in these brain

regions. While we saw a reduction in $A\beta_{1-42}$ plaques that trended towards significance in the PFCTX due to ENR, we did not see any effect by ENR on reducing FA extracted insoluble $A\beta_{1-42}$ in this brain region or any other examined region supporting the hypothesis of maintenance and not clearance of Aβ pathology.

We did not see a reduction in FA extracted $A\beta_{1-40}$ due to VAC, however, an increase of PBS extracted $A\beta_{1-40}$ was seen. This increase in soluble $A\beta_{1-40}$ could be the result of breaking down plaques by the VAC into more soluble forms of Aβ. While we saw an increase in soluble $A\beta_{1-40}$ in the CSF of ENR treated dogs, we did not see the expected decrease in insoluble or soluble $A\beta_{1-40}$ in any brain region of these dogs. As discussed earlier, the increase in CSF $A\beta_{1-40}$ may have been due to the clearance of deposited Aβ in the cerebrovasculature by ENR benefits on vascular health. If this is the case, then any insoluble $A\beta_{1-40}$ deposited in blood vessels would be broken down into a soluble state and be more readily cleared from the brain and into the periphery than that of which is deposited in the parenchyma of the brain. While this clearance of vascular deposited Aβ could lead to a noticeable increase in peripheral $A\beta_{1-40}$, as seen in the CSF of ENR animals, it may not be enough to indicate an apparent decrease in insoluble brain $A\beta_{1-40}$. This would explain the lack of difference in the serial extracted Aβ in the brain due to ENR.

Using the measurements of $A\beta_{1-42}$ and $A\beta_{1-40}$ we calculated the Aβ 42/40 ratio. Generally Aβ 42/40 ratio is an indicator of AD pathology and onset of the disease (95, 217). In dogs, it is expected that this ratio would be higher with age

as pathology and cognitive decline progress. Since the VAC dogs in our study received vaccinations of fibrillar $A\beta_{1-42}$ and had shown reduced levels of $A\beta_{1-42}$, we hypothesized that the Aβ 42/40 ratio would be lower in VAC treated animals than non-treated animals. Aβ 42/40 ratios for PBS and SDS extractable Aβ were lower in the PFCTX of VAC treated animals. With these results we could conclude that the VAC was most productive in reducing Aβ pathology specifically in the PFCTX, which is portrayed in the reduction of plaque forms and serial extracted Aβ in the PFCTX. While no change was seen in Aβ 42/40 ratios for the PCTX, OCTX, or HIPPO of the VAC animals, these regions did still show reduced plaque loads or soluble and insoluble Aβ.

While both VAC and ENR had their respective treatment effects on CSF Aβ, Aβ plaque load, serially extracted Aβ, and overall reduction of Aβ pathology, no significant additive effects were seen in the combination treatment group in further reducing Aβ pathology. Statistically by two way ANOVA, there was a significant additive effect of VAC and ENR in decreasing $A\beta_{1-42}$ plaque load in the PFCTX, decreasing SDS extractable $A\beta_{1-42}$ and Aβ 42/40 ratio in the PFCTX, increasing PBS extractable $A\beta_{1-42}$ and Aβ 42/40 ratio in the HIPPO, and increasing SDS extractable $A\beta_{1-40}$ in the PCTX and HIPPO. However, by post hoc the E/V treatment group did not have greater effects than the C/V group. In the presence of active vaccine, the ENR provides no additional benefit.

The results of our study further support the ability of active vaccination with fibrillar $A\beta_{1-42}$ combined with Alum to reduce Aβ pathology in aged canines. Additionally the vaccine in our study does not promote adverse effects similar to

the meningoencephalitis that was seen in the human clinical trials using an active vaccine with QS-2 in polysorbate 80 as an adjuvant. However, there was still the concern of intracerebral microhemorrhages due to the use of immunotherapies for treatment of AD. Such an increase in microhemorrhages could increase risk of intracerebral microhemorrhage and further cognitive impairment (39, 469). We examined the frequency of microhemorrhages in the PFCTX, OCTX, and HIPPO regions of the brains in all study dogs along with the added pre-treatment archive cases in order to determine if an increase in microhemorrhages would occur with the use of the active vaccination with fibrillar $A\beta_{1-42}$ in Alum. Microhemorrhages appeared to be more frequent in the PFCTX and OCTX than the HIPPO of study dogs. The PFCTX and OCTX are regions of the brain where Aβ deposition is thought to occur first with age (18, 50-52, 445). Since microhemorrhages do occur with age and with greater Aβ deposition in the vasculature of the brain, it was not surprising to see these regions having the greater number of microhemorrhages compared to the HIPPO. Neither VAC nor ENR led to an increase in microhemorrhages in the PFCTX or HIPPO. There was a statistically significant increase in microhemorrhages in the OCTX due to VAC. However, the C/V and E/V treatment groups did not differ in microhemorrhage occurrence compared to the C/C group. What was apparent was the lower number of OCTX microhemorrhages in the E/C treatment group compared to the other groups. It's possible that this lower frequency of microhemorrhages in the E/C drove the statistical increase in microhemorrhages due to VAC since the E/C group is considered part of the non-VAC animals. Using the same baseline age match

archive cases that were in the plaque load analysis, we were able to determine if the lower microhemorrhage frequency in the E/C group was due to a maintenance effect of treatment. Pre-treatment dogs showed a similar frequency of microhemorrhages in the OCTX as the E/C treatment group. This observation suggests that ENR shows maintenance or protective effects against additional microhemorrhages while all other treatment groups experienced more bleeds with age independently of VAC.

Although no significant or consistent cognitive benefits were detected in any treatment group, the active vaccine successfully produced antibody responses against fibrillar $A\beta_{1-42}$. Vaccinated animals also showed a reduction in overall Aβ pathology in multiple areas of the brain, while not showing an increase in CSF Aβ. Previous immunotherapy studies in animals or humans had investigated the potential ability to reduce modified Aβ pathology such as plaques consisting of AβpE3. Here we show that active vaccination with fibrillar $A\beta_{1-42}$ is successful in reducing a toxic and highly aggregated form of modified Aβ in the canine model. In addition to the vaccine treatment, those receiving ENR showed a lack of age-associated increase in Aβ pathology that has not been reported in past ENR studies in dogs. ENR led to an increase in CSF $A\beta_{1-40}$ possibly suggesting ENR aiding in the clearance of deposited Aβ in the vasculature. ENR also slowed the age associated increase of $A\beta_{1-42}$ plaque load in the PFCTX with treatment. In the HIPPO, ENR decreased SDS extractable $A\beta_{1-42}$ in the HIPPO. As for the concern for potential adverse effects of immunotherapy for AD, no serious negative effects were seen and frequency of microhemorrhage occurrence was not increased with

the use of active vaccination with fibrillar $A\beta_{1-42}$ in Alum. Additionally, we found that ENR may actually reduce the risk of microhemorrhages in the OCTX region. While benefits of both VAC and ENR were experienced in reducing Aβ pathology, the combination of both treatments did not cause any additional benefit compared to using only single treatment of either VAC or ENR. Additionally, no synergistic improvement was detected in cognitive function in the combination treatment group compared to individual treatment groups.

Many therapeutic approaches have at best, modestly improved cognitive function in larger animal models of AD. This lack of cognitive improvement is consistent with clinical trials with AD patients. It is possible that once Aβ has begun its damage to neurons it is challenging to reverse it and restore cognition that has already been compromised. Either alternative therapeutic approaches need to be explored, or treatment may need to be initiated earlier as a preventative approach before the Aβ pathology begins or worsens.

The combination treatment we use in this study may better serve as a preventative therapy for AD. Our results from the ENR treatment strongly support a maintenance effect that likely works on a mechanism completely separate to that by which the VAC component of the combination therapy did. While the ENR appears to be acting on the mechanism of Aβ accumulation, the VAC works by clearing pre-existing Aβ pathology. Together, the combination treatment could be a viable therapeutic approach for the prevention of AD. With the age of onset for AD pathology varying between individuals, determining a timeline for administration of a preventative treatment becomes difficult. Using a combination

therapy approach such as ENR with VAC could target both the prevention of Aβ accumulation as well as the clearance of any likely little pre-existing Aβ pathology. This type of treatment approach could allow for some flexibility when determining a timeline for administering the treatment in a given individual. Ultimately, the treatment could act early enough to prevent any possible irreversible damage being done to the neurons by the Aβ pathology and avoid resulting cognitive deficits.

CHAPTER EIGHT: Future Directions

While the approach of using VAC in combination with ENR in this study did not lead to additive effects in reducing Aβ pathology or improving cognition in aged canines, we did see interesting and novel cerebrovascular benefits due to ENR that had otherwise not been seen in the previous canine study. It would be of great interest to measure the amount of blood vessel Aβ pathology in the study animals and determine if there is a correlation between these levels and increased CSF $A\beta_{1\text{-}40}$. We would hypothesize that there would be a correlation between lower levels of blood vessel pathology and increased levels of CSF $A\beta_{1\text{-}40}$ and that this correlation would be seen in animals receiving ENR. Additionally, the changes in Aβ plaque pathology seen due to ENR indicate that a maintenance effect is likely occurring in ENR treated animals. To further investigate this maintenance idea, α- and β-secretase activity could be measured. In a previous canine study exploring the effects of an antioxidant diet and ENR on Aβ load, α- secretase activity was increased in animals receiving ENR (322). For the current study we would hypothesize that ENR is promoting α-secretase activity resulting in ENR animals having lower β-secretase activity and increased non-amyloidogenic processing compared to non-ENR animals.

The focus of this study was to examine effects on cognition and Aβ pathology due to the combination treatment. Other neurological changes that have previously been reported in past ENR studies have not yet been explored in these animals. For instance, although neurogenesis was not increased with ENR in a previous aged canine study, neuron number in the hippocampus and the growth

factor BDNF are maintained and improved, respectively, in ENR treated dogs (116, 278, 279, 407). Future directions of this project would be to explore these changes in the present study animals and comparing those in the E/V treatment group to all other groups. One could hypothesize that the both the E/C and E/V treatment groups would show higher levels of BDNF and potentially enhanced neuron survival and neurogenesis, with the E/V group experiencing greater improvement.

It was noted that the VAC was successful in reducing modified Aβ, AβpE3, pathology that has been previously shown to be more toxic. Additionally, AβpE3 correlates with the hyperphosphorylation of tau. Though canines do not produce NFTs with age, they do show hyperphosphorylation of tau at sites that coincide with those affected in humans with AD. It would be of interest to measure levels of soluble and insoluble tau in our treated canines and see if a correlation exists between these measures and the effects of VAC on AβpE3 plaque pathology. One could hypothesize that levels of insoluble tau in C/C and E/C treatment groups would be higher than those of the C/V and E/V treatment groups. The effects of VAC in reducing AβpE3 pathology could lead to the de-hyperphosphorylation of tau or the prevention of additional hyperphosphorylated insoluble tau.

As mentioned earlier, the combination approach tested in the current study may have exhibited greater additive effects, particularly on cognition, had the treatment been started at an earlier age in the canines. As a future project, this combination treatment could be tested in canines around 7 to 8 years old, just as Aβ begins to accumulate. At this age, Aβ pathology should be minimal and

cognitive changes would be predicted to be mild. ENR started at this age would promote healthier brain aging as earlier studies have shown ENR improve neurogenesis and reduced neuronal loss (116, 408). From our findings in this study, ENR should also reduce the risk of microhemorrhage occurrence which can contribute to cognitive decline. Additionally ENR prevented or slowed the rate of Aβ plaque accumulation and would be hypothesized to aid in preventing Aβ in younger canines. With ENR and VAC acting on separate pathways, one could hypothesized the resulting immune response would further assist in preventing additional Aβ pathology and clearing out any pre-existing early Aβ plaque formation before any neuronal damage could occur.

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VITA

Paulina R. Davis
University of Kentucky College of Medicine

EDUCATION

2010-Present	Ph.D. Candidate	Department of Molecular and Biomedical Pharmacology University of Kentucky College of Medicine Lexington, KY GPA – 3.52
2005-2009	B.S.	Major: Biological Sciences University of California, Irvine Irvine, CA
2001-2005		Temecula Valley High School Temecula, CA

Prior Work Experience

2007-2009	Research Assistant Institute for Brain Aging and Dementia University of California-Irvine, Irvine, CA
2008	Quality Tutoring Services 1585 W Broadway, Anaheim, CA 92802
2006-2007	Research Assistant Department of Biochemistry University of California-Irvine, Irvine, CA

ACHIEVEMENTS, HONORS AND AWARDS

2014	Alzheimer's Association International Conference Volunteer Program Copenhagen, Denmark
2013-Present	Center for Clinical and Translational Science TL1 Program University of Kentucky, Lexington, KY TL1RR033172
2013	Lyman T. Johnson Award University of Kentucky, Lexington, KY

2009-2010	Post-Baccalaureate Research Education Program (PREP) University of California–Irvine, Irvine, CA
2009	Excellence in Research University of California–Irvine, Irvine, CA
2009	Minority Health and Heath Disparities International Research Training (MHIRT) Cavanilles Institute of the Universitat de Valencia, Valencia, Spain NIH T37MD001485
2007-2009	Minority Access to Research Careers (MARC) University of California–Irvine, Irvine, CA NIH T34GM069337
2006-2007	Minorities in Biomedical Research Support (MBRS) University of California–Irvine, Irvine, CA NIH R25GM055246
2006	Academic Dean's List University of California–Irvine, Irvine, CA

PUBLICATIONS

Bruce-Keller, A.J., Gupta, S., Knight, A.G., Beckett, T.L., McMullen, J.M., Davis, P.R., Murphy, M.P., Van Eldik, L.J., St Clair, D., Keller, J.N. Cognitive impairment in humanized APPxPS1 mice is linked to Aβ (1-42) and NOX activation. *Neurobiology of Disease*. 2011: 44: 317-326.

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PLATFORM PRESENTATIONS AND SEMINARS

Davis, P.R. "A Combination Approach in a Canine Model of Aging: Effects of Immunotherapy and Behavioral Enrichment on Beta-Amyloid Pathology" Pharmacology Departmental Seminar Series, University of Kentucky, Lexington, KY. 2013.

ABSTRACTS AND POSTER PRESENTATIONS

Davis, P. R., Beckett, T., GIannini, G., Platt, T., Murphy, M. P., Barrett, E., Head, E. (2014) Beta-Amyloid Immunization with Behavioral Enrichment in a Canine Model of Aging. Annual Translational Science 2014 Annual Meeting, Washington, D.C.

Davis, P. R., Beckett, T., GIannini, G., Platt, T., Murphy, M. P., Barrett, E., Head, E. (2014) Effects of Immunotherapy and Behavioral Enrichment on Beta-Amyloid Pathology in a Canine Model of Aging. Annual CCTS Spring Conference, Lexington, KY.

Davis, P. R., Beckett, T., GIannini, G., Platt, T., Murphy, M. P., Barrett, E., Head, E. (2013) Immunotherapy in Combination with Behavioral Enrichment: Beta-Amyloid Changes in a Canine Model of Aging. W.R. Markesbery Symposium of Aging and Dementia, Lexington, KY.

Davis, P. R., Beckett, T., GIannini, G., Platt, T., Murphy, M. P., Barrett, E., Head, E. (2013) A Combination Approach in a Canine Model of Aging: Effects of Immunotherapy and Behavioral Enrichment on Beta-Amyloid Pathology. Society for Neuroscience conference, San Diego, CA.

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Davis, P. and Head, E. (2009) The Changes in soluble tau as a function of age in dogs comparison with AD. American Association for the Advancement of Science conference, Chicago, IL.

Davis, P. and Head, E. (2008) The Changes in soluble tau as a function of age in dogs comparison with AD. Sigma Xi annual conference, Washington D.C..

Davis, P., Sarsoza, F., Saing, T., Head, E. (2008) Lower Alzheimer's disease pathology in an adult with mosaic Down syndrome - A role for inflammation. American Association for the Advancement of Science conference, Boston, MA.

Davis, P., Sarsoza, F., and Saing, T. (2007) Lower Alzheimer's disease pathology in an adult with Down syndrome with leptomeningitis - A role for inflammation in Alzheimer's disease. Annual Biomedical Research Conference for Minority Students (ABCRMS) Austin, Texas.