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Tau-Directed Immunotherapy for Alzheimer’s Disease

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Tau-Directed Immunotherapy for Alzheimer’s Disease

by

Sulana K Schroeder

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy with a concentration in Neuroscience Department of Molecular Pharmacology and Physiology College of Medicine University of South Florida

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ABSTRACT

Alzheimer’s disease (AD) is the leading cause of dementia, accounting for 50 to 80 percent of dementia cases, and the prevalence of the disease is projected to increase significantly with time. AD is characterized by severe cognitive decline with age, ultimately requiring continued caregiving and eventually death. The pathology of AD is characterized by the presence of extracellular amyloid plaques, intracellular neurofibrillary tangles (NFT) composed of hyperphosphorylated tau protein, neuron loss, and evidence of inflammation indicated by the presence of reactive microglia and astrocytes. Frontotemporal Lobe Dementia (FTLD) is a rare form of dementia that is related to AD, most notably in the pathology of hyperphosphorylated tau and macroscopic brain shrinkage. It has been defined as one of a host of tauopathies, and has a more rapid onset than AD. Symptoms that resemble personality changes, moreso than memory loss, are characteristic of these other tauopathies (FTLD is a representative of a whole class of neurological disorders). Like AD, there are no known treatments or cures for FTLD. AD and FTLD are two manifestations of a class of diseases known as tauopathies, due to the presence of toxic forms of tau.

Tau is a protein normally found in neurons. It functions as a stabilizer for microtubules, and has a role in the trafficking of materials from the cell body to the presynaptic terminal. In AD and FTLD, tau can become hyperphosphorylated, which causes it to form twisted fibrils called NFTs. An emerging area of research is to identify antibodies that target tau as a way to clear tau pathology and hopefully reduce synaptic and neuron loss (Boutajangout et al., 2011b). While these diseases have no known cure or treatment at present, immunotherapy is emerging as a very promising approach
for treatment. The studies presented here investigated a variety of antibodies directed against tau, and incorporated different timeframes and administration routes to identify the best candidate for future clinical investigation of tau immunotherapy.

The mouse model rTg4510, known for expressing cognitive-related tauopathy, was primarily used to evaluate tau antibody effectiveness prior to clinical consideration. Our investigations began by utilizing a more familiar mouse model which was also reported to express tau pathology.

Our studies first examined intracranial injection of a variety of antibodies using an mouse model previously reported to demonstrate tau pathology, to identify short-term clearance of tau pathology and NFTs. Next, we examined a more robust tau-producing mouse line, to further identify a most effective antibody, as well as to examine the time course of effect, after administration. A longer-term administration, and different route of administration was tested using mini-osmotic pump implantation into the mice, which provided for 28-day continuous infusion. This approach was followed with administration of antibodies, systemically. Behavioral analysis, in addition to pathological testing, was incorporated into the longer-term administration studies.
CHAPTER 1: BACKGROUND AND SIGNIFICANCE

1.1 Alzheimer’s Disease

Alzheimer’s disease (AD) is currently the sixth leading cause of death in the United States (US). An estimated 5.4 million people currently are suffering from AD and it is the only cause of death, of the top 10 in the US, which cannot be treated, prevented, or slowed acceptably yet. It is a slowly developing and progressive disease that affects memory such that patients eventually lose their ability to function and interact with the environment. Early clinical symptoms include difficulty remembering names and recent events, as well as frequent cases of apathy and depression. Later symptoms include impaired judgment, disorientation, confusion, behavior changes, and difficulty speaking, swallowing, and walking (Alzheimer’s Association, 2011). A diagnosis of AD is one of the darkest diagnoses a person, and his or her family and friends, can receive. People, (friends, relatives, and caretakers), who surround and support an advanced AD patient, report that they no longer recognize the patient, just as the patient has no memory or recognition of the people in their lives.

AD is not only debilitating and deadly, it is also very costly. There are approximately 15 million unpaid caregivers in the US who incur an annual cost of about $200 billion. Due to the toll on caregivers, about one-third of caretakers report symptoms of depression, and nearly two-thirds of them report high levels of stress.
These burdens significantly increase the cost of health care to the caregivers, themselves.

Our population is aging due to other health care advances, however this aging population, and especially that of the ‘baby boomer generation’, is transitioning into a dramatic rise in symptoms of AD, as well as increasing familial burdens to care for these beloved aging people. It is projected that the cost of care for this disease will be $1.1 trillion by 2050 (Alzheimer’s Association, 2011). Therefore it is of utmost importance for patients and caregivers that treatments for AD are identified.

1.2 Alzheimer’s Disease and other Dementias - Pathology

AD pathology can be described macroscopically and microscopically. Macroscopically, widespread atrophy of the brain and associated cerebral loss is observed, along with cerebral amyloid angiopathy (CAA). Microscopically, there are numerous markers of the disease: extracellular deposition of amyloid β protein (Aβ), in the form of amyloid plaques (dense core plaques), and extracellular soluble Aβ, which are formed by accumulation of the Aβ40 or Aβ42 molecule. Intracellularly, the microtubule stabilizing protein tau becomes hyperphosphorylated, forming neurofibrillary tangles (NFTs), which lead to dystrophic neurites and synapse loss. Evidence of inflammation in the affected areas is also shown as recruitment of microglia and astrocytes. Ultimately there is a loss of neuronal cells in the affected areas, and an additional increase in inflammatory cells presents. Development of plaques, NFTs and
inflammatory indications of degeneration can predict a future of AD or other tauopathies.

The US Food and Drug Administration (FDA) has approved at least five drugs that slow the progression of the disease by 6 to 12 months, however there are no drugs as of yet that delay the onset, or halt progression of the disease (Alzheimer’s Association, 2011). Accordingly, there is a large research effort to identify drugs that can significantly reduce or even stop disease progression. Recently, immunotherapy has shown promise in clearing amyloid pathology or tau pathology in transgenic (Tg) mouse models of the disease. Immune-based drugs, targeting different components of AD and tauopathy, are in clinical trials.

1.3 Immunotherapy Concepts and Mechanisms

Immunotherapy consists of administration of a substance that will provide treatment by way of modulating the immune system: antigens that lead to an endogenous immune response have been examined, also antibodies directly directed against the foreign or unwanted entity are used to remove and destroy the invading entity (plaques and/or tangles).

The use of antigen administration has been described as the “humoral” response, which refers to the fact that antibody-mediated interactions occur in the “humors,” namely the extracellular fluids such as plasma, lymph, and the non-cellular portion of blood. However, it was discovered that cells (in particular, macrophages, as well as T-cells, and natural killer cells, not just serum), are important for eliciting immune
responses. This understanding has lead to a division in definition, between “cellular immunity” and “humoral immunity.”

Lymphocytes are the cells responsible for adaptive immunity. Two main categories of lymphocytes have been identified: B cells, which are derived from bone marrow, and T cells, which are produced in the thymus. B cells synthesize immunoglobulin (Ig) molecules, otherwise known as antibodies, and also inherently possess Igs in their plasma membrane to recognize foreign or unwanted material (antigens). B cells possess Igs for one specific antigen, and only one antigen. B cells act as memory cells after their first encounter with the specific antigen, and remain as memory cells to facilitate the immune system to act more quickly and robustly upon future encounters with the antigen.

T cells, in contrast, possess receptors that recognize glycoproteins on cell membranes to identify ‘foreign’ agents for breakdown and removal. Major histocompatibility complex (MHC) T-helper (T$_H$) cells, also known as CD4+ T cells because they possess CD4 on their surface, become activated when they are presented with antigens possessing MHC class II antigenic components. Once activated, T cells will secrete cytokines to assist in the active immune response. Cytotoxic T cells (T$_C$), also known as CD8+ T cells, possess CD8 glycoprotein on their surface, and recognize antigen associated with MHC class I antigens leading to this immune response. Upon recognition binding, they actively destroy the invading cells or pathogens.
Antibodies are specific immunoglobulin (Ig) entities that act as adapter molecules. They consist of two heavy chains of amino acids and two light chains that are connected by disulfide bonds in a Y configuration. The two antigen-binding regions are defined as the F(ab) regions. Two such binding domains are present on an antibody, thereby requiring binding of either two sites from one antibody, or usually, binding by two antibodies.

The constant region, located on opposite arms of the antibody, is referred to as the Fc region. The Fc region binds to Fc receptors located on phagocytic cells. Fc binding facilitates antigen breakdown and removal with help from the monocytes, or related phagocytic cells possessing proper binding recognition for the Fc portion of the bound antibody.

Each F(ab) region possess two constant regions, one on the outer, antigenic binding region; a heavy chain peptide, with an additional, modifying component comprise the light chain of the F(ab) regions of an antibody. Two, variable, light chain regions are similarly arranged as the heavy chains, within an antibody, however the light chains are only located within the antigen binding region of the antibody, thereby increasing recognition of multiple foreign substances. The F(ab) variable region determines the antigen specificity of the Ig, whereas the Fc region determines the cellular response in result to antibody binding.

Antibodies are classified as either monoclonal (resulting from one B cell specific to one cell specific to antigen, which is replicated or cloned to produce numerous identical antibodies), or polyclonal (a composite of multiple B cells that can recognize
multiple antigens, possibly from one individual protein, and are hybridomas of immune cells with cells exhibiting rapid proliferation, such as cancer cells). Polyclonal antibodies target multiple antigens, and thereby exhibit a greater spectrum of recognition. However, since they are not generated from a specific singular cell polyclonal antibodies possess the potential to simultaneously target a greater multitude of antigens, but also possess a greater potential for inducing “side effect” since they are less discriminating.

Monoclonal antibodies are advantageous for immunotherapy, due to their specificity as a result from cloning of one cell. This minimizes potential side effects, as they target very specific antigens. On the other hand, this specificity can result in monoclonal antibodies ‘missing’ the target antigen and failing to launch an appropriate immune response.

Polyclonal antibodies are produced as hybridomas; hybridomas incorporate immune cells into cancer cells to create multiple, quickly proliferating, copies of a desired antibody. As such, polyclonal antibodies can be preferable to monoclonal antibodies to increase a generalized immunological response to treatment, however they possess lower specificity for single antigens, leading to a greater potential of generating undesired, or unintended, potential for inducing ‘side effects’.

Antibodies utilize the body’s immune system by binding to phagocytic cells, then initiate a cellular response to target the antigen and prepare it for phagocytic destruction an removal. Binding of antigen to antibody leads to an intracellular response. Based on the binding properties, and generated responses of such binding, five classes of
antibodies exist. These are different Ig structures (isotypes) referred to as: IgG, IgA, IgM, IgD, and IgE. These isotypes of antibody vary, based on their Fc region and the cellular events that occur upon binding to their receptor. IgG is the most abundant of the classes, and is the class that is emphasized herein due to the presence of microglia in neuronal tissue.

Antibodies effect a response after forming an antigen-antibody complex. Following antigen binding to a cell with Fc receptors, opsonization of the antigen occurs, which is defined as the coating by adhesion molecule(s), to initiate complement deactivation, or to direct phagocyte engulfment of the antigen. These two processes can work in concert, or independently, to lead to destruction of the bound antigens. Additionally, microglia respond to foreign substances, then are activated to secrete cytokines, which help to stimulate T-cell mediated immune responses and inflammation. Microglial activation, the neuronal component of the immune system, is an important process to evaluate so as to protect the BBB separated CNS.

1.4 Mechanisms of Immunotherapy

Immunotherapy directs the immune system to remove unwanted material from the body. This can be accomplished by amplifying the body’s innate immune system to remove antigen. Immunotherapy can also be implemented to remove an unfamiliar antigen, by activating the acquired immune system (memory immune cells and phagocytes), and by introducing antigen to the affected body to spur creation of new antibodies, which recognize this foreign substance. Exposure to an unfamiliar pathogen
or antigen will lead to the immune response, which will begin to create antibodies within the invaded organism, to eliminate the ‘foreign invader’ and to form memory cells in case this foreign invader is encountered again in the future.

Vaccination is the most common form of immunotherapy. It is an established, safe process to expose humans and other organisms to antigen to induce the recipient to produce their own adaptive antibodies. This is typically accomplished by injection of the foreign antigen, although vaccines can also be inhaled via a nasal spray, absorbed via a dermal patch, or ingested.

Edward Jenner first attempted immunotherapy procedures in 1798 by using cowpox exposure to prevent the more severe smallpox (Sanchez-Sampedro et al., 2015). The cowpox virus was similar enough to the smallpox virus that he realized exposure to one could lead to development of resistance to the other. Louis Pasteur is credited with popularizing active vaccination, and creating the term “vaccination” when he had chickens experiencing cholera. He injected “old” versions of the pathogen into younger chickens whereby they developed immunity to the disease. From there, he moved on to vaccination of sheep against anthrax, and eventually translated these experiences into human vaccination (rabies vaccination) such that people would develop immunity to a given pathogen for future exposures. Since his time, vaccines have nearly eliminated diseases such as measles, mumps, tetanus, polio, and whooping cough.

Immunization can be defined as passive or active, which describes the nature of the substance injected as well as the anticipated physiological response to it. Active
immunization is the administration of an antigen, or an inactivated (attenuated) version, a fragment of the full antigen or a biochemical analogue. Active immunization triggers the body’s adaptive system to respond by producing antibodies against the toxin. A majority of the antibodies will act to remove the toxic agent, however some will help to form memory cells in case of future exposure. These memory cells help produce an exacerbated response with each future exposure to the antigen.

In contrast to active immunization, passive immunization involves administration of already produced antibodies directed against the toxic substance. In humans, the half life of an exogenous antibody is about 21 days (Stowell, 2006). Because the antibodies are being introduced and not endogenously produced, they will essentially get used up, as they bind to antigen, leading to a decline of function. Therefore, passive immunotherapy requires routine and continual inoculation, whereas active immunization can require very few treatments provided the patient responds to the immunogen and launches his or her own immune response, properly (without serious side effects).

An adjuvant is frequently provided with active vaccines to boost the immune system’s response to the foreign substance (the immunogen, or antigen). Adjuvants increase the immunogenicity of the administered compound by activating macrophages (such as microglia) and they increase the antigen-presenting feature of an antigen, thereby helping to increase endogenous production of antibody; adjuvants stimulate the immune system to produce a greater response than the antigen alone would produce. “Freund’s adjuvant,” is commonly used and is well tolerated in animal studies. Most adjuvants consist of aluminum-based compounds such as alum. Alum stimulates Th2
responses (anti-inflammatory), while Freund’s Adjuvant stimulates Th1 responses (pro-inflammatory). Another common adjuvant, Quil A, is often used in veterinary practices, and the equivalent for human clinical studies is called QS-1 (discussed below) (Ghochikyan et al., 2006, Ragupathi et al., 2010).

1.5 Role of Glial cells in Inflammation and Treatment of Neuronal Disorders

Microglia are the primary immune cells in the CNS, and are a neuronal-based class of myeloid-derived monocytes, dedicated to the brain and CNS to function as the privileged phagocyte within the CNS and peripheral nerves. These cells have been demonstrated to present in association with amyloid plaques and other sites of neurological damage or insult. Microglial cells possess Fc receptors: Fcγ-R receptors bind to the Fc portion of the antibody to effect cellular responses. As such, these cells have been closely examined when neuro-immunotherapy is investigated.

1.6 Immunotherapeutic Approaches to Alzheimer’s Disease

Immunotherapy was partially pioneered in the 1980’s (Shenk et al., 1984), and then was applied to AD (Simpson et al., 1987, Schenk et al., 1999b). The initial AD studies focused on clearing Aβ, which forms the extracellular amyloid plaques characteristic of this disease. Aβ is produced by proteolytic cleavage of the amyloid precursor protein (APP) by β-secretase and γ-secretase. The Aβ peptide can be either 40 or 42 amino acids in length, with the Aβ_{42} peptide being more prone to form β-pleated sheets than Aβ_{40}. Aβ accumulation can lead to formation of oligomers, fibrils, and dense-core plaques.
Early treatment approaches for AD have focused on Aβ, in part due to the genetic correlates between AD and Aβ. The E4 allele of apolipoprotein E (ApoE4) is one of the major risk factors for developing AD, and the presence of this allele is associated with increased amyloid burden. Additionally, mutations in the genes for amyloid precursor protein (APP), presenilin 1 (PS1), and presenilin 2 (PS2), are involved in the production of Aβ, specifically the aggregation prone Aβ42 (Citron, 2010).

Studies targeting Aβ, by drug or immunotherapy, have shown great promise in mouse models of AD. The first reported immunotherapy study for treating AD was conducted by Schenk et al, of Elan Pharmaceutical (Schenk et al., 1999b). Synthetic human Aβ42 or serum amyloid-P component (SAP), with Freund’s Adjuvant, were peripherally injected into PDAPP mice, with controls receiving phosphate buffered saline (PBS) plus adjuvant or no treatment. Mice were injected at 6 weeks of age, before AD pathology develops, or at 11 months, when pathology is well established. They found that the mice developed large antibody titres; 8 of 9 of those injected with Aβ had titres greater than 1:10,000, and those injected with SAP ranged between 1:1,000 and 1:10,000 with one mouse exceeding this level. At 13 months, the animals were evaluated for immunohistochemical (IHC) markers of AD pathology and the Aβ42 vaccinated mice showed almost no pathology, while the SAP vaccinated mice exhibited pathology similar to controls. Older vaccinated mice were given a similar vaccination and evaluation schedule, and those vaccinated with Aβ42 showed markedly reduced pathology compared to controls (Schenk et al., 1999b).

This study was followed by work in our own laboratory, using a different mouse model of AD, the APP/PS1 mouse, which has been shown to display cognitive deficits
resembling AD in concurrence with the development of amyloid pathology. Mice were vaccinated subcutaneously starting at 5.5 months of age with either Aβ_{42} or keyhole limpet haemocyanin (KLH) adjuvant, alone, as a control. Vaccinated mice were tested for behavioral/cognitive effects, using the radial arm water maze (RAWM). At 11.5 months, all mice were able to learn the platform location, suggesting that the immune response elicited by the vaccinations did not impair working memory. The vaccinations were continued, and when the mice were re-tested at 15.5 months of age, the Aβ_{42}-vaccinated mice showed superior learning compared to the KLH-injected controls. Pathological testing for Aβ pathology showed that the Aβ_{42}–vaccinated mice exhibited reduced plaque-burden and less Aβ_{42} and Aβ_{40} than the respective controls (Morgan et al., 2000a). This study verified the Schenk study using a different AD mouse model, and extended that study to demonstrate corresponding cognitive improvements in response to active immunotherapy. Additionally, this study demonstrated no detrimental inflammation resulting from the treatment.

Concurrent with the above studies, another group reported results of vaccinating TgCRND8 mice with Aβ_{42}. Approximately 50 percent reduction in dense core amyloid plaques was identified. There was no difference in total Aβ levels and reduced cognitive deficit in the Morris water maze (Janus et al., 2000).

Numerous additional studies followed, using various immunogens, adjuvants, and routes of administration. The majority of these studies demonstrated that B cell epitopes reside within the first 15 amino acids of Aβ, while T cell epitopes exist in the Aβ mid-region and C-terminus. Immunogens can be designed to favor Th2 vs Th1 immune responses (Lemere, 2009). This research addressed concerns of autoimmunity, in that
the administration of a peptide fragment of a protein can elicit both a protective protein-clearing effect via B cells, and a cytotoxic T cell response to the endogenous Aβ.

A consideration when immunizing with peptide fragments, rather than the entire protein, is the tertiary conformation of the antigen. Variation of structures can influence the ability to produce effective antibodies to the immunogen.

Antigen-Ig binding is conformation specific, so it is not surprising that injection of a fragment of amyloid failed to elicit immune responses in some of the animals because its tertiary structure was not identical to the antigenic portion of the full protein. These considerations become important when translating the animal experiments to the clinic, as described below.

1.7 Translation of Immunotherapy Using Animal Models To Human Applications

Initial active immunotherapy studies in animals led to a series of passive immunization attempts. Elan Pharmaceuticals led this endeavor by injecting one of two monoclonal antibodies against Aβ (10D5 or 21F12), or a polyclonal Ig fraction from their Aβ1-42 vaccinated mice, or PBS control. Mice were injected intraperitoneally (i.p.) on a weekly basis for 6 months, then were evaluated for pathology. They found that the polyclonal antibody reduced plaque burden by 93%, and the 10D5 monoclonal antibody reduced it by 81%. They proceeded to examine two additional antibodies, 3D6 and 16C11, finding similar plaque reduction with 3D6, but no effect from the 16C11 antibody. These studies identified no T-cell proliferation, leading to the conclusion that a T-cell response is not necessary for plaque clearance. Fluorescent tagging revealed
that the antibodies had entered the brain and found their way to the plaques. Further investigation revealed that these antibodies had cleared pre-existing plaques, and that microglia were involved in the clearance via Fc receptor-mediated phagocytosis (Bard et al., 2000).

While the mouse data appeared to be a promising method for treating AD pathology and memory loss, complications in translating the mouse studies to human clinical trials has been established. Several drugs that target Aβ immunologically have been or are currently in human trials.

The first clinical trial, the AN1792 trial conducted by Elan and Wyeth, reported administration of an active vaccination by injecting full length Aβ₁₋₄₂. Three hundred patients were vaccinated intramuscularly with full length Aβ₁₋₄₂ in a strong Th1 adjuvant, QS-21 (described above), while 72 participants received a placebo control. A positive antibody response was identified in 59 (22%) of the immunized individuals, while the remaining 241 patients were considered non-responders as determined by plasma antibody titers. Significant reduction of CSF tau was additionally observed in the antibody-responders. However no significant difference was found in a battery of neuropsychological tests, between the vaccinated and placebo groups. In Phase II of the study, 18 of the 300 vaccinated patients (6%) developed meningoencephalitis, as evidenced by magnetic resonance imaging (MRI), while none of the placebo group developed this condition. Thirteen of the antibody responders (22%), and five of the non-responders were found to have enlarged ventricles and reduced overall brain volume. The incidence of meningoencephalitis was not correlated with serum Aβ levels. Therefore it was unclear whether this side effect was due to the Aβ antibody
itself, or to T cell mediated inflammation, or to microglial responses indicative of a possible autoimmune reaction. Additionally, it was thought that the chosen QS-21 adjuvant may have caused the cytotoxic T cell response. Follow-up of two of the subjects revealed elevation of soluble Aβ, which can contain toxic oligomers (Orgogozo et al., 2003, Bayer et al., 2005, Gilman et al., 2005, Patton et al., 2006). A 5-year follow up showed no difference in the rate of overall decline of the patients, and despite plaque reduction, there was no significant difference in survival or the time to development of severe dementia (Holmes et al., 2008). The clinical trials for AN1792 were ultimately terminated due to the negative side effects and the lack of psychological improvement in the patients.

Despite the early termination of the AN1792 study, investigation into anti-amyloid immunotherapy has continued. Currently three passive immunization drugs are in human Phase III trials to clear Aβ: Bapineuzumab—a humanized monoclonal antibody, 3D6, which binds to an N-terminal epitope of Aβ deposits and reduces amyloid load primarily by microglial clearance, and was studied by Elan and Wyeth; Solanezumab—a humanized monoclonal antibody, 266, that binds to the mid-region of soluble Aβ and reduces amyloid load via the peripheral sink method, was evaluated by Eli Lilly and Company; additionally Baxter examined intravenous IgG (IVIG)—a human immunoglobulin preparation that contains endogenous polyclonal antibodies to Aβ and that binds soluble Aβ and reduces amyloid load through the peripheral sink method (Citron, 2010, Joly-Amado et al., 2014).

The extensive body of work to date on Aβ immunotherapy has shown that both active and passive vaccination can result in clearance of dense core plaques and other
Aβ-associated pathology of AD, in mouse models and in human trials. Antibodies to Aβ can cross the blood-brain barrier (BBB) and solubilize plaques, reverse or prevent plaque pathology and even prevent or reverse some associated cognitive loss.

Four potential mechanisms, which may work independently or together, have been proposed to explain the method of Aβ clearance by antibodies (Lemere, 2009, Citron, 2010, Morgan, 2011a):

- **Microglia Mediated Clearance:** This model suggests that circulating antibodies reach parenchymal amyloid deposits and bind to Aβ, then the Fc portion of the antibody binds to the Fc receptor on microglia to facilitate phagocytosis and removal of the plaques and/or Aβ. This hypothesis is supported by the early work reported by Bard et al (Bard et al., 2000) and by the presence of microglia around plaques (Bacskai et al., 2002).

- **Antibody-Antigen Direct Resolution:** This model suggests that the binding of antibody to antigen may prevent aggregation and/or dissolve pre-formed aggregates. It is as if the antibody breaks down the toxic aggregates of Aβ, as if the antibody were an enzyme. This proposed mechanism is supported by work by Solomon et al (1997) who showed in vitro that some site-directed monoclonal antibodies disturb the assembly of fibrillar amyloid, observed in Aβ aggregation and plaque formation.

- **Peripheral Sink:** Antibodies may not be required to cross the blood-brain barrier (BBB) in order to clear amyloid from the brain. This model suggests that antibodies in the bloodstream act on amyloid in the blood, altering the equilibrium between the brain and the vasculature, causing an efflux of amyloid out of the
brain, into circulation for removal. This notion is supported by studies that have shown effective plaque removal in the brain accompanied by high circulating concentrations of Aβ.

- *Alteration of toxic species:* This model suggests that interaction with antibodies modifies the secondary structure of amyloid by breaking down plaques or oligomers into less toxic entities. This process would resemble a catalytic breakdown of amyloid, and is supported by the small ratio of antibody to antigen that is needed. Unlike the ‘direct resolution’ mechanism, this hypothesis just addresses the aggregation or dis-aggregation of the unwanted protein. Support for this hypothesis comes from work conducted by Morgan et al (2011), which revealed antibody to Aβ stoichiometry levels of a magnitude of 1:1000 (antibody to Aβ). If demonstrated, this would be a preferable mechanism because it could produce robust clearance of Aβ and plaques without the involvement of microglia or other immune-system entities.

Some questions remain as to which of these potential mechanisms are the pathways at work in immunotherapy studies. It is possible that multiple pathways are involved, and that the pathways might be antibody-specific.

A potential limitation to Aβ immunotherapy is that amyloid pathology develops well before cognitive and behavioral symptoms appear, so strategies focused on Aβ clearance may prove too late in the pathological process to fully ameliorate the disease (Krishnamurthy and Sigurdsson, 2011, Morgan, 2011a). In addition, Aβ plaque load, unlike NFTs, doesn’t correlate linearly with severity of dementia (Bierer et al., 1995). This became evident when patients from the AN1792 trial were brought to autopsy and
showed post-mortem evidence of significant, if not complete, removal of amyloid pathology, however little or no cognitive benefit from drug administration had been observed (Holmes et al., 2008). Another follow-up study identified the distinction between antibody responders and non-responders in human patients: when the antibody responders were compared to patients that received placebo, a significant reduction in functional decline was observed using the Disability Assessment for Dementia scale and the Dependence Scale (Vellas et al., 2009). Additionally, no further cases of encephalopathy were observed in this follow-up study.

Amyloid deposits and plaques can also be found within the vasculature of the brain, a condition that is referred to as cerebral amyloid angiopathy (CAA) (Beckmann et al., 2016, Diem et al., 2016, Merlini et al., 2016). Amyloid deposits can exist throughout the vasculature, in addition to being observed in the brain. Early treatment approaches for AD focused, in part, on removing Aβ (amyloid) plaques, due to the genetic correlates between AD and CAA. The E4 allele of apolipoprotein E (ApoE4) is one of the major risk factors for developing AD, and the presence of this allele is also associated with increased amyloid burden, as plaques, in both the brain and peripheral vasculature (Miller-Thomas et al., 2016, van Veluw et al., 2016).

CAA may result in the development of microhemorrhages. Therefore clearance of parenchymal amyloid plaques has proven to be a confounder when investigating Aβ immunotherapy. While reports of Aβ immunotherapy have shown significant reductions of soluble amyloid in the parenchyma, concurrent increases of cerebral microhemorrhages associated with amyloid-laden vessels have been observed. Perhaps such vascular components may be due to a weakening of the vessel walls.
caused by a local inflammatory response (Pfeifer et al., 2002). This might suggest that the Aβ vaccines clear amyloid, and specifically plaques, in the parenchyma quickly, resulting in a transfer of amyloid from parenchyma to the blood stream, such that amyloid clearance may compromise the integrity of blood vessel walls, which may present as greater degrees of CAA.

1.8 Introduction to Tauopathies

Recent work for treating dementias (including AD) has turned towards treatments that can target pathological tau. Tau becomes pathologic in AD and other tauopathies, such as frontotemporal lobar degeneration (FTLD), frontotemporal dementia with associated Parkinsonism linked to chromosome 17 (FTDP-17), Pick’s Disease (PiD), corticobasal degeneration (CBD), and agryophilic grain disease (AGD).

Tau is a microtubule-associated protein that is normally soluble and present in the cytoplasm of neurons. It is a cytoskeletal protein that acts to stabilize microtubules and to facilitate intracellular axonal transport. It interacts with kinesin, thereby affecting cellular transport mechanisms, and has multiple binding sites for microtubules, providing structure and function to cells, particularly neurons. It can also regulate signaling pathways and activate or inhibit several enzymes (Morris et al., 2011). It can be associated with the plasma membrane, where it is phosphorylated at tyrosine residues and might play a role in intracellular signal transduction (Wang and Liu, 2008).

Normal tau is usually found in axons, but in tauopathies tau is redistributed to cell bodies and dendrites. Then, it localizes to dendritic spines and impairs trafficking of
NMDA and AMPA receptors to the presynaptic membrane. This impedes signaling and eventually leads to synapse loss (Hoover et al., 2010). During development, tau can undergo several post-translational modifications, including phosphorylation, glycosylation, ubiquitination, truncation, and nitration (Wang and Liu, 2008). The primary post-translational modification, especially as is relevant to AD and other tauopathies, is phosphorylation. Hyperphosphorylated tau leads to conformational changes and aggregation into oligomers and NFTs (Polydoro et al., 2009). The NFTs seen in AD are hyperphosphorylated, aggregated, insoluble complexes of tau molecules. Hyperphosphorylation leads to the formation of paired helical filaments (PHFs), which become the trademark intracellular “tangles” identified in AD.

Tau protein can be subdivided into 4 regions: an N-terminal projection region, a proline rich domain, a microtubule binding domain (MBD), and a C-terminal region. Six isoforms of tau exist in the human nervous system, which result from variations in the splicing of a single gene located on chromosome 17. The protein ranges in size from 352 to 441 amino acids, based on the presence or absence of exon 2 and/or exon 3, which results in a 29- or 58-amino-acid insert in the N-terminal region.

The MBD can consist of repeats of 31 to 32 amino acids encoded by exon 10. Tau variants are named by based on which N-terminal exons are included (N) and the number of MBD-repeats (R) they comprise: eg. 0N has no N-terminal exons, 1N has exon 2, and 2N has exons 2 and 3, while 3R tau has 3 MBD repeats, and 4R tau has a fourth due to the inclusion of exon 10; (Lee et al., 2001, Morris et al., 2011). The number of repeats affects the ability of tau to bind to microtubules, with 4R tau having a
different affinity for microtubule binding (Espinoza et al., 2008, Wisniewski and Sigurdsson, 2010).

The normal brain has similar levels of 3R and 4R tau. Tauopathies are somewhat characterized by the number of MBD repeats they express; for example PiD only has 3R tau, while AD has a combination of 3R and 4R tau. The amino terminal, which is comprised by a large number of acidic amino acids, projects from the microtubules and may play a role in spacing between microtubules. It may also be involved in connecting microtubules to other cytoskeleton proteins (Wang and Liu, 2008).

Tau mutations have been identified in AD, and in other tauopathies, with 32 mutations having been described in more than 100 families with FTDP-17 (Goedert and Jakes, 2005). Two primary types of mutation can occur: alternative splicing of exon 10, which results in different ratios of MBD repeats (3 repeats vs. 4 repeats), and mutations that alter tau’s interactions with microtubules (Krishnamurthy and Sigurdsson, 2011).

Two mutations, some in exon 12 (V337M), and one in exon 13, R406W—lead to the formation of PHFs that can alter all six isoforms of tau, similar to the filaments found in AD. The P301L mutation in exon 10, which only effects 20 to 25 percent of tau molecules, leads to the formation of twisted ribbons containing the 4R isoform (Goedert and Jakes, 2005). It has been suggested that a correct ratio of 3R to 4R tau may be necessary for its normal function, or that an overproduction of 4R tau could lead to an excess of binding to microtubules, leading to cytosolic accumulation and aggregation of tau (Goedert and Jakes, 2005).
Mutation can cause tau to become hyperphosphorylated, leading to misfolding and disruption of function. Tau has more than 30 serine and/or threonine sites that are subject to phosphorylation. Antibodies directed at these sites enable examination of these phosphorylation sites to assess relative tau pathology. For example, the AT8 antibody recognizes phosphorylation at Ser199/202, an early stage of hyperphosphorylation. Phosphorylation at Ser262 is identified using the 12E8 antibody. AT270 recognizes phosphorylation at Thr181. AT180 identifies phosphorylation at Thr231, and PHF-1 recognizes paired helical filaments phosphorylated at Ser396 & Ser404, a late-stage hyperphosphorylation marker. Ser422 is another AD-relevant phosphorylation site (Wisniewski and Sigurdsson, 2010, Xia et al., 2015).

Phosphorylation at most targets leads to a moderate reduction in microtubule binding. However, Ser262 has been identified as a unique phosphorylation site in that its phosphorylation seems to prove instrumental in leading to microtubule detachment and possible PHF aggregation, as is seen in AD; phosphorylation of this amino acid reduces binding affinity by about 300 percent (Mandelkow et al., 1995).

Several kinases lead to tau hyperphosphorylation, including proline-directed mitogen-activated protein kinases (MAPK), glycogen synthase kinase 3 (GSK 3β) and cyclin dependent kinase (cdk5) (Mandelkow et al., 1995). In general, the greater the phosphorylation of tau, the lower its ability becomes to bind to microtubules. Microtubule affinity-regulating kinase (MARK) phosphorylates Ser262 as well as other serine residues, causing tau to become detached from microtubules, leading to a reduction in length of tau, which results in it becoming unstable, and the resulting function can present as if no tau is present (Mandelkow et al., 1996).
Tau can also undergo post-translational modifications such as truncation, nitration, and more. Caspases and other proteases truncate tau at Glu391 or Asp421 of the C-terminal, producing fragments that can form NFTs or lead to apoptosis (Wang and Liu, 2008). Tau can be nitrated at various Tyr residues, and nitrated tau has been found to be co-localized with NFTs, which may represent oxidative damage.

1.9 Early Studies Investigating Tau-Directed Immunotherapy

Tau is an intracellular protein, which suggests that antibodies may not be able to reach and exert an effect on tau pathology. Early studies have demonstrated that both active and passive forms of immunization clear some of the pathological forms of tau. Rosenmann et al (2006) injected full-length recombinant human tau protein with complete Freund’s Adjuvant (CFA) and pertussis toxin (PT) into wild type C57BL/6 mice to see if this might cause an autoimmune response such as encephalomyelitis, which was observed in clinical trials with Aβ vaccination. The CFA+PT adjuvant was chosen for its proinflammatory effect to enable the researchers to focus on immunotherapy safety issues. Using this approach, they detected encephalomyelitis along with NFT formation 1.5 to 5 months post-injection (Orgogozo et al., 2003).

A follow-up study involved injecting a combination of 3 phospho-tau peptides, along with CFA and PT, peripherally into two NFT mouse models, hypothesizing a reduction of pathology. This peptide combination covered 5 pathogenic phospho tau residues. Encephalopathic symptoms were not seen up to 8 months post-injection, however significant reductions were seen in NFTs and phospho-tau by staining for
Gallyas, AT 8 and AT 180, as well as an increase in microglia population in response to the phospho-tau injections. In this study, these effects were still seen 8 months after a single injection followed by one booster (Boimel et al., 2010).

Initial work by Sigurdsson et al (2008) examined the effects of active immunization with 4E6G7, a computer-designed immunogen peptide of tau amino acids 379 to 408, which includes the PHF-1 phosphorylation sites of Ser396 and Ser404 (a late-stage phosphoepitope in NFTs). This immunogen, or alum adjuvant as control, were injected subcutaneously for 2 to 5 months into JNPL3 P301L mice, which express 4R/0N tau via a FTDP-17-associated tau mutation, P301L (Denk and Wade-Martins, 2009), and develop pathology primarily in motor regions of the brain: the motor cortex, brainstem, and spinal cord. Alum was chosen as an adjuvant because it is well tolerated and leads to a Th2-type immune response.

The vaccinated mice were found to have reductions of insoluble tau and increases in soluble tau. The mice also exhibited improved performance on rotarod and balance beam tests. Additionally, a gender difference was observed, with female mice expressing greater levels of tau pathology. These differences were observed both before and after treatment, compared to male mice. It has been suggested pathological tau was cleared at higher rates in females, compared to males (Bayer et al., 2005, Sigurdsson, 2008).

A series of investigations have been conducted to investigate the physiologic location(s), and access of tau antibodies to such locations, within the brain.
Intracellular and extracellular presence (or absence of) antibodies the passage of antibodies from the blood and CSF, into into neurons and other parenchymal tissue.

Sigurdsson’s group collected and purified antibodies generated against the tau P301L immunogen, then FITC-labeled them, and injected the antibodies into the carotid arteries of the JNPL3 P301L mice. Subsequent histochemistry was examined, and identified labeled antibodies to be primarily located in the brain, within neurons, and co-localized with markers for pathologic tau. The labeled antibodies also were injected into non-transgenic animals and no uptake into the brain was observed, suggesting that the BBB likely is compromised in the transgenic animals. These results may extrapolate into humans with tauopathies, thereby facilitating antibody transport from the periphery, into the brain (Sigurdsson, 2009).

The mouse model used by Sigurdsson et al. in these studies does not necessarily represent AD pathology; it is primarily a model of motor pathology that progresses so quickly that cognitive maze testing is not practical. However, this was an early, published study indicating that immunization against tau could be a successful therapeutic approach for treating tauopathies and perhaps could function to treat AD. They followed this with work using a different mouse model that mimics AD more closely and exhibits development of tangles in addition to displaying cognitive deficits associated with AD. Their newer mouse model was created by crossing htau mice with M146L-mutation PS1 mice maintained on a mouse-tau knock-out background. These mice displayed early (before 2 months of age), rapidly progressing tau pathology in cortical and hippocampal areas. The same immunogen 4E6G7 was administered i.p. with alum adjuvant, controls received adjuvant alone. Antibody titers were measured by
ELISA, and were notable, throughout the experiment. Behavioral testing included the same motor tests as described above, and also included the radial arm maze, the closed field symmetrical maze and object recognition testing. Immunization resulted in significant performance improvement in all three cognitive tests compared to controls. IHC for PHF1 revealed significant decreases in the immunized mice vs. controls. Soluble PHF1 tau was also reduced as measured by Western blotting. Most notable is that the behavioral improvements correlated well with the reductions of PHF1-detected pathology. (Boutajangout et al., 2010).

In 2001, Lim et al created a mouse model, VLW, that expresses human 4-repeat tau with 2 N-terminal inserts from exons 2 and 3 along with three mutations, G272V, P301L and R406W, that have been found in FTDP-17 patients. Ultrastructural tissue analysis revealed an increase in the number of lysosomal complexes in neurons of VLW mice compared to age-matched wild types. Lysosomal bodies were also found in tau-positive neurons, which the authors speculated were residual bodies derived from lysosomes (Lim et al., 2001).

1.10 Potential Intracellular Metabolism of Tau

Two primary pathways for protein degradation exist within cells, the proteosome pathway and the lysosome pathway, which includes autophagy. The proteosomal pathway requires ubiquitination—a post-translational modification process in which activated ubiquitin binds to lysine (Lys) residues of a modified protein. Lysine can become acetylated in AD, impeding this route of degradation (Otvos et al., 1994). However, tau can be present as an unfolded protein with three lysines available for
ubiquitination: Lys-6, Lys-11 and Lys-48, with the added Lys-48 being the primary site for polyubiquitination and degradation by the ubiquitin-proteosome-system (UPS). Lys-6 functions as an inhibitory site for the UPS (Wang and Liu, 2008). The UPS is a quality-control system for proteins and helps to eliminate small, misfolded soluble proteins. When misfolded tau is deposited as NFTs there is a potential problem with the UPS.

The lysosomal pathway, or autophagy, is the pathway for degradation of larger aggregates of misfolded proteins, mutant proteins that are known for conformational instability, and proteins that possess the propensity to form oligomers and aggregates. These misfolded proteins reside in the cytoplasm, where they can be easily sequestered into autophagosomes and then enzymatically degraded (Ding and Yin, 2008). This macroautophagy pathway can be overwhelmed in pathologic conditions, leading to accumulation of aggregates in the cytosol which, over time, can form paired helical filaments, as in AD (Sigurdsson, 2009).

In addition, enzyme-activity staining for acid phosphatase, which is a marker for lysosomes, showed an increase in staining, particularly in the cortex and hippocampus of VLW mice compared to wild type, which resembles staining for tau. Further analysis revealed that mutated tau, as opposed to wild type tau, showed greater lysosomal activity (Lim et al., 2001). (Bi et al., 2000).
1.11 Application of Immunotherapy to Potential Treatments for Dementias

Aβ immunotherapy studies began by focusing on investigations on active immunization. These studies utilized various forms of Aβ, first in mice, and then translated into human clinical trials. A similar course of investigation has evolved in tau immunotherapy. Active immunization was examined first, utilizing various mouse models and immunogens (antigens), as described above. Other studies evaluated passive immunization using various antibodies and multiple mouse models, to identify if exogenous antibodies could prove more effective for treatment use.

Sigurdsson et al (2011) led this endeavor by administering PHF1 to JNPL3 mice. Mice that were 2- to 3 months old were given weekly injections of PHF1 or pooled mouse IgG. Blood was collected at various points and was assayed for the tau epitope 4E6G7 (described above) by enzyme-linked immunosorbent assay (ELISA). At 5 to 6 months of age, the mice were administered behavioral testing (traverse or balance beam, rotarod, and open field). The dentate gyrus, motor cortex, and brainstem were evaluated using IHC for PHF1 to compare pathological forms of tau to total tau levels. A significant reduction of PHF1 was identified in the dentate gyrus, along with a strong trend of reduction in the motor cortex, but no difference was noted in the brain stem. Western blotting for PHF1 and CP13/B19 revealed significant decreases of insoluble pathological tau, but no differences in soluble or sarkosyl soluble tau. Behavioral analysis demonstrated fewer footslips on the traverse beam in the treated group, while the other motor testing did not appear to be affected.

Because this mouse model primarily exhibits motor deficits, cognitive performance was not evaluated. The results do, however, show great promise for
passive immunotherapy directed against tau, although the authors noted that this passive-immunotherapy approach seemed to be less efficacious than the prior active-immunization studies, which targeted essentially the same region of phosphorylated tau. This conclusion was based on the absence of an effect in the brainstem, despite effectiveness in the dentate gyrus. They also noted, however, that passive immunization is likely to be safer, by avoiding a possible negative T cell response such as that found in the clinical AN1792 study (Boutajangout et al., 2011a).

Their work was followed by a passive immunization study in the laboratory of Martin Citron at Eli Lilly using two different tau-overexpressing mouse lines, the JNPL3 mouse and P301S mutated mice. The JNPL mice have been discussed above. The P301S mouse strain is a more rapidly progressing line of Tg mice, with pathology becoming evident at younger ages, compared to older mice. It has also proven useful for studying tau pathologies and potential treatments. Chai et al (2011) tested two different types of anti-tau antibodies on these mice, initially with investigating PHF1 antibody administration. In addition, they examined MC-1, which is specific for conformation alterations of tau. The JNPL3 mice were given systemic injections of either antibody or an IgG1 control from 2 months of age until 6 months. Western blotting revealed similar results between the different treatment groups, so this team developed an ELISA assay using the AT8 antibody, which revealed a significant and substantial reduction of phosphorylated tau in response to both administered antibodies.

A subsequent study involved administering the same antibodies to the P301S mice from age 2 to 5 months that were then subjected to motor testing. Treated mice showed a significant improvement on the rotarod for both antibody groups compared to
controls. The rate and progression of pathology and motor deficits was greater for the P301S mice than for the JNPL3 mice. ELISA analysis revealed 45 percent reduction in pathology with PHF1 antibody administration, and a 33 percent reduction in pathology after MC-1 administration. IHC testing also revealed reduced tau pathology after administration of either antibody compared to control, and no significant difference in microglia or astrocyte activation (Chai et al., 2011a).

Immunotherapy directed against tau is likely to encounter some limitations as additional studies are conducted and the field grows. The first potential issue relates to tau being a normal protein with specific cellular functions (unlike Aβ, which currently is not known to have necessity-based biological function). Immunotherapy against tau will have to be investigated with caution, to ensure that normal tau is not disrupted and that clearance of toxic species of tau doesn’t result in depletion of normal tau. Studies to date have avoided this issue by specifically targeting hyperphosphorylated tau species. Studies conducted using tau knock-out animals have shown relatively normal function (Denk and Wade-Martins, 2009), suggesting that the body has a way of compensating for lower or non-existent levels of normal tau when it is cleared inadvertently. If tau immunotherapeutics advance to clinical trials, there should be little risk to control or non-demented individuals whose BBBs should be intact; very little antibody crosses the BBB the under normal circumstances. Tau is an endogenous protein, therefore issues regarding overactivity of the immune system or autoimmunity may present. Such issues have been minimized given the overall results from the Aβ studies.

Immunotherapy treatments for dementias have recently become an attractive means for targeting tau protein, in part because of some of the issues limiting Aβ
efficacy in clinical studies, and in part because tau pathology is more directly linked to
the symptoms and severity of AD. There is a much stronger correlation between
cognitive decline and the development of tau aggregates than Aβ deposition
(Sigurdsson, 2008) The best strategy for treating AD might prove to be one that targets
both Aβ and tau in concert.

1.12 Translation into Human Treatments in the Clinic

Minimization of side effects and other improperly directed effects can partially be
minimized by incorporating lessons from the Aβ clinical trials into experimental and
clinical-trial design. Early studies of tau immunotherapy have shown encephalitis-like
functional deficits in some of the mouse studies, however it is still thought that the
choice of adjuvant may be the major reason for this (Sigurdsson, 2009).

1.13 Future Directions for Tau-Directed Immunotherapy

Early immunotherapy strategies for treating AD, tauopathies, and other
neurodegenerative disorders suggested that this could be a fruitful avenue for future
treatment strategies. Oligomers of Aβ and/or tau are likely to be the subject of
increasing focus, as is further described, below. Many groups have been investigating
oligomeric forms of amyloid and tau and view these to be the most toxic forms of the
proteins. As the groundwork is laid in tau passive immunotherapy studies, it is likely
that the next generation of immunotherapies will be directed at oligomers. Additionally,
a number of experts have suggested the future of immunotherapeutic treatments for AD
and related dementias will likely address Aβ and tau, in concert. It is our hope that the studies presented herein will yield results that could make their way into the clinic in the very near future.

1.14 rTg4510 Mice and Their Role in Studying Tauopathies, Including AD

Transgenic mice have been established, using many techniques, to facilitate scientific understanding of basic biological processes, disease mechanisms, and treatment approaches for the advance of medical science and care. Aβ approaches for AD and related dementias are addressed above. In more recent times, however, tau pathology shows much stronger correlation to the behavioral symptoms of dementias.

One of the most robust tau-producing mouse lines relevant to dementias is the rTg4510 mouse. This mouse line combines a specific tau mutation, P301L, which leads to pathologic tau mutation, with a second genetic line, TTA, a tetracycline-controlled activator that can either turn on, or off, the expression of the mutant P301L gene. As such, the mutant tau can be regulated by a normal “on” state (mutant P301L expression), or can be “turned off” by administration of doxycycline. The TTA transactivator is further described as a CaM++ kinase transactivator (CaMK++) due to regulation by doxycycline, as shown in Figure 1.1.

Our lab has established a population of rTg4510 mice for the following studies. These mice have both a TTA/CaMK++ transactivator, and the p301L mutation. This dual mutation, combined, enables tau pathology to be turned on and off. Additionally, it directs tau pathology to upper/cognitive regions of the brain (whereas the p301L
mutation, alone, tends to express in lower, motor regions without the TTA transactivator, further referred to as Tet). The studies described herein focused on the differential location of the pathology, which occurs in upper cognitive brain regions. Figure 1.1 provides an illustration of the genetic design for how this combination-transgene strain of mice was created.
Figure 1.1: Tet-Off expression system. The expression of $tau_{P301L}$ gene is suppressed in the presence of Dox in bigenic mice derived by crossing parental strains carrying either responder or activator transgenes (a). In Tet-Off expression system, a tetracycline-controlled tTA protein regulates the expression of a target gene, in this case $tau$ gene which is under the transcriptional control of a TRE. The TRE is composed of tetO fused to a promoter [human cytomegalovirus (CMV) immediate-early promoter]. In the absence of CMV, or Dox, tTA binds to tetO of TRE and activates transcription of $tau$ transgene (b). Doxycycline, which is usually delivered in food or drinking water, has higher binding affinity to tTA and thus, when present, prevents tTA from binding to TRE and consequently suppresses the expression of $tau$ gene (c).

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CHAPTER 2: PRELIMINARY STUDIES

2.1 Introduction

Our laboratory has conducted a number of studies using animal models to establish the feasibility of treating tau pathology, and to identify pharmacologic effects and behavioral impacts of various tau treatment strategies. This series of studies initially examined treatment effects using anti-tau antibodies in APP mice with the added transgenes for presenilin1 (PS1) and inducible nitric oxide negative (iNOS), NOS2-/-/APP/PS1 mice. Such mice have been reported to express tau pathology, in addition to Aβ pathology.

Identifying the relatively minor (or “lack of”) toxicity and behavior effects from anti tau treatment to APP/NOS2-/--mice (with and without the added PS1 transgene) led to further examination of the effects of tau treatment in more robust tau-producing mice. The transgenic mouse model rTg4510 is specifically designed and bred to overproduce tau pathology. The combination of the P301L mutation, with the Tet transactivator, specifically leads to tau pathology in higher brain regions (cognitive regions such as cortex and hippocampus), and provides a useful model for identifying tau-based treatments when investigating AD-related tauopathy treatment strategies.

These studies sought to identify the most effective and specific antibody treatment, the best route of administration, and optimal time frames for treating tau
pathology in rTg4510 mice. The goal was to develop a greater perspective of tau antibody treatment. Behavior was also examined, and was compared to observed alterations of brain pathology before and after treatment with tau antibodies.

Initial studies utilized a commercially available antibody, Tau 5. Four tau antibodies that exhibit distinctly different mechanisms of action also were explored: two with demonstrated affinity for pathological tau but not normal tau, one pan-tau antibody that has yet to demonstrate any specificity for pathological tau, and one directed specifically to tau oligomers:

**Tau 5** is specific to tau and binds to the mid-region, amino acids 218 – 225, and as such is able to bind all forms of tau because this region is conserved among all post-translationally modified forms of tau.

**PHF1** is a phosphorylation-dependent monoclonal antibody that recognizes phosphorylated serine (pSer) 396 and pSer404, two phosphorylation sites in pathological tau, which is hyperphosphorylated at the paired helical filaments (PHFs) in what is generally considered to be “late stage” for AD. This antibody is often used in IHC or Western blotting to visually localize and quantify hyperphosphorylated tau. It can recognize either of the pSer epitopes, alone, or both together. It exhibits greater than 10-fold sensitivity when both are present (Otvos et al., 1994).

**MC1** is a conformation-dependent monoclonal antibody that recognizes early-stage pathology conformation changes of PHFs across all 6 isoforms of tau. It has dual epitopes at the N-terminal and between amino acids 312 and 342 in the third microtubule-binding domain (MBD) (Jicha et al., 1997, Oddo et al., 2004) and will only
bind tau that is conformationally modified such that MC1 simultaneously connects the N-terminal epitope with the mid-domain epitope. Given that conformational variation, especially the pathological conformations identified in AD and other neurodegenerative diseases, is an important factor in proteins’ overall function, it was prudent to evaluate a conformational-dependent antibody in these studies. The ability to bind two linearly separated binding domains simultaneously reflects the existence (and possible clearance, via treatment) of early NFTs.

*DA9* is a pan-tau monoclonal sequence antibody that binds to acids 102 through 140 and has no known specificity for pathological tau, thus only binds normal tau, which is necessary for normal functioning. This antibody was included to address the question of whether normal tau is affected or reduced.

*TOMA* (tau oligomeric monoclonal antibody, also called H12) was included in later studies because it has been shown to reduce oligomers and improve behavior in JNPL3 and htau mice (Lasagna-Reeves et al., 2011b, Castillo-Carranza et al., 2014b, Guerrero-Munoz et al., 2014, Lasagna-Reeves et al., 2014). It had not yet been tested in rTg4510 mice.

Anti-GFP was used as a control because it is a similar isotype of IgG. IgG antibodies do not activate complement in the mouse, so any clearance observed from treatment antibodies, when compared to the GFP IgG1, would be due to other mechanisms such as those described above for Aβ (Bacskai et al., 2002).

Behavioral testing before, and after, treatment was conducted to correlate pathology to behavioral effects of the treatments. Intracranial injection of Tau 5 or an
anti-GFP control antibody was first conducted using NOS+/APP/PS1 mice to assess effects of tau treatment and any inflammation that might result from antibody injections. Additionally, an antibody directed against tau and administered to amyloid-producing mice could also affect amyloid pathology, which we therefore also evaluated. Investigation using a more specific tau mouse model, rTg4510, was then conducted using additional antibodies.

2.2 Tau Administration

Several injection paradigms were investigated, using multiple Tg mouse strains and antibodies. Intracranial injections, using convection-enhanced delivery (CED) (Carty et al., 2010) to maximize dispersion of the injected antibodies, were performed at a rate of 2.5 μl/min, for a total of 2 μl (2 μg) per injection site, followed by one minute before needle retraction. Parenchymal injections were administered using stereotaxic equipment, with proper coordinates set for each brain region and adjusted for brain atrophy, given the age of the mouse.

Ventricular injection was also examined, using 15 μl of antibody per animal, injected at a flow rate of 2.5 μl /min into the right ventricle. Gallyas and Nissl staining were completed to identify changes in NFTs or neuronal population. Infusion of antibodies followed, using implanted pumps to deliver longer-term exposure to the treatment antibodies. Subcutaneous (sc) injections were later administered by i.p. into the abdominal cavity.
2.3 Behavioral Testing

General mobility and activity testing: open-field testing, balance beam, rotarod, and Y maze, was conducted to identify potential physical or cognitive performance deficits. The number of arm entries in the Y maze can be used as a measure of general activity levels of the mice. The percent of alternations provides an indication of working memory; it is expected that normal (nTg) mice will remember the most recent arms visited then opt for a different arm, searching for reward (food). This represents a behavior akin to evolutionary foraging strategies.

Cognitive function was examined using the radial arm water maze (RAWM), which tests for learning and memory abilities. RAWM testing was followed by open pool testing to evaluate vision and swimming abilities, as a control, to ensure the mice were capable of conducting the tasks required for the RAWM. These are swimming tests; RAWM consists of 6 different arms radiating from a center area, with one arm containing a hidden escape platform.

On the first day, the first 4 blocks (12 trials) use a visible platform alternated with a hidden platform to assist the mice to use spatial cues, to learn which arm to swim to for safety. The platform location remains constant for each mouse, but is alternated between mice within each group to rule out confounding factors such as odor trails from previous mice. The last block (3 trials) on day 1, and all of the blocks on day 2, used a hidden platform to force the mice to rely on spatial memory cues to find the escape. Throughout testing, the time for the mouse to find the platform is recorded as are the
number of errors (defined as entries into an incorrect arm, or greater than 15 seconds spent in one arm or in the pool center without arm selection). Analysis of the number of errors provides the best evaluation of learning and memory.

Results were verified by open pool testing, where a visible platform is placed in random quadrants of the pool, and the time required for each mouse to find the escape platform is recorded. Open field is a control test for RAWM, to ensure the mice can see the platform and that they are able to swim competently to reach it.

Following intracranial antibody injection, the animals were given a brief recovery period (6 – 7 days). Post-injection RAWM behavioral testing was conducted using procedures described above. For post-injection testing, the escape platform was placed in a different arm than where it had been during the pre-injection testing. Modified arm placement was intended as a “new” test of cognitive ability, not as a long-term retention test, to identify if the antibody injection resulted in any change in learning and memory ability over time.

Post-injection behavioral testing further included fear conditioning (FC). FC consisted of a first day of training, pairing a tone with a foot shock, then a second day of associative memory evaluation. During training, the mice were placed in a chamber and were allowed to freely explore for 2.5 minutes, at which time an audible tone was played. The tone lasted for 30 seconds and ended with the administration of a foot shock. Mice were observed for freezing behavior (an indication of fear), and their movements were recorded digitally. At the 4-minute time point during training, a second 30-second tone was administered that again ended with a foot shock. The mice were
observed for 7 minutes after being placed in the chamber and their movement was
digitally recorded as amperage, recorded by a camera inside the chamber. Freezing
was defined if the motion of the mouse was less than threshold, which was established
as 10 mA, for 2 msec.

One day after training, two tests of association and memory were conducted.
The first of these is referred to as “context” testing, which tests for hippocampal-
dependent memory. The mouse was placed in the same chamber in which it received
the foot shock, for 3 minutes, and similar observations of freezing were recorded. No
shock was administered. This procedure tests for contextual learning and memory. Did
the mouse associate the context with the previous shock, and freeze, simply due to its
environment or context?

The second FC test was “cued” testing, which is a test of amygdala-dependent
associative memory. For this task, a different chamber was used, olfactory cues were
changed, the appearance of the experimenter changed, and lighting to the room was
turned off. These changes were intended to remove all context-based cues from the
previous day’s training. Mice were placed in this novel environment and allowed to
explore for 3 minutes, at which time the original tone was played. The tone remained
on for the duration of the experiment, but no foot shocks were delivered. Again,
freezing behavior was recorded to identify if the mice remembered the association
between tone and shock. This is a test for auditory association with learning. Does
tone, alone, result in freezing? All FC testing results were reported as percentage of
time freezing.
2.4 Tissue Collection

Mice were euthanized after completion of the post-treatment behavioral studies. A 200 mg/kg-body weight injection of Somnasol was administered. Approximately 10 minutes, post-injection, an ear punch was collected for confirmation genotyping. Mice were then intracardially perfused with a 0.9% sodium chloride solution.

Tissue collection consisted of dissecting the anterior cortex, posterior cortex, hippocampus, striatum, thalamus, cerebellum and “rest of brain” (ROB) from the right hemisphere of the brain, freezing them on dry ice and storing at -80 degrees for potential biochemistry evaluation. The left hemispheres were fixed for 24 hours using a freshly made 4% paraformaldehyde (PFA) solution, after which time they were transferred to Dulbecco’s phosphate buffered saline (DPBS) + Azide, until they were cryoprotected for sectioning. Further cryoprotection consisted of a sequential immersion in increasing sucrose solutions, beginning with 10% for 24 hours, 20% for 24 hours, and finally 30% for 24 hours.

The left hemisphere was sectioned for immunohistochemical (IHC) and histological staining. Sections of 25 μm were collected using a sliding microtome with a 4-degree Celsius cooled stage to freeze the brains. Sections were collected into 24-well plates containing DPBS + Azide. Four successive sections were collected, per well. Free floating IHC was conducted on sections spaced 300 μm apart, in the region of the depth of injection. In general, 6 sections were selected per animal. Staining for CD45 was conducted using a rat monoclonal antibody to clone YW62.3 (Thermo), and
staining for diffuse Aβ was conducted using an antibody that was a gift from Paul Gottschall, University of Akansas.

2.5 Western Blot

Biochemistry was examined using Western blot. Dissected brain regions were homogenized, then sonicated in RIPA buffer, with added protease and phosphatase inhibitors, then centrifuged at 40,000 x G for 30 min at 4° C. Supernatant was collected for analysis of RIPA-soluble proteins. The resulting pellet was digested with 70% formic acid, to analyze RIPA-insoluble proteins. Samples were separated using 10% Tris-glycine gels, then were transferred to either nitrocellulose or PVDF membranes, for probing and fluorescent and/or photo-luminescent detection.

*NOS*²⁻/APP/PS1 mice

Antibodies were administered via intracranial parenchymal injection into reported tau producing mice, NOS2⁻⁻ mice crossed with either APP (tg2576) or APP/PS1 mice. Littermate nTg mice were used as controls. Eight mice per group were injected, one group being NOS2⁻⁻/nTg, the second being NOS2⁻⁻/APP, and the third was NOS2⁻⁻/APP/PS1. For each group, half of the mice received Tau 5 antibody, bilaterally, and the other half received anti-GFP bilaterally, into the hippocampus.
Intracranial injections of either Tau 5 antibody or an isotype-specific (IgG1) control antibody against GFP were administered bilaterally into the hippocampus, to half of each genotype group, using rTg4510 mice and their littermates.

Five rTg4510 mice (9-11 month old) were intracranially injected with either Tau 5, or an IgG1 control, anti-GFP, into the hippocampus. Two µg of Tau 5 antibody (directed against the mid domain of the tau peptide) was injected into one hippocampus, and 2 µg of anti-GFP control was injected into the hippocampus on the opposite side.

Tau 5, or anti-GFP, was subsequently injected into contralateral sides of a small population of younger, 3.5 month old, rTg4510 mice. The hemisphere receiving Tau 5 vs. anti-GFP was alternated between different animals to reduce hemispheric influence. Tissue was collected 4 days later. Whole brain sections were collected.

Antibodies were then injected, unilaterally, into the cortex and hippocampus, of rTg4510 mice that were 11 - 13 months of age. Injection was conducted similarly to the Tau 5 antibody injections described, above.

Following the initial pilot studies with Tau 5, antibodies for: PHF-1, MC-1, DA-9, or anti-GFP as an IgG1, control were unilaterally injected into both the hippocampus (HPC) and the anterior cortex (ACx) of 11 – 13 month old rTg4510 mice (8 mice per group). Tissue collection occurred 4 days after injection. The anterior pole of each whole brain was dissected, and was snap frozen for biochemistry.
Tau 5, MC-1, anti-GFP or TOMA were then examined using different administration protocols, as described below.

2.6 Results: NOS2-/-APP/PS1 Mice and Controls

Intracranial injection of Tau 5 or a green fluorescent protein (GFP) control antibody anti-GFP (both murine IgG1), into NOS2-/- mice crossed with either APP (tg2576) or APP/PS1 mice was first investigated. Littermate nTg mice were used as controls. Eight mice per group were injected, one group being NOS2-/-/nTg, the second being NOS2-/-/APP, and the third was NOS2-/-/APP/PS1. For each group, half of the mice received Tau 5 antibody, bilaterally, and the other half received anti-GFP bilaterally, into the hippocampus.

Behavior analysis was forefront in this study to identify if antibody injection, in general (control or treatment), would demonstrate effects on performance. Additionally, we sought to identify any potential benefit from the Tau 5 antibody in neuronal amyloid-producing groups of mice. Pre-injection baseline (first round) behavior testing was conducted; Figures 2 through 4 show the results from the baseline activity behavioral testing.

Figure 2.1 shows motor ability results. Panel A demonstrates the latency to fall off of an elevated balance beam. The NOS2-/-/APP mice had previously exhibited the greatest performance deficit in this task. Our results indicated that this performance difference was not statistically different from the other groups. Panel B shows the time the mice were able to remain on the rotarod, which is a rotating treadmill that gradually increases in speed over time. All mice were able to perform this task, showed
improvement with subsequent trials, and did not perform significantly different based on genotype. NOS2-/-APP/PS1 mice were non-significantly more active (hyperactive), in the Y maze as shown in Figure 2.2., with Panel A presenting the total number of arm entries and Panel B showing the percent of alterations between arms. The NOS2-/- /nTg mice displayed significantly greater percentages of alterations in the Y Maze than the other two genotypes, indicating that they had a better memory of which arms had already been visited. These results are consistent with previous results by our laboratory, using this mouse line, even without the NOS2 gene deletion (Gordon et al., 2001).

Figure 2.3 presents the results of RAWM and open pool tests. Panel A shows RAWM results as a function of time. Each Block represents the average of 3 trials, per block, conducted over two days. This figure shows that all of the mice are able to learn the task. The total number of errors for the three groups (Panel B) was also compared, and here we note that there is significant impairment in the NOS2-/-APP/PS1 mice compared to the NOS2-/- mice. Results are consistent with those previously reported from our laboratory for NOS2 sufficient mice (Arendash et al., 2001). Results in Panel C show that the mice were able to see the platform and were able to perform the tasks required to find it.

Figure 2.4 shows post-injection results for the RAWM, by block. A greater number of errors were initially observed with all mice, since many animals returned to the original platform location. Over time, they learned the new location, and Tau 5 animals appeared to learn the new location more quickly. Statistical analysis did not demonstrate significance, however. The NOS2-/-APP/PS1 mice, in all treatment
conditions, demonstrated the most impaired performance, as was the case in the first, pre-injection RAWM test.

Figure 2.5 divides the pre-injection RAWM testing into the resulting 6 groups, and provides a comparison between pre- and post- injection RAWM testing for all six groups. The most notable results are the increased number of errors at the onset of post-injection RAWM testing. Also notable, for both rounds of testing (pre-, vs. post-injection), was a genotype difference. Tau 5 treated mice made fewer errors, across all genotypes. However extra variability was introduced by comparing multiple genotypes and evaluation of treatment results across all three genotypes. Therefore a treatment-induced difference could not be identified statistically.

Figure 2.6 shows FC training results, post injection, and illustrates NOS2-/-/nTg mice mice appear to exhibit more of a deficit learning the task compared to anti-GFP controls (Panel A). NOS2-/-/APP mice froze similarly by treatment during the course of training (Panel B). NOS2-/-/APP/PS1 mice showed an increase in freezing behavior in response control treatment vs Tau 5 injection (Panel C).

Testing of contextual memory (Figure 2.7) shows no significant differences by either genotype or treatment.

Figure 2.8 shows results from cued testing in NOS2-/- mice and controls. Tau 5 treated mice appeared to minimally increase their associative memory freezing response in nTg mice (Panel A). No other differences were noted between either genotype or treatment with FC.
Figure 2.9 shows the results of CD 45 IHC staining. We focused our attention to the area of injection (essentially CA1) to best visualize relative microglial activation in response to intracranial injection of the 2 antibodies. Specifically, we were trying to identify if the Tau 5 antibody resulted in more, less, or similar levels of inflammation compared to the control. Most notably was greater CD 45 staining, reflective of inflammation, in the NOS2-/-/APP/PS1 mice compared to the other genotypes, however no significant difference between the treated vs. control groups was identified.

Aβ IHC was also examined, on the chance that the tau antibody treatment might show an impact on amyloid pathology in these primarily amyloid-producing mice. As expected, there was no Aβ staining evident in the NOS2-/-/nTg mice, and the greatest staining was observed in the NOS2-/-/APP/PS1 mice. Since we have not found tau pathology in any of these mice, and these mice are known to be primarily amyloid producers, we did not evaluate IHC pathology for tau. Additionally, we weren’t anticipating any significant changes in Aβ IHC in the mice that were treated with Tau 5 vs the anti-GFP controls, since the target is different. Surprisingly enough, Figure 2.10 shows that an increase in Aβ staining was observed in the Tau 5 treated mice. As with the behavior data (particularly the FC data), it must first be noted that the ending number of animals for each unique group was only 4, thereby making the determination of satisfactory statistical significance difficult. It is for this reason that the observed statistical significance was especially surprising. However, as with the behavioral results, the significance that we found is more likely to be genotype-dependent as opposed to being treatment-dependent.
No significant differences in biochemistry were observed by Western blot, as a result of tau treatment. Therefore results are not included here.

rTg4510 mice GFP vs Tau 5

Figures 2.11 and 2.12 present micrograph and statistical results for the microglial markers CD 45 and Iba1, respectively. Figure 2.13 shows similar results for cell density using Nissl staining. No significant differences were noted in any of these examinations, however the Nissl staining results did appear to reveal a greater, but non-significant, number of cells in the treated animals compared to the control animals.

Subsequent to antibody injection, behavioral analysis was conducted as described above. Figure 2.14 provides results from Rotarod activity testing. Figure 2.15 presents results from Y Maze alternations. Both tests revealed no activity differences between the different treatment groups, for this genotype. The open field test was also completed, but it similarly showed no difference between treatments, therefore the results are not included here.

Post-treatment cognitive tests were also conducted to identify any treatment-based effects. Novel Object Recognition (NOR) testing results are reported in Figure 2.16. RAWM results are reported in Figure 2.17. Novel Object Recognition testing showed that the MC-1 treatment restored rTg4510 mice to nTg performance levels. Tau 5 treated mice demonstrated slight improvement in recognition of a foreign object, suggesting that they remembered the original object and avoided the novel object. The
RAWM test revealed a high level of variability between the mice, but by the last block of testing, MC-1 treated mice performed significantly better than the IgG1 controls.

Ventricular Injection

No significant differences were noted as a result of ventricular injection, between any of the treatment groups. This method of administration did not appear to be productive therefore results are not included.

Discussion

We could attempt to dissect the observed differences, or lack thereof, by repeating these experiments using additional mice to increase the statistical power of the results. However, this experiment was just a pilot study, so we chose not to pursue it further. The main goal of this pilot study was to do a thorough behavioral investigation of the mice, both before and after surgery, and to note any substantial differences in microglial activation or amyloid pathology as a result of tau antibody administration (post-surgery). The significance (or lack thereof) reported here could be substantiated, or contradicted, by adding additional animals, or by conducting follow-up studies. However the fundamental goals of the pilot study had been achieved and the decision was made to move forward by studying the effects of anti-tau antibodies on more relevant tau-producing mice.
Preliminary data, reflected initial attempts to utilize an antibody directed to all forms of tau; its epitope is specific and conserved enough to represent binding to total tau and all forms of tau. However, the tau molecule, itself, may not be the most toxic form in the context of AD and tauopathies. Also, as noted above, tau can be hyperphosphorylated at many locations; it can undergo mutation and/or conformational changes, it can undergo other types of post-translational modification, and it eventually forms filaments, which can further aggregate and lead to the formation of NFTs. There are many different “types” of antibodies directed for different forms of tau.

Characterization of the APP/NOS mice, initially, did not reveal the reported tau pathology or neuron loss. They were still useful as preliminary study animals to assess potential inflammation, which might result from antibody injections. An antibody directed against tau, administered to amyloid-producing mice, could also affect amyloid pathology.

2.7 Pilot Test of Total Tau Antibody Using rTg4510 Mice

Initial investigation of toxicity and behavior effects resulting from anti-tau treatment to AP/NOS \(^{+/−}\) mice, (with and without the added PS1 transgene), led to further studies to examine the effects of tau treatment on robust tau-producing mice. The transgenic mouse model rTg4510 is specifically designed, and bred, to overproduce tau pathology. The combination of the P301L mutation, with the Tet transactivator, specifically leads to tau pathology in higher brain regions (cognitive regions), and
provides a very useful model for identifying tau treatments when investigating AD-related tauopathy treatment strategies.

Small groups of rTg4510 mice, ranging in age, were intracranially injected with a commercially available antibody to tau, Tau 5. This antibody binds to the mid-region of tau, namely amino acids 218 – 225, and as such is able to bind all forms of tau since this region is conserved among all post-translationally modified forms of tau. It also binds to the 6 different isoforms of tau.

The intent was to identify if treatment using Tau 5, and binding by this antibody, might indicate whether or not immunotherapy could be effective in directly treating pathological tau, and whether it might be effective in treating pathologically generated tau-mice. This antibody is effective in binding and/or labeling all forms of tau, even ‘normal’ healthy forms of it. Therefore we expected it to demonstrate effectiveness across different breeds of tau mice.

Initially, 5 rTg4510 mice (9 -11 month old) were intracranially injected with either Tau 5, or an IgG1 control, anti-GFP, into the hippocampus by use of convection-enhanced delivery (CED) (Carty et al., 2010). Two µg of Tau 5 antibody (directed against the mid domain of the tau peptide) was injected into one hippocampus, and 2 µg of anti-GFP control (both murine IgG1) was injected into the hippocampus on the opposite side. Anti-GFP is not known to have any biological activity in the mouse, and it has the advantage of being an IgG1 isotype, akin to Tau 5 and the other antibodies investigated herein. The GFP antibody also does not stain normal mouse brain proteins when used in immunostaining reactions.
Tau 5, or anti-GFP, was subsequently injected into contralateral sides of a small population of younger, 3.5 month old, rTg4510 mice. The hemisphere receiving Tau 5 vs. anti-GFP was alternated between different animals to reduce hemispheric influence. Tissue was collected 4 days later. Whole brain sections were collected into two horizontally aligned 24-well plates. Four adjacent sections were placed in each well, then the next well was filled in the first plate, and section collection continued in the first plate, until it continued into the second plate. Sections from the center-most wells were used for IHC and other staining.

After completion of the post-treatment behavioral studies, mice were euthanized using a 200 mg/kg-body weight injection of Somnasol. Approximately 10 minutes, post-injection, an ear punch was collected for confirmation genotyping, then mice were intracardially perfused with a 0.9% sodium chloride solution. Tissue collection consisted of dissecting the anterior cortex, posterior cortex, hippocampus, striatum, thalamus, cerebellum and “rest of brain” (ROB) from the right hemisphere of the brain, freezing them on dry ice and storing at -80 degrees for potential biochemistry. The left hemispheres were fixed for 24 hours using a freshly made 4% paraformaldehyde (PFA) solution, after which time they were transferred to DPBS + Azide until they were cryoprotected for sectioning. Cryoprotection consisted of a sequential immersion in increasing sucrose solutions, beginning with 10% for 24 hours, 20% for 24 hours, and finally 30% for 24 hours. Then, the left hemisphere was sectioned for immunohistochemical (IHC) and histological staining. Sections of 25 μm were collected using a sliding microtome with a 4-degree Celsius cooled stage to freeze the brains. Sections were collected into 24-well plates containing DPBS + Azide, 4 successive
sections were collected, per well. Free floating IHC was conducted on sections spaced 300 μm apart, in the region of the depth of injection. In general, 6 sections were selected per animal. Staining for CD45 was conducted using a rat monoclonal antibody to clone YW62.3 (Thermo), and staining for diffuse Aβ was conducted using an antibody that was a gift from Paul Gottschall, University of Arkansas.

Additionally, we examined biochemistry using Western blot. Dissected brain regions were homogenized then sonicated in RIPA buffer, with added protease and phosphatase inhibitors, then centrifuged at 40,000 x G for 30 min at 4° C. Supernatant was collected for analysis of RIPA-soluble proteins. The resulting pellet was digested with 70% formic acid, to analyze RIPA-insoluble proteins. Samples were separated using 10% Tris-glycine gels, then were transferred to either nitrocellulose or PVDF membranes, for probing and fluorescent and/or photo-luminescent detection.

We could attempt to dissect the observed differences, or lack thereof, by repeating these experiments using additional mice to increase the statistical power of the results. However, this series of experiments were just pilot studies, so we chose not to pursue it further. The main goal of this pilot study was to do a thorough behavioral investigation of these mice, both before and after surgery, and to note any substantial differences in microglial activation or amyloid pathology as a result of tau antibody administration (post-surgery). The significance (or lack thereof) reported here could be substantiated, or contradicted, by adding additional animals, or by conducting follow-up
studies. However the fundamental goals of the pilot study had been achieved and the
decision was made to move forward by studying the effects of anti-tau antibodies on
more relevant tau-producing mice.

FC results must be qualified, based on the low number of experimental subjects
per group at the time of testing. Three genotypes of mice were each divided in half for
the different treatments, resulting in only 4 animals in each genotype-treatment group.
With that noted, there was consistency amongst the results.

After completion of pre-injection behavioral studies, intracranial injections were
administered bilaterally into the hippocampus. Injections of either Tau 5 antibody or an
isotype-specific (IgG1) control antibody against GFP were administered to half of each
genotype group. Anti-GFP is not known to have any biological activity in the mouse,
and it has the advantage of being an IgG1 isotype, akin to Tau 5 and the other
antibodies investigated here.

Results: NOS2\(^{-/}\)APP/PS1 Mice and Controls

Intracranial injection of Tau 5 or a green fluorescent protein (GFP) control
antibody, anti-GFP (both murine IgG1), into NOS2\(^{-/}\) mice crossed with either APP
(tg2576) or APP/PS1 mice was investigated. Littermate nTg mice were used as
controls. Eight mice per group were injected, one group being NOS2\(^{-/}\)/nTg, the second
being NOS2\(^{-/}\)/APP, and the third was NOS2\(^{-/}\)/APP/PS1. For each group, half of the
mice received Tau 5 antibody, bilaterally, and the other half received anti-GFP
bilaterally, into the hippocampus.
Behavior analysis was forefront in this study to identify if antibody injection, in general (control or treatment), would demonstrate effects on performance. Additionally, we sought to identify any potential benefit from the Tau 5 antibody in neuronal amyloid-producing groups of mice. NOS2−/−/APP/PS1 mice were non-significantly more active (hyperactive), as shown in Figure 2.1. Panel A demonstrates the latency to fall off of an elevated balance beam. The NOS2−/−/APP mice had previously exhibited the greatest performance deficit in this task. Our results indicated that this performance difference was not statistically different from the other groups. Panel B shows the time the mice were able to remain on the rotarod, which is a rotating treadmill that gradually increases in speed over time. All mice were able to perform this task, showed improvement with subsequent trials, and did not perform significantly different based on genotype.

Figure 2.2 shows performance in the Y maze, with Panel A presenting the total number of arm entries and Panel B showing the percent of alterations between arms. The NOS2−/−/NTg mice displayed significantly greater percentages of alterations in the Y Maze than the other two genotypes, indicating that they had a better memory of which arms had already been visited. These results are consistent with previous results by our laboratory, using this mouse line, even without the NOS2 gene deletion (Gordon et al., 2001).

Figure 2.3 presents the results of RAWM and open pool tests. Panel A shows RAWM results as a function of time. Each Block represents the average of 3 trials, per block, conducted over two days. This figure shows that all of the mice are able to learn the task and remember where the escape platform is located. The total number of errors for the three groups (Panel B), was also compared, showing that there is
significant impairment in the NOS2\textsuperscript{−/−}/APP/PS1 mice compared to the other two groups. Results are consistent with those previously reported from our laboratory for NOS2 sufficient mice (Arendash et al., 2001). Results in Panel C show that the mice were able to see the platform and were able to perform the tasks required to find it.

Figure 2.4 demonstrates RAWM results, post injection. A new platform was introduced, therefore initial errors were greater, initially. All mice appeared to learn the new escape platform location, over time, with no statistically significant difference, although the Tau 5 treated mice appeared to perform slightly better.

Figure 2.5 shows the results for all six groups (based on genotype and treatment differences) collectively, from pre- vs post-injection RAWM testing. The NOS2\textsuperscript{−/−}/APP/PS1 mice, in both treatment conditions, demonstrated the most impaired performance, as was the case in the first, pre-injection RAWM test, while the NOS2\textsuperscript{−/−}/nTg mice learned the task most quickly and robustly.

Figure 9 (Figure 2.8) divides the pre-injection RAWM testing into the resulting 6 groups, and provides a comparison between pre- and post- injection RAWM testing for all six groups. The most notable results are the increased number of errors at the onset of post-injection RAWM testing. Also notable, for both rounds of testing (pre-, vs. post-injection), was a genotype difference. Figure 2.8 demonstrates little difference due to treatment effects.

After round 2 post-injection RAWM testing, the Tau 5 treated mice made fewer errors, across all genotypes. However extra variability from comparing multiple
genotypes and attempting to evaluate treatment results across all three genotypes, introduced added variability, therefore a treatment-induced difference could not be identified statistically.

FC training results, shows that NOS2−/−/nTg mice increased freezing during the course of training Fig 2.6. Tau 5 injected mice appeared to learn the task better than anti-GFP controls by freezing significantly less (Panel A). NOS2−/−/APP mice froze similarly by treatment during the course of training (Panel B). NOS2−/−/APP/PS1 mice showed a significant decrease in freezing behavior in response to Tau 5 injection (Panel C). It is unlikely that the GFP antibody would have had an effect on freezing or learning abilities.

Testing for contextual memory showed that in NOS2−/−/nTg mice, Tau 5 treatment appeared to improve memory of training, as the mice froze more quickly then maintained freezing for the course of the test, Figure 2.7 shows context results from FC in NOS2−/− mice.

NOS2−/−/APP mice demonstrated a non-significant reduced time of freezing with Tau 5 treatment, and anti-GFP treatment may have led to an improvement in memory or ability to freeze, which was not an expected result. Taken into consideration with performance during training, it would appear that the NOS2−/−/APP genotype is especially resistant to freezing behavior, and that antibody treatment probably made no difference. Surprisingly, the NOS2−/−/APP/PS1 mice seemed to show retention of context, and performed somewhat similarly to the NOS2−/−/nTg mice.
Tau 5 treated mice appeared to minimally increase their associative memory freezing response (Panel A). During training and contextual testing, NOS2^{-/-}/APP mice showed little or no freezing, even in response to the tone. This behavior continued into cued testing (Figure 2.8) which shows results from cued testing in NOS2^{-/-} mice, and no treatment difference was noted. NOS2^{-/-}/APP/PS1 mice demonstrated very little freezing during cued testing. Anti-GFP treated mice appeared to freeze a little more in association with the tone. These responses were not significant, however.

FC results here must be qualified, based on the low number of experimental subjects per group at the time of testing. Three genotypes of mice were each divided in half for the different treatments, resulting in only 4 animals in each genotype-treatment group. With that noted, there was consistency amongst the results. The NOS2^{-/-}/nTg mice performed all components of training and testing much like regular non-transgenic mice would be expected to perform, and the Tau 5 treatment seemed to improve learning and memory performance. NOS2^{-/-}/APP/PS1 mice generally showed impaired freezing responses, and surprisingly the NOS2^{-/-}/APP mice were the most impaired in these tasks. Unexpectedly, anti-GFP control treatment appeared to improve performance in some of the tasks compared to the Tau 5 treatment. Anti-GFP is not known to have biological function, so it would not be prudent to assume it rescued performance. In the end, there was limited statistical significance between any of the genotypes or the treatments, so any apparent improvement in anti-GFP treated mice is
likely an artifact due to the number of different factors being evaluated and to the low number of animals in each unique group. The only statistically significant finding in all of these results was between the no-tone and tone phases of NOS2°/°/nTg cued testing (P < 0.05).

Figure 2.9 shows the results of CD 45 IHC staining. We focused our attention to the area of injection (essentially CA1) to best visualize relative microglial activation in response to intracranial injection of the 2 antibodies. Specifically, we were trying to identify if the Tau 5 antibody resulted in more, less, or similar levels of inflammation compared to the control. Most notably was greater CD 45 staining, reflective of inflammation, in the NOS2°/°/APP/PS1 mice compared to the other genotypes. As for which antibody treatment produced greater indices of inflammation upon injection, slight differences between the genotypes demonstrated no significant difference between the treated vs. control groups.

Aβ IHC was also examined, on the chance that the tau antibody treatment might show an impact on amyloid pathology in these primarily amyloid-producing mice. As expected, there was no Aβ staining evident in the NOS2°/°/nTg mice, and the greatest staining was observed in the NOS2°/°/APP/PS1 mice. Since we have not found tau pathology in any of these mice, and these mice are known to be primarily amyloid producers, we did not evaluate IHC pathology for tau. Additionally, we weren’t anticipating any significant changes in Aβ IHC in the mice that were treated with Tau 5 vs the anti-GFP controls, since the target is different.
Surprisingly, Figure 2.10 shows that an increase in Aβ staining was observed in the Tau 5 treated mice. As with the behavior data (particularly the FC data), it must first be noted that the ending number of animals for each unique group was only 4, thereby making the determination of satisfactory statistical significance difficult. It is for this reason that the observed statistical significance was especially surprising. However, as with the behavioral results, the significance that we found is more likely to be genotype-dependent as opposed to being treatment-dependent.

No significant differences in biochemistry were noted by Western blot, as a result of tau treatment. Therefore results are not included here.

Tau immunotherapy studies were expanded to evaluating treatment of rTg4510 mice. This series of studies evaluated different types of antibodies, targeted to different epitopes, and with different mechanisms of action. Our studies with total tau antibody were furthered to identify other variations of tau that might be as good, or better, at targeting pathological tau in disease states.

Unilateral intracranial injections were administered into both the hippocampus (HPC) and the anterior cortex (ACx). Whole brain sections were collected for IHC analysis, although the anterior pole was dissected and saved, for biochemical analysis by Western Blot. Animals (8 per group) consisted of 11 – 13 month old rTg4510 mice; they received, either, antibodies for: PHF-1, MC-1, DA-9, or anti-GFP as an IgG1, control. Tissue collection occurred 4 days after injection. The anterior pole of each whole brain was dissected, and was snap frozen for biochemistry. Sections of the remaining brain were collected into two horizontally aligned 24-well plates. Four
adjacent sections were placed into each well, the next well was filled in the first plate, until the first plate was filled. Section collection continued into the second plate. Sections from the center-most wells were used for IHC and other staining. Positive staining was quantified; the values of the injected hemisphere were divided by values from the uninjected side, then statistics were computed to compare the results between treatment groups.

Three additional tau antibodies, which exhibit distinctly different mechanisms of action, were explored in hopes of identifying the best antibody treatment to evaluate further. The additional antibodies examined here included two with demonstrated affinity for pathological tau but not normal tau, and one pan-tau antibody that has yet to demonstrate any specificity for pathological tau. These antibodies included:

- **PHF-1**, a phosphorylation-dependent antibody that recognizes phosphorylated Ser396 and Ser404. This antibody is often used in IHC or Western Blotting to visually localize and quantify hyperphosphorylated tau, generally at sites considered to be “late stage” for AD. This antibody can recognize either of the pSer epitopes alone, or both together which results in >10 fold sensitivity when both are present (Otvos et al., 1994).

- **MC-1**, another important tau antibody which recognizes early-stage conformation changes of PHFs across all 6 isoforms of tau. It has dual epitopes at the N-terminal and between amino acids 312-342 in the third microtubule-binding domain (MBD) (Jicha et al., 1997, Oddo et al., 2004). The tau protein needs to be conformationally modified such that MC-1 can simultaneously connect the N-terminal epitope with the mid-domain epitope, otherwise MC-1 won’t bind. Given
that conformational variations of proteins, especially the pathological conformations identified in AD and other neurodegenerative diseases, is an important factor in their overall function, it was prudent to evaluate a conformational-dependent antibody in these studies. The ability to find and bind two linearly separated binding domains at the same time reflects the existence (and possible clearance, from treatment) of early NFT formation, an early toxic form of tau.

- **DA-9**, a pan-tau antibody, has no known specificity for pathological tau and only binds normal tau. This addition is important due to the necessity of normal tau for normal functioning. Here, we addressed the question of whether reduction in normal tau is deleterious. DA-9 is an anti-tau antibody with an epitope at amino acids 102 through 150 (Tremblay et al., 2010). It is not known to interact with known toxic forms of tau, but rather binds normal tau. DA-31, an additional pan tau antibody that binds amino acids 150 – 190, about which less is known, was considered to be an option should DA-9 prove an uninformative treatment alternative. These 4 additional antibodies, PHF1, MC-1, DA-9, and DA-31 were all a gift from Peter Davies of the Albert Einstein College of Medicine in New York. We received these antibodies, solubilized in PBS, at a concentration of 1 mg/ml.

Tau 5, PHF-1, MC-1, and DA-9, are all murine IgG1 antibodies. Anti GFP was used as a control since it is the same isotype of IgG. IgG1 antibodies do not activate complement in the mouse. Any clearance observed from treatment antibodies
compared to the GFP IgG1, would be due to other mechanisms such as those described above for Aβ (Bacskai et al., 2002).

Each antibody, and/or control, was injected intracranially, to ascertain acute benefit. Antibodies were injected unilaterally, into the cortex and hippocampus, of rTg4510 mice that were 11 - 13 months of age. Injection was conducted similarly to the Tau 5 antibody injections described in the Preliminary Results. CED needles maximized dispersion of the injected antibodies, and injection was performed at a rate of 2.5 μl/min, for a total of 2 μl (2 μg) per injection site. Once the injection was complete, one minute elapsed before the needle was retracted.

Figures 2.11 and 2.12 present micrograph and statistical results for the microglial markers CD 45 and Iba1, respectively. Figure 2.13 shows similar results for cell density using Nissl staining. No significant differences were noted in any of these examinations, however the Nissl staining results did appear to reveal a greater, but non-significant, number of cells in the treated animals compared to the control animals.

Subsequent to antibody injection, behavioral analysis was conducted as described above. Figure 2.14 provides results from Rotarod activity testing. Figure 2.15 presents results from Y Maze alternations. Both tests revealed no activity differences between the different treatment groups, for this genotype. The open field test was also completed, but it similarly showed no difference between treatments, therefore the results are not included here.

Post-treatment cognitive tests were also conducted to identify any treatment-based effects. Novel Object Recognition (NOR) testing results are reported in Figure
RAWM results are reported in Figure 2.17. Novel Object Recognition testing showed that the MC-1 treatment restored rTg4510 mice to nTg performance levels. Tau 5 treated mice demonstrated slight improvement in recognition of a foreign object, suggesting that they remembered the original object and avoided the novel object. The RAWM test revealed a high level of variability between the mice, but by the last block of testing, MC-1 treated mice performed significantly better than the IgG1 controls.

Four days after behavioral testing, mice were anesthetized with Somnasol, followed by intracardiac perfusion using a 0.9% saline solution. The left and right cortices (anterior poles of the brain, 3mm in length) were dissected, then snap frozen on dry ice for the purposes of conducting biochemistry such as Western blotting. The posterior portion was immersion-fixed for 24 hours in 4 percent paraformaldehyde for IHC analysis. After 24 hours, the posterior brain portion was cryoprotected using a series of sucrose immersions to remove residual water from the tissue, ending with immersion in 30% sucrose. The brains were frozen for sectioning on a sliding microtome; 25 μm sections were collected and preserved in DPBS + Azide. Sections used for staining were selected every 300 μm, at the approximate depth of antibody injection, for each staining procedure.

Microglial activation was identified using CD45 antibody via IHC (Figure 2.18). NFTs were identified and quantified by Gallyas staining (Figure 2.19). Positive staining was quantified; the values of the injected hemisphere were divided by values from the uninjected side, or were normalized to values from control animals, then statistics were computed to compare the results between treatment groups.
Analysis

Histology data was digitized using a Mirax digital scanning microscope and was analyzed using Mirax software to identify percent staining, by antibody, for a given brain region. Statistical analysis consisted of 1-way ANOVA, computed using Stat View, and the results are presented with means +/- SEM. Positive staining was quantified; the values of the injected hemisphere were divided by values from the uninjected side, or were normalized to values from control animals, then statistics were computed to compare the results between treatment groups.

We could attempt to dissect the observed differences, or lack thereof, by repeating these experiments using additional mice to increase the statistical power of the results. However, this experiment was just a pilot study, so we chose not to pursue it further. The main goal of this pilot study was to do a thorough behavioral investigation of the mice, both before and after surgery, and to note any substantial differences in microglial activation or amyloid pathology as a result of tau antibody administration (post-surgery). The significance (or lack thereof) reported here could be substantiated, or contradicted, by adding additional animals, or by conducting follow-up studies. However the fundamental goals of the pilot study had been achieved and the decision was made to move forward by studying the effects of anti-tau antibodies on more relevant tau-producing mice.
2.8 Investigation of Additional Tau Antibodies, in Additional Brain Regions

Preliminary data, above, reflected initial attempts to utilize an antibody directed to all forms of tau; its epitope is specific and conserved enough to represent binding to total tau and all forms of tau. However, the tau molecule, itself, may not be the most toxic form in the context of AD and tauopathies. Also, as noted above, tau can be hyperphosphorylated at many locations; it can undergo mutation and/or conformational changes, it can undergo other types of post-translational modification, and it eventually forms filaments, which can further aggregate and lead to the formation of NFTs. There are many different “types” of antibodies directed for different forms of tau.

2.9 Ventricular Injection Study

Candidate antibodies were also investigated, via ventricular injection vs. parenchymal injection, to identify whether CSF administration might prove more effective than parenchymal injection. Five or 6 rTg4510 mice were given 15 μl per animal at a flow rate of 2.5 μl/min into the right ventricle. Tissue collection occurred 7 days after injection. Whole-brain sections were collected into two horizontally aligned 24-well plates. Sections from the center-most wells were used for IHC, for microglial markers. Additionally, Gallyas and Nissl staining were completed to identify changes in NFTs or neuronal population. No significant differences were noted as a result of ventricular injection, between any of the treatment groups. This method of administration did not appear to be productive therefore results are not included.
2.10 Tau Aggregation Study

An effort to further understand actions of the administered antibodies was conducted using an aggregation inhibition protocol that relies on the wavelength change of thioflavin T (ThioT), in order to identify the quantities of protein aggregation, with or without, antibody treatment. Recombinant tau (P301L) was used, along with heparin (to induce aggregation), and varying concentrations of each of the antibodies were added to identify aggregation inhibition kinetics.

These in vitro aggregation assays were conducted, using recombinant P301L tau protein (4R0N), to measure the kinetics of tau aggregation. Equal amounts of antibody and tau were added to wells in a 96-well plate. Dithiothreitol and heparin were added to initiate aggregation. Varying antibodies (reported above) were added at different concentrations to identify their effects on aggregation of tau. The resulting fluorescent shift was measured using ThioT. Plates were heated in a 37° water bath (ensuring no water entered the plates), subsequently were read by a plate reader every 12 minutes for a total of 24 hours. ThioT signals from the plate reader quantified protein aggregation.

This study was conducted to identify if the antibodies are effective as aggregation inhibitors, for reducing pathological tau levels, given the questionable results from using them as treatments in vivo. This study was also aimed to understand the dose/response, and time-course, of the potential actions of these antibodies. The antibodies tested for aggregation included: PHF 1, MC-1, DA 9, DA 31 (all graciously received from Peter Davies, PhD.), Tau 5 (purchased), and 2 X TOMA, also known as H12, (a gracious gift from Rakez Kayed, PhD). Proper IgG controls were paired with
each antibody: IgG1 was used as the comparison for all antibodies except for TOMA/H12, which required an IgG2b antibody control.

Each antibody was tested in ½ of a 96-well plate, resulting in 2 antibodies per plate. One antibody was the test antibody of interest, paired with the appropriate antibody control. ThioT was added to all wells (in addition to the recombinant protein, heparin, and dithiothreitol) to visualize aggregation response(s), via the plate reader. Dose-response, and time course of action, was measured for each antibody. The plate reader was programmed to read each plate at selected time intervals to identify aggregation over time. Greater aggregation leads to a higher signal from ThioT binding.

Recombinant P301L tau was the protein, with approximately 20 mM of cell content added, and was prepared to about 5 – 8 mg/ml. This protein content was dialyzed in 100 mM sodium acetate (pH = 7) the night before. A BCA protein analysis was conducted the following day, to prepare the plate(s), as follows:

Dialyzed recombinant P301L tau – 20 mM

20 uM heparin (to induce aggregation)

Varying concentrations of antibody, per plate design

Samples were run in triplicate. The first row, for each antibody, was 10 ug, the second row was 1 ug, followed by the third row at b 0.1 ug, then 0, in terms of the amount of antibody added. Plates were read using a plate reader with proper time points programmed in. Electron microscopy (EM) was used to verify protein identification, using 1 ug/ul or less of the samples. Results were provided after about 40
min per test (10 minutes to generate the picture, the rest involved equilibration, but no hydration).

Experimental design included 4 columns per plate: antibody, IgG control, heat denatured antibody, and either heat denatured IgG control or tau alone (no Ab). Heat denatured antibodies were boiled for 5 minutes; two identical aliquots for each antibody were included, one was heated, the other was used as is (no heat denaturation). The antibody treatments did not appear to inhibit protein aggregation, therefore the results are not included, here.

Figure 2.1: Three genotypes of mice were tested for their general motor abilities by measuring the time they were able to remain on the balance beam (Panel A), then measuring the time they were able to remain on a rotating treadmill that gradually sped up over time (Panel B). No significant difference was noted between the three groups. Error bars represent mean +/- SEM.
Figure 2.2: Mice were placed in a Y maze for 5 minutes, while the arms entered, and the sequence of entry, was recorded. No significant difference was noted in the sequence of arm entries (Panel A) however nTg/NOS2<sup>−/−</sup> mice made more alternations than the other two genotypes (Panel B).
Figure 2.3: RAWM was conducted pre-Injection for all three genotypes of mice to establish a baseline (pre-treatment) level of behavior. Panel A shows the number of errors per block. Panel B shows the total number of errors on all blocks. The NOS2\textsuperscript{−/−} /APP/PS1 mice made a significant greater number of errors compared to the NOS2\textsuperscript{−/−} /APP mice. Open pool testing (Panel C) is a control test to verify that all mice are capable of completing the task.
Figure 2.4: RAWM testing, post injection, using a new platform location. A greater numbers of errors were initially observed, since many animals returned to the original platform location.
Figure 2.5: RAWM testing pre-injection (Panel A) vs post injection (Panel B). More arm-entry errors are identified at the start of the second test, however by the end of testing, mice appeared to learn the new location, and Tau 5 treated mice performed better than anti-GFP treated mice.
Figure 2.6: Fear conditioning was examined post-injection. During training, mice were placed into a chamber where they received a mild foot shock, paired to a novel tone. Freezing is an expected response due to fear of foot shock. Learning the association between the unconditioned tone and conditioned foot shock is indicated by time of freezing.
Figure 2.7: Context testing was conducted 24 hours after FC training. Mice were returned to the original chamber, with no shock or tone administration. Freezing is measured as an indication that mice remembered the associative memory.
Figure 2.8: Cued response post FC training. Mice were placed into novel surroundings, received the tone and freezing response was measured. Freezing response reflects memory of the association between tone and foot shock. NOS2<sup>−/−</sup>/nTg mice showed notable freezing in response to the tone, Tau 5 treatment appeared to increase their associative memory response. NOS2<sup>−/−</sup>/APP mice showed little or no freezing in response to the tone. NOS2<sup>−/−</sup>/APP/PS1 mice also demonstrated very little freezing and again the anti-GFP-treated mice appeared to freeze a little more in association with the tone.
Figure 2.9: Post injection staining results for CD 45 in region CA1 of the hippocampus of NOS2−/− background mice. Tau 5 is compared to anti-GFP to identify differences in microglial activation due to injections. Slight differences were noted, none with statistical significances between the antibodies or genotypes.
Figure 2.10: presents staining results for Aβ in the hippocampus post injection. As expected, the APP/PS1 mice produced the most Aβ, followed by the APP mice, and the nTg mice produced essentially none. The surprise finding here was an increase in Aβ noted in Tau 5 injected mice. This is a likely artifact as the experimental n for this study was 4 animals per group.
Figure 2.11: CD 45 Staining. Microglial activation in rTg4510 mice did not reveal a significant difference as a result of Tau 5 treatment compared to anti-GFP control.
Figure 2.12: IHC staining for microglial activation. Iba1 antibody was used to compare Tau 5 treatment to anti-GFP control. No statistically significant difference as a result of Tau 5 treatment was noted.
Figure 2.13: Nissl staining to identify neuronal cells. No significant difference as a result of Tau 5 treatment compared to anti-GFP treatment was noted in rTg4510 mice.
Figure 2.14: Rotarod testing of general activity levels in post-treated rTg4510 mice. No significant differences in motor performance were observed between any of the treatment groups. H12 is also called TOMA later.
Figure 2.15: Y-maze “alternations” provides another test of general activity. It adds the component of being a simplistic test of working memory; no differences were observed due to any of the antibody treatments to NOS2−/− mice.
Figure 2.16: Novel Object Recognition testing. MC-1 treatment restored rTg4510 mice to nTg performance levels. Tau 5 treatment showed slight improvement.
Figure 2.17: RAWM results for the last block of testing. Variability between mice was high, but by the last block the MC-1 treated mice performed significantly better than the IgG1 controls.
Gallyas Silver Staining

Figure 2.18: Gallyas silver staining for NFTs. Bar graph results are normalized as treated animals vs nTgs. ACX results are demonstrated, taken using MIRAX slide scanner. Graphical Ratios are shown in bar graph. H12 was the initial name for TOMA treatment. Magnification = 20X, Scale bar = 200 μm.
Figure 2.19: pSer199/202 staining: Micrographs show injected (left) side compared to injected (right) side. Graphical representation presents the ratio of treated/untreated (injected/uninjected) sides as a ratio, per different treatments. Scale bar = 200 μm.
Figure 2.18: CD 45 staining did not reveal any significant microglial activation differences in pathology due to antibody treatment. Other anti-tau antibodies tested (results not shown here) yielded similar results. IHC for CD45, however, does show a positive trend to improvement in inflammation in rTg4510 mice, except for the Tau 5 treated group. Scale bar = 200 μm.
Figure 2.19: Gallyas Silver Staining did not reveal any significant differences in pathology in rTg4510 mice due to antibody treatment. Other anti-tau antibodies tested (results not shown here) yielded similar results. IHC for CD45, however, does show a positive trend to improvement in inflammation, except for the Tau 5 treated group.

Scale bar = 200 μm.
CHAPTER 3: SYSTEMIC EVALUATION OF TAU ANTIBODY TREATMENT

Our first published study regarding tau antibody treatment reported the results from 28 days of continuous antibody infusion into the right ventricle of rTg4510 mice. Following infusion, both behavior and pathology were examined. Nine – 11 month old rTg4510 mice were implanted with mini-osmotic pumps to provide continuous delivery of the prospected antibody into rTg4510 mice. Body weight and food intake were measured 3x/week to assess general physiological tolerance to this more aggressive treatment regiment. On average, the mice were approximately 10 months of age at the time of pathology testing. At that time, the groups consisted of 8 nTg mice (controls), 9 rTg4510 mice infused with an IgG1 control treatment, 9 mice who received TOMA (H12) treatment, and 9 mice that were administered Tau 5 treatment. These studies were conducted to provide a more direct comparison between these two antibodies, while changing the route of administration to better assess the effectiveness of antibody treatment. Details, and results from this Infusion study can be found at: (Schroeder et al., 2016), and are included below:
3.1 Abstract

Immunotherapy directed against tau is a promising treatment strategy for Alzheimer’s Disease (AD) and tauopathies. We review initial studies on tau-directed immunotherapy, and present data from our laboratory testing antibodies using the rTg4510 mouse model, which deposits tau in forebrain neurons. Numerous antibodies have been tested for their efficacy in treating both pathology and cognitive function, in different mouse models, by different routes of administration, and at different ages or durations. We report, here, that the conformation-specific antibody MC-1 produces some degree of improvement to both cognition and pathology in rTg4510. Pathological improvements as measured by Gallyas staining for fully formed tangles and phosphorylated tau appeared four days after intracranial injection into the hippocampus. We also examined markers for microglial activation, which did not appear impacted from treatment. Behavioral effects were noted after continuous infusion of antibodies into the lateral ventricle for approximately 2 weeks. We examined basic motor skills, which were not impacted by treatment, but did note cognitive improvements with both novel object and radial arm water maze testing. Our results support earlier reports in the initial review presented here, and collectively show promise for this strategy of treatment. The general absence of extracellular tau deposits may avoid the opsonization and
3.2 Introduction

Dementias are a growing burdensome health condition, affecting an increasingly greater percentage of the world's population. People are living longer, and are surviving conditions such as heart disease and cancer, which are becoming relatively treatable and are declining as 'cause(s) of death'. Alzheimer’s Disease (AD) is the most common and most studied dementia associated with tau accumulation, however there are several other neurodegenerative disorders which are also classified as tauopathies. The term tauopathy suggests that there is some deposition of the protein tau metabolism and pathology occurs in association with this deposition.

AD is the leading cause of dementia, accounting for 50 to 80 percent of dementia cases, and the prevalence of the disease is projected to increase significantly as the baby-boom generation retires and longevity continues to increase. AD is characterized by severe cognitive decline with age, ultimately requiring continuous caregiving and eventually death. The pathology of AD is characterized by the presence of extracellular amyloid plaques, intracellular neurofibrillary tangles (NFT) composed of hyperphosphorylated tau protein, neuron loss, and evidence of inflammation indicated by the presence of reactive microglia and astrocytes, as previously reviewed (Lee et al., 2001, Medeiros et al., 2010). Frontotemporal Lobe Dementia (FTLD) is a rare form of dementia that is somewhat related to AD, most notably in the pathology of
hyperphosphorylated tau and macroscopic brain shrinkage. It has a more rapid onset than AD, with symptoms that reflect personality changes more than memory loss (Lashley et al., 2015). Like AD, there are no known treatments or cures for FTLD. Other tauopathies, where tau becomes pathologic, include frontotemporal dementia with associated Parkinsonism linked to chromosome 17 (FTDP-17), Pick’s Disease, corticobasal degeneration, and argyrophilic grain disease. These diseases have different origins and symptoms, but all share pathologic forms of tau as a major correlative factor underlying the disease (Braak et al., 1993, Utton et al., 2005).

Tau exists as a normal protein within cells to assist in stability of the cytoarchitecture, especially in neurons. It binds to microtubules to provide structural support for axons, and it also facilitates trafficking of important intracellular compounds and organelles, as reviewed by (Morris et al., 2011) and others. It is considered to be a key protein for normal neural functioning; however there are numerous paths by which it can be rendered unstable, or pathological. Post-translational modification is one way that tau can change from beneficial to detrimental; hyperphosphorylation, nitration, acetylation and truncation are examples of post-translational modifications that can significantly alter tau function (Wang et al., 2014). In addition, while considered a natively unfolded protein, tau takes on multiple tertiary conformations, which hinder its ability to perform the intended function and ultimately render it as a toxic entity that leads to neurodegenerative disease (Yu et al., 2012). Tau can become misfolded, leading to aggregation, which can lead to ubiquitination and breakdown by the ubiquitin-proteasomal-system; larger aggregates require the autophagy system for breakdown and removal (Wang and Mandelkow, 2012, Castrillo and Oliver, 2016).
Initial efforts for treating tauopathies have focused on blocking hyperphosphorylation by using kinase inhibitors, which has been thought to be a primary initiating factor for aggregation (Sui et al., 2015). Additional treatment approaches have included: aggregation inhibition using various types of small molecules (O'Leary et al., 2010); degradation of aggregates and clearance by enhancing metabolic processes such as those initiated by heat shock proteins (HSP), the UPS and/or autophagy (Fontaine et al., 2015); direct stabilization of microtubules using paclitaxel, EpoD, or other known microtubule stabilizing agents (Brunden et al., 2010); proteolysis or use of proteases and other methods of aggregate degradation (Guerrero-Munoz et al., 2014) (Utton et al., 2005, Oddo et al., 2009, Gotz et al., 2012, Himmelstein et al., 2012, Mandelkow and Mandelkow, 2012a, Wolfe, 2012, Guerrero-Munoz et al., 2014).

While these potential treatment strategies continue to be explored, an emerging approach that has shown promise in mouse models of tau deposition is immunotherapy. Vaccination is the most common form of immunotherapy, which typically involves administration of antigen, often with adjuvant, to actively increase the body’s production of antibodies. Additionally, immunotherapy can be accomplished by injecting antibodies or antisera against the unwanted substance, thereby eliminating the need for the recipient’s body to produce its own antibodies (passive immunization) (Lee et al., 2001, Wisniewski and Boutajangout, 2010).

An adjuvant is frequently given with active vaccines to boost the immune system’s response to the foreign substance (the immunogen). Adjuvants help improve immunogenicity of the administered compound by activating macrophages and other
innate immune system components, such as microglia in the central nervous system (CNS). They increase the antigen-presentation of the immunogen to lymphocytes, thereby increasing endogenous production of antibodies by stimulating the immune system to produce more of a response than the antigen alone would (Wilcock and Colton, 2008, Agadjanyan et al., 2015, Halle et al., 2015). Most well-tolerated adjuvants used in humans consist of aluminum-based compounds such as alum, which stimulates a Th2 (anti-inflammatory) cytokine response (Ghochikyan et al., 2006). “Freund’s Adjuvant” (either Complete or Incomplete, CFA or IFA) is also commonly used experimentally, and stimulates a Th1 cytokine response (pro-inflammatory) (Billiau and Matthys, 2001). Another common adjuvant, Quil A, is often used in veterinary practices, and the equivalent for human clinical studies is called QS-1 (discussed below) (Ghochikyan et al., 2006, Ragupathi et al., 2010).

Tau has become a focus for immunotherapy studies, in part because of some of the issues and limitations of Aβ immunotherapy in clinical research trials, as previously reviewed (Lemere, 2013), and in part because tau pathology is more directly linked to the symptoms, progression, and severity of AD (Braak et al., 1993, Oddo et al., 2009). There is a much stronger correlation between cognitive decline and the development of tau aggregates than with Aβ deposition (Sigurdsson, 2008). The best strategy for treating AD might prove to be one that targets both Aβ and tau in concert.
3.3 Active Immunization

Aβ immunotherapy began with investigations of active immunization using various forms of Aβ formulated as a vaccine, first in mice and then in human clinical trials. Passive immunization, which consists of injecting purified antibodies, followed preclinical research on active immunotherapy, as previously reviewed (Morgan, 2011a). A similar course has evolved in tau immunotherapy. Active immunization was examined first (Table 1), using various mouse models and immunogens for tau, in addition to multiple adjuvants. The next step was to consider passive immunization with various antibodies and multiple mouse models (see following section). Early studies showed that both active and passive forms of tau-directed immunization clear some of the pathological forms of tau. Even though tau has traditionally been considered an intracellular protein, recent studies have shown it to be released extracellularly and transmit pathology between cells (Lee et al., 2001, Kfoury et al., 2012).

First attempts at tau immunotherapy were performed by a group in Israel (Rosenmann, 2006) who injected full-length recombinant human tau protein with CFA and pertussis toxin (PT) into wild type C57BL/6 mice to see if this might cause an autoimmune response such as encephalomyelitis, which was observed in clinical trials with Aβ vaccination (Orgogozo et al., 2003). The CFA+PT adjuvant was chosen for its proinflammatory effect to identify immunotherapy safety issues. Using this approach, they detected encephalomyelitis along with NFT formation 1.5 to 5 months post-injection. This study demonstrated that by giving tau to non-transgenic animals in the context of severe innate immune activation, tau pathology could be initiated. In an attempt to target toxic forms of tau and avoid deleterious side effects, a follow-up study
involved injecting a combination of 3 phospho-tau peptides, along with CFA and PT, peripherally, into two NFT mouse models (E257T; P301S), hypothesizing a reduction of pathology (Boimel et al., 2010). This peptide combination covered 5 pathogenic phospho-tau residues corresponding to the epitopes for antibodies AT 8, AT 100 and AT 180. Encephalopathic symptoms were not seen up to 8 months post-injection, however significant reductions were seen in NFTs and phospho-tau by staining for Gallyas, AT 8 and AT 180. Microglial population increased in response to the phospho-tau peptide plus adjuvant injections. These effects were still seen 8 months after a single injection followed by one boost.

This group further examined the exaggerated effects of proinflammatory adjuvants (Rozenstein-Tsalkovich et al., 2013) by administering 3 phospho-tau peptide antigens combined with CFA and PT. This combination produces a pro-inflammatory (Th1) response, thereby creating an inflammation-based mechanism of treatment. However, given the initial results from Aβ-targeted immunotherapy in human trials, this type of Th1 biased immunization increased the risk of (apparently) autoimmune encephalitis-like responses. The CFA adjuvant, with added PT, increases blood-brain barrier permeability of antigen, but consistent with the results from anti-Aβ vaccine clinical trials this pro-inflammatory condition can lead to encephalopathic conditions. They noted monocyte infiltration, however no added microglial activation, in response to the adjuvants alone. Antibody production was observed resulting from the p-tau immunizations. Nontransgenic (NTg) mice and double mutant E57T/P301S tau mice revealed significantly more inflammation in the NTg mice compared to the tau mice.
Ittner’s lab reported, in 2011, a study using different adjuvants over the course of administration (Bi et al., 2011). This study examined active immunization using an antigen covering amino acids 395 – 406 (the PHF1 epitope) in pR5 mice overexpressing P301L mutation under the Thy1.2 promoter. Mice, aged 4, 8, and 18 mos, were given three injections of the 12-amino acid antigen conjugated to keyhole limpet hemocyanin (KLH) with CFA adjuvant, followed by injection of antigen with IFA. Four months after they received the initial immunization, the mice showed notable antibody titers. Six to nine months following the initial administration, they collected tissue for anatomical localization and quantification of tau pathology. They identified reductions in Gallyas staining, immuno-staining for PHF1, and pSer422. In addition the oldest group of mice showed a marked significant increase in astrocytosis and there was a trend for increasing levels of GFAP in the middle-aged group of mice.

Following this initial focus on pSer396, Troquier et al (Troquier et al., 2012) studied the pSer422 epitope as a potential therapeutic target. They used THY-1 promoter Tau22 mice, which demonstrate conformationally-based tau pathology at the pSer422 epitope, which is more easily accessible to immunotherapy than other phosphorylated tau epitopes, to investigate the effects of providing active immunotherapy that is specific to pSer422. These mice display pathology starting at 3 months of age. They first tested two immunogens that specifically targeted the pSer422 epitope (one included 7 amino acids, the other 11 amino acids) combined with CFA adjuvant. The mice received immunization for 14 weeks. They then chose the most immunogenic epitope to treat 3.5 mo old mice (15 weeks of age) for 18 weeks followed by analysis for performance in a 2-trial Y-maze test. Pathology was also assessed using
immunohistochemistry analysis (IHC) for AT100 and pSer422, along with Western Blotting for various phospho-tau markers. The behavioral testing showed that vaccinated mice performed similar to NTg animals, based on time spent in the novel arm compared to unvaccinated transgenic controls. The Sarkosyl insoluble tau fraction demonstrated significantly reduced pathology for AT100 and pSer422 by Western analysis. IHC revealed a trend toward reduction of these pathological markers in CA1. They also noticed an apparent efflux of tau from brain parenchyma to the blood sera, suggesting an increase of clearance from brain to systemic mechanisms of clearance, referred to as a peripheral sink (Citron, 2010, Morgan, 2011a).

A very nicely designed series of studies on tau immunotherapy was conducted in the laboratory of Einar Sigurdsson at NYU. Initial work by Sigurdsson’s group (Asuni et al., 2007) examined the effects of active immunization with 4E6G7, a computer-designed immunogen peptide of tau covering amino acids 379-408, which includes the PHF-1 phosphorylation sites of Ser396 and Ser404 (a late-stage phosphoepitope in NFTs). This immunogen, or the Alum adjuvant as a control, was injected subcutaneously between 2 and 5 months into JNPL3 P301L mice (Lewis et al, 2000), which express 4R/0N tau including a FTDP-17-associated tau mutation P301L (Denk and Wade-Martins, 2009) using the mouse prion promoter. These mice develop pathology primarily in the motor cortex, brainstem, and spinal cord, typically resulting in hind limb paralysis after 9 months of age. Alum was chosen as an adjuvant because it is well tolerated and leads to a Th2-type immune response. The vaccinated mice were found to have reductions in insoluble tau and increases in soluble tau, and they exhibited improved performance on rotarod and balance beam tests. Additionally, a
gender difference was noted, with females having more tau pathology before treatment, followed by similar levels to males post-treatment, suggesting that more pathological tau was prevented in the females (Bayer et al., 2005, Sigurdsson, 2008). To address the question of the antibody distribution within the brain and whether or not they could enter neurons, Sigurdsson’s group collected and purified antibodies generated against the tau immunogen, then FITC-labeled them, and injected the antibodies into the carotid arteries of the JNPL3 P301L mice. Later, histochemistry identified labeled antibodies in the brain, within neurons, and co-localized with markers for pathologic tau. Interestingly, the same labeled antibodies also were injected into NTg animals and no uptake into the brain was observed, suggesting that the blood-brain barrier is likely compromised in the transgenic animals, and probably also in humans with tauopathies, which would facilitate antibody passage into the brain (Sigurdsson, 2009). The mouse model used by Sigurdsson et al in their initial studies does not necessarily represent AD pathology; it is primarily a model of motor pathology that progresses so quickly that cognitive maze testing is not practical. However, this was a pioneering study that indicated immunization against tau could be a successful therapeutic approach in tauopathies and perhaps AD.

They followed this with work using a different mouse model that mimics AD more closely, exhibiting development of forebrain tangles and cognitive deficits. This model was created by crossing htau mice with M146L-mutation PS1 mice maintained on a mouse-tau knock-out background. These mice displayed early (before 2 months of age), rapidly progressing tau pathology in cortical and hippocampal areas. The same immunogen 4E6G7 was administered intraperitoneally (i.p.) with alum adjuvant, with
controls receiving adjuvant alone. Antibody titers were measured by ELISA, and were notable, throughout the experiment. Behavioral testing included the same motor tests as described above, and also the radial arm maze, the closed field symmetrical maze, and novel object recognition testing. Immunization resulted in significant performance improvement in all three cognitive tests compared to controls. IHC for PHF1 revealed significant decreases in the immunized mice vs. controls. Soluble PHF1 tau was also reduced as measured by Western blotting. Most notable is that the behavioral improvements correlated well with the levels of PHF1 pathology. (Boutajangout et al., 2010).

Most recently reported work by our research group examined active immunization using different agents, to map relevant epitopes to target with vaccination (Selenica et al., 2014). Specifically, human full-length wild-type tau or P301L tau was administered subcutaneously with Quil A adjuvant into rTg4510 mice for 6 weeks (three bi-weekly injections), rested for 10 weeks then administered 3 additional injections. High titer antibody production in sera was verified and the best antigenic sites on tau were estimated by epitope mapping. The immune response resulted in greatest anti-tau antibody production of isotype IgG1, followed by IgG2b, IgG2a, then IgM. Analysis of IFN-γ demonstrated a strong T-cell response in splenocytes from vaccinated mice. Two N-terminal epitopes, two in the proline-rich domain, and one C-terminal epitope were identified. A 7-amino acid epitope at amino acids 21 – 27 proved to have the most robust binding to antisera, and this epitope corresponds to a caspase-cleavage site. The two epitopes in the proline-rich domain contain phosphorylation sites, perhaps leading to tau dissociation from microtubules and tangle initiation. Microglial activation,
as assessed by CD45 and CD11b IHC revealed decreased inflammation in brains of the vaccinated mice. H150 total-tau staining was reduced in response to the wild-type tau administration, GFAP was reduced in the P301L administration, and AT8 and CD45 staining were reduced in response to both vaccines. A summary of these active immunization studies against tau pathology is reported in Table 1.

3.4 Passive Immunization

Sigurdsson et al (2011) also pioneered passive tau-directed immunotherapy efforts (Table 2), by injecting the monoclonal antibody, PHF1, i.p., into JNPL3 mice. Two to 3-month old mice were given PHF1 weekly, or pooled mouse IgG as a control. At 5 to 6 months of age, behavioral testing (traverse or balance beam, rotarod, and open field) was conducted. The dentate gyrus, motor cortex, and brainstem were evaluated for pathology using IHC for PHF1 to compare pathological forms of tau to total tau levels. They found a significant reduction of PHF1 staining in the dentate gyrus along with a strong trend for reduction in the motor cortex, but no difference in the brainstem. Western blotting for PHF1 and CP13/B19 revealed significant decreases of insoluble pathological tau, but no differences in soluble or Sarkosyl soluble tau. Behavioral analysis resulted in fewer footslips on the traverse beam in the treated group, while the other motor testing did not appear to be affected. Because this mouse model primarily exhibits motor deficits, cognitive performance was not evaluated. The results do, however, show great promise for passive immunotherapy directed against tau, although the authors noted that this passive-immunotherapy approach seemed to be less efficacious than the prior active-immunization approach directed at essentially
the same region of phosphorylated tau. This conclusion was based on the absence of an effect in the brainstem, despite effectiveness in the dentate gyrus. They also noted, however, that passive immunization is likely to be safer, by avoiding a potentially adverse T cell response, such as that found in the AN1792 vaccine study against the amyloid peptide (Boutajangout et al., 2011a).

In parallel work, Martin Citron, then at Eli Lilly was investigating passive immunotherapy directed at tau using two different tau-overexpressing mouse lines, the JNPL3 mouse and P301S mutant PS19 line. The JNPL3 mice received 15 mg/kg of antibody three times per week, while the P301S mice (which demonstrate more rapid progression; pathology becoming evident at younger ages) received 15 mg/kg twice weekly. They tested two different types of anti-tau antibodies: first giving systemic i.p. PHF1 administration or MC-1, an antibody specific to conformational alterations of tau (Chai et al., 2011b). The mice were given antibody or an IgG1 control hybridoma from non-immunized mice, starting at 2 months of age, continuing until 6 months. Western blotting revealed reductions in phosphorylated tau (AT8) in the insoluble fraction, but no change in total tau levels. This was confirmed in an ELISA assay using the AT8 antibody, which revealed a significant and substantial reduction of phosphorylated tau in response to both administered antibodies. They then administered the same antibodies to P301S mice from age 2 to 5 months, tested motor performance and showed a significant improvement on the rotarod for both antibody groups compared to controls. The rate and progression of improvement in motor deficits was greater for the P301S mice, which exhibit a more rapid onset of pathology, than the JNPL3 mice. Similarly, there appeared to be a delayed onset of pathology in the P301S mice. ELISA analysis
revealed 45 percent reduction in pathology with PHF1 administration, and a 33 percent reduction in pathology after MC-1 administration. IHC also revealed reduced tau pathology after administration of either antibody compared to control, and no significant difference in microglia or astrocyte activation.

Work in Peter Davies’ lab has followed on these investigations using JNPL3 mice. PHF1, MC-1, or the pan-tau antibody DA31, were administered to either 4 or 7 month old mice for 4 months. They showed that MC-1 treatment reduced CP-13 staining in CA1 of the hippocampus as well as reducing RZ3 staining, in response to the antibodies directed against pathological tau isoforms. Their study looked for IgG in neurons, but failed to identify any, leading to questions of mechanism of action (d' Abramo, 2013)

Yanamandra et al examined the effects and actions of various extracellular tau-targeting antibodies on P301S mice (Lee et al., 2001, Yanamandra et al., 2013). Ongoing collaborations between Marc Diamond and David Holtzman have resulted in the creation of a spectrum of antibodies with varying activities for binding extracellular tau, with a general emphasis on preventing extracellular aggregation and “seeding” (propagation) of tau pathology. They have created a line of tau antibodies called the HJ8 series, which is raised against full-length human tau, as well as a HJ9 series that are directed against mouse tau (Espinoza et al., 2008). These antibodies were developed in response to observations of extracellular tau appearance was followed by seeding of pathology into nearby cells. They hypothesized that tau can aggregate extracellularly, then become absorbed by adjacent cells, thereby transmitting the pathology. They employed intracerebroventricular (ICV) administration using 3 of their
antibodies: HJ3.4 directed against Aβ, HJ8.5 an anti-human tau antibody, and HJ9.3 & HJ9.4, which are anti-mouse tau. ICV provided continuous delivery of the antibodies, for 3 months, into 6-month old P301S mice. Clearance of antibody from the CNS to the periphery was identified by temporary infusion of biotinylated HJ8.5 followed by analysis of CSF and plasma, during the course of the ICV treatment. Behavioral analysis revealed no differences in motor function, but there was a significant improvement in contextual fear conditioning. The HJ8.5 treated mice exhibited far greater freezing levels than control tau mice, and to a lesser degree the HJ9.4 treated mice also exhibited significantly more freezing.

Pathological evaluation revealed that administration of HJ8.5, HJ9.3 and HJ9.4 reduced immunostaining for AT8 (pSer202/pThr205), in the piriform cortex, amygdala, and entorhinal cortex, with HJ8.5 demonstrating the most robust improvement in pathology. HJ9.4 did not show significant reductions in the piriform cortex, however. These results were consistent between male and female mice, however the males had greater overall pathology. These results correlated with reductions in staining for PHF 1 (pSer396/404) as well as reductions in the microglial marker CD68. Additionally, a semi-quantitative ranking of ThioS staining revealed a reduction of tau deposition in the HJ8.5-treated mice compared to controls.

Biochemical analysis of tau levels was determined using ELISA on 3 fractions from the anterior cortex: an aqueous fraction produced using RAB buffer, a detergent soluble fraction using RIPA buffer, and an insoluble fraction obtained by formic acid (FA) digestion of the resulting pellet. The aqueous and RIPA-soluble fractions did not reveal significant changes in total tau levels as detected using their total-tau antibody
HJ8.7, however the detergent insoluble FA fractions demonstrated a significant decrease (> 50%) of total tau in the HJ8.5 and HJ9.3 treated animals. Western blot analysis, using mouse polyclonal antibodies, revealed similar results to the staining for AT8 and CD68 in response to these two treatments. The HJ9.4-treated animals did not demonstrate such reductions. These results were specific to human, not mouse, tau as assessed by ELISA using species-specific tau markers. AT8 levels followed a similar trend in the FA fractions based on treatment. They further treated HEK293 cells with the RAB lysates from the treated mice; FRET analysis identified significantly lower seeding levels in response to the H8.5 and HJ9.3 (but not HJ9.4) treatments that correlated with the FA-fraction ELISA tau levels. This study emphasized monomeric, extracellular tau in interstitial fluid (ISF), which was previously thought to be a very unlikely location for tau protein until recently. Tau may aggregate extracellularly as well as intracellularly, thereby passing pathology on to nearby cells (Yamada et al., 2011).

Alternatively, Rakez Kayed’s laboratory has been examining antibodies that are specific to oligomeric tau. Oligomers of tau, as well as other proteins, have been identified as possibly being the most pathogenic form of these prion-like proteins (Mandelkow et al., 1996, Hoover et al., 2010, Medeiros et al., 2010, Lasagna-Reeves et al., 2011c). Kayed’s laboratory developed and characterized, novel antibodies, T22 and TOMA, that specifically target tau oligomers, and not monomers or stable NFTs (Lasagna-Reeves et al., 2012a, Lasagna-Reeves et al., 2012b). T22 is a rabbit antiserum that targets oligomers for analysis, whereas TOMA is a mouse monoclonal antibody that has been tested as an immunotherapy agent. Initially, they administered recombinant tau aggregates to 2-month old BALB/c mice along with CFA. The
monoclonal antibodies derived from hybridomas from these mice were screened for specificity of tau oligomer targeting. In vitro testing, by ELISA, Western and dot blot demonstrated that T22 selectively marked oligomeric tau, but not monomeric tau, when compared to Tau 5 which recognizes all forms of tau; T22 did not bind to monomeric tau, while Tau 5 recognized all tau forms, thus demonstrating that T22 is a specific marker for tau oligomers. This original oligomer-specific polyclonal antiserum for tau enabled more specific identification of tau oligomers, so that characterization of tau pathology could be assessed in a new manner, then treatment approaches could be evaluated with a focus on oligomer-based pathology and improvements due to treatments.

Kayed’s lab further created and validated a mouse monoclonal oligomer-specific antibody, TOMA (tau oligomer monoclonal antibody), which they administered to tau transgenic mice to examine its potential as a new immunotherapeutic approach for treating tauopathies (Castillo-Carranza et al., 2014a) TOMA was administered by ICV or IV injection to 8 month-old male JNPL3 mice to examine the effects of oligomer-targeted treatment of tau pathology in vivo. Their ICV protocol was to inject 1 μg TOMA into each lateral ventricle one time. IV injection involved dilating the blood vessels in the tail, then injecting 30 μg of antibody. An additional group of 8 month-old mice received a tail-vein injection of 30 μg of biotinylated TOMA. Blood was collected before, and at several intervals after, injection to measure antibody concentration; brain tissue was collected to identify delivery of the antibody across the blood-brain barrier and into brain parenchyma. In vivo imaging of these animals revealed that the biotinylated TOMA did cross into both the brain and spinal cord. TOMA administration restored
behavioral performance to that of wild-type mice in the tests conducted. The single ICV injection normalized rotarod performance to NTg levels. Additionally, the single IV administration yielded improvements in Y maze performance, which persisted through re-testing 2 months later. Pathology was evaluated using Western Blot, and probing for oligomeric-only tau using the polyclonal T22, in comparison to total monomeric tau, using Tau 5. ICV injection of TOMA led to a strong reduction in T22, but not Tau 5 staining by immunoblot; results were confirmed by immunofluorescent IHC staining, and were further correlated to reductions in pThr231 detection of tau aggregates, which has been shown to be associated with oligomers in human tissue (Lasagna-Reeves et al., 2011a). HT7 results showed increased levels while various other phospho-tau markers were decreased, suggesting a possible transfer of oligomeric tau to monomers and early aggregates. The single IV dose of TOMA, while showing improvements in Y-maze and rotarod performance, also revealed reductions in tau oligomers (assessed by Western blot, probed with rabbit-anti tau antibody), as well as reductions in ELISA levels of tau oligomers. Further anatomical evaluation using immunofluorescence showed reduced levels of oligomers in cell bodies and axons of CA1 neurons compared to controls, and there were no reductions in AT8 or Gallyas staining, suggesting that changes in monomeric and NFT forms of tau were not involved in the improvements demonstrated here. ELISA and Western blot analysis using numerous other phospho-forms of tau did not show differences in either of the treatment protocols. They concluded that oligomeric tau clearance leads to behavioral improvement, whereas earlier monomers, phospho-forms, and even fully formed NFTS did not need to be cleared for these functional improvements.
The next step in AD-related immunotherapy involved passive immunotherapy to 3xTG animals to identify benefits to both tau and Aβ pathologies. Frank LaFerla’s laboratory (Walls et al., 2014) has created a transgenic mouse line with the swAPP/PS1 and tau P301L mutations (3xTg mouse line), with the intent to examine correlated effects and treatments for both Aβ and tau pathology (Walls et al., 2014). Mice aged 15–18 months old were administered the AT8 anti-tau antibody, which recognizes the pSer202 and pThr205 epitopes (Walls et al., 2014). The mice were given AT8, 4G8 (an anti-Aβ antibody) or IgG control, intracranially, into the CA1 region of the hippocampus. A single injection was followed by tissue collection at weekly intervals (1 to 4 weeks post-injection), then IHC to identify changes in pathology. The AT8-injected animals showed a dramatic reduction in tau pathology one-week post-injection (HT7 measurements). This reduction continued through week 2, but starting at week 3 the levels of tau pathology started returning to levels seen in IgG-treated controls. AT8 staining was also reduced 1-week post-injection, as was Gallyas staining for fully-formed NFTs. No changes in Aβ pathology were noted by staining for (6E10 measurements) in response to AT8 injection. However injection of 4G8 resulted in Aβ reductions for 1-2 weeks followed by recovery back to baseline levels, analogous to the transient reductions in tau found with AT8. A summary of these studies is reported in Table 2.
3.5 Mechanisms Of Action

The results summarized above have demonstrated that varied methods of applying anti-tau antibodies are capable of clearing tau pathology, in parallel with improving behavior. The question that obviously arises is: what is the mechanism by which this clearance is occurring?

The earlier work conducted by Sigurdsson’s group has suggested that the tau-antibodies are taken up by neuronal cells. Then autophagy and induction of lysosomal activity degrades and clears the protein. This is the preferred pathway for degradation of misfolded proteins, oligomers and aggregates. These ‘bad’ proteins become sequestered into autophagosomes and then are enzymatically degraded (Ding and Yin, 2008). The autophagy pathway can be overwhelmed in pathologic conditions, leading to accumulation of aggregates in the cytosol, which leads to formation of fibrils, paired helical filaments, and other β-pleated sheet formations such as those seen in AD (Sigurdsson, 2009, Morris et al., 2011).

Yanamandra’s group suggested an extracellular method of tau clearance. Their antibodies were able to impede uptake of extracellular tau aggregates and inhibit prion-like propagation of tau between cells. They demonstrated that, by binding extracellular tau aggregates, intracellular tau pathology could be delayed or prevented from passing on to a neighboring cell (Yanamandra et al., 2013).

Kayed’s group (Castillo-Carranza et al., 2014b) demonstrated that TOMA did not need to be internalized to reduce tau burden. Their results argued for a distinct clearance of tau pathology extracellularly. This extracellular clearance could also result
in a peripheral sink type of mechanism by drawing tau oligomers from the brain and into the circulation, not unlike what has been observed for some anti-Aβ antibodies (Morgan, 2011a).

Another potential mechanism of clearance that has largely been considered for both Aβ and tau immunotherapy involves microglial phagocytosis and removal, quite possibly in conjunction with inflammatory mechanisms. Sigurdsson’s group addressed the role of inflammation and the immune system in tau pathology’s clearance by microglial phagocytosis. They concluded indications did not favor this route of elimination (Sigurdsson, 2008).

Kayed’s group (Castillo-Carranza et al., 2014b) analyzed brain sections from treated and control groups using ELISA and immunofluorescence, for IL-6, IL-1β and Iba1, and found no differences between any of the antibody treatment groups, thereby suggesting that microglial activation and inflammation were not major factors in their model. Their analysis of serum and CSF suggest that oligomeric tau clearance occurs extracellularly and leads to elevated breakdown products in systems designed for full-body elimination, vs simply eliminating these polymers from neuronal cells.

Roche Pharmaceuticals has also examined tau immunotherapy using a triple transgenic model, and has followed on the earlier lines of targeting pSer422 with passive immunotherapy (Collin et al., 2014). Their 3x mouse-line is TauPS2APP; swAPP mice crossed with PS2, then P301L tau mutant mice. These mice received either an acute treatment, or a chronic treatment of their cultured MAb86 antibody (phosphor tau 416 – 430, produced in rabbit) that was either mouse IgG1 (for chronic
administration) or human IgG1 (for shorter administrations). Acute treatment consisted of two i.p. doses of the “human” MAb86, 3 days apart, in 16 month-old mice, with tissue collection 2 days after the last injection. Chronic administration began with 10-month old mice, which received weekly i.p. doses of antibody for 16 weeks, followed by tissue collection a week later. The pSer422 monoclonal antibody, MAb86, specifically binds to tau phosphorylated at Ser422, but not unphosphorylated tau at that location. MAb86 specific binding was located to the CA1 region of the hippocampus of TauPS2APP mice, as shown by immunofluorescence, and it was further shown to be intracellular (somatic or dendritic) binding, suggesting that the antibody is taken into neuronal cells. Double staining with MAb86 and flotillin 1 (marker for lipid rafts) indicated localization in lipid rafts within the plasma membrane. Further localization with lysosomes corroborated the prior studies that suggested anti-tau antibodies are internalized, leading pathologic forms of tau to lysosomes for degradation and removal. They further demonstrated that chronic administration of MAb86 delayed tau pathology, while favoring lysosomal clearance using ELISA and immunofluorescence.

In summary, there are multiple mechanisms anti-tau antibodies might engage when used for the clearance of pathological forms of tau. These include stimulation of intracellular degradation, possibly following internalization of complexes, neutralization of intracellular aggregates, or neutralization of extracellular aggregates, preventing internalization and/or spreading of pathologic tau. Moreover, the peripheral sink mechanism, which reveals increased blood levels of tau aggregates, has been reported and might aid in tau clearance from the brain. At this stage, there is not much evidence favoring an opsonization and enhanced phagocytosis as a mechanism for tau
clearance, as supported by work with anti-Aβ antibodies, but this cannot yet be ruled out. Just as it is likely that different anti-Aβ antibodies employ different or multiple methods of clearing amyloid (Morgan, 2011a), it is equally plausible that different anti-tau antibodies employ different or multiple mechanisms to stem the propagation of, or clearance of tau. Hence, there is not a right or a wrong mechanism of immunotherapy in opposing tau action in tau depositing mice. The apparent mechanistic differences observed by different research teams need not be viewed as conflicts. In fact they might be collectively integrated in attempts to identify the most efficacious monoclonal antibodies (possessing more than one mechanism of action).

3.6 Recent Work In Our Laboratory

Previous work in our laboratory has shown that peripheral administration of anti-Aβ antibody was successful in reducing diffuse amyloid and compact plaques in the brain, and this was accompanied by improved performance in the radial arm water maze (Wilcock et al., 2004b). Multiple microglial markers were modified by immunotherapy suggesting that one likely mechanism of clearance involved opsonization and Fcγ receptor-mediated phagocytosis. Our work in tau immunotherapy has been less conclusive, to date. So far, our results have suggested some positive impacts from tau passive immunotherapy, however the most effective tau antibody remains elusive.

We’ve treated several ages of rTg4510 mice, which demonstrate rapid tau deposition resembling that of advanced AD or FTLD patients (Santacruz et al., 2005a).
This mouse line expresses the 4R0N tau isoform with a P301L mutation under control of a tetracycline (tet) promoter. Crossing tau mice with a CamK\(^ {\text{++}}\) Tet-transactivator-protein mouse drives high-level forebrain tau expression. These mice develop robust, progressive, age-dependent, pre-tangle and NFT pathology starting at 3 months of age. In addition to robust tau pathology, this model develops readily observable brain shrinkage, visible as early as 6 months of age. Concurrent with the tau pathology is neuronal loss and atrophy. Behaviorally these mice exhibit extreme hyperactivity and impairment in a large number of cognitive function tests (Brownlow et al., 2014).

Initial studies examined rTg4510 mice, at an age when robust tau pathology is well established (between 11 and 13 months of age), by intracranial injection of several tau antibodies into the frontal cortex and hippocampus. Injections were performed unilaterally using convection-enhanced delivery (CED), as described in (Carty et al., 2010), at a rate of 2.5 μl/min, resulting in a total of 2 μg IgG per injection site. The contralateral hemisphere was untreated and used to normalize for differences in starting amounts of deposition. Four days post-injection, tissue was collected for IHC and histological staining to compare tau-pathology markers between the groups. NFTs were identified using Gallyas silver staining (Brownlow et al 2014). We also examined total tau using the rabbit polyclonal antibody H150 (Santa Cruz), rabbit-anti pSer199/202 (AnaSpec), and rabbit-anti pSer396 (AnaSpec). CD45 and Iba-1, markers for microglial activation, were also examined, as well as total neuron population via Nissl staining. Sections were imaged with a digital scanning microscope (Mirax) and fractional area stained measured by image analysis of entire regions from 4-6 sections.
per region. Results are expressed as a fraction of the uninjected contralateral hemisphere.

Significant reductions in Gallyas staining were observed (Figure 1) as well as Ser199/202 (Figure 2) and H150 (Figure 3) staining in the hippocampus of mice treated with the antibody MC-1 when compared to mice treated with the antibody directed against the non-mammalian protein green fluorescent protein (GFP), which was not expected to remove tau deposits. We also examined staining for pSer396 and relative microglial activation, because microglial activation is one of the common pathological features of AD (Table 3). No notable increase in microglial activation, as examined by CD45, was identified which is an encouraging finding because over-activation of the innate immune system has proven to be a challenge in clinical studies of Aβ immunotherapy. Nonetheless, all tau-depositing mice have much more CD45 than their non-transgenic littermates. Similarly, we did not observe notable difference in neuronal population between the different treatments when using Nissl staining.

We conducted a subsequent study using 10 – 12 month-old mice, infused with either MC-1, Tau 5 or TOMA, or IgG1 (as a control) antibodies. The mice where implanted with mini-osmotic pumps (Alzet) filled with 20 μg of either MC-1, Tau 5, or TOMA, or IgG control, (all of which were 1 mg/ml) into the right lateral ventricle for continuous delivery of 0.5 μg a day over 28 days, using mini-osmotic pumps (Alzet) as described previously (Selenica et al., 2013, Brownlow et al., 2014). This route of administration allowed for behavioral testing as well as pathology evaluation. Activity testing for open field, revealed a genotype effect but no treatment effect; no differences were noted between the treated mice and controls when comparing tau-depositing
transgenic mice to non-transgenic littermates (Table 4). Cognitive assessment (as in Brownlow et al, 2014) did reveal performance improvements in both novel object recognition testing (Figure 4) and radial arm water maze (RAWM – Figure 5) in response to MC-1 treatment when compared to IgG1 treated mice.

Overall these data suggest that MC-1, a conformation specific antibody developed by Peter Davies, has greater efficacy than several other antibodies when administered ICV. These data encourage consideration of a humanized form of this antibody or related human antibodies for testing in early stage cases of AD.

3.7 Future Directions

Tau provides many different strategies for treatment given its different isoforms, post-translational modifications, conformations, and more. Presently, it appears that targeting oligomers of tau is likely to be the focus for many of these studies. Multiple groups have been investigating oligomeric forms of amyloid and tau and consider these to be the most toxic forms of the proteins. As the groundwork continues to be laid in tau immunotherapy studies, it is likely that the next generation of immunotherapies will be directed at oligomers, as Kayed’s lab has been doing. Additionally, many have suggested a future immunotherapeutic approach targeting Aβ and tau in concert, as noted by recent work in LaFerla’s laboratory (Medeiros et al., 2011). Interestingly, the general success obtained with anti-tau approaches might favor tau immunotherapy over anti-amyloid immunotherapy. The low levels of extracellular tau coupled with the absence of general microglial activation may avoid the edema and hemorrhage
observed with anti-amyloid immunotherapy in mice and humans (Wilcock et al., 2007b, Sperling et al., 2012).

On the clinical front, a tau active vaccine entered into a Phase 1 human clinical trial; an agent named AADvac1. Axon Neuroscience SE began a clinical study in mid 2013, recruiting subjects for a Phase I clinical study on the safety and tolerance, of this active immunotherapy agent: a synthetic tau peptide conjugated to KLH, also using aluminum hydroxide as an adjuvant to further stimulate the immune response. The reported protocol consists of treating patients with mild to moderate AD three times, over a course of three months, then assessing response by measuring neuropsychiatric measures, cognitive testing, MRI, and blood biomarkers. Additional measures of immune response to the treatment will be obtained to verify the responses as immune-mediated.

3.8 Acknowledgements

The authors thank Dr. Peter Davies for the generous gifts of monoclonal antibodies MC-1, PHF1 and DA9, for the treatments reported above. We also thank the vivarium staff at the USF Health Byrd Alzheimer’s Disease Institute for their help in maintaining the health and care of our mouse colony, including maintaining ethical standards and compliance as described in the “Guide for the Care and Use of Laboratory Animals” in an AAALAC-accredited facility. All procedures with mice were approved by the Institutional Animal Care and Use Committee of the University of South Florida. All applicable international, and/or institutional guidelines for the care and use
of animals were followed. This work was supported by NS076308 and by the USF Health Byrd Alzheimer’s Institute.
<table>
<thead>
<tr>
<th>Authors</th>
<th>Antigen, Dose &amp; Adjuvant</th>
<th>Mouse Model, incl. mutation, if present</th>
<th>Age at first inj</th>
<th>Duration</th>
<th>Techniques and markers examined</th>
<th>Details/Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asuni et al, J Neurosci 2007</td>
<td>100 μl Tau379-408 @ 1 mg/ml with Adju-Phos adjuvant, sc bi-weekly for first month, then monthly</td>
<td>JNPL3 (P301L)</td>
<td>2 mo</td>
<td>3 - 5 mo</td>
<td>IHC and Western Blot for MC-1 &amp; PHF1, Rotarod, NOR</td>
<td>↓ MC-1 and PHF 1 by IHC; ↑ PHF 1 in soluble fraction by Western, ↑ time on rotarod; No cognitive change in NOR; noted antibody uptake into neurons</td>
</tr>
<tr>
<td>Boutajangout, et al, J Neurosci 2010</td>
<td>100 μg Tau379-408 + 100 μl Alum, i.p.; 3 injections bi-weekly, then monthly</td>
<td>hTau/PS 1</td>
<td>3-4 mo</td>
<td>5 - 6 mo</td>
<td>IHC for PHF1 &amp; AT8, PHF1 by Western, Rotarod, Traverse Beam RAM, NOR, CFSM</td>
<td>↓ PHF1 and AT8, ↓ soluble PHF1, no change in rotarod performance, improved performance in RAM and NOR</td>
</tr>
<tr>
<td>Boimel et al, Exp Neurol 2010</td>
<td>sc injection of 100 μg each of 3 KLH-linked phos-tau-peptides (aa’s ~ 195 - 238) with i.p. CFA + PT. Adjuvant booster @ 48 hrs, then sc antigen booster 1 wk later</td>
<td>E257T/P301S</td>
<td>4 to 12 mo</td>
<td>2 wks</td>
<td>Clinical evaluation of paralysis; Gallyas staining (NFT); IHC for AT8, AT180, and glial markers GFAP, MAC3, CD45R and CD4; Bielschowsky-Hematoxylin and lectin staining; double immunofluorescence for CD11b, F4/80 and MAC3; &amp; lysosomal staining for Cathepsin D</td>
<td>No ↑ in paralysis; no difference in monocyte infiltration (Bielschowsky Hematoxylin staining), CD45R &amp; CD4 staining showed limited B or T cell infiltration, no macrophages detected by MAC3 &amp; no axonal damage noted by these stains, so encephalogic risk is minimal; injected tau present in sera &amp; blood, not parenchyma; ↓ NFTs, ↓ AT8, ↓ AT180, ↑ microglia by lectin staining, ↓ Cathepsin D</td>
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<tr>
<td>Bi et al, PLOS1 2011</td>
<td>100 μg KLH-linked peptide (aa's 395-406) with CFA (first) then with IFA 2 &amp; 4 wks after</td>
<td>pR5 (P301L)</td>
<td>4, 8, or 18 mo</td>
<td>1 mo</td>
<td>Gallyas staining for tangles, IHC for PHF1 &amp; pSer422, GFAP staining</td>
<td>↓ NFTs, PHF1 and pSer422; ↑GFAP in old mice, trend ↑ in mid-aged mice, no difference in GFAP in young after vaccine</td>
</tr>
<tr>
<td>Troquier et al, Curr Alz Res 2012</td>
<td>100 μg Y14T or Y10A (pSer422 epitope) i.p. + CFA; 2nd injection 2 wks later with IFA, then monthly</td>
<td>THY-Tau22 (4R @ g272v &amp; p301S with Thy 1.2 promoter)</td>
<td>2 or 3.5 mos</td>
<td>14 wks or 18 wks</td>
<td>IHC, Modified Y-maze, ELISA, Western</td>
<td>No changes in AT8, ↓ in AT100 &amp; pSer422, vaccines ↑ performance to wt levels, ↑ tau in sera in correlation with duration of treatment &amp; memory performance, ↓ AT100 &amp; pSer422 in SDS-insoluble fraction</td>
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<td>Rozenstein-Tsalkovich et al, Exp Neurol 2013</td>
<td>Sc injection of 3 phos-tau-peptides @ 100 μg, in IFA and PT, given twice bi-weekly, then 50 μl peptide monthly</td>
<td>E257T/P 301S</td>
<td>6 or 12 mos</td>
<td>8 - 14 wks</td>
<td>Experimental autoimmune encephalomyelitis test, IHC, behavioral observation</td>
<td>↑ monocyte infiltration with memory improvement, no microglial change, treated mice developed paralysis</td>
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Table 3.1: Active Immunization Using Tau Antigens (continued)

<table>
<thead>
<tr>
<th>Study</th>
<th>Adjuvant</th>
<th>Immunization Schedule</th>
<th>Duration</th>
<th>Assays</th>
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<tbody>
<tr>
<td>Selenica et al, J Neuroinflamm 2014</td>
<td>Quil-A</td>
<td>Sc administration of 100 μg tau (human wt or P301L tau) with 20 μg Quil-A adjuvant. 3 bi-weekly injections followed with a 10-week resting period then boosters every 3 weeks for 3 additional times</td>
<td>5 mos</td>
<td>19 wks, including a 10-day resting period</td>
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</tbody>
</table>

↑ antibody production, ↑ cellular response: ↓ CD45, CD11b and GFAP; ↓ H150 & AT8; 5 new immunogenic epitopes identified by microarray: 2 N-terminal (aa's 9-15 and 21-27), two in the proline-rich domain (aa's 168-174 and 220-228) and 1 in the C-terminal (aa's 427-438); 5 new immunogenic epitopes were identified: 2 N-terminal (aa's 9-15 and 21-27), two in the proline-rich domain (aa's 168-174 and 220-228) and 1 in the C-terminal (aa's 427-438)

Table 3.1: Active immunization using tau antigens, notes:

sc = subcutaneous
i.p. = intraperitoneal
IHC = immunohistochemistry
NOR = Novel Object Recognition
RAM = Radial Arm Maze
CFSM = Closed Field Symmetrical Maze
KLH = keyhole limpet hemocyanin
aa’s = amino acids
CFT = complete Freund’s adjuvant
PT = pertussis toxin
IFA = incomplete Freund’s adjuvant
NFT = neurofibrillary tangles
GFAP = glial fibrillary acidic protein
wt = wild-type
<table>
<thead>
<tr>
<th>Authors</th>
<th>Antibody, Dose &amp; Administration</th>
<th>Mouse Models, incl. mutation if present</th>
<th>Age at first injection</th>
<th>Duration</th>
<th>Details/Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chai, et al, JBC 2011</td>
<td>PHF1 or MC-1, 15 mg/kg, peripheral injection (not specified)</td>
<td>JNPL3 (P301L) and/or P301S</td>
<td>2 mos</td>
<td>2 - 3 mos</td>
<td>↓ insol AT8, no change HT 7 by Western, ↑ Time on rotarod, ↓ AT8 &amp; PG5 by IHC; Correlation of AT8 signals in P1 fraction with Sarkosyl-insoluble fractions to show that the preparations were valid</td>
</tr>
<tr>
<td>Boutajang out, et al, J. Neurochem 2011</td>
<td>i.p. injection of PHF1</td>
<td>Female JNPL3</td>
<td>9 - 12 wks</td>
<td>2 - 3 mos</td>
<td>↑ Time on balance beam at 5 - 6 mos, but no difference on rotarod performance; ↓ PHF1/B19 ratio, ↓ PHF1 (alone), ↓ CP13/B19 ratio, ↓ CP13 (alone) in Sarkosyl fraction for Western; ↓ PHF1 in right Dentate Gyrus by IHC</td>
</tr>
<tr>
<td>d’Abramo, et al, PLOS1 2013</td>
<td>i.p. injection of PHF1, MC-1, or DA31: 250 μg/125 μl, for a total of 10 mg/kg weekly</td>
<td>Female JNPL3</td>
<td>3 or 7 mos</td>
<td>3 - 4 mos</td>
<td>↓ CP13 in CA1 with MC-1 treatment by ELISA, ↓ RZ3 in CA1 with DA31 treatment and ↓ CP13 from MC-1 in older mice by IHC; ↓ RZ3 in CA1 of older DA31 treated mice (total tau Western Blot), ↓ in total tau with MC-1, No change with DA3 (Western Blot of insoluble fraction), no changes in survival</td>
</tr>
</tbody>
</table>
Table 3.2: Passive Immunization Using Tau Antibodies (continued)

| Yanamandro, et al, Cell/Neuron 2013 | icv injection of their own tau antibodies | P301S | 6 mos | 3 mos | HJ8.5 ↓ AT8 in piriform & entorhinal Cx, hippocampus & amygdala (by IHC), HJ9.3 ↓ AT8 to lesser degrees in similar regions, HJ9.4 had less ↓, & no change in piriform Cx; ThioS and PHF1 staining yielded similar results. Staining for activated microglia strongly correlated with AT8 staining; HJ8.5 & HJ9.3 ↓ activated microglia in piriform & entorhinal Cx and amygdala. HJ9.4 showed lower effect on microglia; ThioS and PHF1 had similar results. Staining for activated microglia strongly correlated with AT8 staining; HJ8.5 & HJ9.3 ↓ activated microglia in piriform & entorhinal Cx and amygdala. HJ9.4 had lesser effect on microglia. Significant (>50%) ↓ of detergent-insoluble tau (Western) from HJ8.5 and HJ9.3 treatment; > human tau than mouse tau in the FA “insoluble” fractions, ↓ by treatment; “seeding” ↓ by HJ8.5 and HJ9.3, not HJ9.4, in correlation with insoluble tau. No differences in motor testing, Contextual Fear Conditioning ↑ from all treatments with HJ8.5 = most robust freezing, Cued Fear Conditioning not affected by treatments |
| icv injection of their own tau antibodies: Continuous icv infusion of 7.2 μg/d, for 6 weeks of: HJ8 series (anti human tau); HJ8.5 = AA’s 25 – 30; HJ9 series (anti mouse tau); HJ9.3 = AA’s 306 – 320 (repeat domain); HJ9.4 = AA’s 7 – 13 (N-terminal); Or HJ3 series (anti Aβ, mouse) | | | | | |


Table 3.2: Passive Immunization Using Tau Antibodies (continued)

<table>
<thead>
<tr>
<th>Study</th>
<th>Immunization Method</th>
<th>Timepoints</th>
<th>Administration</th>
<th>Immunologic Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Castillo-Carranza, et al, J. Neurosci 2014</td>
<td>icv (1 μl @ 1 mg/ml) or iv (30 μg/animal) injection of TOMA (tau oligomeric monoclonal antibody): single injection; either icv or iv, then 4 days until tissue analysis</td>
<td>JNPL3 4 or 8 mos</td>
<td>single injection</td>
<td>icv ↑ time on rotarod, to near-wild type levels, correlating w/ reductions of oligos; icv treatment ↓ immunofluorescence of Thr231 and T22 as co-localized with DAPI; Single iv injection ↑ time on rotarod and ↑ alternations in Y maze 4 days post-injection; Single icv injection ↓ oligomeric tau (T22); Single iv injection ↑ oligo and HT7 ELISA levels in serum, correlating to ↓ levels in the brain; Single iv injection had no effect on AT8 or Gallyas staining; only T22 staining for oligos was reduced; Single iv injection did not change Western blot analysis for AT8 and/or PHF-13; Western analyses showed ↓ oligo levels, while Tau 5 showed ↑ monomeric tau, by both administrations.</td>
</tr>
<tr>
<td>Walls, et al, Neurosci Letters 2014</td>
<td>i.c. injection of either AT8, 4G8 or IgG; 2 μg/animal</td>
<td>3xTg (swAPP/PS1/P30 1L) 15 - 18 mos</td>
<td>1 - 4 wks</td>
<td>HT7, AT8 and Gallyas staining ↓ 1 - 2 weeks post-injection of AT8; From 3 weeks on, levels of tau (phospho, and normal) returned to IgG-treated (control) levels; 6E10 levels reduced due to 4G8 administration, following a similar pattern; AT8 administration did not effect Aβ levels, and 4G8 did not affect tau</td>
</tr>
</tbody>
</table>
Table 3.2: Passive Immunization Using Tau Antibodies (continued)

| Collin, et al, Brain 2014 | i.p. injection of MAb86 against pSer422-specific antibody, 60 mg/kg with 3 day intervals | TauPS2 APP (P301L tau) | Acute: 16-mo; chronic: 10-mo | Acute = 48 H, chronic = 1 wk | MAb86 specifically binds to tau phosphorylated at Ser422, shown in Western (Sarkosyl fraction); Immunofluorescence in CA1 verified binding; double staining with flotillin showed binding and internalization via lipid rafts; Chronic treatment delayed tau pathology and favored lysosomal clearance as shown by ELISA. |

Table 3.2: Passive Immunization Using Tau Antibodies, notes

IHC = immunohistochemistry
i.p. = intraperitoneal
icv = intracranial ventricular
FA = formic acid extract
iv = intravenous
i.c. = intracranial
Table 3.3 – Additional Staining Results Following Intracranial Injection Of Antibodies

<table>
<thead>
<tr>
<th>STAIN</th>
<th>Anti-GFP Treatment Mean +/- SEM</th>
<th>MC-1 Treatment Mean +/- SEM</th>
<th>PHF1 Treatment Mean +/- SEM</th>
<th>DA9 Treatment Mean +/- SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSer396</td>
<td>1.03 +/- 0.21</td>
<td>0.90 +/- 0.156</td>
<td>0.75 +/- 0.14</td>
<td>1.36 +/- 0.22</td>
</tr>
<tr>
<td>CD45</td>
<td>1.06 +/- 0.13</td>
<td>0.96 +/- 0.12</td>
<td>1.13 +/- 0.11</td>
<td>1.06 +/- 0.10</td>
</tr>
</tbody>
</table>

Table 3.3: Sub-populations of mice were stained with other phospho-epitopes of tau; here we show an example using pSer396, which did not demonstrate significant reduction in pathology, similar to others tested. Additionally, we examined the microglial marker CD45 to note if antibody treatment had any impact on pro-inflammatory response, which was not reflected here.
Table 3.4 – Activity Test Results

<table>
<thead>
<tr>
<th>Activity Test</th>
<th>Non Transgenic mean +/- SEM</th>
<th>rTg4510 + IgG1 mean +/- SEM</th>
<th>rTg4510 + MC-1 Mean +/- SEM</th>
<th>rTg4510 + Tau 5 Mean +/- SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Open Field Distance Traveled</td>
<td>50.6 +/- 5.9</td>
<td>126 +/- 28</td>
<td>149 +/- 26</td>
<td>143 +/- 26</td>
</tr>
<tr>
<td>Rotarod – Total time</td>
<td>40.5 +/- 5.1</td>
<td>57.3 +/- 6.7</td>
<td>44.4 +/- 6.7</td>
<td>48.2 +/- 6.0</td>
</tr>
<tr>
<td>Y-Maze – Number of Entries</td>
<td>38.9 +/- 3.6</td>
<td>51.8 +/- 10.1</td>
<td>44.4 +/- 8.9</td>
<td>46.4 +/- 4.8</td>
</tr>
<tr>
<td>Y-Maze - Alternations</td>
<td>55.2 +/- 3.7</td>
<td>58.3 +/- 6.4</td>
<td>64.0 +/- 5.8</td>
<td>61.4 +/- 4.2</td>
</tr>
</tbody>
</table>

Table 3.4: Our laboratory has observed that rTg4510 mice typically display much greater activity levels than NonTg mice, as mentioned above. Such activity is demonstrated here, by comparing distance traveled in the Open Field test, the time on the Rotarod and the number of entries in the Y-Maze. Slight increases are also shown in the number of alternations in the Y-Maze. Treatment of the rTg4510 mice with either MC-1 or Tau 5 did not significantly impact performance compared to IgG1 treatment.
Fig 3.1: Gallyas silver staining of hippocampal sections, four days after intracranial infusion of anti-tau antibodies into rTg4510 mice. One-year old rTg4510 mice (n = 6 – 8 animals per group) received intracranial injection (by convection enhanced delivery, CED) of potential tau-treatment antibodies into the right hemisphere. The left hemisphere remained untreated. Three different types of antibodies were tested, in comparison to an anti-GFP control injection. PHF-1 is a phosphorylated form of tau, MC-1 is a conformation-dependent antibody, and DA-9 is a pan-tau antibody. MC-1 treatment demonstrated a reduction in NFTs (p < 0.05 by ANOVA), while the other antibodies did not. Scale bar = 100 µm.
Fig 3.2: Analysis of hyperphosphorylated tau (pSer199/202) in the hippocampus, four days post intracranial infusion of antibodies into one-year old rTg4510 mice. The right hemisphere pathology, normalized by the uninjected left hemisphere, showed a marked response to two of the three different antibodies, compared to the anti-GFP control injection. DA 9 treatment yielded a moderate reduction in pSer199/202 staining (p < 0.05), while MC-1 treatment led to an even more dramatic reduction of pSer199/202 (p < 0.01; n = 6 – 8 animals per group)). Statistics were computed using StatView software and ANOVA analysis. Scale bar = 100 µm.
Fig 3.3: H150 immunostaining after hippocampal antibody treatment to rTg4510 mice. One-year old rTg4510 mice (n = 6 – 7 per group) received intracranial injections of treatment antibodies into the right hippocampus. Four days later, tissue was collected and the treated (right) hippocampus was compared to the untreated (left) hippocampus to identify differences in pathology by IHC. Results were compared between antibody treatments and anti-GFP control injections. Both MC-1 and DA 9 treatments led to significant reductions in H150 pathology (p < 0.05) compared to the anti-GFP control treatments. Results were analyzed using StatView software, for the non-parametric Mann Whitney U Test. Scale bar = 100 μm.
Fig 3.4: Spatial memory testing, using the radial arm water maze (RAWM) was used to identify cognitive differences between treated and untreated rTg4510 mice. Mice received two weeks of antibody infusion were subjected to a battery of behavioral testing, with emphasis on cognitive performance. Radial Arm Water Maze testing revealed that the IgG1 control antibody (n = 9) did not lead to performance improvement compared to Non Tg mice (n = 8). In contrast, MC-1 treated mice (n = 8) showed cognitive performance levels that were nearly indistinguishable from the Non Tg mice. ANOVA statistical analysis was performed using StatView software.
Fig 3.5: Cognitive-behavioral results from novel object recognition testing of rTg4510 mice after receiving 2 weeks of continuous infusion of anti-tau antibodies into the right ventricle. Mice that had received two weeks of antibody infusion were subjected to a battery of behavioral testing, with emphasis on cognitive performance. Novel object recognition testing revealed that the IgG1 control antibody (n = 9) did not lead to performance improvement compared to Non Tg mice (n = 8). In contrast, MC-1 treated mice (n = 8) showed cognitive performance levels that were nearly indistinguishable from the Non Tg mice. ANOVA statistical analysis was performed using StatView software.
3.9 References


CHAPTER 4: OLIGOMERIC TAU TARGETED IMMUNOTHERAPY

For these experiments, routine injection of tau antibodies into the peritoneal cavity of rTg4510 mice was performed. Three month-old mice received regular systemic injections at 2-week intervals, for 10 injections. Mice were given i.p. injections of either saline (control), IgG1 (also a control), or TOMA. Additionally, there was a Tet-only control group, which received saline injections. All mice were subjected to a battery of behavioral tests, just prior to tissue collection. Mice were approximately 7 months old after treatment when plasma and CSF and tissue were collected at euthanasiasia. This chapter has been submitted for publication in Alzheimer’s Research and Therapy, 2017.

Oligomeric Tau-Targeted Immunotherapy in rTg4510 mice

Schroeder, S1*, Joly-Amado, A.1*, Soliman A.1, Sengupta, U.2, Kayed, R.2, Gordon, MN.1, Morgan, D.1, 3

4.1 Abstract

Finding ways to reverse or prevent the consequences of pathogenic tau in the brain is of considerable importance for treatment of Alzheimer’s disease and other tauopathies. Immunotherapy against tau has shown promise in several mouse models. In particular,
an antibody with selectivity for oligomeric forms of tau, tau oligomer monoclonal antibody (TOMA), has shown rescue of the behavioral phenotype in several murine models of tau deposition. In this study, we examined the capacity of TOMA to rescue the behavioral, histological and neurochemical consequences of tau deposition in the aggressive rTg4510 model. We treated mice with 60 µg TOMA I.P. from 3.5 to 8 mo of age. Near the end of the treatment, we found that oligomeric tau was elevated in both the CSF and plasma. Further we could detect mouse IgG in rTg4510 mouse brain after TOMA, but not after injection with mouse IgG1 as control. However, we did not find significant reductions in behavioral deficits or tau deposits by either histological or biochemical measurements. These data suggest that there is some exposure of the rTg4510 mouse brain to TOMA, but it was inadequate to affect the phenotype in these mice at the doses used. These data are consistent with other observations that the rTg4510 mouse is very aggressive and therefore more challenging to demonstrate efficacy of tau-lowering treatments than some other preclinical models of tau deposition/overexpression.

4.2 Introduction

Tau is a microtubule binding protein (MBP), which assists in maintaining the physical structure of neurons, primarily the axons. It also facilitates trafficking of organelles and intracellular compounds within the cell in its normal state (Mandelkow and Mandelkow, 2012b). It is an important protein for normal cell functioning, but can become pathological. This pathology is associated with various post-translational modifications, most notably hyperphosphorylation. As tau becomes
hyperphosphorylated, it mis-folds and aggregates into oligomers, and ultimately fibrils. These aggregated forms of tau are associated with a class of neurodegenerative disorders called tauopathies, which include fronto-temporal lobe dementia, Pick's Disease, corticobasal degeneration, argyrophilic grain disease, and Alzheimer's Disease (AD). These diseases have different origins and symptoms, but all have accumulation of aggregated forms of tau as a common feature.

Tau is an attractive target to treat because the progressive pathology of the protein highly correlates with AD symptoms (Braak and Braak, 1995, Nelson et al., 2012). Amyloid Beta (Aβ) was initially investigated, due to the linkage of amyloid metabolism to genetic forms of Alzheimer’s disease (Hardy and Selkoe, 2002). One of the first approaches to reducing brain Aβ was the use of immunotherapy (Schenk et al., 1999a, Morgan et al., 2000b) and this approach has advanced to phase 3 clinical testing (Doody et al., 2014, Salloway et al., 2014). The success of immunotherapy in preclinical models of amyloid deposition led to later attempts to pursue immunotherapeutic approaches to tau deposition as well as reviewed in Chapter 3 (Schroeder et al., 2016).

Traditionally, tau has been considered to be a presynaptic protein, as it stabilizes microtubules and assists in transport through the axon. However, there have been recent studies that have identified mechanisms for tau to be transmitted across synapses to nearby post-synaptic cells, which serves as a means for understanding progressive tau pathology and spread (de Calignon et al., 2012, Liu et al., 2012). Dendritic tau has been associated with synaptic disruption (Ittner et al., 2010, Roberson et al., 2011).
Immunotherapy has become a focus to attempt to treat tauopathies and other protein-based neural diseases (Sigurdsson, 2009), especially given the successes in using this strategy for treating the Aβ component of AD (Morgan, 2011b). Immunotherapy can either be “active,” where an antigen is delivered to the body which then produces its own antibodies to effect healing, or “passive,” in which actual antibodies are administered like a drug. The former is more likely to produce side effects, whereas the latter requires more frequent administration to maintain effective titers of the treatment antibody. Both strategies have been extensively investigated in the past several years.

Our laboratory has examined several antibodies directed against tau (focusing on passive immunization), in effort to find the most successful candidate to ameliorate pathology and behavioral deficits in rTg4510 mice. In this study, we utilized passive immunization of a tau oligomeric monoclonal antibody (TOMA), which was provided by Rakez Kayed's laboratory (Lasagna-Reeves et al., 2011b, Castillo-Carranza et al., 2014b). This antibody had shown positive effects in JNPL3 mice and hTau mice, but had not been tested in rTg4510 mice. JNPL3 mice slowly develop tau aggregation and deposition in spinal cord and brainstem with small amounts of forebrain pathology. The rTg4510 mice rapidly accumulate tau deposits in forebrain regions overlapping CaM kinase II distribution. There is also considerable atrophy and neuron loss in the rTg4510 forebrain with aging, while there is less such pathology in the JNPL3 model. hTau mice develop pathology even later than the JNPL3 mice (Andorfer et al., 2003)
4.3 Materials and Methods

Animals:

The rTg4510 mouse line (0N4R P301L) was chosen for these studies because they are known to have tau pathology (expression of the P301L mutation) directed to the forebrain, which more closely resembles an AD type of tauopathy (Santacruz et al., 2005b). Of note, the mutant human tau expression can be suppressed by application of doxycycline. rTg4510 mice, and their littermates, were bred locally by crossing mice transgenic for a tetracycline-operon-responsive element (tTA), controlled by a calmodulin kinase II (CAMK II) promoter, with a separate mouse line transgenic for human P301L tau driven by the Tet operon, as previously described (Santacruz et al., 2005b). The resulting cross yields approximately 25% non-transgenic (nTg) mice, 25% P301L only (tau only) mice, 25% tTA only (Tet) mice, and 25% rTg4510 (tau-tet) mice. For this study, while the rTg4510 mice were the primary interest, we also used nTg and Tet-only littermates as controls. The mice were approximately 3.5 mo old at first injection and 8 months old at the end of treatment. Groups were as follows: nTg + saline n = 10, Tet + saline n = 10, rTg4510 + IgG1 n = 9, rTg4510 + TOMA n = 9.

Antibody Treatment:

One antibody, tau oligomer monoclonal antibody (TOMA) (Lasagna-Reeves et al., 2011b) was the subject of focus. Effects of systemic administration to rTg4510 mice were examined, to evaluate if the antibody could prevent behavioral and pathological components of the Tg4510 phenotype. Mice were injected I.P with 60 µg TOMA or IgG
control, every two weeks for a total of 10 injections. Tet-only and nTg littermates were also injected at these times with saline to control for effects of biweekly injections on behavioral measures. The experimenter performing the injections was blind with respect to treatment condition. Body weight and food intake were measured at each injection time, to identify if the treatments were altering either of these metabolic measures. We noted that the rTg4510 mice consumed significantly more food, but also exhibited a significant reduction in body weight likely due to their excessive activity as previously described in (Brownlow et al., 2014, Joly-Amado et al., 2016). However, neither body weight nor food intake were modified by the antibody treatment.

Behavioral Testing:

Animals were tested for general activity levels after receiving 8 injections, by open-field testing, followed by rotarod and Y-maze. Y-maze testing indicates overall activity, or hyperactivity, based on the number of arm entries. It can also give a general indication of working memory when one analyzes the arm alternations. They were also tested for cognitive function using: novel object recognition (NOR), spatial navigation memory using the 2 day radial arm water maze (RAWM;(Alamed et al., 2006)) followed by 1 day of reversal training (Brownlow et al., 2013), and associative learning using fear conditioning. Mice were also tested for novel mouse recognition (described in (Brownlow et al., 2014)), a modified version of a social interaction task. Open field and novel object recognition results were quantified using Anymaze software (Stoelting, Wood Dale, IL, USA).
Tissue Collection and Staining:

One day following the last antibody injection, the animals were euthanized using Somnasol (pentobarbital, phenytoin, EtOH, propylene glycol, rhodamine and benzyl alcohol). Anesthetized mice had CSF removed by penetrating the cisterna magna with a glass capillary tube (Sutter Instruments, USA) following the method described in (Liu and Duff, 2008). Blood was collected by intracardiac puncture using EDTA and then centrifuged at 1,000 g for 15 min at 4°C for plasma collection. Mice were then perfused with buffered normal saline on a heated pad to prevent reductions in body temperature and artifactual tau phosphorylation. The brain was exposed and the right hemisphere was collected, preserved in freshly prepared 4% paraformaldehyde in Dulbecco’s phosphate buffered saline. Twenty-four hours later the brains were cryoprotected by successive incubations of 10%, 20% and 30% sucrose solutions for 24 h each. The left hemisphere was dissected into the following regions: anterior cortex, posterior cortex, hippocampus, striatum, thalamus, cerebellum, and midbrain/hindbrain. All dissected tissue samples were snap-frozen on dry ice, stored at -80 degrees until ready for use.

Horizontal sections (25 μm thick) of the left hemisphere were collected into 24-well plates. For each staining performed, 8 sections spaced roughly 600 μm apart were used. Gallyas silver staining for neurofibrillary tangles was conducted as per (Brownlow et al., 2014). Free-floating immunohistochemistry (IHC) was completed for H150 (Santacruz Biotechnology, Dallas, TX, USA), tau phosphorylated at Ser199/202 (pSer199/202, Anaspec, Fremont, CA, USA), pSer396 (Anaspec, Fremont, CA, USA),
and the microglial marker CD45 (Invitrogen, Carlsbad, CA, USA) (Gordon et al., 1997). Additionally, we examined the staining for mouse IgG with biotinylated horse anti-mouse IgG specific secondary antibody (Vector Laboratories, Burlingame, CA, USA) to detect if the systemically injected antibodies may have bound to brain tissue.

Stained anatomical sections were digitally scanned using a Zeiss slide scanner followed by quantification of stained area in user defined regions of interest using custom designed software. Values from all sections from the same mouse were averaged to represent a single value for that region in subsequent statistical analysis.

Biochemistry/Western Blotting:

Dissected brain regions were homogenized then sonicated in RIPA buffer, containing protease inhibitor cocktail and phosphatase inhibitor cocktails I and II (Sigma Aldrich) as per the manufacturer and centrifuged at 40,000 x g for 30 min at 4°C. The supernatant was collected and protein concentrations were determined by BCA protein assay (Pierce, Rockford, IL). The resulting pellet was digested with 70% formic acid according to the wet tissue weight, and then neutralized with NaOH to analyze RIPA-insoluble proteins. Equal amounts of proteins (5μg/well for soluble fraction, 2μg/well for insoluble fraction) were loaded in each well of a 4–12% bis-tris gels, transferred to a 0.2 μm pore size nitrocellulose membrane and immunoblotted with H150 (Santacruz biotechnology, Dallas, TX), pSer199/202 (Anaspec, Fremont, CA), pSer396 and pser262 (Anaspec, Fremont, CA) at 1:1000-fold dilution. Fluorescently- tagged secondary antibodies (LI-COR Biosciences) were used at a dilution of 1:10,000. Western Blot results were
quantified by scanning with a LI-COR Odyssey fluorescent scanner. AlphaEase FC software was used to normalize the tau bands to actin for the soluble fraction. Normalization of the insoluble fraction was conducted using REVERT reagent (LI-COR) to measure total protein. Statistical analysis was then conducted using StatView.

CSF and plasma samples were analyzed for oligomeric tau using both direct and sandwich ELISA, with T22 antibody selective for oligomeric tau (Lasagna-Reeves et al., 2012a)

Statistical Analysis:

In general, results were analyzed by 1-way ANOVA with subsequent means comparisons made using Fisher's test for multiple comparisons. In some cases where tau was measured, the absence of any signal in nTg mice led to using t-tests in Tg4510 groups only.

4.4 Results
Detection of Antibody in Brain

Immunohistochemistry was conducted for mouse IgG by incubating sections in anti-mouse IgG specific secondary antibody. Positive peroxidase reaction product staining was observed in the hippocampus and anterior cortex of rTg4510 mice injected with TOMA, but not in rTg4510 mice injected with mouse IgG1 (Fig 1A,B). There was also dark staining of the ependymal regions of the ventricles seen in both treatment
conditions. When the parenchymal staining in the hippocampus was viewed at higher magnification, there was staining of the neuropil only in rTg4510 mice injected with TOMA (Fig 1 C,D). We quantified immunostaining in the anterior cortex (ACX), posterior cortex (PCX) and hippocampus (HPC). The ratio of positively stained to total area calculated from digitized images is shown in Figure 1E. The rTg4510 mice exhibited significantly higher levels of IgG in the ACX (p < 0.05) compared to all other groups, and in the HPC (p < 0.05 when compared to IgG1, p < 0.01 when compared to the other groups). No significant differences were observed in the PCX.

Detection of Oligomeric Aβ

Plasma and CSF were assayed with an ELISA specific for oligomeric tau. In the CSF, oligomeric tau was detected only in the mice injected with TOMA (P < 0.001; Fig 2A). In the plasma, the TOMA-treated animals demonstrated significantly elevated levels of oligomeric tau compared to the other 3 groups (p < 0.001; Fig 2B). These results suggested that the TOMA was effectively acting as a sink in both CSF and plasma retaining the oligomeric tau, indicating antibody access to both compartments.

Behavioral Testing

There were no overall ANOVA differences in the open field, rotarod or Y maze tasks for any of the comparisons (data not shown). Mice were tested for spatial navigation performance in the radial arm water maze. This is a variant of the Morris
water maze with a submerged platform at the end of one of six swim alleys, permitting errors to be measured rather than time. Over the first two days of training, there was a significant ANOVA effect with both groups of rTg4510 mice requiring more errors to find the platform than nTg or Tet mice (Fig 3A). On the third day of testing, the platform was placed in the arm opposite that used for learning on the first 2 days. Again, the ANOVA was significant with more errors on this reversal task for both groups of rTg4510 mice than the nTg or Tet mice (Fig 3B). In neither the learning nor reversal portions of the training were the TOMA- treated rTg4510 mice different from the IgG1- treated mice.

Mice were also examined for novel object recognition memory. In this task, mice exposed to a novel and a familiar object are expected to spend more time attending to the novel object. Overall ANOVA and means testing determined the rTg4510 mice spent significantly less time attending to the novel object than nTg or Tet mice (Fig 4). Again there was no significant difference between the TOMA- treated and IgG1- treated rTg4510 mice.

Histology

One half of the brain was sectioned for histochemical analysis. One hallmark of AD brain neurofibrillary tangles is their capacity to be stained by silver stains, such as the Gallyas stain. rTg4510 mice at 8 mo of age have numerous Gallyas positive neurons throughout hippocampus in both pyramidal and granule cell layers (Fig 5A, B). No Gallyas positive cells were found in tissues from nTg or Tet mice (not shown). When the amount of staining was quantified in anterior cortex, hippocampus and posterior
cortex, there were small regional differences, but no effect of TOMA treatment on the development of this pathology (Fig 5C). Immunostaining for total tau (antibody H150), or antibodies against phosphorylated forms of tau (pSER396, pSER 199/202) also failed to show an effect of TOMA treatment relative to mice treated with IgG1 antibodies (data not shown).

Western Blotting

The detergent soluble fraction (S1) of homogenized tissue from the right ACX was analyzed by Western Blot (Table 1). We separately quantified the bands at 55 kDa, 64 kDa, and 120-200 kDa (likely representing oligomers) and found no differences between the IgG1 control and the TOMA treatment using detection antibodies for H150, pSer 262 and pSer 396. S1 blots were normalized to actin, and were quantified using AlphaEase FC software.

The detergent insoluble tissue fraction was digested with formic acid then neutralized using NaOH, to permit analysis of tau components as insoluble tangles or other detergent insoluble intermediates. Table 1 also shows the results from Western analysis of the insoluble fractions (FA) from ACX, which similarly show no significant differences between the treatments or controls for any of the forms of tau we examined, including the high molecular weight/oligomeric forms.
4.5 Discussion

Numerous studies have been conducted, to date, utilizing various immunotherapy strategies in an attempt to prevent the development of a tau phenotype in a variety of preclinical models. We have recently reviewed this literature and included some of our own data in investigating intracranial delivery of immunotherapy agents in Chapter 3 (Schroeder et al., 2016). Here, we focused on an oligomeric tau-targeted antibody as passive immunotherapy for rTg4510 mice. We evaluated the tau phenotype in these mice with respect to behavior, histology and biochemical markers of pathology in attempt to identify if TOMA would be a potential therapeutic candidate to pursue in human trials (Gerson and Kayed, 2016). On the whole, our studies showed that in this mouse model, TOMA was not as effective as has been demonstrated in previous studies using different models of tauopathy (Castillo-Carranza et al., 2014a, Castillo-Carranza et al., 2014b) (Castillo-Carranza et al., 2015).

One possible explanation for an absence of impact on the tau phenotype could be failure of the antibody delivery or altered pharmacokinetics. We were able to observe elevated Tau oligomers both in CSF and in plasma of mice treated with TOMA, but not in mice treated with mouse IgG1. The most probable explanation for these elevations is the binding of tau oligomers to the antibody in these compartments and the delay in its clearance due to being bound by antibody. Such a mechanism has been proposed to explain elevated circulating Aβ levels after treatment with some antibodies (DeMattos et al., 2001, Levites et al., 2006, Karlnoski et al., 2009). Other antibodies targeting monomeric tau have also been reported to elevate plasma tau (d'Abramo et al., 2016). However, a different study found antibodies specific for phospho-tau treated for 12
weeks decreased tau levels in CSF (associated with declines in tissue tau levels; (Sankaranarayanan et al., 2015)).

A second explanation may be the poor penetration of antibody into brain parenchyma. However, we detected mouse IgG decorating the neuropil of hippocampus and cerebral cortex, sites of tau expression in rTg4510 mice. This staining in general did not appear to be within neurons, but associated with the molecular layers in hippocampus. It is conceivable that the absence of an effect was due to the inability of this antibody to become internalized by neurons, as appears to be the case for some anti-tau antibodies (Congdon et al., 2013, Shamir et al., 2016). However, given the specificity of the antibody for oligomeric tau, it may represent the locations of this antigen extracellularly or on the cell surface. Again we did not observe this staining in mice injected with IgG1 control antibodies. These results suggest that at least some antibody reached the brains of the rTg4510 mice.

The results from behavioral tests indicated the expected genotype effect of the rTg4510 mice as having worse performance than the nTg or the Tet control mice. We included the Tet group because of prior work showing that the Tet transgene alone can result in a phenotype, including slightly reduced hippocampal volume (Han et al., 2012). When we compared the IgG1 and TOMA treated rTg4510 mice, there was no significant difference, indicating the TOMA was not able to prevent the cognitive deficits in the radial arm water maze, either in the initial learning phase or in the reversal phase. These results were unexpected based on the behavioral improvements in Y-maze performance in the initial publication of this treatment (Castillo-Carranza et al., 2014b). However those studies used the JNPL3 mouse model, not the rTG4510 mice. The novel
object recognition (NOR) results were consistent with the radial arm water maze results indicating a genotype effect, but no treatment effect. These results contrast with those reported by (Castillo-Carranza et al., 2014a), where they did identify that immunotherapy with TOMA led to an improvement in NOR using htau mice seeded with preformed tau aggregates to induce pathology and behavioral deficits.

The results from Gallyas staining do not show any significant differences between the treatments for the three regions inspected, thereby suggesting that there was little to no effect on parenchymal levels of neurofibrillary tangles. Although not shown, we further found no effects on histological measures of total tau, nor several phospho-tau isoforms. Other work with the TOMA antibody has failed to detect reductions in AT8 staining or Gallyas staining in JNPL3 (P301L) tau transgenic mice ((Castillo-Carranza et al., 2014b), or in htau mice injected with tau seeds (Castillo-Carranza et al., 2014a). It was argued that the behavioral deficits were due to tau-oligomers and their removal was sufficient to rescue behavioral deficits.

We examined four markers of tau pathology via Western blot to quantify changes in different tau fractions and molecular weight variants. We examined both detergent soluble tau, and detergent insoluble tau (solubilized with formic acid) fractions. We were unable to detect treatment effects in the two tau monomer bands we could discern (55 and 64kd), nor in the higher molecular weight forms of tau believed to represent oligomers. This latter observation is in contrast to prior work in the JNPL3 and htau models (Castillo-Carranza et al., 2014a, Castillo-Carranza et al., 2014b).
There were several methodological differences between the present results and those demonstrating successful rescue of cognitive changes with TOMA. One is the route of antibody administration. Prior work performed intravenous administration via the tail vein. All of our prior efforts using passive systemic immunotherapy in mouse models of Alzheimer’s pathology (largely anti-Aβ studies) successfully administered antibodies via intraperitoneal injections (Wilcock et al., 2004a, Morgan et al., 2005, Wilcock et al., 2007a, KarlNoski et al., 2009). Other potential explanations for the discrepancies could be that the rTg4510 mouse is sufficiently aggressive that higher antibody doses would be required to effectively lower the tau oligomers to rescue the cognitive impairments. The rTg4510 mouse deposits tau earlier than most other tau transgenic mice and has greater neurodegeneration than most other models (Dujardin et al., 2015). However a third possibility is that the cause of the cognitive impairments in the rTg4510 mouse is different than in JNPL3 or htau models. By 6 mo, there is considerable loss of hippocampal neurons and cortical atrophy in rTg4510 mice (Santacruz et al., 2005b, Nash et al., 2013). The data with TOMA obtained previously results in relatively rapid reversal of the cognitive impairment, implying the rescue is not due to protection from structural changes, but impairments of synaptic function that are reversible. However, it is feasible that the rTg4510 mouse has cognitive deficits that are secondary to neurodegeneration and brain atrophy. These would not be expected to be rapidly reversed, but may require protracted administration from before the age when pathology begins. Thus, treating from 3.5 to 8 mo may not be adequate to provide this protection. It is conceivable had we tested the mice at an
earlier age (e.g. 5 mo) the cognitive deficits might have been more susceptible to clearance of oligomeric tau.

Our overall experience has been that preventing pathology in the rTg4510 mouse is challenging. Some of our negative results have been published (Brownlow et al., 2013, Brownlow et al., 2014, Joly-Amado et al., 2014) while others have not. In the few instances where we have obtained positive effects of treatment, they have required direct and long term application to the brain of genes expressed at high levels resulting in reduced tau deposition (Nash et al., 2013, Hunt et al., 2015). To our knowledge, the only study demonstrating a significant impact of passive immunotherapy in the rTg4510 model was a study by Sankaranarayan et al (Sankaranarayanan et al., 2015). These authors treated rTg4510 mice weekly (as opposed to bieweekly here) with 25 mg/kg (10 times more than used here) of two phospho-tau specific antibodies from 3-6 mo. They detected a 20% decline in soluble AT8. However, there was no decline in detergent insoluble total or AT8 tau isoforms. In parallel studies, they examined the PS19 (P301S) tau mouse and observed much larger reductions in tau pathology, on a percentage basis.

In conclusion, tau immunotherapy remains an attractive area of investigation for the treatment of AD and other tauopathies. However, the wide range of preclinical tauopathy models, each having its own characteristics, and also the wide range of immunogens administered to date, confounds the field. The goal of this avenue of research is to ultimately identify a treatment for tauopathies such as AD, but that bulls-eye treatment has not been identified yet. Synergy between tau and Aβ treatments would likely provide a most useful avenue for further investigation into AD treatments,
as recently TOMA was found to benefit an APP mouse model (Castillo-Carranza et al., 2015) (but see also (Mably et al., 2015). Although the rTg4510 model rigorously replicates the extensive neuron loss and atrophy of Alzheimer’s disease, it may be prudent to reserve this model only for treatments already demonstrated effective in other preclinical tauopathy models and to initiate treatment at very early stages. An alternative is to suppress transgene expression with doxycycline until the mice reach an age when they can receive the treatment.

4.6 Acknowledgments

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Table 4.1: Treatment with TOMA did not affect levels of soluble or insoluble tau in ACx Western blot quantification of tau markers in the soluble fraction (S1) and formic acid treated insoluble fraction (FA) in rTg4510 mice treated with TOMA or IgG1 for 5 months. Values were quantified using alphaEase FC software and normalized to actin for the soluble fraction and total protein using REVERT reagent (LI-COR) for insoluble fraction. Data are presented as mean ± S.E.M.

<table>
<thead>
<tr>
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<th>55 kda</th>
<th>64 kda</th>
<th>Oligomers (120-200 kda)</th>
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<tr>
<td></td>
<td>Tg4510 TOMA</td>
<td>Tg510 IgG</td>
<td>Tg4510 TOMA</td>
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<tr>
<td>S1 (ratio to actin)</td>
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<tr>
<td>H150</td>
<td>1.60 ± 0.13</td>
<td>1.60 ± 0.09</td>
<td>1.61 ± 0.09</td>
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<tr>
<td>Pser262</td>
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<tr>
<td>Pser396</td>
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<td>3.63 ± 0.21</td>
<td>3.25 ± 0.32</td>
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<td>Pser199/202</td>
<td>2.32 ± 0.37</td>
<td>2.32 ± 0.57</td>
<td>0.16 ± 0.05</td>
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<td>FA (ratio to total protein)</td>
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<tr>
<td>H150</td>
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Figure 4.1: rTg4510 mice injected systemically with TOMA present elevated levels of mouse IgG in the brain. Micrographic representation of mouse IgG staining in hippocampus (HPC) of rTg4510 mice treated with TOMA (rTg4510 TOMA, A,C) or IgG1 (rTg4510 IgG1, B,D) for 5 months (from 3.5 to 8 months of age). Panel C and D show magnification of square area in A and B, respectively. No staining was observed in non-transgenic or Tet only littermates. Immunostaining quantification (E) utilizing Mirax software (Zeiss Inc.) in anterior cortex (ACX), hippocampus (HPC) and posterior cortex (PCX) of non-transgenic littermates (nTg, white bars), tet only mice (Tet, grey bars) and rTg4510 mice treated with IgG1 (rTg4510 IgG1, black bars) or TOMA (rTg4510 TOMA, shaded bars) for 5 months. One-way ANOVA showed a significant increase in positive area ratio stained for mouse IgG in the anterior cortex (ACX) and hippocampus (HPC) of rTg4510 treated with TOMA compared to Tg4510 treated with IgG1 and non-transgenic and Tet only control mice. Data are presented as mean ± S.E.M., n = 9-10/group. *p < 0.05, **p<0.01. Scale bar = 200μm (Panels A and B) or 50μm (Panels C and D).
Figure 4.2: rTg4510 mice injected systemically with TOMA present elevated levels of oligomers in both CSF and plasma. Quantitative analysis of oligomeric tau in cerebrospinal fluid (CSF, Panel A) and plasma (Panel B) of non-transgenic (nTg, white bars). Tet only mice (grey bars) and rTg4510 mice treated with IgG1 (rTg4510 IgG1, black bars) or TOMA (rTg4510 TOMA, shaded bars) treated for 5 months. Analysis was performed using both direct and sandwich ELISA. T22 was the antibody used for identifying tau oligomers. One-way ANOVA showed a significant increase in levels of oligomers in CSF of rTg4510 treated with TOMA compared to rTg4510 treated with IgG1 and non-transgenic and tet only control mice. Data are presented as mean ± S.E.M., ***p < 0.001.
Figure 4.3: rTg4510 mice demonstrated spatial memory deficits in the radial arm water maze (RAWM) and reversal trial that were not rescued by TOMA treatment. Number of errors during 2-day RAWM (Panel A) and reversal (Panel B) in non-transgenic mice (nTg, white bars), Tet only mice (Tet, grey bars) and rTg4510 mice treated with IgG1 (rTg4510 IgG1, black bars) or TOMA treated mice (rTg4510 TOMA, shaded bars) for 5 months. rTg4510 mice made significantly more errors attempting to locate a hidden platform in the 2-day RAWM test, compared to nTg mice and Tet only mice, regardless of the treatment. rTg4510 mice were not able to learn a new platform location on the reversal trial. Data are presented as mean ± S.E.M., n = 10/group. *p = 0.05.
Figure 4.4: rTg4510 mice presented memory deficits during novel object recognition test that were not rescued by TOMA treatment. Percentage of exploration (calculated as the time spent with the novel object divided by the sum of times spent with novel and familiar, multiplied by 100) of the novel object presented in the last trial of the Novel object recognition test in non-transgenic (nTtg, white bars) tet only mice (Tet, grey bars) and rTg4510 mice treated with IgG1 (rTg4510 IgG1, black bars) or TOMA (rTg4510 TOMA, shaded bars) for 5 months. Tg4510 mice spent significantly less time exploring the novel object compared to non-transgenic littermates, regardless of the treatment. Data are presented as mean ± S.E.M., n = 10/group. *p = 0.05.
Figure 4.5: Treatment with TOMA did not affect levels of tau deposits when assessed with Gallyas impregnation. Micrographic representation Gallyas silver staining in hippocampus (HPC) of rTg4510 mice treated with IgG1 (rTg4510 IgG1, A) or TOMA (rTg4510 TOMA, B) for 5 months. No signal was detected in non-transgenic or tet only littermates. Immunostaining quantification (E) utilizing Mirax software (Zeiss Inc.) in anterior cortex (ACX), hippocampus (HPC) and posterior cortex (PCX) of rTg4510 mice treated with IgG1 (rTg4510 IgG1, black bars) or TOMA (rTg4510 TOMA, shaded bars) for 5 months. Data are presented as mean ± S.E.M.
4.7 References


CHAPTER 5: DISCUSSION

Initial investigations conducted by our laboratory examined various immunotherapeutic treatments, time course of administration, and routes of administration, for amyloid clearance (Aβ) in amyloid-producing mice (Tg2576). Additionally, microglial activation (inflammation) has been evaluated in response to intracranial administration of antibodies to Aβ. These studies were conducted using the Tg2576 mouse model, which over-produces Aβ, and which provided a useful mouse model for investigating strategies for Aβ treatment by immunotherapy (Wilcock et al., 2011). The studies reported here were designed to target tau clearance by immunotherapy, in pathological tau-producing mice, using both APP/PS1/NOS-/- mice and rTg4510 mice.

The previously reported studies demonstrated that Aβ antibody displayed a broad distribution of effect, after injection. These effects were observed within one day, then diminished over the course of 7 days. At 4 hours post-treatment, diffuse Aβ pathology was not reduced, but by 24 hours it was dramatically reduced, and this effect persisted 3 and 7 days later. Compact plaques were not affected until 3 days post injection, when microglial activation was also identified. At one week post-injection, microglial levels had returned to normal levels, but the reduction of Aβ and plaques persisted. These results suggested that amyloid clearance occurs in two phases, the first occurring between 4 and 24 hours, resulting in clearance of diffuse Aβ that is not
microglial dependent, and the second occurring between 1 and 3 days where microglia are activated, leading to clearance of compact plaques (Wilcock et al., 2003).

The current studies reported herein describe a series of studies directed against the tau protein (typically an intracellular protein, which subsequently has been found to exist extracellularly, as well). The first step was to identify a most effective antibody treatment. Once that was accomplished, it was important to examine various parameters relating to the mechanisms and duration, as well as identifying the most effective route of administration of treatment. The goals herein were to examine numerous antibodies so as to identify a treatment regimen deemed “safe” for clinical administration and testing.

Temporal effects after antibody treatment were important, and relevant, as observed in the Aβ immunotherapy studies (once a select antibody treatment was identified). Routes and duration of administration were also considered to be to be important. Therefore, a similar approach to tau immunotherapy was followed here.

Oddo et al (2004), examined the temporal effect of immunizing 3xTg mice (complex Aβ-producers which have shown added tau pathology), using antibodies directed against Aβ. This mouse model has the advantage of developing Aβ and tau pathology, versus focusing on singular-pathology mouse lines (Aβ producers which have also shown tau pathology). Oddo’s laboratory reported clearance of Aβ by day 3 post-injection, but reported no immediate effect on tau pathology. By day 5, post-injection, both Aβ and tau levels were reduced. Pathological assessments were followed for 45 days post-injection to identify if either of the pathologies re-emerged. At
day 30, Aβ pathology had returned but tau pathology had not. By day 45, tau pathology also returned. Gallyas staining for NFTs was not decreased in response to the antibody treatment, so it was concluded that aggregated tau was not affected due to treatment. In other words, the Aβ antibody was only effective in clearing early tau deposits, but not hyperphosphorylated tau or NFTs (Oddo et al., 2004).

Preliminary tau-based studies reported herein were directed towards identifying an effect from numerous types of tau antibody administration, then to identify the most successful routes of administration. Additionally, it was important to identify the necessary time frames for achieving pathological tau reduction to best establish a potential clinical treatment approach for tauopathy using tau-based immunotherapy.

The initial studies reported here were designed to select a candidate tau antibody for further evaluation and then identify an ultimate treatment regimen. Numerous anti-tau treatments were evaluated to identify which antibody to investigate further by infusion, then systemic administration. The overall evaluations focused on several attempts to identify a preferred antibody treatment, then to determine how effectively and/or how quickly any candidate antibody might provide significant clearance of tau. Additionally, it was important to examine how long such treatment might remain effective.

Throughout the studies reported herein, microglial activation was measured at different time points to assess if tau antibody treatment increases inflammation. This approach was conducted in a similar manner as was reported in the previous Aβ series
of experiments published from this laboratory (Wilcock et al., 2011). The intent was to use the most robustly successful antibody against tau pathology, as determined initially in these studies, for further investigation. Given this approach, we expected to identify a specific antibody that was capable of producing significant clearance of tau pathology at some time point. It was also expected that activation of microglia would be observed at some time point, and might correlate to tau clearance. Further, it was expected that any such microglial activation would eventually subside.

What wasn’t known initially was, at what times these changes would become: a) evident, b) maximized, and c) lasting. Based on our earlier work with Aβ passive immunotherapy, it was predicted that microglial activation would occur between 2- and 7-days post-injection, and that this index of immune response might be gone by the 14-day time point. Considering the studies noted above, tau clearance was expected to become evident around 7-days post-injection, then persist through 28 days or more, then perhaps diminish over time. However, these expectations were based on the identified pathways involved in disease progression and immune system activation for Aβ clearance, which were expected to be different compared to tau clearance.

Concerns existed as to whether the selected tau antibody might fail to demonstrate success in clearing pathologic forms of tau. However, if tau clearance could not be adequately observed, the first step was to re-examine our injection protocols, locations and concentrations of antibody administered, as well as the specific tau antibodies administered. Numerous tau antibodies were investigated, and in two different tau-producing mouse models. Another potential concern regarded whether the
mice could survive to their requisite time points. The rTg4510 mice are quite impaired, so their ultimate survival is of some concern in any study.

Several different antibodies were evaluated, using different mouse models, different routes of administration and time points for their impacts on tau pathology. The post-injection time points for tissue collection were varied to ensure identification of critical time-points to achieve maximal effectiveness of antibody treatment. Injection locations were modified to accommodate for brain shrinkage as pathology advanced (depending on mouse age). Pathology and behavior were evaluated as a function of post-injection to identify amelioration of tau pathology given our various treatment regimens and experimental evaluations. Additionally, microglial activation (inflammation) was monitored to identify the ideal strategy for carefully progressing to more invasive attempts of treatment (infusion then repeated i.p. injection).

Previous work in our laboratory has shown that peripheral administration of anti-Aβ antibody was successful in reducing diffuse amyloid and compact plaques in the brain, and this was accompanied by improved cognitive performance in the radial arm water maze. Different microglial markers were also examined, leading to a likely mechanism of clearance, suggesting that the Aβ becomes opsonized then subject to Fcγ receptor-mediated phagocytosis (Wilcock et al., 2004b). Here, we examined if the selected tau antibody (TOMA) is also able to enter the brain then reduce or prevent intracellular tau pathology, and importantly, to improve cognitive behavior.

Specifically, rTg4510 mice received i.p. injections of the tau antibody or the anti-GFP control, starting at 2 months of age. They received 10 mg/kg of antibody once per
week until 10 injections were administered. A battery of behavioral tests was conducted to identify cognitive improvements or detriments resulting from antibody administration. The behavioral tests included: motor performance and activity level tests using the balance beam, rotarod, and Y maze. Y maze was additionally useful for identification of working memory. Spatial memory was examined using the RAWM test, then FC was conducted to test additional parameters of cognitive functioning. Open pool testing was completed to assess vision and swimming ability (RAWM control). FC examined training, then specific parameters of memory retention in terms of associative memory.

Upon completion of the behavior regimen, the mice were euthanized as described above, then plasma, CSF and tissue were collected. The right hemisphere was dissected into the anterior cortex, posterior cortex, hippocampus, striatum, thalamus, cerebellum and midbrain/hindbrain which were snap-frozen on dry ice then stored at -80 degrees. The left hemisphere was immersion fixed and cryoprotected as described above. IHC was conducted on sections from the left hemisphere, and Western Blotting for tau was conducted on relevant brain sections from the right hemisphere.

It was hoped that peripherally administered antibodies would cross the BBB, and remain functionally intact after doing so. The previous Aβ studies suggested this general approach might prove fortuitous. However, such results were not conclusively demonstrated, as a result of these studies.

The background results presented above were used to assume that antibodies are capable of clearing intracellular proteins. The initial tau studies were conducted in
an effort to establish that antibody treatment could clear tau pathology, via intracellular or extracellular means. Our studies were not designed to evaluate specific mechanisms, but were based on a plethora of previously reported research that indicated that they should prove effective. It remains to be examined if other tau antibodies might prove more effective in crossing the BBB, and/or in treating neurodegeneration. Perhaps, peripheral antibody administration of different antibody treatments might prove effective, and perhaps different mouse models would be a better means for examining such effects. The rTg4510 mouse model did not seem to be treatable, given our antibody studies.

The rTg4510 mouse model posed a concern of overall survival for the mice, as described above. These mice tend to be very hyperactive, and also have a reduced life span. Therefore, tau treatment regimens were started at a variety of ages, in hopes of achieving measurable effect before the inherent transgenes were too severe to experience treatment.

Additional studies could have been conducted, by varying initial mouse ages, treatments, and testing methods. However, it had appeared that an effective antibody had been identified, and we wanted to investigate more extended methods of treatment, by utilizing intracranial injection into multiple brain regions, then examining intraventricular administration, followed by 28-day continuous infusion, then finally by long-term systemic administration.

Our studies demonstrate that administration of tau antibodies to rTg4510 mice does not ameliorate AD or tauopathy symptoms. We have examined behavior,
biomarkers (CSF and blood), as well as key tissue samples, and have failed to strongly demonstrate improvements in tau pathology as a result of anti-tau administration. Much as we would have liked to identify something curative in all of these studies, it would appear that the rTg4510 mice are resistant to treatment.

The purpose of this research was to: a) identify an antibody that is most effective in clearing tau pathology in robust tau-producing mice, b) examine the most effective route of administration for neurological antibody treatment, c) establish the timeframe of antibody activity to identify preventative or therapeutic optimums for it, d) to characterize the role of the immune system in clearing pathologic tau, then e) determine if such an antibody can be administered peripherally, reach the brain, and clear intracellular tau pathology, which would provide a more feasible method for treating humans with tauopathies.

Our laboratory has a well-established history of investigating immunotherapeutic approaches for the treatment of AD, leading to clinical studies targeting Aβ. Tau-based immunotherapy has been demonstrated to be somewhat effective, using different antibodies, immunogens, other animal models, and in other contexts. Once greater therapeutic benefit has been demonstrated, in a safe and effective manner, then it could ultimately be applied to humans in clinical trials. The antibodies examined here do not lead to this future, however antibody treatment (immunotherapy) could become a manner for preventing or treating AD and other tauopathies in the future.
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