Antisense Reduction of the Protein Tau Attenuates Neuronal Hyperexcitability and Permits Clearance of Intraneuronal Tau Accumulations in vivo

Sarah Lorraine DeVos
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Antisense Reduction of the Protein Tau Attenuates Neuronal Hyperexcitability and Permits Clearance of Intraneuronal Tau Accumulations \textit{in vivo}

by

Sarah Lorraine DeVos

A dissertation presented to the Graduate School of Arts and Sciences of Washington University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

August 2014

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always (except for maybe the two minutes when I horribly fumble to try and think of an answer on the spot) thankful. I also appreciate him letting me tag along to lab events as the “Official Unofficial member of the Diamond Lab”. My knowledge in tau has grown drastically because of him and his lab. 2) Dr. David Holtzman has always been a huge source of wisdom when it comes to both my experiments as well as career moves. I have always looked to him as a model for how I would one day want to run a lab. Also, he was the only one to eat my cupcakes when I brought them in for a thesis update several years ago and that appreciation has always stuck with me. 3) Dr. Alison Goate is my power woman committee member. Even while she is so busy, she has always remembered exactly what I am doing and has made me really think about where my project is going and how to make the most of it. And last but most certainly not least, 4) Dr. John Cirrito has been a huge help in thinking about my project and how tau is a far greater protein to study than amyloid-beta. I know that I can always turn to John to help me work through a crazy idea that I have or celebrate awesome results, both now and, I have zero doubts, 20 years from now. I could not have asked for a better thesis committee and look forward to both staying in touch as well as collaborating in the future.

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I am incredibly thankful for all of my friends. Both before and during graduate school. They have helped me keep my sanity and have made my time in St. Louis enjoyable and memorable. There are too many names to list so I will keep this section short. Just know that I owe all of you...
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DEDICATION

I dedicate this dissertation to my grandparents:

**Laverne H. DeVos**
October 10, 1928 - November 4, 2009

**Lorraine A. Schmitt**
July 4, 1929 - January 29, 2010

**Robert T. Schmitt**
February 22, 1927 - January 25, 2012

During my time in graduate school, my paternal grandfather and both of my maternal grandparents passed away from Alzheimer’s disease. While it has not been easy to witness firsthand what this devastating disease can do to both the affected individuals as well as the surrounding loved ones, having this personal connection has fueled in me a deeper fire to continue my work in researching neurodegenerative diseases.
ABSTRACT TO THE DISSERTATION

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Sarah Lorraine DeVos

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Neurosciences
Washington University in St. Louis, 2014
Professor Timothy M Miller, Chairperson

The protein tau is a major contributor in some of the most prevalent neurodegenerative diseases, including the most common form of dementia, Alzheimer’s Disease (AD). As a member of the microtubule-associated protein family, tau is enriched in the axons of mature and growing neurons, though under certain conditions, can become hyperphosphorylated and accumulate into toxic oligomeric species and aggregates. In the studies outlined here, we sought to directly target the protein tau using Antisense Oligonucleotides (ASOs) to reduce total expression of tau in vivo and assess if such a reduction could be therapeutically beneficial. To first test the feasibility of reducing tau in the adult animal, we identified ASOs that reduce endogenous mouse tau in the brain and found no effect on baseline motor or cognitive behavior. We then tested the efficacy of reducing murine tau in the context of hyperexcitability since aberrant neuronal excitability has been linked to AD pathogenesis, both in humans and in amyloid-beta depositing mouse models. We found that mice with reduced tau had significantly less severe seizures than control mice, demonstrating that endogenous tau is indeed integral for regulating neuronal hyperexcitability. While the inducible models are sufficient to assess the roles of endogenous tau, non-transgenic mice do not develop tau aggregates. One of the main pathological AD hallmarks is the presence of tau inclusions, so to better test the effect of tau Reduction of the Protein Tau Attenuates Neuronal Hyperexcitability and Permits Clearance of Intraneuronal Tau Accumulations in vivo

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reduction on pathological tau species, we reduced human tau in a transgenic tauopathy mouse model that develops extensive tau pathology. Following treatment with a human tau ASO, not only did reducing human tau prevent additional tau aggregates from forming, it also allowed for a striking reversal of tau accumulations and hippocampal neuronal loss in aged tauopathy mice. Taken together, the safety of reducing endogenous tau in adult animals, the protective effect against neuronal hyperexcitability, and the ability to clear pre-existing tau aggregates, a tau lowering therapy using ASOs may be a viable and strong therapeutic approach for those human patients with a detrimental hyperexcitability profile, tau inclusions, or even both.
EPIGRAPH

“And the tree was happy.”

- Shel Silverstein
Chapter 1

Introduction and Perspective

This chapter contains parts of a previously published manuscript:

Author contributions for the citation above:
SLD made the figures for the paper. SLD and TM wrote the paper.
The Protein Tau

The protein tau is one member in a larger class of proteins termed microtubule-associated proteins (MAPs), joining MAP1a, MAP1b, and MAP2c. Unlike some of the other MAPs, tau is highly enriched in the axons of mature and growing neurons under normal physiological conditions (Kempt et al, 1996). In humans, six tau isoforms are expressed in the brain due to exon 2, 3, and 10 alternative splicing (Goedert, 1989) (Figure 1.1). Exons 2 and 3 code for N-terminal fragments that interact with microtubules and the plasma membrane (Brandt et al, 1995), while exon 10 includes the second of four repeat domains (RDs) (Goedert, 1989). Because exon 10 encodes an RD, isoforms of tau are classified as either 3R (−exon 10) or 4R (+exon10) (Lee et al, 2001). These two isoforms are tightly regulated during development with only 0N3R expressed during fetal development and a switch to 1:1 4R:3R in the adult human brain (Goedert & Jakes, 1990; Gao et al, 2000).

The role of tau in the brain is often associated with microtubule binding, as that is how it was first described (Weingarten et al, 1975). Tau is capable of promoting tubulin assembly in vitro, forming lengthened microtubules. While binding to and stabilizing microtubules is probably the best known role of tau, emerging evidence places tau in other important roles within the neuron, including binding to DNA, regulating hyperexcitability, and interacting with actin in the dendrites (Hua et al, 2003; Roberson et al, 2007; Frandemiche et al, 2014). While the full physiological role of tau within the cell is still under investigation, it is well characterized that under certain conditions, tau can become hyperphosphorylated and accumulate into Neurofibrillary Tangles (NFTs), resulting in a collection of diseases known as tauopathies (Buee et al, 2000; Lovestone et al, 1997; Billingsley et al, 1997).
**Tau and Primary Tauopathies**

The common denominator of Tauopathies is that intracellular tau inclusions can be found within the brain, though not all inclusions are composed of the same isoform of tau and perhaps not even the same conformation of tau (Clavaguera et al, 2013; Sanders et al, 2014). Clinically, these tauopathies are characterized by dementia, behavior, and/or motor dysfunction and include Alzheimer’s Disease (AD), a subset of Frontotemporal Dementias (ex. FTDP-17), Progressive Supranuclear Palsy, Corticobasal Degeneration, and others (Buee et al, 2000; Rademakers et al, 2004). Of these, AD is the most common and the seventh leading cause of death in the US, with 5.3 million people affected (Alzheimer’s Association). Pathologically, AD is characterized by two hallmarks: the aforementioned intraneuronal NFTs composed of hyperphosphorylated tau as well as extracellular amyloid-β (Aβ) plaques (Greicius et al, 2004). While there are no known AD-associated tau mutations, though there is suggestion of MAPT polymorphism variation (Kauwe et al, 2008), there are over 35 tau mutations that have been directly linked to FTDP-17, providing unambiguous evidence that tau alone is sufficient to induce widespread neurodegeneration and dementia (Schraen-Maschke et al, 2004).

Recently, the concept of tau pathology and NFTs spreading through the brain has gained popularity, based on the idea that NFTs propagate through the AD brain in a predictable manner (Grundke-Iqbal et al, 1986; Braak and Braak, 1991; Morris et al, 2011). While it still unclear exactly how this tau pathology is “spreading” between neurons, emerging evidence has suggested that in neurodegenerative diseases, including AD, misfolded protein aggregates can spread through the brain in a prion like manner (Brundin et al, 2010; Frost and Diamond, 2010; Walker et al, 2013). Numerous studies focused on the protein tau have now shown that tau can both be secreted and endocytosed *in vitro* as well as *in vivo* (Clavaguera et al, 2009; Frost et al, 2009; Yamanada et al, 2011; Kfoury et al, 2012; Holmes et al, 2013). Transgenic mice have been
generated that have been able to recapitulate this propagation of tau pathology through synaptically connected networks, further supporting the idea of tau pathological spread as a possible mechanism for disease pathogenesis (de Calignon et al., 2012; Liu et al., 2012). While we have gained much information on tau spreading using in vivo models, it still remains unclear exactly how tau pathology spreads in people and how that plays a role in diseases pathogenesis, though we are getting closer. For example, tau PET imaging markers may help us better understand the time frame for tau pathology and how its spread varies from one tauopathy to another (Okamura et al., 2014).

There is no current cure for AD and as the baby boomer generation ages, there is even greater need for treatment. Several therapies targeting generation and clearance of the Aβ peptide have unfortunately been unable to slow or reverse the cognitive decline in AD patients. Based on data demonstrating a direct correlation between pathological tau deposition and cognitive decline, we propose to focus on the second major AD pathological hallmark contributor: the protein tau.

**Possible Tau-Targeted Therapies**

Numerous methods exist for targeting a specific gene or protein of interest in the context of disease and neurodegeneration. Even for a group of disorders as specific as tauopathies, there are several different treatment avenues that can be pursued.

Because of the role of tau in binding to and stabilizing microtubules, one area of therapeutic research has been looking at a possible “loss-of-function” disease mechanism, whereby the detachment of tau from microtubules results in axonal transport deficits, disassembly of microtubule networks, and ultimately neuronal death. In an effort to treat this “loss-of-function” disease mechanism, microtubule stabilizers, such as Epothilone D, have been developed and are
capable of restoring axonal transport and preventing the formation of hyperphosphorylated tau pathology in tau transgenic mice (Brunden et al, 2010; Zhang et al, 2012). While Epothilone D was halted in human clinical trials, others are currently being developed in an effort to treat this tau “loss-of-function” disease component.

In line with developing a therapy for tauopathies that doesn’t directly target the protein tau itself, several groups are interested in targeting the hyperphosphorylation of tau by inhibiting kinases, such as glycogen synthase kinase 3-beta (GSK-3β), that are key players in the pathogenic hyperphosphorylation of tau. Tau inclusions that form contain accumulations of hyperphosphorylated tau and it is this hyperphosphorylation that may lead tau to dissociate from microtubules and ultimately promote aggregation. Phosphorylation of tau by GSK-3β is sufficient to induce tau filament formation and when given GSK-3β inhibitors in vivo, mice show a reduction in tau pathology and rescue in neuronal loss (Rankin et al, 2007; Sereno et al, 2009).

In addition to these therapies that indirectly target tau through either stabilizing microtubules or preventing hyperphosphorylation, a more direct approach may be to target the tau protein itself. These therapies include possible tau aggregation inhibitors, such as Methylene Blue, that prevent tau from forming aggregates in some, though not all, tau mouse models (Hosokawa et al, 2012; Spires-Jones et al, 2014; Schirmer et al, 2011), as well as tau immunotherapy, which promotes the clearance of tau, either total tau or specifically oligomeric species (Yamanandra et al, 2013; Castillo-Carranza et al, 2014; Bi et al, 2011).

While all of these therapies in some way target the tau disease pathway, either by interacting with microtubules, kinases, tau aggregates, or the individual tau protein itself, none are so direct
as to decrease total levels of tau. Here, we propose directly targeting the tau gene, reducing total tau mRNA expression and ultimately total tau protein levels. This is the most direct approach to rescuing a tau “gain-of-function” toxicity. In order to reduce total tau levels in vivo, we have employed Antisense Oligonucleotides that are designed to specifically target total tau mRNA.

**Antisense Oligonucleotide Introduction**

“Antisense Oligonucleotide” or ASO, is a broad term, encompassing any relatively short string of nucleic acids. For this specific review, we are focused only on those ASOs that are single-stranded sequences, 8-50 base pairs in length, and bind to the target RNA by means of standard Watson-Crick base pairing. Upon binding, the ASOs can alter the original function of the target RNA through an array of different mechanisms.

**ASO Chemical Modifications**

As they are found in nature, short oligonucleotides possess weak intrinsic binding affinities and are readily degraded by nucleases. However, there are several ways to apply chemical modifications to ASOs in order to overcome these weaknesses. Both the backbone chemistry and sugar moieties are premier targets for ASO design enhancement.

The Phosphorothioate (PS) backbone modification is one of the earliest, and still to date, one of the most widely used modifications for ASO drugs. The non-bridging phosphate oxygen atoms in natural nucleic acids are replaced with sulfur atoms, equipping ASOs with several important properties that support their use as a systemic drug (Eckstein, 2000). The PS backbone significantly increases the stability of the ASO against nuclease degradation (Stein et al., 1988), ensuring that PS-ASOs can reach their target RNA in cells and tissues. Of equal importance, PS-ASOs can recruit the enzyme RNaseH to promote cleavage of the target RNA, a crucial
mechanism of action for many ASOs. In fact, most ASO drugs in development use this exact mechanism to reduce total levels of the target gene (Cerritelli and Crouch, 2009). In addition to increasing stability and recruiting RNaseH, the PS modifications enhance the attachment of ASOs to plasma proteins, facilitating binding to cells and uptake into specific tissues (Brown et al., 1994). While the backbone of the ASOs is an excellent target for manipulation, modifications at the 2’-position of the sugar moiety have also proven to be equally valuable for enhancing drug-like properties of ASOs. Modifications at the 2’ position enhance ASO potency by facilitating target binding. Of the 2’-modifications currently used, the 2’-O-methyl and 2’-O-methoxyethyl (MOE) sugar modifications are the most popular and are in multiple ongoing human clinical trials. MOE modifications not only increase resistance to nucleases, but also reduce nonspecific protein binding, which can in turn reduce the ASO toxicity profile (Teplova et al., 1999).

Although 2’-sugar modifications enhance binding to the mRNA target, almost all significantly reduce or even completely obstruct RNaseH from cleaving the target RNA. One of the most popular strategies used to circumvent this limitation has been to adapt the “gapmer” design, whereby regions of 2’-modified residues flank a longer central unmodified region (Figure 1.2). These 2’-modified “wings” further increase binding affinity and nuclease resistance while still allowing the center gap region to recruit RNaseH.

As with most drugs, ASOs demonstrate dose-dependent transient and mild-to-moderate toxicities in rodents, primates, and humans. Some of the most common toxicities associated with ASOs include inhibition of the clotting cascade and enhanced liver enzymes, typically seen when the concentration of PS-ASOs is high in the plasma (Smith and Miller, 2008; Chan et al., 2006; Jason et al., 2004). In addition, a common subchronic toxicity associated with ASO
administration is the activation of the immune system. Specifically, ASOs interact with Toll-Like receptor 9 (Senn et al., 2005) which can result in splenomegaly, though it appears that rodents are much more sensitive to this particular immune response as compared to primates (Miller et al., 2008). It should be noted that more is known in regards to the toxicities of ASOs in rodents and when delivered to humans peripherally. Thus far, only one paper has been published that has tested the safety of ASOs when delivered directly to the CNS of humans. They found no major side-effects of the second generation ASO in the treated human patients at any of the administered doses (Miller et al., 2013). This bodes well for future second and third generation ASO studies in the CNS, though new modifications are continuously being investigated in an effort to achieve even better tolerated ASOs.

**ASO Mechanisms of Action**

ASOs work through a variety of different mechanisms to achieve target manipulation (Figure 1.3.), though these can be broken into two broad categories: those that recruit RNaseH for degradation and those that do not. RNA degrading ASOs cleave the target mRNA by activating the nuclear enzyme RNaseH (Lorenz et al., 1998). As described above, these ASOs must have at least a portion of the ASO unmodified at the 2' position (Suzuki et al, 2010) (Figure 1.2.). RNaseH is a mammalian enzyme that recognizes RNA-DNA heteroduplexes, cleaves the RNA strand, and releases the intact DNA (Cerritelli et al., 2009). More specifically, RNaseH1 is responsible for mediating RNA cleavage as directed by ASO hybridization (Wu et al., 2004). Because RNaseH1 releases the intact ASO upon cleavage of the bound RNA, it conveniently allows for a single ASO to catalytically cleave many RNA molecules, further increasing ASO potency. Activation of RNaseH is extremely sequence specific, as a single mismatch results in a 3- to 5-fold decrease in RNaseH cleavage (Carroll et al., 2011; Basilion et al., 1999; Lima et al.,
2007), and 3 or more mismatches results in a complete loss of RNaseH degrading activity (Monia et al., 1992).

**ASO Pharmacokinetic Properties**

As might be expected, the highly charged ASOs do not significantly cross the blood brain barrier (BBB) (Smith et al., 2006), thus complicating delivery for neurodegenerative diseases. To circumvent the BBB, ASOs can be delivered directly into the cerebral spinal fluid (CSF) that in turn bathes the brain and spinal cord, allowing for surprisingly efficient distribution of the ASOs in the central nervous system (CNS), both in rodents and Rhesus monkeys (Smith et al., 2006; Kordasiewicz et al., 2012). Staining for the ASOs in the CNS reveals a relatively uniform distribution, suggesting an active ASO uptake mechanism rather than simple diffusion, which would be expected to show the highest concentration near the ventricles.

Upon successful entry into cells, ASOs demonstrate a relatively long duration of action. Following termination of a short ASO infusion period using RNaseH ASOs, target mRNA levels can be suppressed for up to 12-16 weeks (Kordasiewicz et al., 2012; DeVos et al., 2013) and even longer with the fully 2’-MOE modified ASOs (Hua et al., 2010), suggesting that the effect of ASOs is extremely long-lived in tissues, likely due to a lengthy ASO half-life.

**Antisense Oligonucleotides in the Context of Human Neurodegeneration**

There are numerous neurodegenerative disorders that could potentially benefit from a gene reduction therapeutic strategy – primarily those disorders that are a direct result of a single aberrant protein. The first in vivo treatment for a CNS-neurodegenerative disorder using ASOs with a PS backbone and a 2’-O-methyl modification targeted the human SOD1 transgene in the transgenic SOD1G93A rat model of amyotrophic lateral sclerosis (ALS) (Smith et al., 2006), a fatal motor neuron disease. The SOD1 ASOs reduced total human SOD1 mRNA levels by 50%
throughout the brain and spinal cord of the rats, resulting in a 37% extension of survival after the disease onset in vivo. These studies, along with promising toxicology studies in nonhuman primates, prompted the advancement of human SOD1 ASOs into phase I human clinical trials for those familial ALS patients with SOD1 mutations (Miller et al, 2013). Results from this study showed an excellent safety profile after a single 11.5-hour IT infusion of ASOs (Miller et al, 2013) and strongly suggest that CSF delivery of ASOs may be a viable strategy for other neurologic disorders.

With the success of the SOD1 in vivo work, additional neurodegenerative disorders were explored, including Huntington’s disease (HD). HD is an autosomal dominant neurodegenerative disorder that results from a CAG expansion in exon 1 of the huntingtin gene. Those with HD display progressive hyperkinetic involuntary movements, chorea, dystonia, and cognitive deficits. Owing to the direct link between mutated huntingtin and development of HD, a likely therapy may be to decrease the causative mutant huntingtin. When delivered to human huntingtin transgenic mice, human huntingtin ASOs were able to significantly reduce total huntingtin mRNA and protein levels, and were able to rescue the detrimental motor and anxiety phenotypes classically seen in the transgenic line (Kordasiewicz et al, 2012). Interestingly, even after total human huntingtin levels had returned to baseline following ASO treatment, the rescue in behavior persisted, suggesting that a single bolus of huntingtin ASO may have long-lasting beneficial effects as a therapy. With such a striking rescue in both the transgenic mouse behavior and poly-glutamine aggregates, plans for a phase I trial are underway for the treatment of HD with human huntingtin ASOs. Additionally, ASOs have been developed to preferentially bind to the CAG repeat in exon 1 of huntingtin mRNA, allowing for selective reduction of mutated huntingtin while leaving wild-type huntingtin untouched (Hu et al, 2009). This would
eliminate the need to knockdown all huntingtin in the event that too much huntingtin knockdown is detrimental (Nasir et al, 1995; Dragatsis et al, 2000).

The most prevalent neurodegenerative disease that demands therapeutic intervention is AD, with 5.4 million Americans currently afflicted and, if a treatment is not found, 13.2 million by 2050 (Hebert et al, 2003). ASOs against the amyloid precursor protein (APP), the precursor to the toxic amyloid-beta peptides that are generated in the development and progression of AD, have shown in vivo promise. When injected in a mouse model that over expresses hAPP, the APP ASOs resulted in a significant reduction in hAPP protein levels in the brain, and a rescue in learning and memory in both young and aged mice (Kumann et al, 2000; Banks et al, 2001; Erickson et al, 2012).

In addition to targeting amyloid-beta, ASOs against tau, the protein that composes intraneuronal neurofibrillary tangles, may also provide therapeutic benefits. To better understand the possible benefits of such a tau reduction therapy, the tau knockout mouse line has thus far been the only model to study the effects of tau reduction in vivo. Though because of the genetic reduction during all of development, developmental compensation may play an important role. We present here a tau reduction model that begins in adulthood so as to not have any developmental confounds, making our tau reduction model the most relevant and useful in vivo model being currently used.

**Tau Knockout Mouse Model**

The tau knockout model was generated by inserting a GFP construct into exon 1 of the endogenous mouse tau gene, thereby preventing transcription of the tau gene (Tucker et al, 2001). This line lacking all endogenous tau expression has been studied extensively in the
presence of Aβ-deposition and has proven to be protective against a growing number of Aβ-induced insults. The first in vivo study using the mTau\(^{+/-}\) line in the context of AD came from Roberson et al, where they crossed mTau\(^{+/-}\) mice with the hAPP line. By knocking out tau, the J20 mice showed significant improvements in the Morris Water Maze learning/memory task, a rescue of neuronal plasticity markers in the hippocampus, and protection against neuronal hyperexcitability. These exciting results were then repeated by Ittner et al who elegantly showed the same mTau\(^{+/-}\) protective effects in another hAPP line (APP23). Still others report that mTau\(^{+/-}\) protects against additional Aβ-induced insults, including other cognition tests (Andrews-Zwilling et al., 2010; Leroy et al., 2012), hyperexcitability (Roberson et al., 2007, 2011; Ittner et al., 2010; Suberbielle et al., 2013; Li et al, 2014) (see Figure1.4), survival (Roberson et al., 2007, 2011; Ittner et al., 2010), axonal transport deficits (Vossel et al., 2010), cell-cycle re-entry (Seward et al., 2013), and double stranded breaks in DNA (Suberbielle et al., 2013).

One of the best characterized phenotypes in mouse models of AD is the increase in spontaneous seizures and neuronal hyperexcitability. Several human Amyloid Precursor Protein (hAPP) mouse lines and an ApoE4 mouse line have now been generated that display abnormal EEG and increased seizure frequency (Roberson et al., 2007; Ittner et al., 2010; Vogt et al., 2011; Hunter et al., 2012). Similarly, those with familial AD mutations, ApoE4 genotype, and sporadic late-onset AD, also experience an increased incidence in seizures (Takao et al., 2001; Mendez and Lim, 2003; Harden, 2004; Velez-Pardo et al., 2004; Amatniek et al., 2006; Kauffman et al., 2010). This AD associated excitotoxicity has been implicated in the pathogenesis of the disease (Olney et al., 1997; Mattson, 2004). However, because of the limitations in detecting abnormal EEG activity in large populations of AD patients, we currently rely heavily on animal models for predictions regarding hyperexcitability in the context of Aβ. In the hAPP J20 Aβ-depositing line, treatment with the anticonvulsant Levetiracetam returned the aberrant neuronal excitability.
that is classically seen in that line back to NT levels and restored cognition (Sanchez et al., 2012), similar to what was seen with the mTau^−/− genetic cross (Roberson et al., 2007, 2011). This rescue in cognition by means of an anticonvulsant suggests that lowering the abnormal neuronal activity alone may have a positive impact on learning and memory. A similar pilot study was performed in human Mild Cognitively Impaired (MCI) patients, whereby patients were given either placebo or Levetiracetam and then recall memory was tested using functional magnetic resonance imaging methods. The Levetiracetam treatment significantly improved the recall performance of the MCI patients, again providing evidence that reducing the aberrant excitability in MCI and AD patients may help to subsequently restore cognition (Bakker et al., 2012). Perhaps by reducing total tau levels, this same hyperexcitability can be abrogated and cognition be allowed to restored.

Further, in vivo studies of mTau^−/− mice have shown that tau reduction for up to a year does not significantly alter learning/memory (Lei et al, 2012; Tucker et al, 2001). There have been reports of a mild motor parkinsonism phenotype in aged mTau^−/− mice, though the severity of this motor phenotype is debated (Lei et al, 2012; Morris et al, 2013; Li et al, 2014).

In light of these positive tau knockout experiments, translating a similar tau reduction therapy to humans may be a viable approach to treating tauopathies, including AD. However, because all previous studies looking at tau reduction have relied on the genetic ablation of tau, and some have reported a developmental compensation of other MAPs (Harada et al, 1994), the question remains as to whether these beneficial effects are due to an actual decrease in total tau levels, or are an artifact of the tau knockout line. Whenever an in vivo knockout model is made, the possibility exists for other genes to increase expression during development to compensate for the loss of the gene being knocked out. For example, by knocking out the protein tau, other
microtubule associated protein family members may increase in expression to take over the microtubule binding and stabilizing roles of tau. This compensation may then skew any future studies and not be an accurate representation of what is in fact solely a tau-mediated phenotype. The only way around this is to reduce the gene of interest after the animal has developed normally, either with a genetically inducible knockdown model, or by exogenous pharmacological reduction. To answer these questions, we sought to reduce total tau levels in already developed adult mice. If the same protective effect could be seen in mice with reduced tau only in the adult stage of life, it would suggest that the previous protective results using the tau knockout line are in fact tau mediated and not some developmental phenomenon.

While reducing endogenous tau levels greatly helps to address the safety of reducing tau in adult animals as well as whether lowering tau in adults has similar protective benefits as previously demonstrated with the tau knockout line, murine tau does not self-aggregate in Non-Transgenic (NT) mice. Because of this, it is impossible to test whether a tow lowering therapy may also rescue the prominent intracellular NFT phenotype that is one of the two main pathological hallmarks of AD. For this, we need to turn to a transgenic tauopathy mouse model that develops intraneuronal tau inclusions in an age-dependent manner.

**Human Tauopathy Mouse Models**

Several mouse models have been generated now that express the human tau transgene, either with or without mutations, in an effort to better study the accumulation of tau tangles as well as test different mechanisms and drugs that may ameliorate this tau pathology (Noble et al, 2010). Because accumulations of hyperphosphorylated tau are a good predictor of AD cognitive decline due to the negative correlation between the number of NFTs and the Mini-Mental score
(Giannakopoulos et al, 2003), being able to clear such pathology may provide a strong therapeutic avenue for AD and other primary tauopathies.

An obvious choice for a very applicable human tauopathy mouse model in assessing treatment options is the hTau line that contains the full human tau gene under the tau promoter in mice lacking endogenous mouse tau (Duff et al, 2000). These mice express all human tau isoforms, including 3R and 4R tau, though the ratio is not quite the same as in people. While humans express 1:1 4R:3R tau as adults (Goedert et al, 1990; Gao et al, 2000), the hTau line is greatly skewed to 3R tau expression. These mice do develop some tau histopathology, though it takes longer to develop. To really test whether a tau lowering therapy can strongly prevent or perhaps even reverse tau pathology, more robust tauopathy models exist that contain human tau cDNA constructs with known FTD causing tau mutations.

Some of these more robust tau lines have been genetically engineered to allow for transgene suppression in later stages of life. “Turning off” the tau transgene addresses the same question of whether lowering total tau levels may serve as a possible therapeutic strategy. In one of the first regulatable tau transgenic mouse (rTg4510 model), decreasing total tau levels by turning the transgene off after tau inclusions had already started to form was capable of reversing behavior deficits, even though tangles persisted (SantaCruz et al, 2005). Since then, two other groups have also looked at this inducible reduction of human tau and its effect on behavior and tau pathology. In 2011, Sydow et al used a pro-aggregation tau mutant line (Mocanu et al, 2008) and upon inhibiting the expression of the pro-aggregate tau, found that both memory and long term potentiation (LTP) were able to recover, while aggregates of tau were moderately decreased. They also found that while neuron loss persisted, synapses were partially recovered, suggesting that when total tau levels were reduced, it created a less toxic environment where
synapses could once again grown and re-form connections (Sydow et al, 2011). The most recent report of tau suppression demonstrated that when the tau transgene was suppressed in the tau spreading mouse model (rTgTauEC; de Calignon et al, 2012), there was a striking reversal in acetylcholinesterase (AchE) sprouting fibers, a phenotype often seen in the pathogenesis of human AD as well as in this line of mice, in addition to a resolution of NFTs and halting of progressive neuronal loss. They concluded that by reducing tau, the propagation of tau through connected networks in the brain could be reversed (Polydoro et al, 2013).

These three tau repression studies have helped to pave the way to better understand the consequences and benefits of reducing tau on both behavior, as well as the presence of aggregated forms of tau in mouse models of tauopathy. Such a genetic repression is not feasible in humans, however. While one would expect similar outcomes with an exogenously applied tau lowering drug, it is important to still directly test this. One group thus far has been able to reduce total tau levels using a drug in a tauopathy model. Using very high levels of methylene blue, O’Leary et al were able to acutely reduce total levels of soluble tau protein levels. Interestingly, it was only when the soluble levels of tau were reduced that they saw any improvement in cognition in the mice (O’Leary et al, 2010).

The acute decrease in total tau protein levels via methylene blue is very interesting, though the concentration of methylene blue administered to the mice may not be a viable option for human treatment. While we are also interested in reducing total tau levels, antisense oligonucleotides provide a more selective approach to reducing tau, since the knockdown starts at the level of tau mRNA. Additionally, in deciding which mouse model to treat, we chose a line that 1) cultivates strong tau pathology, both hyperphosphorylated tau accumulations as well as neurofibrillary tangles, 2) develops synapse and neuron loss, and 3) is readily accessible and easy to maintain.
Based on these parameters, we chose the TauP301S line (PS19 model). This line contains a human cDNA tau construct that is 1N4R with the P301S tau mutation and overexpresses this mutated form of human tau by five fold in the brain under the prion promoter (Yoshiyama et al, 2007). Several groups have used this P301S model to analyze the efficacy of different tau therapies, including microtubule stabilizers (Brunden et al, 2010; Zhang et al, 2012) and anti-tau antibodies (Yamanandra et al, 2013), allowing us to more directly compare the therapeutic use of ASOs with other tau-based treatments.

**Summary**

The protein tau is a major contributor to Alzheimer’s Disease, a subset of Frontotemporal Dementias, Progressive Supranuclear Palsy, and other primary tauopathies. Accumulations of tau are in direct correlation with a decrease in cognitive decline, implicating the misfolding and aggregation of tau to the pathogenesis of disease in these disorders. One possible therapy may be to target the very source of the problem: total tau expression. To do this, we propose using antisense oligonucleotides (ASOs) to decrease total tau mRNA and protein levels in vivo. By testing the safety of reducing tau in non-transgenic mice and looking at the therapeutic efficacy of reducing tau in the context of hyperexcitability and tau pathology formation, we hope to study the question of whether or not lowering tau in an adult animal would prove beneficial with the ultimate goal of moving this therapy into clinical trials if the *in vivo* data look promising.
Figure 1.1. Isoforms of the Microtubule Associated Protein Tau.

The protein tau can be alternatively spliced into six isoforms that are expressed in the central nervous system. The longest isoform of tau has all three alternatively spliced exons – exons 2, 3, and 10 – whereas the shortest isoform does not contain any of the three spliced exons. Exons 2 and 3 account for the 0N, 1N, and 2N isoforms whereas exon 10 is responsible for 3R and 4R isoforms. Only 0N3R tau is expressed during fetal development followed by a switch to the other isoforms of tau in adulthood.
Figure 1.2. Antisense Oligonucleotide ‘Gapmer’ Design.

Example of a 20 base pair Antisense Oligonucleotide (ASO) that would be designed to support RNaseH activity. The Phosphorothioate backbone is used along the entire length of the ASO to provide nuclease resistance, while the 2’- sugar modification is used exclusively on the first and last 5 nucleotides, leaving the middle 10 nucleotides unmodified at the 2’-sugar position. This provides increased target RNA binding affinity on the outer portions of the ASO, while still allowing RNaseH cleavage at the central region of the ASO.
Antisense Oligonucleotides (ASOs) have been proposed to enter into cells through high- and low-binding plasma protein receptors on the cell surface, resulting in ASO compartmentalization into lysosomes and endosomes. Through a largely unknown mechanism, ASOs are released from the vesicles into the cytoplasm where they can freely move in and out of the nucleus. Upon entry into the nucleus, ASOs can directly bind to mRNA structures and prevent the formation of the 5’-mRNA cap (1), modulate alternative splicing (2), dictate the location of the polyadenylation site (3), and recruit RNaseH1 to induce cleavage (4). ASOs in the cytoplasm can directly bind to the target mRNA and sterically block the ribosomal subunits from attaching and/or running along the mRNA transcript during translation (5). ASOs can also be designed to directly bind to microRNA (miRNA) sequences (6) and Natural Antisense Transcripts (NATs) (7), thereby prohibiting miRNAs and NATs from inhibiting their own specific mRNA targets. This ultimately leads to gene upregulation of the miRNA and NAT targets.
**Figure 1.4. Tau Knockout is Protective against Pentylenetetrazol (PTZ) Induced Global Seizures.**

A, 50mg/kg Pentylenetetrazol (PTZ) was given by intraperitoneal injection to NonTransgenic (+/+) , Tau heterozygous (+/-), and Tau knockout (-/-) mice and the final seizure stage was scored blinded. Kruskal Wallis, Dunns post hoc analysis.

**p<0.01; n=10-12; Error bars represent SEM.**


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Chapter 2

Antisense Reduction of Endogenous Tau in Adult Mice Protects against Seizures
This chapter contains a previously published manuscript:

Author contributions for the citation above:
ABSTRACT

Tau, a microtubule associated protein, is implicated in the pathogenesis of Alzheimer’s Disease (AD), in regards to both neurofibrillary tangle (NFT) formation as well as neuronal network hyperexcitability. The genetic ablation of tau substantially reduces hyperexcitability in AD mouse lines, induced seizure models and genetic in vivo models of epilepsy. These data demonstrate that tau is an important regulator of network excitability. However, developmental compensation in the genetic tau knockout line may account for the protective effect against seizures. To test the efficacy of a tau reducing therapy for disorders with a detrimental hyperexcitability profile in adult animals, we identified antisense oligonucleotides (ASOs) that selectively decrease endogenous tau expression throughout the entire mouse central nervous system – brain and spinal cord tissue, interstitial fluid, and cerebrospinal fluid – while having no effect on baseline motor or cognitive behavior. In two chemically induced seizure models, mice with reduced tau protein had less severe seizures than control mice. Total tau protein levels and seizure severity were highly correlated, such that those mice with the most severe seizures also had the highest levels of tau. Together, our results demonstrate that endogenous tau is integral for regulating neuronal hyperexcitability in adult animals and suggest that an antisense oligonucleotide reduction of tau could benefit those with epilepsy and perhaps other disorders associated with tau-mediated neuronal hyperexcitability.
INTRODUCTION

As a member of the microtubule-associated protein family (Weingarten et al., 1975), the protein tau is enriched in axons of mature and growing neurons (Kempf et al., 1996). However, under certain conditions, tau can become hyperphosphorylated and accumulate into oligomeric species and neurofibrillary tangles (NFTs), resulting in a group of disorders known collectively as tauopathies (Billingsley and Kincaid, 1997; Lovestone and Reynolds, 1997; Buee and Delacourte, 1999), the most common being Alzheimer’s Disease (AD).

While the role of tau in proteinaceous aggregates has long been studied (Iqbal et al., 1975; Brion et al., 1985), a new role has emerged that implicates tau as a regulator of neuronal hyperexcitability. Tau knockout (tau−/−) mice demonstrate substantially reduced seizure severity in models of chemically induced seizures (Roberson et al., 2007, 2011; Ittner et al., 2010) and genetic models of severe epilepsy (Holth et al., 2013). These data collectively suggest that tau plays a role in neuronal hyperexcitability and provide evidence that a tau reducing therapy may be beneficial for those with seizure disorders. Additionally, amyloid precursor protein overexpression/amyloid-beta depositing mouse lines show increased baseline neuronal hyperexcitability and spontaneous seizures. When placed onto a tau−/− background, these AD mouse models show both decreased seizure frequency as well as improved learning and memory (Roberson et al., 2007, 2011; Ittner et al., 2010), suggesting that tau-linked neuronal hyperexcitability may be an important component of AD pathophysiology.

However, whether reducing tau levels in an adult animal will modulate neuronal hyperexcitability similar to genetic deletion remains unknown. For example, developmental compensation could contribute to the protective effect of tau−/−, such as the reported increase in microtubule associated protein 1A (Harada et al., 1994). In the report herein, we directly test the
effect of reducing tau in adult non-transgenic mice by reducing endogenous tau levels and subsequently analyzing the effects on baseline behavior and induced seizure severity. We reduce murine endogenous tau levels using antisense oligonucleotides (ASOs) delivered directly to the cerebrospinal fluid (CSF) (DeVos and Miller, 2013a). Recent data demonstrating safety of CSF-delivered ASOs in humans (Miller et al., 2013) suggests that the strategy used here may be translated into a therapy for seizures and possibly other neurodegenerative disorders.
MATERIAL AND METHODS

Animals. All ASO treated mice were C57BL/6J Non-transgenic mice ordered directly from The Jackson Laboratory. Tau/- mice containing a GFP-encoding cDNA integrated into exon 1 of MAPT gene (Tucker et al., 2001) were obtained from The Jackson Laboratory and maintained on a C57BL/6J background. Characterization and behavioral experiments were performed using gender-balanced groups age 2-4 months (Figures 2.1 – 2.6). Seizure experiments were performed using males age 3-5 months (Fig. 2.7 – 2.10). Mice had access to food and water ad libitum and were housed on a 12 hour light:dark cycle. Experiments involving animals were approved by the Animal Studies Committee at Washington University in St. Louis.

Antisense Oligonucleotides: The ASOs have the following modifications: five nucleotides on the 5’- and 3’-termini containing 2’-O-methoxyethyl modifications and 10 unmodified central oligodeoxynucleotides (DeVos and Miller, 2013b) to support RNaseH activity and a phosphorothioate backbone to improve nuclease resistance and promote cellular uptake (Bennett and Swayze, 2010). ASOs were synthesized as previously described (McKay et al., 1999; Cheruvallath et al., 2003) and solubilized in 0.9% sterile saline immediately prior to use. ASO Sequences: Tau<sup>ASO-1</sup>: 5’-GCAGGAGTCTTAGATGTCT-3’; Tau<sup>ASO-2</sup>: 5’-AAGCAGGTTAGTGACAAGC-3’; Tau<sup>ASO-3</sup>: 5’-ATCACTGATTTGAAATGTCCC-3’; Scrambled ASO: 5’-CCTTCCCTGAAGGTTCCTCC-3’.

Surgical placement of Intracerebroventricular (ICV) Pumps and Tissue Collection: As previously described, (Smith et al., 2006; DeVos and Miller, 2013a) mice were anesthetized with isoflurane and the 28 day osmotic pumps (ALZET) with ASO were implanted in a subcutaneous pocket that was formed on the back of the mouse. The catheter was placed in the right lateral ventricle using the following coordinates based on bregma: -0.5mm Posterior, -
1.1mm Lateral (right), -2.5mm Ventral. For CSF collection, mice were placed on a heating pad and anesthetized with isoflurane. CSF was drawn through the cisterna magna as previously reported and immediately frozen on dry ice (Barten et al., 2011). For tissue collection, mice were anesthetized with isoflurane and perfused using chilled PBS-heparin. Brain and spinal cords were rapidly removed and either snap frozen in liquid nitrogen and stored at -80°C or post-fixed in 4% paraformaldehyde at 4°C and transferred to 30% sucrose 24 hours later. All animal protocols were approved by the Washington University in St. Louis Institutional Animal Care and Use Committee.

Quantitative Real-Time PCR: RNA analyses were performed using quantitative real-time RT-PCR. Total RNA was extracted from brain tissue using a QIAGEN RNeasy Kit (QIAGEN). For total tau analyses, RNA was reverse transcribed and amplified using the EXPRESS One-Step Superscript qRT-PCR Universal Kit (Invitrogen). For Nsmaf mRNA levels, RNA was reverse transcribed and amplified with the Power SYBR Green RT-to-Ct 1-Step Kit (Invitrogen). The qRT-PCRs were run and analyzed on the ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems). Total tau and Nsmaf mRNA expression levels were normalized to GAPDH mRNA levels and analyzed using the ΔΔCt method for relative expression analysis. Primer/Probe sequences: Total Tau: Forward 5' - GAA CCA CCA AAA TCC GGA GA -3'; Reverse 5' - CTC TTA CTA GTG GAT GGT GAC -3'; Probe 5'/56-FAM/CC AAG AAG GTG GCA GTG GTC C/3IABkFQ/-3', Nsmaf: Forward 5' - CTTACTGCTTCTCAACTTGGA -3'; Reverse 5' - GAACATATCTTTAAGGAGCCTC -3', GAPDH: Forward 5' - TGC CCC CAT GT TGT GAT G -3'; Reverse 3' - TGT GTG CAT GAG CCC TTC C - 3'; Probe 5'/56-FAM/ AAT GCA TCC TGC ACC ACC AAC TGC TT /3AHBkFQ/ 3' (IDT).
**Tau Protein Analysis:** Tissues were weighed and homogenized in 10X volume RAB buffer ([100mM MES, 1mM EDTA, 0.5mM MgSO4, 750mM NaCl, 20mM NaF, 1mM Na3VO4, supplemented with protease inhibitor (Complete, Roche) and phosphatase inhibitor (Sigma)]. Homogenate was spun at 21,000xg on a tabletop centrifuge for 10 minutes at 4°C. Supernatant was collected and protein concentration measured using Pierce BCA Protein Assay Kit (Thermo Scientific). For tau protein quantification in brain, ISF, and CSF, tau concentrations were analyzed using the published Tau5-BT2 sandwich ELISA (Yamada et al., 2011). Briefly, 96-half-well plates (Nunc) were coated with the Tau-5 antibody (Millipore) overnight at 4°C. Plates were blocked with 4% BSA for 60 minutes at 37°C, brain homogenate, ISF, or CSF diluted in standard buffer [0.25% BSA, 300mM Tris, 0.05% azide, and 1X protease inhibitor in PBS] was added, and incubated overnight at 4°C. For the standard curve, the longest mouse tau isoform, mTau40, recombinant protein was used. The detection antibody biotinylated BT-2 (Pierce) was added the next day followed by streptavidin poly-horseradish peroxidase-40 (Fitzgerald). Plates were developed using Super Slow ELISA TMB (Sigma) and read on an Epoch Microplate Spectrophotometer (BioTek).

**Immunofluorescence:** Brains post-fixed in 4% paraformaldehyde were sliced at 50µm on a freezing microtome. Brain slices were treated with Citra Plus antigen retrieval (BioGenex) before antibody application. Brains were incubated with the primary antibodies Tau46 (1:300, Cell Signaling) and Pan-ASO (1:1000, Isis) in 3% Horse Serum overnight at 4°C followed by a 1 hour incubation at room temperature with fluorescent-conjugated secondary antibodies (1:3000, DyLight, ThermoScientific). Fluorescent images were captured using the Olympus Nanozoomer 2.0-HT (Hamamatsu) and processed using the NDP viewer software (Hamamatsu).
**In vivo Microdialysis for ISF Collection:** *In vivo* microdialysis experiments to assess brain interstitial fluid (ISF) tau levels from awake and freely moving mice were developed with modifications of our previously described method (Yamada et al., 2011). A guide cannula (Eicom microdialysis) was stereotaxically implanted in the left hippocampus under isoflurane anesthesia, and cemented. After implantation of the cannula and dummy probes (Eicom microdialysis), mice were habituated to microdialysis cages for one more day. After this recovery period, a 2mm 1000kDa cut-off AtmosLM microdialysis probe (Eicom microdialysis) was inserted through the guide cannula. A probe was connected to a microdialysis peristaltic pump with 2 channels (MAB20; SciPro, Sanborn, NY), which was operated in a push pull mode. The perfusion buffer, 25% human albumin solution (Gemini Bio Inc), was diluted to 4% with artificial CSF (aCSF) (1.3mM CaCl₂, 1.2mM MgSO₄, 3mM KCl, 0.4mM KH₂PO₄, 25mM NaHCO₃, and 122mM NaCl, pH 7.35) on the day of use and filtered through 0.1 µm membrane. ISF was collected at 1 µl/min in a refrigerated fraction collector (SciPro).

**Lactate Assay:** Lactate concentration in ISF was determined by YSI2700 biochemistry analyzer (YSI Life Sciences).

**Behavioral Analysis:**

*Sensorimotor Battery and 1-h Locomotor Activity:* All mice were evaluated on a battery of sensorimotor tests designed to assess balance (ledge and platform), strength (inverted screen), coordination (pole and inclined screens) and initiation of movement (walking initiation), as previously described (Wozniak et al., 2004; Grady et al., 2006). Locomotor activity was evaluated in all mice over a 1-h period using computerized photobeam instrumentation as previously described (Wozniak et al., 2004, 2007). General activity variables (total ambulations,
vertical rearings) along with indices of emotionality including time spent, distance traveled and entries made in a $33 \times 11$ cm central zone were analyzed.

**Elevated Plus Maze:** As described previously (Schaefer et al., 2000), the elevated plus maze (EPM) apparatus is a four arm maze shaped like a "plus sign". One set of the opposing arms have walls (closed arms) while the other set is not enclosed (open arms). The number of entries made, time spent, and distance traveled in each set of arms were quantified using a computerized, high-resolution photobeam system (Hamilton-Kinder, LLC). We also analyzed these three variables after normalizing the values to reflect percentages calculated out of the totals measured in both sets of arms.

**Morris Water Maze:** Spatial learning and memory were evaluated in the Morris water maze using a computerized tracking system (ANY-maze, Stoelting Co.) and procedures that were similar to previously described methods (Wozniak et al., 2004, 2007). The protocol included cued, place, and probe trials, and all trials were performed in a 120 cm diameter pool filled with opaque water. Cued trials were performed to identify nonassociative dysfunctions which might affect performance. This involved conducting 4 trials (60 second maximum) per day for 2 consecutive days with very few distal cues being present and with the platform location being moved for each trial to limit spatial learning. Three days later, place trials were initiated to assess spatial learning where the mice were required to learn the single location of a submerged (nonvisible) platform in the presence of several salient distal spatial cues. The place trials were conducted for 5 consecutive days, each day consisting of 2 blocks of 2 trials (60 seconds maximum) separated by 2 hours. Escape path length, latency, and swimming speeds were calculated for the cued and place trials. A probe trial lasting 60 seconds was conducted 1 h after the last trial of the place condition. This involved removing the platform and quantifying the
time spent in the pool quadrant that had contained the platform (target) and each of the other quadrants, as well as the number of crossings a mouse made over the exact location of where the platform had been (platform crossings). Spatial bias for the target quadrant was analyzed by comparing the time spent in it versus the times spent in each of the other quadrants.

**Picrotoxin Seizures and EEG Recording:** *In vivo* picrotoxin reverse microdialysis experiments from awake and freely moving mice were developed with modifications to the previously described method (Cirrito et al., 2008). To record EEG activity, bipolar recording electrodes (Teflon coated, stainless steel wire, 0.0055” coated OD, A-M Systems) were attached to the outside of the microdialysis guide cannula shaft using Elmer’s Super-Fast Epoxy Resin (Elmer’s). The electrodes extended ~1mm beyond the tip of guide, such that the tips of the electrodes would fall in the center of the 2mm microdialysis probe membrane once inserted. The guide cannula with attached electrodes (BR-style, Bioanalytical Systems) was stereotaxically implanted in the left hippocampus under isoflurane anesthesia and cemented. 2mm microdialysis probes (BR-2, 30-kDa MWCO membrane, Bioanalytical Systems) were inserted into the hippocampus. The perfusion buffer comprised artificial CSCF (aCSF) with 0.15% bovine serum albumin filtered through 0.1 µm membrane on the day of use. Once the microdialysis guide cannula and probe were placed into the left hippocampus, 12 hours of basal EEG activity was measured using a P511K A.C. pre-amplifier (Grass Instruments), digitized with a DigiData 1322A Data Acquisition System (Molecular Devices), and recorded digitally with pClamp 9.2 (Molecular Devices). Picrotoxin (Sigma-Aldrich) was diluted to the indicated concentrations in 0.15% BSA-aCSF perfusion buffer and delivered at a flow rate of 1.0 µL/min, with the lowest dose given first. Each increasing dose was delivered for 90 minutes with EEG measured continuously throughout drug delivery. EEG spike frequency was assessed for the last 60 minutes of each PTX dose and normalized to basal EEG of each mouse.
**Experimental PTZ Seizures:** Pentylenetetrazole (PTZ) (Sigma) was dissolved in sterile PBS at a concentration of 5mg/mL. A dose of 55mg/kg or 80mg/kg was delivered intraperitoneally for Figures 2.8/2.9 and Figure 2.10, respectively. A quiet, isolated room was used for all seizures to minimize noise and/or visual distractions. Immediately following PTZ administration, each mouse was videotaped and after 15 minutes, the mouse was sacrificed and the brain was snap frozen for biochemical analyses. Seizures recorded on videotapes were scored in a blinded fashion for severity according to published scales (Löscher and Nolting, n.d.; Racine, 1972). The seizure severity score used was as follows: 0 – normal behavior; 1 – immobility; 2 – spasm, tremble, or twitch; 3 – tail extension; 4 – forelimb clonus; 5 – generalized clonic activity; 6 – jumping or running seizures; 7 – full tonic extension; 8 – death.

**Statistics:** The data were analyzed for statistical significance using the graphing program GraphPad Prism 5. Two-tailed Student t-tests were used for total tau mRNA/protein and Lactate analyses when only one comparison was being made and for analyzing tau mRNA/protein levels in multiple different brain regions. One-way ANOVA with Bonferroni post hoc analyses were used for total tau mRNA and protein expression when more than one comparison was needed, as well as the inverted screen task. Two-way ANOVA with Bonferroni post hoc analyses were used for the duration of action study and ISF tau levels in multiple fractions. Two-way repeated measures ANOVA with Bonferroni post hoc analyses were used to analyze Morris Water Maze, Elevated Plus Maze trials, and Picrotoxin Dose Response. Specifically for the Morris Water Maze and Elevated Plus Maze analyses, the Huynh-Feldt adjustment of alpha levels was utilized for all within-subjects effects containing more than two levels to protect against violations of sphericity/compound symmetry assumptions underlying repeated measures ANOVA models. The Kruskal-Wallis with Dunns post-hoc analyses was used.
for the PTZ Seizure Severity analysis due to the categorical nature of the seizure severity scale. Linear Regression analysis was used to analyze the CSF and Brain tau correlations. While the linear regression was used to generate the “best-fit” and 95% confidence interval lines seen on the graphs in Figures 2.9 and 2.10, the Spearman correlation was used to generate ‘r’ and ‘p’ values for all total tau and EEG/Seizure comparisons. Error bars represent the SEM.
RESULTS

**Tau Antisense Oligonucleotides Reduce Endogenous Tau mRNA and Protein Expression**

To test the functional effect of reducing tau mRNA and protein in vivo, we developed an antisense oligonucleotide (ASO) that reduces endogenous tau mRNA and protein in adult mice. After screening 80 ASOs for ability to reduce tau mRNA in murine B16-F10 cells, we selected the three most potent ASOs to screen in vivo. We infused 50µg of each Tau ASO into the right hippocampus of adult non-transgenic (NT) mice using Saline and a Scrambled ASO as controls. One week post ASO infusion, the hippocampus surrounding the injection site was analyzed for total tau mRNA levels (Figure 2.1, A). All ASOs screened in the hippocampus provided >75% reduction of tau mRNA (ANOVA $F_{(4,22)}=151.4$, p<0.0001). The ASO Tau$^{ASO-3}$ was then tested in a 1 month intracerebroventricular (ICV) Alzet osmotic pump infusion at 100µg/day. As compared to the saline and scrambled ASO controls, those mice treated with Tau$^{ASO-3}$ had substantially less total tau mRNA (ANOVA $F_{(2,14)}=291.8$, p<0.0001) and protein levels (ANOVA $F_{(2,13)}=7.578$, p=0.007) (Figure 2.1, B-C). To test for general off-target ASO effects, we normalized tau mRNA to total RNA input and found no difference between tau mRNA results generated through standard GAPDH normalization or total RNA input (two-tailed t-test: Saline t(12)=0.000, p=1.000; Scrambled t(10)=0.297, p=0.773; Tau$^{ASO-3}$ t(6)=1.317, p=0.236). To further test specificity for tau, we BLASTED the Tau$^{ASO-3}$ sequence against the mouse genome and found that the closest match – Neutral sphingomyelinase activation associated factor (Nsmaf) – had 5 base-pair mismatches. When total Nsmaf mRNA levels were measured, no significant difference between saline, 100µg scrambled, or 100µg Tau$^{ASO-3}$ treated mice was found (ANOVA $F_{(2,14)}=1.914$, p=0.184), reinforcing the specificity of Tau$^{ASO-3}$ for murine tau.
In order to select the optimum dose for behavioral studies, we tested five doses of Tau\textsuperscript{ASO-3}. There was a dose dependent decrease in total tau mRNA expression levels (ANOVA $F_{(5,28)}=48.12$, $p<0.0001$) (Figure2.1, D). The 25µg/day dose was the lowest dose to provide >75% tau mRNA reduction and was selected for subsequent studies. Additionally, to plan treatment paradigms for behavioral studies, we tested the duration of action of a one month ASO infusion. After infusing 25µg/day Tau\textsuperscript{ASO-3} for 1 month, tau mRNA levels were measured in cohorts of mice at 4, 8, 12, and 16 weeks post-pump implantation. Four weeks after ASO infusion had stopped (8 weeks post-pump implantation), tau mRNA levels remained >80% decreased. Even at 12-16 weeks post pump implantation, tau mRNA remained decreased to >50% of the saline control (ANOVA $F_{(1,34)}=225.7$, $p<0.0001$) (Figure2.1, E). The long-term target knockdown results shown here are consistent with what others have reported for RNase-H activating ASOs (Kordasiewicz et al., 2012), highlighting the long duration of action that RNase-H ASOs exhibit in tissue. This lengthy duration of action is likely due to a long half-life of the ASO itself (Yu et al., 2009; Kordasiewicz et al., 2012), allowing a 1 month infusion of 25µg/day Tau\textsuperscript{ASO-3} to provide 4 months of tau mRNA reduction.

**Tau ASOs Reduce tau mRNA and protein Throughout the Brain and Spinal Cord**

To determine the distribution of Tau\textsuperscript{ASO-3} following 1 month ICV infusion of 25µg/day, we used an antibody that recognizes the backbone chemistry of the ASO (Kordasiewicz et al., 2012). Staining for Tau\textsuperscript{ASO-3} demonstrated widespread, diffuse distribution of ASO in the brains of ASO treated mice (Figure 2.2). Co-staining for Tau\textsuperscript{ASO-3} and total tau demonstrated a clear link between the presence of ASO and lack of total tau in the contralateral Frontal Cortex, Hippocampus, and Cerebellum (Figure 2.2) showing that infusing Tau\textsuperscript{ASO-3} using ICV pumps can effectively distribute the ASO throughout the brain and reduce tau protein.
The immunofluorescence results show a qualitative decrease in tau protein levels. To more precisely quantify the amount of tau mRNA and protein reduction in the CNS, we measured total tau mRNA and protein levels in multiple CNS regions 8 weeks after implantation of 1 month ICV pumps with 25µg/day Tau\textsuperscript{ASO-3} (Figure 2.3, A). In both the mRNA and protein analyses, total tau levels were decreased in the left and right brain hemispheres as well as the spinal cord, confirming the widespread reduction of tau expression in the adult mouse CNS (Figure 2.3, B-C).

**Tau Protein Levels are Reduced in the Brain Interstitial Fluid and Cerebrospinal Fluid**

In order to better understand the full effects of Tau\textsuperscript{ASO-3}, we examined tau protein levels in two additional CNS compartments: the brain interstitial fluid (ISF) and cerebrospinal fluid (CSF). Recently, reports have placed tau in the extracellular space under physiological conditions (Yamada et al., 2011; Pooler et al., 2013). To determine whether tau ASOs can decrease tau that is secreted into the ISF, we treated a cohort of mice with a 75µg/day concentration of Tau\textsuperscript{ASO-3} or saline for 1 month with ICV pumps. At the end of ASO infusion, catheters and pumps were removed and microdialysis probes implanted into the left hippocampus two weeks later. We collected ISF for 48 hours, with a new fraction being collected every 90 minutes (Figure 2.4, A). Immediately following ISF collection, the left hippocampus was dissected out and total tau levels measured to confirm that brain tau protein was indeed reduced in the Tau\textsuperscript{ASO-3} cohort (two-tailed t-test t(10)=8.462, p<0.0001) (Figure 2.4, B).

Total ISF tau protein levels were steady across time in both the saline and Tau\textsuperscript{ASO-3} groups and substantially reduced in the Tau\textsuperscript{ASO-3} treated cohort (ANOVA F\textsubscript{(1,36)}=48.22, p<0.0001) (Figure 2.4, C), allowing multiple fractions to be combined from the same mouse. These same 8
fractions were pooled for each animal and total tau protein levels were again measured. In the Tau\textsuperscript{ASO-3} treated group, ISF total tau protein levels were greatly reduced as compared to the saline control (two-tailed t-test t(8)=9.283, p=0.003). ISF lactate levels were not significantly different between saline and Tau\textsuperscript{ASO-3} groups (two-tailed t-test t(8)=0.6169, p=0.555) (Figure 2.4, D). No difference in ISF lactate levels, which usually increase in response to synaptic transmission, suggests that a reduction in endogenous tau does not influence baseline neuronal activity (Bero et al., 2011).

To examine the correlation between brain and cerebrospinal fluid (CSF) tau levels, we treated a cohort of mice with 25µg/day Tau\textsuperscript{ASO-3} or saline for 1 month. Half of the mice were collected 4 weeks post-pump implantation and the other 8 weeks after pump insertion. Immediately prior to collecting the brains, CSF was drawn from the cisterna magna of the mice, averaging 10µL CSF per mouse. As predicted, brain tau protein levels were decreased at both the first time point as well as the second (ANOVA $F_{(2,14)}=20.96$, $p<0.0001$) (Figure 2.5, A). Interestingly, the CSF total tau was not reduced to the same extent as brain tau at the 4 week collection time, though by the 8 week time point, CSF tau was lower in the Tau\textsuperscript{ASO-3} treated mice (ANOVA $F_{(2,24)}=9.720$, $p=0.0008$) (Figure 2.5, B). While the reason for this lag in CSF tau reduction is unknown, it may be in part due to a slow turn-over of intracellular tau protein to the cerebrospinal fluid pool, resulting in a continued decrease in CSF tau while brain tau levels begin to increase. Total brain and CSF tau protein levels were significantly correlated, both in the brain adjacent to the catheter (Linear Regression $R^2=0.867$, $F_{(1,6)}=39.11$, $p=0.0008$) (Figure 2.5, C) and the contralateral frontal cortex (Linear Regression $R^2=0.657$, $F_{(1,6)}=11.49$, $p=0.0147$) (Figure 2.5, D). These data, in addition to showing a reduction of extracellular tau, suggest that CSF tau levels may be an excellent predictor of brain tau levels in a Tau\textsuperscript{ASO-3} treatment paradigm.
Reducing Tau mRNA and protein does not alter Baseline Behavior

Before assessing whether tau reduction can provide protection in experimental behavioral paradigms, we first analyzed the mice for any gross motor or cognitive behavioral abnormalities. Tau knockout mice (tau$^{-/-}$) appear normal on learning/memory tasks for up to one year (Roberson et al., 2007, 2011; Dawson et al., 2010; Ittner et al., 2010) with some minor parkinsonism-motor phenotypes developing around 12 months of age (Lei et al., 2012; Morris et al., 2013). These largely normal behavior phenotypes, however, could be in part due to developmental compensation. We treated a cohort of NT mice with either saline, 25µg/day scrambled control ASO, or 25µg/day Tau$^{ASO-3}$ for 1 month and conducted behavioral assessments for 1.5 months after pump removal. Total tau mRNA (ANOVA $F_{(2,19)}=101.3$, $p<0.0001$) and protein (ANOVA $F_{(2,19)}=13.95$, $p=0.0002$) levels were confirmed to be reduced only in mice treated with Tau$^{ASO-3}$ (Figure 2.6, A-B). The mice with decreased tau levels performed similarly to both the saline and scrambled ASO control groups on all seven measures of the sensorimotor battery including the inverted screen test (ANOVA $F_{(2,19)}=0.116$, $p=0.891$) (Figure 2.6, C), suggesting that the Tau$^{ASO-3}$ mice did not have any gross sensorimotor dysfunctions. Except for a possible hyperactivity in the Tau$^{ASO-3}$ group, Tau$^{ASO-3}$ mice also displayed similar behavior during the 1-hour Locomotor Activity test. The Tau$^{ASO-3}$ mice did not exhibit any significant performance deficits on the place (rmANOVA $F_{(2,76)}=0.006$, $p=0.994$) or probe (rmANOVA $F_{(2,76)}<0.0001$, $p=1.000$) trials in the water maze (Figure 2.6, D-E), thus providing evidence that their spatial learning and memory were intact. Analysis of the Elevated plus maze (EPM) data also showed that the Tau$^{ASO-3}$ did not differ in levels of anxiety-related behaviors compared to the saline and scrambled ASO control groups (%open arm entries rmANOVA $F_{(2,38)}=0.284$, $p=0.756$; %open arm time rmANOVA $F_{(2,38)}=0.134$, $p=0.875$; %open arm distance rmANOVA $F_{(2,38)}=0.211$, $p=0.754$) (Figure 2.6, F-H). There was also no difference between groups in regards to the total distance traveled in the EPM (ANOVA $F_{(2,19)}=0.639$,
Recognizing that the sample size tested was relatively small, the data suggest that at least in the short term, reducing tau mRNA and protein in the adult mouse does not appear to result in behavioral impairments.

Reducing Tau mRNA and protein Protects Against Chemically Induced Seizures

To test whether tau reduction is protective in an induced focal seizure model, we used reverse microdialysis to focally deliver the non-competitive GABA_A receptor antagonist Picrotoxin (PTX) (Olsen, 2006) into the left hippocampus of NT male mice treated with saline, 25µg/day scrambled ASO, or 12-25µg/day Tau^{ASO-3} and simultaneously record the electroencephalography (EEG) activity at the site of PTX delivery. Since this treatment paradigm had not been previously used to study the protective effects of tau reduction, we included a cohort of untreated NT and tau^{-/-} mice to serve as controls. 12 hours of basal EEG activity were recorded followed by continuous infusion of PTX into the left hippocampus with stepwise increase in concentration every 90 minutes (4µM, 20µM, 100µM, and 500µM). Untreated NT and Saline treated mice were combined due to no significant difference between the groups. The tau^{-/-} mice showed a reduction in normalized spike frequency as compared to the NT/Saline group at the 500µM PTX concentration, confirming the protective effect in tau null mice in this new excitation paradigm (rmANOVA $F_{(3,72)}=8.634$, p=0.0009) (Figure 2.7, A-B). The Tau^{ASO-3} treated group compared to both the NT/Saline and Scrambled cohorts also showed a strong protective effect (Figure 2.7, A-B). Furthermore, total tau protein levels in the left hippocampus of pump treated mice were highly correlated with normalized spike frequency at 500µM PTX (Spearman correlation, $r(12)=0.670$, p=0.0087) (Figure 2.7, C). These PTX studies in adult mice support a direct correlation between lower tau protein levels and reduced neuronal hyperexcitability.
In addition to the focal increase in EEG activity, we tested the effects of tau reduction in a widely used seizure paradigm – Pentylenetetrazol (PTZ) intraperitoneal (i.p.) injections. PTZ seizures are considered a “gold standard” when testing the efficacy of anticonvulsant drugs in the early stages of development in vivo (Löschner, 2011). Three month old NT male mice were treated with saline, 25µg/day scrambled ASO, or 25µg/day TauASO-3 for 1 month and pumps then removed. Three weeks later, 55mg/kg of the GABA-antagonist PTZ (Macdonald and Barker, 1977) was administered to the mice by i.p. injection. The mice were videorecorded for 15 minutes and then immediately collected for total tau analyses (Figure 2.8, A). Any mouse that had higher than 50% total tau mRNA levels was eliminated from the analysis of the TauASO-3 group. Those mice treated with TauASO-3 had less severe seizures than both the saline and scrambled ASO control groups (Kruskal Wallis statistic=16.26, p=0.0003) (Figure 2.8, B). Total tau mRNA (ANOVA $F_{(2,62)}=281.5$, p<0.0001) and protein (ANOVA $F_{(2,62)}=45.73$, p<0.0001) levels were confirmed to be reduced specifically in the TauASO-3 treated group (Figure 2.8, C-D). As further confirmation that the effect of TauASO-3 on seizures was secondary to tau reduction and not an unknown effect of the ASO, we correlated the level of tau protein with seizure severity for individual animals. Indeed, seizure severity and tau protein level correlated well in all tested mice (Spearman Correlations, Saline r(24)=0.5889, p=0.0016; Scrambled r(19)=0.6795, p=0.0007; TauASO-3 r(18)=0.504, p=0.0236) (Figure 2.9, A-C), providing evidence in a second inducible seizure model that a reduction in tau protein is protective against seizures.

**Intrinsic Variability in Tau protein levels predicts Susceptibility to Chemically Induced Seizures**

Due to the variability that has been seen with PTZ seizures (Mandhane et al., 2007), we were surprised that the correlation between seizure severity and tau levels persisted even in NT mice treated with only saline (Figure 2.8, A). This correlation suggests that among the NT mouse
population, normal endogenous tau levels predict susceptibility to neuronal hyperexcitability. It may be, however, that a more severe seizure results in an acute increase in brain tau protein expression. To test this possibility, we induced severe seizures in a separate cohort of untreated NT mice using a high dose of PTZ and analyzed total tau protein levels immediately after. There was no difference in tau protein levels in brain homogenate between those mice that underwent severe seizures secondary to PTZ injection as compared to those mice that received a saline injection and did not have seizures (two-tailed t-test t(12)=0.354, p=0.730) (Figure 2.10, A).

Interestingly, in the PTZ injected group, there was an inverse correlation between the time it took to reach a Stage 8 seizure and the amount of endogenous tau protein measured. Those mice that had higher levels of endogenous tau protein progressed to severe seizures more quickly than those mice with lower tau (Spearman correlation r(7) = -0.887, p=0.003) (Figure 2.10, B).

These PTZ data show that the seizure itself does not acutely increase tau protein in brain tissues during the period of the seizure and, together with the TauASO-3 PTX and PTZ seizure data, strongly suggest that those mice with higher levels of endogenous tau are inherently more susceptible to neuronal hyperexcitability.
DISCUSSION

Using antisense oligonucleotide technology directed against endogenous murine tau, total tau mRNA and protein levels were decreased throughout the brain and spinal cord of adult NT mice (Figures 2.1-2.3). In addition extracellular tau in the brain interstitial fluid (Figure 2.4) and cerebrospinal fluid (Figure 2.5) was also reduced following infusion of Tau\textsuperscript{ASO-3} ASO. After tau was reduced in the adult mouse, no significant deviations from baseline were observed in a battery of motor and learning/memory behavior tasks (Figure 2.6), demonstrating that short-term tau knockdown is well tolerated \textit{in vivo}. In the setting of chemically induced seizures, tau reduction protected against seizure severity (Figure 2.7-2.9), on par with what has been reported with the genetic tau\textsuperscript{-/-} mouse model. Further, we noted a significant correlation between total tau protein levels in the brain and seizure severity, both in treated mice (Figure 2.7-2.9) and untreated (Figure 2.10). Combined, these data strengthen the link between total tau expression levels and neuronal hyperexcitability regulation \textit{in vivo} and demonstrate that the tau\textsuperscript{-/-} effect on neuronal hyperexcitability is likely a tau-mediated event and not a developmental phenomenon.

The tau\textsuperscript{-/-} genotype has been shown in numerous studies to be protective against excitotoxic insults (Roberson et al., 2007, 2011; Ittner et al., 2010), implicating tau in the physiological regulation of aberrant neuronal excitability. Additionally, both a complete reduction as well as haploinsufficiency of tau significantly reduced seizures and extended survival in a well-established genetic mouse model of epilepsy, Kv1.1\textsuperscript{-/-} (Glasscock et al., 2010, 2012; Holth et al., 2013). These reports, in conjunction with previous \textit{in vitro} data using tau knockdown ASOs to protect cells from glutamate induced excitotoxicity (Pizzi et al., 1993) and our own \textit{in vivo} tau knockdown data in two different seizure models, support the application of a tau lowering therapy to regulate hyperexcitability in human patients. Compounds that provide protection against PTZ seizures \textit{in vivo} have generally been successful in subsequent human clinical trials.
Though many epilepsy patients respond to one or two anticonvulsants, 20-40% of patients remain untreated (Devinsky, 1999; Brodie et al., 2012). Thus, a tau reduction approach may be an alternative therapy for this refractory population. Given the previous tau\(^{-/-}\) protective findings in multiple seizure paradigms (Roberson et al., 2007, 2011; Holth et al., 2013), we predict that our findings of tau knockdown using two different GABA-antagonists will also apply broadly to epilepsy \textit{in vivo} models and human epilepsy.

Of further interest, the finding that physiological endogenous tau levels in adult mice can affect susceptibility to hyperexcitability not only lends support to the idea that reducing tau may help lower seizure severity, but also implies that endogenous tau levels in humans may influence the risk of developing seizures. While the exact reason for variability in tau protein expression between mice is unknown, other groups have shown similar variability in murine total tau levels (Holth et al., 2013). Further, tau mRNA and protein levels in human brains can vary by greater than 2-fold (Lu et al., 2004; Kauwe et al., 2008; Trabzuni et al., 2012) and CSF total tau levels in control human subjects can differ substantially (Clifford et al., 2009; Fagan et al., 2009; Oka et al., 2013), perhaps due to variability in neuronal excretion rates of tau and different baseline tau levels in the brain. Higher levels of tau protein at baseline may not be detrimental, but upon insult, increased tau expression may predispose human patients to injury-induced seizures. It is well documented that the incidence of seizures increases following different types of brain injury, including ischemic stroke (Camilo and Goldstein, 2004; Kwan, 2010) and traumatic brain injury (Annegers et al., 1998; Vespa et al., 2010). If human patients with higher baseline tau are more prone to developing seizures following an injury, being able to identify such patients through genetic studies of tau polymorphisms (Myers et al., 2007; Kauwe et al., 2008) or tau CSF levels (Palmio et al., 2009; Cruchaga et al., 2013) may help to risk stratify those patients and aid in determining who would benefit from a preventive antiepileptic therapy.
Tau/− has also been studied extensively in the presence of Aβ-deposition and has proven to be protective against a growing number of Aβ-induced insults, including cognition (Roberson et al., 2007; Andrews-Zwilling et al., 2010; Ittner et al., 2010; Leroy et al., 2012), hyperexcitability (Roberson et al., 2007, 2011; Ittner et al., 2010; Suberbielle et al., 2013), decreased survival (Roberson et al., 2007, 2011; Ittner et al., 2010), axonal transport deficits (Vossel et al., 2010), cell-cycle re-entry (Seward et al., 2013), and double stranded breaks in DNA (Suberbielle et al., 2013). Several human Amyloid Precursor Protein (hAPP) mouse lines and an ApoE4 mouse line have now been generated that display abnormal EEG and increased seizure frequency (Roberson et al., 2007; Ittner et al., 2010; Vogt et al., 2011; Hunter et al., 2012). Similarly, those with familial AD mutations, ApoE4 genotype, and sporadic late-onset AD, also experience an increased incidence in seizures (Takao et al., 2001; Mendez and Lim, 2003; Harden, 2004; Velez-Pardo et al., 2004; Amatniek et al., 2006; Kauffman et al., 2010). This AD associated excitotoxicity has been implicated in the pathogenesis of the disease (Olney et al., 1997; Mattson, 2004).

Because of the limitations in detecting abnormal EEG activity in large populations of AD patients, we currently rely heavily on animal models for predictions regarding hyperexcitability in the context of Aβ. In the hAPP J20 Aβ-depositing line, treatment with the anticonvulsant Levetiracetam returned the baseline aberrant excitability back to NT levels and restored cognition (Sanchez et al., 2012), similar to what was seen with the tau/− genetic cross (Roberson et al., 2007, 2011). This rescue in cognition by means of an anticonvulsant suggests that lowering the abnormal neuronal activity alone may have a positive impact on learning and memory. A similar pilot study was performed in human Mild Cognitively Impaired (MCI) patients, whereby patients were given either placebo or Levetiracetam and then recall memory
was tested using functional magnetic resonance imaging methods. The Levetiracetam treatment significantly improved the recall performance of the MCI patients, again providing evidence that reducing the aberrant excitability in MCI and AD patients may help to subsequently restore cognition (Bakker et al., 2012). To test the hypothesis that decreasing aberrant hyperexcitability by means of tau reduction can in turn rescue cognitive decline, we have initiated tau lowering ASO therapy in an Aβ-depositing mouse model of AD. We propose that tau may be involved in both AD-associated hyperexcitability as well as neuronal cell loss by means of tau aggregation and neurofibrillary tangle formation, making tau knockdown a strong therapeutic target for AD.

The human analog of the mouse tau ASO used here may be readily applicable to human patients. ASOs against superoxide dismutase 1 (SOD1) that extended survival in a rat model of Amyotrophic Lateral Sclerosis (ALS) (Smith et al., 2006) recently finished a Phase I clinical trial in human ALS patients. The CSF delivered ASOs demonstrated excellent safety (Miller et al., 2013). Further, ASOs against spinal motor neuron (SMN) that also rescued rodent Spinal Muscular Atrophy (SMA) models (Hua et al., 2010; Passini et al., 2011; Porensky et al., 2012) are currently being used in Phase II studies for children with SMA. These studies and the growing success of ASOs in preclinical models (Kumar et al., 2000; Yokota et al., 2009; Lanford et al., 2010; DeVos and Miller, 2013b) suggest that the tau reduction strategy outlined here has real potential to be translated to the clinic for patients with epilepsy and perhaps tauopathies such as Alzheimer’s Disease, Progressive Supranuclear Palsy, and Frontotemporal Dementia.
Figure 2.1. Antisense Oligonucleotides Reduce Endogenous Tau mRNA and Protein.

A, Saline, Scrambled, or Tau ASOs were infused into the right hippocampus of Non-Transgenic (NT) mice (n=4-6 per group) and the area around the injection site was analyzed 1 week later for total tau mRNA levels. Tau ASO-3 was very potent at reducing total tau mRNA. One-way ANOVA, Bonferroni post hoc analysis.

B,C, Saline, Scrambled, or Tau ASO-3 was infused Intracerebroventricularly (ICV) into NT mice at 100µg/day for 1 month (n=4-7). The right parietal cortex was analyzed for total tau mRNA levels
(B) and the right ventral white matter was analyzed for total tau protein levels by ELISA (C). One-way ANOVA, Bonferroni post hoc analysis.

**D,** Saline or Tau^{ASO-3} was delivered via ICV infusion with increasing concentrations of Tau^{ASO-3} (n=3-13). After 1 month, total tau mRNA levels were measured. One-way ANOVA, Bonferroni post hoc analysis.

**E,** Saline or Tau^{ASO-3} was delivered via ICV infusion at 25µg/day for 1 month. Total tau mRNA levels were analyzed at 4, 8, 12, and 16 weeks after pump implantation (n=3-5). Two-way ANOVA, Bonferroni post hoc analysis.

*p<0.05, **p<0.01, ***p<0.001. Error bars represent SEM.
Figure 2.2. ASOs Distribute Throughout the entire Adult Mouse Brain.

A, Saline or Tau^{ASO-3} was delivered by ICV infusion at 25µg/day into Non-Transgenic (NT) mice for 1 month. In mice collected 8 weeks after pump implantation, brain tissue was co-stained with a total tau antibody (green), an ASO antibody (red), and counterstained with DAPI (blue). Three brain regions – hippocampus, frontal cortex, and cerebellum – on the contralateral side of the brain from the catheter (left hemisphere) were analyzed. Scale bars represent 500µm.
Figure 2.3. ASOs Reduce Tau mRNA and Protein Throughout the Mouse Central Nervous System.

A-C, Saline or Tau\textsuperscript{ASO-3} was delivered by ICV infusion at 25µg/day into Non-Transgenic (NT) mice for 1 month. In mice collected 8 weeks after pump implantation. Brains were dissected into regions as shown in (A) (n=4-6). Tau mRNA (B) and protein levels by ELISA (C) were measured in each region. Two-tailed t-test.

#p<0.1, *p<0.05, **p<0.01, ***p<0.001; Error bars represent SEM.
Figure 2.4. Total Tau Protein in the Brain Interstitial Fluid is Decreased.

A, Experimental Paradigm. Non-Transgenic (NT) mice (n=4-6) were treated with saline or 75µg/day TauASO-3 via ICV infusion for 1 month and the catheters then removed. After 14 days, a guide cannula and microdialysis probe were placed in the left hippocampus. Interstitial Fluid (ISF) was collected for 48 hours. Each ISF fraction comprised a 90 minute collection time.

B, Total brain tau protein levels in the left hippocampus were measured, confirming that brain tau was reduced. Two-tailed T-test.

C, Total tau protein levels were measured in several ISF fractions. Two-way ANOVA, Bonferroni post hoc analysis.
Fractions from 18-34 hours were pooled for each animal to measure total tau protein levels and Lactate as a control for probe function. The concentrations were calculated for the 1µL/min flow rate. There was no significant decrease in Lactate levels demonstrating adequate probe function. Two-tailed t-test.

*p<0.05, **p<0.01, ***p<0.001; Error bars represent SEM.
Figure 2.5. Cerebrospinal Fluid Tau Protein levels are Decreased and Correlate with Brain Tau Levels.

A, B, Non-Transgenic (NT) mice were treated with saline or 25µg/day Tau^{ASO-3} via ICV infusion for 1 month. At 4 weeks and 8 weeks post-pump implantation, total tau protein levels in the brain (n=4-7) (A) and CSF (n=8-10) (B) were measured. One-way ANOVA, Bonferroni post hoc analysis.

C, D, Total brain tau from the right (C) and left (D) sides of the brain were correlated with CSF tau levels for each mouse (n=8). The best-fit line and 95% confidence bands were generated using Linear Regression Analysis. 

#p<0.1, **p<0.01, ***p<0.001; Error bars represent SEM.
Figure 2.6. Tau Reduction does not alter Baseline Behavior in vivo.

A, B, Non-Transgenic (NT) mice (n=7-8) were treated with saline, 25µg/day Scrambled ASO, or 25µg/day Tau\textsuperscript{ASO-3} via ICV infusion for 1 month. Pumps were removed and mice sent for a battery of behavioral tasks, lasting a total of 1.5 months. Immediately following behavior, brain total tau mRNA (A) and protein (B) levels were measured. One-way ANOVA, Bonferroni post hoc analysis.

C, All three treatment groups performed similarly on the inverted screen task from the sensorimotor battery. One-way ANOVA, Bonferroni post hoc analysis.

D, E, No significant differences were observed among the three treatment groups with regard to performance on the place trials in the Morris water maze (D) or with regard to spatial bias for the target quadrant during the probe trial (E). Two-way repeated measures ANOVA (rmANOVA), Bonferroni post hoc analysis.

F, G, H The three treatment groups also performed similarly on the elevated plus maze in terms of open arm entry percentage (F), percent time spent in open arms (G), and open arm distance percentage (H), suggesting that tau reduction does not result in anxiety related behavioral performance deficits. Two-way rmANOVA, Bonferroni post hoc analysis.

**p<0.01, ***p<0.001; n=7-8; Error bars represent SEM.
Figure 2.7. Tau Reduction is Protective against Picrotoxin Induced Hyperexcitability in the Hippocampus

A, Increasing doses of Picrotoxin (PTX) were infused into the left hippocampus of Non-Transgenic (NT) male mice treated with Saline, 25µg/day Scrambled ASO, or 12-25µg/day TauASO-3, or NT and tau−/− male mice with no catheter. Representative EEG traces from both baseline and 500µm PTX are shown for NT/Saline, Scrambled ASO, Tau−/−, and TauASO-3 mice.
**B.** The spike frequency during the last hour of PTX infusion for each dose was calculated and normalized to baseline EEG. Untreated NT and Saline treated mice were combined due to no significant difference between the groups. n=4-8. Two-way rmANOVA, Bonferroni *post hoc* analysis.

**C.** For those mice treated with Saline, Scrambled, and Tau^ASO-3^ ICV pumps, total tau levels in the left hippocampus were plotted against normalized spike frequency at the 500µm PTX concentration (Spearman r = 0.670, p = 0.0087). n=14. The ‘best-fit line’ and 95% confidence bands were generated using Linear Regression Analysis.

***p<0.001; Error bars represent SEM.
Figure 2.8. Tau Reduction is Protective against Pentylenetetrazol (PTZ) Induced Global Seizures.

A, Experimental paradigm. Non-Transgenic (NT) male mice were treated with saline, 25µg/day Scrambled ASO, or 25µg/day Tau ASO-3 for 1 month. Pumps were removed and seizures induced 21 days later. Brains were immediately collected following the seizure.

B, 55mg/kg Pentylenetetrazol (PTZ) was given by intraperitoneal injection to Saline, Scrambled ASO, and Tau ASO-3 treated mice and final seizure stage was scored blinded. Kruskal Wallis, Dunns post hoc analysis.

C, D, Total tau mRNA (D) and protein (E) levels were confirmed to be down in only the Tau ASO-3 treated group. One-way ANOVA, Bonferroni post hoc analysis.

*p<0.05, **p<0.01, ***p<0.001; n=18-26; Error bars represent SEM.
Figure 2.9. Tau Protein levels Directly Correlate with Final PTZ Induced Seizure Stage.

A-C, Final seizure stage for each treated mouse plotted against the total tau protein levels.
(Saline: Spearman r = 0.589, p=0.002; Scrambled: Spearman r = 0.680, p=0.0007; Tau\textsuperscript{ASO-3}: Spearman r = 0.504, p=0.024). The 'best-fit line' and 95% confidence bands were generated using linear regression analysis.
**Figure 2.10. Total Tau Protein Inversely Correlates with Seizure Latency.**

**A**, A cohort of Non-Transgenic (NT) male mice (n=5-9) were injected intraperitoneally with either saline or 80mg/kg Pentylenetetrazol (PTZ). Mice were collected immediately after they reached a Stage 8 seizure and total tau protein levels in right ventral white matter were measured. Two-tailed T-test.

**B**, For each PTZ injected mouse, the time to reach a Stage 8 seizure was plotted versus total tau protein (Spearman r = -0.887, p = 0.003). The ‘best-fit line’ and 95% confidence bands were generated using linear regression analysis. Error bars represent SEM.
REFERENCES


Chapter 3

Human Tau Reduction Reverses Pathological Tau Deposition in a Mouse Model of Tauopathy
PREFACE

This chapter contains a manuscript in preparation:

Author contributions for the citation above:
ABSTRACT

The microtubule associated protein tau is implicated in the pathogenesis of a class of neurodegenerative disorders known as Tauopathies, of which includes Alzheimer's Disease (AD), some forms of Frontotemporal Dementia, and Progressive Supranuclear Palsy. While there are no known tau mutations in AD, numerous tau mutations in other primary Tauopathies directly result in intraneuronal accumulations of tau in the form of Neurofibrillary Tangles accompanied with cognitive decline and dementia. One therapy for these disorders may be to decrease total levels of the protein tau. To test the efficacy of a tau reducing therapy for disorders with tau inclusions, we identified antisense oligonucleotides (ASOs) that selectively decrease human tau expression throughout the entire mouse central nervous system in the TauP301S tauopathy mouse model. Total human tau mRNA levels are reduced by about 50% in all Tau ASO treated ages of P301S mice. We found that by reducing human tau to this level in younger P301S mice, hyperphosphorylated tau deposition was prevented. Even more striking was that by reducing human tau levels in aged P301S mice, pre-existing tau pathology was reversed as measured by AT8 and MC1, in addition to a rescue in hippocampal volume and CA1 hippocampal neuron quantification. Further, inclusions of the autophagic marker p62 were reversed in the aged P301S Tau ASO treated mice, suggesting that by lowering the total amount of human tau expression to even just 50%, neurons are capable of clearing the accumulated p62 inclusions and AT8 positive tau species. Together, these data strongly support the use of a tau lowering therapy for those human patients who have tau positive neuronal inclusions, even after pathological tau species have already started to deposit in the brain.
INTRODUCTION

Numerous neurodegenerative diseases arise from the misfolding of specific proteins, such as huntingtin in Huntington’s disease, alpha-synuclein in Parkinson’s disease, and tau in primary Tauopathies, including Alzheimer's disease (AD). We are focused on the protein tau, a microtubule associated protein that is capable of binding to and stabilizing microtubules under normal physiologic conditions in the neuron, as well as aggregating and forming toxic oligomeric species and intraneuronal neurofibrillary tangles (NFTs) in pathogenic conditions. In the pathogenesis of AD, one of the early histopathologic markers that develops is the presence of NFTs in the entorhinal cortex. These pathogenic accumulations of tau appear to traverse through the brain along connected pathways to the hippocampus and ultimately the neocortex (Hyman et al, 1984; Braak and Braak, 1991; Delacourte et al, 1999). It is damage to these neuronal connections and pathways that is hypothesized to underlie memory loss in AD patients and act as one of the first steps towards an irreversible series of events that ultimately culminates in widespread neuronal loss and dementia (Hyman et al, 1984; Gomez-Isla et al, 1996).

Several methods are currently being tested in an attempt to rescue those deficits induced by the misfolding and accumulation of tau in vivo. Tau aggregation inhibitors (Hosokawa et al, 2012; Schirmer et al, 2011), kinase inhibitors (Rankin et al, 2007; Sereno et al, 2009) microtubule stabilizers (Brunden et al, 2010; Zhang et al, 2012) , and antibodies (Yamanandra et al, 2013; Castillo-Carranza et al, 2014; Bi et al, 2011) are all being explored. We propose to directly target tau expression at the level of mRNA using antisense oligonucleotides (ASOs) to decrease total tau protein levels in the brain. Several groups have used repressible tauopathy mouse models to test the effects of reducing the human tau transgene on cognition, neuron and synapse loss, and tau accumulations. Significant improvements in memory, long term potentiation, synapse loss,
and NFTs were all found to possible when tau levels were suppressed genetically (SantaCruz et al, 2005; Sydow et al, 2011; Polydoro et al, 2013). Surprisingly, pre-existing tau inclusions and tangles could even be reversed with a long enough reduction in human tau levels (Sydow et al, 2011; Polydoro et al, 2013). Lowering total tau levels by means of ASO technology is conceptually very similar to these genetic repression studies, though carries with it the possibility for direct human clinical translation.

We have previously demonstrated that ASOs targeting endogenous murine tau can in fact reduce total tau protein levels throughout the adult mouse brain with no detrimental effects on baseline motor of cognitive behavior (DeVos et al, 2013). The ASO is capable of effectively distributing through all tissues in the adult mouse central nervous system, regardless of sequence, following a one month infusion. Importantly, the ASO is also capable of reducing total tau mRNA and protein levels in all regions of the brain (DeVos et al, 2013). This is an important point to make, that we are not reducing tau just around the infusion catheter, but instead throughout the entire central nervous system. These widespread data demonstrate the feasibility of delivering a Tau ASO to the central nervous system (CNS) as well as that reducing endogenous tau appears to be safe in vivo. Tau knockout mice are surprisingly normal, with a minor parkinsonism phenotype developing in late life (Roberson et al, 2007; Morris et al, 2013; Lei et al, 2012; Li et al, 2014), though because of the genetic nature of the tau reduction, developmental compensation could not be ruled out (Harada et al, 1994). Demonstrating that tau reduction via ASOs in adult mice still shows no behavioral or neuroanatomical abnormalities from baseline lends support for the use of a tau lowering therapy in human patients.

In our attempt to study the therapeutic effects of human tau reduction, we have developed a method for human tau reduction using ASOs that works well in vivo. With this tool, we
ultimately sought to test the idea that tau pathology can be reversed using exogenously applied ASOs, a method that has the potential to be directly applied to people. Using ASOs in the context of neurodegeneration is not a novel concept. Similar ASO knockdown approaches have been successful at rescuing disease phenotypes in a rat model of SOD1-related ALS (Smith et al, 2006) as well as in mouse models of Huntington’s Disease (Kordasiewicz et al, 2012). Further, using ASOs in cell lines of ALS/FTD C9ORF72 patients rescued the toxic RNA foci phenotype (Lagier-Tourenne et al, 2013; Donnelly et al, 2013). Excitingly, administering SOD1 ASOs into the CNS of human ALS patients via the CSF appears to be very well tolerated (Miller et al, 2013), supporting that the ASO mediated tau reduction strategy described here may be applied to a human clinical trial in the near future.
MATERIAL AND METHODS

Animals. The P301S (PS19) mice used in this study were created using a 1N4R tau cDNA construct with a Tau\(^{P301S}\) mutation and express human tau 5-fold over endogenous mouse tau levels (Yoshiyama et al, 2007). Mice were maintained on a B6C3 background. All non-transgenic mice used were littermates of P301S mice. All studies were performed using gender-balanced groups. Mice had access to food and water ad libitum and were housed on a 12 hour light:dark cycle. Experiments involving animals were approved by the Animal Studies Committee at Washington University in St. Louis.

Antisense Oligonucleotides: The ASOs have the following modifications: five nucleotides on the 5’- and 3’-termini containing 2’-O-methoxyethyl modifications and 10 unmodified central oligodeoxynucleotides (DeVos and Miller, 2013b) to support RNaseH activity and a phosphorothioate backbone to improve nuclease resistance and promote cellular uptake (Bennett and Swayze, 2010). ASOs were synthesized as previously described (McKay et al., 1999; Cheruvallath et al., 2003) and solubilized in 0.9% sterile saline immediately prior to use.

Surgical placement of Intracerebroventricular (ICV) Pumps and Tissue Collection: As previously described, (Smith et al., 2006; DeVos and Miller, 2013a) mice were anesthetized with isoflurane and the 28 day osmotic pumps (ALZET) with ASO were implanted in a subcutaneous pocket that was formed on the back of the mouse. The catheter was placed in the right lateral ventricle using the following coordinates based on bregma: -0.5mm Posterior, -1.1mm Lateral (right), -2.5mm Ventral. For CSF collection, mice were placed on a heating pad and anesthetized with isoflurane. CSF was drawn through the cisterna magna as previously reported and immediately frozen on dry ice (Barten et al., 2011). For tissue collection, mice were anesthetized with isoflurane and perfused using chilled PBS-heparin. Brain was rapidly removed.
and either snap frozen in liquid nitrogen and stored at -80°C or post-fixed in 4% paraformaldehyde at 4°C and transferred to 30% sucrose 24 hours later. All animal protocols were approved by the Washington University in St. Louis Institutional Animal Care and Use Committee.

**Quantitative Real-Time PCR:** RNA analyses were performed using quantitative real-time RT-PCR. Total RNA was extracted from brain tissue using a QIAGEN RNeasy Kit (QIAGEN). For total tau analyses, RNA was reverse transcribed and amplified using the EXPRESS One-Step Superscript qRT-PCR Universal Kit (Invitrogen). The qRT-PCRs were run and analyzed on the ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems). Total human and mouse tau mRNA expression levels were normalized to mouse GAPDH mRNA levels and analyzed using the ΔΔCt method for relative expression analysis. Primer/Probe sequences: Human Total Tau: Forward 5’- AGA AGC AGG CAT TGG AGA C-3’; Reverse 5’- TCT TCG TTT TAC CAT CAG CC -3’; Probe 5’- /56-FAM/ACG GGA CTG GAA GCG ATG ACA AAA/3IABkFQ/ - 3’, Mouse Total Tau: Forward 5’- GAA CCA CCA AAA TCC GGA GA -3’; Reverse 5’- CTC TTA CTA GCT GAT GGT GAC -3’; Probe 5’- /56-FAM/CCA AGA AGG TGG CAG TGG TCC/3IABkFQ/ - 3’, GAPDH: Forward 5’ – TGC CCC CAT GT TGT GAT G 3’; Reverse 3’ – TGT GGT CAT GAG CCC TTC C – 3’; Probe 5’/56-FAM/ AAT GCA TCC TGC ACC ACC AAC TGC TT /3AHBkFQ/ 3’ (IDT).

**Tau Protein Analysis:** Tissues were weighed and homogenized in 5X volume RAB buffer [100mM MES, 1mM EDTA, 0.5mM MgSO4, 750mM NaCl, 20mM NaF, 1mM Na3VO4, supplemented with protease inhibitor (Complete, Roche) and phosphatase inhibitor (Sigma)]. Homogenate was spun at 21,000xg on a tabletop centrifuge for 10 minutes at 4°C. Supernatant was collected and protein concentration measured using Pierce BCA Protein Assay Kit (Thermo Scientific). Total Human Tau protein levels were measured on Western Blot by loading 15ug
protein per lane and running on SDS-PAGE. Proteins were transferred to PVDF membrane and blocked for 1 hour RT in 5% Non-fat Milk 1X PBS 0.05% Tween. Membranes were incubated with HT7 (Thermo Scientific) at 1:1000 in blocking buffer overnight at 4°C. Blots were incubated with anti-mouse HRP-secondary for 1 hour at room temperature and developed using ECL Plus (Pierce).

**Immunohistochemistry:** Brains post-fixed in 4% paraformaldehyde were sliced at 50µm on a freezing microtome. Slices were treated with 0.3% (for AT8) or 3% (for MC1) hydrogen peroxide for 10 minutes and then blocked with 3% Non-fat dried milk 1X TBS 0.25% Triton for 30 minutes at room temperature. Slices were then incubated overnight at 4°C with either 1:500 AT8-B (Thermo Scientific) that recognizes tau phosphorylated at ser202 and thr205 (Goedert et al, 1995) or 1:1000 MC1 that only recognizes a misfolded conformation of tau (Jicha et al, 1997) (gift from Peter Davies). For MC1, sections were incubated the next day with 1:2000 anti-mouse IgG1 biotin secondary for 1 hour at room temperature in 20% Superblock 1X TBS 0.1% Triton. For both AT8 and MC1, sections were then incubated with 1:400 Vectastain ABC Elite (Vector Labs) for 2 hours at room temperature. Slices were developed using DAB kit (Vector Labs) with the Nickel addition. DAB was diluted 1:1 in distilled water before applying to. Brightfield images were taken using the Olympus Nanozoomer 2.0-HT (Hamamatsu) and processed using the NDP viewer software (Hamamatsu).

**Tau Pathology Quantification:** All pathology quantification was carried out blinded. For AT8 and MC1 pathology staining, 4 brain sections 300µm apart were used from each mouse. The sections were used to assess the percent area covered by AT8 hyperphosphorylated tau staining. All images used were exported using the NDP viewer software and then quantified in ImageJ as follows. In ImageJ, all images were converted to greyscale and then uniformly
thresholded to quantify the percent area positively stained. The average of all four sections was used as the final percent stained value for that mouse.

**Hippocampal Volume Analysis:** All hippocampal volume analyses were carried out blinded. Starting at the very beginning of the hippocampus, Bregma -1.0mm, 8 serial sections were taken at 300µm apart. Sections were mounted and stained with Crystal Violet before imaging on the Olympus Nanozoomer 2.0-HT (Hamamatsu). Using the NDP viewer software, the hippocampus in each section was outlined to obtain the total hippocampal area. Each of the hippocampal areas was then multiplied by 300µm to obtain a final hippocampal volume in mm³ for each mouse.

**Immunofluorescence:** Brains post-fixed in 4% paraformaldehyde were sliced at 50µm on a freezing microtome. Brains were incubated with the primary antibodies Pan-ASO (1:2000, Isis), AT8 (1:500, Thermo Scientific), NeuN (1:100, Abcam), or p62 (1:500, Abcam) in 3% Horse Serum 1X TBS 0.1% Triton overnight at 4°C, followed by a 1 hour incubation at room temperature with fluorescent-conjugated secondary antibodies (1:400 anti-mouse DyLight 488, 1:400 anti-rabbit DyLight 550, ThermoScientific). All sections were counterstained with DAPI for 5 minutes immediately following secondary incubation. Fluorescent images were captured using the Olympus Nanozoomer 2.0-HT (Hamamatsu) and processed using the NDP viewer software (Hamamatsu).

**NeuN Mean Fluorescence Intensity Analysis:** In ImageJ, a rectangle was placed over the CA1 region. The same size rectangle was used for each section to give the same area. Using the measurement tool, the area, mean fluorescence intensity (MFI) and integrated density (IntDen) were measured. Another area near to CA1 was also selected for background. The background
was subtracted out using the formula: Corrected MFI\textsubscript{CA1} = (\text{IntDen}\textsubscript{CA1} - (\text{Area}\textsubscript{CA1} \times \text{MFI}\textsubscript{background})) / \text{Area}\textsubscript{CA1}.

**Statistics:** The data were analyzed for statistical significance using the graphing program GraphPad Prism 5. Two-tailed Student t-tests were used for total tau mRNA/protein and AT8 pathology quantification when only one comparison was being made. One-way ANOVA with Bonferroni *post hoc* analyses were used for total tau mRNA expression when more than one comparison was needed, as well as hippocampal volume and NeuN MFI analysis. Two-way ANOVA with Bonferroni *post hoc* analyses were used for the duration of action study. Error bars represent the SEM.
RESULTS

Human Tau Antisense Oligonucleotides Selectively Reduce Transgenic Human Tau mRNA in vivo

In order to test the effect of a tau lowering therapy on tau accumulations and neuronal loss in vivo, we screened 100 ASOs for their ability to reduce human tau mRNA in vitro (data not shown) and selected 4 of the most potent ASOs for addition in vivo screening. ASOs were designed to be human specific. To test in vivo tau mRNA reduction, we slowly infused 50µg of each of the human Tau ASOs or saline into the right hippocampus of adult P301S mice. After one week, we collected the hippocampal tissue that surrounded the injection site and analyzed total human and mouse tau levels. None of the ASOs reduced mouse tau levels, confirming the specificity for human tau. However, unlike what we have previously seen with endogenous mouse tau ASOs (DeVos et al, 2013), the human tau ASOs could only achieve a max of 40% reduction in human tau mRNA levels as seen with TauASO-12 (data not shown). We thought one week may not be enough time to reduce the human tau levels since the P301S line overexpresses the human tau cDNA transgene by 5-fold. To test a longer treatment paradigm, we delivered 30µg/day of the ASOs intracerebroventricularly (ICV) into the right lateral ventricle of P301S mice for 1 month using Alzet osmotic pumps. Immediately following the one month of active ASO infusion, we collected the brain tissue for human and mouse tau mRNA analysis (Figure 3.1, A,B). When compared to the saline control, only those P301S mice treated with TauASO-12 had significantly reduced human tau mRNA levels. While TauASO-12 appears selective for human tau, we BLASTED the ASO sequence against the entire mouse genome. There were no known genes that had less than 6 base pair mismatches, reinforcing the specificity for human tau.

With the starting 30µg/day dose of TauASO-12, we were able to achieve 50-60% human tau mRNA reduction. For the purposes of these studies, we didn’t want to use a lower ASO dose than
30µg/day since we were unsure what level of tau reduction we would need to obtain in order to see a phenotypic change in tau pathology. In preparation for longer treatment studies, we also tested the duration of action of a one month Tau\textsuperscript{ASO-12} infusion at 30µg/day. After infusion of either saline or Tau\textsuperscript{ASO-12} for 1 month, total human and mouse tau levels were measured in cohorts of P301S mice at 4, 8, and 12 weeks post-pump implantation. Human tau mRNA levels remained significantly reduced at all three time points (see Figure 3.1, C) while mouse tau mRNA levels dipped slightly at the first collection point, but then quickly rose back to saline levels (see Figure 3.1, D). The long range reduction of the target mRNA that is seen here is in accordance with what other groups have seen when using similar RNase-H ASOs (Kordasiewicz et al, 2012; DeVos et al, 2013), allowing a one-month infusion of ASO to provide at least 12 weeks of human tau mRNA reduction.

**Human Tau ASOs Effectively Distribute Throughout the Adult Mouse Brain**

Because we are slowly infusing the ASO into the cerebrospinal fluid (CSF) via the right lateral ventricle, it is important to demonstrate that the ASO can in fact penetrate into the deeper brain parenchyma and does not just line the ventricles. In order to determine the location of the ASO in the brain, we used an antibody that has been shown to recognize the backbone chemistry of the ASO, regardless of sequence (Kordasiewicz et al, 2012; DeVos et al, 2013). After 1 month of either saline or ASO infusion at 30µg/day followed by an 8 week wash-out period, the hemisphere contralateral to the infusion catheter (left hemisphere) was fixed and sections throughout the entire brain were taken at 600µm apart and stained for the presence of ASO (Figure 3.2, A,B). As can be clearly seen, the ASO is able to not only distribute throughout the entire brain, but also persist 8 weeks after active ASO infusion.
Two Month Reduction of Human Tau mRNA and Protein is Capable of Decreasing Hyperphosphorylated Tau Deposition

Thus far we have been able to demonstrate a selective reduction of human tau mRNA levels in the P301S adult brain as well as widespread distribution of the ASO following infusion into the lateral ventricle. Next, we sought to determine if we were able to reduce total tau protein levels, as well as look at the effects of a shorter 2 month reduction on tau histopathology. We infused either saline or 30µg/day of TauASO-12 into 5 month old P301S mice for 1 month, followed by a one month wash-out period. At 7 months, we collected the right half of the brain for mRNA and protein analysis and post-fixed the left half for immunohistochemistry. Total tau analyses once again showed a selective reduction in total human tau mRNA levels while mouse tau remained unchanged (Figure 3.3, A-B). Running the lysates on Western Blot revealed a visible reduction in total human tau protein levels in the TauASO-12 group as compared to the saline group, confirming that human tau mRNA knockdown does in fact lead to human tau protein reduction (Figure 3.3, C).

Once the human tau mRNA and protein levels were confirmed as being reduced in the TauASO-12 treated cohort, we stained the contralateral brain hemispheres using the hyperphosphorylated tau antibody that recognizes the tau phospho-epitopes ser202 and thr205 and is often used to stain for tau accumulations in human AD brain (Goedert et al, 1995). In staining the P301S brains with AT8, cell body positivity can be seen throughout the brain, though there appears to be a decrease in total AT8 staining in the TauASO-12 P301S mice (Figure 3.3, D). Previous reports using the P301S line have focused on the CA1 of the hippocampus, Entorhinal Cortex, and Amygdala as particularly affected brain regions (Yamanandra et al, 2013). When quantified, there is a significant decrease in AT8 positivity in the Entorhinal Cortex and Amygdala as well as a trend in the CA1 region of the hippocampus. These results were encouraging since we were unsure if 1)
a 50% reduction in human tau mRNA levels was going to be enough to see a change in tau pathology and 2) if 2 months was going to be enough time for tau protein levels to decrease and have an impact on pathological tau deposition. Because the P301S has been reported to develop pathology around the age of 4-6 months, we couldn’t be sure if we were preventing additional tau from being deposited or if we were at all reversing pre-existing pathology. To begin to answer that question, we would need to treat additional P301S mice at younger and older ages.

**Lowing Human Tau mRNA levels Reduces Hyperphosphorylated Tau Inclusions in Multiple Ages of P301S mice**

With the idea in mind to treat different ages of P301S mice, we first settled on treating two different groups of mice: 1) a young group starting at 3 months of age and 2) a middle group starting at 6 months. Additionally, instead of treating the mice for only 2 months, we lengthened the total time to 3 months, keeping the ASO dose and active infusion time the same (Figure 3.4, A). This would allow us to directly compare our younger group with the middle aged group in regards to a treatment start point, i.e. at the end of the treatment timeline, the 3-6 month cohort (pumps implanted at 3 months of age and mice collected at 6 months) can serve as a starting treatment point for the 6-9 month cohort (pumps implanted at 6 months of age and collected at 9 months). Again, the right hemisphere was collected for biochemical purposes and the left hemisphere was collected for histology. Human tau mRNA was significantly decreased in both the 3-6 month Cohort (Figure 3.4, B) as well as the 6-9 month Cohort (Figure 3.4, D), while mouse tau levels were identical in all NT and P301S treatment groups (Figure 3.4, C, E).

As in the previous treatment group (Figure 3.3), brains were stained with the hyperphosphorylated tau antibody AT8. In both the 3-6 month and 6-9 month cohorts, we observed a marked decrease in AT8 staining in the hippocampus, amygdala, and entorhinal
cortex. Reducing human tau with Tau\textsuperscript{ASO-12} is extremely effective at decreasing the number of AT8 positive inclusions throughout the brain. Again, with the young 3-6 month cohort, being able to differentiate between a prevention of pathology accumulation and a reversal in deposition is impossible. The 6-9 month group also has substantially less AT8 positivity in the Tau\textsuperscript{ASO-12} treated group as compared to the scrambled control. Because these mice are older at the start of treatment and there does appear to be AT8 pathology at the 6 month starting time point, reducing human tau may be capable of reversing pre-existing AT8 positive inclusions. However, to be absolute certain that simply reducing total human tau levels by \textasciitilde50\% can reverse pathological tau species \textit{in vivo}, we treated an even older cohort of mice that has substantial amounts of tau pathology at the start of treatment.

**Human Tau ASO Treatment is Capable of Reversing Hyperphosphorylated and Conformation Specific Tau Species in Aged P301S brains**

To better study the possibility of reversing tau pathology, we turned to P301S mice starting at 9 months of age. As with the 3-6 month and 6-9 month cohorts, we treated 9 month old P301S mice with 30µg/day Scrambled or Tau\textsuperscript{ASO-12} for 1 month, followed by a 2 month washout period. The left hemisphere was post-fixed and stained for both hyperphosphorylated AT8 pathology, as well as the misfolded tau conformation specific antibody MC1. As expected, 12 month non-transgenic littermate controls showed no AT8 or MC1 specific staining (Figure 3.5, A-B). P301S mice at 9 months of age show both AT8 and MC1 tau pathology, allowing us to directly compare the 9-12 month treated mice to this 9 month “starting point” in an effort to determine if accumulating tau pathology is halted or if pre-existing accumulations are also allowed to clear. Surprisingly, with both the AT8 and MC1 antibodies, those 12 month P301S mice treated with Tau\textsuperscript{ASO-12} had visibly less cell body staining than both the age-matched 12 month P301S Scrambled mice, as well as the 9 month P301S starting group. This strongly suggests that when
total human tau levels are reduced, pre-existing neuronal accumulations of the protein tau can remarkably be cleared from neurons in vivo. When the ASO was co-localized with AT8 staining, there was very little co-localization in the Tau\textsuperscript{ASO-12} treated P301S mice, unlike the Scrambled control treated P301S cohort (Figure 3.6, C,D). Because total human tau mRNA levels can only be reduced by ~50%, this strong dissociation between ASO and AT8 tau pathology suggests that human tau levels don’t need to be completely reduced in order to abrogate pathogenic tau accumulations.

**GFAP expression is greatly reduced following Human Tau Reduction**

Inflammation has been implicated in the pathogenesis of the Alzheimer’s disease (Lee et al, 2010; Frank-Cannon et al, 2009). The P301S mouse model develops an age-dependent increase in the activation of astrocytes, evident by an increase in glial fibrillary acidic protein (GFAP) levels (Yoshiyama et al, 2007; Saul et al, 2013). To determine the effect of reducing human tau levels on the this inflammatory marker, we stained 9 month, 12 month untreated, and 12 month Tau\textsuperscript{ASO-12} P301S mice for the presence of GFAP (Figure 3.7 A,B). We chose to use untreated P301S mice at 12 months for an age-matched comparison to ensure there was no confounding astrogliosis induced by the ASO backbone chemistry in the scrambled treated 12 month P301S mice. Levels of GFAP were substantially reduced in the P301S Tau\textsuperscript{ASO-12} mice as compared to a 12 month untreated P201S mouse. It is less obvious if we are reversing GFAP expression when compared to the 9 month P301S brains. A more detailed analysis looking at GFAP mRNA or protein levels would better answer whether Tau\textsuperscript{ASO-12} treatment is capable of reversing astrogliosis.
**Hippocampal Volume and Neuronal Loss in aged P301S mice is Fully Restored to Non-Transgenic levels when Human Tau is Reduced**

In addition to an age-dependent increase in GFAP astrogliosis, P301S mice have been shown by several groups to experience a decrease in total hippocampal volume and neuron loss in the hippocampus (Yosiyama et al, 2007; Zhang et al, 2012; Stancu et al, 2014). This loss is first evident at 9 months of age and progresses as the mice age to 12 months. In the genetic suppression Tauopathy models, neuronal stabilization was reported after total tau levels were reduced (Sydow et al, 2011; Polydoro et al, 2013) though never reversed. We also wanted to access whether lowering tau with an ASO could prevent further hippocampal volume and neuron loss. To look at the hippocampal volume, we measured the area of the hippocampus in 8 serial sections, 300µm apart and used those values to calculate the total volume. When non-transgenic (NT) mice at 9 months and 12 months were compared, there was no significant difference in hippocampal volume between the two (data not shown) so they were combined. Further, in our hands, there is a significant decrease in hippocampal volume in P301S mice at 12 months of age when compared to NT mice. At 9 months, there is a trend towards a decreased volume, though it does not reach statistical significance. When we measured the volume of the hippocampi in the 12 month TauASO-12 treated P301S mice, we observed a significant recovery in hippocampal volume as compared to the 12mo P301S mice (Figure 3.8, A-B). In fact, the hippocampi in the TauASO-12 treated cohort reached the same volume as their NonTg littermates.

This surprisingly full recovery in hippocampal volume prompted us to look at the neuronal density in the hippocampus. Using NeuN as a marker for neurons (Wolf et al, 1996), we stained the same 9-12 month cohort of P301S brains (Figure 3.9). NeuN nicely labeled all neurons within the hippocampus and co-labeled well with the nuclear marker DAPI (Figure 3.9, A).
looking at the hippocampi from the different P301S treatment groups, we noticed a qualitative decrease in total hippocampal NeuN staining in the 12mo P301S Scrambled treated mice that appeared to be completely rescued by human tau reduction (Figure 3.9, B). We co-localized the NeuN stain with DAPI to make sure that NeuN wasn’t in some way regulated by tau such that when total tau levels were reduced, NeuN expression increased without actual neuron recovery. NeuN and DAPI co-localized in all treatment groups (Figure 3.9, B). To get a more quantitative look at this possible rescue in neuronal loss, we quantified the NeuN mean fluorescence in the CA1 region of the hippocampus in all treated mice (Figure 3.9, C). There is a significant reduction in NeuN levels in the 12mo P301S mice compared to NT littermates with a reduction trend in 9 month P301S mice. In those 12 month P301S mice that were treated with Tau\textsuperscript{ASO-12}, there is a complete rescue in CA1 NeuN staining, back to the level of NTs. Because there is not a significant decrease in hippocampal volume or NeuN loss in the 9 month P301S, we cannot yet surmise whether the Tau\textsuperscript{ASO-12} treatment is capable of reversing pre-existing neuronal loss.

**Inclusions of the autophagic marker, p62, are reversed in Human Tau ASO treated P301S mice**

In this P301S tauopathy line, no one has yet to investigate whether the autophagy system is activated or in some way impaired. As a very first attempt, we looked at the autophagy marker p62. The protein p62 serves as a receptor in the autophagy pathway, due to its ability to bind to both ubiquitin and microtubule-associated protein 1A/1B-light chain 3 (LC3) (Pankiv et al, 2007; Lamark et al, 2009). The presence of p62 is thought to play a role in the degeneration of misfolded and aggregated protein inclusions (Bjorkoy et al, 2006, Watanabe and Tanaka, 2011) and has even been found to associate with neurofibrillary tangles found in the brains of human tauopathy patients (Kuusisto et al, 2001, 2002; Zatloukal et al, 2002).
To first determine if p62 can be detected in the P301S mice we were using, we stained aged P301S and NT mice at 12 months for p62. In our preliminary stain, the NT brain showed very low diffuse p62 staining throughout the brain, while the 12mo P301S brain revealed strong neuronal inclusions, primarily in regions affected by AT8 pathology (data not shown). These brightly labeled inclusions sparked our interest since p62 is often found co-localized with tau accumulations in human AD brain. Further, we found a report in a separate P301S Tauopathy mouse model that also revealed inclusions of p62 in their model that co-localized with hyperphosphorylated tau inclusions (Schaeffer et al, 2012). In light of this, we stained all of the brains in the 9-12month P301S cohort for p62 and again, found low level general staining in NT mice and strong intraneuronal p62 inclusions in P301s brains (Figure 3.10, A,B). Inclusions of p62 could be found in both 9 month and 12 month P301S Scrambled treated mice, perhaps not surprisingly since p62 appears to co-localize strongly with AT8 tau pathology (Figure 3.10, C). In the Tau^ASO-12 human tau reduction mice, the number of p62 inclusions was drastically reduced, returning almost to NT baseline levels (Figure 3.10, B). This strong reduction in p62 inclusions has previously been shown when the autophagy system was stimulated using the drug trehalose (Schaeffer et al, 2012). A reduction in p62 inclusions suggests that p62 itself may bind directly to tau accumulations before they are degraded by means of autophagy, such that when AT8 pathology decreases, so do p62 inclusions. While there is a clear reversal in p62 accumulations in the Tau^ASO-12 treatment paradigm, whether that is due to a re-activation of the autophagy pathway or some other clearance mechanism will need to be further teased apart.
DISCUSSION

In using antisense oligonucleotides (ASOs) specifically directed against human tau in the P301S Tauopathy mouse model, total human tau mRNA and human tau protein levels were decreased (Figures 3.1, 3.3). Mouse tau mRNA were not changed. Importantly, the ASO was able to diffuse throughout the entire adult P301S brain when delivered intraventricularly (Figure 3.2). After human tau levels were reduced for 2-3 months, we saw a marked decrease in hyperphosphorylated AT8 tau pathology in multiple regions of the brain (Figure 3.3, 3.4). However, it was unclear whether reducing human tau was preventing additional tau accumulations from forming or if it was capable of reversing pre-existing tau inclusions. To address this, aged P301S mice were treated after significant tau pathology had already developed. In this progressed stage, human tau reduction was capable of reversing AT8 hyperphosphorylated tau and MC1 conformation specific tau inclusions (Figure 3.5) as well as substantially reduce astrogliosis (Figure 3.7), hippocampal and neuronal loss (Figure 3.8, 3.9) and even reverse autophagy marker p62 inclusions (Figure 3.10). Combined, these data strengthen the therapeutic potential of a human tau lowering therapy in vivo and support the use of a tau lowering therapy for those human patients with a primary Tauopathy.

In previous studies, human tau has only been reduced by genetic means in suppressible mouse models of Tauopathy. Though genetic in nature, these studies have greatly informed the field as to the possible benefits from a tau lowering therapy. Cognition has been shown to improve, long-term potentiation deficits reversed, neuronal loss stabilized, synapses able to reform, and hyperphosphorylated tau inclusions and neurofibrillary tangles able to be reversed, both at the site of reduction as well as synaptically connected regions, all due to a reduction in total human tau levels by inhibiting the production of newly synthesized tau (SantaCruz et al, 2005; Sydow et al, 2011; Polydoro et al, 2013). Similar to these studies, when we reduce human tau levels in
adult P301S tauopathy mice using an exogenously applied human tau ASO, we see a striking reversal in tau inclusions, both AT8 hyperphosphorylation and MC1 conformation specific, in addition to a reduction in astrogliosis, hippocampal volume and neuronal loss, and even a reversal in autophagy marker p62 inclusions. The level of recovery that we see in aged Tauopathy mice with our ASO is similar to the previous genetic reports, lending even more support for the use of a tau lowering therapy. However, unlike the genetic studies, our study supports the direct translation of the ASOs into a human therapy.

An important aspect of decreasing total levels of tau in human patients is to determine the safety of tau reduction in vivo. While reducing human tau in the P301S line provides us with an excellent model to study the effect on pathological tau inclusions and the effects on neuronal integrity, it is not the best system to study safety in reducing total tau levels. Studying the effects of endogenous mouse tau reduction may be more parallel to tau reduction in people. The mouse tau knockout line (mTau−/−) has been studied extensively both within and outside the presence of Aβ-deposition. Alone, the mTau−/− mice have proven to be phenotypically normal in terms of learning/memory and general cognition (Tucker et al, 2001; Roberson et al, 2007; Morris et al, 2013; Li et al, 2014) with a minor parkinsonism motor phenotype developing in later life, of which the severity is debated between groups (Lei et al, 2012; Morris et al, 2013; Li et al, 2014). Importantly, when endogenous mouse tau levels were reduced in adult mice, no deviations from baseline were seen in any sensory, motor, or cognitive behavior task (DeVos et al, 2013), further supporting the safety of reducing tau levels in the adult brain.

Reducing human tau levels in the P301S mouse model is capable of significantly reducing tau pathology. However, when studying the effects of a tau lowering therapy in the context of Alzheimer’s Disease, where tau pathology is only one of the pathological hallmarks, the P301S
model cannot address the myriad Amyloid-beta induced deficits that have been reported in hAPP mouse models. However, lowering endogenous levels of murine tau using the tau knockout line has proven protective against a growing number of Aβ-induced insults, including cognition (Roberson et al., 2007; Andrews-Zwilling et al., 2010; Ittner et al., 2010; Leroy et al., 2012), hyperexcitability (Roberson et al., 2007, 2011; Ittner et al., 2010; Suberbielle et al., 2013; Li et al, 2014), survival (Roberson et al., 2007, 2011; Ittner et al., 2010), axonal transport deficits (Vossel et al., 2010), cell-cycle re-entry (Seward et al., 2013), and double stranded breaks in DNA (Suberbielle et al., 2013). Further, using ASOs against mouse tau has also convincingly shown that lowering tau levels in adult mice is protective against neuronal hyperexcitability (DeVos et al, 2013).

We propose that tau may be involved in both AD-associated hyperexcitability as well as neuronal cell loss by means of tau aggregation and neurofibrillary tangle formation. By previously showing that endogenous tau reduction can protect against hyperexcitability (DeVos et al, 2013) and now demonstrating that human tau knockdown can reverse tau inclusions and protect against neuronal cell loss, a tau reduction approach may be a strong therapeutic avenue for AD. A comparable human tau ASO as the one used here may be readily applicable to human Tauopathy patients. A similar ASO strategy that targeted superoxide dismutase 1 (SOD1) extended survival in a rat model of Amyotrophic Lateral Sclerosis (ALS) (Smith et al., 2006) and completed a Phase I clinical trial in human ALS patients. The CSF delivered human SOD1 ASOs demonstrated an excellent safety profile (Miller et al., 2013). Further, ASOs against spinal motor neuron (SMN) that also rescued rodent Spinal Muscular Atrophy (SMA) models (Hua et al., 2010; Passini et al., 2011; Porensky et al., 2012) are currently being used in Phase II studies for children with SMA and human huntingtin ASOs that successfully treated mouse models of Huntington’s Disease (HD) (Kordasewicz et al, 2012) are now being considered for human
clinical trial for HD. These successful in vivo and human studies suggest that the human tau reduction approach described here has a strong possibility to be translated to the human clinic for patients with tauopathies such as Alzheimer’s Disease, Progressive Supranuclear Palsy, and Frontotemporal Dementia.
Figure 3.1. Antisense Oligonucleotides Specifically Reduce Human Tau mRNA.

A,B, Saline or Human Tau ASOs were infused Intracerebroventricularly (ICV) into P301S mice at 30µg/day for 1 month (n=2-4). The right parietal cortex was analyzed for total human (A) and mouse (B) tau mRNA levels. Tau^{ASO-12} was the only ASO to significantly reduce human tau mRNA levels after 1 month of ASO delivery. One-way ANOVA, Bonferroni post hoc analysis.

C,D, Saline or Tau^{ASO-12} was delivered via ICV infusion at 30µg/day for 1 month. Human (C) and mouse (D) total tau mRNA levels were analyzed at 4, 8, and 12 weeks after pump implantation (n=3-11). Two-way ANOVA, Bonferroni post hoc analysis.

*p<0.05, ****p<0.0001. Error bars represent SEM.
**Figure 3.2. ASOs Distribute Throughout the P301S Adult Mouse Brain.**

*A,B,* Saline (A) or Tau$^{ASO-12}$ (B) was delivered by ICV infusion at 30µg/day into P301S mice for 1 month. In mice collected 12 weeks post-pump implantation, brain tissue on the contralateral side of the catheter (left hemisphere) was stained with an ASO-antibody (red) and counterstained with DAPI (blue). ASO is able to penetrate throughout the entire adult mouse brain and persist for two months post active ASO infusion.
Figure 3.3. Human Tau ASOs Reduce AT8 Tau Pathology.

A, B, Saline or Tau^{ASO-12} was delivered by ICV infusion at 30μg/day into 5 month old P301S mice for 1 month and the mice were then collected at 7 months of age (n=5 per group). Total human (A) and mouse (B) tau levels were measured from the right parietal cortex. Human tau mRNA was selectively reduced.

C, Total human tau protein was analyzed via Western Blot using a human tau specific antibody. Total human tau was found to be greatly reduced in the Tau^{ASO-12} treated P301S mice.

D, Fixed brain tissue contralateral to the catheter (left hemisphere) were stained for tau pathology using the hyperphosphorylated tau antibody AT8. There is a clear reduction in total AT8 tau staining in the hippocampus, amygdala, and entorhinal cortex of P301S mice treated with Tau^{ASO-12} as compared to saline treated P301S mice.

E, F, G, Percent area of positive AT8 stain in each of the brain regions shown was quantified in a blinded fashion, showing a decrease in AT8 positivity throughout multiple brain regions in the P301S Tau^{ASO-12} mice.

*p<0.05, **p<0.01. Two-tailed Student t-test.
A: Implant Pumps with 30μg/day ASO

B: Relative Human Tau mRNA

C: Relative Mouse Tau mRNA

D: Relative Human Tau mRNA

E: Relative Mouse Tau mRNA

F: Hippocampus

G: Amygdala

Entorhinal Cortex

3-6 Month Treatment

6-9 Month Treatment
Figure 3.4. Human Tau ASOs Markedly Decrease AT8 Tau Pathology in Longer Treatment Paradigm.

A, Experimental Design. P301S mice starting at either 3 or 6 months of age were treated with either Scrambled of Tau\textsuperscript{ASO-12} for 1 month at 30µg/day. Non-Transgenic (NT) mice treated with 30µg/day Scrambled ASO served as the NT control. Pumps were removed after 1 month and mice were collected 2 months after pump-removal. Right hemisphere for biochemistry and Left hemisphere for histology.

B,C, Total human (B) and mouse (C) tau mRNA levels were measured in the 3-6 month treatment group. Only human tau was decreased at the collection point. (n=5-11 per group).

D,E, Total human (D) and mouse (E) tau mRNA levels were measured in the 6-9 month treatment group. Again, human tau was selectively reduced at the 9 month final collection point (n=7-10 per group).

F, Representative Images of AT8 Tau Pathology in the Hippocampus, Amygdala and Entorhinal cortex in 6 month P301S mice that had been treated with either Scrambled or Tau\textsuperscript{ASO-12}. There is a visible decrease in total AT8 staining in the Tau\textsuperscript{ASO-12} treatment group.

G, Representative Images of AT8 Tau Pathology in the same regions as in F in the 9 month P301S mice. Again, there is a very clear reduction in AT8 positivity in the Tau\textsuperscript{ASO-12} treated P301S mice. This later time point suggests not only a prevention of additional tau accumulations, but may also suggest a reversal in pre-existing AT8 positive inclusions.

One-way ANOVA, Bonferroni post hoc analysis. **p<0.01, ****p<0.0001. Error bars represent SEM.
Figure 3.5. Human Tau ASOs Reverse Pathological Tau Staining in aged P301S mice.

A,B, P301S mice starting at 9 months of age were treated with either Scrambled or Tau^{ASO-12} for 1 month at 30μg/day. NonTransgenic (NT) mice treated with 30μg/day Scrambled ASO served as the NT control. Pumps were removed after 1 month and mice were collected 2 months after pump-removal. Right hemisphere for biochemistry and left hemisphere for histology.

Representative Images of AT8 (C) and MC1 (D) tau staining in 12month NT mice, showing a lack of inclusion staining.

C, Representative Images of AT8 Tau Pathology in the Hippocampus and Entorhinal cortex in a 9 month P301S mouse and 12 month P301S mice that had been treated with either Scrambled or
Tau<sup>ASO-12</sup>. There is a visible decrease in total AT8 staining in the Tau<sup>ASO-12</sup> treatment group as compared to both the aged matched 12 month P301S group and 9 month P301S treatment start point.

**D,** Representative Images of MC1 Tau Pathology in the Hippocampus and Entorhinal cortex in a 9 month P301S mouse and 12 month P301S mice that had been treated with either Scrambled or Tau<sup>ASO-12</sup>. There is a decrease in total MC1 conformation tau staining in the Tau<sup>ASO-12</sup> treatment group as compared to both the aged matched 12 month P301S group and 9 month P301S treatment start point.
Figure 3.6. Neurons with Human Tau\textsuperscript{ASO-12} Rarely Co-localize with AT8.

\textbf{A-D,} 9 month P301S mice were treated with 30\(\mu\)g/day Scrambled or Tau\textsuperscript{ASO-12} for 1 month and then collected at 12 months of age. Non-Transgenic (NT) mice treated with 30\(\mu\)g/day Scrambled were treated as a control. There are no AT8 positive accumulations that form in NT mice (A) while there are numerous neurons in CA3 that have AT8 positive tau inclusions in P301S mice at
12 months of age (B). There is significant overlay between the scrambled ASO and AT8 tau pathology as would be expected since the Scrambled ASO does not target human tau (C), while there is very little co-localization between Tau$^{\text{ASO-12}}$ and AT8 (D).
Figure 3.7. Astrogliosis is Reduced with a Human Tau^{ASO-12} Treatment in aged P301S mice.

A, Hippocampal sections from 12 month NTs were stained for the astrogliosis marker, glial fibrillary acidic protein (GFAP). Representative images shown of the full Hippocampus.
B, Hippocampal sections from 9 month P301S mice, 12 month P301S Untreated, and 12 month P301S mice treated with TauASO-12 were stained for GFAP. Representative images shown of the full Hippocampus. The GFAP positivity in TauASO-12 treated aged P301S mice is greatly decreased as compared to the age-matched 12 month P301S untreated mice.
Figure 3.8. Reducing Human Tau rescues hippocampal loss in aged P301S mice.

A, Serial sections 300µm apart were taken from 9 month NonTransgenics (NT), 12 month NTs, 9 month P301S mice, 12 month P301S (both untreated and treated with Scrambled ASO), and 12 month P301S mice treated with Tau<sup>ASO-12</sup>. The area of the sections were measured blindly and combined for a final hippocampal volume number (n=6-13).

B, Representative hippocampi images.

One-way ANOVA, Bonferroni post hoc analysis. *p<0.05. Error bars represent SEM.
Figure 3.9. CA1 Hippocampal Neuron Loss is Rescued following Human Tau$^{\text{ASO-12}}$ Treatment in aged P301S mice.

A, Hippocampal sections from 12 month NTs were stained for NeuN. Representative images shown of the full Hippocampus and CA1 at closer magnification.

B, Hippocampal sections from 9 month P301S mice, 12 month P301S treated with Scrambled ASO, and 12 month P301S mice treated with Tau$^{\text{ASO-12}}$ were stained for NeuN. Representative images shown of the full Hippocampus and CA1 at closer magnification. The NeuN positivity in CA1 of Tau$^{\text{ASO-12}}$ treated aged P301S mice is markedly greater than both the age-matched 12 month P301S Scrambled ASO mice and starting 9 month P301S mice.

C, Quantification of NeuN mean fluorescence intensity in CA1. n=5-8.

Scale bars for full hippocampus: 500µm. Scale bars for CA1 Inset: 100µm.

*p<0.05. One way ANOVA. Bonferonni post hoc analysis.
**Figure 3.10. Inclusions of the autophagic marker, p62, are reversed in Human Tau ASO treated P301S mice**

**A,** Hippocampal sections from 12 month NTs were stained for the autophagic marker, p62. Representative images shown of the dentate gyrus.

**B,** Hippocampal sections from 9 month P301S mice, and 12 month P301S mice treated with either Scrambled ASO or Tau\(^{ASO-12}\) were stained for p62. Representative images shown of the dentate gyrus. The number of p62 inclusions in Tau\(^{ASO-12}\) treated aged P301S mice is greatly decreased as compared to the age-matched 12 month P301S scrambled mice and even the starting 9 month P301S mice.

**C,** Aged P301S brain was co-labeled for p62 and AT8 tau pathology. There is a strong co-localization between the p62 autophagy marker and AT8 hyperphosphorylated tau. Co-labeled neurons are highlighted with arrowheads.
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Chapter 4

Conclusions and Future Directions
Summary of Results

The overall goal of the experiments described herein was to study the effects of total tau reduction, both in terms of safety as well as therapeutic efficacy. In order to best study the full effects of tau reduction, we employed two very different models: inducible seizures in non-transgenic mice and a transgenic mouse model of human tauopathy. Before the safety of lowering tau and any possible benefits could be studied, we first needed to develop a method for reducing total tau levels throughout the brain in adult mice.

While multiple ways exist to knockdown a protein of interest, we used antisense oligonucleotides (ASOs) for all of our studies outlined here. ASOs are 18-20mer DNA-like molecules that can be designed to selectively bind to a target mRNA sequence of interest, recruit the nuclear enzyme RNaseH, and ultimately lead to the degradation of that mRNA and gene knockdown (DeVos and Miller, 2013b). We designed and screened ASOs that targeted either total mouse tau or selectively human tau. When delivered to the adult mouse brain via osmotic pump infusion into the right lateral ventricle (DeVos and Miller, 2013a), the mouse tau ASO (Tau^{ASO-3}) could achieve upwards of 80% total mouse tau reduction (Figure 2.1), while our top human tau ASO was capable of reducing human tau by about 50%, leaving mouse tau levels unaltered (Figure 3.1). Of equal importance, we needed to ensure that the ASOs could effectively distribute throughout the entire brain and not just line the ventricles. Both the mouse tau ASO (Figure 2.2) and the human tau ASO (Figure 3.2) were capable of distributing through the adult mouse brain. We carefully measured total tau mRNA and protein levels throughout the entire brain and spinal cord in adult mouse brains subjected to the mouse tau ASO and confirmed that the ASO was capable of reducing total tau levels not just around the catheter, but everywhere in the central nervous system (Figure 2.3).
We first looked at endogenous mouse tau reduction since that would give us the best glimpse into the full extent of tau reduction and any effects on baseline behavior that may result from reducing total tau levels in the adult brain. We were able to reduce total tau mRNA and protein levels in the brain tissue (Figure 2.2), the interstitial fluid (Figure 2.4), and the cerebrospinal fluid (Figure 2.5). We were able to detect a significant correlation between the brain and CSF total tau levels after ASO infusion (Figure 2.5), suggesting that monitoring CSF tau levels following the infusion of a tau ASO may be a viable way to track the levels of brain tau in a human clinical trial. In addition to demonstrating tau reduction in all mediums in the brain – tissue, interstitial fluid, and cerebrospinal fluid – we subjected mice to a whole battery of sensory, motor, and cognitive tasks following tau reduction to begin to assess the safety of reducing total tau protein levels in the brain. mTau+/− mice are surprisingly normal, though this may be a result of developmental compensation. We saw no deviations from baseline behavior on any of the tasks (Figure 2.6). Though this is not an extensive analysis of safety, it bodes well for future studies looking at tau reduction feasibility.

To study therapeutic benefits using the mouse tau reduction ASO, we took a chapter from the AD mouse world where they observe significant reductions in neuronal hyperexcitability, both spontaneous as well as chemically induced, in the context of genetic tau knockout. Using two different seizure induction models, we tested the effects of tau reduction in adult non-transgenic mice by ASO and noted a significant reduction in EEG hyperexcitability (Figure 2.7) and seizure severity (Figure 2.8). Even more interesting, we noted a strong correlation between total tau protein levels and seizure severity (Figure 2.9) and discovered that baseline tau levels in naïve mice play an important role in seizure latency, with those mice that have higher baseline tau protein levels moving through the seizure stages much more rapidly than mice that have intrinsically lower tau levels (Figure 2.10) (DeVos et al, 2013).
These mouse tau reduction data demonstrate that 1) total tau levels can be reduced throughout the adult mouse brain using ASO technology, 2) reducing endogenous tau appears to be safe, 3) tau levels play an integral role in regulating hyperexcitability and 4) by lowering endogenous tau levels, neuronal hyperexcitability can significantly be prevented.

The endogenous tau reduction in non-transgenic mice was a helpful way to assess safety and physiological roles of tau, though non-transgenic mice do not form tau inclusions. In humans, a tau therapy would ideally prevent or ever reverse tau aggregation. To test whether a tau lowering therapy could provide therapeutic benefit in the context of tau inclusions, we needed to treat a transgenic mouse model that develops pathological tau deposition. For this, we turned to the P301S tauopathy model that overexpresses human tau with the P301S mutation (Yoshiyama et al, 2007). Just as with the mouse tau reduction studies, we developed human tau ASOs that selectively reduced human tau mRNA (Figure 3.1) and distributed widely throughout the adult P301S brain (Figure 3.2).

By reducing total human tau levels in this model, we were able to significantly reduce the number of AT8 positive hyperphosphorylated tau inclusions in multiple regions of the brain (Figure 3.3, 3.4). Even more interesting was that when human tau levels were reduced in aged P301S mice, the presence of both hyperphosphorylated tau deposits and MC1 positive misfolded tau accumulations was markedly reversed (Figure 3.5, 3.6). So with a reduction therapy, not only are new inclusions prevented from forming, pre-existing aggregates of tau are cleared from neurons. In addition to the reversal of tau pathology, as measured by AT8 and MC1 tau antibodies, astrogliosis (Figure 3.7), hippocampal volume loss (Figure 3.8), CA1 neuron loss (Figure 3.9), and p62 autophagic inclusions (Figure 3.10) were all visibly reduced. Impressively, hippocampal volume and CA1 neuron loss were returned to non-transgenic baseline levels.
following treatment with the human tau ASO (Figure 3.8, 3.9). By reducing total human tau levels in the P301S adult brain using an ASO, tau pathological burden could be reversed and hippocampal neuronal loss fully rescued, strongly supporting the use of a tau lowering therapy for those individuals with tau deposition.

Implications for Neurodegenerative Disease

The endogenous mouse tau reduction presented in this report strongly supports a tau lowering therapy for those with a detrimental hyperexcitability profile. Aberrant neuronal hyperexcitability can originate for a multitude of different sources and whether or not a tau lowering therapy would be beneficial for all of them remains to be tested. One of the most obvious neurologic disorders that experiences neuronal hyperexcitability is epilepsy, either genetic forms or environmentally induced. While those patients with epilepsy do not have mutations in the tau gene, a tau lowering therapy may still be beneficial. The tau+/− genotype has been shown in numerous studies to be protective against inducible excitotoxic insults (Roberson et al., 2007, 2011; Ittner et al., 2010; Li et al., 2014), suggesting that tau plays an important role in the physiological regulation of aberrant neuronal excitability. Interestingly, both a complete reduction as well as haploinsufficiency of tau significantly reduced seizures and extended survival in a well-established genetic mouse model of epilepsy, Kv1.1+/−, that experiences spontaneous, not chemically induced, seizures (Glasscock et al., 2010, 2012; Holth et al., 2013). These pure epilepsy reports, in combination with in vitro data using tau knockdown ASOs to protect cells from glutamate induced excitotoxicity (Pizzi et al., 1993) and our own in vivo tau knockdown data in two different inducible seizure models presented here, all support a total tau lowering therapy.
Compounds that significantly protect against PTZ-induced seizures in vivo are often successful when translated to human clinical trials (Rogawski, 2006). Though many epilepsy patients respond positively to one or two anticonvulsants, upwards of 20-40% of the patient population remains untreated (Devinsky, 1999; Brodie et al., 2012). In light of this, a tau reduction approach may be an alternative therapy for this refractory population. Given the previous tau+/− protective findings in multiple seizure paradigms (Roberson et al., 2007, 2011; Holth et al., 2013), we predict that our findings of tau knockdown using two different GABA-antagonists will also apply broadly to epilepsy in vivo models and human epilepsy in an effort to regulate hyperexcitability outside the presence of tau inclusions in human patients.

The human tau reduction experiments herein support a tau reduction therapy for those patients with pathogenic tau accumulations. These Tauopathy disorders include AD, a subset of Frontotemporal Dementias (ex. FTDP-17), Progressive Supranuclear Palsy, Corticobasal Degeneration, and others (Buee et al, 2000; Rademakers et al, 2004). While AD is not a result of any known tau mutations, over 35 mutations in the human tau gene have been directly linked to FTDP-17 disorders, proving that tau alone is capable of inducing widespread neurodegeneration (Schraem-Maschte et al, 2004). Aggregates of the protein tau are toxic to neurons and have been shown in vivo to travel along synaptically connected networks (de Calignon et al, 2012; Liu et al, 2012), a concept brought about by observations showing that tau pathology progresses in a very predictable pattern in human AD brains (Braak and Braak, 1991). With our human tau-lowering ASO, we are able to see a striking reduction and reversal in total tau pathology throughout the P301S brain, suggesting that by preventing the formation of new monomeric tau species, the cells are able to then clear pre-existing tau aggregates that may be toxic. A recent report analyzing tau in the somatodendritic compartment convincingly demonstrated that in its pathogenic state, tau does not re-localize from the axon to the somatodendritic compartment as
previously thought, but instead it is newly synthesized tau that is created and then remains within the neuronal soma and dendrites following the degeneration of the axonal tau (Zempel et al, 2013). With a tau reduction treatment, this newly synthesized tau would no longer be able to form, perhaps making it easier for the neuron to eliminate pre-existing pathological tau species. Further, when Polydoro et al reduced total human tau expression in one brain region in their repressible tau spreading mouse model (de Calignon et al, 2012), they noted a reversal in tau pathology in a distant brain region that did not directly experience the reduction in newly synthesized tau (Polydoro et al, 2013). This would suggest that the point of tau reduction may not even need to occur in all affected brain regions in order to reduce tau pathology throughout the brain, further supporting the use of a total tau reduction treatment paradigm for even those patients who already have signs of tau deposition in the brain.

In the AD brain, the role of amyloid-beta extracellular plaques as well as intraneuronal tau tangles as the two main pathological hallmarks has long been studied (Iqbal et al, 1975; Glenner and Wong, 1984; Brion et al, 1985; Ogomori et al, 1989). Recently, however, there has been accumulating evidence suggesting that neuronal hyperexcitability may also play an important role in AD pathogenesis (Olney et al, 1997; Mattson, 2004). In fact, those with familial AD mutations, an ApoE4 genotype, or sporadic late-onset AD, all experience an increased incidence in seizures (Takao et al., 2001; Mendez and Lim, 2003; Harden, 2004; Velez-Pardo et al., 2004; Amatniek et al., 2006; Kauffman et al., 2010). A small pilot study looking at this effect of increased neuronal activity in the hippocampus of human Mild Cognitively Impaired (MCI) patients studied the effects on this aberrant activity using an anti-convulsant. They administered either placebo or Levetiracetam to the MCI patients and then tested recall memory using functional magnetic resonance imaging techniques. Interestingly, the Levetiracetam treatment brought hippocampal neuronal hyperexcitability down to baseline levels and significantly
improved the recall performance of the MCI patients (Bakker et al, 2012), similar to what had been previously reported in a mouse models of AD treated with Levetiracetam (Sanchez et al, 2012). It should be noted that this same hyperexcitability protection and cognition restoration can be seen with a reduction in total tau protein levels as well (Roberson et al, 2007, 2011; Ittner et al, 2010; DeVos et al, 2013; Li et al, 2014). Further, the toxic tau species that appears in aggregates within the AD brain can be reduced and even reversed following human tau reduction in vivo (SantaCruz et al, 2005; Sydow et al, 2011; Polydoro et al, 2013), again supporting the use of a tau lowering therapy, though for different reasons. Ultimately, we believe that a tau reduction approach to treating AD could therapeutically protect against aberrant neuronal hyperexcitability as well as the formation and persistence of aggregated tau inclusions in the brain, thereby increasing the probability of drug efficacy in human clinical trials for the treatment of Alzheimer's Disease.

**Future Directions**

As with any scientific experiment, with every answer comes at least two more questions. While these tau reduction studies have provided strong evidence for the use of tau lowering ASOs as a therapy for AD and other tauopathy human patients, there are still a number of other experiments that should be done to further our knowledge as to the full therapeutic benefit that could come from such a tau targeted therapy.

While our mouse tau reduction and inducible seizure data would suggest that lowering total tau levels may benefit those patients with epilepsy, it will first be important to test whether tau ASOs can in fact provide protection in a mouse model of epilepsy, such as the Kv1.1-/- genetic mouse model of epilepsy that experiences spontaneous seizures (Glasscock et al, 2010, 2012). We would expect that lowering tau using an ASO would also provide benefit, since when placed
on the tau^{−/−} and even tau^{+/−} background, a significant reduction in seizure activity and increased survival was observed in the Kv1.1^{−/−} line (Holth et al, 2013), though this should be directly tested. Along these same lines, treating an hAPP mouse model with the endogenous mouse tau ASOs will be an important experiment to conduct. It could be that lowering tau in the adult animal is capable of only reducing seizure severity but not other functional or cognitive abnormalities. We have begun experiments in the APP/PS1 mouse line (Jankowsky et al, 2004) to test this exact question of whether reducing endogenous mouse tau can protect against functional connectivity deficits (Bero et al, 2011) and learning/memory decline (Gimbel et al, 2010) that is classically seen in the line.

Testing whether lowering total tau protein levels in epilepsy and hAPP murine models will be an important next step in moving tau ASOs toward the human clinic. However, those experiments will not shed further light as to the mechanism through with decreased tau levels can offer protection against neuronal hyperexcitability. One possibility may be that extracellular tau levels can in some way mediate this neuronal activity. In the tau knockout studies and in our own tau knockdown work, total tau protein is reduced in all compartments of the brain, including the extracellular space (Figure 2.4), making it impossible to tease apart the roles of extracellular versus intracellular tau. Tau is physiologically found in the extracellular space (Yamada et al, 2011; DeVos et al, 2013; Pooler et al, 2013) and rapidly increases in concentration with the increase in neuronal firing (Yamada et al, 2014). Using cell-impermeable antibodies targeted to total tau, extracellular tau levels could be selectively reduced in the hippocampus. After this selective reduction in extracellular tau, GABA-antagonists can be infused into the same region of the hippocampus and EEG activity monitored continuously, similar to what we previously did with our picrotoxin studies (Figure 2.7). If neuronal activity can be decreased similar to what we have seen with total tau reduction via ASOs, this would strongly suggest that extracellular tau
molecules are in some way mediating this hyperexcitable activity. This would greatly add to the mechanistic data of how tau is able to play such a prominent role in modulating neuronal hyperexcitability, a question that largely remains unanswered in the AD field.

In addition to using tau reduction as a treatment, the striking observation that physiological endogenous tau levels in adult mice can affect susceptibility to seizures implies that endogenous baseline tau levels in humans may also influence the risk of developing seizures. Tau mRNA and protein levels in human brains can vary by greater than 2-fold (Lu et al., 2004; Kauwe et al., 2008; Trabzuni et al., 2012) and CSF total tau levels in control human subjects can differ quite substantially (Clifford et al., 2009; Fagan et al., 2009; Oka et al., 2013). While the exact source of variation in CSF tau levels is unknown, possibilities could include differences in neuronal excretion rates of tau and/or different baseline tau protein levels in the brain. Higher levels of tau protein in the brain at baseline may not be detrimental, but upon some injury to the brain, increased tau expression may predispose human patients to injury-induced seizures.

It is well documented that seizure incidence increases after multiple types of brain injury, including ischemic stroke (Camilo and Goldstein, 2004; Kwan, 2010) and traumatic brain injury (Annegers et al., 1998; Vespa et al., 2010). If human patients with higher baseline tau are more prone to developing seizures following an injury, being able to identify such patients through genetic studies of tau polymorphisms (Myers et al., 2007; Kauwe et al., 2008) or tau CSF levels (Palmio et al., 2009; Cruchaga et al., 2013) may help to risk stratify those patients and aid in determining who would benefit from a preventive antiepileptic therapy. The correlation between total CSF and brain tau levels is unknown in the control human patient population, so as a first attempt to try and find a good biomarker to predict brain tau levels in living adults, we are currently analyzing CSF tau and brain tau levels in naïve adult mice. This basic analysis of CSF
and brain tau correlation will allow us to determine if CSF tau levels can predict which mice have higher and lower total brain tau levels. If a strong direct correlation is found, using total tau levels in the CSF of human patients may serve as a possible method for identifying high and low brain tau levels, and ultimately high and low risk seizure patients.

In addition to future mouse tau reduction studies, there are numerous follow-on experiments with the human tau reduction work that could be explored. One of the big questions that emerged from our P301S studies is the mechanism by which tau pathology is cleared. How does a reduction in newly synthesized tau ultimately culminate in a clearing of pre-existing aggregates of tau in neurons? Two other groups have seen similar pathology reversal following a general reduction in human tau levels (Sydow et al, 2011; Polydoro et al, 2013). One possibility may be that human tau accumulations and oligomers within neurons can overwhelm and ultimately hinder the proteasome or macroautophagy protein clearance mechanisms that have previously been implicated in the clearance of tau (Berger et al, 2007; Dickey et al, 2007; Carrettiero et al, 2009; Wang et al, 2009; Tai et al, 2012). Perhaps by decreasing newly synthesized tau levels by even just 50%, these clearance mechanisms can be freed of the overwhelming tau inhibition and allow for the clearance of any pre-existing toxic tau species and ultimately the successful recovery of neurons. We hope to test this idea by measuring autophagy pathway activation levels in our aged P301S treated cohort to see if reducing total human tau levels can re-activate the autophagy system which may then lead to a clearance of tau pathology.

While we know that a ~50% reduction in human tau mRNA over the course of 3 months is sufficient to reverse tau pathology in aged P301S mice (Figure 3.5), tau inclusions may also be reversed with a lower dose of ASO and/or in a shorter treatment time. Knowing the smallest dose that can still achieve pathology reversal and a rescue in neuronal loss will be extremely
useful in thinking about using the ASO in human clinical trials. Ideally, the lower the ED$_{50}$, the fewer the off-target side-effects. Equally important is the timeline for pathology reversal. Does it take the full 3 months for tau protein levels to decrease and pre-existing tau accumulations to be cleared? Or can the same degree of reversal occur in 2 months or even 1 month? This will become more important in calculating how quickly a human tau reduction treatment may be expected to start showing beneficial effects in terms of a reduction in tau pathology, most likely as measured by a Tau PET imaging marker (Okamura et al, 2014). Both of these studies, looking at the ideal human tau ASO dose as well as pathology reversal timeline, are currently underway.

**Concluding Remarks**

The microtubule associated protein tau, when misfolded and aggregated, is a major contributor in a class of neurodegenerative disorders and dementias known collectively as Tauopathies, of which Alzheimer’s Disease (AD) is the most common form. Normally enriched in axons of neurons, tau can become hyperphosphorylated and form toxic oligomeric species and larger aggregates that can ultimately lead to neuronal cell death. In the work presented here, we developed a technique using antisense oligonucleotides (ASOs) to reduce total tau levels and applied the ASOs *in vivo* to determine if such a reduction is therapeutically beneficial. In our first set of experiments as outlined in Chapter 2, we reduced endogenous mouse tau in the brain of adult non-transgenic mice and found no effect on baseline motor or cognitive behavior, ensuring the safety of tau reduction in the adult animal. We also tested the efficacy of reducing murine tau in the setting of seizures and neuronal hyperexcitability due to the fact that increased neuronal excitability has been linked to the pathogenesis of AD. Mice with reduced tau protein levels had significant less severe seizures, demonstrating that endogenous tau is indeed integral for regulating neuronal hyperexcitability. While these non-transgenic studies are sufficient to study the physiological roles of tau, non-transgenic mice do not develop tau
inclusions. One of the two main pathological AD hallmarks is intraneuronal tau accumulations, so in order to better study the effect of total tau reduction on these pathological tau accumulations, we lowered human tau levels in the P301S Tauopathy mouse model. After treating the P301S line with the human tau ASO, not only did this human tau reduction prevent tau aggregates from developing, but it also reversed pre-existing tau aggregates and rescued hippocampal neuronal loss. Taken together, the safety of lowering total endogenous tau levels in adult animals, the protective phenotype against hyperexcitability, and the capability to remove pre-existing tau inclusions, a tau reduction therapy by means of ASO treatment may be a strong therapeutic candidate for those patients with aberrant neuronal hyperexcitability, pathogenic tau inclusions, or both.
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Tai HC, Serrano-Pozo A, Hashimoto T, Frosch MP, Spires-Jones TL, Human BT. The synaptic


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A. Personal Statement

In receiving my PhD in the Department of Neurology at Washington University in St. Louis, I have had the opportunity to work in the field of neurodegeneration with close guidance from some of the top researchers in the field, including my PhD mentor Dr. Tim Miller, as well as members of my thesis committee: Dr. David Holtzman, Dr. Marc Diamond, Dr. Alison Goate, and Dr. John Cirrito. In an ongoing collaboration with Isis Pharmaceuticals, I am currently focused on testing the efficacy of a tau antisense oligonucleotide (ASO) therapy for primary tauopathy disorders in vivo. My previous research internship and undergraduate research project equipped me with several different techniques and ideas, such that upon joining Dr. Tim Miller’s lab, I was able to immediately jump into the tau ASO projects and move them forward quickly. I was recently given the opportunity to present my research in a platform talk at the Cold Spring Harbor Laboratory 2012 Neurodegeneration Meeting and have published an invited review, a methods paper, and a Journal of Neuroscience original research article so far this year. I also work very closely with Marc Diamond’s lab by assisting in their in vivo surgeries, resulting in one published and one submitted second author paper. In addition to my research in the lab, I am the recipient of the 2012 Poletsky Award given by the Alzheimer’s Disease Research Center at Washington University as well as the winner of the 2013 Annual WashU Hope Center Award for a talk I gave during the Hope Center retreat. Since my 2009 matriculation into the Neuroscience PhD program, I have been extremely privileged to be surrounded by so many great and inquisitive minds, allowing me to grown in both my technical skills set as well as scientific thought process.
## B. Positions and Honors

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<td>Dr. Paul Taghert</td>
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1. Washington University St. Louis James L. O’Leary Neuroscience Award Finalist 2014
2. St. Jude’s National Graduate Student Symposium Award 2014
3. 14th Annual Alzheimer’s Drug Discovery Foundation Conference Travel Award 2013
4. Washington University St. Louis Hope Center Award Recipient 2013
5. Washington University St. Louis ADRC Poletsky Award Recipient 2012
7. Markey Pathway Recipient 2010-12
8. Outstanding Senior Project Award at Central Michigan University 2009
9. NSF and The Horace W Goldsmith Foundation support for Jackson Lab internship 2008
11. Sigma Xi Grants-in-Aid of Research Grant 2007
12. Arthur and Loren Kontio Outstanding Young Biologist Award 2007
13. CMU Central Scholar Award (4 years tuition and housing) 2005-2009

### Other Experiences

1. Member of the Washington University in St. Louis Neuroscience Retreat Committee 2010-2013
C. Publications

Research Papers:


Abstracts:


DeVos SL, Plotzker A, Bennett CF, Miller TM. Antisense Reduction of Human Tau in the CNS


Patents:

   Inventors: Bennett CF, DeVos SL, Miller TM, Rigo F. Filed 3/14/13 under Docket Number 012147-PCT1/1.