EVALUATION OF TAU AS A TARGET FOR ALZHEIMER DISEASE AND OTHER CONDITIONS WITH EPILEPTIFORM ACTIVITY

by

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A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham, in partial fulfillment of the requirements for the degree of Doctor of Philosophy

BIRMINGHAM, ALABAMA

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GRADUATE BIOMEDICAL SCIENCE NEUROSCIENCE THEME
ABSTRACT

Alzheimer disease (AD) is a devastating neurological condition that slowly destroys memory and thinking skills of the affected patients. No disease-modifying medications are available. While all the treatment against Aβ (major component of one hallmark pathology of AD) failed in clinical trials, tau (major component of the other hallmark pathology of AD) is emerging as a better alternative.

Germline knockout of tau does not cause overt abnormalities in young mice and prevents AD-like deficits in mouse models of AD. Germline knockout of tau also confers resistance to epileptiform activity in models of both AD and epilepsy. Therefore tau is proposed as a drug target for AD and other conditions with epileptiform activity. However, the long-term effects of tau reduction in aged mice are controversial. The efficacy and safety of tau reduction in adulthood is unknown.

To test the long-term effects of tau reduction, we comprehensively characterized a cohort of aged mice with germline knockout of tau. We demonstrated that partial tau reduction does not cause any detectable abnormality in aged mice, and complete loss of tau causes a phenotype of hyperactivity in aged mice but no motor impairment and no neurodegeneration. We also demonstrated that seizure protective effects of tau reduction persist with aging.

To test the whether tau reduction in adulthood is beneficial and safe, we developed a new line of inducible knockout mice. This enables us to maintain normal level of tau expression during development and to reduce tau levels in adult mice. By characterizing those mice with adulthood tau knockout, we demonstrated that tau
reduction in adulthood does not cause overt abnormalities, and that tau reduction in adulthood confers resistance to epileptiform activity in two seizure models.

Overall, our findings support the long-term safety of tau reduction as well as safety and benefits of tau reduction in adulthood. Our work further validates tau as a target for Alzheimer disease and other conditions with epileptiform activity.

Keywords: Alzheimer disease, Tau, Epilepsy, Seizure
DEDICATION

I dedicate my thesis to those souls wandering in the mountains of my home town. They may have lived with Alzheimer disease or other neurological conditions in their late life; but they were never diagnosed or tended and were perceived as old misbehaving children before they died. May this work help bring attention and potential treatment to Alzheimer disease and other neurological conditions, so people who are affected by Alzheimer disease like those souls in my home town can retain dignity in their late life.
ACKNOWLEDGMENTS

I would like to thank my wife and daughter for their support during my pursuit of a PhD. Without their support and company, I am not sure I could finish this 6-year long marathon. I also would like to thank my parents. Though they themselves even did not finish elementary school, they encouraged me, and supported me both morally and financially to pursue higher education in China and in the Unites States. Without them, I would never be able to come this far from a rural village deep in the mounts of China.

I would like thank my mentor Dr. Erik Roberson. He brought me into the exciting research on Alzheimer disease, tau, and mouse behavioral testing for which I will continue to pursue in the future. He also transformed my way of thinking during his daily mentoring and weekly writing class. I also want to thank my committee members, Dr. David Standaert, Dr. John Hablitz, Dr. Lori McMahon and Dr. Scott Wilson for their guidance, constructive suggestions, and concrete help on experiments of their expertise. I also want to thank Patricia Matthews for her always prompt reminder and support.

I would also like to thank all the current and past members of Roberson lab for their help in the lab and other matters. Their support and fellowship makes the lab an enjoyable place to stay. I would also thank everyone in the neuroscience community of UAB. I have been roaming from one city to another over the past years. UAB is by far the longest and most enjoyable place I have ever stayed.
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DISCUSSION

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INTRODUCTION

Alzheimer disease

Alzheimer disease (AD) is a neurological condition that slowly destroys memory and thinking skills. In those affected by AD, symptoms like memory problems usually appear after the age of 60, but can start as early as age of 40 in rare familial forms of AD. With the progression of disease, those affected by AD gradually start to have difficulties in managing finances, finding a home, learning new information and recognizing family members, even those beloved ones who have been living with the affected for decades. Eventually the affected lose the ability to carry out even the simplest functions of living, and start to depend on others. Because AD attacks memory and thinking skills, the very basis of us as human beings, it is a big intellectual burden for those who care about self-identity and self-independence.

AD also stands for a huge practical burden for the society financially. AD is currently the most common form of dementia. According to the Alzheimer Association, more than 5 million Americans now live with AD, a lot of them have to stay in nursing homes and depend on care givers because of their inability to live alone. The total cost mounts to $200 billion in 2013, with a lot of care givers being unpaid. With the general population ageing, unless some treatment is developed, the projected total cost in 2050 stands at $1.2 trillion, a number easily bankrupts the society (http://www.alz.org/alzheimers_disease_facts_and_figures.asp).

Unfortunately no cure or disease-modifying treatments are available (Roberson and Mucke, 2006). Five drugs have been approved by FDA to treat AD (Table 1). They generally fall into two categories: cholinesterase inhibitors and NMDA receptor antagonist. Cholinesterase
inhibitors were designed to slow down the degradation of acetylcholine. Loss of cholinergic neurons was once believed to be the major underline mechanism of AD pathogenesis (Auld et al., 2002). An antagonist of the NMDA receptor, a subtype of glutamate receptors, was designed to prevent over-stimulation of NDMA receptor. The possible excitotoxicity caused by over-activation NMDA receptor may attribute to the pathogenesis of AD and many other neurodegenerative diseases. However, the effectiveness of all approved drugs is modest (Trinh et al., 2003; Rive et al., 2013). Though the lack of effective treatment is discouraging, heavy investment into research has yielded a great deal of the biology underlying AD pathogenesis, upon which new treatment might be developed.

**Table 1: FDA approved treatment for AD and effectiveness**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Approved Usage</th>
<th>Mechanism</th>
<th>Side Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donepezil</td>
<td>All stages</td>
<td>Cholinesterase inhibitors</td>
<td>Nausea, vomiting, loss of appetite and increased frequency of bowel movements</td>
</tr>
<tr>
<td>Galantamine</td>
<td>Mild to moderate</td>
<td></td>
<td>Nausea, vomiting, loss of appetite and increased frequency of bowel movements</td>
</tr>
<tr>
<td>Rivastigmine</td>
<td>Mild to moderate</td>
<td></td>
<td>Nausea, vomiting, loss of appetite and increased frequency of bowel movements</td>
</tr>
<tr>
<td>Tacrine</td>
<td>Mild to moderate</td>
<td>NMDA receptor antagonist</td>
<td>Possible liver damage, nausea, and omitting</td>
</tr>
<tr>
<td>Memantine</td>
<td>Moderate to severe</td>
<td></td>
<td>Headache, constipation, confusion and dizziness</td>
</tr>
</tbody>
</table>

Adapted from: [http://www.alz.org/research/science/alzheimers_disease_treatments.asp](http://www.alz.org/research/science/alzheimers_disease_treatments.asp)

The pathogenesis of Alzheimer disease

Our understanding into the etiology and pathogenesis of AD is largely driven by the hallmark pathologies of AD and the genetics of both familiar and sporadic forms of AD. During post mortem analysis, brains from patients affected by AD (both familiar and sporadic forms) exhibit two hallmark pathologies: plaques and neurofibrillary tangles. The major component of plaques was then purified and determined as amyloid beta (Aβ) (Glenner and Wong, 1984a). And mutations in genes responsible for production of Aβ were predicted to cause familiar form
of AD (Glenner and Wong, 1984b). This prediction was soon confirmed by the genetic linkage of familiar form AD and genetic markers on chromosome 21 in the vicinity of APP, the precursor protein of Aβ (Goldgaber et al., 1987; Kang et al., 1987; Tanzi et al., 1987). Since then, more than 200 mutations in the genes (APP, PS1, and PS2) responsible for production of Aβ have been identified (http://www.molgen.ua.ac.be/ADMutations). The majority of those mutations increase the ratio of Aβ42/Aβ40, with Aβ42 being more amyloidogenic. The rest of those mutations either increase the production of both Aβ42 and Aβ40, or increase the amyloidogenicity of Aβ42/Aβ40 (Table 2). Of note, a mutation in APP that protects against AD was recently demonstrated to reduce the production of Aβ42/Aβ40 (Jonsson et al., 2012). In sporadic form AD, ApoE4, the biggest risk factor of AD, was demonstrated to decrease the clearance of Aβ42/Aβ42 (Castellano et al., 2011).

**Table 2: Mutations causing familiar form of AD**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Mutation Counts</th>
<th>Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>APP</td>
<td>Amyloid b precursor protein</td>
<td>33</td>
<td>Increase Aβ_{42/40} ratio</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Increase Aβ production</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Increase Aβ aggregation</td>
</tr>
<tr>
<td>PSEN1</td>
<td>Presenilin 1</td>
<td>185</td>
<td>Increase Aβ_{42/40} ratio</td>
</tr>
<tr>
<td>PSEN2</td>
<td>Presenilin 2</td>
<td>13</td>
<td>Increase Aβ_{42/40} ratio</td>
</tr>
</tbody>
</table>

Note: mutation in PSEN1 and PSEN2 were reported have implications unrelated to their enzymatic activity on Aβ production (Pimplikar et al., 2010). Adapted from: http://www.molgen.ua.ac.be/ADMutations/

Given the indistinguishable nature of familiar and sporadic forms of AD in both clinical and post mortem analysis, a unifying hypothesis, the Aβ cascade hypothesis, was formed (Hardy and Higgins, 1992; Hardy and Selko, 2002; Selko, 2011): the accumulation of Aβ42/Aβ40 due to either over production or decreased clearance starts a chain reaction that eventually leads to synaptic failure, neurodegeneration, and dementia (Figure 1).
Epileptiform activity in Alzheimer disease

Epileptic seizures in AD have been long documented but generally not given a great deal of attention. Though the absolute incidence of unprovoked seizures in those affected by AD is low, the risk of seizures in those affected by AD is 8 times higher than general population of the same age (Scarmeas et al., 2009); in those affected by early-onset familiar form of AD, the incidence ratio can be as many as 87 times higher (Amatniek et al., 2006). Therefore, AD is usually considered a risk factor of seizures and epileptic seizures are thought to a complication of AD due to neuronal loss.

Fig. 1: Aβ cascade hypothesis. In this hypothesis, changes in metabolism of Aβ peptides leads accumulation formation of toxic species Aβ peptides. The toxic species of Aβ, presumably soluble oligomeric Aβ, starts a cascade of reaction that eventually leads to dementia in Alzheimer patients.
Table 3: Epileptiform activity in AD mouse models

<table>
<thead>
<tr>
<th>Transgene</th>
<th>Mouse background</th>
<th>Seizure observation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human APP&lt;sub&gt;SWE&lt;/sub&gt;</td>
<td>FVB/N</td>
<td>Generalized tonic-clonic seizures, occasionally terminated with sudden death</td>
<td>(Hsiao et al., 1995)</td>
</tr>
<tr>
<td>Murine Aβ&lt;sub&gt;42&lt;/sub&gt;</td>
<td>FVB/N</td>
<td>Generalized tonic-clonic seizures, reduced life span</td>
<td>(LaFerla et al., 1995)</td>
</tr>
<tr>
<td>Human APP/RK</td>
<td>FVB/N</td>
<td>Brief seizures of variable severity in young mice, extended generalized tonic-clonic seizures in old mice</td>
<td>(Moechars et al., 1996)</td>
</tr>
<tr>
<td>Human APP/RK</td>
<td>C57BL</td>
<td>Very rare seizure incidence</td>
<td>(Moechars et al., 1996)</td>
</tr>
<tr>
<td>Human APP&lt;sub&gt;RK&lt;/sub&gt;</td>
<td></td>
<td>Mild to severe tonic-clonic seizure in 15% of mice older than 6 month</td>
<td>(Kumar-Singh et al., 2000)</td>
</tr>
<tr>
<td>Human APP SWE</td>
<td>C57BL/6J</td>
<td>Tonic-clonic seizures in 40% of mice</td>
<td>(Lalonde et al., 2005)</td>
</tr>
<tr>
<td>Human APP&lt;sub&gt;SWE,Ind&lt;/sub&gt;</td>
<td>C57BL/6J</td>
<td>Frequent generalized- sharp- synchronous-electrographic discharges intermittent non-convulsive; electroencephalographic seizures</td>
<td>(Palop et al., 2007)</td>
</tr>
<tr>
<td>Human APP&lt;sub&gt;SWE&lt;/sub&gt; + PS1dE9</td>
<td>C57BL/6J</td>
<td>65% mice had at least one electrographic seizure in 3 weeks</td>
<td>(Minkeviciene et al., 2009)</td>
</tr>
<tr>
<td>Human APP intracellular domain + Fe65</td>
<td>C57BL/6J</td>
<td>Increased electrographic spikes</td>
<td>(Vogt et al., 2011)</td>
</tr>
</tbody>
</table>

Recent advances in animal models of AD have however suggested a different view regarding the relationship between epileptic seizures and AD: instead of being sorely a consequence of progression of AD, epileptiform activity including both convulsive and non-convulsive seizures might be a contributing factor to the cognitive impairment (Leonard and McNamara, 2007). As summarized in table 3, seizures have been documented in a variety of different mouse models of AD. In one particular model, hAPP-J20, continuous video-electroencephalographic (EEG) monitoring revealed frequent epileptiform activity including sharp waves and spikes and intermittent unprovoked seizures at the time or before cognitive impairment in a mouse model that produces high level of Aβ peptides (Palop et al., 2007). This mouse model also revealed that those epileptiform events trigger inhibitory compensatory remodeling in hippocampal circuits including depletion of calbindin in granule cells and
increased expression of NPY in molecular layer of dentate gyrus and mossy fibers (Palop et al., 2007). Earlier studies in hAPP-J20 mice also revealed that high level of Aβ peptides trigger sprouting of inhibitory axonal terminal in molecular layer of dentate gyrus, enhance synaptic inhibition, and alters expression of several activity or calcium dependent proteins like Arc and Fos (Palop et al., 2003; Chin et al., 2004; Chin et al., 2005; Palop et al., 2005). These molecular and cellular alteration and cognitive impairment also tightly correlate with each other (Palop et al., 2003; Chin et al., 2004; Chin et al., 2005; Palop et al., 2005; Palop et al., 2007). This timing and tight correlation between these alterations suggested a causative relationship between epileptiform activity and cognitive impairment in pathogenesis of AD (Figure 2).

The concept that epileptiform activity contributes to AD pathogenesis provides a new framework for development of treatment for AD. On one hand, FDA-approved medications for epilepsy could be repurposed for AD. Along this line, one antiepileptic drug, levetiracetam, was demonstrated to be able to improve hippocampal dependent memory function of age-impaired rats (Koh et al., 2010). Levetiracetam was later shown to suppress aberrant EEG activity, inhibit compensatory remodeling in hippocampus, and ameliorate cognitive deficits in a mouse model of AD (Sanchez et al., 2012). The same drug, levetiracetam, was also demonstrated to be able to improve cognitive function of patients with aMCI (a stage preceding to AD) patients (Bakker et al., 2012). On the other hand, while mouse models of AD usually involve complex breeding and long time to develop AD-like deficits, new treatment for AD can also be tested in mouse models of seizures which can be rapidly induced with excitotoxins.
Modeling Alzheimer disease and epilepsy in mice

Mouse models of AD have been particularly useful for drug discovery of AD, particularly in target identification and validation (Hall and Roberson, 2012). All mouse models bear one or another mutation in genes responsible for the production of Aβ peptides, and capture certain aspects of AD including the cardinal features like Aβ plaques and cognitive deficits. Many novel treatments, like immunotherapies for Aβ clearance, and β-/γ-secretase inhibitors that block the production of Aβ peptides, were discovered and developed in those mouse models. These treatments then advanced into clinical trials and probably into some effective medication in the future. As mentioned earlier, epileptic activity was identified as a new target in mouse
models first and then a medication directed against epileptic activity was later proved to be beneficial in human patients (Bakker et al., 2012).

While epileptiform activity presents in mouse models of AD along with other AD-like deficits, epileptic seizures can be also rapidly induced by excitotoxins in mice (Loscher, 2011). Two commonly used excitotoxins are: pentylenetetrazole (PTZ) and kainic acid. PTZ is an antagonist for GABA$_A$ receptors. Acute application of PTZ in mice induces stereotyped behavioral reactions that mimic absence seizures in human (Dhir, 2001). PTZ-induces seizures have been the gatekeeper for the development of antiepileptic drugs (Loscher, 2011). Kainic acid is agonist of kainic acid receptors. Local injection of kainic acid into amygdala, hippocampus or ventricle, or systemic administration through intraperitoneally injection induces a syndrome of seizures that mimic human temporal lobe epilepsy (TLE) (Nadler, 1981; Ben-Ari, 1985, 2012). Recurrent tonic-clonic discharges and long-lasting status epilepsy can also be monitored by EEG recording. Because of their simplicity and robustness, those two models, PTZ and kainic acid induced seizures, were selected to evaluate the effectiveness of tau reduction against seizures.

Microtubule associated protein tau as target for Alzheimer disease

Possible roles of tau in AD pathogenesis

The role of tau in AD pathogenesis has been long considered critical but elusive. On one hand, tau is the major component of neurofibrillary tangles, one of the hallmark pathologies of AD. The tempo-spatial distribution and progression of tau pathology correlates well with the onset and progression of symptoms of AD (Serrano-Pozo et al., 2011), which suggests certain critical role of tau in the pathogenesis of AD. On the other hand, no mutations in tau have been associated with familiar form of AD so far. In contrast, more than 200 mutations in genes
responsible for Aβ production have been demonstrated to cause familiar form of AD (Tanzi, 2012). Mutations in tau are causative to a different type of dementia, frontotemporal dementia (Goedert et al., 2012).

The first theory is that loss of function of tau contributes to the pathogenesis of AD (Lee et al., 2011). Tau has been long considered critical for the normal function of brain. Tau was initially identified as a microtubule binding protein and demonstrated to stabilize microtubules in vitro (Bunker et al., 2004) among other possible important functions like regulating axonal transport (Dixit et al., 2008) and neurite outgrowth (Caceres and Kosik, 1990). During the course of AD pathogenesis, tau becomes hyperphosphorylated, then dissociates from microtubules (Mandelkow and Mandelkow, 2012). The dissociation of tau from microtubule is then hypothesized to contribute to neuronal degeneration in AD. Along this line, several agents that stabilize microtubules and therefore compensate the loss of function of tau are being developed (Zhang et al., 2005; Brunden et al., 2010; Zhang et al., 2012). Though these agents showed some beneficial effects in tau transgenic mice, they may function through some complementary pathways unrelated to tau, because in vivo experiments from tau knockout mice do not support the hypothesis that tau is critical for the stability of microtubules. No abnormalities were observed in microtubules of tau knockout mice (Morris et al., 2011; Ke et al., 2012), and no dysfunction in axonal transport was observed (Vossel et al., 2010).

A second theory is that tau gains toxic function due to excessive amount of Aβ peptides that present in AD brains. This notion has been supported by intensive in vitro and in vivo studies. In dishes, tau becomes hyperphosphorylated, translocates into dendrites upon addition of Aβ peptides (Hoover et al., 2010; Jin et al., 2011; Zempel and Mandelkow, 2012; Zempel et al., 2013). In mouse models, tau relocates from soma to dendrites and directs other effectors like Fyn
kinase into dendrites through scaffolding (Ittner et al., 2010). Oligomers of tau were recently isolated from AD patients (Lasagna-Reeves et al., 2012) and were demonstrated to compromise membrane integrity (Flach et al., 2012).

A third theory is that the normal function of tau could be to serve as an enabling factor downstream of Aβ peptides. The supporting evidence majorly came from study of AD mouse models. The majority of mouse models that over produce Aβ peptides do not have hyperphosphorylated tau or neurofibrillary tangles while those models still capture other features of AD like cognitive deficits and Aβ plaques (Laferla and Green, 2012). Therefore, at least in mouse models, hyperphosphorylation of tau and formation of neurofibrillary tangles are not necessary for the development of AD-like deficits. Since no biochemical changes in tau protein is observed in those mouse model, tau is unlikely to gain toxic functions.

Table 4: Role of tau in AD pathogenesis

<table>
<thead>
<tr>
<th>Role</th>
<th>Evidence</th>
<th>Targeting strategy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loss of function</td>
<td>Tau regulates microtubules in vitro; Disociation of tau from microtubule in AD;</td>
<td>Compensating function of tau Microtubule stabilizing agents</td>
</tr>
<tr>
<td>Gain of function</td>
<td>Hyperphosphorylation of tau; Formation of oligomers; Toxic effects of Hyperphosphorylated /oligomeric tau;</td>
<td>Block formation of toxic form of tau; Clearing toxic form of tau;</td>
</tr>
<tr>
<td>Normal function</td>
<td>Absence of changes in tau in AD mouse model</td>
<td>Reducing level of tau Blocking certain function of normal tau</td>
</tr>
</tbody>
</table>

Along the lines of second and third theories, gain of toxic function and normal function of tau are responsible for the pathogenesis of AD, the strategy targeting tau could be reducing the level of tau (the normal, hyperphosphorylated or oligomeric form) or blocking the function of tau. This strategy is inspired and backed by genetic manipulation of tau in several mouse models of AD.
Tau reduction as a treatment strategy for Alzheimer disease

Early work in cultured neurons demonstrated that the cytotoxicity of Aβ peptides depends on tau expression. High levels of Aβ peptides induced degeneration in cultured neurons that express either murine tau or human tau but no degeneration in tau-depleted neurons (Rapoport et al., 2002). Aβ peptides also reduced viability of differentiated/tau-expressing neuronal culture but not of undifferentiated/non-tau expressing neurons, reducing tau levels in differentiated neurons with antisense oligoes blocked the neuro-toxicity (Liu et al., 2004). These early seminal studies were later confirmed with low level but more potent oligomeric Aβ (Jin et al., 2011; Nussbaum et al., 2012; Seward et al., 2013). These findings prompted the possibility that tau reduction might be beneficial in vivo.

The beneficial effects of tau reduction were then further confirmed in mouse models of AD. Genetically reducing levels of tau protein prevented deficits in spatial learning which is a cardinal feature of AD as well as other AD-like deficits like premature mortality and hyperactivity (Roberson et al., 2007). These findings were later reproduced in other mouse models of AD and by different researchers (Ittner et al., 2010; Roberson et al., 2011). Interestingly, genetically reducing levels of tau also prevented epileptiform activity presented in a mouse model of AD (Roberson et al., 2011) and inhibitory compensatory remodeling induced by either over-production of Aβ peptides or excitotoxin kainic acid (Palop et al., 2007). This in turn further confirms that epileptiform activity might be contributing to AD pathogenesis.

Genetically reducing levels of tau also conferred resistance to epileptiform activity induced by other factors in non-AD animal models. When challenged with excitotoxin pentylenetetrazole (PTZ), mice with reduced levels of tau reached less severe stages of seizure
and were slower to reach later stages of seizure (Roberson et al., 2007; Ittner et al., 2010). When challenged with a different type of excitotoxin, kainic acid, fewer mice with reduced level of tau exhibited tonic-clonic seizure (Roberson et al., 2007). In a genetic model of epilepsy, genetically reducing levels of tau prevented Kcna1 (a sodium channel) deficiency-induced megencephaly and reduced seizure frequency and mortality (Holth et al., 2013). This suggested tau could also serve as target for antiepileptic drugs (Holth et al., 2013).

The findings that partially reducing levels of tau is also beneficial in preventing AD-like deficits and epileptiform activity is very encouraging (Roberson et al., 2007; Ittner et al., 2010; Roberson et al., 2011; Holth et al., 2013). What is also encouraging regarding the prosperity of tau as drug target is that tau knockout mice are largely normal, despite all proposed critical functions of tau based on in vitro studies. All four different lines of tau knockout mice are viable and no overt abnormalities have been reported (Harada et al., 1994; Dawson et al., 2001; Tucker et al., 2001; Fujio et al., 2007). Given this, tau reduction could be a potential treatment strategy for AD and other conditions with epileptiform activity.

Unanswered questions on tau as target for Alzheimer disease and other conditions with epileptiform activity

*The safety of long-term tau reduction*

Patients affected by AD usually live as long as 10 years or even longer after diagnosis. In this regard, any treatment for AD could be potentially administered for a long period of time. Therefore the long-term effects of any potential treatment need to be evaluated beforehand in animal models. Though no overt abnormalities were found in young tau knockout mice, possible detrimental effects due to loss of tau could accumulate and become evident over time with
ageing. Indeed, aged mice with no tau protein were recently reported to develop parkinsonism including impairment in motor functioning, neuronal loss, and perturbation in dopamine and iron homeostasis (Lei et al., 2012). Although complete loss of tau protein or function is unlikely to happen due to any tau-directed treatment, this report does prompt us to carefully evaluate the safety of long-term tau reduction, particularly long-term- and partial-tau reduction.

The efficacy and safety of tau reduction in adulthood

Up to this point, all manipulations of tau levels in mice were achieved through germline knockout, a technique that reduces or eliminates tau protein from conception, which conceivably could result in compensatory changes during development. Indeed, changes in expression of MAP1A, another member of microtubule associated protein, and microtubule organization and stability in small-calibre neurons were observed in young tau knockout mice, which is likely due to change in neurodevelopment (Harada et al., 1994). Also, the prevention of Kcna1–deficiency-induced megencephaly by tau reduction happens during neurodevelopment (Holth et al., 2013). Although this coincidence in timing does not necessarily mean this prevention is a developmental effect, it does raise the possibility that conventional tau knockout does have certain developmental effects. The developmental effects of germline tau knockout have implications with both the efficacy and safety of tau reduction as treatment.

On the efficacy of tau reduction, it could be these very developmental effects that confer resistance to Aβ peptides and excitotoxins later (Ashe, 2007). Meanwhile, tau-directed treatment will only be administered into adults and may no longer be beneficial when those compensatory changes of germline knockout of tau will conceivably not in place.
On the safety of tau reduction, the change of MAP1A protein expression and other unreported changes after loss of tau protein could be a compensatory mechanism that ensures normal function of brain in later adulthood. When tau-direct treatment acutely reduces level of tau protein or block the functions of tau in adulthood, when such compensatory mechanism will unlikely be in place, detrimental effects undetectable in germline tau knockout could become evident.

In summary, the long term safety of tau reduction and efficacy and safety of tau reduction in adulthood need to be further evaluated before targeting tau as an actual treatment. This thesis study is set to fill this gap and pave the foundation for development of tau-directed medications.
Seizure Resistance without Parkinsonism in Aged Mice after Tau Reduction

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ABSTRACT

Tau is an emerging target for Alzheimer disease and other conditions with epileptiform activity. Genetic tau reduction (in \( Tau^{+/}\) and \( Tau^{-/}\) mice) prevents Alzheimer-like deficits and has an excitoprotective effect, increasing resistance to seizures, without causing apparent neuronal dysfunction. However, most of these studies were conducted in mice less than one year of age and the effects of tau reduction in aged mice are less clear. Specifically, whether the seizure-protective effects of tau reduction persist with aging is unknown and whether tau reduction causes major neuronal dysfunction, including parkinsonism, with aging is controversial. Here we performed a comprehensive analysis of two-year-old \( Tau^{+/+} \), \( Tau^{+/}\), and \( Tau^{-/-} \) mice. In aged mice, tau reduction still conferred resistance to pentylenetetrazole-induced seizures. Moreover, tau reduction did not cause parkinsonian abnormalities in dopamine levels or motor function and did not cause iron accumulation or impaired cognition, although \( Tau^{-/-} \) mice had mild hyperactivity and decreased brain weight. Importantly, the excitoprotective effect in aged \( Tau^{+/+} \) mice was not accompanied by detectable abnormalities, indicating that partially reducing tau or blocking its function may be a safe and effective therapeutic approach for Alzheimer disease and other conditions with increased excitability.
INTRODUCTION


While tau reduction is beneficial and does not cause major neuronal dysfunction in mice up to one year of age (Ikegami, Harada et al. 2000, Dawson, Ferreira et al. 2001, Tucker, Meyer et al. 2001, Roberson, Scearce-Levie et al. 2007, Lei, Ayton et al. 2012), the long-term effects of tau reduction have been less well studied. It is unclear whether the beneficial effects of tau reduction persist and whether long-term tau reduction has detrimental effects with aging. In fact, $\text{Tau}^{+/-}$ mice were recently reported to develop parkinsonism after one year, including impaired motor coordination, reduced dopamine levels, and neuronal loss related to iron accumulation (Lei, Ayton et al. 2012). However, others have been unable to reproduce these findings (Morris, Hamto et al. 2013), and it is unknown whether the previously described excitoprotective effects
persist with aging. To address these questions regarding the long-term effects of tau reduction, we performed a comprehensive analysis of two-year-old $\text{Tau}^{+/+}$, $\text{Tau}^{+/−}$ and $\text{Tau}^{−/−}$ mice.

**METHODS**

*Animals*

The tau knockout mice were originated from a line developed by Dr. Dawson (Dawson, Ferreira et al. 2001). While mice used in a prior study of Lei et al (2012) were on a mixed background of 129/Sv and C57BL/6, and control mice in that study seemed have been bred separately; mice used in this study were on a congenic C57BL/6J background, and heterozygous tau knockout mice ($\text{Tau}^{+/−}$) were bred to generate littermate $\text{Tau}^{+/+}$, $\text{Tau}^{+/−}$ and $\text{Tau}^{−/−}$ mice. Mice were then aged to around 2 years. As in younger mice, aged $\text{Tau}^{+/−}$ mice had about 50% tau protein of $\text{Tau}^{+/+}$ mice while $\text{Tau}^{−/−}$ mice had no detectable tau protein when checked by western blot (Figure S1).

Both male and female mice were used in experiments and no sex-dependent effects were observed. Mice were housed in a pathogen-free barrier facility on a 12-hour light/dark cycle with ad libitum access to water and food (NIH-31 Open Formula Diet, #7917, Harlan). For postmortem analyses, mice were anesthetized by Fatal-plust (Vortech) and perfused with 0.9% saline. Brains were then removed, weighed, and dissected for processing as described below. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

*Pentylenetetrazole-induced seizures*

Pentylenetetrazole (PTZ)-induced seizure susceptibility was measured as previously described (Roberson, Halabisky et al. 2011). Briefly, PTZ (Sigma) was dissolved in PBS at a
concentration of 4 mg/ml. PTZ at dose of 40 mg/kg body weight was injected intraperitoneally. Immediately after PTZ injection, each mouse was placed into a cage for 20 minutes and observed by an investigator blind to its genotype, with video recording. The following scale of seizure stages was used: 0, normal behavior; 1, immobility; 2, generalized spasm, tremble, or twitch; 3, tail extension; 4, forelimb clonus; 5, generalized clonic activity; 6, bouncing or running seizures; 7, full tonic extension; 8, death (Racine 1972, Loscher, Honack et al. 1991). The latency to reach each stage and the maximum stage each mouse reached were recorded.

Dopamine measurement

Immediately after sacrifice and perfusion, the striatum was dissected and a punch was removed using a Pasteur pipette. The sample was flash frozen on dry ice and dopamine levels were determined by high-performance liquid chromatography at the Vanderbilt Neurochemistry Core Lab.

Behavioral tests

For all behavioral tests, experiments were carried out during light cycle at least one hour after the lights came on. All mice were transferred to testing room for acclimation at least one hour prior to experiments. Testing apparatuses were cleaned by 75% ethanol between experiments and disinfected by 2% chlorhexidine after experiments were finished each day. All mice were tested in all the behavioral tests in the same order. Mice became sick or injured during the course of testing were not tested in the following tests. Investigators were blind to the genotype of individual mouse at the time of experiments.
**Rotarod**

Mice were placed onto an accelerating rod (4–40 rpm over 5 min; Med Associates). The duration that each mouse remained on the rod was recorded. Each mouse was tested 5 times with at least 4 hours between each test and the average of 5 trials was reported.

**Pole test**

A wooden pole (3/8 inch diameter, 2 feet long) was placed perpendicular to ground into a cage with 1 inch deep bedding. Rubber bands were wrapped around the pole every 1.5 inches to provide grip. Each mouse was placed on the top of pole facing downwards and then released. All mice were trained for five trials on the first day and then tested with 5 consecutive trials on the next day. The amount of time each mouse needed to walk down into the cage was recorded and the best (shortest) trial test was reported.

**Beam walk test**

A 1-yard long narrow beam was placed on two stands, with one end 15 cm off the ground and the other end 20 cm off the ground. A bright light was placed close to the lower end of beam, and a container with food pellets was placed at the higher end. The ceiling lights in the testing room were turned off during testing. When put at the lower end that is close to the bright light, a mouse usually walks as fast as it can to the other end of the beam. Training started with three consecutive trials on a 1.25-inch diameter beam, followed a week later by three consecutive trials on a 1-inch diameter beam. Testing was conducted a week after the end of training and consisted of three consecutive trials on a 0.75-inch diameter beam. The time it took each mouse to cross the beam was measured based on recorded video. The average of the three trials on the 0.75-inch beam was reported.
**Open field**

Each mouse was placed into the corner of an open field apparatus (Med Associates) and allowed to walk freely for 15 minutes. Minute by minute ambulatory distance and rearing (vertical counts) of each mouse were determined using the manufacturer’s software. Average velocity of each mouse was manually calculated by dividing total ambulatory distance by total ambulatory time.

**Y-Maze**

The Y-Maze apparatus consisted of three 15-inch long, 3.5-inch wide and 5-inch high arms made of white opaque plexiglass placed on a table. Each mouse was placed into the hub and allowed to freely explore for 6 minutes, with video recording. An entry was defined as the center of mouse body extending 2 inch into an arm, using tracking software (CleverSys). The chronological order of entries into respective arms was determined. Each time the mouse entered all three arms successively (e.g. A-B-C or A-C-B) was considered a set. Percent alternation was calculated by dividing the number of sets by the total number of entries minus two (since by definition, the first two entries cannot meet criteria for a set). Mice with 12 or fewer total entries were excluded from spontaneous alternation calculations due to insufficient sample size.

**Contextual fear conditioning**

Experiments were conducted in Quick Change Test chambers (Med Associates). For the acquisition of fear memory, each mouse was trained by the following protocol: 2 minute acclimation to the chamber, three mild foot shocks (0.5 mA, 2 seconds duration, 1 minute apart) each co-terminating with a 20-second auditory cue (white noise, 75 dB), and 3 minutes of association time after the last foot shock. For testing, each mouse was returned to the same chamber 24 hours after training and video-recorded for 5 minutes. The percentage of time
freezing was calculated using the manufacturer’s software. The following parameters were used to calculate the percent of freezing of each mouse: Motion Threshold, 20; Min Freeze Duration, 1s; Method, linear.

Iron measurement

A triple quadrupole ICP-MS (Agilent 8800 ICP-QQQ) instrument was utilized for its enhanced sensitivity and interference control in MS/MS mode. Samples were first digested in ultra-pure nitric acid, hydrogen peroxide and hydrochloric acid, and then diluted to volume with deionized water. The resulting digestate was then introduced to the instrument via a quartz concentric nebulizer and a Scott spray chamber. Standards and samples were analyzed in MS/MS mode, monitoring multiple Fe masses (m/z) to include 54, 56, and 57. In MS/MS, or mass shift mode, the precursor ion is reacted with oxygen under vacuum resulting in the formation of a product ion (i.e. Fe-54 + O-16 = FeO-70). For reporting purposes, $^{57}\text{Fe}^{16}\text{O}$ (73 m/z) was chosen as the reportable mass. Prior to analysis, a ten point calibration was performed along with QA/QC samples to verify the accuracy of the resulting curve. A minimum of one aliquot of each tissue type was analyzed in duplicate, in addition to a matrix spike, to verify analytical precision and accuracy in this sample matrix. To monitor instrument stability, a Continuing Calibration Verification (CCV) and Continuing Calibration Blank (CCB) was analyzed evenly in ten replicates to confirm accuracy and background. The resulting sample concentrations were then reported as μg of iron per gram of wet tissue (PPM).

Statistical analysis

Data are presented as mean +/- standard error of the mean. Statistical analyses were performed using Prism 6.0 (GraphPad). Each dataset was evaluated for normality. Normal datasets were evaluated using ANOVA with post hoc Dunnett’s multiple comparison test vs.
wild-type. Nonparametric datasets were evaluated using the Kruskal-Wallis test and Dunn’s multiple comparison test vs. wild-type. The survival data was analyzed by Kaplan-Meier statistics and post-hoc Log-rank (Mantel-Cox) test. A threshold of $p < 0.05$ was considered significant.

RESULTS

Excitoprotective effects of tau reduction persist in aged mice

Aberrant excitatory neuronal activity induced by high levels of Aβ contributes to downstream cognitive impairment and other AD-like deficits in mice (Palop and Mucke 2010). Because tau reduction in young mice confers resistance to seizures induced by PTZ (Roberson, Scearce-Levie et al. 2007, Ittner, Ke et al. 2010), kainate (Palop, Chin et al. 2007, Roberson, Scearce-Levie et al. 2007), Aβ (Roberson, Halabisky et al. 2011), and potassium channel dysfunction (Holth, Reed et al. 2011), decreasing neuronal excitability is considered a possible mechanism by which tau reduction prevents AD-like deficits in mouse models. To determine whether these excitoprotective effects of tau reduction seen in younger mice persist in aged mice, we evaluated seizure susceptibility of $\text{Tau}^{+/+}$, $\text{Tau}^{+-}$ and $\text{Tau}^{-/-}$ mice at 23–25 months.

We used the PTZ-induced seizure model. In this model, mice progress through stereotypic stages of seizure severity. The latency to each stage and the maximum stage reached are indications of the susceptibility to seizures. In young mice, tau reduction increases latency and decreases maximal seizure stage (Roberson, Scearce-Levie et al. 2007, Ittner, Ke et al. 2010). After intraperitoneal injection of PTZ, aged $\text{Tau}^{+/+}$ and $\text{Tau}^{+-}$ mice had longer seizure latencies than $\text{Tau}^{+/+}$ mice (Fig. 1A). Tau reduction also reduced the maximal seizure stage in aged mice (Fig. 1B). Thus, the excitoprotective effects of tau reduction previously observed in young mice persist in aged mice.
Absence of parkinsonian phenotypes in aged mice with tau reduction

Next we evaluated potential adverse effects of tau reduction with aging. There were no differences in survival into old age between \(\text{Tau}^{+/+}\), \(\text{Tau}^{+/-}\), and \(\text{Tau}^{-/-}\) mice (Fig. 2).

A prior study reported parkinsonism (dopamine deficiency and motor deficits including decreased duration remaining on accelerating rod, slower descent from the top a thin pole and decreased travel distance in an open field) associated with iron accumulation in aged (\(\geq 12\) months) \(\text{Tau}^{-/-}\) mice (Lei, Ayton et al. 2012), but these findings were not reproduced by another group (Morris, Hamto et al. 2013). We first measured levels of dopamine and its metabolite, homovanillic acid (HVA), in the striatum of aged mice. Striatal dopamine and HVA levels were not different from \(\text{Tau}^{+/+}\) mice in both aged \(\text{Tau}^{+/-}\) and \(\text{Tau}^{-/-}\) mice (Fig. 3). We next examined motor function using rotarod, pole test and open field which were used in the prior study of (Lei, Ayton et al. 2012) plus beam walk which is also a common test for motor function. On the accelerating rotarod, aged \(\text{ Tau}^{+/-}\) and \(\text{ Tau}^{-/-}\) mice had performance not different from \(\text{ Tau}^{+/+}\) mice(Fig. 4A). On the pole test, aged \(\text{ Tau}^{+/-}\) mice were not different and aged \(\text{ Tau}^{-/-}\) mice actually descended slightly faster than \(\text{ Tau}^{+/+}\) mice (Fig. 4B), opposite the slower descent expected with parkinsonism. On the beam walk, aged \(\text{ Tau}^{+/-}\) and \(\text{ Tau}^{-/-}\) mice had no abnormalities in time to cross (Fig. 4C). Finally, in the open field, ambulation was not different from \(\text{ Tau}^{+/+}\) mice in both aged \(\text{ Tau}^{+/-}\) and \(\text{ Tau}^{-/-}\) mice, while aged \(\text{ Tau}^{-/-}\) mice reared more than normal (Fig. 4D–F).

Age-appropriate cognitive function in aged mice with tau reduction

We next examined the long-term effects of tau reduction on cognition. We first evaluated short-term working memory using the Y maze. Because of their tendency to explore novel environments, mice freely exploring the Y maze tend to alternate between the three arms, which
requires intact short-term working memory. There were no differences in percent alternation between aged $\text{Tau}^{+/+}$, $\text{Tau}^{+/−}$, and $\text{Tau}^{−/−}$ mice (Fig. 5A).

We next evaluated long-term spatial associative memory using classical fear conditioning. When a mouse enters an environment in which it previously had an aversive experience (mild foot shock), it tends to freeze as a manifestation of fear. Thus, percent time spent freezing is a gauge of spatial memory. Aged $\text{Tau}^{+/−}$ and $\text{Tau}^{−/−}$ mice exhibited level of fear conditioning comparable to $\text{Tau}^{+/+}$ mice, indicating no deficit in long-term associative memory (Fig. 5B).

Iron levels comparable to control in aged mice with tau reduction

Our behavioral and neurochemical results differ from those of Lei et al (2012), who observed parkinsonism and cognitive impairment due to iron accumulation in aged $\text{Tau}^{−/−}$ mice. One difference between the studies was genetic background; our mice were congenic C57BL/6 while those of Lei et al. were on a mixed C57BL/6 and Sv129 background. C57BL/6 mice are reportedly resistant to iron overload–induced cardiotoxicity (Musumeci, Maccari et al. 2013). This raises two possible explanations for the discrepancy in results: either our mice do not have iron accumulation, or they are resistant to the neurological effects of iron accumulation due to their greater proportion of C57BL/6 background. To distinguish between these possibilities, we measured total iron levels in several brain regions. There were no abnormalities in iron levels in cortex, hippocampus, or substantia nigra in aged $\text{Tau}^{+/−}$ or $\text{Tau}^{−/−}$ mice (Fig. 6). Thus, in our aged $\text{Tau}^{−/−}$ mice on a congenic C57BL/6 background, tau reduction does not lead to iron accumulation.
Age-independent decrease in brain weight of Tau\(^{-/-}\) mice

Finally, we examined the effects of long-term tau reduction on brain size. Aged Tau\(^{-/-}\) mice were reported to have decreased gross brain weight and enlarged ventricles (Lei, Ayton et al. 2012). Consistent with those findings, we also observed a small (8%) but significant reduction in total brain weight in aged Tau\(^{-/-}\) mice (Fig. 7A). This was not due to a generalized size difference, as there were no differences in total body weight (Fig. 7B). Brain weight was not decreased in aged Tau\(^{+/+}\) mice (Fig. 7A).

Because the behavioral phenotype we observed in aged Tau\(^{-/-}\) mice (mild hyperactivity) had been previously described in younger mice (Ikegami, Harada et al. 2000) along with some subtle developmental changes (Harada, Oguchi et al. 1994), we asked whether the changes in brain weight were in fact age-dependent. We measured brain weight in a cohort of young (2.5-month-old) mice and observed a similar small but significant decrease in brain weight in Tau\(^{-/-}\) mice, with no change in Tau\(^{+/+}\) mice (Fig 7C). Again, the decrease in brain weight in young Tau\(^{-/-}\) mice was independent of a body weight difference (Fig. 7D). There was no significant interaction between tau and age on brain weight (F (2,97) = 0.9252, \(p = 0.44\)), suggesting that the reduced brain weight in Tau\(^{-/-}\) mice may be more likely a neurodevelopmental than neurodegenerative effect.

To further test whether decrease in brain weight of aged mice is a neurodevelopmental or neurodegenerative effect, we measured the sizes of lateral ventricle at Bregma 0.26mm of both aged. If the decrease of brain weight is a result of neurodegeneration, then the size of lateral ventricle will be larger due to shrinkage of surrounding regions. In aged Tau\(^{-/-}\) mice, size of lateral ventricle is significantly smaller than Tau\(^{+/+}\) mice (42% of wild type control); sizes of cortex, caudate and entire section are also smaller than Tau\(^{+/+}\) mice to a lesser degree (85–88%
of wild type) (Figure 7E). In young $\text{Tau}^{-/-}$ mice, the sizes of lateral ventricle and cortex are trending smaller than $\text{Tau}^{+/+}$ mice (Data not shown).

In summary, we observed an age-independent decrease of brain weight in $\text{Tau}^{-/-}$ mice and the decrease is more likely a result of neurodevelopment than neurodegeneration.

**DISCUSSION**

The goal of this study was to determine if the excitoprotective effects of tau reduction seen in younger mice persist with aging and if they are accompanied by significant adverse effects. We found that aged (>22-month-old) $\text{Tau}^{-/-}$ mice were resistant to seizures, just as younger $\text{Tau}^{-/-}$ mice are. Moreover, while the aged $\text{Tau}^{-/-}$ mice had mild hyperactivity as previously described in younger $\text{Tau}^{-/-}$ mice, we found no behavioral or neurochemical evidence of parkinsonism and no cognitive deficits. Perhaps even more important are our findings in $\text{Tau}^{+/-}$ mice with ~50% tau protein of $\text{Tau}^{+/+}$ mice. Aged $\text{Tau}^{+/-}$ mice were also resistant to seizures and did not have even the relatively minor abnormalities seen in aged $\text{Tau}^{-/-}$ mice. Thus, partial tau reduction is excitoprotective without adverse effects, even over a span of almost two years, most of the lifetime of the mouse.

There have been conflicting reports on adverse effects in aged $\text{Tau}^{-/-}$ mice. Lei et al (2012) found iron accumulation, dopaminergic cell loss, parkinsonian motor impairment, and Y-maze memory deficits whereas Morris et al. (Morris, Hamto et al. 2013) found only mild motor impairment with dopamine levels and cognition comparable to $\text{Tau}^{+/+}$ mice. Our study, finding only mild hyperactivity in aged $\text{Tau}^{-/-}$ mice but no parkinsonism or cognitive impairment, is more consistent with Morris et al. (Morris, Hamto et al. 2013). The reason for the discrepancy between studies is not fully clear, since all three studies examined mice from the same origin.
(Dawson, Ferreira et al. 2001) using many of the same tests. One reason could be the difference in genetic background; the mice in both Morris et al. (Morris, Hamto et al. 2013) and our study were on a congenic C57BL/6J background, while those in Lei et al. (2012) were mixed C57BL/6 and 129/Sv. While C57BL/6 mice show some resistance to the cardiac effects of iron overload (Musumeci, Maccari et al. 2013), this would not explain the discrepant results, as there was no evidence of iron overload in either our study (Fig. 4) or in Morris et al. (Morris, Hamto et al. 2013). Another reason might be how the experimental and control mice were bred. The strength of both Morris et al. (Morris, Hamto et al. 2013) and our study is the strategy of breeding $\text{Ta}^{+/−}$ mice to generate littermates of the three genotypes, enabling direct comparison without concerns of genetic drift between separate colonies of $\text{Ta}^{+/+}$ and $\text{Ta}^{−/−}$ mice. Experimental and control mice in study of Lei et al. (2012) seemed have been bred separately.

$\text{Ta}^{−/−}$ mice are of interest because they provide a tool for studying the effects of tau loss of function, which has been considered one potential mechanism in tauopathies because phosphorylation and aggregation of tau may limit its ability to stabilize microtubules. However, for the most part, $\text{Ta}^{−/−}$ mice are surprisingly not that different from $\text{Ta}^{+/+}$ mice, without abnormalities in gross morphology and expression of synaptic and astrogliosis marker (Dawson, Ferreira et al. 2001, Tucker, Meyer et al. 2001), survival (Roberson, Scoearce-Levie et al. 2007 and Fig. 2). Although Deficits in long term depression was recently reported in $\text{Ta}^{−/−}$ mice(Kimura, Whitcomb et al. 2014), but the implication of this deficit in synaptic plasticity has yet to be understood because no abnormalities have been reported in learning and memory on a variety of tests including radial arm maze, Morris water maze, T-maze, Y-maze, novel object, and fear conditioning (Ikegami, Harada et al. 2000, Roberson, Scoearce-Levie et al. 2007, Ittner, Ke et al. 2010, Roberson, Halabisky et al. 2011, Morris, Hamto et al. 2013 and Fig. 5). The only
consistent observation in $\text{Tau}^{-/-}$ mice is mild motor deficits generally reflecting hyperactivity (Ikegami, Harada et al. 2000, Morris, Hamto et al. 2013 and Fig. 3), although it is interesting that tau reduction actually prevents – not exacerbates – the hyperactivity seen in human amyloid precursor protein (hAPP) transgenic models of AD (Roberson, Scearce-Levie et al. 2007). But the more important point to emphasize regarding tau loss of function as a potential disease mechanism is that in tauopathies, on top of contribution from gain of toxic function, any loss of tau function would be only partial, and partial loss of tau in $\text{Tau}^{+/-}$ mice has not been associated with abnormalities in any of these studies, even with aging. Thus, the data from studies of tau-deficient mice does not provide strong support for the loss-of-function hypothesis.

The induction of seizure resistance by tau reduction has emerged as one of the most consistent phenotypes in tau-deficient mice. $\text{Tau}^{-/-}$ and $\text{Tau}^{+/-}$ mice are resistant to epileptiform activity induced by hAPP/Aβ (Roberson, Scearce-Levie et al. 2007, Roberson, Halabisky et al. 2011), GABA antagonists (Roberson, Scearce-Levie et al. 2007, Ittner, Ke et al. 2010), glutamate agonists (Roberson, Scearce-Levie et al. 2007), and potassium channel mutations (Holth, Reed et al. 2011). Tau antisense oligonucleotides also induce seizure resistance (Devos, Goncharoff et al. 2013). In addition, hippocampal slices from $\text{Tau}^{-/-}$ mice are resistant to epileptiform bursting induced by bicuculline (Roberson, Halabisky et al. 2011). And the observation is not limited to mice, as tau-deficient flies were also resistant in two Drosophila seizure models (Holth, Reed et al. 2011). These findings suggest that tau plays an important role regulating neuronal excitability in the adult brain. The molecular basis of this effect, including whether it is related to or independent of tau’s microtubule-binding role, is a critical goal for future research.
ACKNOWLEDGEMENT

We thank James Black, Dheepa Sekar, and Miriam Roberson for help maintaining the mouse colony, genotyping, and tissue processing. We thank Dr. Ashley S. Harms for demonstrating substantia nigra dissection and Dr. Raymond Johnson of the Vanderbilt Neurochemistry Core lab for measuring biogenic monoamines. This work was supported by the NIH (R01NS075487), the BrightFocus Foundation, and a scholarship from the UAB Comprehensive Center for Healthy Aging.

APPENDIX

Supplemental data associated with this article can be found in the online version.

REFERENCE


**FIGURES**

![Graph A](image1.png) ![Graph B](image2.png)

**Fig. 1:** Excitoprotective effects of tau reduction persist in aged mice. 23–25-month-old mice were tested for susceptibility to PTZ-induced seizures ($N = 13$–20 mice per genotype). (A) Latency to each stage. Tau reduction increased the latency of PTZ-induced seizures (ANOVA, tau genotype x stage interaction, $p < 0.05$; * $\text{Tau}^{+/−}$ mice differed from $\text{Tau}^{+/+}$ at stages 5–8 and $\text{Tau}^{−/−}$ mice differed from $\text{Tau}^{+/+}$ mice at stages 6–8 on post hoc tests, $p < 0.05$). (B) Maximal seizure stage. Tau reduction decreased the severity of seizures (Kruskal-Wallis test, $p < 0.05$; * = $p < 0.05$, † $p = 0.078$ on post hoc tests).
Fig. 2: Normal survival in aging tau-deficient mice. Kaplan-Meier analysis showed no differences in spontaneous mortality between aging $\textit{Tau}^{+/+}$, $\textit{Tau}^{+/-}$, and $\textit{Tau}^{-/-}$ mice ($N = 44–97$ mice per genotype).
Fig. 3: Normal striatal dopamine levels in aging mice with tau reduction. There were no differences in levels of (A) dopamine (DA) or (B) the end product of its metabolism, homovanillic acid (HVA) measured in the striatum of mice aged 23–25 months ($N = 10–13$ mice per genotype).
Fig. 4: Tau reduction does not cause parkinsonism in aged mice. 22–24-month-old mice were tested for parkinsonian motor phenotypes on four tests (N = 13–22 mice per genotype). (A) Rotarod. There were no differences in the duration mice remained on the accelerating rod. (B) Pole test. There were no differences in \( \text{Tau}^{+/-} \) mice. \( \text{Tau}^{-/-} \) mice descended the pole more quickly than \( \text{Tau}^{+/+} \) controls (ANOVA, \( p < 0.01 \); * \( p < 0.05 \) on post hoc tests). (C) Beam walk. There were no differences in the time needed to cross. (D–F) Open field. (D) There were no differences in the ambulatory distance on minute by minute basis. (E) There were no differences in the average velocity. (F) There were no abnormalities in rearing in \( \text{Tau}^{+/-} \) mice, but aged \( \text{Tau}^{-/-} \) mice had more rearings than \( \text{Tau}^{+/+} \) controls (ANOVA, \( p < 0.01 \); * \( p < 0.05 \) on post hoc tests).
**Fig. 5:** Normal cognitive function in aged mice with tau reduction. 22–25-month-old mice were tested for short-term working memory and long-term spatial memory. (A) Y-Maze. There were no differences in percent alternation, a measure of short-term working memory ($N = 12–15$ mice per genotype). (B) Contextual fear conditioning. There were no differences in percent time freezing 24 hours after training ($N = 13–20$ mice per genotype).
Fig. 6: Normal levels of iron in brains of aged mice with reduced tau levels. There were no differences in total iron content in (A) cortex, (B) hippocampus, or (C) substantia nigra in 23–25-month-old mice ($N = 10–11$ mice per genotype).
Fig. 7: Age-independent decrease in brain weight of $\text{Tau}^{-/-}$ mice. (A) Brain weight was decreased in aged (23–25-month) $\text{Tau}^{-/-}$ mice (ANOVA, $p < 0.005$; ** $p < 0.005$ on post hoc tests), but not in $\text{Tau}^{+/-}$ mice ($N = 19–24$ mice per genotype). (B) There were no differences in body weight in aged tau-deficient mice. (C) Brain weight was decreased in young (2–5-month) $\text{Tau}^{-/-}$ mice (ANOVA, $p < 0.005$; *** $p < 0.005$ on post hoc tests), but not in $\text{Tau}^{+/-}$ mice ($N = 11–16$ mice per genotype). (D) There were no differences in body weight in young tau-deficient mice. (E) Sizes of different regions of brain sections at Bregma 0.26mm. Lateral ventricle, caudate, cortex and whole section were smaller in aged $\text{Tau}^{-/-}$ mice (ANOVA: $p = 0.0105$ in lateral ventricle, $p = 0.0213$ in caudate, $p = 0.0092$ in cortex, $p = 0.0175$ in whole section; # $p = 0.0847$, * $p < 0.05$ on post hoc tests), but not in $\text{Tau}^{+/-}$ mice ($N = 7–12$ mice per genotype).
Fig. S1: Tau levels in aged mice. Tau levels in frontal cortex of 23–25-month-old mice were measured by western blotting and quantified. \( \text{Tau}^{+/+} \) mice have ~50% of tau protein while \( \text{Tau}^{-/-} \) mice have no detectable tau protein (ANOVA, \( p < 0.0001 \); **** \( p < 0.0001 \) on post hoc tests; \( N = 10–11 \) mice per genotype).
Seizure Protective Effects of Tau Knockout in Adult Mice

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INTRODUCTION

Microtubule associated protein tau is emerging as a better alternative target for Alzheimer disease (AD) after a series failures of Aβ-directed clinical trials (Giacobini and Gold, 2013). Between two hallmark pathologies of AD, tau pathology (neurofibrillary tangles) is closely correlated to the onset and progression of memory deficits in AD patients (Serrano-Pozo et al., 2011); while Aβ-pathology (plaques) starts decades before and also reaches a plateau before onset of AD symptoms. Therefore, targeting tau might be more effective than Aβ-directed treatment.

Germline knockout of tau has been demonstrated to be beneficial and safe in mouse models of both AD and seizures. Genetically reducing tau in mice prevents impairment in learning and memory, epileptiform activity and other AD-like abnormalities in multiple mouse models of AD (Palop et al., 2007; Roberson et al., 2007; Meilandt et al., 2008; Andrews-Zwilling et al., 2010; Ittner et al., 2010; Roberson et al., 2011; Leroy et al., 2012; Nussbaum et al., 2012). Also, genetically reducing tau protects against both pharmaceutically induced seizure (Roberson et al., 2007; Ittner et al., 2010) and genetic model of seizure (Holth et al., 2013). Meanwhile, no overt abnormalities have been reported in young mice with germline knockout of tau (Ikegami et al., 2000; Dawson et al., 2001; Tucker et al., 2001; Roberson et al., 2007; Lei et al., 2012). Although parkinsonism like phenotype was recently reported in aged mice of germline knockout of tau (Lei et al., 2012), this finding was not reproduced in another two following studies (Morris et al., 2013) and might be artifact of genetic drift [the present study]. Therefore, reducing levels of tau has been proposed as a treatment for AD and other conditions with epileptiform activity.
What is unknown is whether tau reduction in adulthood is beneficial and safe. Tau is abundantly expressed since early on during embryonic development, reducing level of tau from conception could trigger compensatory changes in brain circuit development (Ashe, 2007; Holth et al., 2013). Indeed changes in the expression level of other microtubule associated proteins, microtubule organizations and gross brain weight have been reported in mice with germline knockout of tau (Harada et al., 1994; Holth et al., 2013). This type of changes in development could render resistance to excessive Aβ and excitotoxins, and ensure the absence of overt abnormalities. In adulthood, compensatory mechanism is largely unavailable. Therefore, acutely reducing tau in adulthood may or may not be beneficial and safe. To test these two possibilities, we developed a new line of inducible tau knockout mice (Feil et al., 2009), and performed a comprehensive analysis of the effects of adulthood knockout of tau in mice.

METHOD AND MATERIAL

Cloning of targeting vector

The targeting vector was constructed by recombineering as previously described (Liu et al., 2003). Briefly, a 9kb fragment spanning exon 1 of mouse tau allele was retrieved by homologous recombination into vector PL253 which contains the HSV-Thymidine kinase gene for negative selection. Homologous recombination was then used to target a loxP site 5’ of exon 1 and a FRT-neomycin-FRT-LoxP cassette 3’ of exon 1. A BamH I restriction site was introduced downstream of the 3’ loxP site to facilitate the southern blot analysis. The final targeting vector was confirmed by sequencing.

Gene targeting in embryonic stem cells

The targeting vector was linearized by restriction digest with the enzyme Not I, gel purified, and transfected into C57BL/6-derived embryonic stem cells by electroporation. The
embryonic cells that were resistant to antibiotic G418 (indicating integration of neomycin cassette) and not susceptible to ganciclovir (indicating the absence of HSV-TK cassette) were selected for further screening by Southern blot and PCR. Genomic DNA from embryonic stem cells were first digested with restriction enzyme BamH I and Sac I respectively, resolved on agarose gel, transferred under alkali conditions onto a hybond XL membrane (GE healthcare life science) and visualized by $^{32}$P labeled probes. The probes were labeled with Prime It primer labeling kit (Agilent) according to manufacturer’s instruction. A PCR based method confirmed the presence of the 5’ loxP site and ruled out the possibility of recombination between exon 1 of tau and 5’ frt site. The following two primers were used for PCR: F10, GCAATCACCTCCCTCCATAACTAC; R13, TCAGCATCCACACTAAAGCAGG.

**Animals**

All mice were housed in a pathogen-free barrier facility on a 12-hour light/dark cycle with ad libitum access to water and food (NIH-31 Open Formula Diet, #7917, Harlan). For postmortem analyses, mice were anesthetized by Fatal-plus (Vortech) and perfused with 0.9% saline. Brains were then removed, weighed, and dissected for processing as described below. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

**Generation of Tau$^{\text{flo}}$ mice**

Embryonic cells that passed southern blot and PCR analyses were further examined for their morphology, chromosome numbers and in mycoplasma test to rule out possible infection. Three clones that contained floxed tau allele were then selected, expanded, and used for blastocyst injection. Two ES clones were injected into albino C57BL/6 mice to produce black on white chimeras. Germline transmission of the floxed tau allele was confirmed by PCR analysis.
(with primer F10 and R13). Mice containing the floxed tau allele were crossed with flipase transgenic mice (Jax#: 009086) to delete the neomycin cassette. The deletion of neomycin cassette was confirmed by PCR analysis. Mice containing two loxP sites flanking exon 1 of tau allele are termed \( \text{Tau}^{\text{flox}} \) mice.

**Tamoxifen-induced tau adulthood knockout (tau aKO) in mice**

\( \text{Tau}^{\text{flox/flox}} \) mice were crossed with a CAGG-Cre-ER\(^{\text{TM}} \) transgenic mice (Jax#:004682, C57BL/6) (Hayashi and McMahon, 2002) to generate \( \text{CreER}^{\text{TM}}: \text{Tau}^{\text{flox/flox}} \) and \( \text{Tau}^{\text{flox/flox}} \) mice. \( \text{CreER}^{\text{TM}}: \text{Tau}^{+/+} \) and \( \text{Tau}^{+/+} \) mice were used as control for the effects of CreER\(^{\text{TM}} \) expression and activation. All mice were injected intraperitoneally with tamoxifen (Sigma) at dose of 160 mg/kg body weight at age and number of times as indicated in each figure. Tamoxifen was prepared in the following steps: 100mg tamoxifen powder was first dissolved in 10ml absolute ethanol with brief sonication, and then 90ml of corn oil (Sigma) was added. The resulted mixture was sonicated until it became completely clear. The resulted tamoxifen solution was then aliquoted and stored at \(-20^\circ\text{C}\) until used. During injection, 27G needles were used to minimize inflict to mice and back flow of tamoxifen solution.

**Behavioral testing**

All behavioral tests were carried out at least one hour after the beginning of light cycle. Mice were first brought into testing room for acclimation for at least 1 hour prior to experiments. Testing apparatuses were cleaned by 75% ethanol between experiments and disinfected by 2% chlorhexidine after experiments were finished each day. Investigators were blind to the genotype of individual mouse at the time of experiments. Both male and female mice were used, and no sex-dependent effects were observed.
**Roto rod**

Mice were placed onto an accelerating rod (4–40 rpm in 5 min; Med Associates). The duration that each mouse remained on the rod was recorded. Each mouse was tested in two sessions on the same day with 4 hours break between each session. During each session, each mouse was tested in three consecutive trials. The group average for each trial was reported.

**Pole test**

A wooden pole (3/8 inch diameter, 2 feet long) was placed perpendicular to ground into a cage with 1 inch deep bedding. Rubber bands were wrapped around the pole every 1.5 inches to provide grip. Each mouse was placed on the top of pole facing downwards and then released. All mice were trained for five trials on the first day and then tested with 5 consecutive trials on the next day. The amount of time each mouse needed to walk down into the cage was recorded and the best (shortest) trial test was reported.

**PTZ-induced seizure**

Pentylenetetrazole (PTZ)-induced seizure susceptibility was measured as previously described (Roberson et al., 2011). Briefly, PTZ (Sigma) was dissolved in PBS at a concentration of 4 mg/ml. PTZ at dose of 40 mg/kg body weight was injected intraperitoneally. Immediately after PTZ injection, each mouse was placed into a cage for 20 minutes and observed by an investigator blind to its genotype, with video recording. The following scale of seizure stages was used: 0, normal behavior; 1, immobility; 2, generalized spasm, tremble, or twitch; 3, tail extension; 4, forelimb clonus; 5, generalized clonic activity; 6, bouncing or running seizures; 7, full tonic extension; 8, death (Racine, 1972; Loscher et al., 1991). The latency to reach each stage and the maximum stage each mouse reached were recorded.
Kainic–acid-induced electroencephalographic (EEG) seizure

Surgical Procedure: All mice were anesthetized and maintained with 2.5% isofluorane. Six burr holes were drilled bilaterally through the skull, 2, 4, and 6 mm posterior to Bregma, and 2, 4, and 2 mm lateral to midline, respectively, using a dental drill equipped with a 1.0 mm drill bit. Three 1.6 mm stainless steel screws (Small Parts, Inc.) were screwed halfway into alternate holes. Then, an EEG electrode (Plastics One, Inc.) with 2 lead wires and a ground wire, cut to ~1.5 mm, was inserted into the remaining three drill holes. The lead wires were placed bilaterally to record from each hemisphere. Dental acrylic was then applied to form a stable cap on the skull that cements the electrode in place. When the acrylic became dried, the scalp was closed with skin glue (3M Vetbond).

EEG/video recording: One week after electrode implantation, mice were transferred to specially constructed EEG monitoring cages where they were single housed. EEG data was acquired using Biopac Systems amplifiers (Biopac EEG100C) and AcqKnowledge 4.2 EEG Acquisition and Reader Software (BIOPAC Systems, Inc.). Data was stored and analyzed in digital format. Each cage was also equipped with an IR Digital Color CCD camera (Digimerge Technologies) that records each animal for the duration concurrent with EEG monitoring; recordings were acquired for review using security system hardware and software (L20WD800 Series, Lorex Technology, Inc.). All collected data was visually screened for epileptic events by an experienced observer blinded to genotype or treatment parameter. Abnormalities in the recordings indicative of epileptic activity were aligned chronologically with the corresponding video in order to confirm spiking or seizures.

Kainic acid injection: kainic acid (Tocris) was dissolved in PBS at concentration of 2mg/ml with intensive sonication. Of note, NaOH was not added to increase solubility of kainic
acid. Kainic acid was injected intraperitoneally into each mouse at dose of 17.5mg/kg body weight. Immediately after injection, mice were returned to recording chamber and recorded for at least 24 hours.

**Western blot**

Flash frozen brain hemispheres were dissected in hippocampus and cortex, and then homogenized in Tris-buffered salt with protease and phosphatase inhibitors. The homogenate was then centrifuged twice at max speed for 20 minutes and supernatant was transferred into new ependorf tube after each centrifuge. Equal amount of protein was then loaded onto 4-12% Bis-Tris gel (Invitrogen), transferred onto PVDF-FL membrane (Minipore), blotted with either antibody against tau or a-tubulin, and visualized on Licor imager.

**Data analysis**

Data are presented as mean +/- standard error of the mean. Statistical analyses were performed using Prism 6.0 (GraphPad). Each dataset was evaluated for normality. Normal datasets were evaluated using ANOVA with post hoc Dunnett’s multiple comparison test vs. control (tamoxifen injected Tauflox/flox mice). Nonparametric datasets were evaluated using the Kruskal-Wallis test vs. control. A threshold of p < 0.05 was considered significant.

**RESULTS**

**Generation of Tauflox mice**

To avoid developmental effects of germline knockout of tau, we utilized tamoxifen-CreER™ inducible knockout system (Feil et al., 2009). In this system, the timing of tau knockout can be controlled by the timing of tamoxifen injection. Upon injection tamoxifen, CreER™ protein translocates into nucleus, excises Tau allele from mouse genome, thus shuts
down the expression of tau protein. While CerER\textsuperscript{TM} transgenenic mice are readily available from Jaxon lab, we developed \textit{Tau}\textsuperscript{flox} mice in which tau allele is flanked by two loxP sequences. Two loxP sequences together with frt flanked selection marker neomycin cassette were introduced into tau allele through homologous recombination (Figure 1A). After electroporation of targeting vector, embryonic stem cells that went through homologous recombination were screened and confirmed with southern blot and a tertiary PCR analysis (Figure 1B-1D). Selection marker neomycin cassette was removed by crossing germline transmission mice with \textit{Flp} transgenic mice. The removal was confirmed with PCR analysis against neomycin sequence (Figure 1E). To confirm that \textit{Tau}\textsuperscript{flox} allele can be excised by active Cre recombinase, a new tau knockout line was generated by breeding \textit{Tau}\textsuperscript{flox} and beta-actin driven Cre transgenic mice. The excision of \textit{Tau}\textsuperscript{flox} allele was confirmed by PCR (figure 1F).

\textit{Rapid and wide-spread reduction of tau induced by tamoxifen}

We next tested the feasibility of tamoxifen to induce knockout of tau in the newly developed \textit{Tau}\textsuperscript{flox} mice. The presence of loxP and frt sequences in the vicinity of exon 1 of tau allele does not affect the expression level of tau and CreER\textsuperscript{TM} does not decrease tau expression in the absence of tamoxifen (data not shown). 10 or 15 daily injection of tamoxifen reduced about 80\% of tau protein in both cortex and hippocampus (Figure 2A). The levels of tau were reduced by 50\% in 6-8 days starting from the first injection of tau (Figure 2B). Therefore, tamoxifen-induced knockout of tau mimics acute reduction of tau as a treatment. While 10 or more daily injection of tamoxifen induces consistent reduction of tau expression, 5 daily injection of tamoxifen induced considerable amount of tau reduction but in a more variable fashion (Figure 12B); and 20 daily injection of tamoxifen did not further increase the extent of tau reduction (data not shown).
The safety of tau adulthood knockout

We then evaluated the safety of tau adulthood knockout. Tau is abundantly expressed in adult brain, kidney, lung, and testis (Gu et al., 1996). Though the function of tau in adulthood is largely unknown, acutely reducing levels of tau could possibly cause overt abnormalities like death when compensatory mechanism is largely unavailable. Also, germline knockout of tau was reported to decrease gross brain weight and to impair motor coordination. We therefore looked into possible mortality, brain weight decrease, and motor impairment.

We first analyzed the mortality rate in Tau aKO mice and mice with CreER\(^\text{TM}\) activation (tamoxifen-injected \textit{CreER}^\text{TM}.\textit{Tau}^{+/+} mice). Similar to a prior report (Higashi et al., 2009), activation of \textit{CreER}^\text{TM} by tamoxifen induced about 20% of mortality. On top of the toxicity of \textit{CreER}^\text{TM} activation, adulthood knockout of tau didn’t cause additional mortality (Figure 3A). We also measured the brain weight of Tau aKO mice. While decrease in brain weight is evident in 3-month old mice with germline knockout of tau, no such decrease was observed in mice with 5month long adulthood knockout of tau (Figure 3B). This confirmed our prior argument that the decrease in brain weight is most likely a developmental effect of germline knockout of tau.

We finally tested Tau aKO mice in two motor coordination assay to see if they develop parkinsonism as reported in aged germline knockout of tau (Lei et al., 2012). The performance of Tau aKO mice was indistinguishable from control in both pole test and rota rod.

In summary, we conclude that Tau aKO does not cause overt abnormalities in mice, similar to germline knockout of tau. The lack of abnormalities in tau knockout mice is probably not due to development compensation.
Excitoprotective effects of Tau adulthood knockout

We lastly evaluated the resistance of Tau aKO mice to excitotoxins-induced seizures. We first challenged Tau aKO mice with pentylenetetrazol (PTZ), an antagonist of GABA receptor. Upon intraperitoneal injection of PTZ, mice rapidly go through several stereotypical stages of behavioral manifestation of seizure; the worst stage a mouse reaches and the latency to reach each stage serve as gauge of resistance to PTZ. After injection of PTZ, Tau aKO mice reached a less sever stage of seizure (Figure 4A), and also the latency for Tau aKO mice to reach different sates was significantly longer (Figure 4B), suggesting higher resistance to PTZ-induced seizure in Tau aKO mice. These effects were not due to the activation of CreER\textsuperscript{TM}, because no difference in both the worst stage and latency to each stage was observed between control and mice with CreER\textsuperscript{TM} activation (Figure 4C-4D).

We next evaluated the resistance of Tau aKO mice to kainic–acid-induced seizure. Kainic acid is cyclic analog of L-glutamate and an agonist of ionotropic kainic receptors. Intraperitoneal injection of kainic acid induces incidental death and in survived mice robust long lasting epileptiform discharges which can be monitored through subdural electroencephalographic recording (Buckingham et al., 2011). To reflect both death and seizures of survived mice, we developed a clinical score scale based on the latency to death and duration of seizure (Table 1). The clinical scores were less severe in Tau aKO mice (Figure 5A). Also, among Tau aKO mice, the severity was correlated with the residual level of tau protein (Figure 5B) with the higher level of tau the severer seizure is.

In summary, Tau aKO conferred resistance to both PTZ and kainic-acid-induced seizures, suggesting an excitoprotective effect of tau adulthood knockout.
DISCUSSION

This study was aimed to evaluate the safety and efficacy of tau reduction in adulthood as treatment for Alzheimer disease and other conditions with epileptiform activity. Taking advantage of an inducible knockout system, we were able to reduce the level of tau in adult mice by up to 80% while maintaining normal level of tau during development, therefore avoiding developmental effects associated with germline knockout of tau. We demonstrated that persistent reduction of tau in adulthood does not impair motor functioning of mice, decrease brain weight, and does not incur additional death on top of mortality carried over from activation CreER™. We also demonstrated that tau reduction in adulthood increased seizure resistance in two different pharmaceutically-induced seizure models. Given the close relationship between epileptiform activity and pathogenesis of AD (Palop et al., 2007; Palop and Mucke, 2009), we argue that tau reduction can be a safe and effective treatment for Alzheimer disease and other conditions with epileptiform activity.

This study confirmed findings from a recent study on adulthood knock-down of tau by antisense oligoes. Devos et al. demonstrated that transient reduction of tau from neuronal system by antisense oligoes conferred resistance to PTX-infusion-induced EEG activity and PTZ-induced global seizures, without affecting baseline behavior (Devos et al., 2013). Here, through a genetic approach, we confirm that persistent and systematic reduction of tau in adult mice also confers resistance to PTZ-induced global seizures and kanic–acid-induced epileptiform activity in EEG recording. Devos et al. also demonstrated that levels of residual tau positively correlate with severity of PTZ-induced seizures and frequency of PTX-induced EEG spike. We didn’t observe this correlation in PTZ-induced seizures. This is most likely due to the difference in dosages of PTZ (40mg/kg body weight in this study vs. 55mg/kg and 80mg/kg in Devos et al.).
We used a lower dosage and observed less variable seizure severity in both control and Tau aKO mice. But we did observe a positive correlation between residual levels of tau and kainic acid induced epileptiform activity. In this experiment, the seizures were much more severe, and a lot of mice died. This prompts us argue that the correlation between tau level and resistance to seizures may be more evident in severer seizures.

This study also highlighted the in vivo function of tau in regulating neuronal excitability. Based on early experiments in vitro, tau was proposed to stabilize microtubule and to regulate neurite outgrowth and axonal transport. However later studies in vivo have demonstrated otherwise: germline tau knockout mice are largely viable and healthy; no abnormalities in microtubule stability and axonal transport were found in tau knockout mice. The lack of overt abnormalities in tau knockout mice was then attributed to developmental compensation. In this study, as well as a prior study using antisense oligos, tau levels were kept normal during development and acutely reduced in adulthood therefore not triggering possible developmental compensation. Still, no major neuronal dysfunctions were observed. We therefore argue that tau is unlikely to stabilize microtubule and to regulate axonal transport, because both functions are too critical to be disrupted. On the other hand, regulation of neuronal excitability is emerging as the most robust function of tau. Different lines of mice with germline tau knockout (Roberson et al., 2007; Ittner et al., 2010; Roberson et al., 2011), mice with adulthood knockout in this study, mice with reduced level of tau by antisense oligoes (Devos et al., 2013), and slices from tau knockout mice (Roberson et al., 2011), all exhibited higher resistance to excitotoxins. The mechanism how tau regulates neuronal excitability is unclear and shall be further elucidated.

In summary, we provide further evidence that tau reduction could be a safe and effective treatment for Alzheimer disease and other conditions with epileptiform activity.
REFERENCE


Table 1: Clinical scale for kainic acid induced seizure

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<tr>
<th>Scale</th>
<th>Readout</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No EEG Abnormality</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Seizure Duration in 24 hours</td>
<td>&lt;= 500 Seconds</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>500 – 1000 Seconds</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>1000 – 5000 Seconds</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>5000 – 10000 Seconds</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>&gt;= 10000 Seconds</td>
</tr>
<tr>
<td>6</td>
<td>Latency to Death</td>
<td>1 – 1.5 hours</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>0.5 – 1 hour</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>&lt;= 0.5 hour</td>
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Fig. 1: Construction of Tauflox mice. (A) schematic of targetting vector, wild type, targeted, Tauflox and Tau knockout allele. (B-D): confirmation of correct homogue recombination in the three clones of embryonic stem cells that were used to generate \( \text{Tau}^{\text{flox}} \) mice. B: genomic DNA digested with Bam HI and bloted with 3’ probe. (C) genomic DNA digested with Sac I and bloted with 5’ probe. (D) PCR reaction with primers F10 and R13 to confirm presence of loxP sequence on 5’ side of tau exon 1. (E) PCR genotyping to confirm the removal of neomycin
cassatte by Flp from \( Tau^{\text{flax}} \) mice. (F) PCR with primers F10 and R10 to confirm successful removal of tau exon 1 by Cre recombinase.
Fig. 2: Tamoxifen induced rapid tau reduction in adult mice. (A) 2.6–3.9-month old $\text{Tau}^{\text{lox/lox}}$ and $\text{CreER}^{\text{TM}}:\text{Tau}^{\text{lox/lox}}$ mice were injected daily with tamoxifen for 10/15 times in 5 injection blocks. Mice were taken down 5 month after the first injection and level of tau protein were determined by western blot. The residual level of tau was measured at 23% in cortex and 19% in hippocampus of control mice. No difference in residual level of tau between 10 and 15 injections were observed. (Student $t$-test, $*** = p < 0.0001$) (B) Time course of tau reduction from the 1st injection of tamoxifen. Mice were taken down at indicated time points after the 1st injection of tamoxifen and residual levels of tau in cortex and hippocampus were determined with western blot. Time needed for 50% reduction of tau was estimated at 6-8 days. No difference in residual levels of tau between cortex and hippocampus were observed.
Fig. 3: Safety of tau reduction in adulthood. (A) Mortality analysis of $\text{Tau}^{+/+}$, $\text{CreER}^\text{TM}:\text{Tau}^{+/+}$, $\text{CreER}^\text{TM}:\text{Tau}^{\text{flox/flox}}$ mice after tamoxifen injection. ($\chi^2$-square, control vs. $\text{CreER}^\text{TM}$: $p = 0.0286$; $\text{CreER}^\text{TM}$ vs. Tau aKO: $p = 0.5663$). (B) 2.6–3.9-month old $\text{Tau}^{\text{flox/flox}}$ and $\text{CreER}^\text{TM}:\text{Tau}^{\text{flox/flox}}$ mice were injected daily with tamoxifen for 10/15 times in 5 injection blocks. Mice were taken down 5 month after the first injection of tamoxifen. Brains were dissected out and gross weight was measured. (Student $t$-test, $p = 0.3037$). (C-D) Mice treated as described in B were tested in 3 month after first injection of tamoxifen in pole test and rota rod. (C) Pole test (Student $t$-test, $p = 0.4981$). (D) Rota rod test (Two-way ANOVA, control vs. Tau aKO: $p = 0.7718$).
Fig. 4: Tau reduction in adulthood increased resistance to PTZ-induced seizures. (A-B) 2.6–3.9-month old \( \text{Tau}^{\text{flax/flox}} \) and \( \text{CreER}^\text{TM}:\text{Tau}^{\text{flax/flox}} \) mice were injected daily with tamoxifen for 10/15 times in 5 injection blocks. Mice were intraperitoneally injected with PTZ at dose of 40mg/kg body weight of mouse 5-month after 1st injection of tamoxifen. (A) Max stage of each group of mice reached (student t-test, \( p = 0.0482 \)). (B) Latency to each stage (two-way ANOVA, \( p = 0.0488 \), ** = \( p < 0.01 \) in Holm-Sidak's multiple comparisons test). (C-D) 1.7–4.9-month old \( \text{Tau}^{+/-} \) and \( \text{CreER}^\text{TM}:\text{Tau}^{+/-} \) mice were injected with 10 daily tamoxifen in 5-injections block and 1-week interval. Mice were then intraperitoneally injection with PTZ at dose of 40mg/kg body.
weight of mouse 4-month after the first injection. (C) Max stage of each group of mice reached (Student t-test: \( p = 0.4959 \)). D: latency to each stage (two-way ANOVA, \( p = 0.6258 \)).
Fig. 5: Tau reduction in adulthood increases resistance to kainic–acid-induced seizures and death. 3–7-month old (average 4-month old) $\text{Tau}^{\text{flax/flax}}$ and $\text{CreER}^{\text{TM}}.\text{Tau}^{\text{flax/flax}}$ mice were injected daily with tamoxifen for 5 consecutive times. Mice were intraperitoneally injected with kainic acid at dose of 17.5mg/kg body weight of mouse 3–4 month after 1st tamoxifen injection. (A) Clinical score reflecting both latency to death and seizure severity in survived mice (Student $t$-test: $p = 0.0373$ when mice in Tau aKO group with greater than 25% of residual tau were excluded; $p = 0.0819$ when the mouse in Tau aKO group with 87% residual tau was excluded). (B) Correlation between clinical score and residual level of tau in Tau aKO group.
DISCUSSION

Tau reduction is further validated as a treatment strategy for Alzheimer disease and other conditions with epileptiform activity.

This study was aimed to test the feasibility of tau reduction as a treatment strategy, particularly the safety of long-term tau reduction, and both efficacy and safety of tau reduction in adulthood. By comprehensively characterizing a cohort of aged mice with germline knockout of tau, we demonstrated here that long-term tau reduction does not cause severe deficits like parkinsonism as reported in a prior study (Lei et al., 2012), while the excitoprotective effects of tau reduction persists with ageing. Of note is lifetime partial tau reduction in heterozygous tau knockout mice does not lead to any detectable abnormality in any of the behavioral, anatomical, and neurochemical tests we conducted. As partial reduction or partial blocking of tau function is what is most likely to happen during treatment, the normality observed in aged heterozygous tau knockout mice highlights the safety of long-term tau reduction.

By developing a new line of inducible tau knockout mice, we were able to maintain a normal level of tau expression during development, therefore avoiding developmental effects of germline tau reduction and then to reduce tau levels in adulthood. By challenging those mice with reduced level of tau in adulthood, we were able to demonstrate the excitoprotective effects of tau reduction in adulthood in two different seizure models. By characterizing the baseline behavior of those mice with reduced levels of tau in adulthood, we demonstrated here that tau
reduction in adulthood does not cause additional abnormalities in addition to abnormalities carried over from activation of CreER™.

In summary from both our data in aged mice with germline tau knockout and mice with tau knockout only in adulthood, we conclude that tau reduction is an effective and safe treatment strategy for Alzheimer disease and other conditions with epileptiform activity.

The role of tau in AD pathogenesis

There has been a great deal of debate regarding the role of tau in the pathogenesis of AD. As summarized in the Introduction, generally there are three theories: 1) loss of function; 2) gain of toxic function; 3) normal enabling function. At one point, loss of function was the predominant theory. When germline knockout of tau turned largely benign in young mice, and genetically reducing tau levels from AD mouse models ameliorated instead of exacerbating AD-like phenotypes, arguments were made that insufficient time was given for the detrimental effects to develop, and germline tau knockout may have triggered compensatory mechanisms.

In the present study, we demonstrated that lifelong loss of tau protein, either partially or completely, does not lead to impairment in learning and memory, a cardinal feature of AD. In fact, lifelong tau protein loss does not induce major neuronal dysfunction. In the present study, we also demonstrated that tau reduction in adulthood when developmental compensatory mechanism(s) is (are) largely not in place, still no overt abnormalities were observed. We therefore argue that it is unlikely that loss of tau function contributes to the AD pathogenesis. Instead, it is more likely the normal function of tau and/or in combination with gain of toxic function of tau that mediates the toxicity of Aβ peptides.
Strategies to target tau for treatment of AD and other conditions with epileptiform activity

With tau reduction being demonstrated to protect against epileptiform activity without causing major neuronal dysfunction, the strategy to actually target tau in clinical trials then becomes either reducing overall level of tau protein/pathology or surgically blocking certain function of tau.

To reduce the overall level of tau, we can either speed the degradation, directly clear tau protein, or reduce the level of tau mRNA. Tau is degraded through both ubiquitin-proteasome and autophagy-lysosome pathways (Wang and Mandelkow, 2012). Targeting ubiquitin-proteasome pathway to speed up tau degradation is currently under investigation. Degradation of tau through ubiquitin-proteasome pathway is regulated by chaperone system HSP70/HSP90. Deletion or inhibition of HSP90 on the other hand led to decreased level of p-Tau protein (Dickey et al., 2007). Therefore, inhibition of HSP90 through small compounds can be an indirect way to target tau. Mono-clonal antibodies are widely used to clear toxic proteins such as Aβ in immune-therapy. Though tau is generally considered an intra-cellular protein, tau does exist in extra-cellular spaces such as cerebrospinal fluid. In fact, tau level in cerebrospinal fluid is being proposed as biomarker for the diagnosis of AD (Jack and Holtzman, 2013). Interestingly, the release of tau into extra-cellular space is regulated by neuronal activity both in vitro and in vivo (Pooler et al., 2013; Yamada et al., 2014). While mono-clonal antibodies are generally better at clearing extra-cellular proteins, the release of tau into extra-cellular space makes immunotherapy very promising. While this dissertation is being prepared, an independent study utilizing antisense oligoes against tau mRNA was published. Infusion of tau antisense oligoes into ventricles greatly reduced the level of tau in central neuronal system, and conferred
resistance to excitotoxins such as PTZ (Devos et al., 2013). This on one hand confirmed findings of the present study, on the other hand validated a useful way to target tau for treatment.

An emerging way to target tau is to block the transmission of tau pathology. In human patients, tau pathology starts in entorhinal cortex and spreads to the whole brain in a very stereotypic way (Serrano-Pozo et al., 2011). In mice, restricted expression of tau in entorhinal cortex was recently demonstrated to spread tau pathology all-over the brain (de Calignon et al., 2012; Liu et al., 2012). Therefore, the progression of AD could be delayed or halted by stopping the spread of tau pathology. Actually some antibodies have been recently demonstrated to greatly decrease tau pathology in a tau transgenic mouse model (Yanamandra et al., 2013). Because of the tight correlation between tau pathology and onset and progression of AD symptoms, intervening in the spread of tau pathology stands for a very unique and attractive opportunity.

Ideally, tau needs be surgically targeted to avoid side effects as much as possible. Though tau reduction does not cause major neuronal dysfunction, some abnormalities, mainly behavioral hyperactivity, have been observed. While the cellular and molecular mechanism of both the normal and pathological function is just beginning to be elucidated, the mechanism of the pathological function of tau is possibly different from the normal function of tau. Tau interacts with quite a few different binding partners and has recently been proposed as a scaffold protein (Morris et al., 2011). Conceptually, some binding partners are responsible for the pathological functions. The interaction between tau and those binding partners could be surgically targeted.

The Changing landscape of drug discovery for AD: tau as a better alternative to Aβ

Since the identification of Aβ as a major component of plaques 30 years ago (Glenner and Wong, 1984b, a), Aβ has been the primary target for AD drug discovery. However, despite
heavy investment in this avenue, clinical trials trying to block production of Aβ through β-γ-secretase inhibitors, clearing Aβ plaques/oligomers through immunotherapy or blocking Aβ aggregation through small molecules, have all failed epically (Karran et al., 2011; Sperling et al., 2011). Although the failure of each particular clinical trial may have its own specific reasons, the uniform failure of all Aβ-directed trials prompts us to consider a different target and rethink the rationale behind all Aβ-directed clinical trials: the Aβ cascade hypothesis.

Based on Aβ cascade hypothesis which was originally proposed by Dr. Hardy and Higgins, then reiterated and aired by Dr. Selkoe (Hardy and Higgins, 1992; Hardy and Selkoe, 2002; Selkoe, 2011), the accumulation of Aβ peptides represents the first insult to the neuronal system, leads to the formation of tau pathology (hyperphosphorylation, oligomers and neurofibrillary tangles), synaptic damage, neuronal dysfunction, and ultimately dementia. Although supported by human genetics and transgenic mouse models of AD, the Aβ cascade hypothesis does not explain the dissociation between the appearance of Aβ plaques and the onset/progression of AD symptoms. The accumulation of Aβ plaques starts decades before and reaches a plateau before the onset of AD symptoms (Jack et al., 2013). The spatial distribution of Aβ plaques in AD brains does not correlate with neuronal damage and tau pathology (Giannakopoulos et al., 2003). One prediction based on the Aβ cascade hypothesis would be that removal of excessive Aβ shall shut down the whole chain reaction and shall stop the progression of AD. This prediction has been disproved by the failures of all Aβ-directed clinical trials.

One explanation for poor predictive power of the original Aβ cascade hypothesis is that the role of tau is underestimated and not fully reflected. Compared to Aβ pathology, tau pathology correlates better with the onset/progression of AD symptoms, and also better with the
spatial distribution of neuronal damage inside AD brains (Giannakopoulos et al., 2003). Further, a recent study demonstrated that tau pathology can self-propagate from one region of the brain to another, in a way much like prion protein (de Calignon et al., 2012; Liu et al., 2012). Tau pathology, presumably tau oligomers, could incur neuronal dysfunction by itself in the absence of Aβ pathology (Lasagna-Reeves et al., 2011; Flach et al., 2012; Lasagna-Reeves et al., 2012). If these new findings are incorporated, a modified version of Aβ cascade hypothesis could be: while accumulation of Aβ peptides serves as a trigger, the formation of tau pathology acts as a downstream effector to exert the actual damage to the neuronal system in a self-sufficient way (Figure 1). If this is the case, then it is not surprising that all the Aβ-directed clinical trials failed to improve cognitive function of AD patients of later stage, because removal of Aβ peptides does not block the toxic function and propagation of tau pathology.
Realizing the possibility that pathogenesis can progress independent of Aβ peptides beyond certain point, one way forward is to start the intervention earlier even before the accumulation of Aβ pathology (Selkoe, 2012). Indeed several efforts of this kind are under way. While Aβ-directed preventative intervention could possibly do some good, it also bears high financial cost and has limited audience. As the accumulation of Aβ can start decades before the onset of AD symptoms, preventative interventions for that duration of time will be a huge financial burden. Also, the accumulation of Aβ does not always lead to AD (Jack et al., 2013).
Therefore, the preventative intervention will be limited only to those mutation bearers who are doomed to develop AD, which accounts less than 5% of the total AD patients.

The other way forward would be targeting an effector downstream of A\(\beta\) peptides. In this regard, tau is an apparent candidate of choice. Tau-directed treatment could ameliorate both A\(\beta\)-dependent toxicity and the toxicity of tau pathology itself. Tau-directed treatment could also have better chance to be effective in later stages of AD and wider audience.

Given this big picture and the fact that a lot of tau-directed treatments like immunotherapy to clear toxic tau species are under development, we expect the present study to contribute to the transition from A\(\beta\)-directed to tau-direction drug discovery for AD.

**FUTURE DIRECTIONS**

Region specific knockout of tau in AD model mice

Like any other type of neurodegenerative disease, cells in certain brain regions are of strategic importance or are particularly vulnerable in AD. The construction of \(\text{Tau}^{\text{flox}}\) mice enables region-specific tau knockout at desired time points by targeted delivery of Cre-expressing viral vector. While ubiquitously knockout of tau from the entire brain is protective against AD-like deficits, knockout of tau from individual region of brains could shed lights into the mechanism of AD pathogenesis and help devise new treatment for AD.

One example would be hippocampus. Hippocampal neurons are of particular interest for AD pathogenesis because the cardinal feature of AD is impairment in memory and hippocampal formation is critical for memory functioning. Shrinkage of hippocampal volume in MRI imaging is considered an early marker of AD (Schuff et al., 2009). Brain circuits are organized through different networks, manipulation of certain hubs or strategic network nod can influence the
dynamics of entire network (Buckner et al., 2008; Bonifazi et al., 2009). Even manipulation of a single neuron can modulate the behavioral state of an animal (Li et al., 2009). This opens up the possibility to treat AD by intervening within certain hubs. As a proof of concept, restoring expression of Eph2B in a sub-region of hippocampus, the dentate gyrus, has been previously demonstrated to reverse memory impairment in a mouse model of AD (Cisse et al., 2011). Knowing whether tau reduction in hippocampus is sufficient to prevent or reverse AD-like deficits could further test the hypothesis that hippocampus or more specifically the dentate gyrus is indeed a hub critical for the AD pathogenesis.

Another example would be entorhinal cortex. Tau pathology appears first in the transentorhinal region, then spreads into entorhinal region, Ammon's horn, and entire neocortex (Braak and Braak, 1995). It would be interesting to know whether genetic deletion of tau specifically from entorhinal cortex is sufficient to prevent or stop progression of AD-like deficits.

To test whether tau reduction in hippocampus/entorhinal cortex is sufficient to prevent and/or reverse AD-like deficits can also further answer the general question whether tau reduction in adulthood is able to prevent and/or reverse AD-like deficits. The present study demonstrated that tau reduction in adulthood confers resistance to excitotoxin-induced seizures. We then argued that tau reduction could be also beneficial in AD model mice based on the tight correlation between beneficial effects of germline knockout of against both excitotoxin-induces seizure and other AD-like deficits (Roberson et al., 2007; Ittner et al., 2010; Roberson et al., 2011). However, this argument could have been better directly tested in AD model mice such as hAPP J20 and APP23 in which germline knockout of tau has been demonstrated beneficial.
Two hurdles prevented us from utilizing tamoxifen-CreER™ system to study the effects of tau adulthood knockout in AD mice: 1) activation of CreER™ by tamoxifen induces about 20% deaths and impairs spatial learning of survived mice. This toxicity is most likely associated with CreER™ (a fusion of Cre and estrogen receptor binding domain) instead of Cre itself, because no such mortality and impairment of spatial learning has been reported in transgenic mice of regular Cre recombinase which is constitutively active. 2): tamoxifen selectively kills hAPP (J20) transgenic mice (13/44 in hAPP tg vs. 5/58 in non-hAPP tg mice). The reason why hAPP (J20) mice are particularly susceptible to tamoxifen is not clear.

Delivering viral vectors that express Cre recombinase into brain regions like hippocampus or entorhinal cortex of adult mice can then achieve the goal of tau reduction in adulthood without bearing the side effects associated with tamoxifen-CreER™.

Cell type specific knockout of tau to study the mechanism how tau regulates neuronal excitability

Tau has been well demonstrated to regulate neuronal excitability by prior studies and further by the present study in both AD and non-AD mouse models (Palop et al., 2007; Roberson et al., 2007; Ittner et al., 2010; Roberson et al., 2011). Tau reduction confers resistance epileptiform activity induced by both excessive Aβ and excitotoxin like PTZ and kainic acid. However, the mechanism is largely unknown.

In AD model mice, the aberrant excitatory neuronal activity is most likely caused by relative decrease in synaptic inhibition (Palop and Mucke, 2010). In APP-PS1 transgenic mice, when neuronal activity was monitored by in vivo two-photon Ca2+ imaging, more hyperactive neurons were observed in the vicinity of Aβ plaques than in normal mice (Busche et al., 2008).
The hyperactivity in those neurons was not due to increased intrinsic excitability but decreased inhibitory input (Busche et al., 2008). In ApoE4 knock-in mice, number of GABAergic neurons decreased with aging (Andrews-Zwilling et al., 2012). However, in pharmacologically induced seizure models, tau reduction protects both enhanced excitatory and decreased inhibitory neuronal activity caused seizure.

Therefore, at the network level, the mechanism becomes a question as to whether tau reduction in inhibitory neurons or excitatory neurons mediates the regulatory role of tau in neuronal activity. This question can be then answered by genetic removal of tau selectively from inhibitory or excitatory neurons by crossing cell-specific promoter driven Cre transgenic mice with \( \text{Tau}^{\text{flox}} \) mice.

Normal functions of tau and their mechanism

Because of the high profile association between tau and AD, the primary focus of the research on tau relates to the role of tau in AD pathogenesis and how tau could be targeted to treat AD. The normal function of tau is largely under-investigated which is unfortunate. Research into the normal function shall also shed light into the pathological function of in AD and other tauopathies.

Several functions of tau emerged from recent studies: 1) regulation of neuronal excitability; 2) possible role in regulating brain development or neuronal genesis. Also some minor behavioral abnormalities have been consistently observed in either germ-line or adulthood knockout, such as more frequent rearing in open field. Both the implication of these abnormalities for higher cognitive functions and mechanism why tau deficiency causes these abnormalities are completely unknown.
LIST OF GENERAL REFERENCES


