Tau aggregation: role in neurodegeneration and inhibition with small molecules

DISSERTATION

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AGGREGATES OF THE MICROTUBULE-ASSOCIATED PROTEIN TAU CHARACTERIZE SEVERAL NEURODEGENERATIVE DISORDERS, INCLUDING ALZHEIMER’S DISEASE AND THE FRONTOTEMPORAL LOBAR DEGENERATIONS. BECAUSE THE TEMPORAL AND SPATIAL PROGRESSION OF TAU LESIONS CORRELATES WITH THE CLINICAL PROGRESSION OF SYMPTOMS, AN UNDERSTANDING OF FACTORS THAT CONTRIBUTE TO TAU AGGREGATION IS OF UTMOST IMPORTANCE. FURTHERMORE, IDENTIFYING SMALL MOLECULES THAT CAN INTERFERE WITH THE AGGREGATION OF TAU MAY PAVE THE WAY FOR DEVELOPMENT OF THERAPEUTICS FOR THESE NEURODEGENERATIVE DISEASES.

MUTATIONS IN THE CODING SEQUENCE FOR TAU PROTEIN ARE ASSOCIATED WITH HEREDITARY FAMILIAL TAUOPATHIES. SOME OF THESE MUTATIONS WERE KNOWN TO HAVE EFFECTS ON CELLULAR REGULATION OF TAU, BUT WHETHER THESE MUTATIONS DIRECTLY AFFECT THE ABILITY OF TAU TO AGGREGATE WAS UNKNOWN. THE DATA SHOWED THAT MOST MUTATIONS WERE ABLE TO INCREASE TAU’S INTRINSIC PROPENSITY TO AGGREGATE. FURTHERMORE, DIFFERENT MUTATIONS ACTED AT DIFFERENT STEPS IN THE AGGREGATION PROCESS, PROVIDING A POSSIBLE MECHANISM FOR THE CLINICAL HETEROGENEITY OF THESE MUTATIONS.

TAU PROTEIN IS OFTEN FOUND HYPERPHOSPHORYLATED IN DISEASE-ASSOCIATED AGGREGATES. THE USE OF A PSEUDOPHOSPHORYLATION MUTANT ALLOWS FOR ANALYSIS OF THE CONTRIBUTION OF A SPECIFIC PHOSPHORYLATION SITE TO AGGREGATION PROPENSITY. DATA SHOWED THAT
pseudophosphorylation of tau at site T212 increased tau aggregation propensity at multiple steps, similar to the effect of coding sequence mutations.

Pseudophosphorylation and exonic mutations, both disease-causing agents, show a common ability to promote tau aggregation. This suggests that interventions that can interfere with tau aggregation may be useful as therapeutic agents that can slow or halt the advance of neurodegeneration. We extended our lab’s previous work on a small molecule inhibitor of tau aggregation, N744, to determine a structure-activity relationship in inhibition of tau aggregation. Out of the panel of related cyanine dyes tested, one compound had inhibitory potency similar to that of N744 while showing improved predicted pharmacokinetic properties. These data show that it is possible to maintain tau inhibitory activity while developing more druglike molecules for potential future use.
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MAPT mutations differentially modulate tau aggregation propensity at nucleation

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3R, 3-repeat tau isoforms; 4R, 4-repeat tau isoforms; AD, Alzheimer’s disease; ECL, enhanced chemiluminescence; FTLD, frontotemporal lobar degeneration; HRP, horse radish peroxidase; PHF, paired helical filament; PVDF, polyvinylidene fluoride; SAR, structure-activity relationship; SEE, standard error of the estimate
1. INTRODUCTION

1.1. Tau and neurodegenerative disease

Alzheimer’s disease (AD) can only be definitively diagnosed on the basis of pathology. Characteristic neuritic brain lesions include neurofibrillary tangles (corresponding to affected nerve cell bodies), neuropil threads (affected neuronal processes), and dystrophic neurites (swollen and misshapen nerve cell processes associated with Aβ plaques). All neuritic morphologies involve intracellular deposition of aggregated forms of tau, a microtubule-associated protein that normally functions as a monomer in conjunction with its physiological binding partner, tubulin. Because the transformation of tau from monomer to aggregate along with changes in its state of post-translational modification correlates with neurodegeneration and cognitive decline (Gomez-Isla, Hollister et al. 1997), neuritic lesion formation can serve as a marker of AD neurodegeneration. In fact, the hierarchical appearance of neuritic lesions is commonly used to stage AD progression (Braak and Braak 1991).

Alzheimer’s like changes in tau structure occur in certain frontotemporal lobar degeneration (FTLD) diseases as well, where they can involve glial cells in addition to neurons, and also in inclusion body myositis, where they accumulate in muscle cells. In
some familial cases of FTLD, disease is conferred by mutations in the tau gene, suggesting a closer link between tau misfunction and neurodegeneration than implied solely by histological correlation studies (Hutton, Lendon et al. 1998; Spillantini, Crowther et al. 1998; Spillantini, Van Swieten et al. 2000; Neumann, Schulz-Schaeffer et al. 2001; Hayashi, Toyoshima et al. 2002; Poorkaj, Muma et al. 2002). However, the majority of AD and some FTLD cases are sporadic (not genetically-linked). Abnormal post-translational modifications of tau, such as hyperphosphorylation and truncation, have been proposed to promote tau aggregation in these cases.

Several hypotheses exist as to the role of tau protein in neurodegenerative diseases. Aggregation of tau could result in either loss-of-function or gain-of-function toxicity. The loss of tau’s normal function to bind to and stabilize the microtubule could result in cytoskeletal collapse, and it has been shown that cytoskeletal changes exist in disease (Braak, Braak et al. 1994). Conversely, aggregation of tau could be actively toxic to the cell. Neurons with tau fibrils show signaling defects (Callahan and Coleman 1995) and tau aggregates may interfere with normal proteasome function (Keck, Nitsch et al. 2003). Thus, a clearer understanding of the factors that contribute to tau aggregation is of paramount importance in continuing research on these neurodegenerative diseases.

1.2. Modeling the tau aggregation reaction

Modeling of tau aggregation in vitro allows the analysis of the modulating effects of various interventions on tau aggregation away from the complexity of in vivo systems. However, modeling tau aggregation in vitro is not straightforward. Specifically, tau does not aggregate in experimentally tractable time periods under physiological conditions of pH, ionic strength, temperature, and concentration (Ko, DeTure et al. 2002). In biological
models, this limitation has been overcome by high level tau overexpression (Gotz, Probst et al. 1995; Duff, Knight et al. 2000; Wittmann, Wszeek et al. 2001; Andorfer, Acker et al. 2005), incorporation of aggregation-promoting missense mutations or deletions (Lewis, McGowan et al. 2000; Gotz, Tlnoy et al. 2001; Lim, Hernandez et al. 2001; Tanemura, Akagi et al. 2001; Tatebayashi, Miyasaka et al. 2002; Santacruz, Lewis et al. 2005; Rosenmann, Grigoriadis et al. 2008), or through aggressive post-translational modification (Sato, Tatebayashi et al. 2002; Sze, Su et al. 2004; Sahara, Murayama et al. 2008).

In vitro, resistance to aggregation can be overcome in the presence of polyanions (e.g., heparin (Goedert, Jakes et al. 1996; Perez, Valpuesta et al. 1996)) or anionic surfactants (arachidonic acid or alkyl sulfate detergents). Although surfactants are small monomers in dilute solution, they readily aggregate to form micelles above a critical concentration. The presence of protein depresses surfactant critical micelle concentration and leads to formation of tau-surfactant complexes (Chirita, Necula et al. 2003). These are predicted to vary in size depending on the surfactant employed and its concentration, ranging from small micelles arranged along the natively unfolded tau polypeptide chain (Shirahama, Tsujii et al. 1974) to larger protein-bound bodies visible in the electron microscope (Chirita, Necula et al. 2003). Thus, despite their much smaller monomeric size relative to heparin, surfactants may share a limited ability to achieve aggregation-inducing concentrations in intact cells.

Recent studies show that the kinetic barrier to fibrillization also can be overcome by addition of agonist dyes such as Congo red, Thiazine red, and Thioflavine S (Chirita, Congdon et al. 2005). The resultant tau filaments display a twisted-ribbon morphology and a mass-per-unit-length similar to that of authentic tissue-derived filaments (Congdon,
Kim et al. 2008). Congo red can populate partially-folded amyloidogenic conformations of proteins (Kim, Randolph et al. 2003), suggesting that agonists may function through thermodynamic linkage of binding with the aggregation reaction. Regardless of mechanism, the performance of small-molecule aggregation agonists differs substantially from macromolecular and micelle-based inducers. For example, the extent of the aggregation reaction does not depend on dye/tau ratios, thereby simplifying kinetic analysis (Chirita, Congdon et al. 2005). In addition, agonists facilitate fibrillization of full-length 4-repeat tau isoforms at submicromolar concentrations (Congdon, Kim et al. 2008) which is well within physiological levels. These properties make small-molecule based tau aggregation inducers ideal for kinetic characterization of tau aggregation. *In vitro*, Thiazine red-induced aggregation follows a nucleation-elongation mechanism consistent with a dimeric nucleus, a monomeric extension reaction, and a submicromolar critical concentration (Fig. 1.1). Preliminary estimates of elementary rate constants derived from this model were consistent with the time dependent evolution of total filament length and also filament length distribution (Congdon, Kim et al. 2008). Thus, the use of small molecule inducers such as thiazine red allows for a direct, quantitative analysis of various factors on tau aggregation, such as whether changes directly increase the aggregation propensity of tau and at which steps these changes might act.

1.3. Antagonists of tau aggregation

The association of aggregation-promoting missense tau mutations with neurodegenerative disease (Rademakers, Cruts et al. 2004), combined with toxicity associated with tau aggregation in model systems (Mocanu, Nissen et al. 2008), suggests that the accumulation of tau aggregates may contribute to neurodegeneration. If so, then
direct interference with aggregation may halt disease progression in affected individuals. The strategy is attractive because tau aggregation is associated with diseased neurons but not normal biology. Early screens for tau aggregation antagonists focused on the ability of aggregation-prone tau fragments to recruit full-length tau into aggregates. These assays identified methylene blue (tetramethylthionine chloride) and other phenothiazine derivatives as inhibitors of this reaction at low or even sub-micromolar concentrations (Wischik, Bentham et al. 2008). Treatment of mild-to-moderate AD sufferers with methylene blue (30 – 60 mg twice daily) has been reported to halt cognitive decline, improve cerebral blood flow, and improve fluorodeoxyglucose uptake relative to placebo over a ~1 year treatment period (Wischik, Bentham et al. 2008). Thus at least one tau aggregation antagonist may have therapeutic utility for AD. Nonetheless, methylene blue also is a redox agent with affinity for the heme moieties of several proteins. In fact, this activity has been leveraged to treat methemoglobinemia in humans (i.e., to reduce heme-associated iron from the abnormal ferric state to the oxygen-carrying ferrous state) (Wendel 1939). Moreover, it has antioxidant activity owing to direct effects on the electron transport chains of mitochondria (Atamna, Nguyen et al. 2008). Currently, it is not clear whether the therapeutic benefits of methylene blue stem from its aggregation antagonist activity, its antioxidant effects on mitochondria, or its direct binding to and modulation of heme-containing proteins.

Additional non-phenothiazine aggregation inhibitors will help clarify this issue. Toward this end, the ability of heparin and anionic surfactants to induce the aggregation of recombinant tau or tau fragments *in vitro* has been leveraged to screen for novel aggregation antagonists. High throughput screening has identified over 400 candidate
inhibitors composed of diverse heterocycle scaffolds (Taniguchi, Suzuki et al. 2005; Mandelkow, Mandelkow et al. 2006; Larbig, Pickhardt et al. 2007). Many of these are polyalcohols, which when incubated under nonreducing conditions generate Michael acceptors capable of covalently reacting with tau protomer. For example 5,6,7-trihydroxyflavone (baicalein) oxidizes at pH 7.5 to form protein-reactive baicalein quinone with $t_{1/2} \sim 15$ h (Zhu, Rajamani et al. 2004). Covalent modification effectively sequesters tau, thereby raising the apparent minimal concentration required to support the aggregation reaction (Fig 1.1). Many of these compounds inhibit Aβ and α-synuclein aggregation as well, suggesting that the mechanism can be widely generalized to different aggregating proteins. Inhibition of protein aggregation through covalent modification has been shown for other scaffold classes as well (Crowe, Ballatore et al. 2007). Further studies will be required to determine whether this mechanism can modulate tau aggregation in vivo.

Other antagonist scaffolds include phenylthiazolyl-hydrides (Pickhardt, Larbig et al. 2007), N-phenylamines (Pickhardt, Biernat et al. 2007), rhodanines (Bulic, Pickhardt et al. 2007), and thiacarbocyanines (Chirita, Necula et al. 2004). The first two classes are reportedly active down to the micromolar range, whereas some rhodanines and cyanines are active at submicromolar concentrations. The mechanism of action of these compounds on tau is not established, but they may act to stabilize off-pathway aggregates, thereby depleting tau available to support the nucleation and extension reactions (Fig. 1.1). Closer inspection of phenothiazine and thiacarbocyanine antagonists, representing two of the most potent antagonist scaffolds, reveals that both not only are cationic, but
share push-pull electronic character (i.e., they contain an electron donating substituent separated from an electron accepting substituent by a conjugated ring system). As a result, both scaffolds are highly polarizable (i.e., charge can be localized to specific parts of the extended \( \pi \) system). This may facilitate \( \pi-\pi \) interactions with a complimentary set of one or more residue side chains present on tau. If so, then additional scaffolds with drug-like properties will be capable of supporting tau aggregation antagonist activity.

1.4. Unresolved Issues

As mentioned, the discovery of mutations within the tau gene that were directly linked to neurodegenerative disease provided powerful evidence that tau misfunction itself can contribute to neurodegeneration. Nevertheless, it is unclear how precisely these mutations cause disease. Disease-causing mutations have often been linked to enhanced rates and/or extents of tau fibrillization. However, it is unknown whether these mutations do so by increasing the available pool of tau, such as by decreasing tau binding to the microtubule, decreasing its proteolysis, or increasing its expression, or whether these mutations may have direct effects on tau aggregation. Herein we use the nucleation-extension model as described above to examine the precise role of FTLD mutations in disease.

Similarly, tau in disease-associated lesions is often found in a hyperphosphorylated state. Again, it is unclear as to exactly how hyperphosphorylation contributes to the disease state. It is known that hyperphosphorylation can increase the free pool of tau by decreasing tau binding to tubulin, but direct effects on tau aggregation have been harder
to determine. Herein, we use a pseudophosphorylation mutant model to analyze the effects of phosphorylation on tau aggregation propensity.

With evidence that tau aggregation contributes to neurodegeneration, research into small molecules that can interfere with the tau aggregation process generates much interest. However, methods used to screen for these compounds remain underdeveloped. Assays that rely on fluorescent compound screens such as thioflavin S may be confounding, as thioflavin S is associated with tau conformational change and not necessarily aggregation. Electron microscopy is a powerful method that allows direct visualization and quantification of tau aggregation, but it is slow and unsuitable for screening of large numbers of compounds and conditions. Several groups have used filter assay methods to combine the semi-high-throughput nature of plate assays with a more accurate measurement of tau aggregation. Herein, we optimize and characterize a filter assay for tau aggregation.

Finally, preliminary clinical trials with methylene blue as described above provides validation of tau as a clinical target of interest. Although many inhibitors of tau aggregation have been identified through high-throughput screening, it is unclear as to how these small molecules actually work. Knowledge of how small molecules interfere with tau fibrillization could enhance optimization of candidate compounds destined for eventual use in humans. Our lab has previous experience with a thiocarbocyanine inhibitor of tau aggregation, N744. Herein we present an analysis of a family of carbocyanine compounds similar to N744 and attempt to build a structure-activity relationship for this class of compound as a first step towards a hypothesis for a general model by which small molecules may inhibit tau aggregation.
1.5. Summary

The appearance of proteinaceous deposits of tau protein is characteristic in several neurodegenerative diseases. The clear correlation of the appearance and spread of these tau lesions with the clinical progression of disease makes tau aggregation a useful marker of disease as well as a potential mediator for neurodegeneration. However, the causes and triggers of tau aggregation in human disease remain unknown. Herein we provide detailed quantitative analyses of how disease-associated processes like tau missense mutations and tau hyperphosphorylation may promote tau aggregation. In addition, the correlation between disease-causing agents and aggregation suggest that inhibitors of tau aggregation are of vital interest in the future development of therapies for AD and other tauopathies. The results presented herein characterize the SAR for a particularly potent class of tau inhibitors as well as point the way towards a hypothesis for the mechanism of action of small molecule tau inhibitors. Overall, the data shown here represent significant findings on the causes of tau polymerization and the way in which compounds can interfere with this polymerization.
1.6 Figures

**Figure 1.1. General tau aggregation scheme.** We hypothesize that tau must undergo several steps before it can form filaments. First, the free concentration of tau protein in the cytoplasm must exceed a certain minimum critical concentration required for aggregation. This can be accomplished through several means, whether an increase in tau expression, a decrease in tau degradation, or a decrease in tau’s natural affinity for tubulin. Second, tau protein undergoes a conformational change into an assembly-competent conformation. This change can be promoted by small molecule inducers of tau aggregation. Third, a rate-limiting nucleation process occurs, thought to be a dimerization. Finally, after nucleation further tau monomer addition becomes energetically favorable in the extension step.
2. PATHOGENIC MISSENSE MAPT MUTATIONS DIFFERENTIALLY MODULATE TAU AGGREGATION PROPENSITY AT NUCLEATION AND EXTENSION STEPS

2.1 Introduction

Frontotemporal lobar degeneration (FTLD) is a clinically and pathologically heterogeneous group of diseases associated with cognitive decline and degeneration of prefrontal and anterior temporal neocortex (Neary, Snowden et al. 2000). FTLD is often familial, with ~20% of inherited cases caused by mutations in the MAPT gene located on chromosome 17 (Rademakers, Cruts et al. 2004). MAPT encodes the microtubule associated protein tau, which exists in human brain as a mixture of six isoforms owing to alternative splicing of exons 2, 3, and 10 from MAPT transcripts (Fig. 2.1). Tau isoforms normally bind to and function in conjunction with the microtubule cytoskeleton, but they aggregate to form filamentous inclusions in FTLD. To date more than 40 pathogenic MAPT mutations that lead to tau lesion formation and FTLD have been identified in 127 families worldwide (Rademakers, Cruts et al. 2004) 34 of which change tau primary structure at one of 27 different amino-acid residues (Fig 2.1). These residues are found
predominantly in the C-terminal half of tau isoforms (*i.e.*, those encoded by exons 9 – 12), where the 31-32 residue imperfect repeats that mediate both microtubule binding (Goode, Chau et al. 2000) and tau fibrillization (Novak, Kabat et al. 1993) are located. However, pathological mutations have been discovered in exon 1 as well, leading to amino acid substitutions at residue five near the N-terminus (Hayashi, Toyoshima et al. 2002; Poorkaj, Muma et al. 2002), whereas others have been found in exon 13, which encodes the segment C-terminal to the repeat region. Each of these mutations could affect lesion formation through different mechanisms. For example, they could promote tau lesion formation directly by increasing the aggregation propensity of tau isoforms, or indirectly by altering tau-microtubule binding equilibria, levels of tau post-translational modification, or levels of tau expression. Because sequences encoded by exons 1, 9, 11, 12, and 13 affect protein segments common to all tau isoforms, mutations in these regions may affect all six tau splice variants. In contrast, mutations that change tau residues encoded by exon 10, which encodes a fourth imperfect repeat sequence, are expressed only in four-repeat (4R) tau isoforms.

Previous studies support a direct effect of mutation on tau aggregation propensity, with certain tau mutants supporting increased rate (P301L, (Nacharaju, Lewis et al. 1999; Gamblin, King et al. 2000) and/or extent (I260V, (Grover, England et al. 2003); L266V, (Hogg, Grujic et al. 2003); P301L and P301S, (Barghorn, Zheng-Fischhofer et al. 2000; Gamblin, King et al. 2000; Spina, Murrell et al. 2007); and Q336R (Pickering-Brown, Baker et al. 2004)) of aggregation *in vitro*. Some mutations have been reported to support an increase in the numbers of filaments formed (R5H, (Hayashi, Toyoshima et al. 2002); K257T, (Rizzini, Goedert et al. 2000); G272V, (Goedert, Jakes et al. 1999);
P301L and P301S, (Goedert, Jakes et al. 1999); and K369I (Neumann, Schulz-Schaeffer et al. 2001)), suggesting that tau structure may influence the rate limiting step in fibrillization. Many of these studies were only semiquantitative and did not address the mechanism of aggregation enhancement. In addition, tau aggregation in vitro requires addition of an aggregation inducer, typically in the form of an anionic substance, which adds another layer of complexity to analysis of the reaction (Kuret, Congdon et al. 2005). A more systematic study of the kinetics of the tau aggregation reaction is needed to address intrinsic tau mutant aggregation propensity and the mechanism through which the aggregation reaction may be affected.

Recently we showed that small-molecule inducers such as Thiazine red drive aggregation of full-length tau at submicromolar concentrations in the absence of macromolecular inducers such as heparin (Chirita, Congdon et al. 2005). The reaction approximates a homogeneous nucleation scheme characterized by initial formation of an unstable dimeric nucleus, followed by filament elongation through monomer addition (Congdon, Kim et al. 2008). Under these conditions, it is possible to fit the reaction to a mathematical model and estimate the elementary rate constants for tau aggregation. Thus, the inherent aggregation propensity for mutant tau may be quantified and mechanistically localized under near physiological tau protein concentrations.

Here we examine the effects of tauopathy mutants R5L, G272V, P301L, V337M, and R406W on aggregation propensity. The results suggest that some but not all tauopathy mutants increase tau aggregation propensity, and that they can do so at both the nucleation and elongation reaction steps of the reaction.
2.2 Experimental Procedures

2.2.1 Materials

Recombinant polyhistidine-tagged human 2N4R tau, and mutants G272V, P301L, V337M, and R406W in 2N4R background, were prepared as described previously (Carmel, Mager et al. 1996; Gamblin, King et al. 2000). R5L was generated by site-directed mutagenesis (Stratagene QuikChange XL) of the pT7C-htau40 vector using the following primers: forward (5’-CTG AGC CCC TCC AGG AGT TCG AAG TGA TGK 3’) and reverse (5’-CAT CAC TTC GAA CTC CTG GAG GGG  CTC AGK 3’). Successful mutagenesis was confirmed by DNA sequence analysis. R5L mutant tau was then expressed and purified as described for wild-type 2N4R tau (Carmel, Mager et al. 1996).

Aggregation inducer Thiazine red (Chemical Abstract Service registry number 2150-33-6) was obtained from TCI America (Portland, OR, USA). Formvar/carbon-coated copper grids (300 mesh), glutaraldehyde, and uranyl acetate were obtained from Electron Microscopy Sciences (Fort Washington, PA, USA).

2.2.2 Tau Fibrillization Assay

Purified tau preparations were incubated at 37ºC without agitation in assembly buffer (10 mM HEPES pH 7.4, 100 mM NaCl, and 5 mM dithiothreitol) containing aggregation inducer Thiazine red (100 µM final concentration) for various times up to 24 h. Reactions were then fixed with 2% glutaraldehyde, adsorbed onto Formvar/carbon-coated copper grids, stained with 2% uranyl acetate, and examined by transmission electron microscopy as described previously (Necula and Kuret 2004). At least three fields were captured for each reaction condition with a Tecnai G2 Spirit BioTWIN transmission electron
microscope (FEI, Hillsboro, OR, USA) operated at 80 kV and 23,000 – 49,000x magnification, and tau filaments >10 nm in length were quantified with ImageJ software (National Institutes of Health). Total filament length is defined as the sum of the lengths of all resolved filaments per field and is reported ± SD.

2.2.3 Critical Concentration Determination

$K_{\text{crit}}$ values were determined from linear regression analysis of the tau concentration dependence of aggregation by inverse prediction of abscissa intercepts (Congdon, Kim et al. 2008). The accompanying standard error of the estimate, $S_x$, was calculated as:

$$S_x = \frac{C.I.}{2(t_{0.975,n-2})}$$  \hspace{1cm} (Eq. 2.1)

where C.I. is the length of the Fieller 95% confidence interval of each regression, and $t_{0.975,n-2}$ is the Student’s $t$ distribution percentage at 1 - $\alpha = 0.975$ and $n - 2$ degrees of freedom.

2.2.4 Dissociation Rates

Tau filaments prepared as described above for 24 h were diluted 10-fold into assembly buffer containing 100 µM Thiazine red and incubated at 37°C. Aliquots were withdrawn as a function of time up to 5 h post dilution and then assayed for filament length. The resultant disaggregation time series was fit to an exponential decay function to obtain $k_{\text{app}}$, the pseudo-first order rate constant describing the time-dependent decrease in filament length, and $L_0$, the total filament length at time zero. The rate constant $k_e$ was estimated from $k_{\text{app}}$, $L_0$, and the number of filaments at time zero as described previously (Kristofferson, Karr et al. 1980; Necula and Kuret 2005). The association rate constant for elongation, $k_{e+}$, was then obtained from the relationship (Congdon, Kim et al. 2008):
2.2.5 Aggregation Time Series

Aliquots of tau aggregation reactions prepared as described above were removed as a function of time and assayed for filament formation by electron microscopy. Aggregation lag times, defined as the time when the tangent to the point of maximum aggregation rate intersects the abscissa of the sigmoidal curve (Evans, Berger et al. 1995), were obtained ± SE from each time series by Gompertz regression as described in (Necula and Kuret 2004). To determine the nucleation dissociation equilibrium constant, $K_n$, filament length data was converted to protomer concentration, $c_p^*$, by assuming that all protein above the critical concentration formed filaments (Congdon, Kim et al. 2008), and that the resultant filaments were composed of two tau protomers per β-sheet spacing (Congdon, Kim et al. 2008). Data were then fitted to the simplified homogeneous nucleation scheme of Wegner and Engel (Wegner and Engel 1975) assuming a dimeric nucleus (Congdon, Kim et al. 2008):

$$c_1 = c_{total} - c_{p^*} \quad \text{(Eq. 2.3)}$$

$$\frac{dc_p}{dt} = \frac{k_{e+} (k_{e-} c_1 - k_{e-}^2) c_1}{k_{e-} + k_{e+} c_1 - k_{e-}} \quad \text{(Eq. 2.4)}$$

$$\frac{dc_p^*}{dt} = (k_{e+} c_1 - k_{e-}^2) c_p \quad \text{(Eq. 2.5)}$$

where $c_{total}$, $c_1$, and $c_p$ represent bulk tau, tau monomer, and tau filament concentrations, respectively. Parameter estimates were obtained by fitting experimentally determined values of $c_{total}$, $c_{p^*}$, $k_{e-}$, and $k_{e+}$ to equations 3-5 in JACOBIAN™ modeling software (Numerica Technology, LLC, Cambridge, MA). The simulation yielded estimates of
forward and reverse nucleation rate constants \( k_{n+} \) and \( k_{n-} \), with the ratio \( k_{n-}/k_{n+} \) recorded as \( K_n \).

### 2.2.6 Statistical Analysis

The probability \( (p) \) of obtaining the observed results, assuming the null hypothesis, was assessed by \( z \)-test:

\[
z = \frac{x_1 - x_2}{\sqrt{(S_{x_1})^2 + (S_{x_2})^2}} \quad \text{(Eq. 2.6)}
\]

where \( x_1 \pm S_{x_1} \) and \( x_2 \pm S_{x_2} \) are the pair of estimates \( \pm \) SE being compared, \( z \) is the \( 1-\alpha \) point of the standard normal distribution, and \( p \) is \( 2\alpha \). All statistical analyses were carried out using JMP 7.0 (SAS Institute, Cary, NC).

### 2.3. Results

#### 2.3.1 Initial Characterization

Missense tau mutants R5L, G272V, P301L, V337M, and R406W, were selected for analysis because their association with FTLD is well established and because all except R5L have been shown to promote neurofibrillary lesion formation in transgenic mice (Denk and Wade-Martins 2007). Furthermore, the mutations span the length of the tau molecule (Fig. 2.1A) to include the N-terminal projection domain (R5L), the first, second, and third microtubule-binding repeats (G272V, P301L, and V337M), and the C-terminal tail (R406W). Because P301L can be accommodated in 4R but not 3R tau isoforms, all mutants were prepared in a 2N4R background to facilitate direct comparison to each other and also wild-type 2N4R prepared by identical methods.
When incubated in the presence of Thiazine red aggregation inducer under near physiological conditions of pH, reducing conditions, and ionic strength, sub-micromolar concentrations of wild-type 2N4R form twisted ribbons (Chirita, Congdon et al. 2005) with a mass-per-unit length similar to authentic brain-derived PHFs (Congdon, Kim et al. 2008). When the missense mutants were incubated under identical conditions up to 1 µM concentration, all formed filaments of similar width and morphology as wild-type 2N4R (Fig. 2.1B-G). Filament length distributions were also similar to wild-type 2N4R tau (data not shown). These data indicate that all five missense mutations retain the fundamental aggregation properties of wild-type tau and can be characterized at physiological bulk tau concentrations.

To quantify the effects of primary structure on relative aggregation propensity, the critical concentration ($K_{\text{crit}}$) of each mutant was estimated in the presence of Thiazine red inducer. In nucleation dependent reactions, $K_{\text{crit}}$ approximates the equilibrium dissociation constant for elongation, $K_e$ (Congdon, Kim et al. 2008). Because $K_{\text{crit}}$ also represents the highest protein monomer concentration that does not support aggregation, it can be estimated from the abscissa intercept of the tau concentration dependence of plateau fibrillization in the submicromolar concentration regime. Results showed that $K_{\text{crit}}$ values for R5L, V337M, and R406W were all ~200 nM (Fig. 2.2A), and did not differ from wild-type 2N4R tau at $p < 0.05$ (Fig. 2.2B; Table 2.1). In contrast, both G272V and P301L mutations reduced $K_{\text{crit}}$ to below 90 nM (Fig. 2.2B; Table 2.1). These data indicate that tauopathy mutants G272V and P301L lead to increased aggregation propensity by depressing the minimum concentration of tau needed to support fibril
formation. However, $K_{\text{crit}}$ depression could potentially result from changes in sensitivity to Thiazine red inducer as well. To address this possibility, the concentration-effect relationship for Thiazine red mediated fibrillization was quantified for all tau preparations at constant tau supersaturation (*i.e.*, the net difference between bulk concentration and $K_{\text{crit}}$ was held constant, so that the amount of aggregation at equilibrium was approximately the same for all tau species). Results showed that all mutants resembled wild-type 2N4R tau with respect to both the potency and efficacy of Thiazine red (Fig. 2.3). Together these data indicate that tauopathy mutants do not display differential sensitivity to Thiazine red, and suggest that the decreases in $K_{\text{crit}}$ observed with G272V and P301L reflect intrinsic differences in aggregation propensity relative to wild-type tau.

### 2.3.2 Effect of tau mutation on extension rates

$K_{\text{crit}}$ approximates the ratio of dissociation ($k_{e-}$) and association ($k_{e+}$) rate constants for filament elongation (Eq. 2.2). Thus decreases in $K_{\text{crit}}$ may result from decreases in $k_{e-}$, corresponding to filament stabilization, from increases in $k_{e+}$, reflecting a more efficient association reaction, or from both. To determine the mechanism of $K_{\text{crit}}$ depression, $k_{e-}$ was estimated for each tau preparation by diluting preassembled filaments in assembly buffer containing Thiazine red but no tau protein and following the time-dependent loss of filament length by electron microscopy. Loss of filament length was first order as predicted for endwise depolymerization from a Poisson-like length distribution (Kristofferson, Karr et al. 1980) (Fig. 2.4A). On the basis of the relationship between tau mass and filament length established for wild-type 2N4R tau (Congdon, Kim et al. 2008), the dissociation elongation constant $k_{e-}$ was derived from the disaggregation rate of each
mutant (Table 2.1). Rate constant $k_{e^+}$ was then calculated from estimates of $k_e$ and $K_{crit}$ for each mutant through Equation 2.2 (Table 2.1). Pairwise comparison of any mutant with wild-type 2N4R tau failed to show a significant difference in $k_e$ at $p < 0.05$ (Fig. 2.4B). In contrast, both G272V and P301L aggregated with significantly greater $k_{e^+}$ values than wild-type tau. These data indicate that G272V and P301L mutations depress $K_{crit}$ by increasing the efficiency of monomer addition to nascent filament ends.

### 2.3.3 Effect of tau mutation on nucleation rates

In the presence of Thiazone red inducer, tau aggregation approximates an equilibrium nucleation-elongation reaction, where assembly-competent monomer rapidly equilibrates with a thermodynamically unstable species termed the nucleus (Ferrone 1999). Once the critical nucleus cluster size is reached, subsequent additions to the nascent filament ends are energetically favorable and elongation proceeds efficiently. As a result, aggregation rate depends not only on the rate of filament elongation, but on the efficiency of the nucleation step as well. To assess the effects of primary structure on nucleation rate, the time course of aggregation was quantified for each tau construct at constant supersaturation. Under these conditions, differences in reaction rates primarily reflect differences in rates of nucleation and protein concentrations (Fesce, Benfenati et al. 1992). All resultant reaction progress curves were sigmoidal with lag, exponential growth, and equilibrium phases (Fig. 2.5A). Lag times, which vary inversely with nucleation rate (Evans, Berger et al. 1995), were obtained after fitting the data to a 3-parameter Gompertz growth function as described in Experimental Procedures. The values are summarized in Table 2.1 and compared graphically to wild-type 2N4R tau in Fig. 2.5B. The data show that all tauopathy mutants except R406W aggregated with significantly
shorter lag times than wild-type 2N4R tau, suggesting that R5L, G272V, P301L, and V337M mutations can directly accelerate the nucleation phase of the aggregation reaction.

To quantify the nucleation reaction, all reaction progress curves were fit to the approximation of Wegner and Engel (Wegner and Engel 1975) as described previously for wild-type isoform 2N4R (Congdon, Kim et al. 2008). This approach simplifies the family of differential equations describing nucleation and extension of all aggregated species to just three equations (Eq. 2.3 – 2.5) that relate \( c_p^* \) (tau protomer concentration) and \( c_p \) (tau filament concentration) to \( c_{\text{total}} \) (bulk tau concentration). Length measurements were converted to molar units by assuming all tau above \( K_{\text{crit}} \) was fibrillar and all tau below \( K_{\text{crit}} \) was a monomer having concentration \( c_1 \) (Congdon, Kim et al. 2008) The time series in molar units was then fit to equations 2.3 – 2.5 assuming a dimeric nucleus (Congdon, Kim et al. 2008) Equations 4 and 5 predict that filament number concentration (\( c_p \)) should be a function of tau concentration and all four rate constants that govern the nucleation and extension phases of the aggregation reaction. Therefore, filament number is not a direct measure of any kinetic parameter, including nucleation rate. However, because both R5L and V337M display decreased \( K_n \) relative to wild-type 2N4R while their elongation rate constants remain unchanged, they would be predicted to yield more filaments relative to wild-type 2N4R tau when compared at identical tau concentrations. A simulation of this hypothetical reaction at 400 nM bulk tau concentration using the kinetic constants summarized in Table 2.1 is shown in Fig. 2.6A. R5L and V337M were predicted to produce ~1.4 – 1.6 fold increases in filament number relative to wild-type 2N4R at reaction plateau. To test this prediction, R5L, V337M, and wild-type 2N4R were subjected to aggregation conditions at identical bulk concentrations.
(400 nM), then subjected to transmission electron microscopy to quantify filament number. The results showed that R5L and V337M yielded 1.5 ± 0.2 and 1.7 ± 0.1 fold increases in filament number relative to wild-type 2N4R tau, respectively (Fig. 2.6B). These data are consistent with the increases in nucleation rates predicted by mathematical simulation, and support the hypothesis that missense tauopathy mutants can differentially modulate the nucleation phase of the aggregation reaction.

The ratio of elongation and nucleation equilibrium constants, termed $\sigma$, provides an index of reaction cooperativity (Zhao and Moore 2003). Expressed in terms of free energy, $\Delta G_\sigma$ represents the difference in energy that accompanies contacts formed in the elongation step relative to the nucleation step. $\Delta G_\sigma$ varied over the narrow range of 6.2 to 6.9 Kcal/mol for wild-type and mutant tau preparations (Table 2.1), indicating that the free energy of nucleation was consistently less favorable than that for elongation. Overall these data indicate that tauopathy mutants can strongly and directly increase aggregation propensity at nucleation and extension steps while retaining the cooperative aggregation mechanism of wild-type tau.

2.4 Discussion
2.4.1 Significance
Certain tau missense mutations cause FTLD, indicating that tau dysfunction alone can induce neurofibrillary lesion formation, neurodegeneration, and cognitive decline. The findings here, along with those from previous studies, are consistent with the disease promoting activity of some missense mutations being related to augmentation of tau
aggregate formation. In the case of the R5L, G272V, P301L, and V337M, aggregation was promoted directly at the level of intrinsic aggregation propensity. The largest effects on aggregation were observed with G272V and P301L mutations, which are located in exon 9 and 10 regions that encode the first and second binding repeats, respectively. As exon 10 is alternatively-spliced, P301L only affects four-repeat isoforms of tau whereas G272V affects both three- and four-repeat isoforms. Both mutants promote filament nucleation by decreasing the dissociation equilibrium constant for dimerization and support aggregation at low tau concentrations by lowering the dissociation equilibrium constant for filament extension. For both mutants, effects on the extension reaction are driven primarily by an increase in the association rate constant, $k_{e^+}$, governing association of tau monomer with filament ends. Therefore, the aggregation of G272V and P301L tau is predicted to be highly sensitive to changes in free cytosolic tau concentrations. Although bulk tau levels are reportedly low micromolar (Drubin, Feinstein et al. 1985; Khatoon, Grundke-Iqbal et al. 1994), normal free cytosolic concentrations are well below this owing to high-affinity microtubule binding and sequestration (Ackmann, Wiech et al. 2000; Makrides, Massie et al. 2004). Thus, the ability of G272V and P301L to support rapid tau aggregation at submicromolar concentrations is likely to be especially important in the early stages of disease when tau dissociation from microtubules is incomplete.

With respect to structure, both mutations affect PGGG segments located in the repeat region of several microtubule-associated proteins (Lewis, Wang et al. 1988; Olson, McIntosh et al. 1995); G272V alters the segment in the first repeat to $^{270}$PGVG$^{273}$, whereas P301L alters the segment in the second repeat to $^{301}$LGGG$^{304}$. Each segment lies adjacent to hexapeptide motifs involved in transition of the repeat region to cross-$\beta$-sheet
structure (von Bergen, Friedhoff et al. 2000). Both mutations increase local hydrophobicity, which is a major determinant of aggregation propensity (Pawar, Dubay et al. 2005; Rojas Quijano, Morrow et al. 2006; Tartaglia, Pawar et al. 2008), and a hypothetical model linking the conformation of PGGG segments to local hexapeptide β-sheet formation has been proposed (von Bergen, Barghorn et al. 2001). The similarity in aggregation propensity profile – reduced $K_{crit}$, increased $k_e^+$, and reduced lag time and $K_n$ – are consistent with these two mutations targeting homologous segments of the repeat region.

In contrast, the R5L mutation led to increased nucleation rate but no change in extension kinetics relative to wild-type tau. The effects on nucleation are consistent with the ability of another missense mutation at the same site, R5H, to increase numbers of filaments formed at high concentrations in vitro relative to wild-type tau (Hayashi, Toyoshima et al. 2002). As the core of the PHF is mainly composed of the repeat region of tau (Novak, Kabat et al. 1993), it is perhaps surprising that an N-terminal mutation can result in increased aggregation propensity. However, conformation-sensitive antibody binding studies suggest that the N-terminus is in close proximity to or influenced by the microtubule binding repeat region (Carmel, Mager et al. 1996). In fact, the N-terminus of tau may contact the repeat domain in tau monomers (Jeganathan, von Bergen et al. 2006), and elimination of this interaction through truncation results in decreased aggregation rates in vitro (Gamblin, Berry et al. 2003). The change of Arg to Leu eliminates a positive charge and again introduces a hydrophobic residue, both of which are predicted
Mutation V337M is found in the third repeat, which composes part of the PHF core (Novak, Kabat et al. 1993). Nonetheless, this region is not essential for arachidonic acid induced fibrillization in vitro (Abraha, Ghoshal et al. 2000). V337M resembles R5L in terms of aggregation propensity, displaying an increased nucleation rate without change in $K_{\text{crit}}$, $k_{n_+}$, or $k_{e+}$. Consistent with these observations, a previous study showed an increase in aggregation rate but not in plateau levels in the presence of heparin (Nacharaju, Lewis et al. 1999). These data are consistent with V337M favoring faster nucleation rate with little effect on the extension reaction. A similar spectrum of activity may accompany K257T, which reportedly increases filament number (Rizzini, Goedert et al. 2000) but not total filament mass at reaction plateau (Grover, England et al. 2003).

R406W is located in the C-terminal segment flanking the repeat region. Although truncation of this region reportedly increases extent of aggregation (Abraha, Ghoshal et al. 2000), here it was found that R406W did not differ from wild-type tau with respect to aggregation propensity. Therefore, the biological effects of R406W on aggregation are most likely indirect, and depend on differential interaction with cellular factors, such as phosphotransferases (Goedert, Satumtira et al. 2000; Alonso Adel, Mederlyova et al. 2004).

2.4.2 Interplay of tau mutations with cellular factors

Tau aggregation in cells is a complex process, where the intrinsic aggregation propensity of tau proteins is modulated by interactions with other factors. We have proposed that the pathway involves four principal steps that must be overcome for filamentous
aggregates to accumulate in disease (Congdon, Kim et al. 2008) (Fig. 2.7). First, tau must
dissociate from microtubules so that its free cytosolic concentration exceeds the minimal
tau concentration necessary to support aggregation. Although marked decreases in
microtubule assembly kinetics have been observed for many missense mutations,
including G272V, P301L, V337M, and R406W (Hasegawa, Smith et al. 1998; Barghorn,
Zheng-Fischhofer et al. 2000; DeTure, Ko et al. 2000; Poorkaj, Muma et al. 2002), their
binding affinity for microtubules, which may more closely reflect normal tau function
(Qiang, Yu et al. 2006), is only weakly affected \textit{in vitro} and remains high affinity
(Barghorn, Zheng-Fischhofer et al. 2000; DeTure, Ko et al. 2000). So long as the
dissociation equilibrium constant for binding remains lower than the concentration of
available tau binding sites on microtubules, small changes in affinity may not appreciably
affect free tau concentrations. In MCF7 cells, for example, mutant tau (i.e., G272V,
P301L, or R406W) microinjected at physiological concentrations colocalizes with
microtubules much like wild-type tau (Bunker, Kamath et al. 2006). Similarly, bulk tau
levels raised by increased expression or decreased degradation of tau may have limited
effects on free tau levels. R5L is associated with \textsim 2-fold bulk tau overexpression
(Poorkaj, Muma et al. 2002), whereas V337M and R406W stabilize tau against
proteolytic degradation by calpain I (EC 3.4.22.52) (Yen, Easson et al. 1999). Although
the magnitude of these changes may be insufficient to overcome the large excess of
tubulin in cells (Drubin, Feinstein et al. 1985), both increased expression and decreased
turnover could make more tau available for aggregation once liberated from microtubules
by other means. Binding affinity is modulated by post-translational modifications such
as phosphorylation (Biernat, Gustke et al. 1993; Bramblett, Goedert et al. 1993), which
may be the primary gatekeeper controlling the amount of free tau available for aggregation. The missense tauopathy mutants examined here (R5L, G272V, P301L, V337M, and R406W) all have been reported to be better protein kinase substrates \textit{in vitro}, whereas G272V, P301L, V337M, and R406W are weaker phosphoprotein phosphatase 2A (EC 3.1.3.16) binding partners than wild-type tau isoforms (Goedert, Satumtira et al. 2000; Alonso Adel, Mederlyova et al. 2004). Both of these interactions would be expected to increase occupancy of phosphorylation sites mediating tau-microtubule affinity, and to increase amounts of cytosolic tau available for aggregation (regardless of any direct affects of phosphorylation on the aggregation reaction). Overall, changes in microtubule binding affinity, expression levels, and degradation may synergize with the effects of posttranslational modifications on tau-microtubule equilibria to increase the amount of free tau available for aggregation. These interactions may be especially important for aggregation of R406W which, as shown here, does not directly modify intrinsic aggregation propensity.

The second step involves a conformational change to an assembly competent state (Fig. 2.7). This step is proposed to be a barrier to aggregation because high concentrations (\textit{i.e.}, up to 100 µM) of free tau alone are insufficient to support aggregation or seeding reactions \textit{in vitro} (Ko, DeTure et al. 2002). In contrast, tau proteins readily aggregate in the presence of anionic inducers such as Thiazine red. The effects of missense mutations on this step are not clear, but on the basis of infrared spectroscopy, neither G272V, P301L, V337M, nor R406W increases secondary structure
content of tau monomers relative to wild-type constructs (von Bergen, Barghorn et al.

Once aggregation-competent conformations are adopted, the rate-limiting step in filament formation becomes dimerization, which is energetically disfavored at physiological tau concentrations, and therefore a third key point of control (Fig. 2.7). As shown here, missense mutations can directly modulate this step. Because of its dimeric nature, nucleus structure may be a key determinant of whether 3R or 4R forms of tau predominate in aggregates. For example, disulfide bond formation can favor nucleation of 3R isoforms while inhibiting nucleation of 4R isoforms (Schweers, Mandelkow et al. 1995).

The final step in filament formation is extension. Although not rate limiting, equilibria at filament ends dictate the minimal concentration of tau required to support aggregation. In addition to promoting microtubule dissociation, the negative charge associated with phosphorylation can enhance the extension reaction by inhibiting filament dissociation (Necula and Kuret 2004; Necula and Kuret 2005). This reaction may synergize with the effects of P301L and G272V on extension to further augment the rate of monomer association with filament ends.

Overall, the findings here are consistent with a growing literature suggesting that missense tau mutations promote tau aggregation by acting at multiple steps along a single pathway. For some mutations, these include direct effects on the rate and/or extent of aggregation. The differential activity of missense mutations on individual steps in the pathway may influence how tau misfunction leads to clinically and histopathologically distinct diseases.
2.5 Tables

<table>
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<tr>
<th></th>
<th>$^a K_{crit}$</th>
<th>$^a k_{r-}$</th>
<th>$^a k_{r+}$</th>
<th>Lag Time</th>
<th>$K_n$</th>
<th>$^b \Delta G_\sigma$</th>
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<td></td>
<td>(nM)</td>
<td>(s$^{-1}$)</td>
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<td>(h)</td>
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<td>(Kcal/mol)</td>
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<td>2N4R</td>
<td>200 ± 15</td>
<td>0.019 ± 0.001</td>
<td>94.8 ± 12.8</td>
<td>0.54 ± 0.09</td>
<td>15.0</td>
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<tr>
<td>R5L</td>
<td>226 ± 7</td>
<td>0.021 ± 0.002</td>
<td>92.9 ± 9.9</td>
<td>0.37 ± 0.12*</td>
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<td>6.3</td>
</tr>
<tr>
<td>G272V</td>
<td>89 ± 8**</td>
<td>0.020 ± 0.001</td>
<td>229.0 ± 22.0**</td>
<td>0.10 ± 0.05**</td>
<td>2.2</td>
<td>6.2</td>
</tr>
<tr>
<td>P301L</td>
<td>80 ± 12**</td>
<td>0.019 ± 0.001</td>
<td>240.0 ± 38.0**</td>
<td>0.23 ± 0.04**</td>
<td>3.0</td>
<td>6.5</td>
</tr>
<tr>
<td>V337M</td>
<td>205 ± 12</td>
<td>0.019 ± 0.002</td>
<td>90.5 ± 9.3</td>
<td>0.33 ± 0.10*</td>
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<tr>
<td>R406W</td>
<td>186 ± 14</td>
<td>0.016 ± 0.001</td>
<td>87.1 ± 8.5</td>
<td>0.47 ± 0.15</td>
<td>11.8</td>
<td>6.8</td>
</tr>
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$^a$Overall constants reflecting events at both filament ends

$^b\Delta G_\sigma = -RT \ln \sigma$, where $\sigma = K_{crit}/K_n$

*, $p < 0.05$; **, $p < 0.01$ versus 2N4R tau.

Table 2.1: Summary of FTLD mutant aggregation parameters
2.6 Figures

Figure 2.1. FTLD mutant filament morphology. (a) Distribution of 27 amino acid residues affected by pathological missense tau mutations currently tabulated at http://www.molgen.ua.ac.be/FTDMutations (Rademakers, Cruts et al. 2004) and depicted on isoform 2N4R. This isoform contains alternatively spliced exons 2 and 3 (E2 and E3), each of which encodes an acidic 29-residue segment, and exon 10 (E10), which encodes an additional microtubule binding repeat sequence. Mutants R5L, G272V, P301L, V337M, and R406W modeled herein span the tau molecule and are distinguished graphically with raised hollow symbols. (b-g) Full-length wild-type 2N4R tau (b) and mutants R5L (c), G272V (d), P301L (e), V337M (f), and R406W (g) were incubated (1 µM concentration) without agitation in the presence of 100 µM Thiazine red (24 h at 37°C), spotted onto copper Formvar-carbon mesh grids, stained with 2% uranyl acetate, and viewed by transmission electron microscopy. All tau species produced unbranched filaments ~16 nm in diameter with no obvious differences in morphology or length distribution. Scale bar = 200 nm.
Fig. 2.2. Effect of tau mutations on critical concentration. Wild-type 2N4R tau (●) and mutants R5L (○), G272V (▼), P301L (△), V337M (■), and R406W (□) were incubated at varying bulk concentrations in the presence of Thiazine red inducer for 24 h at 37°C, then assayed for filament formation by electron microscopy. (a) Plot of total filament length against bulk protein concentration, where each data point represents the mean ± SD of triplicate determinations and the solid lines represent best fit of the data points to linear regression. The abscissa intercept, which was obtained by extrapolation (dotted lines), was used to estimate critical concentration ($K_{\text{crit}}$). (b) Replot of data from Panel A, where each bar represents the $K_{\text{crit}}$ for examined tau species relative to wild-type 2N4R (dashed line). Both G272V and P301L tau mutants aggregated with significantly lower $K_{\text{crit}}$ values than did wild-type 2N4R. **, $p < 0.01$ versus wild-type 2N4R tau.
Fig. 2.3. Tau mutants share a common sensitivity to aggregation inducer Thiazine red. Wild-type 2N4R (●) and mutants R5L (○), G272V (▼), P301L (Δ), V337M (■), and R406W (□) were incubated (24 h at 37°C) at constant supersaturation (i.e., 0.5 µM above $K_{\text{crit}}$) in the presence of varying concentrations of Thiazine red, and then assayed for filament formation by electron microscopy. Each data point represents mean total filament lengths/field from triplicate determinations, whereas the solid curve is drawn solely to aid visualization. Under these conditions, the concentration effect relationship for Thiazine red was similar for all tau species.
Fig. 2.4. Estimation of extension association and dissociation rate constants. (a) Tau filaments prepared from wild-type 2N4R (●) and mutants R5L (○), G272V (▼), P301L (△), V337M (■), and R406W (□) in the presence of 100 µM Thiazine red at 37ºC were diluted 10-fold into assembly buffer containing Thiazine red, and the resultant disaggregation was followed as a function of time by electron microscopy. Each data point represents total filament length per field ± SD (n = 3 observations), whereas the solid lines represent best fit of the data points to linear regression. The first order decay constant $k_{app}$ was estimated from each regression, and used in conjunction with measured $K_{crit}$ values to estimate $k_e$ and $k_{e+}$ as described in the Experimental Procedures section. (b) Replot of data from Panel A, where each bar represents $k_e$ and $k_{e+}$ values for each examined tau species relative to wild-type 2N4R (dashed line). Filaments composed of mutants G272V and P301L extended significantly faster than did 2N4R tau. **, p < 0.01 versus wild-type 2N4R tau.
Fig. 2.5. Effects of tau mutations on aggregation time course. (a) Wild-type 2N4R (●) and mutants R5L (○), G272V (▼), P301L (△), V337M (■), and R406W (□) were incubated (37°C) at constant supersaturation (i.e., 0.2 µM above \(K_{\text{crit}}\)) in the presence of 100 µM Thiazine red, and then assayed for filament formation as a function of time. Each data point represents mean total filament lengths/field (expressed as % plateau length) calculated from triplicate electron microscopy images whereas each normalized curve represents best fit of the data points to a three parameter Gompertz growth function. The fits were used to calculate lag time as described in the Experimental Procedures section. (b) Replot of data from Panel A, where each bar represents the lag time ± SE calculated from each Gompertz regression normalized to wild-type isoform 2N4R (dotted line). Mutants R5L, G272V, P301L, and V337M aggregated significantly faster than wild-type 2N4R when compared at constant supersaturation. *, \(p < 0.05\); **, \(p < 0.01\) versus wild-type 2N4R tau.
Fig. 2.6. Mutants R5L and V337M yield increased filament number concentration. (a) Mathematical simulation of reaction time course for R5L, V337M, and WT 2N4R tau at 400 nM bulk concentration using the kinetic parameters summarized in Table 1. Each curve represents $c_p$ (the predicted number concentration of filaments) as function of time. The simulations predict that the decreases in $K_n$ associated with R5L and V337M should yield increases in filament number concentration relative to 2N4R tau. (b) R5L, V337M, and wild-type 2N4R were incubated (37°C) at 400 nM concentration in the presence of 100 µM Thiazine red, and then subjected to electron microscopy analysis at 24h. Each column represents mean total filament number/field ± S.D. (normalized to wild-type 2N4R tau) calculated from quadruplicate electron microscopy images. Consistent with mathematical simulation, R5L and V337M yielded significantly more tau filaments relative to wild-type 2N4R tau when assayed at identical concentration. **, $p < 0.01$; n.s., $p > 0.05$. 
Fig. 2.7. Effect of FTLD mutations on the fibrillization pathway. Normal tau binds tightly to microtubules, but dissociates upon phosphorylation to form free tau, which exists as a natively disordered, assembly incompetent monomer (Uₓ). A conformational change to an assembly competent state accelerates polymerization (Uₜ). Once assembly competent species form, the rate-limiting step in tau fibrillization is formation of dimer, which represents the thermodynamic nucleus (N). Following nucleation, extension occurs through further addition of assembly competent monomers to the filament (F) ends. Tau mutations promote fibrillization at multiple points in the pathway. Reactions characterized herein are shown in green lettering, whereas those reported in the literature are shown in red lettering. See text for details.
3.1 Introduction

The neurofibrillary lesions of Alzheimer’s disease develop intracellular aggregates of the microtubule-associated protein tau (Ballatore, Lee et al. 2007). Although certain familial tauopathies result from mutations in the tau gene (MAPT), the pathogenesis of Alzheimer’s disease is not associated with changes in tau amino acid sequence. Rather, lesion formation is accompanied by a 3-4 fold increase in tau phosphorylation stoichiometry (Ksiezak-Reding, Liu et al. 1992; Kopke, Tung et al. 1993). The covalently bound phosphate is distributed among ~30 sites within and adjacent to the tau microtubule-binding domain (Morishima-Kawashima, Hasegawa et al. 1995; Hanger, Betts et al. 1998; Hanger, Byers et al. 2007). Occupancy of these sites may influence tau aggregation in two ways. First, occupancy of certain sites modulates tau-tubulin affinity (Lindwall and Cole 1984), fostering an increase in the levels of free cytoplasmic tau available to nucleate and support the aggregation reaction (Biernat, Gustke et al. 1993; Bramlett, Goedert et al. 1993; Patrick, Zukerberg et al. 1999; Li, Yin et al. 2004). Second, hyperphosphorylation can increase tau aggregation propensity directly (Alonso,
Grundke-Iqbal et al. 1997; Alonso, Zaidi et al. 2001). However, the precise mechanism of these direct effects has been difficult to establish. Challenges to overcome include the difficulties of recapitulating the complex phosphorylation patterns observed in disease tissue, and of quantifying the aggregation reaction under controlled conditions. The challenge of site occupancy has been addressed through phosphorylation mimicry, where phosphorylatable hydroxy-amino acids are converted to negatively-charged Asp or Glu residues. The approach fosters site-specific incorporation of negative charge at full occupancy. Resultant pseudophosphorylation mutants have been shown to mimic phosphorylation-induced changes in tau structure and function (Leger, Kempf et al. 1997; Eidenmuller, Fath et al. 2001; Bielska and Zondlo 2006), and to be recognized by phosphorylation-sensitive anti-tau antibodies (Haase, Stieler et al. 2004). The challenge of aggregation kinetics has been addressed by the development of agents that drive efficient aggregation \textit{in vitro} over tractable time periods and near physiological concentrations of tau protein (Kuret, Congdon et al. 2005).

Despite these advances, aggregation kinetics in the presence of exogenous inducers can be difficult to analyze with explicit models. For example, the effects of some inducers, such as heparin, depend on the concentration ratio between inducer and tau protein (Friedhoff, Schneider et al. 1998). Other inducers, such as anionic surfactants, micellize on contact with tau (Chirita, Necula et al. 2003). When aggregation reactions are initiated with octadecyl sulfate (ODS), for example, the rate of micellization is slow relative to aggregation, and so the early stages of aggregation may be obscured (Necula and Kuret 2004; Necula and Kuret 2004).

Recently we found that aggregation of full-length tau at submicromolar
concentrations can be achieved with Thiazine red (Chirita, Necula et al. 2003). Thiazine red mediated aggregation can be explicitly modeled as a homogeneous nucleation scheme involving the formation of an unstable dimeric nucleus followed by monomer addition to growing filament ends (Congdon, Kim et al. 2008). Under these conditions, the nucleation and extension phases of aggregation can be assessed and quantified. Thus, the inherent aggregation propensity of pseudophosphorylated tau can be quantified and compared to that of wild-type tau.

Here, we examine the aggregation propensity of a tau mutant pseudophosphorylated at residue T212 in 2N4R background. T212 composes part of the AT100 epitope (Hoffmann, Lee et al. 1997; Yoshida and Goedert 2006), which is recognized by multiple protein kinases (Drewes, Lichtenberg-Kraag et al. 1992; Illenberger, Zheng-Fischhofer et al. 1998; Zheng-Fischhofer, Biernat et al. 1998; Reynolds, Betts et al. 2000; Woods, Cohen et al. 2001), and is selectively occupied in disease (Matsuo, Shin et al. 1994). The results show that the introduction of negative charge at this position directly promotes tau fibrillization by acting at multiple points along the aggregation pathway.

3.2 Experimental Procedures

3.2.1 Materials

Recombinant polyhistidine-tagged 2N4R tau and pseudophosphorylation mutant 2N4R-T212E were prepared as described previously (Carmel, Mager et al. 1996; Necula and Kuret 2004). Aggregation inducer Thiazine red (Chemical Abstract Service registry number 2150-33-6) was obtained from TCI America (Portland, OR, USA).
Formvar/carbon-coated copper grids, glutaraldehyde, and uranyl acetate were obtained from Electron Microscopy Sciences (Fort Washington, PA, USA).

### 3.2.2 Tau fibrillization assay

Purified tau was incubated without agitation in assembly buffer (10 mM HEPES, pH 7.4, 100 mM NaCl, and 5 mM dithiothreitol) for up to 24 h at 37°C. Aggregation inducer Thiazine red was included at 100 µM final concentration. Reactions were terminated with 2% glutaraldehyde, adsorbed to Formvar/carbon-coated copper grids, stained with 2% uranyl acetate, and viewed in a Tecnai G2 Spirit BioTWIN transmission electron microscope (FEI, Hillsboro, OR, USA) operated at 80 kV and 23,000-49,000x magnification. At least three viewing fields were captured for each reaction condition in which filaments >10 nm in length were counted and quantified with ImageJ software (National Institutes of Health, Bethesda, MD, USA). Total filament length is defined as the sum of the lengths of all resolved filaments per field and is reported as ± SD.

### 3.2.3 Critical concentration

Critical concentrations ($K_{\text{crit}}$) were determined by inverse prediction of the abscissa intercept on a plot of the concentration dependence of tau aggregation, as described previously (Congdon, Kim et al. 2008). The accompanying standard error of the estimate ($S_x$) was calculated as:

$$S_x = \frac{C.I.}{2(t_{0.975,n-2})}$$

(Eq. 3.1)

where C.I. is the Fieller 95% confidence interval of each regression, and $t_{0.975,n-2}$ is the Student’s $t$ distribution percentage at 1- $\alpha = 0.975$ and $n - 2$ degrees of freedom.
3.2.4 Dissociation rates

Assembled tau filaments prepared as described above were diluted 10-fold into assembly buffer containing 100 µM Thiazine red and incubated at 37°C. Aliquots were removed as a function of time up to 5 h post-dilution and assayed for total filament length. The disaggregation time course was fit to an exponential decay function in order to determine $k_{app}$, the pseudo first-order rate constant describing the decrease in filament length over time, and $L_0$, the total filament length at time zero. These values, combined with the number of filaments at time zero, and the mass-per-unit length of Thiazine red-induced tau filaments (Congdon, Kim et al. 2008), were used to determine the dissociation rate constant $k_e$. (Kristofferson, Karr et al. 1980; Necula and Kuret 2005). The association rate constant $k_{e+}$ was then determined from the relationship (Congdon, Kim et al. 2008):

$$K_{crit} = k_e / k_{e+}$$  \hspace{1cm} (Eq. 3.2)

3.2.5 Aggregation time series

Aggregation lag times, defined as the time when the tangent to the point of maximum aggregation rate intersects the abscissa of the sigmoidal curve (Evans, Berger et al. 1995), were obtained ± SE from each time series by Gompertz regression as described in (Necula and Kuret 2004). Filament length data was converted to protomer concentration based on the assumptions that all protein above the critical concentration formed filaments (Congdon, Kim et al. 2008) and that the resultant filaments were composed of two tau protomers per β-sheet spacing (Congdon, Kim et al. 2008). Data were then fitted to the simplified homogeneous nucleation scheme of Wegner and Engel (Wegner and
Engel 1975) in JACOBIAN™ modeling software (Numerica Technology, LLC, Cambridge, MA) assuming a dimeric nucleus as described previously (Congdon, Kim et al. 2008).

### 3.2.6 Statistical analysis

The probability of differences between kinetic parameters was assessed by z-test:

\[
z = \frac{x_1 - x_2}{\sqrt{(S_{x_1})^2 + (S_{x_2})^2}}
\]

(Eq. 3.3)

where \(x_1 \pm S_{x_1}\) and \(x_2 \pm S_{x_2}\) are the pair of estimates ± SE being compared, and \(z\) is the 1-\(\alpha\) point of the standard normal distribution. All statistical analyses were carried out using SigmaPlot 10.0 (Systat Software, Chicago, IL) and JMP 7.0 (SAS Institute, Cary, NC).

### 3.3 Results

#### 3.3.1 Effect of pseudophosphorylation on critical concentration

Approximately thirty phosphorylation sites have been mapped to the microtubule-binding repeat region on authentic tau filaments isolated from AD brain (Fig. 3.1a). Previously, we showed that phosphorylation mimicry at some of these sites modulated the aggregation propensity of recombinant tau preparations in the presence of anionic surfactant inducer (Necula and Kuret 2004). Among these mutants, T212E showed the greatest effect and so was selected for detailed analysis in the presence of a second inducer, Thiazine red. When full length recombinant 2N4R tau (≤1 µM) was incubated with Thiazine red at near-physiological conditions of pH, ionic strength, and reducing environment, filaments with twisted ribbon morphology formed (Fig. 3.1b). These
filaments have a mass-per-unit length similar to authentic brain-derived paired helical filaments (Congdon, Kim et al. 2008). When incubated under identical conditions, 2N4R-T212E produced filaments that were morphologically identical to those formed by wild-type 2N4R tau (Fig. 3.1c). These data indicate that T212E shares the fundamental aggregation characteristics of wild-type 2N4R tau and can be studied at physiological bulk tau concentrations.

Characterization of aggregation propensity began with estimation of the minimal tau concentration required to support aggregation of 2N4R and 2N4R-T212E. In nucleation-dependent reactions, the minimal concentration is termed the critical concentration \((K_{\text{crit}})\), and approximates the dissociation equilibrium dissociation constant for elongation \((K_e)\) (Congdon, Kim et al. 2008). The minimal concentration of 2N4R estimated from the abscissa intercept of a plot of aggregation versus tau concentration was \(~200\) nM (Fig. 3.2a; Table 3.1). In contrast, the minimal concentration of 2N4R-T212E was below \(100\) nM (Fig. 3.2a; Table 3.1), differing from wild-type tau at \(p < 0.01\) (Fig. 3.2b). These data indicate that phosphorylation mimicry at residue 212 leads to increased aggregation propensity by depressing the minimum concentration of tau needed to support fibril formation. To confirm that the decrease in \(K_{\text{crit}}\) was a result of increased ability to aggregate and not a change in sensitivity to Thiazine red induction, 2N4R and 2N4R-T212E and were incubated with different concentrations of Thiazine red at constant tau supersaturation \((i.e.\ at\ a\ constant\ tau\ concentration\ above\ K_{\text{crit}})\) (Fig. 3.3). The concentration effect relationship was similar for both tau forms with maximal efficacy near \(100\ \mu\text{M}\), indicating that the response of T212E to Thiazine red was not perturbed. Together these data indicate that the depression of \(K_{\text{crit}}\) observed with
pseudophosphorylation reflects differences in aggregation propensity, and not differential sensitivity to Thiazine red inducer.

3.3.2 Effect of pseudophosphorylation on the extension reaction

From Equation 3.2, $K_{crit}$ approximates the ratio of the rate of monomer dissociation from filament ends ($k_e$) to the rate of monomer addition to filament ends ($k_{e+}$). A decrease in $K_{crit}$ could result from a stabilization of filaments (i.e., a decrease in $k_e$), enhanced monomer addition (an increase in $k_{e+}$), or a combination of both. To distinguish these possibilities, $k_e$ was estimated from the disaggregation rate of preassembled filaments composed of 2N4R and 2N4R-T212E tau. Rate constant $k_{e+}$ was then calculated from estimates of $k_e$ and $K_{crit}$ for each mutant through Equation 3.2. Results show that disaggregation followed first order kinetics as predicted for a Poisson-like distribution of filament lengths undergoing endwise depolymerization (Kristofferson, Karr et al. 1980) (Fig. 3.4a), and that $k_e$ for 2N4R-T212E is decreased nearly two-fold relative to wild-type 2N4R tau (Fig. 3.4b). In contrast, there was no significant difference in calculated values of $k_{e+}$ (Fig. 3.4b). These data indicate that filaments formed by 2N4R-T212E are inherently more stable and less prone to disaggregate than filaments formed by wild-type 2N4R tau. Furthermore, these observations were consistent for two different filament morphologies formed from two distinct inducers (Thiazine red, herein; octadecyl sulfate, (Necula and Kuret 2004; Necula and Kuret 2005)).

3.3.3 Effect of pseudophosphorylation on the nucleation reaction

In the presence of Thiazine red inducer, the aggregation reaction of tau is driven by the rapid equilibration of assembly competent monomers with a thermodynamic nucleus,
defined as the least stable species reversibly interconverted with monomer (Ferrone 1999). Since elongation can proceed efficiently only after the nucleus has formed, the tau aggregation rate depends not only on protein concentration and the rate of elongation, but on the nucleation rate as well. To determine whether pseudophosphorylation affected nucleation rate, tau aggregation time course was quantified for both wild type and T212E at constant supersaturation. Under these conditions, differences in reaction rates primarily reflect differences in rates of nucleation and of protein concentrations (Fesce, Benfenati et al. 1992). Both reaction progress curves displayed lag, exponential growth, and equilibrium phases (Fig. 3.5a). The data were fit to a 3-parameter Gompertz growth function in order to obtain the lag times, which were then normalized to wild-type 2N4R and graphically compared in Fig. 3.5b. Results show that 2N4R-T212E significantly shortened lag time relative to wild-type tau despite being present at lower bulk concentrations, indicating that pseudophosphorylation accelerates the nucleation phase of tau aggregation reaction. To quantify the effect of pseudophosphorylation on the nucleation dissociation equilibrium constant $K_n$, time series were fit to the model of Wegner and Engel as described in (Congdon, Kim et al. 2008) using elongation rate constants $k_{e+}$ and $k_{e-}$ as constraints. The calculations revealed that pseudophosphorylation mutant T212E increases the efficiency of nucleation by decreasing $K_n \sim 4$ fold (Table 3.1). Together these data indicate that incorporation of negative charge at residue 212 directly increases aggregation propensity at the nucleation step by decreasing $K_n$ and at the extension step by decreasing $k_{e-}$. 

45
3.4 Discussion

3.4.1 Summary

These results confirm that incorporation of negative charge into tau protein can directly modulate its aggregation propensity. Two mechanisms have been proposed to account for the effect. The first posits that charge neutralization decreases the isoelectric point of tau protein so as to lower its solubility at physiological pH (Ruben, Ciardelli et al. 1997; Alonso Adel, Mederlyova et al. 2004). The second mechanism predicts that specific tau conformations induced by phosphorylation and phosphorylation mimicry promote self-association (Bielska and Zondlo 2006). These mechanisms need not be mutually exclusive. In fact, multiple phosphorylation sites may exert their effects in tandem, with some modifications enhancing fibrillization ((Haase, Stieler et al. 2004; Necula and Kuret 2004; Ding, Matthews et al. 2006; Kuhla, Haase et al. 2007; Gohar, Yang et al. 2008) and herein) and others having neutral or inhibitory effects (Schneider, Biernat et al. 1999; Liu, Li et al. 2007).

Previously we reported that pseudophosphorylation mutants, including T212E, did not modulate filament nucleation rate (Necula and Kuret 2004). The discrepancy may result from the use of octadecyl sulfate (ODS) as inducer in the previous report. ODS is a surfactant that functions in micellar form by presenting a negatively-charged surface for filament nucleation (Chirita, Congdon et al. 2005). As a result, the rate of filament formation is limited by the rate of micellization, which potentially masks any difference in nucleation rate between T212E and wild-type tau. The results presented herein also disagree with equilibrium aggregation measurements made with pseudophosphorylation mutants including T212E and heparin inducer (Yoshida and Goedert 2006). The
discrepancy could result from differences in protein concentration, which were submicromolar range in the present study, and ~60 µM in the heparin-induced study. In a nucleation-dependent reaction, the amount of aggregation at equilibrium is proportional to the net concentration of protein above the critical concentration rather than the bulk protein concentration. Thus, when bulk concentrations are high relative to critical concentrations, net concentrations may not differ substantially among tau constructs, thereby masking potential differences in aggregation propensity.

3.4.2 Implications of tau aggregation mechanism

On the basis of tau aggregation kinetics in the presence of exogenous inducer Thiazine red (Congdon, Kim et al. 2008), we have proposed that four key steps must be overcome for tau to aggregate in disease (Fig. 3.6). First, the concentration of free tau in the cytoplasm must be sufficient to support filament formation. This can be accomplished through increased MAPT expression, decreased tau degradation, or decreases in tau-microtubule binding affinity. The role of tau phosphorylation in modulating tau-microtubule affinity is well established (Biernat, Gustke et al. 1993; Bramblett, Goedert et al. 1993; Patrick, Zukerberg et al. 1999). For example, T212E reportedly has diminished ability to promote microtubule assembly (Yoshida and Goedert 2006), consistent with an impaired ability to bind tubulin. In addition, tau phosphorylation has been reported to decrease proteasome-mediated tau turnover in a neuronal cell model (Poppek, Keck et al. 2006). Thus, occupancy of certain tau phosphorylation sites may raise free cytoplasmic tau concentrations through multiple mechanisms.

The second step involves the transition of dissociated tau monomers to an assembly-
competent conformation (Fig. 3.6). This step is proposed to be a barrier to aggregation because high concentrations (i.e., up to 100 µM) of free tau alone are insufficient to support aggregation or seeding reactions in vitro (Ko, DeTure et al. 2002). Phosphorylation of tau at multiple sites within the proline-rich region of tau (including T212) can induce local polyproline II helix conformation (Bielska and Zondlo 2006). Adoption of such conformations, which are associated with protein-protein interfaces (Kay, Williamson et al. 2000), may help overcome the resistance of monomeric unmodified tau proteins to aggregation.

Once aggregation-competent conformations are adopted, the rate-limiting step in filament formation becomes dimerization (Congdon, Kim et al. 2008), which is energetically disfavored at physiological tau concentrations, and therefore a third key point of control (Fig. 3.6). In the case of T212E, increased aggregation propensity included acceleration of filament nucleation rate. Consistent with this observation, tau dimerization can be promoted in vitro by NCLK/cdk5 (Paudel 1997), which includes T212 as a target (Illenberger, Zheng-Fischhofer et al. 1998).

The final step in fibrillization is mediated by an extension reaction. Although not rate limiting, equilibria at filament ends dictate the minimal concentration of tau required to support aggregation. T212E enhanced filament elongation by decreasing the rate at which monomers dissociated from filament ends. These effects are not unique to the twisted ribbon morphology induced by Thiazine red, also having been observed with the filamentous morphologies induced by anionic surfactants (Necula and Kuret 2005). These results indicate that the effects of T212E on filament stability are not inducer
specific. However, its effects on filament elongation differ from that of certain frontotemporal dementia linked missense mutations, some of which act to increase the rate of monomer addition to filament ends without affecting filament stability ((Chang, Kim et al. 2008) and Chapter 2).

Together, these data suggest that occupancy of specific tau phosphorylation sites could potentially modulate key rate-limiting steps along the fibrillization pathway. This reinforces the contribution of tau hyperphosphorylation to neurological disease and provides further support for hyperphosphorylation as a target for pharmacological efforts in treatment of tauopathies.
### 3.5 Tables

<table>
<thead>
<tr>
<th>Protein</th>
<th>$^aK_{\text{crit}}$ (nM)</th>
<th>$^a\nu_{\text{c-}}$ (s$^{-1}$)</th>
<th>$^a\nu_{\text{c+}}$ (mM$^{-1}$s$^{-1}$)</th>
<th>Lag Time (h)</th>
<th>$K_n$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2N4R</td>
<td>186 ± 25</td>
<td>0.020 ± 0.001</td>
<td>105 ± 15</td>
<td>0.81 ± 0.06</td>
<td>21.7</td>
</tr>
<tr>
<td>T212E</td>
<td>84 ± 28**</td>
<td>0.011 ± 0.001**</td>
<td>133 ± 46</td>
<td>0.28 ± 0.05**</td>
<td>5.1</td>
</tr>
</tbody>
</table>

$^a$Overall constants reflecting events at both filament ends

**, $p < 0.01$ versus 2N4R tau.

**Table 3.1.: Summary of Aggregation Parameters**
3.6 Figures

Fig. 3.1. Filament morphology. (a) Distribution of ~30 hydroxyamino acid residues affected by phosphorylation depicted on isoform 2N4R. This isoform contains alternatively spliced exons 2 and 3 (E2 and E3), each of which encodes an acidic 29-residue segment, and exon 10 (E10), which encodes an additional microtubule binding repeat sequence. Pseudophosphorylation mutant T212E is distinguished graphically by a raised hollow symbol. (b-c) Full-length wild-type 2N4R tau (b) and mutants T212E (c) were incubated (1 µM concentration) without agitation in the presence of 100 µM Thiazine red (24 h at 37°C), spotted onto copper Formvar-carbon mesh grids, stained with 2% uranyl acetate, and viewed by transmission electron microscopy. T212E produced unbranched filaments ~16 nm in diameter with no obvious differences in morphology or length distribution from wild-type 2N4R. Scale bar = 100 nm.
Fig. 3.2. Effect of pseudophosphorylation on critical concentration.

Wild-type 2N4R tau (●) and mutant T212E (○) were incubated at varying bulk concentrations in the presence of Thiazine red inducer for 24 h at 37ºC, then assayed for filament formation by electron microscopy. (a) Plot of total filament length against bulk protein concentration, where each data point represents the mean ± SD of triplicate determinations and the solid lines represent best fit of the data points to linear regression. The abscissa intercept, which was obtained by extrapolation (dotted lines), was used to estimate critical concentration ($K_{\text{crit}}$).

(b) Replot of data from Panel (a), where the bars represent the $K_{\text{crit}}$ for T212E relative to wild-type 2N4R. T212E aggregated with significantly lower $K_{\text{crit}}$ values than did wild-type 2N4R. **, $p < 0.01$ versus wild-type 2N4R tau.
Fig. 3.3. Tau mutants share a common sensitivity to aggregation inducer Thiazine red. Wild-type 2N4R (●) and mutant T212E (○) were incubated (24 h at 37°C) at constant supersaturation (i.e., 0.5 µM above $K_{crit}$) in the presence of varying concentrations of Thiazine red and then assayed for filament formation by electron microscopy. Each data point represents total filament length per field ± SD from triplicate determinations, whereas the solid curve is drawn solely to aid visualization. Under these conditions, the concentration effect relationship for Thiazine red was similar for both tau species.
Fig. 3.4. Association and dissociation rate constants for filament extension. (a) Tau filaments prepared from wild-type 2N4R (●) and mutant T212E (○) in the presence of 100 µM Thiazine red at 37°C were diluted 10-fold into assembly buffer containing Thiazine red, and the resultant disaggregation was followed as a function of time by electron microscopy. Each data point represents total filament length per field ± SD (triplicate observations), whereas the solid lines represent best fit of the data points to linear regression. The first order decay constant $k_{app}$ was estimated from each regression, and used in conjunction with measured $K_{crit}$ values to estimate $k_e-$ and $k_e+$ as described in the section 3.2. (b) Replot of data from Panel (a), where each bar represents $k_e-$ and $k_e+$ values for T212E relative to wild-type 2N4R (dashed line). Filaments composed of T212E were more stable than those composed of 2N4R tau. **, p < 0.01 versus wild-type 2N4R tau.
Fig. 3.5. Effects of tau mutations on aggregation time course. (a) Wild-type 2N4R (●) and mutants T212E (○) were incubated (37°C) at constant supersaturation \( (\text{i.e.,} \, 0.2 \, \mu\text{M above} \, K_{\text{crit}}) \) in the presence of 100 \( \mu\text{M} \) Thiazine red, and then assayed for filament formation as a function of time. Each data point represents mean total filament lengths/field (expressed as % plateau length) calculated from triplicate electron microscopy images whereas each normalized curve represents best fit of the data points to a three parameter Gompertz growth function. The fits were used to calculate lag time as described in the section 3.2. (b) Replot of data from Panel (a), where each bar represents the lag time ± SE calculated from each Gompertz regression normalized to wild-type isoform 2N4R (dotted line). Mutant T212E aggregated significantly faster than wild-type 2N4R when compared at constant supersaturation. *, \( p < 0.05 \); **, \( p < 0.01 \) versus wild-type 2N4R tau.
Normal tau binds tightly to microtubules but dissociates upon phosphorylation to form free tau, which exists as a natively disordered, assembly incompetent monomer ($U_x$). A conformational change to an assembly competent state accelerates polymerization ($U_c$). Once assembly competent species form, the rate-limiting step in tau fibrillization is formation of dimer, which represents the thermodynamic nucleus (N). Following nucleation, extension occurs through further addition of assembly competent monomers to the filament (F) ends. Introduction of negative charge at residue T212 in the form of pseudophosphorylation, and potentially phosphorylation, affects multiple points in the pathway. See text for details.

Fig. 3.6. Effect of T212E mutation on the tau fibrillization pathway. Normal tau binds tightly to microtubules but dissociates upon phosphorylation to form free tau, which exists as a natively disordered, assembly incompetent monomer ($U_x$). A conformational change to an assembly competent state accelerates polymerization ($U_c$). Once assembly competent species form, the rate-limiting step in tau fibrillization is formation of dimer, which represents the thermodynamic nucleus (N). Following nucleation, extension occurs through further addition of assembly competent monomers to the filament (F) ends. Introduction of negative charge at residue T212 in the form of pseudophosphorylation, and potentially phosphorylation, affects multiple points in the pathway. See text for details.
CHAPTER 4

4. DETECTION AND QUANTIFICATION OF TAU AGGREGATION USING A MEMBRANE FILTER ASSAY

4.1 Introduction

Abnormal aggregates of the microtubule-associated protein tau are found in several progressive neurodegenerative diseases, including Alzheimer’s disease (AD) and frontotemporal dementia (Buee, Bussiere et al. 2000). In AD, clinical progression of symptoms correlates well with the temporal and spatial spread of tau aggregates in the brains of affected individuals (Braak and Braak 1991; Ghoshal, Garcia-Sierra et al. 2002; Royall, Palmer et al. 2002). Thus tau aggregation is a useful marker of disease, and characterization of its mechanism of formation can yield information on underlying pathological processes. In addition, in vitro modeling of tau fibrillization can be used to clarify the kinetics of the process as well as identify compounds potentially capable of modulating lesion formation (Chirita, Necula et al. 2004).

Tau aggregation has been quantified using various methods, including dye-based fluorescence spectroscopy (LeVine 1993; Friedhoff, Schneider et al. 1998), laser light scattering (Gamblin, King et al. 2000; Necula and Kuret 2004), high speed centrifugation (Bandyopadhyay, Li et al. 2007), and electron microscopy (Necula and Kuret 2004).
Each approach has its own advantages and disadvantages. For example, transmission electron microscopy provides direct visualization of the aggregates, which establishes morphology, as well as the length distribution of filaments, which reflects aggregation mechanism. However, this method is low throughput and may be subject to measurement bias depending on conditions of experimentation (Necula and Kuret 2004).

Recently, filtration methods have been used to quantify the products of protein aggregation reactions (Scherzinger, Lurz et al. 1997; Wanker, Scherzinger et al. 1999). In this approach, reaction products are filtered through a membrane that traps and retains large protein aggregates while small species including protein monomers pass through. When combined with solid-phase immunodetection, the approach can yield a highly sensitive estimation of protein aggregation. Early versions of the assay used cellulose acetate as the capture membrane (Wanker, Scherzinger et al. 1999), which proved capable of trapping tau aggregates in extracts of human and transgenic mouse brain tissue (Xu, Gonzales et al. 2002). Subsequent assay of tau aggregation in cultured cell extracts and also in vitro using purified protein preparations employed filters with greater protein binding affinity, including nitrocellulose (Dou, Netzer et al. 2003) and PVDF (Pickhardt, von Bergen et al. 2005). However, a full characterization of any filter-based assay for tau, including the effect of membrane composition and porosity, has not been reported. Moreover, the relative sensitivity and linearity of these assays have not been disclosed.

Here we characterize a vacuum-based 96-well format filter assay for assessment of tau fibril formation in vitro. Results indicate that while assay sensitivity is a function of both filter composition and porosity, analyte concentration dependence is non-linear under all conditions tested. However, control for non-linearity through use of calibration
standards yields a quantitative assay capable of estimating aggregation parameters with a precision equal to microscopy-based methods.

4.2 Experimental procedures

4.2.1 Materials

Recombinant htau40 was prepared as described previously (Carmel, Mager et al. 1996). Primary mouse monoclonal antibodies Tau1 (Binder, Frankfurter et al. 1985) and Tau5 (LoPresti, Szuchet et al. 1995) were the gifts of L. I. Binder (Northwestern University), whereas Alz50 (Wolozin, Pruchnicki et al. 1986) was the gift of P. Davies (Albert Einstein College of Medicine). HRP-linked goat anti-mouse IgG and goat anti-mouse IgM were from Kirkegaard and Perry (Gaithersburg, MD). Filter membranes used included 0.45 µm cellulose acetate from Sterlitech Corporation (Kent, WA), 0.2 µm and 0.45 µm nitrocellulose from Bio-Rad Laboratories (Hercules, CA), and 0.45 µm PVDF from Millipore (Billerica, MA). Formvar/carbon-coated copper grids (300 mesh), glutaraldehyde, and uranyl acetate were obtained from Electron Microscopy Sciences (Ft. Washington, PA). Thiazine red was from ICN Biomedicals (Aurora, OH). Octadecyl sulfate detergent (Lancaster Synthesis, Pelham, NH) was dissolved in 1:1 isopropanol/H$_2$O before use.

4.2.2 Fibrillization

Tau protein was incubated (50 µl final volume) at 37ºC for up to 24 h in assembly buffer (10 mM HEPES, pH 7.4, 100 mM NaCl, and 5 mM dithiothreitol) in the presence and absence of either Thiazone red (100 µM) or ODS (50 µM) fibrillization inducers.
Reactions were immediately subjected to either filter or electron microscopy assays described below.

4.2.3 Filter Assay

Tau fibrillization reaction products were diluted up to 10-fold in 2% SDS to prepare a series of descending tau filament concentrations. All samples underwent a further 1:3 dilution in 2% SDS before vacuum filtration through a 96-well dot blot apparatus (Bio-Rad Laboratories, Hercules, CA) containing nitrocellulose, PVDF, or cellulose acetate membranes. The resultant membranes were washed twice with 2% SDS, blocked in 4% nonfat dry milk dissolved in blocking buffer (100 mM Tris-HCl, pH 7.4, 150 mM NaCl) for 2 h, and then incubated with primary antibody at 1:1000 dilution for 1.5 h. Membranes were washed twice in blocking buffer, then incubated with HRP-linked secondary antibody for 1.5 h. The membranes were washed again twice in blocking buffer, then developed with the ECL Western Blotting Analysis System (GE Healthcare, Buckinghamshire, UK). Chemiluminescence was recorded on an Omega 12iC Molecular Imaging System and quantified using UltraQuant software (UltraLum, Claremont, CA).

4.2.4 Transmission electron microscopy

Tau fibrillization reactions were terminated with the addition of 2% glutaraldehyde and then adsorbed onto 300-mesh Formvar/carbon-coated copper grids for 1 min. The grids were rinsed with water, negatively stained for 1 min with 2% uranyl acetate, and washed again with water. Images were captured on a Tecnai G2 Spirit BioTWIN transmission electron microscope (FEI, Hillsboro, OR) operated at 80kV and 23,000x magnification, then analyzed with ImageJ software (National Institutes of Health).
Average total filament length was determined as described previously (Necula and Kuret 2004).

### 4.2.5 Analytical methods

Regression analysis was performed with Sigmaplot software (Systat Software Inc., San Jose, CA). Analyte concentration dependence of the filter assay was fit to the power function

\[
y - y_0 = ax^b
\]  
(Eq. 4.1)

where \(y\) is the signal intensity produced in the presence of aggregation inducer at tau concentration \(x\), \(y_0\) is the background signal produced in the absence of tau aggregation inducer at tau concentration \(x_0\), and \(a\) and \(b\) are constants.

The critical concentration for fibrillization was estimated from the abscissa intercept after least-squares linear regression and is reported \(\pm\) SEE (Necula and Kuret 2004). \(Z’\)-factor for evaluating assay performance was calculated as described previously (Zhang, Chung et al. 1999).

### 4.3 Results

#### 4.3.1 Initial characterization

To generate a population of tau filaments for testing in filter assays, 1 \(\mu\)M full-length, four-repeat tau protein (htau40; (Goedert, Spillantini et al. 1989)) was incubated (16 h at 37°C) in the presence and absence of Thiazine red inducer. Stable, plateau levels of fibrillization are induced under these conditions (Chirita, Congdon et al. 2005). In contrast, tau protein incubated in assembly buffer without inducer does not produce
detectable aggregates (King, Ahuja et al. 1999). In a preliminary test of the filter trap assay, tau samples prepared as described above were diluted in 2% SDS to form a descending concentration series, then equal volumes of each dilution were vacuum-filtered through a 0.2 µm nitrocellulose membrane in 96-well format. SDS was used as diluent because authentic tau filaments are relatively stable in detergents (Greenberg and Davies 1990), including SDS (Xu, Gonzales et al. 2002). Trapped tau protein was then labeled with Tau1 monoclonal antibody in conjunction with an HRP-linked secondary antibody and chemiluminescent substrate. Chemiluminescence was captured using an Omega 12iC Molecular Imaging System. Tau1 was used as the labeling antibody because it binds to a well-characterized linear epitope in non-phosphorylated tau protein with high affinity (Szendrei, Lee et al. 1993; Carmel, Mager et al. 1996). Results showed that the nitrocellulose filter detected much stronger chemiluminescent signals from the tau sample treated with Thiazine red compared to the non-treated control reaction (Fig. 4.1), indicating that retention of unaggregated monomer on the membrane was minor relative to trapping of filaments under these conditions. The mean signal to background ratio ($i.e.$ signal from sample with Thizine red inducer compared to sample without inducer) was $29.7 \pm 6.9$ ($n = 3$ replicates) for neat sample, and decreased in parallel with the amount of tau aggregate subjected to filtration (Fig. 4.1). The $Z'$-factor for assays spanning the 5-fold relative concentration range 0.2 – 1 (Fig. 4.1) was $0.66 \pm 0.15$ ($n = 9$ concentrations). These data indicate that the tau filter trap assay is adequate for high-throughput screening applications under these experimental conditions (Zhang, Chung et al. 1999).

To assess assay linearity, net chemiluminescence ($i.e.$, the difference between Thiazine red-induced and non-induced tau samples) was determined by densitometry and
then plotted as a function of relative tau concentration. The resultant curves were nonlinear (Fig. 4.2A), but could be fit to a simple power function (Eq. 4.1). Nonlinearity did not result from choice of inducer, because similar data was obtained for tau filaments prepared in the presence of anionic surfactant octadecyl sulfate (data not shown). These data suggest that tau aggregates can be selectively trapped and detected by nitrocellulose filters, but that chemiluminescent signal is not linearly related to tau filament concentration under these experimental conditions.

4.3.2 Membrane chemistry

Published filter trap assays have employed cellulose acetate (Wanker, Scherzinger et al. 1999; Xu, Gonzales et al. 2002), nitrocellulose (Dou, Netzer et al. 2003), and PVDF (Pickhardt, von Bergen et al. 2005) membranes. To compare the performance of these membranes at constant porosity, each was subjected in parallel to the assay described above using Tau1 monoclonal antibody in conjunction with an HRP-linked secondary antibody and chemiluminescent substrate. Net chemiluminescence was then plotted as a function of relative tau concentration (Fig. 4.2A). Because dependence of chemiluminescence on analyte concentration was non-linear under all conditions, data were fit to eq. 1, with parameter $a$ taken as a measure of relative sensitivity (Fig. 4.2B). Results showed that 0.45 µm cellulose acetate had by far the weakest ability to trap and retain tau aggregates. In contrast, 0.45 µm PVDF improved retention by more than an order of magnitude (Fig. 4.2B). Filters of the same porosity but composed of nitrocellulose improved sensitivity by a further 1.5 ± 0.1 fold. Finally, narrowing nitrocellulose porosity to 0.2 µm increased sensitivity 1.7 ± 0.1 fold more. These data...
suggest that filaments prepared *in vitro* from purified full-length tau preparations are best captured and retained by membranes with high protein binding activity such as nitrocellulose and PVDF. In addition, while smaller pore sizes are more efficient at trapping tau aggregates in the context of nitrocellulose, they do not improve assay linearity.

**4.3.3 Primary antibodies**

Many monoclonal antibodies are available for detection of tau protein, including those that bind epitopes dependent on conformation. To assess relative performance of the filter trap assay, two additional antibodies, Tau5 and Alz50, were tested as detection reagents. Tau5 is an IgG that binds a linear tau epitope independent of phosphorylation state (Carmel, Mager et al. 1996; Porzig, Singer et al. 2007), whereas Alz50 is an IgM with anti-tau binding affinity selective for filamentous tau (Carmel, Mager et al. 1996). A comparison of the performance of Tau1, Tau5, and Alz50 is shown in Fig. 4.3. All three antibodies displayed parabolic concentration dependence curves, with only minor differences in sensitivity. These data suggest that selection of primary antibody has only minor effects on the performance of the filter trap assay.

**4.3.4 Comparison with electron microscopy**

When corrected for non-linearity, the filter trap assay could potentially provide sensitive and high-throughput measurement of tau aggregation parameters. To test this approach, tau critical concentration was estimated by both electron microscopy and corrected filter trap assays. Critical concentration is the highest concentration of tau protein that does not support fibrillization, and therefore corresponds to the abscissa intercept when aggregate mass or total length is plotted as a function of bulk protein
concentration (Kuret, Congdon et al. 2005). To determine tau critical concentration, varying concentrations of tau protein (0.3 – 0.7 µM) were incubated in the presence and absence of Thiazine red inducer, with one aliquot used to determine total filament length by electron microscopy while an identical aliquot was subjected to the filter trap assay (with 0.2 µm nitrocellulose as the filter and Tau1 as the primary detection antibody). For the latter measurements, net chemiluminescence was corrected for non-linearity using Eq. 4.1 fit to a descending concentration series similar to that shown in Figs. 4.1 and 4.2. Under these conditions, total filament length as determined by electron microscopy was linearly related to bulk tau concentration (linear regression $r^2 = 0.995$), with an abscissa intercept of 0.23 ± 0.02 µM (Fig. 4.4). When corrected using Eq. 4.1, net chemiluminescence also was linearly related to bulk tau concentration (linear regression $r^2 = 0.994$), with an abscissa intercept of 0.20 ± 0.02 µM (Fig. 4.4). The critical concentration values determined by electron microscopy and corrected filter trap assay were in good agreement with previous determinations (Chirita, Congdon et al. 2005), and were not statistically different from each other ($p = 0.37$). This result suggests that the precision of the filter assay is comparable to the electron microscopy assay for tau aggregation, and that it may be used to quantify of aggregation kinetics provided a standard curve is used to correct for non-linearity.

4.4 Discussion

Filter trap assays can play an important role in the characterization of tau aggregation reactions owing to their sensitivity and high-throughput capability. Results presented
here identified areas of concern in employing this method. First, the relationship between amounts of analyte trapped from \textit{in vitro} aggregation reactions did not correlate linearly with amounts applied to the filter. Nonlinearity was independent of the nature of tau aggregation inducer, membrane composition, membrane porosity, or detection antibody isotype or conformational binding selectivity, suggesting it was intrinsic to the assay methodology. Nonlinear immunoassays can result from concentration-dependent aggregation of analyte as found, for example, with prion protein PrP$^\text{C}$ (Meyer, Lustig et al. 2000). However, the tau aggregates employed in the current study were harvested at reaction plateau and therefore were no longer rapidly changing aggregation state (Chirita, Congdon et al. 2005). In addition, the dissociation rate of tau filaments is slow relative to sample preparation and assay time (Necula and Kuret 2005), suggesting that changes in tau aggregation state do not contribute to non-linearity. Alternatively, non-linearity may stem from the use of immunological detection methods. Both the IgGs and the IgM used for detection of bound tau aggregates are multivalent, and therefore subject to avidity effects. Indeed, immobilization of antigen on a solid support is known to enhance avidity effects (Harlow and Lane 1999). Finally, it is conceivable that trapped tau aggregates help recruit and retain additional aggregates on the filter resulting in apparent binding cooperativity.

Regardless of its source, non-linear concentration-dependence of signal intensity was well modeled as a power function. Therefore, it was possible to correct raw signal intensity by including a dilution range of tau fibrils and interpolating the resultant standard curve. When so corrected, the relationship between analyte concentration and filter trapping became linear, and the filter trap assay was capable of determining a value
for critical concentration in the presence of Thiazine red inducer that was statistically indistinguishable from the value estimated by transmission electron microscopy. In terms of precision, the ability of the corrected filter assay to quantify the extent of tau fibrillization at reaction plateau was comparable to that of microscopy methods.

Although different membrane chemistries can be used for trapping tau aggregates, the most sensitive detection was found with nitrocellulose followed closely by PVDF when porosity effects were controlled. Cellulose acetate performed poorly with tau filaments prepared from purified recombinant tau, although it efficiently captures tau aggregates from human and animal brain extracts (Xu, Gonzales et al. 2002). The difference may be attributable to porosity (0.2 µm vs. the 0.45 µm used here) and also the size and complexity of the aggregates. For example, aggregates trapped from brain samples contain β-amyloid as well as tau protein, suggesting that trapped aggregates represent a heterogeneous mixture of protein species. In contrast, tau filaments formed in vitro are free of other proteins and as a result may be retained less efficiently by cellulose acetate.

Of the antibodies tested in this assay, Tau1 was the most sensitive at lower concentrations of aggregates; however all three of the antibodies tested showed similar signal curves with minimal backgrounds. Thus, labeling antibody may be chosen to fit the needs of a particular application (e.g., phosphorylation- or conformation-sensitive antibodies).

When corrected for nonlinearity, and using Tau1 as detection antibody, the performance of the filter trap assay was comparable to electron microscopy (Necula and Kuret 2004) and laser light scattering methods (Necula and Kuret 2004). However,
filtration has special utility for high-throughput quantitation of tau aggregation. Potential applications include determination of the potency and efficacy of aggregation inhibitors (Pickhardt, von Bergen et al. 2005), the structure-activity relationship of aggregation inducers (Chirita, Necula et al. 2003), or as shown here, critical concentration. The commonality in these measurements is constant incubation time. In principle the filter assay could also be used for time-dependent applications (such as estimation of lag time) provided that reactions are stopped at the appropriate time points before they are filtered through the membrane.

Regardless of the application, trapping of aggregates produced \textit{in vitro} is limited by porosity of the membrane. As a result, the assay will underestimate the extent of aggregation depending on the length distribution of reaction products. For example, retention of the small soluble oligomers is expected to be poor. These species are most abundant at very early time points during filament formation (Hu, Matzavinos et al. 2007), can appear at any time owing to off-pathway reactions (Necula, Kayed et al. 2007). Were the latter large enough to be retained on filters, then it may be possible to selectively detect them using conformation selective antibodies (Kayed, Head et al. 2003). Alternatively, the fibrillized state could be selectively detected in the presence of off-pathway aggregates with fibril-selective antibodies such as Alz50 (Carmel, Mager et al. 1996). The difficulty of detecting small aggregates is common to electron microscopy, light scattering, and centrifugation methods as well (Kuret, Congdon et al. 2005).

In summary, the filter trap assay described above can yield rapid and quantitative assessment of tau aggregation state. The assay is suitable for a number of membranes and antibodies and so can be modified to fit the particular needs of the experimenter.
Quantification can be improved by calibrating against dilution standards, which facilitates estimation of aggregation parameters such as critical concentration.
4.5 Figures

**Fig. 4.1. Results from the filter trap assay.** Purified recombinant htau40 (1 µM) was incubated (16 h at 37°C) in the presence and absence of 100 µM Thiazine red inducer, then diluted in 2% SDS to create a descending concentration series of reaction products. These dilutions were vacuum-filtered through a 0.2 µm porosity nitrocellulose membrane, and then stained with Tau1 primary and HRP-conjugated secondary antibodies. Tau immunostaining (chemiluminescence) was then visualized on an Omega 12iC Molecular Imaging System. Under these conditions, substantially more tau is trapped in the presence of aggregation inducer than in its absence.
Influence of membrane composition and porosity on detection of tau aggregates. The dilution series described in Fig. 1 were vacuum-filtered through 0.2 µm nitrocellulose, 0.45 µm nitrocellulose (NC), 0.45 µm PVDF, or 0.45 µm cellulose acetate (CA) membranes. Membrane-bound tau was then detected with Tau1 monoclonal antibody followed by densitometric quantification of chemiluminescence. A, Net chemiluminescence, corresponding to the difference between signals generated in the presence and absence of Thiazine red inducer, were plotted versus relative tau filament concentration (where undiluted analyte has a concentration of 1). Each data point represents the mean ± SD of triplicate densitometric measurements whereas the solid curves represent best fits of the data points to a power function (Eq. 4.1). All membranes yielded non-linear concentration dependence curves. B, parameter $a$ estimated from fits to eq. 1 were replotted ± SEE as a measure of assay sensitivity. Assay sensitivity depended on membrane composition and porosity.
Fig. 4.3. Comparison of anti-tau monoclonal antibodies for detection of tau aggregates. The dilution series described in Fig. 1 was vacuum-filtered through a 0.2 µm nitrocellulose membrane. Net chemiluminescence was quantified using Tau1 (A), Tau5 (B), or Alz50 (C) primary antibodies and appropriate HRP-conjugated secondary antibodies (goat anti-mouse IgG for Tau1 and Tau5, goat anti-mouse IgM for Alz50) and plotted versus relative tau filament concentration (undiluted analyte = 1). Each data point represents the mean ± SD of triplicate measurements, whereas the solid curves represent best fits of the data points to a power function (Eq. 4.1). All primary antibodies yielded non-linear concentration curves.
Fig. 4.4. **Use of calibrated filter assay to estimate critical concentration.** Varying concentrations of purified recombinant htau40 (0.3 – 0.7 µM) were incubated 16 h at 37ºC in the presence and absence of 100 µM Thiazine red inducer. One aliquot of each reaction product was subjected to the filter trap assay (○), while an equal aliquot was subjected to the transmission electron microscopy assay (●). Net chemiluminescence measured in the filter assay was corrected for nonlinearity using a dilution series, whereas total filament length was by electron microscopy measurements. Both were then plotted as a function of bulk tau concentration. Each data point represents the mean ± SD of triplicate measurements whereas the solid lines represent best fits to a linear regression. Critical concentrations, which were determined from the intercepts of these regressions with the abscissa (*dotted line*), were not statistically different under these conditions (*p* = 0.37).
5.1 Introduction

Alzheimer’s disease (AD) is defined in part by the accumulation of aggregated tau proteins within neuronal cell bodies and processes (Buee, Bussiere et al. 2000; Ballatore, Lee et al. 2007). Because tau aggregate formation correlates with cognitive decline and neurodegeneration (Congdon and Duff 2008; Honson and Kuret 2008), inhibitors of aggregation are under investigation as a means of controlling pathogenesis and preserving neuronal function in disease (Kuret 2007). Several inhibitory scaffolds capable of supporting tau aggregation antagonist activity in vitro have been identified for this purpose, including (but not limited to) phenothiazine (Wischik, Edwards et al. 1996), triarylmethane (Honson, Johnson et al. 2007), acridine (Wischik, Edwards et al. 2005), phenazine (Wischik, Edwards et al. 2005), xanthene (Wischik, Edwards et al. 2005), and thiacarbocyanine (Chirita, Necula et al. 2004) derivatives. All are polymethine members of the broad $\pi$-delocalized lipophilic cation (DLC) family that passively cross cell membranes and accumulate intracellularly depending in part on membrane polarization (Modica-Napolitano and Aprille 2001). A Phase II clinical trial completed with one
phenothiazine having aggregation antagonist activity, Methylene blue (3,7-bis(dimethylamino)phenothiazin-5-i um chloride), reported decreased cognitive decline, improved cerebral blood flow, and improved fluorodeoxyglucose uptake relative to placebo over a ~1 year treatment period (Wischik, Bentham et al. 2008). Thus at least one tau aggregation antagonist may have therapeutic utility for AD. However, the actions of Methylene blue \textit{in vivo} are not limited to antagonism of tau aggregation. Methylene blue efficiently enters into cyclic redox reactions, during which it is reversibly reduced to Leucocyte blue (Atamna, Nguyen et al. 2008), and this cycling may contribute to its biological activity (Buchholz, Schirmer et al. 2008). Thus while Methylene blue is an oxidant capable of modulating the activity of both heme- and non-heme containing enzymes (including nitric oxide synthase (Mayer, Brunner et al. 1993)), Leucocyte blue has antioxidant activity and is the active form of the dye during treatment of methemoglobinemia (Yusim, Livingstone et al. 2007). Moreover, as a DLC, Methylene blue accumulates in mitochondria where it and its reduced form may exert antioxidant effects through interactions with resident electron transport chains (Atamna, Nguyen et al. 2008). It is not clear whether the therapeutic benefits of Methylene blue stem from its aggregation antagonist activity, its antioxidant effects in mitochondria, its redox modulation of iron-containing proteins, or from other effects.

Recently we introduced thiacarbocyanines as structurally distinct members of the DLC family possessing tau aggregation antagonist activity (Chirita, Necula et al. 2004). The scaffold has several advantages for validating tau aggregation as a therapeutic target. First, certain symmetrical thiacarbocyanines, such as N744 (Table 5.1, compound 1), are among the most potent tau aggregation inhibitors yet described \textit{in vitro}, with
submicromolar IC$_{50}$ under near physiological buffer conditions and tau concentrations (Chirita, Necula et al. 2004). Second, because cyanines lack an electron sink analogous to the thiomorpholine ring of Methylene blue, they have much lower reduction potentials (Zigman and Gilman 1980) and do not enter readily into cycling redox reactions. Third, many carbocyanine derivatives have been prepared over the years, offering a facile route to building SARs. Finally, as with other DLCs, carbocyanine dyes are readily taken up and concentrated in cells where they can contact tau. On the other hand, carbocyanines also present special handling difficulties. First, they are the quintessential aggregation-prone dyes, and readily form face-to-face dimers and higher order aggregates at low concentration (Congdon, Necula et al. 2007). Dye aggregation is a major cause of nonspecific inhibitory effects on unrelated biological targets (Feng, Simeonov et al. 2007) which can complicate target validation. Second, many carbocyanines form stable complexes with DNA at micromolar concentrations through groove binding (Hannah and Armitage 2004) and at high concentrations (~100 µM) through direct intercalation (Biver, De Biasi et al. 2005). These interactions could potentially lead to genotoxicity through frameshift mutagenesis or chromosome breakage. Finally, carbocyanines inhibit various enzymes including DNA primase (Li, Liu et al. 2004) and electron transport complex I (NADH-ubiquinone reductase) of mitochondria (Anderson, Chambers et al. 1991; Anderson, Delinck et al. 1993; Anderson, Wood et al. 1993). Any or all of these actions may contribute to the cytotoxicity reported with carbocyanine exposure, including collapse of the microtubule cytoskeleton (Lee, Wu et al. 1995) and induction of apoptosis (Li, Liu et al. 2004; Li, Jiang et al. 2006). Here we identify the structural features of carbocyanine
dyes responsible for tau aggregation inhibitory activity, and demonstrate the activity of
one family member in organotypic slice culture. The results suggest potent inhibition of
tau aggregation can be achieved in vitro and in the biological model at low nanomolar
concentrations. Furthermore, bioactivity can be separated from dye aggregation. The
results suggest that carbocyanines may have utility for validating tau aggregation as a
therapeutic target for AD.

5.2 Experimental Procedures

5.2.1 Materials

Recombinant His-tagged wild-type 2N4R tau (i.e., the longest human central nervous
system isoform of tau (Goedert, Spillantini et al. 1989)) was prepared as described
previously (Carmel, Mager et al. 1996). Mouse monoclonal antibodies Tau5 (LoPresti,
Szuchet et al. 1995) and CP27 (Espinoza, de Silva et al. 2008) were gifts from L.I. Binder
(Northwestern University, IL) and P. Davies (Albert Einstein College of Medicine, NY),
respectively, whereas rabbit polyclonal anti-cleaved PARP1 and mouse monoclonal anti-
α-tubulin antibodies were from Cell Signaling (Danvers, MA) and Sigma-Aldrich (St.
Louis, MO), respectively. Nitrocellulose membrane (0.2 µM porosity) was from Bio-Rad
Laboratories (Hercules, CA). Protease inhibitors (1x complete contained: 1 mM 4-(2-
aminoethyl)-bezenesulfonylfluoride, 0.85 µM aprotinin, 40 µM bestatin, 20 µM leupeptin,
15 µM pepstatin A, 14 µM trans-Epoxy succinyl-L-leucylamido[4-guanidino]butane)
were from Sigma-Aldrich. Formvar/carbon-coated copper grids, glutaraldehyde, and
uranyl acetate were obtained from Electron Microscopy Sciences (Fort Washington, PA).
Aggregation inducer ODS was obtained from Lancaster Synthesis (Pelham, NH) and was dissolved in 1:1 water:isopropanol before use. Screened compounds included 1 (Chirita, Necula et al. 2004; Congdon, Necula et al. 2007) cyanines 2 - 13 (Sigma-Aldrich), and Methylene blue (Acros Organics, Morris Plains, NJ). All compounds, which were at least 95% pure on the basis of argentometric titration (5), high-performance capillary electrophoresis (6), thin-layer chromatography (8, 9, 10), or HPLC analysis (all others), were dissolved in DMSO prior to use.

5.2.2 Tau aggregation in vitro

Tau preparations were incubated (37°C) without agitation in assembly buffer (10 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM dithiothreitol) for 24 h in the presence or absence of fibrillization inducer ODS (50 µM) and candidate inhibitors. Control reactions contained DMSO vehicle, which was limited to 1% (v/v) final concentration in all aggregation reactions. After incubation, reactions were immediately assayed by either filter trap assay or electron microscopy as described below.

5.2.3 Filter trap assay

This was performed as described previously ((Chang and Kuret 2008) and Chapter 4). Briefly, reactions were diluted in 2% SDS before vacuum filtration in triplicate through a 96-well dot blot apparatus onto nitrocellulose membranes. Membranes were washed twice with 2% SDS, blocked with 4% nonfat dry milk in blocking buffer (100 mM Tris-HCl, pH 7.4, and 150 mM NaCl) for 2 h, and incubated with Tau5 primary antibody for 1.5 h. They were then washed twice in blocking buffer and then incubated with HRP-linked secondary antibody for 1.5 h. The membranes were washed again in blocking
buffer and developed with the ECL (Enhanced Chemiluminescence) Western Blotting Analysis System (GE Healthcare, Buckinghamshire, UK). Chemiluminescence was recorded on an Omega 12iC Molecular Imaging System and quantified using UltraQuant software (UltraLum, Claremont, CA) and is reported ± SD.

5.2.4 Electron microscopy

Reaction aliquots were removed, treated with 2% glutaraldehyde (final concentration), mounted on formvar/carbon-coated 300 mesh grids, and negatively stained with 2% uranyl acetate as described previously (Necula and Kuret 2004). Random fields were viewed with a Tecnai G2 Spirit BioTWIN transmission electron microscope (FEI, Hillsboro, OR) operated at 80kV and 23,000-49,000x magnification. At least three viewing fields were captured for each reaction condition and filaments >10 nm in length were counted and quantified with ImageJ software (National Institutes of Health, Bethesda, MD). Total filament length is defined as the sum of the lengths of all resolved filaments per field and is reported as ± SD.

5.2.5 Dye aggregation

Compounds were incubated (1 h at 37°C) in assembly buffer, then subjected to absorbance spectroscopy (Varian Cary 50 Bio) over the wavelength range 400 - 700 nm. Concentrations of dye monomer \( (C_m) \) were estimated as described previously (West and Pearce 1965; Necula, Chirita et al. 2005). Briefly, the extinction coefficient for dye monomer was established in methanol, and then used to estimate monomer concentration in aqueous solutions on the basis of the Beer-Lambert law:

\[
A = \varepsilon C_m l
\]  
(Eq. 5.1)
where $A$ is absorbance, $\varepsilon$ is the molar extinction coefficient, and $l$ is the path length of the sample. Dimer concentration ($C_d$) was estimated from total dye concentration ($C_t$) assuming (West and Pearce 1965):

$$C_d = \frac{(C_t - C_m)}{2}$$  
(Eq. 5.2)

The dissociation constant for dimerization ($K_{\text{dim}}$) was then calculated from the function (West and Pearce 1965):

$$K_{\text{dim}} = \frac{C_m^2}{C_d}$$  
(Eq. 5.3)

$K_{\text{dim}}$ values were derived from at least five spectra and reported as mean ± S.D.

**5.2.6 Organotypic slice culture**

Brains were harvested from JNPL3 mice (Lewis, McGowan et al. 2000) at postnatal day ten. After removal of cerebellum and brainstem, the cerebral cortex and hippocampus were sectioned at 400 $\mu$m as described previously (Duff, Noble et al. 2002). Slices from each hemisphere were then separated in ice cold buffer (pH 7.1), transferred to membrane inserts containing 0.4 $\mu$m pores (Laboratory Disposable Products; Wayne, NJ), and then maintained in culture for 14 days in media containing 25% serum (media was changed every two days). After the culture period, slices prepared from one hemisphere were treated with varying concentrations of compound for seven days, whereas slices prepared from the contralateral hemisphere were treated with DMSO vehicle alone (0.008% final concentration of DMSO in media). Thus, the vehicle-treated hemispheres served as within-animal controls for each treatment condition.

**5.2.7 Tissue fractionation**

Treated and control slices were separately pooled and homogenized by sonication in
cold RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1 mM Na$_3$VO$_4$, 1x protease inhibitors, 1µg/mL phosphatase inhibitors) (Greenberg and Davies 1990). Homogenates were then centrifuged at 20,000g at 4°C for 20 min. The resulting supernatant fractions (crude extracts) were collected and assayed for α-tubulin and cleaved PARP1 immunoreactivity using a Fujifilm LAS3000 imaging system.

To assess tau aggregation, extracts were diluted in RIPA buffer as necessary to achieve equivalent total protein levels. An aliquot of low-speed supernatant was incubated in 1% sarkosyl for 30 min at room temperature. Samples were then centrifuged for 1 h at 100,000g at 20°C. Supernatant was discarded and the pellet, termed the sarkosyl-insoluble fraction, was then resuspended in 20 µL O+ buffer (a modified O buffer (O'Farrell 1975) composed of 62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 5% 2-mercaptoethanol, 2.3% SDS, 1 mM EGTA, 1 mM EDTA, 1 mM PMSF, 1 mM Na$_3$VO$_4$, 1 mM NaF, 1x protease inhibitors), boiled for 3 min, then subjected to immunoblot analysis using monoclonal antibody CP27.

5.2.8 Analytical methods

Concentration effect data from either filter or electron microscopy assays were normalized to DMSO vehicle control reactions and fit to the function:

\[
y = y_{\text{min}} + \frac{y_{\text{max}} - y_{\text{min}}}{1 + 10^{\left(\log IC_{50} - \log x\right)n}}
\]

(Eq. 5.4)

where \(y\) and \(y_{\text{max}}\) represent the minimum and maximum aggregation measured in the presence and absence of inhibitor (at concentration \(x\)), respectively, \(n\) is the Hill coefficient, and \(IC_{50}\) is the concentration of inhibitor that results in 50% of maximal inhibition. \(IC_{50}\) values are reported ± standard error of the estimate.
5.3 Results

5.3.1 In vitro activity

Symmetrical carbocyanine dyes consist of two identical heterocycles connected by a polymethine bridge (i.e., by a system of conjugated double bonds) (Table 5.1). Each heterocycle contains an alkyl N-substituent, whereas the bridge can contain hydrogen or other substituents (Table 5.1). To determine the contribution of these four structural components to tau aggregation antagonist activity, a small library of dyes was subjected to a filter binding assay for ODS-induced fibrillization of human 2N4R tau under near physiological conditions of pH, ionic strength, and reducing conditions. The first series (2 – 5, Table 5.1) evaluated the influence of polymethine bridge length on inhibitory activity. Bridge length influences the photophysical properties of cyanine dyes (West and Pearce 1965) and is a major determinant of molecule length. The series contained identical ethyl N-substituents, only hydrogen bridge substituents, and two benzothiazole heterocycles, whereas the polymethine bridge was systematically varied in length from zero to three methine units (i.e., the bridges varied from one to seven carbons in length) (Table 5.1). All four compounds inhibited tau aggregation but with weaker potencies than starting compound 1 (Figure 5.1A). A replot of IC$_{50}$ versus polymethine bridge length revealed a clear optimum centered on a bridge length of three carbons (Figure 5.1B). Therefore, further studies were limited to compounds containing a polymethine bridge of this length.

To evaluate the contribution of heterocycle composition, the second series (3, 6, 7, and 8) contained ethyl N-substituents and a hydrogen meso group in the presence of
either benzothiazole, benzoaxazole, dimethylindole, or quinoline heterocycles. Heterocycle composition influences compound polarizability and also ability to form H-bonds with solvent (Vranchev, Andreev et al. 1978). All heterocycles were as potent and efficacious as benzothiazole 3 except benzoaxazole 6, which had essentially no inhibitory activity at the highest concentration tested (Figure 5.2). These data show that inhibitory activity is not uniquely associated with the benzothiazole heterocycle, and can be extended to other heterocycles lacking sulfur.

Cyanine N-substituents consist of alkyl or other groups (including ionizable groups) that do not participate in extended conjugated bond structure. Nonetheless, substituent structure influences compound pharmacokinetics by modulating binding affinity for serum albumin (Tatikolov and Costa 2004) and by affecting net compound charge (and hence ability to serve as substrates for organic anion/cation transporters (Klaassen and Lu 2008)). The contribution of N-substituent to inhibitory activity was evaluated in both benzothiazole and dimethylindole heterocycle-containing dyes. In the dimethylindole background, elongation of the alkyl chains from two carbons (8) to three carbons (9) reduced inhibitory potency ~3-fold (Figure 5.3). In a benzothiazole background, elongation of the alkyl chains from two carbons (3) to six carbons (10) completely eliminated activity at up to 10 μM concentration (Figure 5.3). These data indicate that short N-alkyl substituents are most compatible with inhibitory activity.

Finally, the contribution of the meso substituent was investigated. Meso groups modulate the configuration of the polymethine bridge, with a hydrogen substituent favoring s-trans conformation whereas larger substituents favor s-cis conformation (West,
The SAR for symmetrical cyanine dyes summarized above indicates that it is possible to retain the potency and efficacy of parent compound 1, but with molecular weight, calculated octanol:water partition coefficient (ClogP), and topological polar surface area (tPSA) values more consistent with potential CNS penetration (Hou and Xu 2003) (Table 5.1). To confirm these findings, the most potent compound in the cyanine series (11) was assayed by an independent method (quantitative electron microscopy) and compared to established inhibitor Methylene blue. Results showed that the efficacy and potency of 11 was consistent in both filter trap and electron microscopy assay formats (Figure 5.4). In comparison, Methylene blue behaved as a partial antagonist with an IC\textsubscript{50} of 610 ± 50 nM. These data indicate that cyanine inhibitors with superior \textit{in silico} physical properties are available that exceed the potency and efficacy of Methylene blue \textit{in vitro}.

### 5.3.2 Dye aggregation

Many planar heterocycles aggregate depending on pH, ionic strength, temperature,
solvent polarity, and compound concentration (Murakami 2002). Symmetrical carbocyanine dyes are especially aggregation prone in water owing to strong dispersion forces between their nearly planar faces (Herz 1977) and H-bond formation with solvent (Vranchev, Andreev et al. 1978). Shifts in absorbance spectra accompany thiacarbocyanine aggregation depending on the quaternary structure of the aggregate formed. Hypsochromic shifts to shorter wavelengths are generally referred to as H-bands (Herz 1974). In solution, these correspond primarily to small aggregates (e.g., dimer) although much larger sizes can be attained at high concentration or in the presence of certain surfaces (Maskasky 1991). In previous studies, formation of large H-aggregates correlated with loss of inhibitory activity (Congdon, Necula et al. 2007). However, substantial amounts of H-dimer were present at inhibitory concentrations suggesting these were candidate inhibitory species. To test this hypothesis, the concentration dependent aggregation of the carbocyanine dye series shown in Table 1 was assessed by absorbance spectroscopy and compared to the SAR for tau aggregation inhibition. In neat methanol (which does not support dye oligomerization (West and Pearce 1965)), 11 absorbance appeared as a major band at 544 nm with a weak vibrational shoulder at ~510 nm (Figure 5.5A). At low micromolar concentration in aqueous solution (i.e., assembly buffer), monomer appeared centered at 541 nm, consistent with the solvatochromic behavior of many thiacarbocyanine derivatives (Seifert, Connor et al. 1999). As concentration increased, a second absorbance maximum centered at 502 nm also became apparent. On the basis of absorption spectra of other thiacarbocyanine dyes (West and Pearce 1965), the 502 nm band corresponds to an H-dimer (Figure 5A). To confirm this prediction, the amount of monomer as a function of bulk 11 concentration was
determined by absorbance spectroscopy and used to calculate the concentration of putative dimer by use of Eq 5.1. A double log plot of monomer versus dimer concentration was linear with a slope of $2.3 \pm 0.1$, confirming that the reaction was consistent with dimerization (Figure 5.6). On the basis of Eq. 5.3, the dissociation equilibrium constant for dimerization ($K_{\text{dim}}$) was $20.9 \pm 0.7$ µM. Thus, 11 readily dimerizes at low micromolar concentrations under near physiological buffer conditions, but at $10.0 \pm 0.2$-fold higher concentrations than 1 under identical conditions (Necula, Chirita et al. 2005). These data suggest that dimerization can be dissociated from inhibitory activity. Indeed, certain cyanines with weak or no inhibitory activity, such as 5 ($K_{\text{dim}} = 0.68 \pm 0.04$ µM) and 12 ($K_{\text{dim}} = 7.5 \pm 0.4$ µM) dimerized more efficiently than 11 (Figs. 5.5B and 5.6). Conversely, other compounds, such as dimethylindocarbocyanine 8, inhibited tau aggregation at micromolar concentrations (Table 5.1) but did not dimerize at any concentration tested up to 96 µM (Figure 5.5C). Overall, there was no discernible correlation between dimerization propensity of compounds listed in Table 5.1 and inhibitory potency, suggesting that the active inhibitory species in the low concentration regime is the cyanine monomer.

### 5.3.3 Biological activity

The *in vitro* SAR described above suggests that cyanine dyes should inhibit tau aggregation in biological models at sub-micromolar concentrations. To test this prediction, organotypic slices prepared from JNPL3 transgenic mice were exposed to a broad concentration range (0.1 nM – 1 µM) of 11 over a period of seven days after which levels of sarkosyl-insoluble tau were estimated. Compound 11 was chosen for study
because it was the most potent member of the carbocyanine series. The JNPL3 model was selected as host because it accumulates tau aggregates in the diencephalon, brainstem, and cerebellar nuclei and is well characterized (Lewis, McGowan et al. 2000; Lin, Lewis et al. 2003; Lin, Lewis et al. 2003; Arendash, Lewis et al. 2004; Lin, Zehr et al. 2005). The model is driven by expression of tau above normal physiological levels, which leads to increased nucleation and extension rates (Congdon, Kim et al. 2008) and by insertion of the P301L missense mutation, which increases both the rate and extent of aggregation still further compared to wild-type tau protein ((Chang, Kim et al. 2008) and Chapter 2).

The effects of 11 in this model were biphasic (Figure 5.7AB). In the low concentration regime (1 – 10 nM) the compound decreased tau aggregation to ~50% of that observed in the presence of DMSO vehicle alone. Inhibitory activity was lost at 100 nM, whereas tau aggregation was induced at 300 nM and above.

In cell culture models, >300 nM 11 reportedly initiates apoptosis (Li, Liu et al. 2004; Li, Jiang et al. 2006) and collapse of the microtubule cytoskeleton (Lee, Wu et al. 1995). To determine whether 11 induced similar toxicity in neurons, organotypic slices exposed to 11 were assayed for changes in levels of cleaved PARP1 (an enzyme that is proteolytically inactivated by caspases during apoptosis (Oliver, de la Rubia et al. 1998)) and α-tubulin (the structural component of microtubules). Levels of cleaved PARP1 were low in the presence of vehicle alone and did not increase at any tested concentration of 11, suggesting that pro-apoptotic caspases were not activated under these conditions (Fig. 5.8). Similarly, levels of α-tubulin were unchanged in cultures incubated with concentrations of 11 (1 nM) that inhibited tau aggregation (Fig. 5.8). However, at high 11
concentrations (1 µM), depletion of α-tubulin from extracts was detected, suggesting misfunction of the microtubule cytoskeleton under these conditions (Fig. 5.8). Together these data indicate that a cyanine identified on the basis of *in vitro* methods can modulate tau aggregation in a biological model, and is effective at concentrations well below those associated with toxicity in the form of induction of apoptosis or misfunction of the microtubule cytoskeleton.

### 5.4 Discussion

Despite promising effects reported for Methylene blue on AD progression, the significance of tau aggregation as a therapeutic target requires further validation. The SAR summarized above suggests that cyanine dyes may have utility for this purpose. In particular, the availability of active and inactive cyanines sharing very similar structure will help clarify whether direct (herein) or indirect (Honson, Jensen et al. 2009) mechanisms mediate clearance of tau aggregates in biological models.

Both cyanines and Methylene blue consist of strong electron donor and acceptor substituents separated by a conjugated bridge composed of an odd number of carbon atoms. This organization supports the ideal polymethine state characterized by alternating π-electron density distribution along the molecular chain and by high polarizability (Dähne 1978). These properties may be important for tau aggregation inhibitory activity. Consistent with this hypothesis, the benzoxazole heterocycle, which had the lowest polarizability in the series (owing to the electronegative oxygen atom at position X; Table 5.1) lacked inhibitory activity. Polarizability also gives rise to strong dispersion forces.
that support efficient compound self association (Dähne 1978). However, as reported previously for a macrocyclic thiacarbocyanine (Honson, Jensen et al. 2007) and through SAR here, the propensity for face-to-face association can be separated from inhibitory activity. Dimethylindocyanines, which contain geminal methyl groups that interfere with ordered stacking even in the presence of templates such as DNA (Garoff, Litzinger et al. 2002), offer a scaffold that is particularly dimerization resistant. Elimination of stacking interactions with itself or with DNA may be important for limiting potential toxicity associated with chronic cyanine exposure.

One symmetrical carbocyanine, indocyanine green (14; Table 5.1), is approved for diagnostic applications (Caesar, Shaldon et al. 1961; Brancato and Trabucchi 1998) but its molecular properties are not appropriate for tau aggregation inhibitor activity. In particular, its high molecular weight precludes efficient uptake into CNS, and its extended polymethine bridge, long sulfonated N-substituents, and hydrophobic benzindole heterocycles, confer tight binding to serum albumin. As a result, 14 resides primarily in blood plasma after administration (Meijer, Weert et al. 1988). In contrast, the molecular properties associated with cyanine aggregation antagonists are predicted to support better CNS bioavailability than currently possible with 14. Nonetheless, it will be necessary to address the short half-life of cyanines in plasma. For example, compound 14 is rapidly cleared unchanged through the hepatobiliary route (Cherrick, Stein et al. 1960). It enters hepatocytes through organic anion-transporting polypeptides in the sinusoidal (basolateral) membrane, and is excreted through the canalicular (apical) membrane through the P-glycoprotein efflux transporter (Huang and Vore 2001). Other cyanines, such as 6, share affinity for this efflux transporter (Li, Yuan et al. 2008) but not
necessarily the passive transporters in the sinusoidal membrane owing to differences in net charge. Chemical strategies for decreasing the rate of hepatic clearance are under investigation (Licha, Riefke et al. 2000) and may be applicable to tau aggregation inhibitors as well.

Compound 11 inhibited tau aggregation in organotypic slices at media concentrations as low as 1 nM. This high apparent potency may arise from several considerations. First, DLCs including cyanine dyes are readily taken up and concentrated in cells and tissues. For example, cultured K562 cells (a human erythroleukemic cell line established from a patient with chronic myelogenous leukemia in blast transformation) reportedly accumulate intracellular indo-, thia-, and oxo-carbocyanines two orders of magnitude over applied concentrations in media (Sima and Kanofsky 2000). These cells accumulate DLCs because their mitochondrial membrane potentials are hyperpolarized relative to non-transformed cells (Modica-Napolitano and Aprille 2001; Modica-Napolitano, Kulawiec et al. 2007) and because they lack the P-glycoprotein efflux transporter (Marques-Santos, Oliveira et al. 2003). Second, cytoplasmic DLCs accumulate near membranes, which are putative sites of tau filament nucleation (Gray, Paula-Barbosa et al. 1987; Galvan, David et al. 2001). Thus, compound concentrations at local intracellular sites of action may be far higher than implied solely on the basis of bulk concentrations in media.

Compound uptake into organotypic brain slices may follow similar principles. In brain, the P-glycoprotein efflux transporter is predominantly located on the luminal membrane of endothelial cells lining brain microvessels associated with the blood-brain barrier. The slice preparation bypasses this barrier and facilitates direct exposure of
neurons undergoing tau aggregation to compounds. Because neurons do not express P-glycoprotein under basal conditions (Volk, Burkhardt et al. 2004), the slice preparation is particularly useful for assessing the activity of cyanines in neurons until analogs with improved pharmacokinetics are available.

In summary, symmetrical carbocyanines are more potent tau aggregation antagonists than Methylene blue. Their structures are consistent with favorable \textit{in silico} pharmacokinetic predictions, whereas confounding activities such as dye aggregation, DNA intercalation, and high affinity serum albumin binding can be attenuated without destroying inhibitory potency. Therefore, these compounds may have utility for target validation. Organotypic slices prepared from transgenic mouse models will help this effort. In particular, their ability to support robust aggregation over a period of weeks while preserving CNS organization is ideally suited to development of intracellular protein aggregation antagonists.
5.5 Tables

Table 5.1. Summary of molecular properties of cyanine salts used in SAR studies.

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<sup>a</sup>tPSA and CLogP values were calculated from the website http://www.molinspiration.com/

<sup>b</sup>organic component of the salt.

<sup>c</sup>Accurate estimates of SE could not be determined owing to incomplete concentration-effect relationship.

<sup>d</sup>1-ethylpyridinium.

<sup>e</sup>Naphthothiazole heterocycle

<sup>f</sup>Naphthoindole heterocycle.
5.6 Figures

**Figure 5.1. Polymethine bridge length modulates inhibitory activity.** Tau protein (4 µM) was incubated with ODS (50 µM) without agitation (24 h at 37°C) in the presence of either thiacarbocyanines 2 - 5 or DMSO vehicle alone, then assayed for aggregation by filter trap assay. (a) Concentration dependence of inhibition, where each point represents aggregation expressed as a normalized percentage of aggregation measured in the presence of DMSO vehicle alone (triplicate determination ± SD), and each solid line represents best fit of the data points to eq 4. All tested compounds were active, but less so than lead compound 1. (b) Replot of IC\textsubscript{50} values determined in Panel (a) versus bridge length for compounds 2 - 5. Inhibitory potency was maximal when the polymethine bridge consisted of three carbon atoms. *, \( p < 0.05 \); **, \( p < 0.01 \) when compared with compound 3 by Student’s \( t \) test.
Figure 5.2. Multiple heterocycles support inhibitory activity. Tau protein (4 µM) was incubated with ODS (50 µM) without agitation (24 h at 37°C) in the presence of either 3, 6, 7, 8 (composed of benzothiazole, benzoxazole, quinoline, and dimethylindole heterocycles, respectively) or DMSO vehicle alone, then assayed for aggregation by filter trap assay. Each point represents normalized aggregation relative to the DMSO vehicle control (mean of triplicate determination ± SD), whereas each solid line represents best fit of the data points to Eq 5.4. All heterocycles except benzoxazole supported inhibitory activity under these conditions.
Figure 5.3. Meso- and N-substituents influence antagonist potency. Tau protein (4 µM) was incubated with ODS (50 µM) without agitation (24 h at 37°C) in the presence of either thiacarbocyanines 9 - 13 or DMSO vehicle alone, then assayed for aggregation by filter trap assay. Each point represents normalized aggregation relative to the DMSO vehicle control (mean of triplicate determination ± SD), whereas each solid line represents best fit of the data points to Eq. 5.4. Data for compounds 1 and 3 are replotted from Figure 5.1 for comparison. Extension of the N-substituent beyond two carbons decreased potency, whereas introduction of a methyl meso substituent increased potency. The combination of benzothiazole nucleus, three carbon bridge, methyl meso substituent, and ethyl N-substituents yielded an inhibitor (compound 11) with the efficacy and potency of the starting compound 1, but with physicochemical properties more appropriate for biological experimentation.
Figure 5.4. Thiacarbocyanine 11 is a stronger aggregation antagonist than Methylene blue. Tau protein (4 μM) was incubated with ODS (50 μM) without agitation (24 h at 37°C) in the presence of either 11, Methylene blue, or DMSO vehicle alone, then assayed for aggregation. Each point represents normalized aggregation relative to the DMSO vehicle control (mean of triplicate determination ± SD), whereas each line represents best fit of the data points to Eq 5.4. Compound 11 was equally efficacious when assayed by either filter trap assay (○) or direct measurement of total filament length by quantitative electron microscopy assays (●), confirming that inhibitory activity did not depend on assay modality. However, 11 was significantly more potent and efficacious than Methylene blue (■) (electron microscopy assay format).
Figure 5.5. Inhibitory potency does not correlate with cyanine aggregation propensity. When dissolved in methanol at 5 µM concentration, compounds 11 (a), 12 (b), and 8 (c) yield simple spectra (dotted lines) consistent with monomeric structure (M). In assembly buffer (solid lines), increasing concentrations of compounds 11 and 12, but not 8, revealed the presence of both monomeric (M) and aggregated (D) species, with the latter more pronounced at high concentrations. These data suggest that 11 and 12, but not 8, underwent aggregation in aqueous solution at the concentrations tested.
Figure 5.6. The principal cyanine aggregate formed in solution is a dimer. Concentrations of monomeric ($C_m$) and aggregate ($C_t$-$C_m$) forms of 11, 12, and 5 were calculated from absorbance spectra and replotted on double logarithmic scales. Each point represents a spectrum collected at a different bulk compound concentration, whereas each line represents best fit of the data points to a linear regression. Regression slopes for 11, 12, and 5 were 2.3 ± 0.1, 1.8 ± 0.1, and 1.7 ± 0.1, respectively, consistent with dimerization being mostly responsible for the spectral shifts of all three compounds.
Figure 5.7. **Compound 11 inhibits tau aggregation in organotypic slices.** Slice cultures prepared from JNPL3 transgenic mice were incubated with either DMSO vehicle control or varying concentrations of 11 for seven days, then extracted and analyzed for levels of sarkosyl-insoluble tau (a measure of tau aggregation). (a) Two representative immunoblots collected after incubation with DMSO vehicle control (C) or thiacarbocyanine 11 (T) are shown. The presence of 1 nM 11 reduced the amount of tau aggregation in the slices relative to DMSO vehicle control, whereas 1 µM 11 exacerbated aggregation. (b) Sarkosyl-insoluble tau immunoreactivity was quantified by densitometry ($n =$ three observations) and plotted as a normalized percentage of aggregation measured in the presence of DMSO vehicle alone. Tau aggregation was suppressed at 11 concentrations below 100 nM, but was aggravated at higher concentrations (≥300 nm). *, $p < 0.05$; **, $p < 0.01$ when compared to slices treated with vehicle alone by Student’s $t$ test.
Figure 5.8. Compound 11 does not induce apoptosis in organotypic slices. Slices prepared from JNPL3 transgenic mice were incubated with either DMSO vehicle control or varying concentrations of 11 for seven days, then extracted and analyzed for levels of α-tubulin and cleaved PARP1. (a) Representative immunoblots collected after incubation with DMSO vehicle control (C) or 11 (T) are shown. (b) Levels of cleaved PARP1 (hollow bar) and α-tubulin (solid bar) immunoreactivity were quantified by densitometry ($n = 3$ observations) and plotted as a normalized percentage relative to treatment with vehicle alone. Cleaved PARP1 (CP) levels were unaffected after exposure to 11, suggesting that executioner caspase activity was not activated even at the highest concentration tested. Similarly, efficacious concentrations of 11 (1 nM) did not modulate levels of α-tubulin immunoreactivity in tissue extracts. However, high concentrations of 11 associated with induction of tau aggregation (1 µM) led to marked decreases in α-tubulin immunoreactivity. *, $p < 0.05$; **, $p < 0.01$ when compared to slices treated with vehicle alone by Student’s $t$ test.
Alzheimer’s disease and other tauopathies are devastating diseases. These diseases have a long, steadily declining course, leading to staggeringly high costs of care as well as significant emotional distress on loved ones and family members. As the number one risk factor for many of these diseases is simple age, the demographic shift of the United States population towards a more elderly one means that these diseases are a priority health care concern. Unfortunately, the current state of treatment for these diseases is poor. A few treatments are available, but these treatments only alleviate some of the symptoms, and are rarely effective for more than a year.

The lack of effective treatment for tauopathies stems from the fact that the pathogenic mechanisms underlying these diseases are largely unknown. The factors that cause tau to aggregate are a mystery, as are the precise means by which tau aggregates contribute to neurodegeneration and clinical decline. Certain modifications to tau protein, such as missense mutations or hyperphosphorylation, are known to cause disease, but the ways in which they do so are in question.

The unique nature of protein aggregation diseases also makes development of therapeutics challenging, as there is no target enzyme to inhibit, nor is there a pathogenic
organism or cell to target. A recent report that one compound, methylene blue, was effective in delaying the progression of AD in a Phase II clinical trial is encouraging. However, it is not clear how this compound exerts its effects in the brain. Several other classes of tau aggregation inhibitors have also been identified, but to date no clear mechanism has been proposed as to how certain compounds can exhibit tau aggregation antagonist activity.

The data presented herein clarify some of these issues. With the use of thiazine red, tau protein can be induced to aggregate in vitro under near-physiological, biologically-relevant conditions (Chirita, Congdon et al. 2005). Furthermore, this reaction can be modeled mathematically as a simple nucleation-extension reaction, allowing calculation of the basic kinetic rate constants involved with a few simple experiments (Congdon, Kim et al. 2008). The data showed that tau missense mutations associated with FTLDs can differentially modulate aggregation propensity. Some mutations, such as G272V and P301L, enhance both the nucleation and extension steps of the reaction, whereas mutations R5L and V337M enhance only the extension step. In all cases the extension enhancement is a result of a greater monomer association rate to growing filament ends. Another mutation, R406W, had no effect on intrinsic mutation propensity and likely functions through interaction with other cellular factors. The differences in how these mutants affect tau aggregation propensity could in part explain the clinical and pathological heterogeneity of the FTLDs.

We used the same methods to analyze a pseudophosphorylation mutant of tau. Previous work in this lab established that pseudophosphorylation of tau could depress the critical concentration needed for tau aggregation, and that different residues promoted
aggregation to different extents (Necula and Kuret 2004; Necula and Kuret 2005). Herein, the nucleation-extension model was used to determine at which steps in the process T212E, the most potent of these pseudophosphorylation mutants, acted. The data revealed that T212E also enhanced both nucleation and extension steps, but unlike the FTLD mutations analyzed, enhancement extension was a result of decreased dissociation of monomer from filament ends. In other words, pseudophosphorylation resulted in more stable filaments. The results support the hypothesis that phosphorylation can act in a direct manner to promote tau aggregation.

Missense mutations and phosphorylation are not the only disease-related alterations to tau protein. Mutations in the intronic regions of tau have been shown to cause disease, even without altering the basic sequence of tau. These intronic mutations appear to function by altering the splicing of tau, leading to an imbalance of its six isoforms (Hutton, Lendon et al. 1998). Furthermore, several FTLDs show perturbed ratio of tau isoforms, some being predominantly 4R FTLDs with relative overexpression of four-repeat tau while a few are 3R-predominant. As with the missense mutations, the way in which the different isoforms of tau differ in their aggregation propensity is of interest. The same quantitative methods described above can be applied to this question. By applying these methods to the full complement of tau’s six isoforms, the ways in which these isoforms differ in their nucleation and extension reactions can be determined. Furthermore, through pairwise comparisons of the isoforms, it should be possible to determine the exact contributions of exons 2, 3, and 10 to tau aggregation propensity.

With the correlation between disease-causing agents and enhanced aggregation of tau thus strengthened, we turned our attention to small molecules that can inhibit aggregation.
Our lab had previously identified a thiacarbocyanine, N744, as a potent compound active to submicromolar concentrations (Necula, Chirita et al. 2005; Congdon, Necula et al. 2007). We selected a panel of cyanines similar in structure to N744 and subjected them to semi-high-throughput filter assay to build a structure-activity relationship for this class of compounds. The data revealed that it was possible to maintain inhibitory potency while improving the calculated pharmacokinetic parameters of the N744-like scaffold significantly. One particularly active compound, 11 (Table 5.1), was active in a biological model down to 1 nM. Furthermore, we were able to show how specific alterations to the cyanine scaffold affected potency.

Of more overarching interest, the analysis of the cyanine scaffold revealed some hints as to how small molecules in general may be able to inhibit tau aggregation. Moving from a benzothiazole scaffold such as that of compound 3 (Table 5.1) to a benzoxazole 6 eliminated all inhibitory activity, even though perturbation of the benzothiazole to other aromatic heterocycles such as dimethylindole or quinoline did not affect potency. This suggests that a change to the benzoxazole eliminates some essential quality necessary for tau antagonist activity. Replacing the benzothiazole sulfur with the more electronegative oxygen reduces the polarizability of the molecule. The cyanines are known for their high ideal polymethine character (Dähne 1978) and a reduction in molecular polarizability also results in a decrease in polymethine character as the molecule is less able to delocalize electrons along its length. The nearly-identical but inactive compound 6 thus turns out to be quite valuable as it directly suggests that polarizability and/or the polymethine state is of paramount importance for inhibitory activity. Indeed, several other identified tau aggregation antagonists including methylene blue also display high
polymethine character. With this in mind, a focused screening effort can be undertaken to
test the hypothesis. Compounds from families with identified tau antagonists with high
polymethine character, such as the phenothiazines with methylene blue (Wischik,
Edwards et al. 1996) or triarylmethanes (Honson, Johnson et al. 2007), will be selected.
These compound families have the advantage of having numerous commercially
available members, allowing for selection of a range of molecules within the family with
differing amounts of polymethine character. These compounds will then be assayed for
tau inhibitory activity with the methods presented herein. These studies will be able to
strengthen the hypothesis that polymethine character correlates with inhibitory activity
against tau aggregation, or identify other molecular properties that are important for
inhibitory activity. These proposed future studies will build our understanding of the
factors that contribute to tau aggregation and how these factors can be manipulated with
the ultimate goal of treating human disease.
LIST OF REFERENCES


