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Cyclophilin 40 as a Novel Disaggregase

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Cyclophilin 40 as a Novel Disaggregase

by

Jeremy Dustin Baker

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

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DEDICATION

To my family, for providing unending, unconditional support.
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The discovery presented herein is the culmination of years of work and ideas born in the lab of Dr. Chad Dickey, who tragically passed away far too young. I specifically thank my mentor, Dr. Laura Blair for her guidance and determination in preserving Chad’s legacy. To Dr. Lindsey Kirkland, for the countless hours spent helping me in this endeavor. To Dr. Dali Zheng, for teaching me most of what I know at the bench. And finally, to Dr. Bob Deschenes, thank you for always being in my corner and having my best interests at heart.
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ABSTRACT

The negative health and economic impacts of neurodegenerative diseases on Americans is astounding and accelerating with an aging population. The Alzheimer’s Association reports that 5.7 million Americans suffer from Alzheimer’s disease (AD), a number which is expected to increase to 14 million by 2050. In economic terms, AD and other neurodegenerative disorders will cost the US over $275 billion in 2018, rising to over $1 trillion annually by 2050. AD causes gross brain atrophy and is most damaging throughout the cortex and the hippocampus, regions required for higher cognitive function and memory. AD presents as tangles within neurons composed of the tau protein, and plaques outside of neurons consisting of amyloid beta. Aggregates have been clearly shown to have associated toxicity.

The past three decades of drug development targeting amyloid beta, an aggregating protein responsible for plaques seen in AD, has failed and currently we have no treatments beyond ameliorative options. Therapeutic alternatives with novel targets are desperately needed in our fight against AD. Therefore, the ultimate goal of this research is to identify novel enzymes involved in aggregation and provide a potential therapeutic option for neurodegeneration capable of lessening tau tangle burden, protecting against the loss of neurons, and preventing cognitive decline.

In AD, both amyloid beta and tau proteins aberrantly aggregate and promote neuronal loss, gross brain tissue degeneration, and cognitive decline. Although the interplay between these two proteins is not well understood, it is clear that targeting amyloid beta has not worked thus far despite over 100 phase III clinical trials targeting amyloid beta. Additionally, it has been
repeatedly shown that tau neurofibrillary tangles are correlated with disease severity and not amyloid beta plaques. Finally, tau tangles are present in many other neurodegenerative dementia disorders termed tauopathies including progressive supranuclear palsy, corticobasal degeneration, Pick’s disease, and frontotemporal lobar dementia. Strategies that target tau have a higher chance of success over amyloid beta, and if a therapeutic is found will have a broad impact as tau is involved in so many other devastating neurodegenerative diseases.

Here we show that a human peptidyl prolyl-isomerase (PPlase), cyclophilin 40 (CyP40), dissolves fibrillized tau amyloid fibril in recombinant protein assays, in a human cell model, and in a mouse brain overexpressing human tau. PPlases play an important role in normal cell physiology as catalysts of protein folding at structurally important proline residues for both newly synthesized proteins and in conformational changes for molecular switches. Intrinsically disordered amyloid-forming proteins like tau have proline-rich regions which may regulate aggregation propensity. Our work reveals human CyP40 protein, on its own, possesses remarkable disaggregase activity towards amyloidogenic tau and α-synuclein through interaction with proline residues. CyP40 disaggregase activity is ATP-independent, using its peptidyl-prolyl isomerase activity and untangles amyloid fibrils in vitro. Additionally, CyP40 decreases both tau fibril and oligomer accumulation in a transgenic mouse tauopathy model and delivery of CyP40 to neurons early in the pathogenic progression of the mouse model results in significant improvements in learning and memory as assessed by radial arm water maze and fear conditioning paradigms. This demonstrates a novel disaggregation mechanism for CyP40 making it a potential therapeutic target for neurodegenerative disease.
CHAPTER ONE:

AGGREGATION AND NEURODEGENERATIVE DISEASE

1.1 Abstract

The misbehavior of key proteins results in aberrant misfolding or misassembly and leads to neuronal loss, producing motor or cognitive impairments found in many neurological disorders, including Alzheimer’s disease (AD), Parkinson’s disease (PD), Amyotrophic Lateral Sclerosis (ALS), and Huntington’s disease (HD). These amyloid aggregates are distinguished by characteristic β-sheet structures that can form long fibrils as well as recruit and convert protein that has not yet misfolded. Indeed, because amyloid formation results in a heterogeneous mix of many oligomeric, prefibrillar and fibrillar structures, determining the toxic aggregated species has been difficult, and current therapeutics have failed to prevent the pathology caused by these proteins. Here we provide background on protein folding regulatory processes as well as illustrate certain determinants for protein misfolding. We will also describe current hypotheses on how protein aggregates cause cellular stress and neurodegeneration and provide an overview of new early detection and therapeutic strategies.

1Portions of this work were previously published (Baker, J.D. et al., 2018) and are used with permission of the publisher.
1.2 Introduction

Neurodegeneration is a result of modification and actions key proteins that are aberrantly misfolded or misassembled, leading to neuronal loss producing motor or cognitive impairments. Misfolding and subsequent aggregation of proteins is linked to many neurological disorders, including Alzheimer’s disease (AD), Parkinson’s disease (PD), Amyotrophic Lateral Sclerosis (ALS), and Huntington’s disease (HD), to name a few. Generally, these protein aggregates form a special structure known as amyloid, which has the distinguishing characteristic of containing β-sheet structures that can aggregate into long fibrils as well as recruit and convert protein that has not yet misfolded. How the formation of amyloid aggregates leads to neuronal dysfunction and neuron loss despite cellular machinery and processes evolved to ensure proper protein folding and regulation is an area of active research\(^1\). Indeed, because amyloid formation results in a heterogeneous mix of many oligomeric, prefibrillar and fibrillar structures, determining the toxic aggregated species has been difficult, and current therapeutics have failed to prevent the pathology caused by these proteins. This chapter will provide background on protein folding regulatory processes as well as illustrate certain determinants for protein misfolding. We will also describe current hypotheses on how protein aggregates cause cellular stress and neurodegeneration and provide an overview of new early detection and therapeutic strategies.

1.3 Discussion

1.3.1 Roles for protein folding, modification and degradation

Proteins are essential macromolecules of all living cells. Their functions are crucial and diverse, ranging from key structural components such as keratins, which form external protective layers of all land vertebrates, and tubulin, which forms the eukaryotic cytoskeleton and plays a
central role in neuronal transport\textsuperscript{2}, to various enzymes catalyzing a multitude of biological reactions, such as acetylcholinesterase, a key protein within the synaptic cleft. Mammalian immune defense relies on antibody proteins, while cellular, tissue, and organ messaging depends on growth hormone proteins for communication\textsuperscript{3}. Obviously, these listed examples represent only a miniscule fraction of the immense universe of protein functions. The functional diversity of proteins arises from the multitude of polypeptide chains, whose sequences are uniquely assembled from a set of 20 major amino acids. Alternative splicing and RNA editing ensures diversification of mRNA and plays an important role in increasing the assortment of functional proteins encoded in the genome. Some proteins are folded into unique structures, whereas others exist as dynamic conformational ensembles. Biological activities of many proteins require further assembly of individual chains to large functioning protein complexes. Furthermore, synthesized proteins can be further diversified via various chemical modifications introduced by other proteins\textsuperscript{4}. These so-called post-translational modifications (PTMs) are important for regulating the wide spectrum of protein functions that play important roles in various biological processes, ranging from cell signaling to translocation of newly synthesized proteins. In fact, although DNA typically encodes 20 primary amino acids, proteins contain more than 140 different residues, because of various PTMs. As illustrated below, abnormal PTMs can lead to neurodegeneration. Phosphorylation, the transfer of a phosphoryl group to an amino acid mediated by a variety of kinases, is the most common PTM. Phosphorylation plays a key role as a biochemical “switch” for some proteins, for instance activating or deactivating certain enzymes. A host of neurodegenerative diseases known as tauopathies may arise after the abnormal hyperphosphorylation of tau, a protein known to stabilize axonal microtubules\textsuperscript{5}. 
Acetylation, another common PTM where acetyl groups are covalently linked to an N-terminal amino group or lysine residue, generally results in changes in the stability of proteins. Histone acetylases (HATs) and histone deacetylases (HDACs) control the balance of acetylation of specific substrates. When the cellular environment changes and a neuron loses acetylation homeostasis, due to a change in HAT:HDAC equilibrium, neurodegenerative disease can result\(^6,7\). Another common PTM, glycosylation, or the addition of carbohydrates to amino acid residues, is crucial to the proper folding and translocation of many cell surface and secreted proteins\(^8\). Traumatic brain injury (TBI) often results in an altered pattern of glycosylated amyloid precursor protein, which may be linked to its pathological clinical presentation in the form of cerebral plaques\(^9,10\).

In addition to PTMs playing a role in proper folding of nascent proteins and regulation of protein function, a class of proteins known as molecular chaperones assist in forming the correct three-dimensional structure of proteins and help to assemble large protein complexes\(^11\). Many molecular chaperones are activated in response to cellular stress, such as elevated temperature or changes in pH or salt concentration\(^12\). Cellular stress, like heat, causes a much higher incidence of misfolding, which may lead to protein aggregation. In humans, some of the more important cellular chaperones use ATP as an energy source to actively support the folding or unfolding of proteins and are termed foldases or unfoldases\(^13-15\). For example, heat shock protein 70 (Hsp70), a chaperone expressed in response to causes of neuronal stress, including stroke and neurodegenerative disease, may play an important role in the suppression of aggregation and resulting toxicity of some neuropathic proteins\(^16,17\). Some other chaperones, like the small heat shock proteins \(\alpha\)B-crystallin and Hsp27, are ATP-independent and do not actively fold proteins,
but rather hold proteins in certain conformations and are referred to as holdases\textsuperscript{18}. These small heat shock proteins have been shown to reduce toxicity caused by $\alpha$-synuclein in Parkinson’s disease\textsuperscript{19}. One of the more exciting recent discoveries is that of a chaperone complex of Hsp70, a DnaJ co-chaperone, and the nucleotide exchange factor Hsp110, which work in concert and harnesses energy from ATP to physically pull apart and disassemble amyloid fibrils. This complex disassembles $\alpha$-synuclein amyloid fibrils leading to a non-toxic end-product\textsuperscript{20}. Research into harnessing the power of this human disaggregase machinery to disentangle pathological amyloids is just beginning but presents a novel therapeutic approach.

Protein homeostasis (or proteostasis) is required for cellular health and is controlled not only by the rate of synthesis of proteins, their proper folding and trafficking, PTMs and the action of molecular chaperones, but also by regulated degradation\textsuperscript{21,22}. Degradation processes within the proteostasis network include the ubiquitin-proteasome system (UPS), chaperone mediated autophagy (CMA) and macroautophagy\textsuperscript{23}. Misfolded proteins can be marked for proteosomal degradation by covalent tagging with chains of ubiquitin protein. A downregulation of the UPS is associated with progressive onset of several neurodegenerative diseases, and it is thought that aggregated proteins can physically interfere with the proteasome machinery to reduce its activity\textsuperscript{24,25}. Autophagy refers to the cellular sequestration of proteins into lysosomes, an organelle containing hydrolytic enzymes capable of degrading diverse proteins. Like the UPS, down regulation or genetic inactivation of autophagy has been linked to numerous degenerative diseases\textsuperscript{26,27}. 
The remainder of this chapter will focus on the precise causes of the misfolding of proteins, how they result in toxic oligomeric and amyloid assemblies, and current explanations for how these proteins may cause disease.

1.3.2 **What is protein misfolding and why does it occur?**

Misfolding of certain proteins leads to neurodegenerative disease, due to loss of native protein function or a gain of deleterious function, the latter resulting from aggregation into toxic structures\(^{28-31}\). This chapter will focus on protein aggregation as a toxic gain of function phenomenon associated with many neurological disorders.

Generally, in the case of structurally ordered proteins, nascent polypeptide chains fold into properly functioning three-dimensional structures, either spontaneously or with the assistance of molecular chaperones. Resulting structures can be further tuned with the help of post-translational modifications. Structural integrity of a folded protein is maintained via an interplay of weak conformational forces (such as hydrophobic interactions, hydrogen bonds, van der Waals interactions and electrostatic interactions) opposing the action of conformational entropy. Folded globular proteins are characterized by structures where hydrophobic residues are generally buried deep within the protein core and amino acid side chains are engaged in multiple interactions to maintain conformation. However, because of fluctuating environmental conditions, such as changes in pH or temperature, native state proteins may partially unfold, and the resulting intermediates may begin to aggregate. Misfolding and aggregation can also be triggered by point mutations, aberrant PTMs, and many other factors. Pathological proteins undergo a series of steps from their native monomeric form to multi-unit aggregates, typically rich in β-sheet structure. During this conformational change from properly folded to misfolded
and aggregated forms, often involving a shift from native α-helical secondary structure to β-sheet structure, some key hydrophobic residues, originally buried within the protein core, are exposed\textsuperscript{23,32}. These hydrophobic residues located on the protein surface cause inter-chain attraction manifested in multiple protein monomers coming together to form aggregates. Often, the assembly of these β-sheet-rich structures has a fibrillar morphology, and the resulting aggregated forms are termed amyloid fibrils. One should keep in mind though that, in addition to amyloid fibrils, many other types of pathological protein aggregates can be formed, ranging from various assemblies of soluble oligomers and protofibrils, to amorphous aggregates and spherulites\textsuperscript{33-36}.

Amyloid fibrils have been associated with more than 30 human diseases, including several neurodegenerative disorders as shown in Table 1.1\textsuperscript{37-39}. Fibrils (or at least their core) are normally composed of polypeptides taking on a β-sheet secondary structure, which then self-associate to form long fibers\textsuperscript{40,41}. Using atomic force microscopy and transmission electron microscopy, fibrils have been shown to consist of a cross-β-sheet secondary structure within the core of the fibrils, where the parallel chains of β-stranded peptides are arranged in an orientation perpendicular to the axis of the fiber and linked by an array of inter-backbone hydrogen bonds\textsuperscript{42,43}. This structure is incredibly resistant to cellular degradation mechanics as well as experimental means of protein structure disruption\textsuperscript{44-47}. Amyloid may consist of hundreds and often thousands of the original monomeric protein subunits, resulting in large bodies visible by microscopy within neurons and brain tissue.

Although amyloid fibrils are the most commonly detected form of aggregation, these pathogenic proteins form a heterogeneous mixture of oligomers, protofibrils and fibrils\textsuperscript{48,49}. The
structures and associated pathology of these assemblies is of great interest as we look for multiple toxic species responsible for the associated neurological diseases. Generally, the end-products of aggregation are insoluble fibrillar amyloid aggregates; however there are many intermediary structures along the pathways of aggregation\textsuperscript{50-53}. Because the aggregate structures of proteins like Aβ and tau are disordered and consist of a heterogeneous population of species, traditional methods to study structure, such as x-ray crystallography and nuclear magnetic resonance, has not yielded high-resolution structures for many smaller aggregates. However, recent advances in solid-state NMR have given us key insights into aggregate structures specifically in regard to Aβ\textsuperscript{52,54,55}.

1.3.3 Pathological misfolding of intrinsically disordered and mutant proteins

Many pathological amyloidogenic proteins that cause neurodegeneration are intrinsically disordered. That is, they present not as classical globular three-dimensional structures, but as a highly dynamic conformational ensembles\textsuperscript{56,57}. The flexibility of intrinsically disordered proteins allows them to assume numerous conformations and increases binding affinity for a much larger range of substrates compared to fixed-conformation globular proteins. However, the flexibility and lack of a three-dimensional structure, along with an enhanced propensity to be post-translationally modified and engaged in multiple physiological and pathological interactions makes some of these proteins more prone to aggregation and amyloid formation\textsuperscript{58,59}. Intrinsically disordered proteins which aggregate in neurological diseases include the microtubule binding protein tau and amyloid beta (Aβ)\textsuperscript{58,60,61}, both involved in Alzheimer’s disease, as well as α-synuclein, which aggregates to form Lewy bodies in Parkinson’s disease pathology\textsuperscript{62,63}.
Meanwhile other misfolded proteins involved in neurodegeneration develop a propensity for misfolding resulting from a genetic expansion of nucleotide repeats. Expanded CAG repeats in the coding region of certain genes results in expanded polyglutamine (PolyQ) tracts within the translated protein, which have a propensity to misfold into β-sheet-containing amyloids. For example, Huntington’s disease and spinocerebellar ataxias are caused by expanded polyQ tracts within the huntingtin or ataxin proteins. Additionally, expanded nucleotide repeat sequences in the noncoding region of the C9orf72 gene have been linked to frontotemporal dementia and amyotrophic lateral sclerosis. This nucleotide repeat expansion becomes translated in the absence of a start codon into a mixture of 5 aggregating dipeptide repeat (DPR) proteins translated from all 6 reading frames.

Although it has been challenging to study the mechanisms of self-assembly of these pathological proteins by experimentation, with the help of molecular dynamic simulations and advanced biochemical techniques, a more robust picture of what drives aggregate formation is emerging. Monitoring fibril growth with a quartz crystal oscillator has revealed that the native state of these proteins is thermodynamically more stable than the amyloid state as long as a critical concentration of amyloid is not reached. Once a critical concentration of amyloid is present in the cell, the native state will inevitably and spontaneously be driven by thermodynamics toward the amyloid state unless cellular mechanisms prevent the transition. Experiments using small amyloid-forming fragments of several proteins have given insights into the kinetics of amyloid formation. Three kinetic stages occur in amyloid formation. Firstly, nucleation of monomers results in the formation of a metastable critical nucleus, which can shift the protein into a growth phase. This growth phase consists of rapid assembly of oligomers into
protofibrils which self-associate. Ultimately, a final dynamic stabilization phase is reached, in which mature fibrils are formed\textsuperscript{68,69}.

The triggers that start the aggregation process vary and range from post-translational modifications to changes in environmental factors within the cell. Additionally, certain amino acid changes due to genetic variation within aggregation-prone proteins increase the propensity for self-assembly into amyloid\textsuperscript{70-73}. It should be noted that intrinsically ordered proteins “breathe”, that is they have an inherent fluctuation in conformation which may transiently expose residues normally not exposed to the cellular environment, increasing chances for aggregation\textsuperscript{74,75}, environmental stressors like pH changes, pressure changes, salt concentration changes, and temperature changes can drive partial unfolding of proteins and cause an aggregation cascade\textsuperscript{76-80}.

Under normal cellular conditions, environmental factors are kept relatively constant, and other factors may be predominantly responsible for triggering amyloidogenesis. A recognized trigger of amyloidogenesis of tau protein is its hyperphosphorylation, indicating suggesting regulation via cell-signaling pathways. Tau is rich in phosphorylation sites and is regulated by a number of kinases. When tau is heavily phosphorylated, its association with microtubules is disrupted. This disruption from microtubules facilitates self-association and the formation of aggregates.

\textbf{1.3.4 How do misfolded proteins and aggregates cause neurodegeneration?}

Increasing evidence supports oligomers as the causative toxic species in a number of neurodegenerative disorders, whereby fibril generation is thought to be a protective mechanism in neurons, sequestering the smaller toxic aggregates\textsuperscript{81-83}. It is well known that Aβ aberrantly
accumulates in Alzheimer’s disease (AD); however, the presence of deposited extracellular Aβ plaques do not generally correlate well with disease progression\textsuperscript{84}, and many healthy brains carry an Aβ plaque burden without associated cognitive deficits. This has led to the hypothesis that a smaller soluble aggregated form of Aβ is somehow pathogenic. In a mouse model of tauopathy, turning off tau expression halted neuronal loss and prevented cognitive decline, even though neurofibrillary tangles remained present and further accumulated\textsuperscript{85}, providing evidence for a soluble oligomeric intermediate as the toxic species. Additionally, TDP-43, a protein associated with ALS, has been shown to form a heterogeneous population of oligomers, and these oligomers exist in patients with TDP-43-positive frontotemporal lobar dementia (FTLD-TDP)\textsuperscript{86}, a form of dementia not associated with tau or Aβ. It should be pointed out again that amyloid aggregation results in the formation of a heterogeneous population of oligomers with a diverse range of structure and size. Determining the toxicity of specific oligomer assemblies is challenging, but it has been postulated that there may be an inverse correlation between oligomer size and toxicity\textsuperscript{81}.

Oligomers have important biochemical properties which make them more toxic than mature amyloid fibrils. Hydrophobic residues of the more inert fibrils are buried within its cross-β-sheet core; however, these residues are more readily accessible in oligomers and provide an interaction surface for other cellular structures and proteins. Oligomers, but not fibers, have been shown to interact with and disrupt membranes\textsuperscript{87}. Lastly, oligomers have been shown to seed the production of oligomers of other amyloid proteins, initiating a cascade of aberrant assembly of toxic proteins within cells\textsuperscript{88}. Indeed, very recent and convincing evidence has indicated that these misfolded structures of various proteins might spread from neuron to neuron in a way that
resembles the pathological spread of prion proteins. Precisely how these oligomers are causing disease is just now being brought into focus, and a number of well-regulated pathways being disrupted by oligomer activity may be pathogenic. In this section, we will discuss new research illustrating just how oligomers may promote toxicity within neurons as shown in Figure 1.1.

Astrocytes and microglia regulate neuroinflammation through a host of cytokines, small signaling proteins that include chemokines, interferons, interleukins, and tumor necrosis factors (TNF). It is well known that a number of neurodegenerative diseases are associated with increased inflammation throughout the brain, and increased activity and accumulation of microglia and astrocytes precedes deposition of insoluble amyloid, such as Aβ or tau fibrils, in AD patients. Cytokines released by glial cells interact with neuronal receptors to promote signaling pathways that have been implicated in several neurodegenerative disorders. Chemokines like IL-1, IL-6, and TNFα, released by glial cells are significantly more abundant in AD brain tissue than healthy brains, and cytokines can upregulate pathways promoting activation of proteins associated with AD, such as protein kinase C or p38 mitogen-activating protein kinase. Prolonged, chronic activation of glial cells associates well with brain atrophy in AD. Protein oligomers may be directly responsible for interacting with glial cells, causing a cascade of increasing inflammation and glial activity contributing to neuron loss.

It has been proposed by the Kayed group that tau oligomers may induce a positive feedback loop by causing neuroinflammation, which in turn induces increased formation of oligomeric species. Their studies indicate that tau oligomers co-localize near astrocytes and may be responsible for astrocyte activity, leading to increased inflammation. Additionally, the
group found that tau oligomers co-localized with microglia, neural macrophages activated in inflammation. In contrast to astrocytes, where oligomers seem to interact near the cell surface, microglial cells apparently engulf the oligomers. Phagocytosis and subsequent release through exosomes has been reported and may play a role in neural spreading of tau\textsuperscript{102,103}. The authors propose that activation of microglia may result from oligomer interaction with the Receptor for Advanced Glycation Endproducts (RAGE) receptor, which leads to NFκB activation and subsequent upregulation of HMGB1, a pro-inflammatory cytokine. Importantly, RAGE is itself upregulated by this process, leading to chronic inflammation. It is also well known that Aβ can also induce release of cytokines from astrocytes. A 2011 study by the Noble group showed that Aβ treatment of astrocytes increased the amounts of cytokines CINC2α/β, IFN-γ, IL-1β, IL-1ra, IL-6, IL-13, IL-17, IP-10 and MIG\textsuperscript{104}. This is important because IL-1β, IL-6, and IP-10 are all associated with plaque and tangle pathology in both human AD brains and in mouse models of AD. Further studies are needed to identify which receptors oligomers are acting on and to clarify the mechanism by which they influence inflammatory pathways.

As previously mentioned, proteostasis refers to numerous pathways and cellular organelles which function to regulate protein translation, folding, localization and degradation. It is well known that proteins abnormally accumulate in numerous neurodegenerative diseases, indicating a disruption of degradation and de-aggregation processes required for neuronal survival. Oligomers of various proteins associated with neurodegenerative diseases interact with these degradation pathways and subsequently inhibit clearance of toxic proteins by the cell. We will discuss two major pathways of cellular protein degradation and highlight new areas of
research showing how misassembled oligomers may be interacting with and disrupting these crucial processes.

The UPS is a two-step degradation pathway, whereby proteins targeted for degradation are first covalently conjugated by to a polyubiquitin chain before being translocated to the proteasome. The proteasome is a catalytic complex responsible for the degradation of misfolded proteins. The complex consists of a 20S catalytic core along with 19S regulatory subunits on either end, which direct misfolded proteins to the catalytic core subunit. Proteolytic activity of the proteasome is reduced by proteins central to neurodegenerative diseases, including tau in tauopathies, by α-synuclein in PD, and Aβ in AD\textsuperscript{105}.

Oligomers of diverse neurotoxic proteins interact with and inhibit proteolytic activity of the UPS. For example, in an \textit{in vitro} proteasome activity assay, oligomeric Aβ inhibited proteosomal catalytic activity in a dose-dependent manner, whereas the Aβ monomer had no effect on proteasome activity\textsuperscript{105}. In a human cell model, Aβ interacted with and sequestered the α-subunit of the proteasome into aggregates. Further, in an \textit{in vivo} mouse model overexpressing Aβ, administration of an anti-Aβ antibody results in a reduction of oligomer levels in the brain while simultaneously increasing proteasome activity\textsuperscript{105}. In mice overexpressing a pathologically aggregating form of tau containing the P301L mutation, known to cause frontotemporal dementia, aggregates of tau directly inhibited the activity of the 26S proteasome\textsuperscript{106}. Finally, α-synuclein oligomers directly interact and disrupt the activity of the 26S proteasome, indicating a common pathology among different oligomer forming proteins\textsuperscript{107}.

Another major pathway through which neurons remove or degrade cellular components is through macroautophagy, whereby unneeded components are sequestered to a vacuole. This
vacuole subsequently fuses to a lysosome, which contains diverse hydrolytic enzymes capable of breaking down proteins and other cellular components. Inhibition of macroautophagy leads to accumulation of toxic proteins, such as tau, Aβ, and α-synuclein oligomers\textsuperscript{108}; however, the mechanism by which neurotoxic proteins interact and hinder the macroautophagy-driven degradation pathway is unclear. Recent work suggests that Aβ has a direct conformation-specific role in modifying autophagy and subsequent apoptosis of cells. Beclin-1 is a crucial protein in the autophagy pathway, as it initiates the formation of phagosomes through recruitment of other proteins. However, if Beclin-1 interacts with bcl-2, it prevents autophagosome formation and leads to apoptosis, or neuronal death\textsuperscript{109}. Interestingly, while monomers of Aβ inhibit this interaction, Aβ oligomers actually enhance the formation of Beclin-1:bcl-2 complexes and cause neuronal death\textsuperscript{110}. Because oligomers of diverse toxic proteins seem to have similar effects on cellular pathways and organelles, amyloidogenic proteins other than Aβ may also directly impair autophagy.

Neuronal damage precedes fibrillary tau aggregates, suggesting that prefibrillar oligomers may be neurotoxic. Loss of neurons ultimately causes cognitive and motor impairments in animal models and in human neurodegenerative diseases. However, oligomeric forms of amyloidogenic proteins cause disruptions in synaptic activity and plasticity well before cellular degeneration. Synaptic dysfunction rather than neuron loss may be the cause of motor impairment and memory loss early in neurodegenerative disease progression\textsuperscript{111,112}. Aβ oligomers have been shown to inhibit hippocampal long term potentiation and enhance long term depression well before cellular degeneration\textsuperscript{113,114}, which suggests a potential means of reversal of neurodegenerative symptoms if diagnosed early\textsuperscript{115,116}. Soluble oligomeric amyloids associate
with synaptic proteins, resulting in impairments in synaptic function. Aβ oligomers associate with post-synaptic density complexes containing N-methyl-D-aspartate (NMDA) receptors and cause impairment of NMDA receptor-mediated calcium entry and decreased dendritic spine density\textsuperscript{117,118}. Aβ oligomers have been demonstrated to cause impaired synaptic trafficking of ionotropic and metabotropic glutamate receptors\textsuperscript{45,112} and to induce abnormal glutamate release from hippocampal neurons, which may contribute to excitotoxic signaling\textsuperscript{119}. Soluble amyloid oligomers also induce dysregulation of calcium sequestration and signaling through a mechanism of increased membrane permeability independent of Ca\textsuperscript{2+} channel activation\textsuperscript{120}. Aβ oligomers and tau oligomers have also been shown to disrupt axonal transport mechanisms\textsuperscript{121,122} that are critical for the maintenance of healthy synapses. Impaired neuronal signaling resulting from oligomeric amyloid proteins should be reversible prior to neuron loss, providing hope that eventually an appropriate therapeutic combined with early detection could one day facilitate reversal of disease progression.

1.3.5 **How can protein misfolding be targeted?**

Detection and diagnosis of neurodegenerative diseases continue to move away from post-mortem analysis of protein aggregates in the brain towards non-invasive early detection. Many methods from genetic testing\textsuperscript{123}, molecular and functional imaging\textsuperscript{124}, and blood and cerebrospinal fluid (CSF) biomarkers\textsuperscript{125}, to retinal scanning\textsuperscript{126,127} and exhaled volatile biomarker fingerprints\textsuperscript{128} involve non-invasive tests to seek correlations between specific biomarkers and disease. Here we focus on the non-invasive detection of misfolded proteins or interacting chaperones rather than numerous other potential correlative biomarkers.
In AD, the intracellular accumulation of misfolded species of amyloidogenic proteins such as Aβ and tau is also accompanied by the secretion of these proteins from neurons. Remarkably, these proteins can be detected in the CSF and have even been detected in blood. Multiple studies have evaluated the detection of amyloidogenic proteins in the blood or CSF\textsuperscript{123}. The focus of peripheral Aβ detection has been on the ratio of Aβ40/Aβ42\textsuperscript{129}, and, more recently, on smaller Aβ fragments, such as Aβ17\textsuperscript{130}. Similarly, CSF and blood tau detection efforts have included both total and phosphorylated tau\textsuperscript{131}. The extracellular chaperone clusterin has also emerged as a potential biomarker for AD\textsuperscript{123,132}. Similarly, in Parkinson’s disease (PD), changes in the levels of α-synuclein (an intrinsically disordered protein found in pathological intraneuronal hallmarks of PD, Lewy bodies and Lewy neurites) in various biofluids, such as cerebrospinal fluid (CSF), plasma, blood, and saliva, are used as disease-specific and early-stage biomarkers\textsuperscript{133}. Interestingly, the best fitting predictive models for discriminating PD patients from controls can be built using a combination of multiple CSF α-synuclein species, such as total-, oligomeric- and phosphorylated α-synuclein, with classical AD biomarkers, such as phosphorylated tau\textsuperscript{134}. Nevertheless, consistency and reproducibility of using amyloidogenic proteins as blood biomarkers can be challenging. For example, variability in blood-brain barrier integrity will have a profound effect on blood detection of selected biomarkers of brain origin, leading to poor correlation with stage of disease. Additionally, quantitation of proteins with disordered regions possessing high binding potential and/or a propensity to aggregate can be challenging, as the target protein may stick to other proteins on vascular and blood cells as well as to undergo self-aggregation that would mask epitopes used for detection.
Molecular imaging of amyloidogenic protein accumulation has become a promising noninvasive detection method for correlation with neurodegenerative disease progression. Three PET imaging agents targeting Aβ accumulation have been approved by the FDA for the estimation of Aβ neuritic plaque density. However, some patients present as Aβ positive with no dementia symptoms, making the hope of a simple imaging diagnosis more complicated. Therefore, initial approval was designed to rule out AD in Aβ-negative patients with dementia, but longitudinal studies of cognitively normal patients with significant Aβ burden may help to determine whether progression to AD is inevitable. A large multicenter clinical study in the United States, the Imaging Dementia-Evidence for Amyloid Scanning (IDEAS) study, aims to evaluate how amyloid imaging affects diagnosis, patient management, and outcomes. Several tau-targeted PET imaging agents are emerging and entering clinical trials as well as becoming incorporated into clinical trials evaluating potential therapeutics. The pattern of tau tracer retention appears to correlate with disease progression and with the Braak staging pattern of tau deposition in autopsy studies. Many PET imaging strategies for Parkinson’s Disease focus on uptake of agents through monoamine transporters, however there is an intense focus on the development of agents targeting aggregates of α-synuclein.

Overall, treatment options for neurodegenerative diseases have been very disappointing, with the majority of them failing to produce any significant benefits, while others end up doing more harm than good. FDA-approved therapeutic strategies are limited to pharmacological modulation of neurotransmission to counteract a loss in synaptic connections and/or neurons. Future strategies for the development of therapeutics for neurodegenerative protein misfolding disorders are varied and include stem cell replacement, immunotherapy, gene
therapy, targeting modulators of protein aggregation, and degradation and targeting of misfolded proteins themselves. Here we will highlight a few therapeutic strategies that are focused on modifying misfolded or aggregated protein.

Active immunization research through vaccination with amyloidogenic protein epitopes has shown some promise in certain animal models of neurodegeneration and in early clinical trials. Vaccination against N-terminal peptides of Aβ\textsuperscript{144,145} or peptides and phosphopeptides representing tau protein\textsuperscript{146-148} have been evaluated. However, active vaccination carries the risk of inducing an excessive immune response in the brain, evidenced by occurrences of meningoencephalitis in clinical trials. Mimetics of protein epitopes are also being used for vaccination strategies designed to induce a therapeutic immune response to Aβ\textsuperscript{149} and α-synuclein\textsuperscript{150} in hopes of reducing the risk of autoimmunity associated with native antigens. Additionally, adjuvant-free immunotherapies, including passive and DNA immunization, may avoid undesirable immune reactions.

Several antibodies developed against certain species of amyloidogenic aggregates have been extensively tested clinically as passive immunotherapy. For example, aducanumab is a human monoclonal antibody that selectively targets aggregated Aβ. Tested in patients with mild AD in a small, Phase I trial, Aducanumab showed a reduction of Aβ in a dose- and time-dependent manner, as well as a clinical improvement as measured by the Clinical Dementia Rating – Sum of Boxes and Mini Mental State. Aducanumab is currently being tested in a phase III clinical trial on patients with early AD\textsuperscript{151} (NCT02477800). Crenezumab, an antibody targeting multiple forms of Aβ, and gantenerumab, an antibody that specifically recognizes Aβ fibrils, are both currently being investigated in phase III clinical trials for their efficacy as AD
treatments (NCT02565511, NCT02670083, NCT02051608, NCT01224106). Solanezumab was developed as a humanized monoclonal IgG1 antibody recognizing soluble Aβ, in the hopes of targeting the smaller, more toxic Aβ species. Although solanezumab has been investigated in several phase III clinical trials without producing clinically significant differences on primary outcomes (NCT00904683), it is still being tested as a combination therapy with other potential AD treatments (NCT01760005). Less specific to Aβ, a phase III clinical trial examining the role of intravenous immune globulin (IGIV) as an add-on treatment for AD, a part of the Gammaglobulin Alzheimer’s Partnership (GAP) study, failed to meet the primary outcome measures (NCT00818662).

Similarly, antibodies specific for particular species of tau are in clinical development for passive immunotherapy\(^\text{152}\) (NCT02820896, NCT02880956, NCT02460094). Most of these antibodies recognize tau phosphorylated at specific sites\(^\text{152}\), while others target particular peptide sequences\(^\text{153}\) or specific conformations\(^\text{154}\). Certain antibodies in clinical and preclinical development specifically target only prefibrillar oligomeric forms of tau\(^\text{155-157}\).

The development of antibodies against α-synuclein are progressing for treatment of PD and other synucleinopathies by reducing aggregation and toxicity\(^\text{144,158}\). PRX002, a monoclonal antibody against α-synuclein is now being investigated for its role in reducing α-synuclein levels in patients with PD (NCT02157714).

The delivery of therapeutic genes into the cells of patients with disease, usually using viral vectors, is yet another therapeutic strategy. One gene therapy strategy involves the expression of genes encoding single-chain antibody fragments (intrabodies) into cells\(^\text{159}\). In this way, some of the same antibodies developed for passive immunotherapy could be reengineered
for intracellular expression. Intrabodies have been developed to target Aβ160, α-synuclein161, and regions adjacent to the polyQ tract in huntingtin162-164. Similarly, an engineered protein not based on an antibody scaffold that also binds to and modulates amyloid aggregation, Aβ-binding Affibody ZAβ3, represents another potential gene therapy strategy165. Gene therapy may also be used to deliver genome-editing technologies, such as CRISPR and zinc-finger nucleases. This has particular promise for a purely genetic disease like Huntington’s disease, where silencing or editing out the expanded CAG repeats at the DNA level could halt production of the aggregation-prone protein166,167. Additionally, gene therapy delivery may allow the expression of proteins that have a propensity to reduce the aggregation of amyloidogenic protein. Certain chaperone proteins interfere with amyloid aggregation, including αB-crystallin, clusterin, Hsp27 and Hsp70168-170. Likewise, a number of non-chaperone proteins have been found to reduce amyloid aggregation, including catalase, pyruvate kinase, and the prolyl isomerase PIN1171,172. While natural proteins that reduce amyloid aggregation may be promising targets for gene therapy, other endogenous proteins that exacerbate amyloid aggregation may be potential therapeutic targets for inhibition.

Chaperones and enzymes that associate with misfolding proteins, like tau and Aβ, are potential therapeutic targets in neurodegenerative disease. Hsp90 is a major chaperone which helps fold, refold, and stabilize various proteins. Though aberrantly misfolded tau is normally degraded in the proteasome, Hsp90 in concert with co-chaperones stabilize tau, preventing normal degradation and thereby leading to accumulation. Inhibition of Hsp90, in vitro, allows for uninterrupted tau degradation173. Furthermore, Hsp90 increases the formation of β-sheet-rich tau fibrils and in complex with the co-chaperone FKBP51, produces neurotoxic oligomeric tau174.
Because Hsp90 helps maintain levels of kinases and other proteins required for cancer cell survival, it has become a promising target in the fight against cancer, and well-characterized drugs developed for Hsp90 inhibition may also have implications in neurodegenerative disease. Conversely, because of the importance of Hsp90 in proteostasis and the extensive list of Hsp90-interacting proteins\textsuperscript{175}, targeting of Hsp90 co-chaperones, like FKBP51, may prove to be an alternative therapeutic approach with less toxicity due to a smaller subset of chaperone client proteins.

Beta-secretase (BACE-1) is the enzyme that cleaves amyloid precursor protein (APP) into the C99 fragment, which can then be cleaved by gamma-secretase into Aβ fragments. Reducing the amount of Aβ fragments using a BACE inhibitor is thought to be a good therapeutic strategy for AD. Several BACE inhibitors are currently being tested in phase III clinical trials. For example, small molecules AZD3293, CNP520, and JNJ-54861911 are all BACE-1 inhibitors being investigated for their efficacy at various stages of AD (NCT02245737, NCT02783573, NCT02565511, NCT02569398). Verubecestat is a small-molecule inhibitor of BACE-1 and BACE-2 and is currently being used in a phase III clinical trial to examine its efficacy in mild to moderate cases of AD\textsuperscript{176} (NCT01739348, NCT01953601).

There are several other potential therapeutics being tested for the treatment of AD, such as an inhibitor of Aβ polymerization and inflammation (ALZT-OP1) and an inhibitor of tau aggregation (TRx0237). ALZT-OP1 is a combination therapy of two FDA-approved drugs: cromolyn and ibuprofen. Both drugs have anti-inflammatory mechanisms: cromolyn stabilizes mast cells and suppresses cytokine release, whereas ibuprofen is a widely used non-steroidal anti-inflammatory drug. Cromolyn has also been shown to inhibit aggregation of Aβ monomers
in vitro, and decrease soluble Aβ levels in vivo\textsuperscript{177}. ALZT-OP1 is currently being investigated in a phase III trial in patients with early AD. TRx0237 is a reduced form of Methylene Blue (methylthioninium chloride, MTC) that has been designed to stabilize this dye to improve its absorption, bioavailability, and tolerability\textsuperscript{178}. TRx0237 is thought to both prevent tau aggregation and dissolve existing aggregates\textsuperscript{178}. Several phase III trials have examined the effects of TRx0237 on cognition (NCT01626378, NCT01689246, and NCT01689233). Two of the clinical trials failed to meet their primary outcomes, and the results of another has are not yet to published its results. Additional phase III therapeutics are summarized in Table 1.2.

1.4 Conclusions

Maintenance of cellular proteostasis is essential for cellular health and is regulated by a variety of proteins involved in proper protein folding, trafficking and degradation pathways. Thousands of different proteins, each with multiple isoforms and PTMs, are continuously translated, properly folded, associated with binding partners, shuttled to required subcellular locations and degraded as needed. A handful of proteins develop a propensity to misfold due to an inherently disordered structure, mutation or PTM. In the brain, these proteins cause the appearance of large fibrillar aggregates and neurodegeneration, leading to cognitive and motor deficits, such as dementia and dystonia. However, smaller prefibrillar oligomeric aggregates also continue to accumulate throughout disease progression. These toxic oligomers can have deleterious effects on neuronal function, including synaptic dysregulation, neuroinflammation, and the disruption of protein degradation via the UPS and autophagy. Dysregulation of neuronal function may be reflected by cognitive or motor deficits in an early phase of disease progression, prior to significant neuron loss. This could represent a window of therapeutic opportunity that
could be enabled by early diagnosis and oligomer-modifying drugs. Ongoing research towards an understanding of the structural determinants of toxic oligomers and the cellular pathways that modulate their formation will facilitate the development of new diagnostic and therapeutic strategies as well as the refinement of strategies currently in clinical trials.
<table>
<thead>
<tr>
<th>Disease</th>
<th>Misfolding or Aggregating Protein</th>
<th>Manifestation</th>
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<tbody>
<tr>
<td>Alzheimer’s Disease</td>
<td>Amyloid-β</td>
<td>Plaques, Neurofibrillary tangles</td>
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<td></td>
<td>Tau</td>
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<td>Amyotrophic Lateral Sclerosis</td>
<td>SOD-1, TDP-43, FUS, Dipeptide repeat proteins</td>
<td>Inclusion bodies</td>
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<td>Corticobasal Degeneration</td>
<td>Tau</td>
<td>Fine filamentous inclusions, glial plaques</td>
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<tr>
<td>Frontotemporal Dementia</td>
<td>Tau, TDP-43, FUS</td>
<td>Pick bodies, Inclusion bodies</td>
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<td>Huntington’s Disease</td>
<td>Huntingtin</td>
<td>Inclusion bodies</td>
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<tr>
<td>Lewy Body Dementia</td>
<td>α-synuclein, May co-occur with (Aβ and/or tau)</td>
<td>Lewy Bodies</td>
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<tr>
<td>Progressive Supranuclear Palsy</td>
<td>Tau, May co-occur with α-synuclein</td>
<td>Neurofibrillary tangles, Tau tufted astrocytes, Lewy bodies</td>
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<td>Parkinson’s Disease</td>
<td>α-synuclein</td>
<td>Lewy Bodies</td>
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<td>Familial amyloid polyneuropathies</td>
<td>transthyretin</td>
<td>Amyloid fibrils</td>
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**Figure 1.1 Toxic gain of function.** Some proteins can form oligomers that have deleterious effects on neurons and cause neurodegenerative disorders. Certain events like post-translational modification or transient unfolding of proteins can lead to self-association and soluble oligomer formation. It has been shown that these oligomers can seed the formation of long amyloid fibers. Additionally, increasing evidence supports that misfolded proteins involved in neurodegeneration, like such as tau, spread throughout the brain and propagate in a prion-like manner. Oligomers are known to directly act with key regulatory proteins throughout the brain and have been shown to increase neuroinflammation and disrupt degradation pathways, including the ubiquitin-proteasome pathway and autophagy. Lastly, oligomers can interact at the synapse to impede neurotransmission.
<table>
<thead>
<tr>
<th>Therapeutic target</th>
<th>Examples (drug – disease)</th>
<th>Clinical phase</th>
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<tbody>
<tr>
<td><strong>Active immunotherapy</strong></td>
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<td>AADvac1 – AD</td>
<td>Phase II</td>
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<td>ACI-35 – AD</td>
<td>Phase I</td>
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<tr>
<td>CAD106 – AD</td>
<td>Phase II</td>
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<td>AFFITOPE PD01A – PD</td>
<td>Phase I</td>
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<tr>
<td><strong>Passive immunotherapy</strong></td>
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<tr>
<td>Aducanumab – AD</td>
<td>Phase III</td>
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<td>Crenezumab – AD</td>
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<td>Gantenerumab – AD</td>
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<td>Immune globulin – AD</td>
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<td>Solanezumab – AD</td>
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<tr>
<td>BMS-986168 – Tauopathies</td>
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<td>C2N-8E12 – Tauopathies</td>
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<td>PRX002 – PD</td>
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<td><strong>Gene therapies</strong></td>
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<td>Intrabodies – AD, PD, HD</td>
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<td><strong>Aggregation modulating proteins</strong></td>
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<td>AZD3293 – AD</td>
<td>Phase III</td>
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<td>CNP520 – AD</td>
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<td>JNJ-54861911 – AD</td>
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<td>Verubecestat – AD</td>
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<td>Hsp90 inhibitors – AD</td>
<td>Phase III</td>
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<td><strong>Targeting misfolded proteins</strong></td>
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<tr>
<td>ALZT-OP1 – AD</td>
<td>Phase III</td>
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<td>TRx0237 – AD</td>
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CHAPTER TWO ¹:

CYCLOPHILIN 40 UNRAVELS NEUROTOXIC AMYLOIDS

2.1 Abstract

The accumulation of amyloidogenic proteins is a pathological hallmark of neurodegenerative disorders. The aberrant accumulation of the microtubule associating protein tau (MAPT, tau) into toxic oligomers and amyloid deposits is a primary pathology in tauopathies, the most common of which is Alzheimer’s disease (AD). Intrinsically disordered proteins, like tau, are enriched with proline residues which regulate both secondary structure and aggregation propensity. The orientation of proline residues is regulated by cis/trans peptidyl-prolyl isomerases (PPIases). Here we show that cyclophilin 40 (CyP40), a PPIase, dissolves tau amyloids in vitro. Additionally, CyP40 ameliorated silver-positive and oligomeric tau species in a mouse model of tau accumulation, preserving neuronal health and cognition. NMR revealed that CyP40 interacts with tau at sites rich in proline residues. CyP40 was also able to interact with and disaggregate other aggregating proteins which contain prolines. Moreover, CyP40 lacking PPIase activity prevented its capacity for disaggregation in vitro. Finally, we describe a unique structural property of CyP40 which may permit disaggregation to occur in an energy-independent manner. This study identifies a novel human protein disaggregase and, for the first time, demonstrates the capacity of dissolving intracellular amyloids.

¹Portions of this work were previously published (Baker, J.D. et al., 2017) and are used with permission of the publisher.
2.2 Introduction

Most mammalian proteins have intrinsic sequences that promote amyloid fibril formation 179. This is believed to be a natural process to prevent the formation of more toxic amorphous, intermediate structures 179,180. However, in some cases such as neurodegenerative disease, aberrant amyloid formation promotes proteotoxicity 181. Recent evidence suggests endogenous protein complexes have the ability to dissolve these potentially toxic amyloidogenic structures 20. This work has not only shed light on novel cellular pathways of disaggregation, but also suggests that these proteins could be exploited to mitigate disease pathogenesis. For example, the yeast disaggregase, Heat shock protein (Hsp)104, utilizes ATP hydrolysis to disaggregate a variety of human proteins in a yeast model 182,183. More recently, the human Hsp70/DnaJ/Hsp110 complex was shown to facilitate the disaggregation of amyloid substrates in vitro in an ATP-dependent manner 184. But the dynamic and ubiquitous nature of aggregation and disaggregation in cells suggests that a more energy-efficient process might exist, one not dependent on ATP. However, a mammalian ATP-independent amyloid unraveling enzyme has yet to be identified.

For many proteins, amyloidogenesis is significantly affected by structurally rigid proline residues 185,186, which unlike other amino acids have a more energetically favorable cis-conformation 187. This unique nature of proline residues enables major changes in protein tertiary structure through cis-trans isomerization 188. In neurodegenerative disorders, such as Alzheimer’s disease (AD) and Parkinson’s disease (PD), amyloidogenic proteins (Aβ and tau in AD and α-synuclein in PD) form stacked β-sheets 187-189 containing β-turns 190. Because proline residues, which are enriched in tau and other intrinsically disordered proteins, are frequently
found in β-turns\textsuperscript{191,191,190}, we hypothesized that enzymes capable of twisting these residues could unravel amyloids.

The family of \textit{cis/trans} peptidyl prolyl isomerases (PPIases) is diverse\textsuperscript{192}, and, perhaps most importantly, does not require the use of ATP to isomerize proline residues\textsuperscript{193}. Previous work, by our group as well as others, demonstrated PPIases regulate tau aggregation\textsuperscript{194-196}. Despite the fact that β-turns often contain prolines, amyloid disaggregation via PPIases has never been demonstrated. Thus, we speculated that discrete PPIases may disaggregate amyloids in a proline-dependent manner. Here, we describe the isomerase-dependent effects of human cyclophilin 40 (CyP40) in amyloid disaggregation. CyP40 is a member of the cyclophilin family, which along with FK-506 binding proteins (FKBPs) and parvulins, comprise a group of proteins known as immunophilins\textsuperscript{197}. Cyclophilins (CyPs) are defined by their ability to bind Cyclosporin A (CsA). Upon binding CyPs suppress the immune system through deactivation of calcineurin, a regulator of inflammatory inducing transcription factors\textsuperscript{198}. CyPs also play a key role in the folding of nascent proteins by catalyzing the \textit{cis} to \textit{trans} conformation of proline residues, a rate limiting step in proper folding\textsuperscript{199,200}. Recent work has shown that the isomerase activity of CyP40 is regulated through its interaction with Hsp90 and when CyP40 is bound to Hsp90, isomerase activity is reduced. Cellular stress can release CyP40 from Hsp90 subsequently increasing CyP40 isomerase and chaperone activity\textsuperscript{201}.

In this study, we identified a novel disaggregation mechanism. For the first time, we showed that a PPIase is capable of disaggregating amyloids. We demonstrated that CyP40 exhibits disaggregation activity dependent on interactions with proline residues and independent of co-factors such as ATP. Further, two conformational states of CyP40, found in the Protein
Data Bank, provided evidence for a potential mechanism by which amyloid binding and subsequent conformational changes in CyP40 may drive ATP-independent disaggregation activity. Transduction of CyP40 in a tauopathic brain reduced tau oligomers and tangles, yielded significant improvements in neuronal health, and preserved cognitive function. Together, these data implicate CyP40 as a potential therapeutic intervention for tauopathies and other amyloidogenic disorders.

2.3 Materials and Methods

2.3.1 Study approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All animal handling and procedures were carried out in accordance with the University of South Florida’s Institutional Animal Care and Use Committee (IACUC) in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) regulations.

2.3.2 Protein Purification

Recombinant human tau P301L, CyP40, CyP40 H141E, FKBP51, and FKBP52 were cloned into bacterial expression vectors, protein was expressed in *E.coli* BL21 cells, purified and dialyzed. Proteins were estimated to be >95% pure by coomassie staining. The details are given in the Supplemental Experimental Procedures. Amyloid Beta protein fragment 42 was purchased from Sigma Aldrich (107761-42-2), and mature α-synuclein fibrils were generated according to the established protocol by shaking the α-synuclein solution, at a concentration of 0.3 mg/ml (20.8 μM), in 10 mM HEPES buffer (pH 7.5), 100 mM NaCl.
2.3.3 Fibril formation

50 µM recombinant human tau P301L was incubated in the presence of 12.5 µM low molecular weight heparin at 37°C in 100 mM Sodium Acetate pH 7.0 buffer without agitation for 7 days. Aβ42 fibrils were formed by suspending the lyophilized protein in minimal 60 mM NaOH and then diluting to 50 µM in PBS buffer. The solution was then shaken at 700 rpm at 37°C for 5 days. Thioflavin T fluorescence was recorded throughout fibrillation in order to confirm a plateau of fluorescence.

2.3.4 Thioflavin T Fluorescence Assay

7.5 µM pre-fibrillized tau was incubated with 7.5 µM CyP40 (wt or mut where indicated) protein (in 100 µM Sodium Acetate pH 7.0 buffer for tau or PBS for α-synuclein and Aβ42) in 100 µL volumes in 96-well black clear-bottom plate (Fisher #07-200-525). At set time points (0, 1, 3 hrs), 7.5 µM Thioflavin T (final concentration) was added and fluorescence read at 440 nm excitation and 482 nm emission in a BioTek Synergy H1 plate reader. All conditions were performed in at least duplicate.

2.3.5 Transmission Electron Microscopy

10 µL of protein samples were adsorbed onto square mesh copper grids (EMS300-Cu) for 30 seconds, washed twice with 10 µL of deionized water and excess water removed. Samples were negatively stained with 4% uranyl acetate for 30 seconds and dried overnight. Grids were viewed using a JEOL 1400 Digital Transmission Electron Microscope and images were captured with a Gatan Orius wide-field camera. Fields shown are representative.
2.3.6 Nanoparticle tracking analysis

Samples were diluted 10,000 fold into 1 mL of 0.02 µm filtered deionized water. Approximately 300 µL of sample was loaded onto the Malvern Nanosight LM10 equipped with a 633 nm red laser. Protein particle data was captured with a Marlin CCD camera in duplicate. Graphs were generated by Nanosight software.

2.3.7 Circular dichroism (CD) spectroscopy

Far-UV CD measurements were taken using a JASCO J-815 spectropolarimeter. wt CyP40 and mut CyP40 were dialyzed into 10 mM sodium phosphate buffer, pH 7.5 and readings were taken at 25°C. Each spectrum is an average of 3 scans from 190-260 nm at 50 nm/min. Buffer spectral curves were subtracted for each protein.

2.3.8 Nuclear magnetic resonance spectroscopy

$^{15}$N-labeled human tau and α-synuclein proteins were expressed and purified as described previously $^{202,203}$. NMR samples contained 15 µM $^{15}$N single-labeled α-syn protein in 50 mM HEPES buffer (pH 6.8), 100 mM NaCl, 0.02% NaN$_3$ and 10% (v/v) D$_2$O or 20 µM $^{15}$N single-labeled tau protein in 50 mM phosphate buffer (pH 6.8), 0.02% NaN$_3$ and 10% (v/v) D$_2$O.

Unlabeled CyP40 and FKBP51 samples were dialyzed in the same buffers. NMR experiments of α-synuclein and tau were recorded at 15°C and 5°C, respectively on 600, 700, 800 and 900 MHz spectrometers (Bruker) equipped with cryogenic or room-temperature probes. Final concentrations of CyP40 and FKBP51 were 200 µM while tau was 20 µM. Two-dimensional $^1$H–$^{15}$N heteronuclear single-quantum coherence (HSQC) spectra were acquired in the case of α-synuclein using 256 complex points in the indirect dimension and 64 increments with spectral widths of 8417.5 and 1776.1 Hz in the $^1$H and $^{15}$N dimensions or with 512 complex points in the
indirect dimension and 40 scans with spectral widths of 8417.5 and 1705.1 Hz in the case of tau. Spectra were processed with NMRPipe and analyzed using Sparky (T. D. Goddard and D. G. Kneller, SPARKY 3, University of California San Francisco). NMR intensity ratio plots were reported with a 3-residues averaging window.

2.3.9 iHEK P301L and sarkosyl-insoluble analysis

iHEK cells expressing tau P301L for 9 days were transfected PCMV6-CyP40 (wild-type and H141E mutants) plasmids using Lipofectamine 2000 (Invitrogen). 48 h after transfection, the sarkosyl-insoluble fraction of tau was prepared as previously described. The details are given in the Supplemental Experimental Procedures.

2.3.10 Mouse studies and tissue processing

Transgenic mice overexpressing human tau P301L under a CaMKII promoter were injected with AAV9 vector at 6-months old (N = 8 [4 male, 4 female] for CyP40, N = 8 [4 male, 4 female] for GFP), and brain was harvested at 8-months old after perfusion using 0.9% saline as previously described. Hippocampi was dissection from the right hemisphere and was snap frozen for biochemical processing. The left half of the brains were fixed in 4% paraformaldehyde overnight. Sucrose gradients up to 30% were used and tissue was sectioned with a sliding microtome to 25 µm thickness. rTg4510 along with non-transgenic control mice were injected with AAV9 at 3-months of age (N = 20 [10 non-transgenic; 5 male, 5 female], [10 transgenic; 5 male, 5 female] for Cyp40, N = 18 [9 non-transgenic; 5 male, 4 female], [9 transgenic; 5 male, 4 female] for GFP). These mice were used for behavioral testing at 5-months.
2.3.11 Stereology

Neurons were stained with anti-NeuN and cresyl violet, and those positive for both were counted in the CA1 of the hippocampus. A computerized stereological system, connected to a Leica DM4000B microscope with a Prior motorized stage, was used to outline the area using distinct landmarks in the brain at 4x magnification. Neurons were counted in this region by using randomly designated areas in the computer-generated grid using a 100x oil immersion lens. Neurons were counted when they were located within the three-dimensional dissectors or touching the inclusion lines, and the top and bottom 1 μm of tissue were excluded.

2.3.12 Viral injection

Using stereotaxic equipment, mice were injected bilaterally into the hippocampus at X=±3.6 Y=-3.5 Z=+2.68. Each injection delivered 2 μL of $1 \times 10^{12}$ particles/mL of AAV9-GFP or AAV9-CyP40.

2.3.13 Immunohistochemistry and immunofluorescence

Tissue staining, imaging and quantification are described in detail in the Supplemental Experimental Procedures.

2.3.14 Radial-arm water maze

A circular black tank with a six arm metal insert was filled with water and a platform was submerged 1 cm below the surface of the water at the end of a designated goal arm. Animals were permitted 60 seconds to locate the platform, during which time an observer blind to treatment manually scored the number of errors. An error was defined as an entry into an incorrect arm or the absence of an arm choice within 15 seconds. Mice were trained over 2 days with 15 trials per day, which were divided into 5 sessions of 3 trials each.
2.3.15 Fear conditioning

Two mild foot shocks (0.5 milliamps) were paired with an auditory conditioned stimulus (CS, white noise, 70 decibels) within a novel environment. The CS was given for 30 s before each foot shock (2 s). Twenty-four hours later, mice were placed into a novel context for 3 min without CS and then exposed to the CS for 3 min (cued).

2.3.16 Intrinsic disorder analysis

The intrinsic disorder predisposition of human CyP40 (UniProt ID: Q08752) was evaluated by four algorithms from the PONDR family, PONDR® FIT \(^{208}\), PONDR® VLXT \(^{209}\), PONDR® VSL2 \(^{210}\), and PONDR® VL3 \(^{211}\), as well as by the IUPred web server with its two versions for predicting long and short disordered regions \(^{212}\). The consensus disorder propensity of human CyP40 was evaluated by averaging disorder profiles of individual predictors.

2.3.17 Statistical Analysis

Analyses were performed using GraphPad Prism version 5.02. Group differences were analyzed with Student’s t-test, one-way or two-way ANOVA as indicated in figure legends. P-values less than 0.05, 0.01, or 0.001 are marked by one, two or three asterisks (*), respectively. Error bars represent standard error of the mean (SEM). Data were examined for normal distribution and variance to determine if any datasets needed further analysis.

Sample sizes were not predetermined by statistical methods. Sample size estimates for animal studies were chosen based on previous experience with the rTg4510 mouse model. Data met assumptions for each test as analyzed by Shapiro-Wilk normality tests. Variation within groups was not significantly different as analyzed by F-test of equality of variances. Animals
were randomized into experimental groups by an investigator blinded to genotype and treatment. Researchers were blinded for tissue analysis, stereology, and mouse behavior

2.3.18 Recombinant Protein Purification

Recombinant human tau P301L, CyP40, CyP40 H141E, FKBP51, and FKBP52 were cloned into PET28A plasmids with a TEV protease sequence, and then transformed into competent *E.coli* BL21 cells and plated on kanamycin-agar plates and incubated at 37°C overnight (~16 hrs). A single colony was used to inoculate a 10 mL starter culture of LB broth supplemented with kanamycin. After 8-12 hrs, starter cultures were used to inoculate 1 L of LB-kanamycin broth and cultures were grown to an OD600 of 0.8 (~3 hrs). At this point, cultures were induced with 1 mM (final concentration) IPTG and grown a further 3 hrs. Pellets were spun down at 3500 x g for 30 min and supernatant was discarded by aspiration. Pellets were then resuspended in Nickel chromatography running buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 10 mM Imidazole) and frozen for up to 3 months at -80°C.

Pellets were then thawed on ice and subsequently lysed by sonication and spun at 50,000 x g for 1 hr. After filtration of the supernatant with a 0.02 µm filter, a standard gravity nickel column using Ni-NTA resin (Fisher #PI88222) was performed. TEV protease was then added to the elution fraction, placed into a 3,000 MW cutoff dialysis bag and into TEV buffer overnight at 4°C. The solution was then dialyzed back into nickel chromatography running buffer and a 2nd nickel purification column was ran. We then performed size exclusion chromatography using a HiLoad 16/600 Superdex 200pg column and fractions were pooled and concentrated. Proteins were then dialyzed into appropriate buffer (PBS or Sodium Acetate). Proteins were estimated to be >95% pure by coomassie staining.
2.3.19 Chymotrypsin-coupled assay

PPIase activity was measured by a chymotrypsin-coupled assay using a synthetic peptide succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (Sigma) as previously described with some modifications. Briefly, the reactions were set up in precooled 1 ml reaction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM CaCl$_2$) by adding 5 µl 1 mM peptide, 1 µl chymotrypsin (1 mg/ml, Sigma), and 0.5 µM purified protein. The absorbance was measured every 20 seconds for 10 min at 410 nm in a Genesys 10S UV-Vis spectrophotometer (Thermo) at 4°C.

2.3.20 iHEK$_{P301L}$ and sarkosyl-insoluble analysis

The tetracycline-inducible HEK293 cell line (iHEK) was generated by the insertion of human tau P301L 4R0N DNA into a pCDNA 4/TO plasmid vector (Invitrogen). The tau/TO plasmid construct was transfected into the T-REx™ HEK cell line (Invitrogen) which stably expresses the tetracycline repressor protein. After zeocin selection (200 µg/ml), the colonies were picked up and the expression of Tau was detected by Western blot with and without tetracycline (1 µg/ml). Cell lines were found to be free of mycoplasma contamination.

To investigate the effect of CyP40 on the level of aggregated, insoluble tau, tau expression was induced for 9 days and then transfected with PCMV6-CyP40 (wild-type and H141E mutants) plasmids using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. 48 h after transfection, the sarkosyl-insoluble fraction of Tau was prepared as previously described.

2.3.21 Immunohistochemistry and immunofluorescence

Tissue was stained free floating. Tissue sections were incubated in PBS supplemented with 10% MeOH and 3% H$_2$O$_2$ to block endogenous peroxidases. Following PBS washes, tissue
was permeabilized by 0.2% Triton-X-100 with 1.83% lysine and 4% serum in PBS for 30 min.
Tissue was then incubated at room temperature overnight in primary antibody. The following
primary antibodies were used: anti-CyP40 (1:2000, Pierce PA3-023), T22 (1:700, Dr. Rakez
Kayed), and anti-H150 tau (1:30000, Santa Cruz SC-5587). Following three PBS washes,
biotinylated goat anti-rabbit (Southern Biotech) secondary (1:10000) was added for 2 hrs. Prior
to peroxidase development, an ABC kit (Vectastain) was used to increase visibility. Following
PBS washes, tissue was incubated with 0.05% diaminobenzidine plus 0.5% nickel and developed
with 0.03% H₂O₂. Tissue sections were then mounted and allowed to dry overnight before
dehydration in alcohol gradients. Slides were cleared by Histoclear then coverslipped with DPX
(distyrene, plasticizer, and xylene) mountant as a synthetic resin mounting media.

Sections stained for stereology were blocked and permeabilized as described above and
incubated overnight with biotinylated NeuN (1:3000, EMD Millipore MAB377B) at room
temperature. Following PBS washes, ABC conjugation, and peroxidase development tissue was
mounted on glass slides and dried overnight. These sections were then counter-stained with
cresyl violet (nissl) by incubating with 0.05% cresyl violet briefly and then quickly destaining
with 0.3% acetic acid in water prior to dehydration.

For Gallyas silver staining, tissue slices were mounted on glass slides and dried
overnight. These slides were then incubated in a 0.003% potassium permanganate solution for
10 min. After rinsing with water the sections were incubated from 1-2 min in a 2.0% oxalic acid
solution then rinsed thoroughly in water. Slides were then incubated in a 5.0% sodium
metaperiodate solution for 5 min and again rinsed in water. Slides were then treated with an
alkaline silver iodide solution (1 M sodium hydroxide, 0.6 M potassium iodide, 0.053% silver
nitrate) for 1 min, then rinsed three times with a 0.5% acetic acid solution. Staining was developed by combining solutions A (5% sodium carbonate), B (0.024 M ammonium nitrate, 0.012 M silver nitrate, 0.003 M tungstosilicic acid), and C (0.024 M ammonium nitrate, 0.012 M silver nitrate, 0.003 M tungstosilicic acid, 0.25% formaldehyde) in a 2:1:1 ratio, adding B and C to solution A and incubating for 10-30 min. The slides were then rinsed three times in 0.5% acetic acid, then water. The slides were then incubated in gold tone for 3-4 min and again rinsed in water, then a 1% sodium thiosulphate solution for 5 min and a final rinse in water before they were dehydrated and coverslipped using DPX.

2.3.22 Tissue imaging and quantification

An Axio Scan.Z1 (Zeiss) slide scanning microscope was used to image all tissue. Bright field analysis was performed using Zeiss Neuroquant IAE analysis software. This program was used to outline regions of interest from the entire slide. Then, thresholds were set manually until only positive cells, as determined by the analyzer, were selected with as little non-specific areas selected as possible. Using the batch process option, the Area Ratio of positive cells within the regions of interest was automatically calculated for each stained group.

2.4 Results

2.4.1 CyP40 disaggregates tau amyloids

To investigate the activity of PPIases on tau fibrils, we purified three Hsp90 co-chaperones that possess PPIase activity: CyP40, FK506-binding protein (FKBP) 51, and FKBP52. Individual PPIases were incubated with fibrillized recombinant P301L tau for 3 hours, and Thioflavin T (ThT) fluorescence was measured periodically. CyP40 decreased ThT fluorescence, indicating a reduction in β-sheet secondary structure (Figure 2.1A). It should be
noted that ThT signal was not entirely ablated indicating tau species formed still maintain beta-sheet content. An additional ThT assay showed that this effect was concentration dependent, since increasing the molar ratio of CyP40 to tau fibrils further decreased ThT fluorescence (Figure S2.1). Moreover, we determined that tau did not re-aggregate after CyP40 was inactivated by Cyclosporin A (Figure S2.2). Furthermore, FKBP51 and FKBP52, despite having a more robust PPIase activity relative to CyP40 (Figure S2.3), did not lower amyloid content. This is possibly due to differential substrate binding between tau and PPIases as will be discussed later.

We then further characterized the tau species formed following incubation with CyP40. CyP40 dramatically altered the morphology and reduced the size of tau fibrils as observed by transmission electron microscopy (TEM) (Figure 2.1B). We then utilized Nanoparticle Tracking Analysis (NTA) to analyze the particle size profile of recombinant tau fibrils in the presence of CyP40. While mature tau P301L fibrils exist as an array of particles up to 850 nm in size, CyP40 co-incubated samples lacked particles greater than 150 nm in length (Figure 2.1C and 2.1D), corroborating the results of the ThT and TEM assays. CyP40 alone showed a minimal number of particles (Figure S2.4). To further confirm CyP40 disaggregase activity, we utilized an inducible tau cell model capable of forming insoluble tau. sarkosyl-insoluble tau was reduced by the overexpression of CyP40 (Figure 2.1E and 2.1F). An AlamarBlue assay showed that CyP40 was not toxic in the iHEK\textsubscript{P301L} model (Figure S2.5), confirming insoluble tau reduction was not caused by CyP40-associated toxicity. Taken together, these data indicate that CyP40 is capable of disaggregating amyloid fibrils \textit{in vitro}. 

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2.4.2  CyP40 converted tau from sarkosyl-insoluble to sarkosyl-soluble \textit{in vivo}  

The capacity of CyP40 to lower insoluble tau levels was then evaluated in a mouse model of tauopathy. Hippocampi of rTg4510 mice were injected with AAV9-GFP or AAV9-CyP40 at 6 months of age and were harvested at 8 months for biochemical and histological analyses (Figure 2.2A, Figure S2.6). These ages were chosen so that the disaggregation activity of CyP40 on insoluble tau fibrils could be investigated. CyP40 and GFP protein expression was achieved throughout the hippocampus (Figure S2.6). CyP40 was expressed 9.2x higher in mice transduced with AAV9-CyP40 compared to GFP-injected control mice (Figure S2.7). CyP40 overexpression caused a significant decreased in the amount of sarkosyl-insoluble tau, while significantly increasing soluble tau levels (Figure 2.2B and 2.2C), suggesting CyP40 disaggregated insoluble tau \textit{in vivo}.

2.4.3  CyP40 reduced pathologically relevant tau, preserved neuron viability  

To further characterize the state of tau following CyP40 overexpression, immunohistochemical analyses were conducted. Transduction of CyP40 significantly reduced the levels of Gallyas silver-positive tau tangles (Figure 2.3A and 2.3B), a hallmark of tauopathies\cite{214,215}. However, it has been suggested that soluble tau oligomers, like those recognized by T22 tau antibody \cite{216}, are more closely associated with neurodegeneration than tau tangles\cite{122,206,217,218}. To determine if the transition of tau from insoluble to soluble was producing toxic oligomers, we quantified the levels of T22-positive tau. CyP40 decreased T22-positive tau levels (Figure 2.3C and 2.3D) demonstrating that CyP40 can affect numerous multimeric tau species regardless of solubility. To determine if Cyp40 overexpression was neuroprotective, neuron counts were performed using unbiased stereology. CyP40 overexpression significantly
preserved CA1 neurons (Figure 2.3E and 2.3F), suggesting that CyP40 may disaggregate tau into non-toxic species.

### 2.4.4 CyP40 overexpression rescued tau-induced cognitive deficits

To evaluate the effects of CyP40 overexpression on learning and memory, rTg4510 and wild-type (WT) mice were injected with AAV9-GFP or AAV9-CyP40 at 3 months of age. CyP40 overexpressing rTg4510 mice showed a significant reduction in errors compared to GFP overexpressing rTg4510 mice in the two-day radial-arm water maze paradigm (Figure 2.4A). This reduction in errors suggests that spatial learning and memory is more intact in rTg4510 mice overexpressing CyP40. Significant differences were not found in CyP40 or GFP overexpressing WT mice. Additionally, CyP40 overexpression in rTg4510 mice rescued tau-induced freezing deficits in the cued fear conditioning task (Figure 2.4B). This increase in freezing is indicative of a preservation of associative learning and memory, suggesting CyP40 disaggregated existing tau fibrils or prevented the accumulation of aggregating tau prophylactically. Collectively, these data suggest that CyP40 decreased tau aggregates, reduced toxic tau oligomers, increased neuronal survival, and improved cognitive function in vivo.

### 2.4.5 CyP40 disaggregates α-synuclein but not β-amyloid

To investigate the potential for CyP40 to disaggregate amyloids other than tau, we generated recombinant fibrils of A53T α-synuclein (α-syn) and β-amyloid1-42 (Aβ42). Similar to tau, CyP40 decreased ThT fluorescence when incubated with α-synuclein fibrils (Figure 2.5A); TEM and NTA corroborated these results (Figure 2.5B, S2.8). Conversely, CyP40 was unable to disaggregate Aβ42 fibrils (Figure 2.5C and 2.5D, and Figure S2.8). Both tau and α-synuclein
contain proline residues, Aβ42 does not. This suggests CyP40 disaggregation activity is proline-dependent, thus supporting a PPIase-dependent mechanism.

2.4.6 CyP40 selectively binds proline-containing regions of tau and α-synuclein

Next, we acquired 2D heteronuclear single quantum coherence (HSQC) spectra of $^{15}$N-labeled α-synuclein and tau in the presence and absence of unlabeled CyP40. This provides a spectral signature encompassing all amide bonds within a protein, allowing us to map interaction sites by recording variations in position and/or intensity of individual peaks. Upon Cyp40 addition to α-synuclein, the residues present at the C-terminus of the protein displayed a significant intensity loss together with changes in NMR signal position (Figure 2.6 A-C). In the case of tau, a subset of signals mostly contained in the proline-rich region were attenuated with increasing CyP40 concentration (Figure 2.6 D-F), indicating that the binding between the two proteins is specific to this region. The reduction in signal intensity in protein NMR is typically caused by the combined effects of an increase in molecular weight due to complex formation, which accelerates transverse relaxation and chemical exchange at the contact surface \(^{221-223}\). The reduction in intensity seen here is consistent with an intermediate exchange time scale of CyP40-tau binding. Based on these findings, CyP40 selectively recognized proline-rich regions in both proteins. Interestingly, proline-containing β-turn structures are thought to be exposed in mature fibrils \(^{224}\), providing a possible mechanism for how CyP40 accomplishes its disaggregation function. Conversely, HSQC spectra of both proteins in the presence of FKBP51, indicate that FKBP51 does not interact with monomeric α-synuclein (Figure 2.6 G-I) and suggests that FKBP51 binds to the microtubule binding region of tau instead of proline rich domains (Figure 2.6J and 2.6K). This offers a possible explanation for the lack of FKBP51 disaggregation activity.
relative to CyP40. To further support the need for CyP40 binding to the proline rich region of tau in order to disaggregate amyloid fibrils, CyP40 was incubated with fibrillized K18 fragment of tau consisting of only the microtubule binding domain repeats. CyP40 was unable to disaggregate this substrate (Figure 2.6L).

2.4.7  CyP40-mediated disaggregation is diminished in a PPIase-null CyP40 mutant

To further explore the mechanism of CyP40-mediated disaggregation, a mutant of CyP40 lacking PPIase activity was constructed. Mutation of histidine 141 to glutamate (H141E) ablated PPIase activity as shown by a chymotrypsin-coupled assay (Figure S2.9). Importantly, the mutation did not significantly alter secondary structure of the protein as evidenced by circular dichroism (Figure S2.9). Wild-type (wt) and mutant (mut) CyP40 were incubated with tau P301L fibrils, and ThT fluorescence was recorded. Cyp40 lacking PPIase activity was unable to disaggregate tau fibrils (Figure 2.7A). Corresponding TEM showed fibril morphology was maintained following incubation with mut CyP40, while, as expected, wt CyP40 decreased fibrils (Figure 2.7B). Similarly, CyP40 PPIase activity was essential for A53T α-synuclein fibril disaggregation (Figure 2.7C and 2.7D). Collectively, these data indicate that the PPIase activity of CyP40 is necessary for disaggregation activity in vitro. These findings also rule out the possibility that the disaggregation activity of CyP40 was the result of any technical artifacts caused by production or purification of recombinant proteins.

We further explored the putative molecular mechanism of CyP40-mediated disaggregation through the analysis of its intrinsic disorder propensity and through available structural information. Structural data in the Protein Data Bank (PDB) is available only for bovine CyP40, which shares 93.78% sequence identity along with similar disorder plot profiles.
to human CyP40 (Figure S2.10) Structural analysis of bovine CyP40 revealed that this protein can be crystallized in the monoclinic and tetragonal forms. These forms differ dramatically in the spatial organization of the C-terminal domain containing three tetratricopeptide (TPR) motifs (residues 223-256, 273-306, and 307-340)\textsuperscript{226}. Figure 2.8Aa shows that although the monoclinic form is characterized by the presence of seven α-helices of variable length (residues 216-235, 239-259, 269-285, 289-300, 307-319, 323-336, and 341-362) packed in a relatively compact domain (PDB ID: 1ihg), the structure of tetragonal form (PDB ID: 1iip) is remarkably different (Figure 2.8Ab). Figure 2.8Ac shows that the structures of PPIase cyclophilin-type domain and TPR-1 motif remain mostly unperturbed. In fact, in multiple structural alignment, the RMSD for this region spanning residues 1-259 is remarkably low, 0.53 Å. On the other hand, two α-helices of TPR-2 have straightened out, and, together with the second helix of the TPR-1, form one extended α-helix (residues 239-297) that protrudes out from the protein. Furthermore, this structure does not contain coordinates for the C-terminal region of this protein (residues 298-369) which includes the TPR-3 and the C-tail containing putative calmodulin binding site\textsuperscript{226}. Since the C-terminal half of CyP40 (residues 185-370) is responsible for the chaperone activity as well as the interaction with Hsp90 and several other binding partners, it was suggested that a partially folded form of the TPR-containing domain may be related to the functionality of this protein\textsuperscript{226}. The ability of CyP40 to undergo dramatic conformational changes from more closed, monoclinic-like structure to a widely open, tetragonal-like form (see Figure 2.8A) potentially resides in the specifics of the intrinsic disorder propensity distribution within its sequence. In agreement with this hypothesis Figure 2.8B shows that regions linking the PPIase and TPR-containing domains and linkers between the individual TPR motifs are predicted to be either high
disordered (have disorder score above 0.5) or at least flexible (have disorder scores between 0.25 and 0.5), thereby providing means for high conformational flexibility. Though the exact mechanism by which CyP40 disaggregates fibrils remains unclear, this is the first demonstration of a single human protein to display disaggregate activity both in vitro and in vivo.

2.5 Discussion

In this study, we describe the disaggregation activity of the human cyclophilin, CyP40. This is the first description of a PPIase disaggregating neurodegenerative amyloids, such as tau and α-syn. This disaggregation is dependent on the PPIase activity of CyP40 and is ATP-independent. The distinct structural properties of CyP40 may enable this ATP-independent process to occur, and thus may be unique to CyP40 among other PPIases. Disaggregation of tau fibrils by CyP40 reduced pathogenic tau, concomitant with decreased neuronal toxicity and increased cognitive performance.

While being the first human PPIase to display disaggregation activity, CyP40 is not the first disaggregate to be identified. Certain chaperone complexes have been shown to facilitate the disaggregation of oligomers and fibrils \(^{20,225,227}\). For example, the Hsc70/DNAJB1/Apg2 containing complex was shown to reverse the formation of α-syn fibrils in vitro. Identification of this complex was notable because, while previous attempts at chaperone-mediated amyloid disassembly required weeks of incubation in vitro \(^{190}\), this complex solubilized α-syn fibrils in under an hour \(^{20}\). Disaggregation by the Hsc chaperone complex required ATP, however, our study demonstrated that fibrils could be disaggregated with similar kinetics by CyP40 in the absence of ATP. Regardless of energy dependence, the existence of amyloid disaggregases presents a new avenue for therapeutic strategies. The pro-cognitive effects of CyP40
overexpression in the tauopathic brain suggests that strategies to either induce or deliver disaggeregases to the central nervous system could halt or even rescue cognitive deficits associated with neurotoxic amyloids.

We hypothesize two possible mechanisms for amyloid disaggregation by CyP40. Based on our structural data (Figure 2.8A), it is possible that the PPIase domain interacts with specific proline residues in β-turns and twists these residues. Given that CyP40-mediated disaggregation occurs without the input of external energy, it is possible that the disaggregation reaction energy comes from the PPIase domain of CyP40 binding to key residues within the amyloid substrate followed by a conformational switch within the TPR domain (Figure 2.8C). There is precedent for disaggregation independent of external energy as described for the plant chaperone, cpSRP43. Due to the equimolar amount of substrate and CyP40 used in the ThT-monitored disaggregation experiments an alternative mechanism could be proposed. In this hypothesis, CyP40 may interact with and sequester small oligomers and monomers away from the aggregation equilibrium (see Figure 2.8D). However, this hypothesis does not explain the lack of disaggregation activity in the PPIase-null CyP40. Therefore, further experiments are required to determine the precise mechanism by which CyP40 disaggregation occurs.

Though CyP40 can directly interact with Hsp90 through a TPR domain, the effects described here do not appear to be Hsp90-dependent. In fact, the PPIase activity of CyP40 is reduced upon interaction with Hsp90. This may explain that while CyP40 is expressed in our models of tauopathy, tau fibrils were only disaggregated upon the overexpression of CyP40; potentially providing a pool of free CyP40 to interact with amyloids. In addition to CyP40, there are currently 41 known human PPIases within the cyclophilin, FKBP and parvulin families.
Therefore, future screening may reveal additional PPIases with activities similar to CyP40, including ATP-independent disaggregation. Additionally, CyP40 and other PPIases should be further characterized for disaggregation activity against proline-containing amyloids, especially those associated with disease.
Figure 2.1 CyP40 disaggregated tau fibrils. (A) Preformed tau fibrils were monitored for Thioflavin T (ThT) fluorescence intensity over 4 h. CyP40, FKBP51, FKBP52 were added at 1 h and monitoring continued for 3 h (bars represent SEM, unpaired t-test, p = 0.0446, n=2 independent protein preparations). (B) Representative 60,000x transmission electron microscopy (TEM) images of tau fibrils or CyP40-treated fibrils (scale bar 400 nm, n=2 independent preparations of recombinant protein). (C) Nanoparticle tracking analysis (NTA) size distribution of tau fibrils and CyP40-treated tau fibrils. (D) Representative images of particles from the NTA. (E) Inducible HEK cells overexpressing tau P301L (iHEK_P301L) were induced with tetracycline on day 1 and transfected with either control vector or CyP40 on day 9. Sarkosyl-soluble and insoluble fractions were collected at day 11. Sarkosyl-insoluble fractions were probed by Western blot with Tau 12 antibody. Sarkosyl-soluble fractions were probed by Western blot with H-150 anti-tau, anti-CyP40 and anti-GAPDH antibodies. (F) Quantification of insoluble tau levels on day 11 (unpaired t-test, n=2 independent experiments).
Figure 2.2 CyP40 overexpression decreased sarkosyl-insoluble tau. (A) Schematic depicting the timeline of rTg4510 tau pathology and experimental design. (B) Western blot comparing sarkosyl-insoluble and soluble fractions from hippocampi of AAV9-GFP (n = 8) and AAV9-CyP40 (n = 8) injected rTg4510 mice. Each lane indicates an individual transgenic mouse. (C) Quantification of the relative insoluble and soluble tau for AAV9-GFP and AAV9-CyP40 injected mice (unpaired t-test, n = 8 for each group).
Figure 2.3 CyP40 reduced tau deposits and preserved hippocampal CA1 neurons in vivo
(A) Representative images of Gallyas silver-stained hippocampi from AAV9-GFP and AAV9-CyP40 injected mice (scale bar 200 µm). (B) Quantification of Gallyas silver-staining (unpaired t-test, n=8 for each group) (C) Representative images of T22-oligomeric tau stained hippocampi from AAV9-GFP and AAV9-CyP40 injected mice (scale bar 200 µm). (D) Quantification of T22-oligomeric tau staining (unpaired t-test, n=8 for each group) (E) Representative images of CA1 neurons stained with NeuN (brown) and Nissl (violet) are shown (scale bar 125 µm). (F) Neuron counts from unbiased stereology of CA1 neurons in hippocampal sections from AAV9-GFP and AAV9-CyP40 injected mice (unpaired t-test, n=8 for each group).
Figure 2.4 CyP40 rescued tau-induced cognitive deficits (A) Radial-arm water maze spatial memory analysis of AAV9-GFP injected wildtype (wt) (n=9), AAV9-CyP40 injected wt (n=10), AAV9-GFP injected rTg4510 mice (n=9), AAV9-CyP40 injected rTg4510 mice (n=10). Data were analyzed by two-way ANOVA and significant results were followed by Tukey post hoc tests (*p < 0.05). (B) Percent freezing in the cued fear conditioning task (n=5 for each group). Data were analyzed by two-way ANOVA and significant results were followed by Tukey post hoc tests (*p < 0.05).
Figure 2.5 CyP40 disaggregated α-synuclein, but not Aβ42 fibrils. (A) ThT fluorescence of A53T α-synuclein (α-syn) fibrils ± CyP40 protein (unpaired t-test, n=4). (B) Representative TEM images of α-synuclein A53T fibrils ± CyP40 (60,000x magnification; scale bar 400 nm) (C) ThT fluorescence of Aβ42 fibrils ± CyP40 protein (unpaired t-test, n=4). (D) Representative TEM images of Aβ42 fibrils ± CyP40 (60,000x magnification; scale bar 400 nm).
Figure 2.6 CyP40 binds proline-containing regions of tau and α-synuclein. (A) A selected region of the 2D [1H-15N]-HSQC experiment of 15N-labeled α-synuclein before (blue) and after (red) addition of CyP40 (molar ratio 1:15). Y136 is marked. (B) Normalized residue-specific NMR intensities in the presence of a 5-fold (red) and 15-fold (blue) excess of CyP40; the location of proline residues is indicated. (C) Combined [1H-15N] chemical shift perturbation analysis upon addition of CyP40 to α-synuclein. (D) A selected region of 2D [1H-15N]-HSQC experiment of tau before (blue) and after (red) addition of CyP40 (molar ratio 1:10) is shown. Residues displaying significant intensity loss are labeled. (E) Normalized residue-specific NMR intensities of tau in the presence of a 5-fold (red) and 10-fold (blue) excess of CyP40; tau’s domain organization is shown and proline residues are marked with red. (F) Amino acid sequence of the proline-rich regions P1 (top) and P2 (bottom); residues significantly broadened upon addition of CyP40 are colored red. (G) A selected region of the 2D [1H-15N]-HSQC experiment of 15N-labeled α-synuclein before (blue) and after (red) addition of FKBP51 (molar ratio 1:10) is displayed. (H) Normalized residue-specific NMR intensities in the presence of a 5-fold (red) and 10-fold (blue) excess of FKBP51. (I) Combined [1H-15N] chemical shift perturbation analysis upon addition of FKBP51 to α-synuclein. (J) Selected region of a 2D [1H-15N]-HSQC experiment of tau before (blue) and after (red) addition of FKBP51 (molar ratio 1:10). Final concentrations of CyP40 and FKBP51 for NMR experiments are 200 µM. (K) Tau residue-specific NMR intensities in presence of a 10-fold excess of FKBP51. (L) Thioflavin T fluorescence of K18 tau aggregates in the presence or absence of CyP40 (unpaired t-test, p > 0.05, n=3).
Figure 2.7 CyP40-mediated disaggregation assay of wt and PPIase null mutant CyP40 (A) Thioflavin T (ThT) fluorescence of tau ± wt CyP40 or mut CyP40 (unpaired t-test, n=3 for each condition). (B) Representative transmission electron microscopy (TEM) images of (A) (scale bar 200 nm). (C) Thioflavin T (ThT) fluorescence of A53T α-synuclein (α-syn) ± wt CyP40 or mut CyP40 (unpaired t-test, n=3 for each condition). (B) Representative TEM images of (C) (scale bar 200 nm).
Figure 2.8 CyP40 conformation, disorder prediction, and putative disaggregate mechanisms. (A) Structural characterization of bovine CyP40: a, Crystal structure of the monoclinic form of CyP40 (PDB ID: 1ihg); b, Crystal structure of the tetragonal form of CyP40 (PDB ID: 1iip). Structures in plots a and b are colored according to the secondary structure content; c, Multiple structural alignment of the monoclinic (blue) and tetragonal (red) forms of CyP40 conducted by MultiProt platform. (B) Evaluating intrinsic disorder propensity of human CyP40 (UniProt ID: Q08752) by series of per-residue disorder predictors. Disorder profiles generated by PONDR® VLXT, PONDR® VL3, PONDR® VSL2, IUPred_short, IUPred_long, and PONDR® FIT are shown by black, red, green, yellow, blue, and pink lines, respectively. Light pink shadow around the PONDR® FIT shows error distribution. Cyan shaded area shows position of the region with chaperone activity. Positions of three TPR motifs are shown as gray shaded areas. In these disorder analyses, the predicted intrinsic disorder scores above 0.5 are considered to correspond to the disordered residues/regions. (C) Schematic representation of hypothesized enzymatic activity-based CyP40 disaggregation. (D) Schematic representation of hypothesized interaction-based CyP40 disaggregation.
Figure S2.1 Ratio-dependent CyP40 tau disaggregation. Incubations of increasing molar ratios of CyP40 to tau fibrils, as indicated, were monitored by Thioflavin T fluorescence. Samples were run in triplicate (n = 3).
Figure S2.2 Inactivation of recombinant CyP40 with cyclosporin A (CsA) after disaggregation of tau fibrils. Cyclosporin A (dashed line) or DMSO (solid line) was administered to tau fibrils in the presence (red) or absence (black/grey) of CyP40 at 16 hours. Samples were run in duplicate (n = 2).
Figure S2.3 Chymotrypsin-coupled PPIase activity assay. Curves represent No Enzyme (black), CyP40 (red), FKBP51 (blue), FKBP52 (purple) incubated with chymotrypsin (6mg/mL, pH 8.0) and substrate (Suc-AAPF-pNA, 100uM) over 300s. (One-way ANOVA, p < 0.0001, n = 2 independent preparations).
Figure S2.4 Nanoparticle tracking analysis of CyP40. Nanoparticle tracking analysis assay of buffer (green), 7.5uM CyP40 (red), and 15uM CyP40 (orange).
Figure S2.5 CyP40 did not reduce cell viability. iHekP301L<sub>P301L</sub> cell viability was monitored using an AlamarBlue assay following CyP40 (red) or vector (black) transfection ± tau induction by tetracycline. Results are expressed relative to vector without tau induction. Samples were run in triplicate (n = 3).
Figure S2.6 AAV9 hippocampal injections and expression. (A) AAV9-GFP (green) or AAV9-CyP40 (red) injection locations within the hippocampus are indicated. Hippocampal regions are denoted, CA1, CA2, CA3, and Dentate Gyrus (DG). (B) A representative images of AAV9-GFP and CyP40 expression in hippocampi 2 months post-injection (scale bar 200 μm).
Figure S2.7 Expression levels of CyP40 protein from individual mouse brain lysates. Western blot analysis of CyP40 expression in AAV9-GFP and AAV9-CyP40 injected mice are compared to a standard curve generated with recombinant CyP40 protein (ng quantities indicated). Each lane represents an individual mouse. Western blot probed with anti-CyP40 antibody.
Figure S2.8 Nanoparticle analysis of α-synuclein and Aβ42 fibrils after CyP40 co-incubation. (A) Nanoparticle tracking analysis size distribution of A53T α-synuclein fibrils ± CyP40. (B) Representative images of (A). (C) Nanoparticle tracking analysis size distribution of Aβ42 fibrils ± CyP40. (D) Representative images of particles of (C).
Figure S2.9 Nanoparticle analysis of α-synuclein and Aβ42 fibrils after CyP40 co-incubation. (A) Coupled chymotrypsin assay of isomerase activity of wt CyP40 (red), mut CyP40 (teal), and No Enzyme (black) (n = 2 independent preparations). (B) Circular dichroism of wt CyP40 (red) and mut CyP40 (teal).
Figure S2.10 Nanoparticle analysis of α-synuclein and Aβ42 fibrils after CyP40 co-incubation.

(A) Evaluating intrinsic disorder propensity of human CyP40 (UniProt ID: Q08752) by a series of per-residue disorder predictors. (B) Evaluating intrinsic disorder propensity of bovine CyP40 (UniProt ID: P26882). In these plots, disorder profiles generated by PONDR® VLXT, PONDR® VL3, PONDR® VSL2, IUPred_short, IUPred_long, and PONDR® FIT are shown by black, red, green, yellow, blue, and pink lines, respectively. Light pink shadow around the PONDR® FIT shows error distribution. (C) Comparison of the mean disorder propensity of human black solid curve) and bovine CyP40 (red dashed curve). In plots A, B, and C, cyan shaded area shows position of the region with chaperone activity. Positions of three TPR motifs are shown as gray shaded areas. In these disorder analyses, the predicted intrinsic disorder scores above 0.5 are considered to correspond to the disordered residues/regions. (D) Pairwise sequence alignment of human and bovine CyP40 proteins (UniProt IDs Q08752 and P26882, respectively). Identical residues are indicated by star symbol, whereas colon and period symbols show similar residues. Sequences are colored according to the major physic-chemical properties of their residues, with red and green symbols corresponding to hydrophobic and polar residues, respectively, and with positively and negatively charged residues shown by pink and blue symbols, respectively.
CHAPTER THREE:
FUTURE STUDIES AND FINAL CONSIDERATIONS

3.1 Introduction

Although the previous study demonstrating the disaggregase capacity of cyclophilin 40 (CyP40) is exciting, further work is needed to answer several key questions. This chapter will discuss a number of continuing studies designed to provide mechanistic insight into CyP40 disaggregation, provide putative strategies to increase the disaggregase activity of CyP40, to evaluate the end products of CyP-40 mediated disaggregation, and finally will discuss the challenges in moving forward with therapeutic CyP40-mediated tau reduction.

3.2 Mechanistic questions

CyP40 belongs to an immunophilin subfamily known as cyclophilins. In an effort to determine if disaggregase activity was unique to CyP40, we performed an in vitro assay measuring thioflavin T fluorescence over a 16-hour time period for several cyclophilins incubated with mature tau fibrils (Figure 3.1). Our data suggests that CyP40 disaggregation activity is unique within the cyclophilin 40 family, as it was the only cyclophilin able to disaggregate tau fibers and maintain lowered amyloid content over an extended timeframe. Interestingly, PP1E, another prolyl-isomerase reduced amyloid content of tau fibrils, but only briefly. This suggests that this enzyme may disaggregate fibrils into unstable oligomers which readily reaggregate. Further follow-up studies including electron microscopy, size-exclusion chromatography and standard toxicity assays are needed to elucidate end product differences.
resulting from disaggregation by these two prolyl-isomerases. Additionally, data from Alzheimer’s patients reveals that CyP40 may have a physiological role in AD, as mRNA levels are lower in AD brains compared to non-demented controls, specifically in the entorhinal cortex (Figure 3.2).

As of now, we have little insight into the mechanism of CyP40-mediated tau disaggregation. Our screen of cyclophilins provides evidence that the prolyl-isomerase activity of CyP40 is not sufficient to disaggregate amyloid substrates, as PPIA, which consists of solely the prolyl-isomerase domain of CyP40 is devoid of disaggregase activity. To this end, we propose generating a series of constructs consisting of CyP40 truncations in order to pin-point domains required for disaggregation. CyP40 is composed of an N-terminal PPIase domain, a short linker domain, and finally a TPR domain that allows interaction with Hsp90. We will generate constructs consisting only of the prolyl-isomerase domain of CyP40 with or without the linker domain as well as the C-terminal TPR domain as illustrated in Figure 3.3. We will then test the disaggregase propensity for each of these constructs using previously discussed assays including thioflavin t fluorescence, electron microscopy, and human cell models. Further studies will be required to determine enzymatic residues responsible for disaggregation within associated protein domains.

Additionally, although our work described the activity of CyP40 with mature tau fibers, we have not demonstrated its affinity for different aggregation states of tau including oligomers. To this end, we will incubate tau oligomers with CyP40 in order to determine the interaction potential as well as to assess CyP40 effect on oligomeric toxicity. This will be evaluated by
externally applying CyP40-treated or untreated oligomeric tau to neuronal cells in vitro and by measuring toxicity through standard assays including LDH enzymatic activity.

Finally, as of now, we do not know the tau fiber residues required for CyP40-mediated disaggregation. To answer this question, we will mutate key prolines within the aggregation and proline rich domains of tau (Table 3.1) and test for CyP40 binding by NMR-analysis and disaggregation activity by thioflavin t fluorescence and electron microscopy.

3.3 Engineering a better disaggregase

A future goal of these studies is to engineer a powerful disaggregase capable of therapeutic applications in neurodegenerative disorders including Alzheimer’s disease and other tauopathies. We will therefore attempt to increase disaggregation activity by generating chimeric constructs consisting of various prolyl-isomerase domains of other cyclophilins (See Figure 3.3) and testing for disaggregase activity as previously described in this manuscript. A chymotrypsin-coupled assay measuring prolyl-isomerase activity shows that cyclophilin family members present differing enzymatic activity (Figure 3.4), so this recombination may impact disaggregation. Finally, as an alternative approach, random mutagenesis of CyP40 along with a high-throughput disaggregation screen may be invaluable in increasing enzymatic activity or substrate recognition of the disaggregase. The therapeutic challenge for CyP40 arises from a need to upregulate its activity specifically in brain tissue. Although gene therapy capable of delivering an enhanced enzyme directly is possible, recent work has shown that when not bound to Hsp90, CyP40 activity is increased. To this end, we propose screening for small molecules specifically capable of inhibiting the interaction of CyP40 and Hsp90, and to follow up a series of studies for inhibitor toxicity and in vivo and in vitro assays of disaggregation within inhibitor treatment.
3.4 Overall conclusions

Work within this manuscript has provided strong evidence for CyP40 as a disaggregase with substrate recognition toward amyloid substrates including tau and α-synuclein. The major findings presented herein are that CyP40 disaggregates mature tau fibers \textit{in vitro}, as shown by multiple methods including thioflavin T fluorescence, transmission electron microscopy, and nanoparticle tracking analysis. Furthermore, for the first time, overexpression of this disaggregase was shown to reduce insoluble tau in a human cell model of tauopathy. Finally, a major discovery presented here is the capacity for CyP40 overexpression to decrease insoluble tau load, preserve neurons, and rescue behavioral deficits in a mouse model of tauopathy.
Figure 3.1 A disaggregate assay of various cyclophilins.
Figure 3.2 CyP40 mRNA levels in entorhinal cortex and hippocampus in young, aged, and Alzheimer’s disease brains.
Figure 3.3 CyP40 truncations and chimeras
**Figure 3.4** Prolyl-isomerase enzymatic activity assay.
Table 3.1 Tau Proline Mutations

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Appendix B: IACUC Approval

MEMORANDUM

TO:    Chad Dickey, Ph.D.

FROM:  Farah Moulu, MSPH, IACUC Coordinator
        Institutional Animal Care & Use Committee
        Research Integrity & Compliance

DATE:  11/28/2016

PROJECT TITLE:  The Hsp90 Co-chaperone FKBP51 Regulates Tau Structure and Function
                 Investigating the role of Odc37 in AD pathogenesis

FUNDING SOURCE:  National Institute of Neurological

IACUC PROTOCOL #:  R:500003103

PROTOCOL STATUS:  APPROVED

The Institutional Animal Care and Use Committee (IACUC) reviewed your application requesting the use of animals in research for the above-entitled study. The IACUC APPROVED your request to use the following animals in your protocol for a one-year period beginning 11/28/2016.

Mouse:  Tg152 (1-14 months / Male and Female)

Please take note of the following:

- IACUC approval is granted for a one-year period at the end of which, an annual renewal form must be submitted for years two (2) and three (3) of the protocol through the eIACUC system. After three years all continuing studies must be completely re-described in a new electronic application and submitted to IACUC for review.

- All modifications to the IACUC Approved Protocol must be approved by the IACUC prior to initiating the modification. Modifications can be submitted to the IACUC for review and approval as an Amendment or Procedural Change through the eIACUC system. These changes must be within the scope of the original research hypothesis, involve the original species and justified in writing. Any change in the IACUC-approved protocol that does not meet the latter definition is considered a major protocol change and requires the submission of a new application.

- All costs invoiced to a grant account must be allocable to the purpose of the grant. Costs allocable to one protocol may not be shifted to another in order to meet deficiencies caused by overruns, or for other reasons convenience. Rotation of charges among protocols by month without establishing that the rotation schedule credibly reflects the relative benefit to each protocol is unacceptable.