TAUOPATHIES, NOVEL OPTOGENETIC TOOLS, AND THE FUTURE OF ARTIFICIAL INTELLIGENCE IN MEDICINE.

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TAUOPATHIES, NOVEL OPTOGENETIC TOOLS, AND THE FUTURE OF ARTIFICIAL INTELLIGENCE IN MEDICINE.

by

JESSICA LEE BINDER

B.S., University of New Mexico, 2013

DISSEPTION

Submitted in Partial Fulfillment of the Requirements for the Degree of

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The University of New Mexico Albuquerque, New Mexico

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TAUOPATHIES, NOVEL OPTOGENETIC TOOLS, AND THE FUTURE OF ARTIFICIAL INTELLIGENCE IN ALZHEIMER’S DISEASE.

by

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DISSERTATION

Doctor of Philosophy Biomedical Sciences

Abstract

To this day, there is no cure for Alzheimer’s disease (AD) and related dementias (ADRD). With the daunting rise at an exponential rate of ADRD burden and related deaths, the necessity to find a new line of attack is vital. Pathological accumulation of microtubule associated protein tau in neurons is a major neuropathological hallmark of Alzheimer’s disease (AD) and related tauopathies. Attempts have been made to promote clearance of pathological tau (p-Tau) from neurons via autophagy. Transcription factor EB (TFEB) has shown to clear p-Tau from neurons via autophagy. However, sustained TFEB activation and autophagy can create burden on cellular bioenergetics and can be deleterious. Here, we engineered previously described two-plasmid systems of Light Activated Protein (LAP) from bacterial transcription factor – EL222 and Light Responsive Element (LRE) to encode TFEB. Upon blue-light (465nm) illumination, the conformation changes in LAP induced LRE-driven expression of TFEB, its nuclear entry,
TFEB-mediated expression of autophagy-lysosomal genes and clearance of p-Tau from neuronal cells and AD patient-derived human iPSC-neurons. Turning the blue-light off reversed the expression of TFEB-target genes and prevented p-Tau clearance. Together, these results suggest that optically regulated TFEB expression unlocks the potential of opto-therapeutics to treat AD and other dementias.
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Chapter 1: Introduction Part I: All things Tau

1.1 Overview of Alzheimer’s Disease

In 1907, a German psychiatrist and pathologist, who went by the name Alois Alzheimer, published his landmark paper about one of his patients. Auguste D, a 51-yr-old patient at the Frankfurt mental hospital, who displayed severe dementia. It was upon her post-mortem autopsy that he described morphologically and histologically to what is now known as senile plaques and neurofibrillary tangles (Hippius and Neundörfer). Over a hundred years later, the cause to the pathology of Alzheimer’s disease (AD) is still unknown, and yet it is the 6th leading cause of death in the United States, costing over a $280 billion every year (source: Alzheimer’s Association).

There are several genetic mutations that cause AD (termed Early-onset Familial Alzheimer disease (EOFAD)). Studies have shown that single point mutations in APP, PSEN1, or PSEN2 genes are sufficient to predispose certain individuals develop EOFAD. However, the prevalence of EOFAD is considerably less than spontaneous/sporadic AD. On the other hand, many studies have associated genes wherein certain allelic polymorphisms can predispose individuals to develop Late-onset Alzheimer’s disease (LOAD). For example, variants of APOE ε4 or TREM2 are with highest risk of predisposing LOAD. While the prevalence of sporadic LOAD is more common, these relative risks are less severe than EOFAD.

Complex genetics coupled with various co-morbid risk factors including aging, makes AD and related dementia as one of the complex and perplexing diseases of our time. Because of this, there is no preventative or therapeutic remedies till date. Recently, a new theory of omnigenetics suggests the risk of certain diseases are not a subset of
genetic variants or a collection of a few up/down-regulated genes, but the work of hundreds to thousands of genetic variants and de/activated genes working in concert. The more we look at multi-etiologies of AD, finding one gene as the critical/sole player to disease pathology is profoundly likely be false. The consensus seems the pathology is derived from various combinations of genes and environment factors.

Currently, two major pathological features in the brain characterize AD: The aggregation of amyloid beta (Aβ), forming into senile (or amyloid) plaques outside of cells; and the accumulation of aberrant form of the tau protein called ‘neurofibrillary tangles’ (NFTs) inside neurons. For decades, the AD research leaned heavily on targeting extracellular aggregated plaques. Unfortunately, many if not all recent amyloid focused clinical trials have not been successful.

Thus, the AD field is headed in finding new drug targets to develop therapeutic intervention against AD and related dementias.

1.2 Neurodegenerative tauopathies

Tauopathies are driven by abnormalities in microtubule-associated protein tau (MAPT). As the name suggests, its primary function is known to promote nucleation and stabilization of microtubules (MTs), as well as neuronal growth and function. MAPT or tau is predominantly found along the axons of neurons and constitutes over 80% of the neuronal MAPs. Tau has six different isoforms in adult human brain expressed via alternative splicing of single mRNA and play unique roles in various neuronal functions. When post-translational modification of tau occurs, it is thought to be the beginning stages of neurodegenerative tauopathies.
Phosphorylation of tau is one of the very well-studied post-translational modifications and known to affect tau’s ability to bind to microtubules and stabilize their structure\textsuperscript{13}. Studies have suggested that tau phosphorylation is a normal physiological process to regulate different extent of tau-MT interactions during various stages of central nervous system (CNS) development\textsuperscript{11}. For example, during early development, when the neurons are still establishing network connections, it is important to keep the microtubule in a dynamic stage and it is achieved via keeping the tau hyperphosphorylated on multiple different residues\textsuperscript{14}. As the CNS develops, phosphorylation of tau on majority of serines, threonines and tyrosines are significantly reduced to maintain axonal microtubules in the most stable conditions. However, tau seems to undergo extensive hyperphosphorylation on almost all of its phospho-sites during disease conditions, including AD and related tauopathies as well as upon brain trauma following concussions or traumatic brain injuries\textsuperscript{15}. Strikingly, studies have shown that the tau phosphorylation during disease condition, occur in a priming manner. That means, for certain serine/threonine variants in the C-terminal half of protein (For example Ser396/Ser404 – recognized by the antibody PHF-1) to occur, it is essential to have phosphorylation of certain N-terminal residues (for example Ser199/Ser202/Thr205 – recognized by the antibody AT8 and Thr231 – recognized by the antibody AT180) first\textsuperscript{16,17}. Furthermore, recent studies have suggested that excessive hyperphosphorylation of tau on these residues could permanently alter tau’s conformation to facilitate tau aggregation, assembly into paired helical filaments (PHFs) and eventually into neurofibrillary tangles (NFTs). For example, increasing evidence from Lu’s and other groups have observed a phenomenon called ‘cistauosis’ where Thr231 hyperphosphorylated tau undergoes from
‘trans’ conformation into pro-aggregation ‘cis’ conformation that can lead to seeding and assembly of tau into NFTs\textsuperscript{18}. Therefore, assessing the phosphorylation of tau on AT8, AT180 and PHF-1 residues are important determinants of early disease events relevant to tauopathies.

Other examples of neurodegenerative tauopathies include those which are sporadic: 1) Progressive Supranuclear Palsy (PSP), 2) Corticobasal Degeneration (CBD), 3) Tangle-Predominant Senile Dementia (TPSD), 4) Pick’s disease (PiD) and 5) Frontotemporal Lobar Dementia – Tau (FTLD-Tau) or familial (Frontotemporal Dementia and Parkinsonism Linked to Chromosome – 17 tau type (FTDP-17T), which includes over 30 different intronic and exonic disease-causing mutations in \textit{MAPT}, such as a well-studied P301L mutation in the second microtubule binding repeat of tau and known to cause fronto-temporal dementias\textsuperscript{19}. The mutation P301L lies near the hexapeptide motifs and increases tau’s tendency to aggregate\textsuperscript{19}. Thus, the accumulation of the aberrant forms of tau is essentially the basis of neurodegenerative tauopathies.

\textbf{1.3 Current Tauopathy models}

In order to conduct fundamental research, it is imperative to use a disease-relevant model for better understanding of mechanisms driving pathogenesis. Alternatively, certain molecular changes can also be assessed in post-mortem brain tissue of neurodegenerative tauopathy. While the post-mortem tissue has been utilized by many studies to gain important insights into the transcriptomics, protein analysis and genome-wide association studies (GWAS), due to varying post-mortem intervals coupled with different independent variables and co-morbid conditions, it requires a large group size to
make any meaningful conclusions. Albeit, these studies illuminated on our understanding on the hyperphosphorylation of tau, tau oligomerization NFT burden associated with AD, leading to establishment of Braak staging and post-mortem diagnosis (PMID: 29228201). Various cell lines (in vitro) and mouse models (in vivo) have also been generated in efforts to recapitulate the pathology of tauopathies. 

Neuroblastoma cell lines (mouse and human neuroblastoma cells called Neuro 2a or N2a, and SH-SY5Y, respectively) are commonly used as in vitro models. Our group and others have designed tauopathy in vitro models that involve SH-SY5Y’s or N2a’s overexpressing human tau via P301L mutation, phosphorylation-mimicking mutations, cis/trans tau, or with inflammation-inducible phosphorylation of Tau. We have utilized inflammation-induced phosphorylation of tau models by treating murine N2a or human SHSY5Y cells, expressing human wild-type or mutant tau, with condition media (CM) derived from lipopolysaccharide (LPS)-primed mouse RAW 264.7 macrophage or BV-2 cells (murine neonatal microglia that were raf/myc-immortalized).

![Diagram](image)

**Figure 1.1** | Schematic of inflammation-induced MAPT hyperphosphorylation in neuronal cells.

More recently, induced pluripotent stem cells (iPSCs) have made streamline of disease models, specifically in neurogenesis or neurodegenerative diseases. The exquisiteness of using iPSCs is that when they are differentiated into the cells of interest,
they display the same phenotype characteristics their donor, thus representing a relevant *ex vivo* disease model when the iPSCs are obtained from a person with the disease\textsuperscript{29}. iPSC-based models hold tremendous potential for the study of human development and disease. iPSC-derived cells are providing a physiologically relevant model for drug discovery, cell therapy validation, and disease research. Previous studies have utilized AD patient-derived iPSCs to study various mechanisms of the disease\textsuperscript{30}.

Since 1995, many *in vivo* mouse models for tauopathies have been generated\textsuperscript{31,32,33}. These include cDNA-based model where the human tau (*MAPT*) transgene with or without disease-relevant mutations (e.g. FTDP-17) are driven by the promoters specific to neurons\textsuperscript{34}. In addition, there are genomic mouse models where the human *MAPT* is driven under the control of endogenous human *MAPT* promoter\textsuperscript{35}. The benefits of using genomic mouse models over cDNA models are that the transgene is not overexpressed beyond their normal physiological level and therefore can be more relevant to human disease conditions. Nonetheless, most of these disease animal models do not exhibit complex disease pathologies but rather a small fraction thereof. More specifically, it is challenging to recapitulate disease pathology when the initiation of the disease is still unknown. Furthermore, animal’s epigenetics regulation of human transgene may become a confounding factor and can make the interpretation difficult. Our group has investigated in various tauopathy mouse models, for example, a genomic hTau\textsuperscript{MaptKO(Duke)} model (where human *MAPT* is driven by the endogenous human *MAPT* promoter and therefore expresses all six different isoforms of human tau in the endogenous mouse *Mapt* background\textsuperscript{36}). By performing whole genome gene-expression analysis in the hippocampus, we have uncovered that the hTau\textsuperscript{MaptKO(Duke)} mice show
altered levels of a novel regulator of taupathy called methyl CpG binding protein 2 (MeCP2) – a neuronal protein which has been shown to play an important role in Rett Syndrome\textsuperscript{35} (discussed in Appendix A). In addition, we have also utilized a doxycycline regulatable mouse model of tauopathy, rTg4510, which express human tau carrying P301L FTDP-17 tau mutation, which can be suppressed by treating mice with doxycycline\textsuperscript{37}. We have shown that suppression of mutant P301L human tau can reduce neuroinflammatory responses in the brain by showing reduced expression of several makers in the IL-1β signaling pathway (discussed in Appendix B). However, AD mammalian models can only unravel few characteristics of the disease. Altering one or two genes in an entire system will very unlikely provide a complete picture on the impact of human transgene overexpression on the disease pathogenesis. Therefore, it is difficult to corroborate any conclusion from these models. Nevertheless, it is essential and necessary to investigate these types of models, as the results have revolutionized our understanding of various diseases. Moreover, with the vast number of research published, assembling these individual studies and their results together would provide more insight into disease pathologies. Intergrating our understanding arising from various types of cell/animal models to gain better insights into the patho-etioloogy of tauopathies, which will eventually help us develop new preventive therapies and cures.

\subsection*{1.4 Tauopathy Interventions}

Unfortunately, there is no way to prevent or cure AD and related tauopathies at this time. On the pharmacological level, cholinesterase-inhibitors (ChEIs)\textsuperscript{38}, N-methyl-d-aspartate (NMDA) antagonist memantine, or lithium and propentofylline (PPF) have
been approved by the FDA for treatments to AD. However, all of them provide symptomatic relief and have only shown to only slightly delay loss of mental abilities in people who have mild to moderate AD\textsuperscript{39}. Other studies are suggesting combinations of drug therapy with exercise may slightly help prolong disease onset\textsuperscript{40}.

Specific to Aβ and tau, several studies have attempted to target β- and γ-secretases (that cleaves amyloid-precursor protein or APP to generate Aβ), kinases (that hyperphosphorylate tau) or aggregation inhibitors as prevention of Aβ, tau aggregates or NFTs with little to no success\textsuperscript{41}. Other investigators have explored avenues upstream, i.e., the triggers of tau-specific kinase activity\textsuperscript{42} or downstream phosphatase activity\textsuperscript{43}. However, the tau kinases and phosphatases are also crucial for other complex pathways, therefore targeting them has proved to be challenging and therefore, these are unlikely favorable targets to develop therapy against tauopathies, or it might require a combinatorial approach in testing multiple targets. Given that more and more studies failed to meet a successful end-point with the Aβ–targeted therapy and that NFT pathology seem to better correlate with cognitive decline, the rest of this write-up will primarily focus on tau/NFT-targetting approaches. Accordingly, some studies are still targeting certain kinases, such as Cyclin-dependent kinase-5 (CDK5)\textsuperscript{44}, or Glycogen synthase kinase (GSK-3beta)\textsuperscript{45}. Furthermore, tau aggregation inhibitors have also been seriously considered as a choice. Rhodanines are small compounds capable of disrupting the β-sheet structure within the microtubule-binding region of tau, the hexapeptide motif\textsuperscript{46}. However, the approach of a single-target approach to treat AD is considerably flawed as we have yet to identify components that halt AD progression in majority of patient population with tauopathies.
More recently, researchers have been investigating possible clearance enhancing mechanisms to target pathological tau. An interesting strategy to prevent p-Tau from becoming pathological is to promote its degradation via autophagy in “at risk” neuronal populations. As such, there are clinical trials underway to promote clearance of tau and other aggregated proteins in patients with AD and Parkinson’s disease (NCT02947893, NCT02281474). Moreover, impairment of autophagic processes has been implicated in several neurodegenerative disorders\cite{47,48,49,50,51,52,53}, which further supports autophagy’s role in clearing p-Tau and maintaining homeostasis as a potential strategy. Previous work from Dr. Deretic’s group provided compelling evidences that autophagy prevents spurious inflammasome/interleukin-1β (IL-1β) activation\cite{54,55}, which when left uncontrolled, could drive tau pathology and cognitive impairment\cite{56}. Other studies have also suggested that promotion of autophagic processing can enhance clearance of p-Tau and rescue neurotoxicity in a mouse model of tauopathy\cite{57}. We have demonstrated that induction of autophagy via chemical or genetic means lead to the clearance of inflammation-induced p-Tau\cite{58} in neuronal cells. Transcription Factor EB (TFEB) regulates transcription of an entire CLEAR (Coordinated Lysosomal Expression and Regulation) network, which consists of a consensus site predominately found in the promoter regions of autophagy-lysosomal genes\cite{59,60}. Thus, when TFEB localization is nuclear, it leads to a robust increase in lysosome biogenesis, and results in accelerated degradation of autophagic substrates\cite{61,62}. Phosphorylation of Ser211 in TFEB by mammalian target of rapamycin complex 1 or mechanistic target of rapamycin complex 1 (mTORC1) is one of the key regulators of nuclear localization, as the S211 phosphorylation prevents TFEB from entering into the nucleus\cite{63}. However, the limitation
of pro-autophagy studies is their focus on the continual activation of autophagy. While autophagy is generally thought to promote survival as discussed above, under certain conditions sustained autophagic-flux can lead to cell death.  

Furthermore, prolonged activation of autophagy proteins (e.g., LC3 and BECN1) and vacuoles in response to ischemic stroke/reperfusion \textit{in vivo}, or oxygen-glucose deprivation \textit{in vitro} lead to significant cell death. Interestingly many autophagic processes do not significantly affect cell health until days after the injury, indicating that prolonged activation is critical for cell death to occur. Another example, constitutive activation of the δ2 glutamate receptor was demonstrated to cause Purkinje cell death in Lurcher mice via activation of autophagy. Thus, for elderly tauopathy patients with co-morbid conditions such as ischemia and vascular dementia, sustained activation of autophagy could exacerbate cell death rather than reduce it. Therefore, it is crucial to develop a tunable system to turn-on/turn-off autophagy in neurons with optimum temporal control.
2.1 Rationale The rationale for the proposed research is that the use of light-induced regulation of autophagy could reduce p-Tau with minimal side effects. Therefore, our study will validate this novel platform for optogenetic-based strategies to regulate gene expression and target multiple cellular signaling cascades thought to underlie a variety of disorders. Regardless of the outcome, the positive impact of these studies will be the development of a novel and flexible system to rapidly regulate one or more signaling cascades relevant to neurodegenerative disorders using light-based methods.

2.2 Hypothesis Our central hypothesis is that light-induced expression of a master regulator of autophagy, Transcription Factor–EB (TFEB), will accelerate the autophagy process, clear NFTs within neurons, prolong cell survival, and improve cognitive function.
2.3 Specific Aims The above hypothesis was tested under two specific aims:

**Specific Aim 1:** Optimization of optogenetic TFEB gene expression system in a neuronal cell line. 1.1 What are the parameters necessary for optimal light induced gene expression within neurons? 1.2 Can TFEB target various forms of Tau? 1.3 Does TFEB need to be modified for optimal induction of autophagosomal/lysosomal transcription factors upon light stimulation?

**Specific Aim 2:** Light-Induced TFEB-mediated reduction of hyperphosphorylated and aggregated p-Tau in cell line models of tauopathy. 2.1 Does light-induced TFEB expression reduce p-Tau in cellular models of tauopathy? 2.2 Examine the temporal dynamics and autophagocytic activity in human inducible-pluripotent stem cell-derived neurons.
Chapter 3: Optogenetic gene expression systems

3.1 Overview of Optogenetics

In the last fifteen years, researchers have been taking advantage of various opsins\textsuperscript{69} and their unique light-sensitivity functions. The unique function of opsins has transpired into a plethora of light responsive engineered systems\textsuperscript{70,71,72}. More specifically, the discovery of channelrhodopsin\textsuperscript{73,74} led to optical control within mammalian neuronal excitability functions\textsuperscript{68,75,76,77,78}. These genetically engineered channelrhodopsins have contributed to our understanding of neuronal-basis to various behavioral functions\textsuperscript{79}. To date, optogenetic technology has been primarily utilized to alter membrane excitability in neurons using microbial opsins that gate ion channels\textsuperscript{80}. However, an underutilized application of this technology is that of reversible optical regulation of transgene expression\textsuperscript{81}. From the original chemical regulation, e.g., the tetracycline-regulated transcription system\textsuperscript{82} emerged the use of plant flavoproteins to activate transcription factors, therefore utilizing control of their dimerization mechanism and DNA binding sites with light stimulation\textsuperscript{83}. Thus, in contrast to chemical-based transcriptional regulators that suffer from complex pharmacokinetics and a primarily one-way regulatory scheme (ON or OFF), optical approaches offer rapid, reversible induction of gene expression, cell type and/or area specific, and represent an exciting tool for dissecting pathological signaling pathways.

Most optogenetic transcription regulation systems are responsive to ranges between green, blue, and red/NIR wavelengths\textsuperscript{69}. Therefore, this ability to switch genes ‘on’ and ‘off’ with high spatial precision has become a valuable tool in many fields of basic research\textsuperscript{84,85,86,87,88,89,90,91,92}. Despite the origin of optogenetics in the neuroscience
field, only recently has the regulation of an endogenous mammalian transcription factor by light stimulation in neuronal cell lines been shown\(^93\). Importantly, from this dissertation research, another neuronal targeting optogenetic gene expression system is developed.

3.2 Red Light (Müller et al. “Control of gene expression using a red- and far-red light–responsive bi-stable toggle switch.”)\(^94\)

Our group first tried an optogenetic gene expression system that consists of a red light (660nm) 2-3 plasmid system\(^95\). First, a bicistronic ‘light response element’ (LRE) plasmid, is driven by a simian virus 40 (SV40) promoter, where a single transcript encodes for the light responsive PhyB-VP16 protein as well as the DNA binding PIF6-TetR element.

![Diagram of recombinant DNA vectors that encode for the light-response element (LRE; left) and the Reporter (right); activation of transcription occurs in response to red (660nm) light, and can be terminated by exposure to far red (740nm) light. (B-E) HEK293T cells expressing both the LRE+reporter as well as addition of purified PCB. Cells maintained in the dark, showed little background expression of the mCherry reporter (D-E) compared with the](image)

**Figure 3.1 (A)** Diagram of recombinant DNA vectors that encode for the light-response element (LRE; left) and the Reporter (right); activation of transcription occurs in response to red (660nm) light, and can be terminated by exposure to far red (740nm) light. (B-E) HEK293T cells expressing both the LRE+reporter as well as addition of purified PCB. Cells maintained in the dark, showed little background expression of the mCherry reporter (D-E) compared with the
Briefly, *Arabidopsis thaliana* red/far-red light receptor phytochrome B (PhyB) is linked to the herpes simplex VP16 transactivation domain and a nuclear localization sequence (NLS), on the same plasmid separated by a polioviral internal ribosome entry site (IRES<sub>PV</sub>), codes for the phytochrome-interacting factor 6 (PIF6) fused to a tetracycline repressor (TetR). The ‘Reporter’ plasmid contains a tetracycline operator sequence (tetO) motif upstream of a minimal promoter and the reporter gene. Therefore, the PIF6 fused to the TetR will covalently bind to the tetO. This system is activated within milliseconds upon exposure to red light (660nm), converting it to its active state (Pr) where it can interact with phytochrome interacting factors (PIFs). Engineered PIFs that are covalently linked to DNA- or transcription factor-binding domains can recruit Polymerase II to activate transcription of transgenes within minutes when bound to the Pr protein (Fig. 3.1A). This process can be reversed by exposure to far-red light (740nm), converting the PhyB(NT) to its inactive state (Pfr), and dissociating the PhyB-PIF complex. Exposure to inactivating 740nm light causes significant reductions in transgenic mRNA species within hours, and both activation/inactivation can be regulated with light exposures as short as one minute.

However there was one pitfall to this optogenetic system. PhyB requires the binding of the chromophore phycocyanobilin (PCB), which is not found in mammalian cells but can be purified from *Spirulina* or purchased commercially. Addition of purified PCB to culture medium is necessary before illumination. This requires significant optimization between various cell lines, via optimal dose dependence and imbalance of media characteristics. More so, this does not bode well for future *in vivo* studies.
Otherwise, a third plasmid coding for two enzymes that catalyze the biosynthesis of PCB from heme can be tri-transfected, which stirs-up the debate of multiple transfection efficiency concerns. To establish feasibility of the optical gene expression system we used HEK293T cells with a control, constitutively-active mCherry overexpression vector (Figure 3.1 F-I), or the light-responsive and reporter system with addition of purified PCB to the media, as shown in Figure 3.1 A. Cells that received the LRE and reporter were either exposed to 660nm light (~1mW/mm²) for 3h, or maintained in a dark condition, and then assayed for mCherry expression 24h later. Figure 3.1 B-C shows paired bright-field and fluorescent images for HEK293T cells that were light-exposed, and demonstrate little to no mCherry expression equivalent to cells maintained in the dark (Figure 3.1 D-E). Expression was not comparable to cells that received the constitutively-active mCherry vector in either light/dark condition (Figure 3.1 F-I). This system showed great promise for a few reasons. First, it is in the red/far-red spectrum, which is shown to be less toxic to cells. Secondly, the short time frame it takes to initiate gene expression (3 hrs). However, PCB is the most burdensome factor, due to a third component (plasmid or purified), lowers the efficacy of success rate for light induced gene expression. This deems the red-light based optogenetic-based gene expression method is less favorable in comparison to the traditional tetR and tetO system with the addition of doxycycline. Therefore it is important to find an optogenetic gene expression system with fewer components and involve addition of little to none external factors.

3.3 Blue Light (Motta-Mena et al. “An optogenetic gene expression system with rapid activation and deactivation kinetics”.)
We then analyzed an optogenetic gene expression system that consists of a two-plasmid system reactive to blue light (450nm)\textsuperscript{98}. This light-inducible gene expression system utilizes an engineered bacterial transcription factor EL222, containing a Light-Oxygen-Voltage (LOV)\textsuperscript{99,99,100,101} protein and N-terminal VP16 transcriptional activation domain. This system has been shown to induce transcription of target genes with >100 fold dynamic range and rapid activation (<10 s) and deactivation (<50 s) kinetics. While this system has been tested in various mammalian cells and zebrafish embryos, its functional utility in a human disease model system remains untested.

The corresponding DNA binding region to EL222 was previously optimized with five copies of a specific EL222 DNA-binding region, [Clone 1–20 base pairs (C120)]\textsuperscript{94} (Figure 3.2 A). This consensus site acts as a promoter region for the EL222 binding and drives the expression of any genes inserted downstream of C120 allowing for transient expression of the transgene due to relatively fast reductions in expression upon cessation of light exposure (Figure 3.2 A). For further terminology, pVP-EL222 is referred to as the ‘light activated protein’ or ‘LAP’, and the pC120 is referred to as the ‘light-response element’ or ‘LRE’).
A

Figure 3.2 (A) Schematic of previously established gene expression system derived from an EL222 bacterial transcription factor, termed Light-Activated Protein (LAP); activation of transcription occurs in response to red (450nm) light. (B-E) HEK293T cells expressing both the LAP and LRE-mCherry. Cells maintained in the dark, showed little background expression of the mCherry reporter (D-E) compared with the mCherry control plasmid (H-I). In contrast, 12h light exposure was sufficient to induce mCherry expression to levels similar to the control overexpression plasmid (compare upper left panels with lower left panels)

First, we successfully recapitulated the previous studies of 450nm light-inducible gene expression in HEK293T cells (Figure 3.2). However, upon fluorescence-activated cell sorting (FACS) analysis, it is evident that the transfection efficiency was rather low (Figure 3.3). Compared to ~20% of cells expressing mCherry from a
constitutive promoter, the LRE-mCherry light induction is rather low, showing only ~7% of cells were expressing mCherry, and ~1% of cells expressing in the Dark.

Figure 3.3 | Transfection efficiency and light induction analysis via FACS. HEK293T cells expressing both the LAP and LRE-mCherry or LRE-mCherry alone, comparative to pCMV-mCherry control. Cells maintained in the dark, showed little background expression of the mCherry reporter compared with the mCherry control plasmid. In contrast, 12h light exposure was sufficient to induce mCherry expression to about a third comparative to the control overexpression plasmid.

3.4 Successful optogenetic gene expression system in a neuronal cell line.

Next, we verified that co-transfection of Neuro 2a (N2a) cells with both the pSV40SV40NLS-LAP and the pLRE-Firefire Luciferase reporter (pLRE-FLuc) resulted in optically-induced expression of the firefly luciferase reporter. Under this more sensitive assay\textsuperscript{102}, we observed robust luciferase expression and activity driven by the LAP-LRE interaction in HEK293T cells (Figure 3.4 B). However, luciferase expression and activity were more than a two-fold lower in N2a’s compared to HEK293T cells (Figure 3.4 B-C).

In an attempt to optimize the LAP-LRE system for gene expression in neurons we replaced the SV40 promoter with a stronger cytomegalovirus (CMV) promoter\textsuperscript{103,104}. We also included an additional cMyc nuclear localization signal (1xNLS or 2xNLS) sequences\textsuperscript{105,106} (Figure 3.4 A). The addition of the different promoter was sufficient to
significantly improve luciferase expression upon blue-light illumination compared to Dark controls in both HEK293T and N2a cells (Figure 3.4 B-C). We also observed a different degree of luciferase expression with the different promoter and dual NLS combinations, pCMV-LAP-2xNLS, showing the most robust induction of luciferase expression in N2a cells (Figure 3.4 B-C). Due to the notable light-induced transgene expression by pCMV-LAP-2xNLS in both cell lines, we used this LAP construct for all optical experiments. However, the question still remains can optically-expressed TFEB reduce pathological tau in neurons?
Figure 3.4 | Optogenetic gene expression system in neuronal cell line. A. Schematic of our optimized changes made to the LAP construct for successful neuronal transfection/induction as well as TFEB cloned into the LRE construct. B-C. Quantitative comparison of various versions of LAP constructs using pLRE-Firefly Luciferase reporter, (pLRE-FLuc) in HEK293T’s and Neuroblastoma cell line N2a’s, measuring luciferase activity units (RLU) via radiance levels detected by IVIS (mean ± s.e.m, Student’s t test or one-way ANOVA with Tukey multiple comparison test, ****p<0.0005 n=5)
Chapter 4: Optogenetic TFEB

4.1 TFEB clears multiple forms of pathological tau with equal efficiency in cellular models of tauopathy.

To test the ability of optically induced transgene expression to clear p-Tau, we chose TFEB, which is a well-established regulator of autophagy, and previously implicated in clearing tau via constitutive activation\(^{40}\). As a first step, we decided to confirm whether TFEB could clear p-Tau and determine whether TFEB can target multiple forms of p-Tau via autophagic flux in neuronal cells. The \(MAPT\) gene in humans encodes six different isoforms that differ based on inclusion or exclusion of exons 2, 3 and 10\(^{107}\). Exon 10 encodes the second microtubule binding repeat, thereby resulting in tau with either three (3R) or four (4R) microtubule binding repeats of 31–32 amino acids in the C-terminal half of tau\(^{47}\). Exons 2 and 3 encode one (1N), two (2N), or zero (0N) amino terminal inserts of 29 amino acids each in the N-terminal half of the protein\(^{47}\). In normal adult brain, the relative amounts of 3R tau and 4R tau are approximately equal. However, in many neurodegenerative tauopathies, the 3R:4R ratio is often altered\(^{108}\). Besides altered isoform ratios, post-translational modifications such as phosphorylation can also affect tau’s function and contribute to disease pathogenesis. We tested whether TFEB can clear following types of p-Tau: (1) 0N3R – non-mutant tau, when over-expressed can lead to Pick’s Disease (PiD)\(^{109}\), (2) 0N3R (T231D/S235D) tau, which mimics hyperphosphorylation on T231/S335 sites and is known to disrupt tau’s interaction with microtubules\(^{110}\), (3) 0N4R – non-mutant tau, but over-expression can lead to progressive supranuclear palsy (PSP)\(^{111}\), and (4) 0N4R-P301L mutant tau, which cause FTDP-17\(^{112,113}\). Others and our group have previously shown that TFEB-induced autophagic
flux degrades p-Tau via beclin-1 dependent autophagy pathway\textsuperscript{31,32}. However, it is unclear whether TFEB can target and clear various pathological forms of tau. Here we co-transfected N2a cells with constitutive TFEB expressing vectors and each tau constructs (0N3R, 0N3R(T231D/S235D), 0N4R, or 0N4R-P301L) individually at a 1:1 TFEB:p-Tau ratio. As revealed by western blot, TFEB expression caused a significant reduction in all forms of tau in N2a cells with T231D/S235D phosphorylation-mimicking tau showing the most significant reduction (Figure 4.1 A-B). Together, these results suggest that TFEB can consistently clear different types of p-Tau in neuronal cells. Furthermore, since the T231 mutation causes a potent neurotoxic conformation called cis-p-Tau (or ‘Cistauosis’, as a result of phosphorylation of tau at T231)\textsuperscript{114,115,116}, TFEB’s role in significantly reducing T231D/S235D levels supports the therapeutic potential of targeting TFEB against tauopathies.

Next, we also determined whether or not TFEB with different tags (FLAG versus GFP) would affect its ability to clear p-Tau via autophagy. Co-expression of T231D/S235D tau with either pCMV-TFEB3xFLAG or pCMV-TFEB-GFP showed that GFP tagged TFEB has better efficiency in inducing p-Tau reduction than 3xFLAG tagged TFEB (Fig. 4.1C-D).
Figure 4.1 | TFEB differentially targets various forms of pTau.

A-B. Western blot and quantification showing significant reduction in various forms of tau via WT – 0N3R, (0N3R) T231D/S235D, (0N4R) P301L, and WT – 0N4R with the addition of constitutive overexpression of TFEB activity. Results indicated most forms of tau are equivalently reduced by TFEB, however (0N3R) T231D/S235D shows highest significance in expression and reduction. Total tau/GAPDH ratio (mean ± s.e.m, Student’s t test, **p<0.01 n=3)

C-D. Western blot and quantification showing significantly reduced (0N3R) T231D/S235D with the addition of various forms of constitutive TFEB overexpression; pCMV-TFEB3xFLAG, pCMV-TFEB-GFP, pCMV-TFEB(S211A)GFP. Results indicate pCMV-TFEB(S211A)GFP holds the best yield in total tau reduction. Total tau/GAPDH ratio (mean ± s.e.m, one-way ANOVA with Tukey multiple comparison test, ***p<0.005 n=4)

4.2 Different variants of TFEB clear pathological tau with equal efficiency in cellular models of tauopathy.

We observed that GFP-TFEB/3xFLAG-TFEB distribution appeared homogenous throughout cells, indicating that overall nuclear entry of the TFEB was relatively low.
Nonetheless, this lower level of TFEB within the nucleus was sufficient to promote p-Tau clearance. However, to achieve better nuclear entry of TFEB, we tested S211A mutation in TFEB, which was previously shown to prevent phosphorylation by mTORC1\(^{117}\) thereby facilitates TFEB’s nuclear entry. Given that the transcriptional promotion of genes in the CLEAR network requires nuclear localization of TFEB, we next assessed the effects of S211 phosphorylation in TFEB in clearing mutant p-Tau. We observed robust reduction of T231D/S235D mutant p-Tau when they were co-expressed with TFEB(S211A)-GFP (Figure 4.1 C-D). While the quantification showed that the reduction in p-Tau by TFEB(S211A)-GFP was comparable to that of TFEB-GFP, however the level of significance (p<0.01 vs p<0.005 for TFEB-GFP and TFEB(S211A)-GFP, respectively) was different. To confirm this trend, we next used AMNIS/FACS automated image-stream analysis. We quantified the percentage of nuclear TFEB+ cells between WT and S211A (Figure 4.2). TFEB(S211A)-GFP were averaged to be a 92.5% population of nuclear TFEB+ cells compared to 85.3% in WT TFEB tranfection. Together, these results suggest that genetically facilitating the nuclear entry of TFEB via introduction of S211A single point mutation does provide an added advantage in enhancing the autophagic clearance of T231D/S235D tau.
Figure 4.2 | Nuclear comparison of constitutive TFEB(S211A)GFP in a neuronal cell line. Quantitative immunofluorescence, FACS, AMNIS showing significant increase in TFEB(S211A)-GFP nuclear localization comparative to WT. In TFEB (WT), 85.3% of nuclear TFEB+ cells, where as with S211A mutation, this number increased to 92.5%.

4.3 Optogenetically expressed TFEB activates CLEAR network genes in neuronal cells.

To determine the efficiency of optogenetically-driven TFEB (Opto-TFEB) in N2a cells, we co-transfected N2a cells with either pCMV$_{SV40}$NLS-LAP or pCMV-LAP$_{2x}$NLS and pLRE-TFEB(S211A)-GFP plasmids.
First, we assessed the timeline for the optimal light induction, (Figure 4.3), and concluded 12 hr’s is the optimum timepoint for light-induced gene expression. After the cells were stimulated 465nm light for 12h, they were fixed and immunostained to detect the levels of LAP (VP16) and LRE (TFEB(S211A)-GFP). Substitution of the SV40 promoter for a CMV promoter, along with the addition of a second cMyc NLS resulted in a significant increase of TFEB expression (revealed by the GFP signal) with light stimulation compared to the ‘Dark’ control (Figure 4.4 A-B). As expected, the VP16 staining was detectable and localized primarily to the nucleus in cells expressing pCMV-LAP-2xNLS (Figure 4.4 A).
Figure 4.4 | Optogenetic TFEB induction in neuronal cell line and CLEAR activity readout. A-B. Quantitative immunocytochemistry showing significant increase in TFEB expression in Light control vs Dark, comparison of various versions of LAP constructs using pLRE-TFEB-(S211A)GFP. Scale bar: 10 µm. C-D. Quantitative comparison of various versions of LAP constructs using pCLEAR-Firefly Luciferase reporter, (pCLEAR-Fluc) in N2a’s measuring luciferase activity units (RLU) via radiance levels detected by IVIS (mean + s.e.m, Student’s t test or one-way ANOVA with Tukey multiple comparison test, ****p<0.0005 n=4)

As mentioned, many of the target genes activated by TFEB have been identified, and all carry the consensus CLEAR motif (\(^{5'}\)GTCACGTGAC\(^{3'}\)) in their promoter regions\(^ {25} \). To determine whether optically-expressed TFEB (or “Opto-TFEB”) is functionally active, we used a firefly luciferase (Fluc)-based reporter assay to assess the expression of CLEAR-dependent genes\(^ {118} \). The pCLEAR-FLuc plasmid consists of four
replicates of the CLEAR consensus sequence upstream of the luciferase gene, thus representing TFEB transcriptional activity. We transiently co-transfected pCLEAR-FLuc with pLAPs, and pLRE-TFEB(S211A)-GFP in N2a cells and stimulated with blue light overnight (12h). Then the cells were treated with D-luciferin (1:100), and culture plates were immediately (3 - 4 mins after addition of substrate) imaged using luminometer to detect light output from the oxidation of D-luciferin as a measure of luciferase activity. As expected, the CMV-driven constitutively active TFEB produced the highest levels of CLEAR-luciferase signal (Figure 4.4 C-D) that was present even in cells maintained in the Dark control condition. Interestingly, we observed significantly higher levels of CLEAR-luciferase signal in cells that expressed Opto-TFEB and were light exposed, but minimal CLEAR-luciferase signal from samples maintained in the Dark (Figure 4.4 C-D). Together, our results suggest that Opto-TFEB expression is induced by blue light exposure and can functionally activate transcription of downstream targets in the CLEAR network.

4.4 Opto-TFEB reduces pathological tau in neuronal cells

If light-induced Opto-TFEB can bind the CLEAR motif and drive transcriptional regulation, we hypothesized that it would be sufficient to induce autophagic flux and reduce levels of misfolded p-Tau. We first overexpressed human tau carrying the 0N3R-T231D/S2345D double mutation along with pCMV-LAP2xNLS and pLRE-TFEB(S211A)-GFP in N2a cells. Analysis of TFEB(S211A)-GFP and Tau12 through western blot revealed statistically significant increases in TFEB expression (Figure 4.5 A-B) and reduction in the levels of total tau (Tau12) (Figure 4.5 A-C) in light-exposed cells.
Confirmatory, unbiased quantitative morphometry analysis for Tau12 levels using high-content, automated Cellomics® microscopy, revealed a significant decrease in the overall Tau12 intensity in light-exposed Opto-TFEB+ cells compared to Dark controls (Figure 4.5 D-F). Confocal analysis further confirmed that the fluorescence signals for Tau12 and GFP (from TFEB(S211A)-GFP+ cells) were mutually exclusive and non-overlapping (Figure 4.5 G). Together, these results demonstrate that light-induced expression of TFEB is capable of reducing overexpressed phospho-mimicking (T231D/S235D) Tau levels in neurons.

Figure 4.5 | Optogenetic TFEB induction in neuronal cell line reduces neuronal pathological mimicking tau. A-C. Western Blot analysis showing overall total protein levels are reduced when Opto-TFEB is expressed via light stimulation compared to dark. D-F. Cellomics®-based high-content imaging analysis of the effects on total Tau levels within Dark and Light controls. Cells were automatically identified based on nuclear staining (DAPI), then cells were selected for positive nuclear green fluorescence (TFEB(S211A)GFP) to further analyze for Tau12 (RED) intensity levels within 100 pixel
radius per cell. Briefly, white lines represent cell boundaries, red lines represent positive cytosolic Tau12, and yellow lines indicate nuclear TFEB(S211A)GFP-positive cells, then subjected by automated image analysis. G. Representation of colocalization profile for Tau12 (red) and LRE-TFEB(S211A)GFP (green) analysis. Quantitative confocal immunocytochemistry using N2a cells overexpressing human 0N3R-T231D/S235D tau show lack of colocalization of optogenetically induced TFEB expression with Tau12 positive cells. Quantitative morphometric data (mean ± s.e.m, Student’s t test, ****p<0.0001, n=3)

To test the efficacy of this system in human-disease relevant model system, we test Opto-TFEB in induced pluripotent stem cells (iPSC) line from a patient with sporadic AD (sAD2.1)\(^{119}\). We first confirmed that iPSC-derived neurons (iPSNs) from the sAD2.1 line display robust p-Tau expression (positive for AT8, AT180, and Tau12; Figure 4.6 A-C), similar to previous reports\(^{118}\), compared to iPSNs derived from a healthy control line (AX0018).
Figure 4.6 | Characterization of sAD2.1 iPSNs compared to healthy control iPSNs. A. Quantitative immunocytochemistry showing significant increase of p-Tau (AT8) within betaIII-tubulin (neurons) in sAD2.1 iPSNs compared to normal control AX0018 iPSNs. B-C. Western blot and quantification showing significantly increased levels of pMAPT (AT8 and AT180) (mean ± s.e.m, one-way anova, ***p<0.0005, n=3).

It is vital to assess the optimal time of transgene transduction efficiency in iPSNs, therefore we performed a timeline experiment for constitutive pLenti-CMV-mCherry virus and concluded that Day 7 is the optimal day to induce with light (Figure 4.7), since the lenti-viral driven expression of mCherry was highest as this stage.
Figure 4.7 | Lentiviral transduction of iPSNs timeline. Quantitation of pLenti-CMV-mCherry viral transduction rate. iPSNs were at day 40 when infected with an MOI of 2.

To assess the efficacy of Opto-TFEB in sAD2.1 cells we created lentiviral Opto-TFEB constructs (pGF1-CMV-LAP$_{2x}$NLS and pGF1-LRE-TFEB(S211A)-GFP) and co-transduced sAD2.1 iPSNs (see methods). Similar to results in N2a cells, light-exposed iPSNs displayed a significant increase in TFEB-GFP expression and a consequential decrease in both AT8 and AT180 p-Tau levels compared to Dark controls (Figure 4.8 A-B). Lastly, it has been established that the LAP spontaneously gets inactivated in the Dark, thus turning-off the LRE-mediated gene expression$^{120}$. Therefore, to assess the temporal dynamics of Opto-TFEB, we analyzed the light-Dark activity across two days. On day one, a plate of iPSNs was stimulated with light overnight and an identical plate of iPSNs was left in the dark. After the first time point of light stimulation, a row of cells was collected for analysis. The following day, the light was left off and another row of cells were collected for analysis 24 hrs after the first collection. First, we measured the mRNA levels of three well-known TFEB targets; $PTEN^{23}$, $CTSF^{25}$, and $MCOLN1^{25}$ (Figure 4.8 C). On Day one, we observed a significant increase in TFEB expression with light and up-regulation of TFEB target genes compared to Dark (Figure 4.8 C). The mRNA levels of TFEB-target genes reduced back to basal levels after a day of no light.
Western blot analysis to detect total protein levels revealed p-Tau (AT8 and AT180) was significantly reduced (Figure 4.8 F). Notably, while the total tau levels were unaltered, Tau12$^+$ bands showed slightly faster migration (Figure 4.8 E), likely because of the loss of hyperphosphorylated tau pool due to autophagy in the cell lysate. On day two, levels of TFEB(S211A)-GFP and TFEB targets were down to Dark levels, however the AT8$^+$ and AT180$^+$ p-Tau levels seem to have gradually raised up, but still lower than their starting levels (Figure 4.8 E). Taken together, for the first time, these results suggest that light-induced, optogenetic-based expression of TFEB can reduce p-Tau in a human disease-relevant iPSN cell culture model of tauopathy.
Figure 4.8 | Optogenetic TFEB clears pTau in human induced pluripotent stem cells derived into neurons (iPSNs). A-B. Quantitative immunocytochemistry showing significant increase in TFEB expression with subsequent lower levels of p-Tau (AT8 and AT180) within betaIII-tubulin (neurons) in Light control compared to Dark, using viral-particle versions, pGF1-CMV-LAP-2xNLS and pGF1-LRE-TFEB-(S211A)GFP (Scale bars: 10 µm. Mean + s.e.m, Student’s t test, *p<0.05, n=8). C. Two-day timeline using RT-qPCR analysis of TFEB gene expression and TFEB targets (PTEN, CTSF, and MCOLN1). Compared to Dark, each sample was taken 24 hours of subsequent time-point. On Day-1, 12-hour light stimulation; Day-2 from same sample, light was off. D-F. Corresponding to RT-qPCR time-point samples, western blot and quantification showing significantly increased in GFP (TFEB) levels and congruently reduced pMAPT (AT8 and AT180) with the transduction of viral optogenetic TFEB and subsequent light stimulation. (mean + s.e.m, one-way anova, ***p<0.0005, n=3-6).

4.5 Summary of results

Here we demonstrate the utility of an optical system to transiently control the expression of TFEB, which is a master transcriptional regulator of autophagy to reduce the load of pathological forms of tau in neurons. We had to optimize the promoter and NLS of the original described system in order to promote efficient gene expression not only in HEK293 cells, but also in N2a and iPSN neuronal models of tauopathy. We also observe that constitutively active TFEB has the capability of inducing the autophagy-mediated clearance of multiple forms of p-Tau. In addition to promoting autophagy and lysosome biogenesis, TFEB has been shown to promote a variety of biological functions including the inflammatory process, stress-responsive pathways, oxidative stress, and metabolic regulation. Therefore, considering TFEB as a potential therapeutic target has to be a cautious move, as it cannot remain in the nucleus and be constitutively active. Our study described here is aimed towards achieving the transient ‘on/off’
activation/deactivation mechanism using a novel blue-light inducible TFEB gene expression system that works well in mouse neuronal cell lines and human AD iPSCs derived into mature neurons. Using disease-relevant iPSCs will have significant translational value because of they being derived from human fibroblasts and the disease phenotype is displayed when differentiated into another cell type\textsuperscript{124,125,126}. Accordingly, the benefits of using sAD2.1 iPSCs is that they are derived from a patient with sporadic AD and when these iPSCs were differentiated into neurons, they display major hallmarks of AD, including elevated levels p-Tau phosphorylated at Thr231\textsuperscript{59}. Therefore, using sAD2.1 avoided tri-plasmid transfection, which often tend to show poor efficiency. Previous studies have utilized iPSNs to assess the role of autophagy in regulating AD-endophenotypes. For example, Reddy et al. generated human forebrain cortical neurons from iPSCs derived from familial AD patients carrying presenilin-1 (PS-1) mutations (M146L and A246E) and PS-1 knockdowns in neurons\textsuperscript{127}. Using the identical CLEAR-luciferase reporter assay as our group did, they found a reduction in CLEAR activity in AD, but not PS1 knockout, forebrain cortical iPSNs. These results suggest the utility of CLEAR-luciferase assay in determining the reduction of autophagy flux in disease-relevant iPSN models of AD. In another study, exposure of iPSC-derived forebrain cortical neurons with the amino acid metabolite homocysteine caused reduced autophagic activity via elevation of mTORC1 activity. Therefore, reduction in TFEB activity was suggested to be due to hyper-phosphorylation of TFEB by mTORC1\textsuperscript{128}.

Not only have we shown successful light controlled expression of TFEB, but we also effectively enhanced the autophagy flux via mutation of mTORC1 site - S211A, which facilitated nuclear entry of TFEB and robust clearance of p-Tau in the human AD
derived iPSNs. Considering the rise of p-Tau levels one day after light was turned off, proves a spatio-temporal dynamic with our Opto-TFEB system and we hypothesize when turning off autophagy, the potential kinases are likely activated again and/or likelihood of re-accumulation of hyperphosphorylated tau. However, to achieve sustained suppression of p-Tau, precise titration of light-dosing is necessary. It is also essential to induce Opto-TFEB at various time-points during the course of p-Tau pathogenesis to assess the reversibility of tau pathology, which will have high clinical significance. One of the potential limitations of inducing transcription factors is likelihood of transcriptional regulation and compensation\textsuperscript{129} as well as cellular fatigue. Nonetheless, our study demonstrates the expression and functional efficacy of neuronal Opto-TFEB in inducing the expression of CLEAR network genes for the induction of autophagy-lysosomal pathways and p-Tau reduction.
Chapter 5: Discussion

5.1 Discussion of Opto-TFEB

When studying a complex disease like AD, it becomes overwhelming to consider all the patho-etiologic factors that could drive the disease and come-up with a plausible strategy to intervene. For example, in the present study, despite developing an optically controlled TFEB expression as a tool, this may or may not completely cure AD. This is because our understanding of all mechanisms of causation is still at its infancy. There is still a lack of consensus on the primary etiology of AD. With the many failed clinical trials on eliminating protein aggregates we still are searching for answers. As with any scientific study, there are some limitations to the work presented in this dissertation. For one, TFEB is highly regulated. We successfully show that when light stimulates TFEB expression, when turned off, levels of TFEB target genes are brought back down to endogenous levels. However, it is important to continue the ‘on’ and ‘off’ switch to assess optimum Opto-TFEBs capability over a longer time period to achieve successful end-point of reducing p-Tau. Another potential limitation of the system is its low transfection and transduction efficiency, which perhaps require a robust gene-delivary strategy to achieve best possible efficiency.

Another component to consider, Opto-TFEB is that this system is a “transcription factor” driving another transcription factor. There is an assumed waste of energy between these two steps. Elimination of one of the transcription factors would deem more probable for reoccurring light stimulation. Perhaps engineer TFEB to be light inducible, or potentially the use of CRISPRa targeting CLEAR network genes (discussed in more detail later in this chapter).
Lastly, Blue 450-465nm has been shown to be somewhat toxic to cells\textsuperscript{130}. Additionally, this wavelength depth of penetration, through brain tissue, is roughly around 0.3mm – 0.5mm\textsuperscript{131}. Superlatively far-red provides close to 10-fold deeper (\textasciitilde\,2-3mm) penetration to the tissue without the need for invasive insertion of optical fibers. Additionally, potential design and use of an infrared responsive optogenetics system – which might sound like “Infrared-TFEB”, could provide even deeper penetration in the brain tissue, which are the ultimate goal in optogenetics and this would avoid the need of invasive fiber optic implants.

Overall, our results suggest that Opto-TFEB expression unlocks the potential of opto-therapeutics to treat AD and other dementias. However, it is too far from becoming therapeutics as the optogenetic technology is still premature. There are a number of requirements still necessary for the feasibility and testing in complex cells like neurons and eventually \textit{in vivo} is prudent.

\textbf{5.2 Future Directions}

\textbf{5.2.1 Aging and Neuroinflammation}

One of the well-established risk-factors for AD is age. The AD risk increases with age, so is the weakening of the immune system\textsuperscript{132}, hence the name ‘immunosenescence’. Immunosenescence is implicated in older adults from decline in adaptive immunity; results in failure to respond to age-related self-antigens, thus dramatically reduce immune response and affects longevity\textsuperscript{133}. However, the underlying mechanisms of immunosenescence are unclear and limited\textsuperscript{134}. In addition to decreased sensitivity of B cell antibody production and T cell responsiveness, increased pro-inflammatory cytokines and inflammatory responses are altered during aging\textsuperscript{135}. Taken together, these findings
suggest that aging causes an increasingly pro-inflammatory state and reduced immunity to infections, rendering individuals more susceptible to disease such as AD.

As mentioned above, aging is the primary risk factor for AD, and the effect aging has on the immune system is highly negative\textsuperscript{136,137}. Numerous genome-wide association studies (GWAS) in patients with dementia have implicated many genes in the immune system and neuroinflammation are contributing factors for AD and related dementias\textsuperscript{138}. More specifically, studies suggest a strong correlation between tauopathies and microglia activity\textsuperscript{139,140}. Microglia and astrocytes are arguably the primary sources of cytokines in AD. However, there seems to be a debate about whether a pro-inflammatory response to protein aggregates in the brain potentially providing protective characteristics\textsuperscript{141} or exacerbate\textsuperscript{142,143} the disease in a negative feedback loop. In a previous study, our group has demonstrated that genetically accelerating microglia-specific neuroinflammation in the brain accelerated AD-related tangle pathology and cognitive impairment in a manner dependent upon the activation of IL-1β-p38 mitogen activated protein kinase (p38 MAPK) signaling pathway\textsuperscript{144}. Notably, humanized MAPT transgenic mice deficient of the microglia-specific chemokine (fractalkine) receptor, CX3CR1, exhibited enhanced tau phosphorylation and aggregation as well as behavioral impairments that correlated with increased levels of active p38 MAPK\textsuperscript{145}. Thus, targeting biological process (e.g. proteosomal degradation or autophagy) that reduce pathological tau (p-Tau) levels or blocking neuroinflammation may serve as a potential strategy in treating tauopathies. For example, chronic administration of rapamycin (inducer of autophagy) significantly reduced microglial activation, Aβ and p-Tau and improved cognitive function in 3xTg
mouse model of AD\textsuperscript{146}. Autophagy also has the ability to suppress neuroinflammation by preventing inflammasome activation.\textsuperscript{147}

It is not clear whether TFEB-mediated autophagy can suppress microglial inflammation that otherwise can induce MAPT pathology and cognitive impairment. Thus, it is important to study induction of autophagy as a means to block inflammasome activation (Figure 5.1; required for IL-1\(\beta\) maturation and neuroinflammation) and can clear intracellular p-Tau aggregates in microglia and neurons, respectively.

Figure 5.1 | Future Direction Hypothesis schematic
5.2.2 Linking Opto-TFEB to neuroinflammation

One potential future direction is to examine optically-mediated autophagy induction can reduce p-MAPT in microglia cell lines and reduce inflammatory responses. First, activate immortalized murine microglial cell line BV2 cells\textsuperscript{148}, by priming with LPS\textsuperscript{137}, and transduce with pLenti-CMV-TFEB(S211A)-GFP [overexpression of TFEB] and assess inflammatory responses (IL-1β, Caspase 1, and TNF-α as well as other cytokines/chemokines via multiplex cytokine array). Second, transduce BV2 cells or primary microglia with pLenti-CMV-TFEB(S211A)-GFP or control pLenti-CMV empty plasmids (as described above) and directly treat them with PHFs purified from human AD brain (Appendix B). After 24 and 48 h, the media will be collected to measure cytokine levels via multiplex analysis and the cells will be fixed with 4% PFA for immunofluorescence analysis to detect phagocytized PHF/MAPT aggregates. In a separate condition, the cells will be processed for biochemical analysis to detect TFEB expression and other inflammatory proteins (via western blot analysis) or mRNA levels (via qRT-PCR). Transiently transfect the dual pGF1-CMV-LAP-2xNLS and pGF1-LRE-mCherry optogenetic plasmid system with mCherry reporter to test viability and optimal light stimulation. Finally, to validate the read-outs from murine microglial cells models, transduce primary human microglia (from ScienCell® – we currently have in Bhaskar lab) or develop microglia-like cells

\begin{figure}
\centering
\includegraphics[width=\textwidth]{image.png}
\caption{\textit{in vivo} lentiviral-mediated gene expression. (A) Diagram of cortical region targeted for injection. (B) Combined phase contrast and fluorescent images indicating LV-GFP expression at 10x (left panel), 20x (middle panel), and 40x (right panel) magnification. Scale bar 50\,µm. (C) Intracerebral administration of LV-Flag-TFEB in mice show specific expression of Flag (green) in numerous neurons of layers III-V of the cortex. Scale 50\,µm.}
\end{figure}
from iPSCs\textsuperscript{149}, transduce them with pGF1-CMV-LAP-2xNLS and pGF1-LRE-TFEB-(S211A)GFP and co-culture with sAD2.1 and isogenic control iPSNs and assess whether Opto-TFEB expression in microglia could promote autophagy flux and efficiently clear p-Tau derived from sAD2.1 neurons to prevent cytokine production/secretion and reduce p-Tau-induced neuroinflammation.

We predict that Opto-TFEB will result in significantly increased (>10-fold induction) TFEB expression, and that expression levels will correlate with increased autophagy flux and reduce LPS or PHF/p-Tau induced inflammatory responses in BV2, primary microglia and primary microglia+iPSN co-culture systems.

5.2.3 \textit{Bringing Opto-TFEB in vivo:}

We have successfully delivered pLenti-CMV-GFP into the brains of C57BL/6j mice via stereotaxic injection. Notably, 10 days post-delivery, several neurons in the orbito-frontal cortex (OFC) displayed intense GFP expression suggesting successful incorporation of lenti-derived GFP into neuronal genome and subsequent expression of GFP (Figure 5.2 A-B). In a separate experiment, we delivered LV-TFEB-FLAG into the cortex of 2-month-old mice and observed that numerous cortical neurons were Flag positive (Figure 5.2 C). However, further \textit{in vivo} testing of Opto-TFEB, which is beyond the scope of the present study, is necessary in order demonstrate the potential utility of light-induced autophagy flux in clearing p-Tau in mouse model(s) of tauopathy.
5.3 Genetic engineering potential for AD

Numerous GWAS studies have identified many risk genes show strong predisposition to developing AD. For example, amyloidogenic mutations in APP, PSEN1, or PSEN2, FTD mutations in MAPT, and single-nucleotide polymorphisms (SNPs) in sporadic AD genes (i.e. APOE ε4 or TREM2) all could drive and/or increase the risk of AD and related dementias. There are just too many contributing factors that constitute high risk for AD development. Therefore, genetic engineering of fixing single gene/set of genes will very unlikely be interventional strategy for AD. However, from this dissertation work it suggests that promoting cell’s own clearance mechanisms and/or immune systems, via enhancing the autophagy flux or suppressing pro-inflammatory responses, respectively could open-up future research possibilities for testing AD therapeutics.

While the gene therapy approach may not be the best strategy to test in all forms of AD, it could still be tested in prodromal AD cases that are familial, autosomal dominant and carry single point mutations in APP, PSEN1, PSEN2 and MAPT genes. Furthermore, gene therapy may unlikely be useful in sporadic AD cases where certain SNPs increases the risk (unlike autosomal dominant traits). Repairing such SNPs may or may not provide a cure. Nonetheless, it is important to consider efficient gene delivery system for the current studies to promote efficient integration of LRE/LAP into the neuronal genome to promote light responsive induction of autophagy flux and clearance of p-Tau aggregates in neurons. Historically, the first gene therapy clinical trial, in 1990, involved ex vivo gene transfer to umbilical cord blood cells or to T lymphocytes of patients with severe combined immune deficiency (SCID) due to mutations in the
adenosine deaminase (ADA) gene\textsuperscript{150,151,152}. The concept of gene therapy for genetic disease usually consists of \textit{ex vivo} functionality. However, later one of the patients died from a violent innate immune response to the intravenous delivered adenoviral vector in 1999\textsuperscript{153}. However, in 2000, another successful gene therapy was reported. This attempt used an \textit{ex vivo} retroviral gene transfer, rather than previous intravenous delivery, of the $\gamma_c$-chain to CD34$^+$ bone marrow cells to cure patients with X-linked SCID\textsuperscript{154}. However, half of the patients in this trial developed leukemia\textsuperscript{155}. Unfortunately, retroviral vectors will integrate indiscriminately into the host genome, mostly in transcriptionally active genes because of their open/unmethylated formation. Even today, the methodology of gene therapy delivery is not at its full potential, leaving questions and concerns unanswered. As the method used in chapter two, lentiviral transductions of transgenes have shown problematic in gene therapy. Therefore, there is still a desparate need for the gene therapy research for a successful CNS-directed and neuron-targeted delivery system.

Currently, nucleases employed for genome editing purposes consist of zinc finger nucleases (ZFN), or transcription activator-like effector nucleases (TALENs) from microbial origin. However, in 2015, the discovery of CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9, RNA-guided Cas9 endonuclease) made its iconic entrance to the genetic engineering world. CRISPR technology has proven faster, higher specificity, straightforward, and a more affordable way for genome editing, in comparison to traditional ZFN and TALENs approaches. Furthermore, research groups have engineered systems based on Cas9 providing extraordinary versatility and capabilities for genome engineering/manipulation\textsuperscript{156,157,158}. 

Currently, there are several clinical trials emerging for gene therapies using CRISPR/Cas9 technology (source: clinicaltrials.gov). The CRISPR/Cas9 system has also been adapted to generate technologies called CRISPRi (CRISPR interference)\textsuperscript{159} and CRISPRa (CRISPR activation)\textsuperscript{160}. These utilize nuclease-deactivated Cas9 (dCas9) that cannot generate a double-stranded breaks (DSB) (Figure 1A), but much like optogenetic gene expression systems, target genomic regions resulting in RNA-directed transcriptional control. CRISPRa is very similar to our LAP, in that dCas9 is fused to a transcriptional activation domain, which can be directed to promoter regions by guide RNA (gRNA). Utilizing CRISPRa to target CLEAR sites found in promoter regions, would avoid TFEB regulation as mentioned above.

5.4 Overall Conclusions

This dissertation successfully demonstrates an engineered genetic tool that provides positive spatio-temporal regulation of p-Tau levels in an AD iPSN model.

It does so by first providing a comprehensive outline on the burden of tauopathies and their pathology. Traditional tauopathy models were briefly examined, followed by the importance of current and past clinical approaches to the treatment of AD. An attempt to alleviate AD pathology was suggested by Opto-TFEB as well as potential other cell types and mechanisms the autophagy could potentially be beneficial. It may be interesting to see if tunable Opto-TFEB expression system via light would work in other cell types within the CNS. Conversely, it is also important to determine whether or not such regulation is applicable to other genes of interest (example, protein phosphatases, which could dephosphorylate hyperphosphorylated tau). While these questions are very likely be attempted in future studies, our data strongly suggest that Opto-TFEB efficiently
expresses in AD iPSNs, up-regulates TFEB target genes, and efficiently facilitates the clearance of p-Tau. Though it has been a hundred years since AD was first described, there is still much to be learned about the tauopathy and neuroimmune interface. Therefore, the field also needs a more diligent way, or “bigger processor”, than our human minds to comprehend these very complex diseases. Leaning on computational problem solving will be the way of the future. As it was said by Prof. Ehud Keinan, "All biological systems, and even entire living organisms, are natural molecular computers. Every one of us is a biomolecular computer, that is, a machine in which all components are molecules "talking" to one another in a logical manner. The hardware and software are complex biological molecules that activate one another to carry out some predetermined chemical tasks. The input is a molecule that undergoes specific, programmed changes, following a specific set of rules (software) and the output of this chemical computation process is another well defined molecule."
Methods:

Vector construction

All constructs were cloned using NEB HIFI Assembly Kit (NEB # E5520S) with restriction enzymes and PCR amplification. Briefly, the original episomal plasmids gifted by Motta-Mena, (pVP-EL222 and pGL4-C120-mCherry) were cloned into different backbones with subsequent promoters and/or gene of interest; pN1-CMV-TFEB-GFP (Addgene # 38119). Newly cloned episomal plasmids were then additional cloned into lentivector backbone, pGF1-NfkB-EF1-Puro (SystemsBio # TR012PA-P). Q5® Site-Directed Mutagenesis Kit was used to make mutations (S142A and S211A) in TFEB gene (NEB # E0445S). All Tau constructs used: 1) pRC/CMV-0N3R-tau (human tau with three microtubule-binding repeats with no N-terminal inserts); 2) 0N4R-tau (human tau with four microtubule-binding repeats with no N-terminal inserts); 3) 0N4R-P301L (human tau with four microtubule-binding repeats with P301L FTDP-17T mutation); 4) 0N3R-T231D/S235D. See supplemental Table 1 for all cloned vectors and their corresponding names.

Cell Lines

HEK293T and Neuro-2a (ATCC # CRL-3216 and #CCL-131, respectively) cells were maintained at 37°C in 5% CO2 in DMEM supplemented with 10% FBS, 5% penicillin/streptomycin, and grown in 24-well plates. For transient transfections, cells were split the day before ~ 1- 4 × 10^5 cells/well, therefore 70-80% confluence the following day. Before transfection, media was replaced with phenol red free media, (FluoroBrite DMEM; ThermoFisher # A1896701). Cells were then transfected with
Lipofectamine 2000 (Invitrogen) as per company’s protocol. Dilutions of various plasmid concentrations were as followed for a 24-well plate; pLAP’s – [2000ng/µL], pLRE’s – [500ng/µL], pCMV-TFEB’s – [500ng/µL], pCMV-hTau’s – [1000ng/µL], pCLEAR-FLuc – [500ng/µL]. Therefore 1:4 ratio of LRE to LAP.

**Induced pluripotent stem cells**

**sAD2.1; Coriell # GM24666,** *(iPSCs from Fibroblast NIGMS Human Genetic Cell Repository Description: ALZHEIMER DISEASE; AD Affected: Yes. Gender: Male. Age: 83 YR (At Sampling). Race: Caucasian.)*

Briefly, iPSCs were maintained in mTESR + supplement (StemCell # 85850) Neuron differentiation followed the StemCells neuronal differentiation kit/protocol; (StemCell #05835, #05833, #08500, #08510). Later medium was changed to BrainPhys™ without Phenol Red (StemCell #05791) for optical induction. (Neural progenitor cells seeded at 1.5x 10^4 cells/cm^2 for maturation)

**Light Induction**

12hrs post transfection, an in-house blue LED device (465 nm, strip of LEDs glued to PCB board; Amazon) was placed 8cm or 16cm above the plate. Note, the constraints of the light source also had to be altered (twice the distance than our cell lines; 16cm) due to higher sensitivity of iPSNs to the blue-light and the heat it produces, compared to N2a cell lines. The intensity of the light received by cells was measured to be to 8 W/m^2; as previously reported by^42. Verified, using the LI-190 Quantum Sensor and LI-250A light meter (LI-COR Biosciences). The LED strips were connected to SLBSTORES 3528
5050 12V DC Mini Remote Controller (Amazon) for variations of on/off patterns to best match a cycle of 20 s on and 60 s off as recommended per Motta-Mena et al. The control plate was kept in a PCB blackout box with breathable air slots, (a shelf in the incubator, above and away from the light source shelf). For transiently transfected cells, 24hrs post-transfection, samples were collected/ fixed for analysis.

**Lentivirus production and luciferase assay**

Using HEK293T’s, seeded in 100mm plates. Lentiviral Transgenes were cloned into the pGF1-EF1-Puro backbone. Lentiviral packaging vectors: pMD.2, pPAX2 (Invitrogen cat. no. K4975-00). Cells were transfected with plasmid mix using CaPO4 precipitation method, as per protocol Tiscornia et al. 2006 Nat Protocols “Production and purification of lentiviral vectors”. After 48-hrs interval, the viral supernatant was then filtered through 0.45 µm membranes and mixed overnight with cat#631232 Lenti-X™ Concentrator. The next day, samples were centrifuged at 1,500 x g for 45 minutes at 4°C. An off-white pellet is then resuspended in subsequent media, ex: if iPSNs, then neurobasal. Lentiviral titer was measured using cat#631280 Lenti-X™ GoStix™ Plus.

Lentiviral Transduction on iPSNs – an IFU of 1x10^6/mL were added to the neurons to make ~MOI = 2. We transduced sAD2.1 neural progenitor cells 24 hours after plating on poly-ornithine/laminin coated coverslips following StemCell maturation protocol. Subsequently, two weeks after transduction, (Day 40) iPSNs are subjected to light stimulation (12 hours) or kept in the dark, samples were then collected/ fixed for analysis.

For Firefly luciferase activities, 4XCLEAR-luciferase reporter plasmid #66800, purchased from addgene. D-Luciferin, Potassium Salt (ThermoFisher # L2916) was
reconstituted in water and was added (1:100) to each well, 3 - 4 mins after addition of substrate, 24-well plate samples were analyzed through the IVIS Lumina Series II with system software.

**Western blotting (WB) and immunocytochemistry (ICC)**

**WB** - Cells were lysed by RIPA buffer (Thermo #89900), incubated on ice for 30 mins then centrifuged at 20,000 × g for 15 min. Cell lysate supernatants were then sonicated for 20secs at 30%, then subjected to SDS-PAGE usage, transferred to PVDF membranes and detected using the ECL method (Pierce). Protein levels were quantified using ImageJ (National Institute of Health). Antibodies included; tau12, GAPDH, FLAG, GFP, TFEB, AT8, AT180, LC3B, LAMP1.

**ICC** - Cells were plated on coverslips coated with laminin, once cells were ready for fixation, they were fixed in 4% PFA, blocked with 0.2%triton and 10% donkey serum, incubated in primary overnight in 4°C (5% DS), secondaries were incubated for 1hr at RT. Incubated in DAPI for 10 mins, and mounted to slides using fluoromount(CAT#). Immunofluorescence confocal microscopy was carried out using Zeiss LSM 510 Meta microscope. Histo and profile analysis was performed using ZEISS ZEN imaging Software.

**Gene expression analysis**

RNA from cells was extracted using the TriZOL reagent as described by the manufacturer (Thermo Fisher Scientific). Total RNA (20 ng/µL) was converted to cDNA using the High Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific) and
amplified using specific TaqMan assays (catalog # 4331182; Thermo Fisher Scientific). GAPDH (catalog # 4352339E, Thermo Fisher Scientific) was used as a housekeeping gene for normalization. qRT-PCR assays were run on the StepOnePlus® Real-Time PCR System (Thermo Fisher Scientific) and the statistical analyses were performed using Prism.

**Cellomics®-based high-content imaging analysis**

Cells were plated in 96 well plates transiently transfected with pCMV-T231D/S235D (phospho-mimicking tau), pCMV-LAP2xNLS, and pLRE-TFEB(S211A)-GFP. Twenty-four hours later, cells were incubated with conditioned medium from BV2’s, as previously described, then subsequently induced with light (470nm) for 12 hours. Cells were fixed in 4% PFA, blocked with 0.2%triton and 10% donkey serum, incubated in primary for one hour at RT (5% DS), secondary was incubated for 1hr at RT. Incubated in DAPI for 10 mins and analyzed through cellomics machine.

**Statistics**

Unless otherwise indicated, comparisons between the two groups were done via unpaired t test; comparisons between multiple treatment groups were done via one-way or two-way analysis of variance (ANOVA) with indicated multiple comparisons post-hoc tests. All statistical analyses were performed using GraphPad Prism®.
Table 1: Plasmid list

<table>
<thead>
<tr>
<th>Plasmids used:</th>
<th>Description</th>
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<tbody>
<tr>
<td>Motta-Mena et al</td>
<td>pGL4-SV40-VP-EL222</td>
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<td>Motta-Mena et al</td>
<td>pC120-MCH</td>
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<tr>
<td>Motta-Mena et al</td>
<td>pC120-FLuc</td>
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<tr>
<td>Addgene #38119</td>
<td>pN1-CMV-TFEB-GFP</td>
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<td>Binder et al.</td>
<td>pN1-CMV-TFEB(S211A)-GFP</td>
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<td>Light response element</td>
<td>pN1-LRE-TFEB3xFLAG WT</td>
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<td>Light activated protein</td>
<td>pN1-CMV-EL222</td>
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<tr>
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<td>pN1-CMV-EL222-2x-NLS</td>
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<td>Lenti constructs</td>
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<tr>
<td>Lenti constructs</td>
<td>pGF1-LRE-TFEB(S211A)-GFP</td>
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Table 2: Antibodies

Antibodies used in western blotting (WB) or immunohistochemistry (IHC) are listed below.

<table>
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<th>Antibody</th>
<th>Species</th>
<th>Company and Catalog #</th>
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<td>AT180</td>
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<td>AT8</td>
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<td>Millipore, CB1001-500UG</td>
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<tr>
<td>Tau12</td>
<td>Mouse</td>
<td>Abcam, ab74137 Millipore, MAB2241</td>
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Abbreviations Used

AD  Alzheimer’s disease
ADRD  Alzheimer’s disease and related dementias
Aβ  amyloid beta
BV2’s  murine neonatal microglia cell line
CBD  Corticobasal Degeneratio
CDK5  Cyclin-dependent kinase-5
ChEIs  cholinesterase-inhibitors
CLEAR  Coordinated Lysosomal Expression and Regulation
CM  condition media
CMV  cytomegalovirus
CNS  central nervous system
CRISPR/Cas9  clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9, RNA-guided Cas9 endonuclease
EOFAD  Early-onset Familial Alzheimer disease
FACS  fluorescence-activated cell sorting
FTDP-17T  Frontotemporal Dementia and Parkinsonism Linked to Chromosome – 17 tau type
FTLD-Tau  Frontotemporal Lobar Dementia – Tau
GSK-3beta  Glycogen synthase kinase
GWAS  genome-wide association study
IL-1b  interleukin-1b
iPSCs  induced pluripotent stem cells
iPSNs  induced pluripotent stem cells derived neurons
<table>
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<th>Acronym</th>
<th>Description</th>
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<td>IRES&lt;sub&gt;PV&lt;/sub&gt;</td>
<td>polioviral internal ribosome entry site</td>
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<td>LAP</td>
<td>Light Activated Protein</td>
</tr>
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<td>LOAD</td>
<td>Late-onset Alzheimer’s disease</td>
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<tr>
<td>LOV</td>
<td>Light-Oxygen-Voltage</td>
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<td>LPS</td>
<td>lipopolysaccharide</td>
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<td>LRE</td>
<td>Light Responsive Element</td>
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<td>MAPT</td>
<td>microtubule-associated protein tau</td>
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<td>methyl CpG binding protein 2</td>
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<td>microtubules</td>
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<td>NFTs</td>
<td>neurofibrillary tangles</td>
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<td>nuclear localization sequence</td>
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<td>pathological tau</td>
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<td>phycocyanobilin</td>
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<td>paired helical filaments</td>
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<td>phytochrome B</td>
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<td>Pick’s Disease</td>
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<td>phytochrome-interacting factor 6</td>
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<tr>
<td>PPF</td>
<td>propentofylline</td>
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<tr>
<td>PSP</td>
<td>Progressive Supranuclear Palsy</td>
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<td>qRT-PCR</td>
<td>quantitative real time PCR</td>
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<td>Serine</td>
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<tr>
<td>SH-SY5Y’s</td>
<td>human neuroblastoma cell line</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
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<tr>
<td>SV40</td>
<td>simian virus 40</td>
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<td>T/ Thr</td>
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<tr>
<td>TFEB</td>
<td>Transcription factor EB</td>
</tr>
<tr>
<td>TPSD</td>
<td>Tangle-Predominant Senile Dementia</td>
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</table>
LIST OF APPENDICES

1. Published article in Frontiers in molecular neuroscience co-authored by Jessica Binder

Appendix A: Whole genome expression analysis in a mouse model of tauopathy identifies MECP2 as a possible regulator of tau pathology.

2. “In Submission” article co-authored by Jessica Binder

Appendix B: Proteopathic tau activates inflammasomes via ASC and contributes to tauopathies.
Appendix A. Published article in Frontiers in molecular neuroscience co-authored by Jessica Binder

Whole genome expression analysis in a mouse model of tauopathy identifies MECP2 as a possible regulator of tau pathology

Nicole Maphis¹, Shanya Jiang¹, Jessica Binder¹, Carrie Wright², Banu Gopalan³, Bruce T. Lamb⁴ and Kiran Bhaskar¹*

Abstract

Increasing evidence suggests that hyperphosphorylation and aggregation of microtubule-associated protein tau (MAPT or tau) correlates with the development of cognitive impairment in Alzheimer’s disease (AD) and related tauopathies. While numerous attempts have been made to model AD-relevant tau pathology in various animal models, there has been very limited success for these models to fully recapitulate the progression of disease as seen in human tauopathies. Here, we performed whole genome gene expression in a genomic mouse model of tauopathy that expressed human MAPT gene under the control of endogenous human MAPT promoter and also were complete knockout for endogenous mouse tau (referred to as ‘hTau⁵ Mapko(Duke)’ mice). First, whole genome expression analysis revealed 64 genes, which were differentially expressed (32 upregulated and 32 down-regulated) in the hippocampus of 6-month old hTau⁵ Mapko(Duke) mice compared to age-matched non-transgenic controls. Genes relevant to neuronal function or neurological disease include upregulated genes: PKC-alpha (Prkca), MECP2 (MeCP2), STRN4 (Strn4), SLC40a1 (Scl40a1), POLD2 (Pold2), PCSK2 (Pcsk2), and down-regulated genes: KRT12 (Krt12), LASS1 (Cers1), PLAT (Plat) and NRXN1.
(Nrxn1). Second, network analysis suggested anatomical structure development, cellular metabolic process, cell death, signal transduction and stress response were significantly altered biological processes in the hTau<sup>MaptKO(Duke)</sup> mice as compared to age-matched non-transgenic controls. Further characterization of a sub-group of significantly altered genes revealed elevated phosphorylation of MECP2 (methyl-CpG-binding protein-2), which binds to methylated CpGs and associates with chromatin, in hTau<sup>MaptKO(Duke)</sup> mice compared to age-matched controls. Third, phospho-MECP2 was elevated in autopsy brain samples from human AD compared to healthy controls. Finally, siRNA-mediated knockdown of MECP2 in human tau expressing N2a cells resulted in a significant decrease in total and phosphorylated tau. Together, these results suggest that MECP2 is a potential novel regulator of tau pathology relevant to AD and tauopathies.
Figure 5. MECP2 regulates tau pathology \textit{in vitro}. N2a cells transiently transfected with human tau 0N3R isoform (‘+Tau’) or a control plasmid (‘-Tau’) were nucleofected with siRNA (scramble siRNA (siScr) or MECP2 siRNA). After 24 h of siRNA nucleofection, the cells were harvested and probed for AT180, PHF-1, Tau5, Tau12 and total MECP2. (A-B) Note that siMECP2 significantly reduced levels of MECP2 in both ‘-Tau’ and ‘+Tau’ N2a cells (*p<0.01 unpaired t test; n=3 replicates; mean ± SEM). (C-D) siMECP2 treatment also significantly (*p<0.01 unpaired t test; n=3 replicates; mean ± SEM)
reduced the levels of both total tau (Tau5/GAPDH) and human tau (Tau12/GAPDH) ratios in the ‘+Tau’ N2a cells compared to scramble siRNA treated conditions. (E-H) siMECP2 knockdown resulted in statistically significant (*p<0.01 unpaired t test; n=3 replicates; mean ± SEM) increase and decrease in AT180/Tau5 and PHF1/Tau5 ratios, respectively. Note that the ratio for β-actin/GAPDH was not altered either in ‘-Tau’/‘+Tau’ conditions or with/without siMECP2 conditions.

Appendix A examines the importance of deep characterization within each tauopathy models. We explicitly used a hTau\textsuperscript{MaptKO(Duke)} in which we identify novel gene(s) that differentially regulate the expression of human MAPT. More over, we found significant upregulation of the MECP2 (\textit{MeCP2}) as well as phosphorylation of MECP2 (methyl-CpG-binding protein-2), which binds to methylated CpGs and associates with chromatin.
Appendix B: Submitted article to Neuron co-authored by Jessica Binder

Proteopathic Tau Primes and Activates Interleukin-1β (IL-1β) via MyD88- and NLRP3-ASC-Inflammasome Dependent Pathways

Shanya Jiang¹, Nicole Maphis¹, Jessica Binder¹, Devon Chisholm¹, Lea Weston¹, Walter Duran¹, Crina Floruta³, Amber Zimmerman³, Stephen Jett⁴, Eileen Bigio⁵, Changiz Geula⁵, Nikolaos Mellios³, Jason Weick³, Eicke Latz⁶,⁷, Michael T. Heneka⁶,⁷,⁸, and Kiran Bhaskar¹,²*

Abstract

Tauopathies are a group of neurodegenerative diseases characterized by the aggregation of tau protein as neurofibrillary tangles (NFTs) within the brain, where microglia also show activated phenotype(¹). Interleukin-1β (IL-1β) is a potent pro-inflammatory cytokine known to play an important role in the pathophysiology of various tauopathies(²-⁷) including Alzheimer’s disease (AD)(⁸-¹¹). Microglial activation is associated with increased IL-1β in AD(⁶, ¹²). Polymorphisms in the IL1B show significant risk for AD(¹³-¹⁶). However, it is not clear how pathologically modified tau (pTau) uniquely contributes to neuroinflammation in non-AD tauopathies. Here we show that elevated pTau strongly correlates with increased levels of both active IL-1β and apoptosis-associated speck-like protein containing a CARD (ASC) in human fronto-temporal lobar degeneration – tau type (FTLD-Tau) autopsied brains. Suppression of human tau expression, via doxycycline, reduced both priming and activation of
inflammasome markers (ASC and NLR family pyrin domain 3 or NLRP3) in rTg4510 mouse model of tauopathy. Microglial cells treated with pTau containing neuronal conditioned media or purified exosomes, led to microglial uptake of pTau, increased expression and/or activation of ASC, NLRP3 and IL-1β. Purified NFTs from either human FTLD-Tau or rTg4510 mouse brains activated IL-1β in microglia, which was dependent upon ASC. Genetic deficiency of ASC within microglia reduced tau pathology, blocked IL-1β activation, and improved memory in hTau mouse model of FTLD-Tau. The RNA-sequencing analysis of human primary microglia activated by human NFTs suggested that, surprisingly, pTau also induced IL-1β expression via the upregulation of NF-κB pathway. Finally, pTau-driven NF-κB activation and IL-1β maturation were microglial MyD88-dependent. These results demonstrate that pTau could prime microglial NF-κB, trigger inflammasome activation and neuroinflammation in non-AD tauopathies, suggesting that the MyD88/ASC/inflammasome network could be a potential therapeutic target against FTLD-Tau.
Figure 2 | Microglial cells take up pathological tau secreted from neuronal cells and lead to expression and activation of inflammasome-related genes. A-B. LPS-primed (1µg/ml LPS for 6h) BV2 cells show internalization of human tau when incubated with conditioned media (CM) from phosphorylation-mimicking tau (0N3R-T231D/S235D) transfected Neuro 2a (N2a) cells for 24 h prior compared to CM from vector-transfected (“Mock”) or wild type tau (0N3R-WT) transfected N2a cells (Tau12- red). Scale 20mm. C-D. Western blot and quantification of BV2 cell lysates shows significant uptake of
human tau (Tau12/GAPDH ratio) and increased protein levels of ASC, pro- and cleaved(c)-IL-1b, and NLRP3 in BV2 cells treated with CM from WT (0N3R) and/or (0N3R-T231D/S235D)-expressing N2a cells. E. Quantitative real time PCR analysis of unprimed BV2 cells shows significantly elevated the expression of mRNA for ASC (Pycard) mRNA, but not NLRP3/IL-1b, when treated with CM from WT (0N3R) and/or (0N3R-T231D/S235D) expressing N2a cells. F-H. Immortalized mouse macrophages expressing ASC-mCerulean or murine primary microglia show significant increase in the number of intracellular (white arrows) or extracellular (white arrowheads) ASC-specks when treated with CM from N2a cells expressing 0N3R-T231D/S235D tau compared to WT tau or Mock. Scale 10mm. Data displayed as mean ± SEM, one-way ANOVA with Tukey multiple comparison test, *p<0.05, **p<0.01, ***p<0.005, n=6 (C, D, E, G, J and K).
**Figure S4: Characterization of tau in exosomes purified from N2a.** (related to Figure 2). **A-B.** Purified exosomes from N2a CM show vesicles ranging from 50-200nm in diameter with distinct lipid bilayer and were positive for exosome marker Alix (10 nm conjugated gold particle (in B) detected via immune-electron microscopy or IEM). These exosomes were also positive for Tau12 (arrows showing 5 nm gold particles). Scale 100 nm. **C-F.** Western blot analysis with quantification shows presence of human tau (Tau12⁺) in lysates and exosomes of N2a cells transfected with 0N3R-WT or 0N3R-T231D/S235D tau. Ratio of Tau12/GAPDH and Tau12/CD81 (CD81 is an exosome marker) show significantly elevated human tau in both lysates and exosomes compared to mock (vector only) transfected lysate/exosomes. Data shown are mean ± s.e.m, one-way ANOVA with Tukey multiple comparison test, *p<0.05, n=4 (D); n=3 (F); unpaired Students t test).
Appendix B takes a deeper look into connections between tauopathy and neuroinflammation. In particular, our group has previously shown that microglial activation is associated with increased IL-1β, which leads to increased p38 MAPK, and in turn intensifies pTau. However it is not clear if pTau stimulates and uniquely contributes to neuroinflammation, or vise versa. Thus this article takes a multi-angle approach to show that neuron-derived pathological tau activates ASC/NLRP3 inflammasomes and IL-1β in microglia and leads to brain inflammation relevant to tauopathies.
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