Impact of UBQLN2^{P506T} on autophagy, bioenergetics, and inflammation in iPSC-derived cortical neurons, lower motor neurons and microglia

Background: Amyotrophic lateral sclerosis and frontotemporal dementia (ALS/FTD) are devastating diseases caused by the progressive degeneration of motor neurons in the cerebral cortex and spinal cord, and neurons in the frontoinsular cortices. These diseases currently lack effective therapeutics; three FDA approved drugs, Riluzole, Edaravone and Relyvrio, extend survival of ALS patients by 3-6 months, and only Riluzole has a validated mechanism of action. FTD has no approved therapies. Therefore, there is a severe need to understand the biology of ALS/FTD progression to identify targets for rational drug design.

Two hallmarks of ALS/FTD that contribute to neuron loss are sustained inflammation and disrupted neuronal energy homeostasis. These two hallmarks converge at the mitochondria: ATP production by mitochondria is critical to support the high bioenergetic demands at the synapse, and mitochondrial DNA (mtDNA) and its associated proteins are potent proinflammatory molecules when mislocalized. In my postdoctoral research, I have discovered that mitochondria enriched for mtDNA are among the most common cargo cleared by the cellular recycling pathway autophagy in healthy neurons (Goldsmith et al., Neuron, 2022). *In this proposal I will investigate how disruptions to autophagy caused by an ALS/FTD associated mutation affects both neuronal bioenergetic capacity and propensity to promote neuroinflammation.*

Biological Rationale: Mutations affecting the autophagy pathway are strongly genetically linked to ALS/FTD via familial and GWAS studies. UBQLN2^{P506T} is one such mutation at the intersection of the ubiquitin-proteasome system and autophagy that is sufficient to cause both ALS and FTD. To determine the effect of UBQLN2^{P506T} on autophagy in different cell types, I will use human induced pluripotent stem cells (hiPSCs) that have CRISPR knock-in UBQLN2^{P506T}, differentiated into cortical-like neurons (iCN), lower motor neurons (iLMN) which I have previously cultured and validated, as well as microglia (iMG). This model system has many benefits: the hiPSC cell line is highly characterized; UBQLN2^{P506T} has a matched corrected revertant (WT^{R}) so controls are isogenic; precise spatial and temporal live imaging experiments are possible in vitro; the ability to expand the hiPSCs allows for flexibility in experimental set up; human instead of murine biology will be studied; and in the future the experimental paradigms can be extended into patient derived iPSC models.

<u>Aim 1 Neurons</u>: Autophagy of mitochondria in WT, UBQLN2^{P506T}, and WT^{R} iCN and iLMN. UBQLN2^{P506T} impairs the clearance of ubiquitinated proteins by the proteasome, resulting in the accumulation of ubiquitinated proteins in iLMNs and iCNs (Fig.1A). These proteins are alternatively targeted to autophagosomes, that stresses the autophagy pathway shown by the accumulation of LC3-postive puncta (Fig.1B). *I predict that the* UBQLN2^{P506T} *neurons cannot efficiently degrade their normal cargo, in particular mitochondria.*

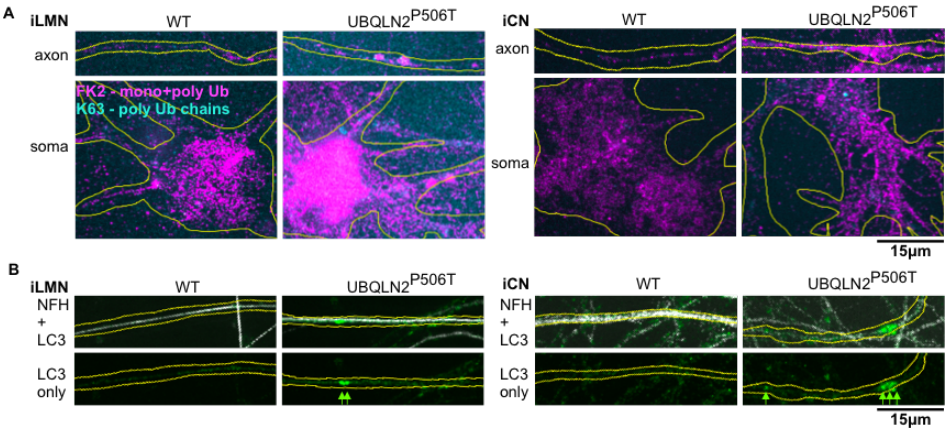

Figure 1: Accumulation of ubiquitinated proteins and LC3 puncta in UBQLN2^{P506T} iNeurons. A. iLMN and iCN (days post induction = DPI14) stained with antibodies to mono and poly ubiquitin. The cell outline is traced in yellow, and axon and soma are annoated. **B**. iLMN and iCN stained with antibodies to Neurofilament H (gray) to mark the axons, and LC3B (green) to identify autophagosomes. Arrows highlight puncta accumulating in the axon

Aim 1A: Characterize the rates of autophagy. Autophagy delivers cargo to the acidic lysosome for degradation. The observed accumulation of LC3 in UBQLN2^{P506T} neurons could be caused by increased autophagosome biogenesis or impaired degradation. To address which occurs, I will perform two common autophagy flux assays. First, I will immunoblot for endogenous LC3 in the presence and absence of Bafilomycin A (BafA) to inhibit autophagosome degradation. If biogenesis is increased in the UBQLN2^{P506T} cells, BafA treatment will raise LC3-II levels. If degradation is impaired, then BafA will not increase LC3-II levels. Second, I will perform live imaging in axons expressing mCh-GFP-LC3, a reporter that distinguishes between newly formed autophagosomes (mCherry and GFP positive) and acidic autophagosomes (GFP is quenched, mCherry positive only). I will quantify the rate of formation of autophagosomes, and the percent of trafficking acidified autophagosomes per micron per second. *I predict that there is more autophagosome biogenesis in* UBQLN2^{P506T} *neurons to compensate for the increased demand placed on the pathway.* If there is delayed acidification of autophagosomes in the UBQLN2^{P506T} iCN or iLMN, I will measure secretion by immunoblotting conditioned media for TSG101, as I have recently found this to be a compensation pathway in a Parkinson's disease model (Goldsmith et al., BioRxiv, 2022).

Aim 1B: Autophagic engulfment of mitochondria containing mtDNA/TFAM. Autophagy engulfs mitochondria containing mtDNA at steady state in iCNs (Goldsmith et al., Neuron, 2022). I will determine whether it also occurs in iLMNs and how it may change because of UBQLN2^{P506T}. *I predict the increase in ubiquitinated cargo caused by UBQLN2^{P506T} disrupts the ability to efficiently remove mitochondria containing mtDNA.* To test this, I will perform two experiments that I have optimized during my postdoctoral training: First, I will quantify the percent of autophagosomes (LC3+) that contain mitochondria (COX8A+) with mtDNA (TFAM+) by live imaging in WT, UBQLN2^{P506T}, and WT^{R} iCN and iLMN co-expressing COX8A-BFP, SNAP-TFAM, and GFP-LC3; Second, I will quantify the amount of TFAM in autophagosomes following an enrichment protocol from the WT, UBQLN2^{P506T}, and WT^{R} iCN and iLMN. I expect less TFAM within the autophagosomes in the UBQLN2^{P506T} compared to control neurons by both assays. Next, I will measure the total levels of mtDNA by qPCR and quantify the number of mtDNA and TFAM puncta within mitochondria in the axon and soma by immunocytochemistry. Preliminary data suggests there is more mtDNA in the axons, although to different degrees in iLMNs and iCNs (Fig.2). However, mtDNA copy number is very tightly regulated, indicating there may be compensation if autophagic engulfment is impaired. I will measure rates of mtDNA replication (predicted decrease) using the Click-iT EdU kit to pulse label mtDNA, and amount secreted (predicted increase) by immunoblotting for TFAM in conditioned media.

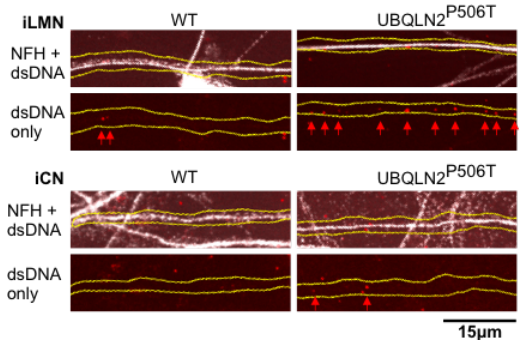

Figure 2: Accumulation of mtDNA in UBQLN2^{P506T} iLMN. iNeurons (DPI21) were stained with Neurofilament H (gray) to mark the axons, and dsDNA (red) to identify mtDNA. Arrows highlight puncta accumulating in the axon.

Aim 1C: Autophagic clearance of mitochondria after damage. Clearance of damaged mitochondria by mitophagy is a separate pathway to the basal autophagic engulfment of mitochondria containing mtDNA. *I predict that* UBQLN2^{P506T} *neurons will be less efficient at clearing damaged mitochondria, which will negatively impact the ability of the neurons to metabolically recover.* I will perform live imaging experiments in WT, UBQLN2^{P506T}, and WT^{R} iCN and iLMN expressing GFP-LC3, COX8A-BFP, SNAP-TFAM and stained with the membrane potential sensor TMRE to discern damaged mitochondria, before and up to 6 hours following a 1hr pulse treatment with the mitochondrial damaging agent Antimycin A (AA; 100nM). I expect that damaged mitochondria will persist longer unengulfed by autophagosomes. To test metabolic recovery, I will measure the baseline mitochondrial respiration and recovery after mitochondrial stress using the Agilent Seahorse XF Mito Stress kit, and monitor local ATP levels before and recovering from mitochondrial damage using the GoATeam ATP sensor.

<u>Aim 2 Microglia</u>: Autophagy of mitochondria in WT, UBQLN2^{P506T}, and WT^{R} iMG. Higher levels of mtDNA or slower clearance of damaged mitochondria in UBQLN2^{P506T} cells should increase the likelihood of promoting a pro-inflammatory response. However preliminary data suggests that there is little change in the inflammatory response in neurons (Fig.3). Microglia are the resident immune cells of the CNS that regulate neuroinflammation. An intense microglia immune response has been associated with motor neuron degeneration in ALS patients and in a mouse model of FTD (Ashford et al., Neuropathol. Appl. Neurobiol, 2021; Lui et al., Cell, 2016). Furthermore, dysregulation of autophagy affects the innate immune functions of microglia (Plaza-Zabala et al., Int. J. Mol. Sci, 2017). *Thus, I predict that disrupted autophagy caused by* UBQLN2^{P506T} *will impair the phagocytic capacity and the inflammatory response of iMG.*

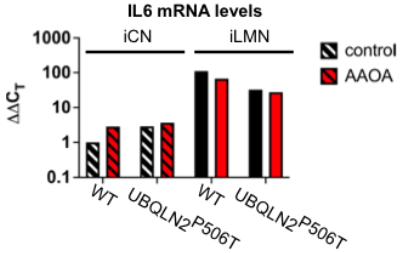

Figure 3: *Il6* is not consistently increased in UBQLN2 mutant iNeurons. iCN or iLMN (DPI21) were mock treated or treated with the mitochondrial poisons Antimycin A (100nM) and Oligomycin A (5µM) (AAOA) for 6hr and mRNA levels of the proinflammatory molecule IL6 were quantified by qPCR, normalized to *Gapdh*.

Aim 2A: Characterize the rates of autophagy. As described in Aim1A, I will immunoblot for endogenous LC3 with and without BafA treatment, and live imaging the mCh-GFP-LC3 reporter in control and UBQLN2^{P506T} iMG. Additionally, I will perform immunocytochemistry for LC3 and ubiquitin puncta. *I predict* UBQLN2^{P506T} *stresses the autophagy pathway in iMG*.

Aim 2B: Does UBQLN2^{P506T} affect microglial function? I will characterize the ability of the WT, UBQLN2^{P506T}, and WT^{R} iMG to phagocytose and degrade using a DQ-BSA assay. In this experiment, the DQ fluorescent signal is quenched until the BSA is phagocytosed and cleaved in the lysosome. I will monitor the amount of fluorescent signal produced over a timecourse of 8 hours. *I predict that impaired autophagy will delay the ability of the UBQLN2^{P506T} microglia to efficiently degrade their cargo.* I will use ELISA and qPCR to monitor production of pro-inflammatory molecules IL-1β, IL6, TNFα and complement C1q following lipopolysaccharide (LPS) stimulation, a canonical proinflammatory molecule, in WT, UBQLN2^{P506T}, and WT^{R} iMG. *I predict that impaired autophagy will lead to the impaired release of pro-inflammatory cytokines*, as the secretion of IL-1β from macrophages relies on functional autophagy (Dupont et al., EMBO, 2011).

Aim 2C: Quantify autophagic engulfment of mitochondria. Autophagic engulfment of mitochondria containing mtDNA occurs in iCN but does not occur in pre-differentiated hiPSCs (Goldsmith et al., Neuron, 2022). *I predict that the engulfment of mitochondria with mtDNA, marked by TFAM, is unique to neurons and will not occur in iMG.* To test this hypothesis, I will perform autophagosome enrichment from WT, UBQLN2^{P506T}, and WT^{R} iMG and immunoblot for mitochondrial markers and TFAM. I expect that there will not be an enrichment of TFAM in iMG, and this will not be affected by UBQLN2 ^{P506T} highlighting the cell type specificity of this process. However, if there is engulfment of mtDNA containing mitochondria in iMG, and it is impaired by UBQLN2^{P506T}, I will measure the total mtDNA levels by qPCR, and the sensitivity of WT, UBQLN2^{P506T}, and WT^{R} iMG to activating the cGAS-STING pathway or NLRP3 inflammasome following LPS stimulation by immunoblotting for phosphorylated TBK1 and cleaved caspase 1.

Aim3 Neuron-Microglia interaction: Inflammation and synapse loss caused by UBQLN2^{P506T}. I will investigate how control and UBQLN2^{P506T} neurons and microglia respond to secreted signals.

Aim 3A: Secreted factors from neurons affecting iMG inflammatory response. If I observe secretion of TFAM, mtDNA or production of IL-1β, IL6, or TNFα from UBQLN2^{P506T} iCN or iLMN at baseline or following low levels of mitochondrial damage (Results from Aim1), I will perform conditioned media transfer experiments from WT^{R} and UBQLN2^{P506T} iCN or iLMN to WT^{R} and UBQLN2^{P506T} iMG. I expect that the media from the UBQLN2^{P506T} neurons will cause iMG to become proinflammatory, marked by increased Iba1 levels assayed by immunocytochemistry. Instead of using AA to damage mitochondria, that will remain in the media transferred, I will alternatively transfect the neurons with MitoKillerRed targeted to mitochondria, which will damage the mitochondria by a rapid burst of reactive oxygen species when exposed to the inducing wavelength of light. A caveat is that most microglia in culture will express Iba1 at baseline therefore the increase may be limited in detection. I will also assay for increases in IL-1β, IL6, TNFα and complement C1q secretion by ELISA in WT, UBQLN2^{P506T}, and WT^{R} neuron conditioned media pre-transfer and post-transfer on WT, UBQLN2^{P506T}, and WT^{R} iMG. If UBQLN2^{P506T} iMG have impaired release of pro-inflammatory molecules, I will investigate whether boosting autophagosome biogenesis using rapamycin, spermidine, or rilmenidine increases release.

Aim 3B: **Secreted factors from iMG causing deleterious synapse pruning**. Microglia contribute to synapse loss by complement C1q production. I expect that conditioned media from iMG stimulated with LPS will cause synapse loss on WT iCN or iLMN neurons, assayed by the quantification of the presynaptic marker synaptotagmin with the post-synaptic marker PSD-95 using SynapseJ automated analysis. Then, I will test whether conditioned media from UBQLN2^{P506T} iMG is as efficient at synapse pruning as control following LPS stimulation. To test whether secretion of C1q is responsible for synapse loss, I will mix the C1q inhibitor chondroitin 4-sulfate proteoglycan or the nanobody-based inhibitor C1qNb75 into the iMG conditioned media before transfer. If conditioned media from UBQLN2^{P506T} neurons contains proinflammatory molecules (Aim3A), I will test whether it is sufficient to induce aberrant synapse pruning when transferred to iMG. A potential roadblock is that iNeurons in culture do not form many synapses. If so, I will culture iCN on primary rat astrocytes to increase synapse number before iMG conditioned media transfer occurs. In the future, I will examine direct co-culture of iNeurons with iMicroglia to investigate the contribution of phagocytosis to synapse pruning.

Statement of relevance to ALS therapeutic strategies and/or biomarkers: While autophagy has long been considered an attractive therapeutic target, enhancers of autophagy have broadly failed in clinical trials. Thus, these cell biology studies may explain some of the challenges and identify potential for therapeutic intervention. For example, if autophagosome biogenesis is increased, then trying to boost autophagy further may be ineffective. If there is disrupted clearance of the autophagosomes, enhancing the autophagosomes-lysosome fusion or lysosome function may be a better target. Furthermore, these studies will suggest whether targeting autophagy in microglia would be beneficial, aiding in the degradation of pathological proteins and organelles, or harmful, increasing the secretion of proinflammatory molecules. Recently, PIKFYVE inhibition has been shown to increase secretion and ameliorate neuron survival in various ALS associated mutations (Hung et al., Cell, 2023). It is of critical importance to understand if there is secretion of mtDNA from autophagy impaired neurons, and how further increasing secretion might impact microglia activation and CNS inflammation.

Description of planned career path in ALS research: My long-term scientific goal is to study how perturbations to autophagy contribute to ALS/FTD progression. The experiments proposed here will establish a foundation for an independent investigator position, distinguished from my current supervisor, studying the impact of disrupted autophagy in ALS/FTD on neuronal-microglial interactions and inflammation. I will be able to bring the tools and reagents I create to my new position. My preliminary data was supported by the Healey Center, which has been an incredible platform for critical feedback and has provided excellent training in ALS/FTD biology. Future directions of my independent laboratory will be to translate these findings using control and patient derived hiPSC-derived neurons and microglia from idiopathic disease and other known mutations.