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UNIVERSITY OF CALIFORNIA,
IRVINE

PPAR δ Regulation of Microglia Function in Health and Neurodegenerative Disease

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biomedical Sciences

by

Jacob Sahag Deyell

Dissertation Committee:
Professor Albert Russell La Spada, Chair
Professor Kim Green
Professor Elizabeth Head
Professor Edwin Monuki
Associate Professor Vivek Swarup

2024

DEDICATION

A thank you to family, friends, mentors, colleagues and others who have helped me along this journey. It certainly takes a village - and I am eternally grateful for mine.

TABLE OF CONTENTS

	Page
LIST OF FIGURES	iv
LIST OF TABLES	vi
ACKNOWLEDGEMENTS	vii
VITA	viii
ABSTRACT OF THE DISSERTATION	ix
INTRODUCTION	1
CHAPTER 1: PPAR δ Primes Microglia to Properly Respond to AD Pathology	15
CHAPTER 2: Phase Separation of PPAR δ	53
CHAPTER 3: Selective vulnerability in Huntington's Disease: From excitotoxicity, mitochondrial dysfunction, and transcription dysregulation to therapeutic opportunity	65
CHAPTER 4: Discussion	80
REFERENCES	86

LIST OF FIGURES

		Page
Figure 1	Expression of PPAR δ in various CNS cell types	9
Figure 2	Expression of the three PPARs in microglia	10
Figure 3	Design of experiment for KD3010 RNA-seq experiment	15
Figure 4	Bulk RNA-seq analysis of mice treated with PPAR δ agonism	17
Figure 5	PPAR δ agonism reduces inflammation in iTF-Microglia stimulated with LPS	20
Figure 6	PPAR δ agonism upregulates the phagocytosis of disease-relevant substrates	22
Figure 7	PPAR δ agonism reduces migration toward ADP and dying neurons	25
Figure 8	Brain-powder treatment induces a DAM-Like state in iTF-Microglia	28
Figure 9	Verification of PPAR δ agonist and antagonist treatment	29
Figure 10	Clustering of iTF-Microglia after treatment	32
Figure 11	Populations in Homeostatic II and Enrichr analysis of the Homeostatic II population	34
Figure 12	Enrichr analysis of DAM-Like vs Homeostatic II Treated iTF-Microglia in the DAM-Like population	35
Figure 13	Differential gene expression based on PPAR δ treatment in iTF-Microglia	37
Figure 14	Gene ontology analysis of PPAR δ agonist treated cells compared to vehicle treated cells in the DAM-like cluster	38
Figure 15	PPAR δ antagonism in the DAM-Like cluster downregulates lipid metabolism	40
Figure 16	PPAR δ interacts with PU.1	42
Figure 17	PPAR δ sequence analysis reveals PPAR δ is capable of phase separation	54

Figure 18	PPAR δ displays typical nuclear speckling of phase-separated proteins	55
Figure 19	PPAR δ activation of targets is dampened in the presence of 1,6-Hexanediol	57
Figure 20	PPAR δ activation of targets is dampened in the absence of Mediator 1	59
Figure 21	Knockdown of Med1 in iTF-Microglia blunts response to PPAR δ agonism	60
Figure 22	PPAR δ interacts with Mediator 1, and this interaction is disrupted in the presence of PU.1	61
Figure 23	PU.1 sequence analysis reveals PU.1 is capable of phase Separation	62
Figure 24	Selective vulnerability in neurodegenerative disease	67
Figure 25	Mitochondrial toxicity in Huntington's disease	70

LIST OF TABLES

	Page
Table 1	
Candidate Therapies for Huntington's Disease Mitochondrial Dysfunction	76

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VITA
Jacob Sahag Deyell

2017	B.S. in Chemical and Biomolecular Engineering, Johns Hopkins University
2019-2022	Medical Student Tutor, University of California, Irvine
2020	Fannie J. and Paul J. Marasco Outstanding Student Award, University of California, Irvine
2024	Ph.D. in Biomedical Sciences, University of California, Irvine

FIELD OF STUDY

Neurodegeneration and Neurotherapeutics

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ABSTRACT OF THE DISSERTATION

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by

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University of California, Irvine, 2024

Professor Albert La Spada, Chair

Alzheimer's disease (AD) is a devastating neurodegenerative disease characterized by progressive memory loss¹. Microglia, the resident immune cells of the central nervous system (CNS), have been heavily studied in their relation to the disease, as they exhibit both neuroprotective and neurodegenerative effects²⁻⁴. Our lab has shown the transcription factor PPAR δ to be neuroprotective in Huntington's disease⁵, and microglia are among the highest expressers of PPAR δ in the CNS^{6,7}. Additionally, PPAR δ is currently the focus of a Phase II clinical trial for AD⁸, thus understanding the effects of PPAR δ in microglia is paramount. To address this, we began by taking an unbiased transcriptomic approach. Mice were treated with PPAR δ agonist for six weeks and gene expression changes in microglia were assessed. We then moved to carry out experiments in an iPSC-derived model of microglia. We utilized experimental procedures including cytokine arrays, phagocytosis assays, migration assays, and single-cell transcriptomics to further characterize the microglial response to PPAR δ agonism.

Preliminary transcriptomic results revealed that long term treatment with PPAR δ agonism reduced inflammatory processes in microglia *in vivo*. Follow-up studies in the

iPSC models of microglia further bolstered that PPAR δ agonism reduces secretion of pro-inflammatory mediators, reduces migration of microglia, and increases phagocytosis of disease relevant substrates (e.g., amyloid-beta and synaptosomes). Single-cell transcriptomics of the iPSC-derived microglia tie these findings together and demonstrate that PPAR δ agonism shifts microglia to a state that is high in lipid-processing and phagocytic genes, but represses inflammatory mediator production.

We also set out to understand the mechanistic basis of PPAR δ activity in microglia after preliminary data revealed that PPAR δ is capable of phase separation. Though more experimentation in this area is necessary, preliminary results indicate that PPAR δ undergoes phase separation with the transcriptional coactivator Mediator 1 (Med1) to carry out its function. Additionally, PPAR δ interacts with PU.1, a master transcription factor for microglia that is a genetic risk factor for AD⁹, and this interaction is decreased by PPAR δ agonist treatment.

The culmination of these experiments suggests that PPAR δ agonism may be beneficial in the AD brain because it appropriately activates microglia to respond to AD pathology (e.g., phagocytosis) while reducing subsequent inflammation, and that these functions are related to PPAR δ interactions with Med1 and PU.1.

INTRODUCTION

Alzheimer's disease

Alzheimer's disease (AD) is a devastating neurodegenerative disorder that is characterized by progressive memory loss and impairment of behavioral functions¹. Neuropathologically, it is classified by the accumulation of amyloid-beta (A β) plaques and neurofibrillary tau tangles in the brain¹. The accumulation of these proteins can result in neuronal dysfunction and death, with eventual deterioration of cognitive function. Though AD is the most common cause of cognitive decline, there remains no known cause for sporadic cases of AD, which make up about 90% of the cases¹⁰, and no cure. There have been many recent efforts to develop effective therapies for AD, with some focused on targeting A β itself by using monoclonal antibodies directed against the protein¹¹. This is largely because of the amyloid hypothesis of AD, which posits that the accumulation of A β is a central driver of disease¹². One such drug, Lecanemab, has shown some efficacy in slowing the progression of AD in the early stages of disease¹³; however, targeting A β for clearance does not cure a patient of disease, which further bolsters the point that the root cause of AD is not yet understood. In addition to attempts to remove A β from the brain, there is much ongoing research surrounding other key players in the disease, including microglia¹⁴.

Microglia: From function, to development, to states

Microglia are the resident immune cells of the central nervous system (CNS)⁴. Under physiological conditions, their typical functions are to surveil the brain for any foreign material, pathogens, or other "danger signals". Upon encountering such a signal,

microglia become active and mount a response to address the issue, carrying out functions such as cytokine secretion, chemotaxis, and phagocytosis¹⁴. The role of neuroinflammation becomes controversial in the context of neurodegenerative diseases because while there is evidence for microglial protection against disease, microglia can also contribute to the disease state of the brain⁴. Microglia were traditionally viewed as hyperactive in the setting of neurodegenerative disease and were seen to have the ability to take on several distinct disease-related phenotypes. Two of the first of these states of microglia to have been described are Disease Associated Microglia (DAM) and Microglial Neurodegenerative Phenotype (MGnD)^{15,16}. Overall, these microglial states are characterized by a downregulation of homeostatic markers (e.g. *P2RY12* and *TMEM119*) and upregulation of pro-inflammatory/“activated” markers (e.g. *CLEC7A* and *SPP1*). Importantly, microglial phenotypes in neurodegenerative disease are cell autonomous, shown through studies in induced pluripotent stem cell (iPSC)-derived microglia that harbor genetic risk alleles relevant to AD and other neurodegenerative conditions^{17,18}.

However, ongoing research has demonstrated that microglial states are much more complicated than simply “homeostatic” versus “activated”. A recent review article written by many leading microglia experts calls for the language surrounding microglia to be more specific¹⁹. For example, instead of writing that microglia are in an activated state, one should refer to the exact sort of stimulus that actually activated the microglia. Many research groups have conducted single-cell sequencing on microglia to analyze the many different states of microglia that exist, both *in vitro* and *in vivo*. A recent paper by Dr. Li Huei Tsai’s lab demonstrates 12 different states of microglia in the human AD brain²⁰.

This highlights the complexity of microglia and that although they are a single cell type, at any point in time, many different cells may exist in varying states and modes of operation. This also underscores how important single-cell transcriptomics are for understanding microglia. Bulk-sequencing, while still powerful and capable of showing large changes, can average out some of the changes of microglial state that might be experienced after a treatment. Single-cell transcriptomics allows for visualization of all of the microglial states in a population and how treatments with various stressors or therapeutics lead to changes in the populations.

To touch on the origin of microglia, they are of the myeloid lineage and originate in the embryonic yolk sac²¹. Microglia colonization of the brain happens during the same time as does vascularization of the brain, radial glia formation, neuronal migration and myelination²¹. This colonization occurs through a variety of mechanisms, including traveling through the vasculature as well as through brain ventricles. These cells are maintained separately from the other myeloid lineage cells in the blood. During development, microglia also carry out critical roles. These include neurogenesis, synaptic remodeling and myelination to name a few. After the cells have completely populated the brain during development, microglia continue in a manner with slow turnover²². This changes in the presence of a stimulus such as AD, where they experience a rapid response of proliferation to encountering such pathology, and this proliferation has been seen to contribute to disease progression as they enter replicative senescence²².

A major source of controversy in the field pertains to whether microglia are protective against AD and other neurodegenerative conditions or whether they contribute to the ongoing pathology. This is further complicated by the fact that there is solid data for both sides. Microglia have been shown to aid in the propagation of A β through the mouse brain²³. Additionally, when microglia are eliminated from the 5XFAD mouse brain by treatment with a CSF1R inhibitor, the plaque burden of the mice is significantly reduced, as is the neuronal loss^{2,3}. These two examples point to microglia contributing negatively to disease. However, at the same time, there is data pointing to microglial neuroprotection. They are the main cells in the brain to help clear debris build-up⁴. They also form barriers around A β that can help prevent the spread throughout the brain²⁴. The fact that there is strong evidence on both sides supporting a beneficial and detrimental role suggests that there may be some sort of temporal dependence on these actions.

Before further describing these roles, it is important to consider what makes microglia detrimental to disease. As described above, this is still an area of active study in the field and is heavily debated. However, in this controversy, one can look at the genetics of the disease. Harboring the *APOE4* allele or *TREM2* mutations are two of the greatest risk factors for developing sporadic cases of AD²⁵. *APOE4* microglia have been extensively characterized and have been shown to have many deficits, including impaired uptake of amyloid-beta and the inability to take on a “DAM” state^{17,26}. The inability to become properly activated is thought to be mediated through the transcription factor PU.1²⁶. Similarly, *TREM2* knockout microglia have been shown to be “locked” in a homeostatic state¹⁸. These microglia that harbor these disease risk genetic changes are

unable to properly carry out their role of responding to pathology in the brain, suggesting that the ability of microglia to respond to pathology is a neuroprotective role, at least in the beginning stages of disease. Later on in disease, it is possible that microglia become overwhelmed, hyperactive and hyperinflammatory²⁷.

Additionally, what actually activates microglia into their more activated states is a topic of recent intrigue. Certainly, intercellular cytokine signaling and other mechanisms play a role, but in an AD brain, what is causing microglia to take on an activated state? An interesting study was carried out by the Stevens lab where they utilized single-cell sequencing to interrogate whether different stressors had the capacity to induce a DAM state in iPSC-derived microglia²⁸. The different stressors used in the study include A β , apoptotic neurons, myelin and synaptosomes. For many years prior to this study, a lot of focus has been given to the interaction of microglia with A β and tau, two of the proteins that can accumulate in the brains of AD patients. This is the area of focus for many other neurodegenerative diseases as well, such as α -Synuclein in Parkinson's disease²⁹. However, what this study demonstrated is that the interaction of microglia with brain substrates, rather than A β , induced more of a diseased state in the microglia²⁸. Dying neurons and myelin debris led to more of an activated phenotype than did the pathologic proteins A β and tau. This is an interesting finding because it shows more of a connection between neurodegenerative diseases than focusing on the protein accumulating. The microglia are responding to the damaged brain.

Leaning back on genetics, one of the reasons why microglia have been so heavily studied in AD is because genome-wide association studies (GWAS) have linked several genes in AD that are either heavily or exclusively expressed in microglia^{30,31}. Several of these genes are regulated by the master transcription factor PU.1, which itself has also been identified as a genetic risk factor for AD, as higher expression of PU.1 is associated with increased risk for AD^{9,32,33}. PU.1 is encoded for by the *SPI1* gene, and conversely, there are polymorphisms in this gene that can lower the expression of PU.1, thus reducing the risk of AD. Because of the implication of PU.1 in AD, many groups have attempted to develop therapies targeting PU.1. One such study was carried out by Dr. Tsai's group and identified a molecule that was able to recruit a repressive complex to PU.1 in order to inactivate the protein³⁴. Through this inactivation, they found a decrease in neuroinflammation, neuronal loss and AD pathology in their mice. Side effects of this compound, however, have yet to be elucidated. How exactly PU.1 contributes risk to AD remains unknown; however, it is likely due to its roles in regulating inflammatory processes as well as its role in maintaining a homeostatic microglial signature^{26,34}.

The above studies demonstrate that, while microglia can certainly play beneficial roles in the AD brain, they are also active players in the disease. Treatments targeting these cells to promote their beneficial functions while preventing them from carrying out their detrimental functions is paramount. One such potential target is Peroxisome Proliferator-Activated Receptor delta (PPAR δ), which was also the focus of a Phase 2 clinical trial for mild-to-moderate AD⁸.

PPAR δ function and previous studies

PPAR δ belongs to the PPAR family, which consists of two other members - PPAR α and PPAR γ . The PPARs are ligand-activated transcription factors that are part of the nuclear hormone receptor superfamily and were initially heavily studied in the context of hyperlipidemia, metabolic syndrome and diabetes due to their role as lipid sensors³⁵. Beyond this action, however, the PPARs exhibit anti-inflammatory effects³⁶. PPAR γ is the most well-studied PPAR and there is an entire class of drugs dedicated to PPAR γ agonism that are used for patients with diabetes mellitus. Because PPAR γ has been so well studied in the field of metabolic conditions, it is also the leading PPAR being studied in the brain. However, the La Spada lab became interested in PPAR δ as an interactor with PGC-1 α in particular because it was discovered to be highly expressed in the CNS³⁷ - more so than PPAR γ . This led to a published study where my lab implicated PPAR δ in Huntington's disease (HD)⁵. My lab showed that PPAR δ interacts with huntingtin protein, that disrupting the function of PPAR δ in medium spiny neurons (a major site of HD pathology) caused mice to develop motor and neurodegenerative abnormalities, and that treatment of HD mice with a PPAR δ agonist rescued disease phenotypes. To disrupt PPAR δ function in the medium spiny neurons, a dominant negative mouse model was used. Dominant negative models work by expressing an inactivate version of the protein which one desires to manipulate. In the case of the PPAR δ dominant negative model, the transgene inserted into the mouse had an E411P mutation. Position 411 is located in the AF2 region of PPAR δ which is important for transactivation of target genes, dimerization and recruitment of coactivators⁵. This makes PPAR δ ^{E411P} ineffective at gene regulation

and diminishes PPAR δ activity in the cells where it is expressed. The transgene was also designed to be under the control of Cre-recombinase. It is preceded by a stop codon that is released in the presence of Cre. When the mice were crossed with CMV-Cre mice, the result was embryonic lethality³⁸. When the mice were crossed with Nestin-Cre mice, the result was severe neurologic abnormalities. The most interesting finding in these mouse crosses was that even disruption of PPAR δ activity solely in medium spiny neurons using Rgs9-Cre resulted in HD-like pathology. Transcriptomic studies done on these mice revealed that their aberrant transcriptional signatures compared to wild-type mice matched the transcriptional signatures of HD patients⁵.

Given the previous data from my lab, PPAR δ is likely very important for HD pathology, and all of this previous work demonstrated a neuroprotective role of PPAR δ in neurons. However, PPAR δ is much more highly expressed in microglia than it is in neurons (**Figure 1**)^{6,7}, leading us to become interested in its function in microglia. PPAR δ is also much more highly expressed than PPAR γ and PPAR α in microglia (**Figure 2A**)³⁹. This is incongruent with the number of studies focused on each PPAR in microglia, as PPAR γ is the most heavily studied member of the family (**Figure 2B**). While microglial PPAR δ is understudied, one group has shown that it is anti-inflammatory and neuroprotective in a mouse model of experimental autoimmune encephalomyelitis (EAE)⁴⁰. However, how PPAR δ carries out its functions and whether it applies to microglia in AD remains elusive.

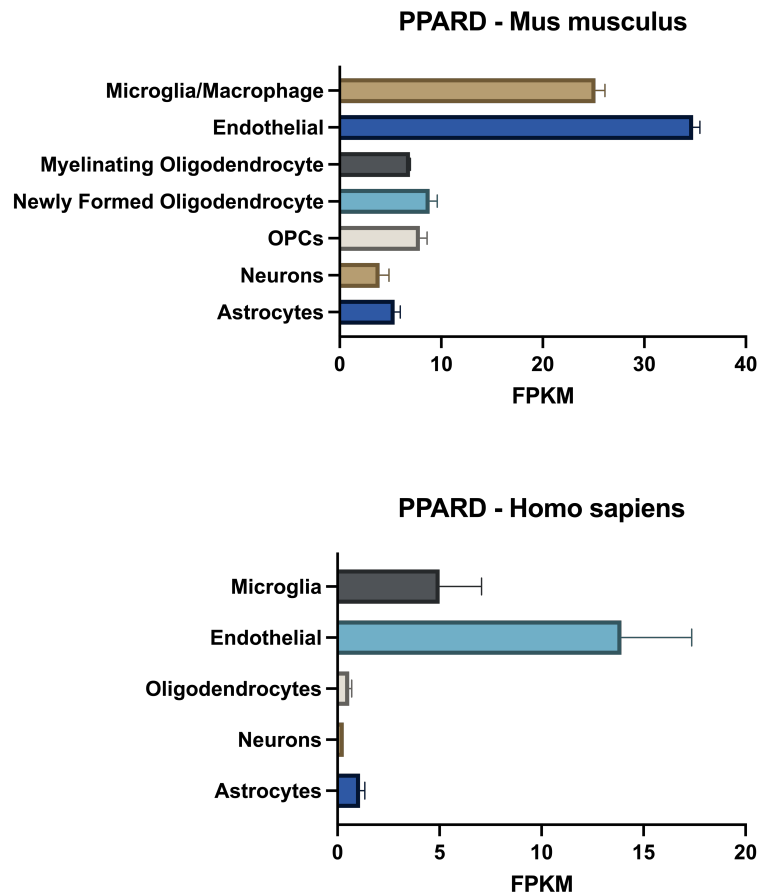
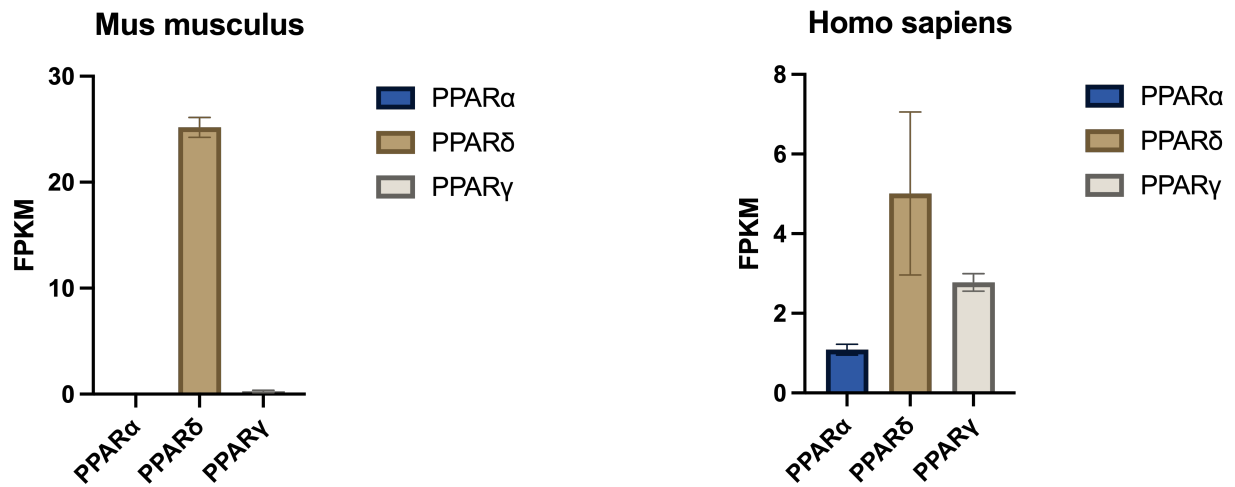


Figure 1. Expression of PPAR δ in various CNS cell types.

FPKM values of PPAR δ in CNS cell types. This data is adapted from Brain-seq resource from the Barres lab. These graphs reveal that microglia are among the highest expressers of PPAR δ in the CNS.

A



B

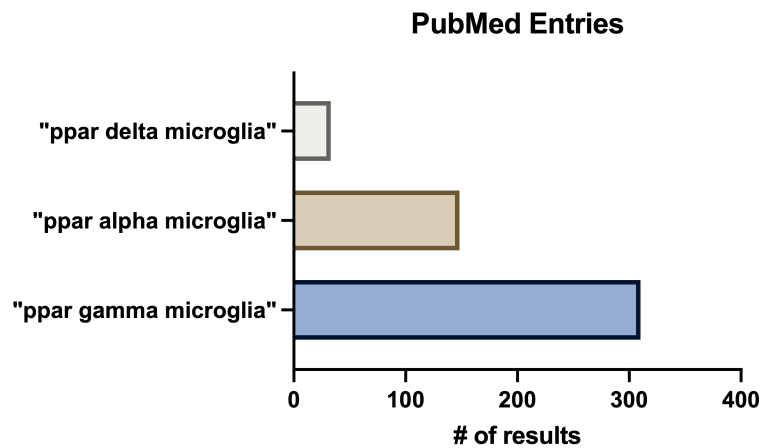


Figure 2. Expression of the three PPARs in microglia.

A) Here we see that PPARδ is the most highly expressed PPAR in both human and murine microglia. These FPKM values are taken from previous sequencing datasets from the Barres lab.

B) PubMed entries for searches of the different PPARs in microglia as of March 2024 reveals that PPARδ is the least published PPAR in microglia compared to other PPARs.

Phase separation

Mechanistically, the PPARs are capable of undergoing phase separation, a process by which the biophysical properties of molecules, such as proteins or nucleic acids, drives their self-organization into distinct membrane-less compartments in the cell, termed condensates⁴¹. Of particular interest to my research, a recent paper analyzed the phase separation of PPAR γ ⁴¹, showing that PPAR γ forms phase separated condensates *in vitro* and that phase separation was important for PPAR γ function. In the same paper, they also described that PPAR α and PPAR δ are capable of phase separation, but no further functional studies were carried out regarding the other PPARs.

Many transcription factors, including nuclear hormone receptors, phase separate with coactivators such as Mediator 1 (Med1) to carry out efficient gene regulation^{42,43}. This finding arose due to the fact that Med1 physically interacts with many different transcription factors. It led researchers to ponder whether instead of conserved sequences shared among several transcription factors, there might be a different interaction at play. Phase separation involves weak, multivalent interactions that form between different biomolecules, such as proteins and RNA⁴³. These interactions typically occur via the activation domains (ADs) of the transcription factor⁴³. In essence, the DNA-binding domain of the transcription factor is bound to DNA and the AD recruits coactivators and other molecules to regulate transcriptional control. Phase separation is a complex, dynamic process. There are some simple methods that can be used to preliminarily investigate phase separation, though. For example, some phase separated condensates are driven to form because molecules contain regions of disorder⁴³. These

can be regions of a protein that are more acidic in nature, that have a high concentration of certain amino acids like glutamine, serine or threonine, and can include low complexity domains, which are amino acid regions in a protein that, instead of utilizing more of a combination of the available 20 amino acids, utilize only a few⁴³. Tools can be used online to analyze these sequences such as the PONDR tool⁴⁴.

Models of microglia

When looking to study microglia, there are several different models as options, each with their own pros and cons. In terms of immortalized cell lines, BV2 cells are the most widely used line and are derived from mouse. However, there are several caveats to this model and it has been shown that they are not always a robust physiological representation^{45,46}. Primary microglia from mice provide a useful *ex vivo* model with which to study microglial function; however, once microglia are removed from the brain, they tend to adopt a more activated phenotype and lose their true microglial identity⁴⁷. Studying microglia *in vivo* in a mouse is a robust model⁴⁸. It provides a biologically relevant system to study function of cells where they are in their natural context. Still, there is always the question of how well findings in murine microglia might translate to human microglia. Thus, the advent of protocols to derive microglia-like cells from iPSCs began. These models allow for questions to be answered about human microglia specifically and are remarkably robust. Their gene expression signatures are strikingly close to those of human microglia, but this model is still qualified in that it does lack the surrounding environment of the CNS^{49,50}. A more recent model has involved the transplantation of human iPSC-derived microglia-like cells (iMGLs) into the brains of mice that have their

own microglia depleted⁵¹. These microglia populate the brain of the mouse and carry out their normal functions.

The model that I have elected to use for the majority of my studies is an iPSC-derived microglia-like cell from the lab of Dr. Martin Kampmann⁵². These cells are called iTF-Microglia. The cells are derived by the overexpression of six key microglial transcription factors that are important for microglial identity, function and development: PU.1, IRF5, IRF8, CEBP α , CEBP β and MAFB. The expression of these transcription factors is regulated by a doxycycline inducible system. This is combined with exposing the cells to 4 different cytokines: IL-34, M-CSF, GM-CSF and TGF β -1. After eight days, the cells are termed iTF-Microglia and are ready for use. These cells have been characterized transcriptionally by the Kampmann lab and have been shown to be similar to microglia. Additionally, these cells exhibit certain microglial properties, such as the ability to phagocytose various substrates, their response to stressors such as lipopolysaccharide (LPS) and their ability to be co-cultured with neurons. Altogether, these cells represent a rapid manner in which to study microglial function. What makes these cells especially useful – though I have yet to utilize this capability – is the incorporation of inducible CRISPRi and CRISPRa machinery into the cells. Thus, one can conduct CRISPRi screens and more easily manipulate the expression of genes in these microglia-like cells as compared to other models.

The iTF-iPSCs (the name for the iPSC precursor to iTF-Microglia) that I have for my experimentation is on the KOLF2.1J background⁵³. The KOLF2.1J cells are an iPSC

cell line that was recently derived by a large collaboration with the iPSC Neurodegenerative Disease Initiative sponsored by the NIH. It was designed as a single reference line that could be used for the study of neurodegenerative diseases. The line lacks any “obvious” disease mutations and was found to be readily differentiable into a variety of cell types. What makes this line especially useful is that the creators have used CRISPR to engineer many derivatives that harbor risk alleles for different neurodegenerative diseases. These include a whole host of single nucleotide variants (SNVs) that correspond to different diseases, such as an “APOE4” variant for AD. Each variant can be purchased as homozygous, heterozygous and as a revertant control to account for off-target changes due to CRISPR editing. The ability for further experimentation with these cells with a variety of SNVs was the main driver for the selection of these cells as a model. These engineered KOLF 2.1J iTF-iPSCs were graciously provided to us by Dr. Bill Skarnes.

Chapter 1: PPAR δ Primes Microglia to Properly Respond to AD Pathology

Introduction

PPAR δ is a ligand-activated transcription factor highly expressed in microglia that has been shown to be neuroprotective in models of HD and EAE^{5,6,40}. PPAR δ was the focus of a Phase II clinical trial for mild-to-moderate AD⁸, so we set out to explore the role that PPAR δ plays in microglia and how this role plays into its value as a therapeutic target. We began this work *in vivo* and then moved to an iPSC-derived microglial model as described in the introduction.

Results

PPAR δ Agonism Reduces Inflammation in Microglia in vivo

We utilized an unbiased approach to begin our study of the effect of PPAR δ agonism in microglia. Wild-type mice between the ages of 6 and 8 months were injected intraperitoneally for 6 weeks with either the potent PPAR δ agonist KD3010 or vehicle control (**Figure 3**). After the treatment period, microglia were isolated from the brains of these mice using CD11b magnetic beads, and RNA was extracted and prepared for bulk

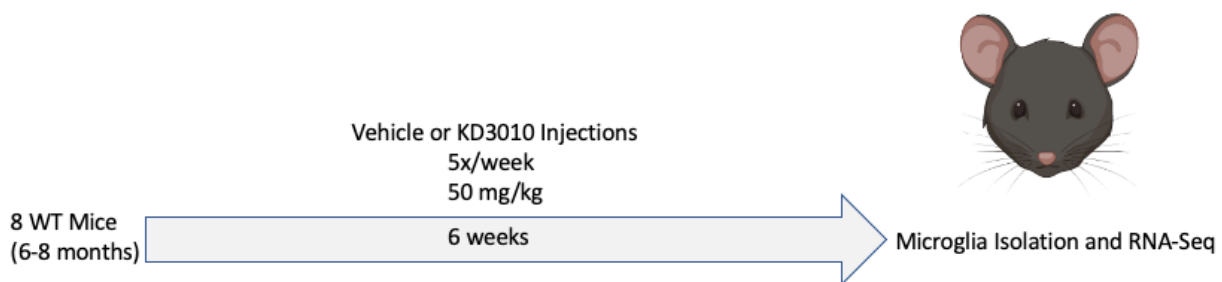


Figure 3: Design of experiment for KD3010 RNA-seq experiment.

Schematic for the treatment of the mouse injections of the PPAR δ agonist KD3010. 8 WT mice between the age of 6 and 8 months were treated with KD3010 for 6 weeks. Microglia were isolated, RNA was extracted and sent off for sequencing.

RNA-sequencing. Analysis of the data from this experiment revealed a total of 1,947 differentially expressed genes (DEGs) – 931 of which were upregulated and 1,016 of which were downregulated (**Figure 4A**). qRT-PCR verification was carried out for some targets to assess the validity of the experiment (**Figure 4B**). Interestingly, gene ontology (GO) analysis of the DEGs revealed a significant downregulation of immune activation, specifically with terms such as “cell migration” and “immune system process” (**Figure 4C**). This is in line with previous data showing that PPARs have anti-inflammatory effects in addition to their more traditional roles of lipid metabolism^{36,54}. Additionally, KEGG analysis on the data showed an upregulation of endocytic processes, which pointed to an increase in the ability of the microglia to phagocytose. A closer look at the list of DEGs showed that several inflammatory mediators are significantly downregulated (e.g., *IL-1 β* and *ITGAX*), as well as some traditional disease associated microglia (DAM) markers (e.g., *CLEC7A* and *SPP1*) and AD associated genes (e.g., *TYROBP*). The directionality of these changes was particularly interesting because excessive inflammation is typically considered to contribute to neurodegeneration²⁷, and PPAR δ agonism appeared to blunt that. Additionally, with an increase in endocytic processes, it may indicate that clearance of disease-relevant substrates might be enhanced.

As an interesting secondary finding, when analyzing the DEGs from this experiment with the online gene set search engine Enrichr^{55–57}, a significant predicted transcription factor from the ChEA transcription factor database for our DEGs was PU.1. ChEA is a widely used tool that works by computing over-representation of targets in a

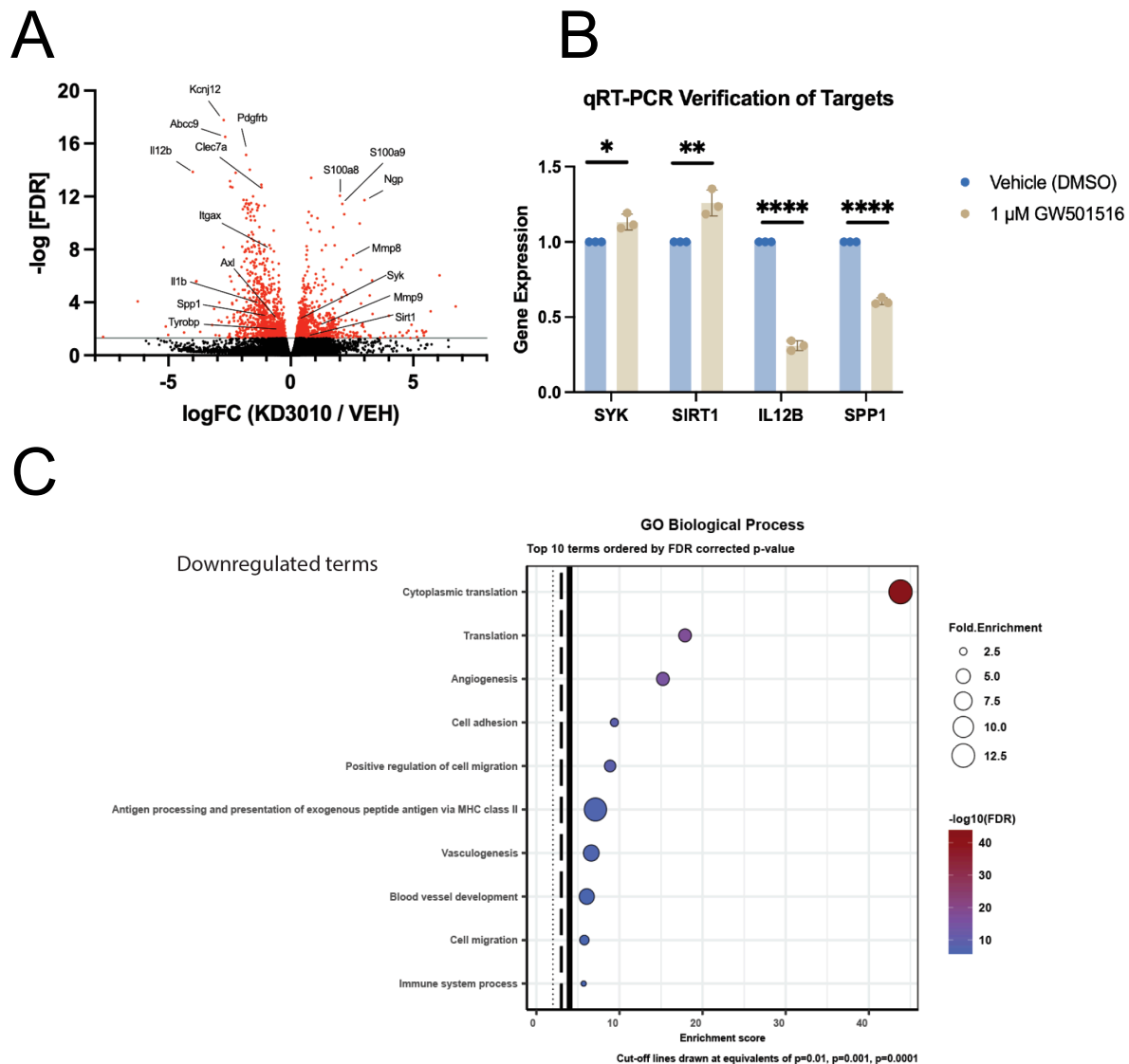


Figure 4: Bulk RNA-seq analysis of mice treated with PPAR δ agonism.

A) Volcano plot showing up- and down-regulated genes, with select genes labeled. Significance level of $p < 0.05$.

B) qRT-PCR verification of the effects of PPAR δ agonism in microglia. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$.

C) GO analysis of down-regulated biological processes by PPAR δ agonism *in vivo* after 6 weeks of treatment.

gene list for different transcription factors based on the ChIP-X database⁵⁸. Roughly 20% of the DEGs from our PPAR δ agonist experiment share a signature with the transcription factor PU.1, a master transcription factor in microglia that has been repeatedly identified as an AD risk gene^{33,34}. When these overlapping 20% of genes (between PU.1 and

PPAR δ) from the transcription factor prediction analysis were run through GO analysis, inflammatory processes emerged as being altered. This is consistent with the role of PU.1 in regulating immune processes in microglia³⁴.

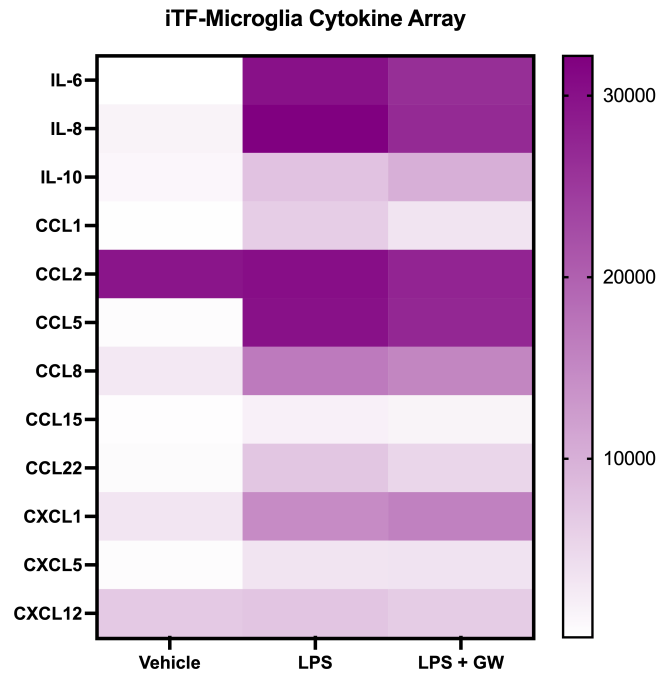
Altogether, the changes observed in this RNA-sequencing experiment demonstrated that PPAR δ agonism significantly altered the signature of microglia and led us to investigate PPAR δ agonism further using an iPSC-derived model of microglia.

PPAR δ Agonism Reduces the Release of Proinflammatory Cytokines from iTF-Microglia

From our transcriptomic analysis, we noted that PPAR δ agonism reduced the expression of a variety of inflammatory genes. To better characterize this at a phenotypic level, we assayed the release of cytokines from our iPSC-derived microglia model (iTF-Microglia) when stimulated with lipopolysaccharide (LPS) with or without the presence of PPAR δ agonism. LPS is a bacterial endotoxin widely used as a potent inducer of microglial inflammatory pathways through the NF- κ B pathway and Toll-Like Receptor 4 pathway⁵⁹. We incubated the conditioned media from iTF-Microglia with these different conditions on a human cytokine array from Abcam for 42 different targets. From this, roughly 29% (12/42) of cytokines had robust secretion when the iTF-Microglia were exposed to LPS (**Figure 5A**). Compared to LPS condition, treatment with the PPAR δ agonist GW501516 (GW) reduced the secretion of several of these cytokines. GW is used for all *in vitro* assays because it is more cost effective than KD3010 and blood-brain permeability is not required. Additionally, it has been shown to have similar beneficial effects to KD3010 in previous work⁵. The heatmap demonstrates those that those

cytokines that increased in the presence of LPS experienced an overall decrease in the presence of PPAR δ agonism, with the exception of IL-10 and CXCL1, which increased. IL-10 is a potent anti-inflammatory cytokine, which fits with the overall decrease in inflammatory response we observed in the iTF-Microglia upon PPAR δ agonism. Notably, several are members of the chemokine and interleukin families. Those with significant changes are graphed in **Figure 5B**. These genes have to do with chemotaxis and inflammatory pathways, and the fact that there is an overall downregulation in expression of these markers is in line with our transcriptomic data and ontology analysis.

A



B

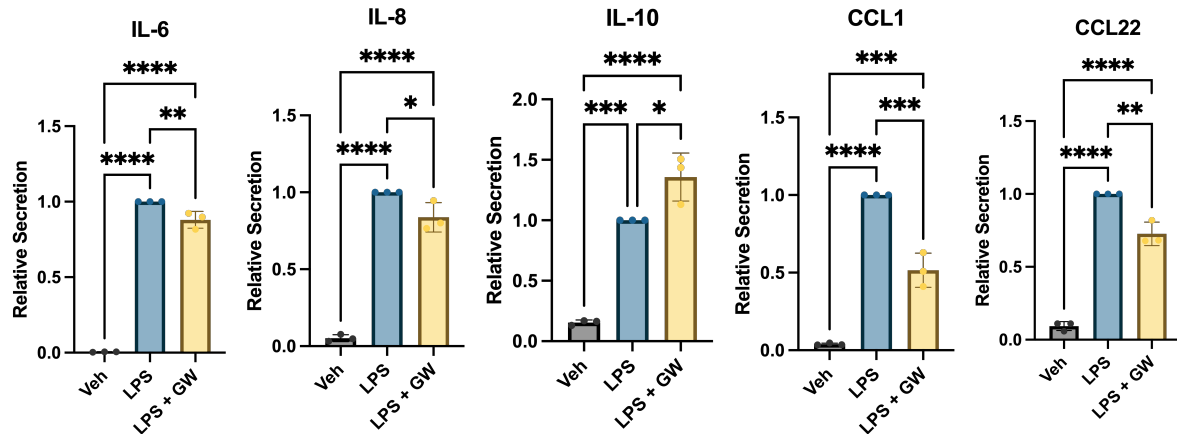


Figure 5: PPAR δ agonism reduces inflammation in iTF-Microglia stimulated with LPS.

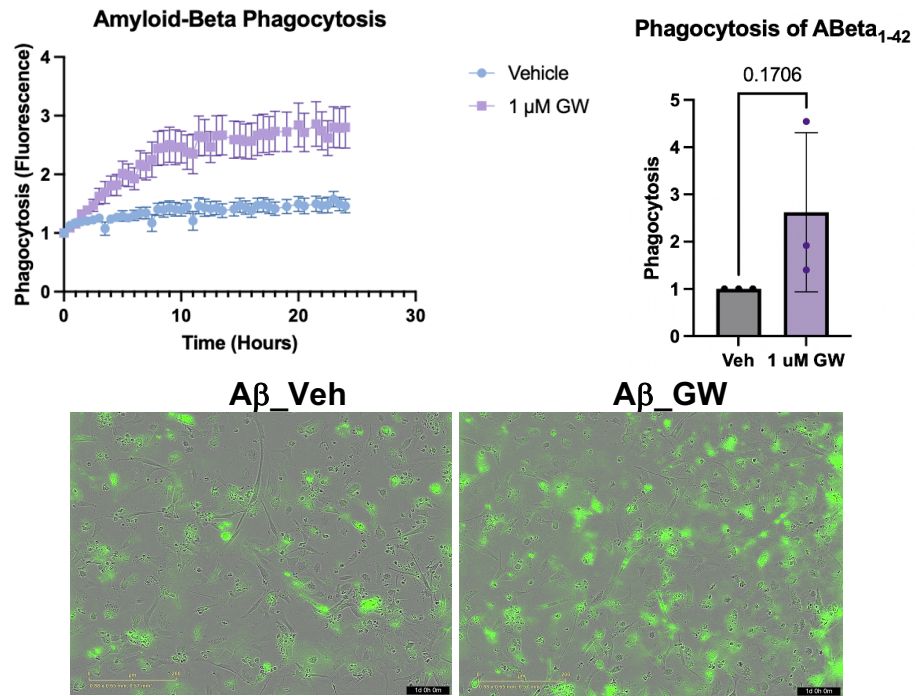
A) Heatmap showing relative secretion of cytokines that were significantly altered by LPS and how those changed with PPAR δ agonism.

B) Analysis of genes showing significant changes with PPAR δ agonism following LPS treatment. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$

PPAR δ Agonism Increases Microglial Phagocytosis of AD-Relevant Substrates

Microglia that harbor risk alleles for AD (e.g., TREM2 mutations or the APOE4 allele) are not able to properly phagocytose and clear the brain of debris. This can allow for accumulation of these substrates in the brain and can precipitate greater inflammation and cell death. We turned to look at how PPAR δ agonism affected the ability of iTF-Microglia to phagocytose disease relevant substrates. We incubated iTF-Microglia that were treated with PPAR δ agonist or vehicle (DMSO) with fluorescent fibrillar A β ₁₋₄₂ and pHrodo red-labelled rat synaptosomes. Images were captured over the course of 24 hours using an Incucyte S3, and the fluorescence of these molecules within the cell was used as a proxy for the amount of phagocytosis carried out by the cells. We observed that PPAR δ agonism increased the phagocytosis of both A β and synaptosomes (**Figure 6**). This is seen when tracing the fluorescence over a 24-hour time period, as well as measuring the relative fluorescence between biological replicates at the 24-hour time period, with representative images shown. This increase in phagocytic capacity is important for the AD brain because it would allow microglia to clear A β that has deposited more efficiently, as well as clear up debris to prevent subsequent inflammatory cascades⁶⁰.

A



B

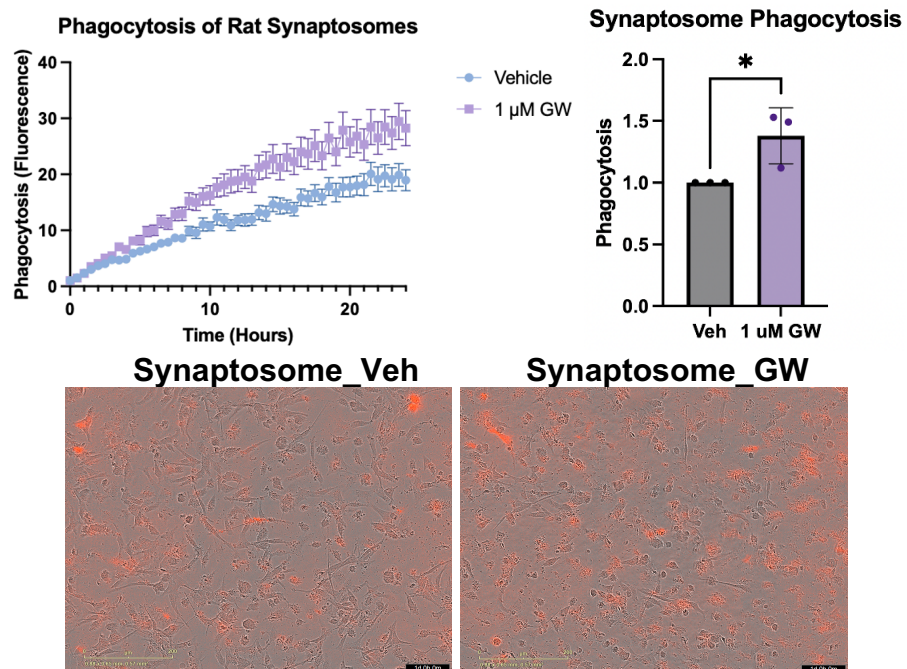


Figure 6: PPAR δ agonism upregulates the phagocytosis of disease-relevant substrates.

A) PPAR δ agonism increases the phagocytosis of A β by iTF-Microglia. Representative phagocytosis (fluorescence) plot over a 24-hour period of 4 technical replicates per condition. The bar plot shows quantification of phagocytosis (fluorescence) across 3 biological replicates at the 24-hour timepoint. At the bottom is a representative image of cells at the 24-hour timepoint, with vehicle treated cells on the left and GW treated cells on the right, and A β labelled in green.

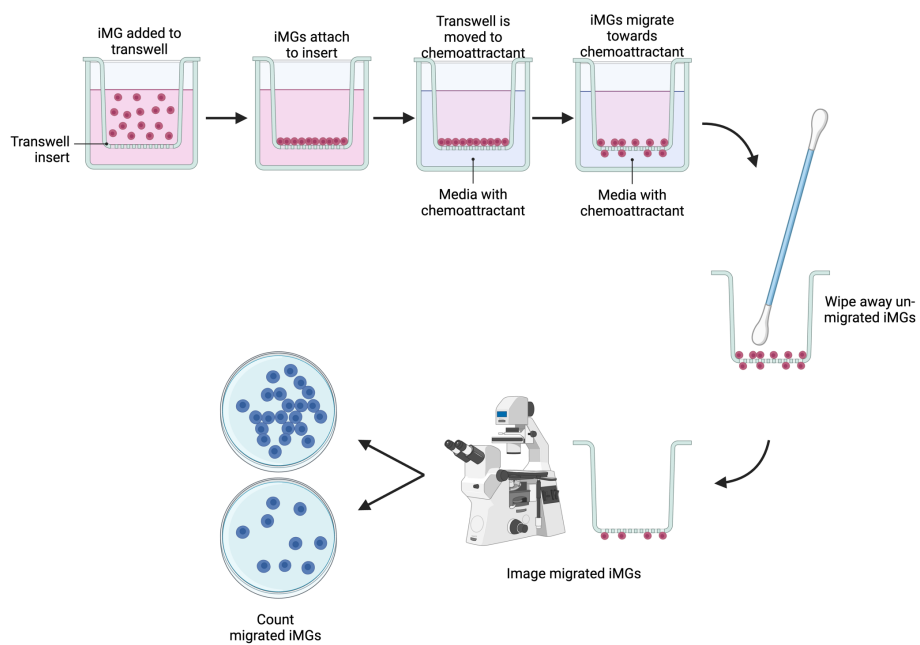
B) PPAR δ agonism increases the phagocytosis of synaptosomes by iTF-Microglia. The same plots are provided as they were for A β (described above). In the images, synaptosomes are labelled in red.

PPAR δ Agonism Reduces Migration Toward ADP and Apoptotic Neurons

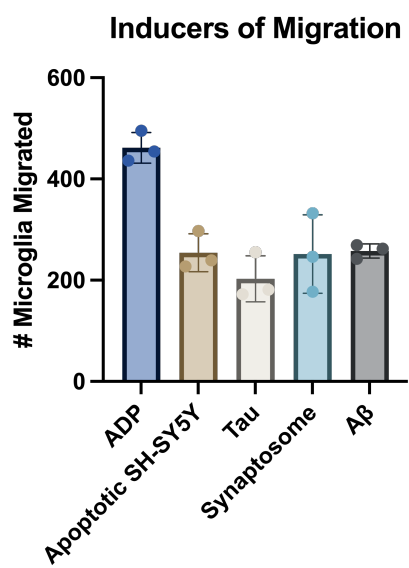
Another key function of microglia is migration, which is involved in the microglial response to injury. Two of the top downregulated GO terms from the bulk-seq experiment were “Positive regulation of cell migration” and “Cell Migration” so we decided to test the response of iTF-Microglia to different stressors. To do this, we utilized a transwell assay in which iTF-Microglia were replated onto a removable mesh insert, which was subsequently placed into a well that contained an attractant – with or without the presence of PPAR δ agonist. The number of microglia that migrated through the pore were then counted, as is represented by the schematic (**Figure 7A**). As migration assays had not yet been done on this model of iPSC-derived microglia, we tested a variety of potential attractants and found that microglia did indeed migrate toward different attractants, and that the strongest inducer of migration was adenosine diphosphate (ADP) – which is used as an attractant because dying neurons release ADP (**Figure 7B**). We then tested how PPAR δ agonism affects the migration capacity of the iTF-Microglia by pretreating the cells with GW501516 for 1 hour prior to their exposure to the attractant. We found that PPAR δ agonism reduced the migration of iTF-Microglia toward ADP and apoptotic SH-SY5Y cells (**Figure 7C**). This is congruent with the GO analysis of the DEGs and makes sense with the fact that microglia sense ADP via the purinergic receptor P2RY12. As expression of this receptor is downregulated with PPAR δ agonism, so is the ability of microglia to

respond to ADP. Of note, PPAR δ agonism did not drastically affect the migration of iTF-Microglia toward A β , Tau or synaptosomes. The biological significance of this finding can be interpreted in a variety of ways; however, this fits with our other data in that PPAR δ agonism blunts the inflammatory response of microglia. It does not prevent migration to different stressors, but it does reduce the migration and overall response. It also fits with the decreased release of chemokines as observed in the cytokine array. Migration of microglia *in vivo* would be necessary to better elucidate the physiological relevance.

A



B



C

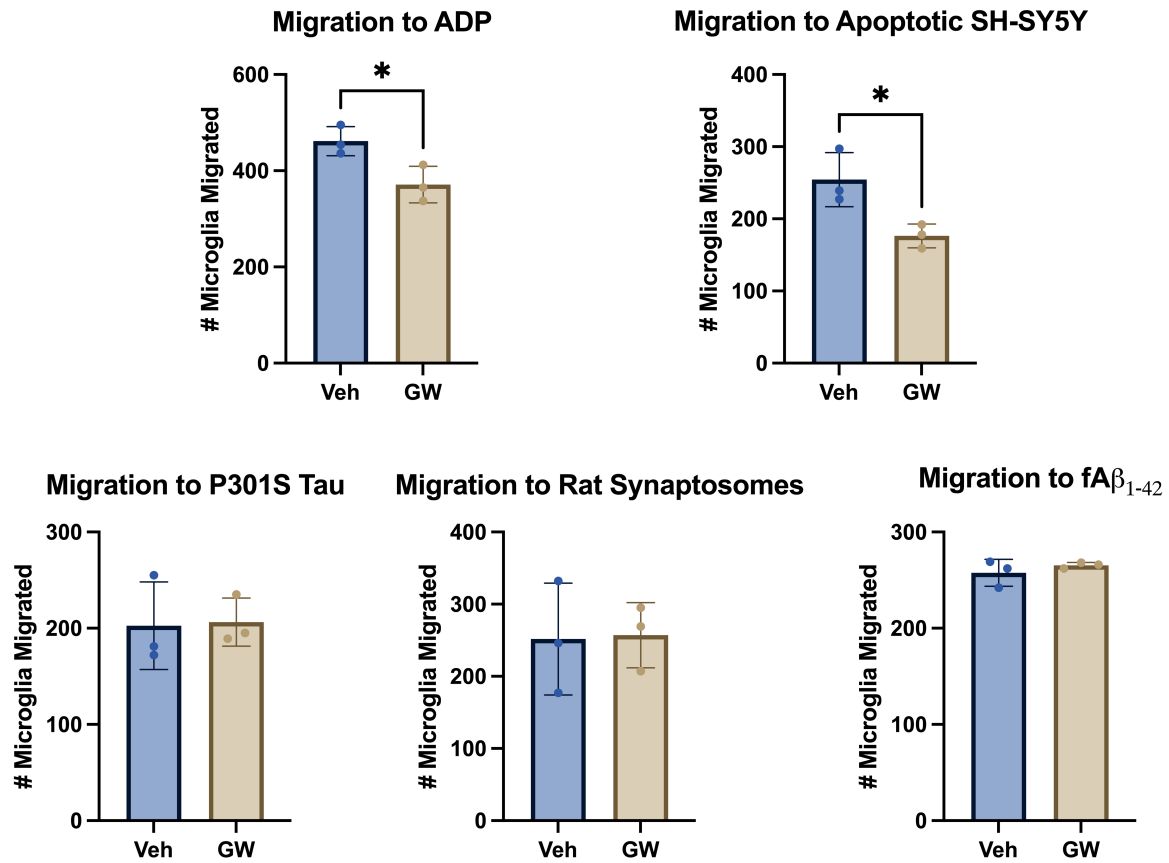


Figure 7: PPAR δ agonism reduces migration toward ADP and dying neurons.

A) Schematic describing the experimental set-up for migration assays.

B) Comparison of iTF-Microglia migration toward different stressors/attractants.

C) Effects of PPAR δ agonism on migration of TF-Microglia toward each substrate. * $p < 0.05$

PPAR δ Agonism Shifts Microglia to a Phagocytic, Lipid Processing State

To more robustly assess the effects of PPAR δ agonism in microglia, we turned to a single-cell RNA-sequencing experiment. In this experiment, we utilized pulverized mouse brain powder as a stressor for microglia. This is based off of the fact that CNS substrates are more effective at activating microglia than are neurodegeneration-associated proteins²⁸, as well as our conversations with collaborators from the Blurton-

Jones lab. As described in the introduction, the Stevens lab used synaptosomes, myelin, apoptotic neurons and A β to activate microglia *in vivo*⁶¹. Myelin, apoptotic neurons and synaptosomes were most effective at inducing a DAM state, so we took a more general approach by using a pulverized wild-type mouse brain to include multiple components.

We treated Day 9 iTF-Microglia with 6 different treatment conditions: +/- Brain Powder with vehicle (DMSO), PPAR δ agonist, or PPAR δ antagonist. This setup allowed for conceptualization of how PPAR δ affects microglial function both in the context of activation (with brain powder) and at baseline (with saline). Additionally, the use of the antagonist allows us to see which processes are dependent upon PPAR δ . Brain powder treatment is an established activation paradigm in the Blurton-Jones lab and functioned in our hands as well with initial qRT-PCR experimentation. For example, brain powder increased the expression of activation markers such as *GPNMB* and *ABCA1* while decreasing the expression of the homeostatic marker *P2RY12*.

After running our single-cell data through the analysis pipeline, our first step with the data was to discern whether brain powder treatment was indeed effective as an activator of iTF-Microglia in a DAM-like manner. As seen in **Figure 8**, brain powder treatment upregulated many DAM-related genes such as *ABCA1*, *CLEC7A*, *IL1 β* , *MERTK*, *CTSD*, and *PLIN2*^{51,62}. Many of these genes have to do with the canonical M2 state of microglia, a term which is no longer in use, but points to a more protective state of microglia, where they upregulate genes that have to do with phagocytosis and lipid processing. In other terms, these cells are focused on clearing debris from the brain and

effectively processing it. Additionally, these cells do upregulate some inflammatory genes and processes, such as *IL1 β* . Altogether, these initial results demonstrated that the cells did in fact respond to the brain powder and that this was an appropriate mode of activation.

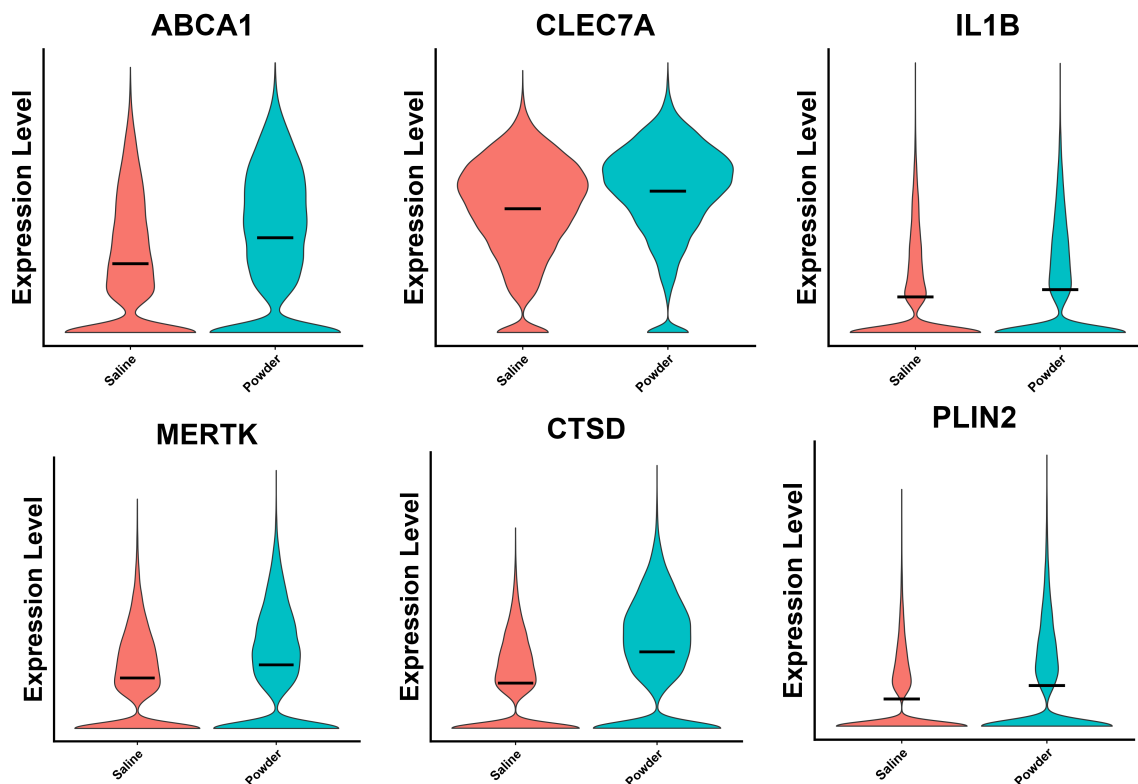


Figure 8: Brain-powder treatment induces a DAM-Like state in iTF-Microglia.

Violin plots showing gene expression data for iTF-Microglia that were either treated with saline or pulverized mouse brain powder or antagonist (GSK).

Next, we looked to verify that we were indeed targeting $\text{PPAR}\delta$ with our agonist and antagonist treatment. We began by looking at established $\text{PPAR}\delta$ target genes such as *PDK4* and *ANGPTL4* and saw an upregulation by agonism, as well as genes of interest such as *ABCA1* and *PLIN2* from our previous experiments (**Figure 9A**). As can be seen,

these genes are upregulated as expected and follow the same directionality change as the brain powder induced. The antagonist opposed many of these increases. Additionally, we specifically examined the DEGs between agonist and antagonist treatment with the

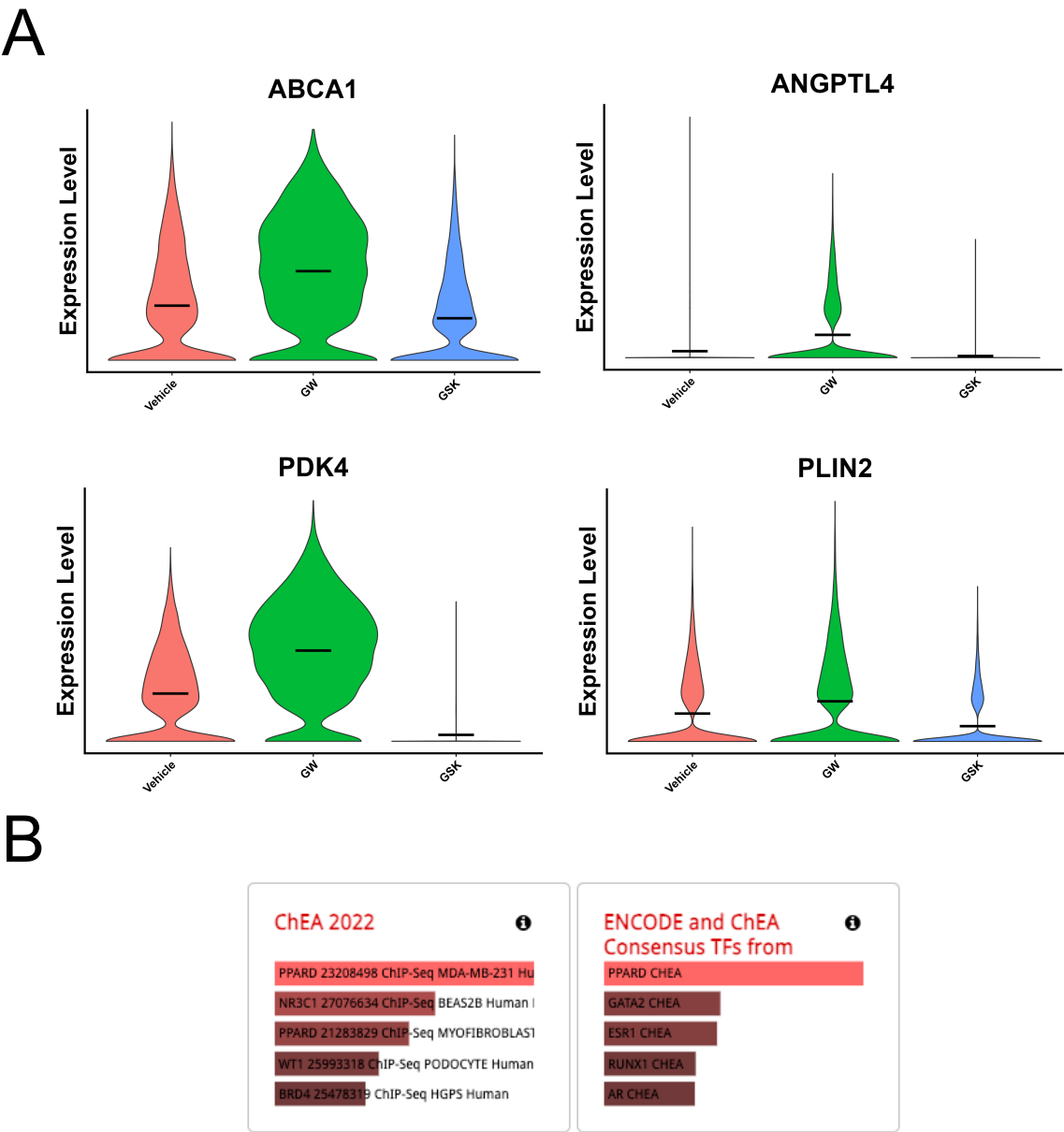
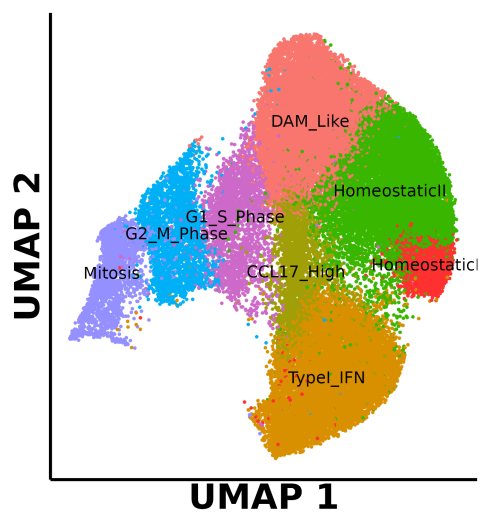


Figure 9: Verification of PPAR δ agonist and antagonist treatment.
A) Violin plots showing gene expression data for iTF-Microglia that were either treated with PPAR δ agonist (GW) or antagonist (GSK). Black bar represents mean expression.
B) Enrichr analysis plots demonstrating PPAR δ was indeed targeted when analyzing DEGs between GW and GSK in the saline groups.

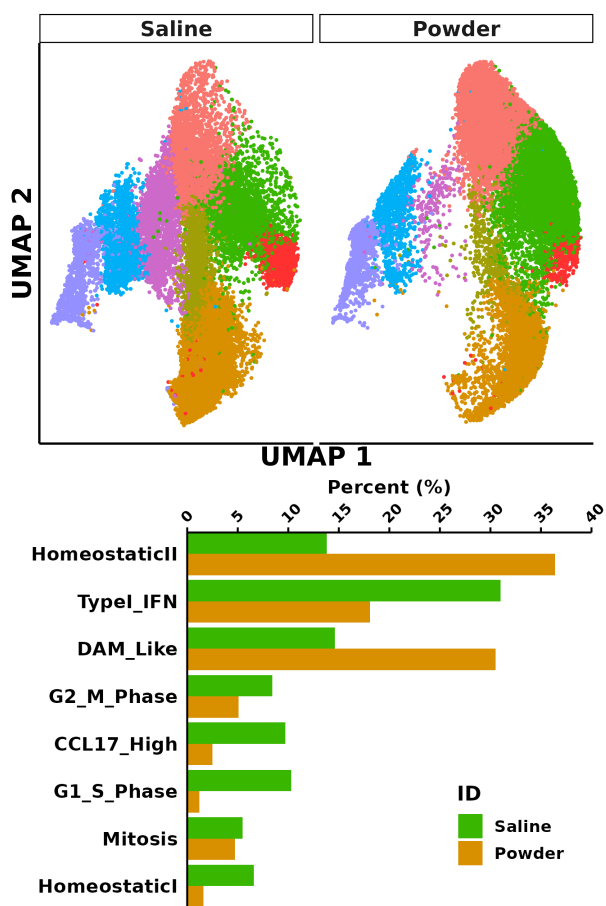
saline condition. When running the DEGs through EnrichR, there is an overwhelming PPAR δ signature (**Figure 9B**), assuring us that we were indeed targeting PPAR δ .

Unbiased clustering of the cells revealed 8 clusters: Homeostatic I, Homeostatic II, DAM_Like, Type I_IFN, CCL17_High, G1_S_Phase, G2_M Phase and Mitosis (**Figure 10A**). When looking at the different clusters, we see states that are often typical of microglia – homeostatic states, DAM-like states, interferon (IFN) states, and dividing states. As is often seen with microglial cultures *in vitro*, the cells exist in a state that is more activated at baseline. This means that all the microglial states are represented at baseline without powder treatment. Regardless, we still observed substantial shifts in the populations with our treatments (**Figure 10B & 10C**).

A



B



C

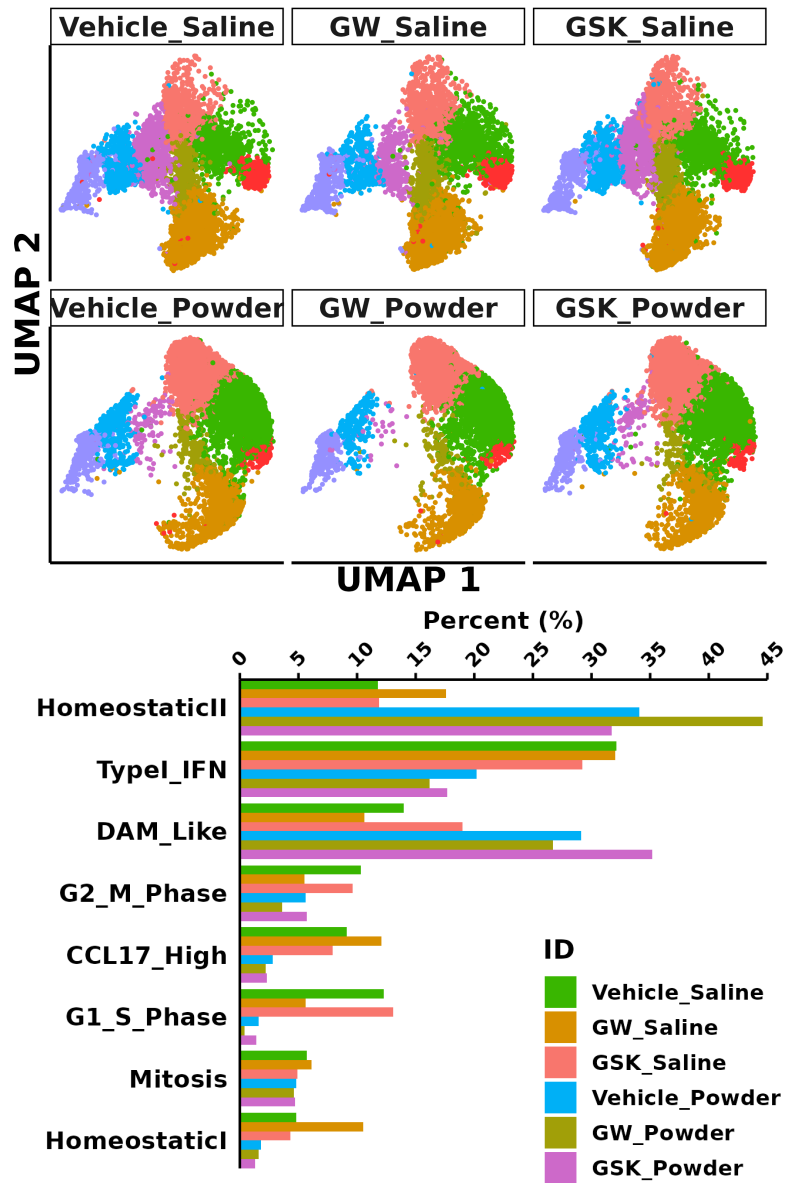


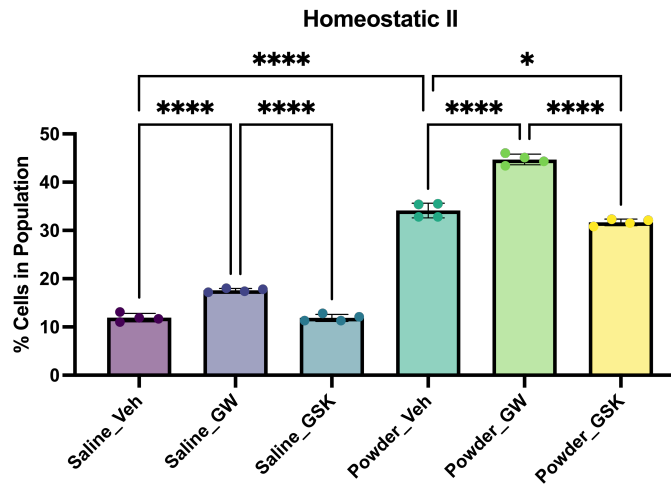
Figure 10: Clustering of iTF-Microglia after treatment.

- A) UMAP showing all 8 clusters of iTF-Microglia.
- B) UMAP showing how clusters changed in response to treatment with pulverized mouse brain powder, with a bar plot to display percent changes.
- C) UMAP showing how clusters changed in all treatment groups with a bar plot demonstrating percentages below.

As described before, and as we expected, treatment with brain powder drove cells into a DAM-like cluster. Interestingly, however, the largest shift in population was into the Homeostatic II population (**Figure 11A**). The Homeostatic II population is particularly interesting for several reasons. For one, it represents a potential “primed” or “pre-activated” state that contains genes that are enriched for lipid processing (e.g., *PLIN2* and *ABCA1*) and phagocytosis (e.g., *CD36* and *SYK*), but it lacks the more inflammatory signature that is seen in DAMs (e.g., *IL1B* and *CCL2*). Additionally, it is the cluster that PPAR δ agonism most drastically affects. Treatment with PPAR δ agonism significantly pushed cells into this cluster, whereas treatment with a PPAR δ antagonist significantly opposed this (**Figure 11A**). Running the genes that define the Homeostatic II state through Enrichr, the top gene ontology hit for biological processes is receptor-mediated endocytosis (**Figure 11B**). Pathway hits from the Elsevier Pathway Collection also demonstrate “Macrophage M2-Related Phagocytosis”. The Homeostatic II cluster is certainly not homeostatic, but is not quite a DAM either because it lacks a lot of the inflammatory signaling as mentioned above. Regardless, MERTK signaling is present in the Homeostatic II cluster, and MERTK is often seen with DAMs⁵¹. It appears to almost be in a “primed” state – activated, yet more in the sense of lipid processing and phagocytosis and not in the sense of inflammatory cytokine secretion.

The DAM-like cluster retains many of these phagocytic and lipid processing genes. Comparing the DAM-like cluster to the Homeostatic II cluster, the DEGs reveals that NF κ B1 signaling is a predicted transcription factor of the transition from Homeostatic II to

A



B

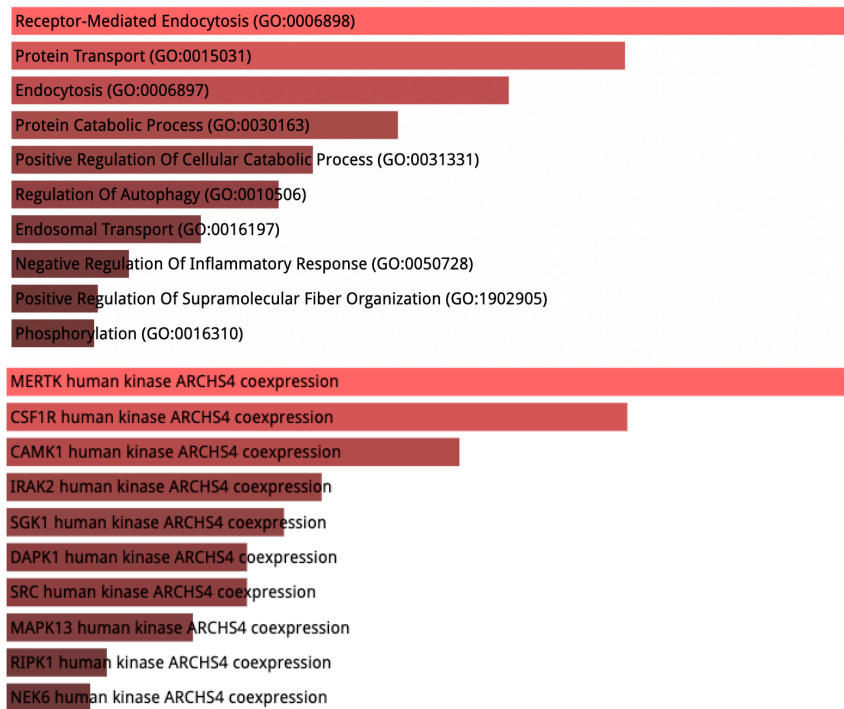


Figure 11: Populations in Homeostatic II and Enrichr analysis of the Homeostatic II population.
A) Distribution of iTF-Microglia in Homeostatic II based on treatment group. n=4 per group. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.
B) Plots from the Enrichr tool that demonstrate top biological processes represented in the Homeostatic II population as well as ARCHS4 coexpression analysis. Log2 fold change>0.5

DAM-Like, with inflammatory processes being a top GO hit (**Figure 12**). This is in line with what I have previously shown, where PPAR δ agonism activates microglia in the way

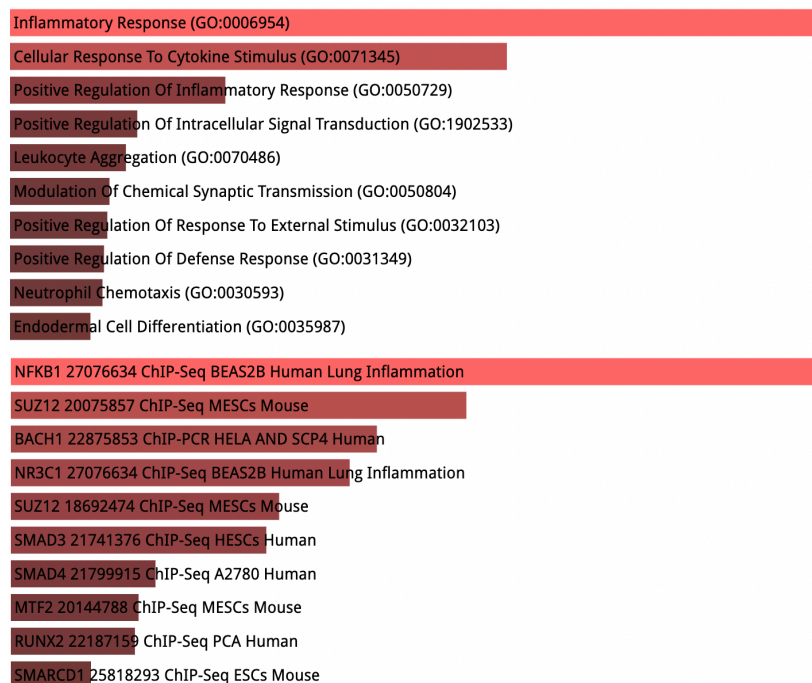


Figure 12: Enrichr analysis of DAM-Like vs Homeostatic II Treated iTF-Microglia in the DAM-Like population.

Plots from the Enrichr tool that demonstrate top biological processes that are upregulated in the DAM-like cluster compared to the Homeostatic II cluster, as well as ChEA analysis for predicted transcription factor binding. Log2 fold change > 0.5

of phagocytic and lipid catabolic processes, but represses the secretion of inflammatory mediators. PPAR δ agonism shifts microglia more into the Homeostatic II cluster, with the antagonist having the opposite effect. In other words, this further supports that PPAR δ regulates microglia to be activated in some sense, but anti-inflammatory at the same time.

The Type I interferon response cluster is a cluster seen in many microglial single cell RNA-seq experiments⁵². It is characterized by interferon genes and is typically seen in response to tauopathies and is viewed as a more detrimental, hyper-inflammatory state – akin to the antiquated “M1” state of microglia⁶³. At baseline, PPAR δ agonism does not

have much of an effect on this cluster; however, when activated with powder, PPAR δ agonism does further reduce the size of this cluster, suggesting another beneficial aspect.

In addition to these shifts in populations, there are some interesting changes in gene expression with PPAR δ agonism. First of all, we looked at how PPAR δ agonism affected cells with and without powder treatment. A volcano plot showing powder+agonist-treated versus powder+antagonist-treated cells is shown in **Figure 13**. As expected, *PDK4*, which is an established PPAR δ target gene⁵, is one of the highest hits. However, the largest change in this grouping is in *IL22RA2*, which codes for Interleukin-22 binding protein. *IL22RA2* is significantly upregulated when cells were treated with PPAR δ agonist. This protein is known to bind IL-22 with an inhibitory effect⁶⁴, which further fits with PPAR δ agonism reducing inflammation in microglia. Additionally, *PLIN2* is starkly upregulated. *PLIN2* is involved with lipid droplet formation in microglia. *ABCA1*, which was also upregulated with PPAR δ agonism, is involved in cholesterol efflux for microglia. Thus, lipid processing genes are being tightly regulated by PPAR δ , and are important for microglia as they clean up debris from the brain and process all of the contents. Looking at the downregulated genes, *CCL2* pops up as being tightly regulated by PPAR δ . A decrease in *CCL2* expression with PPAR δ agonism is in line with previous findings of decreased inflammatory response.

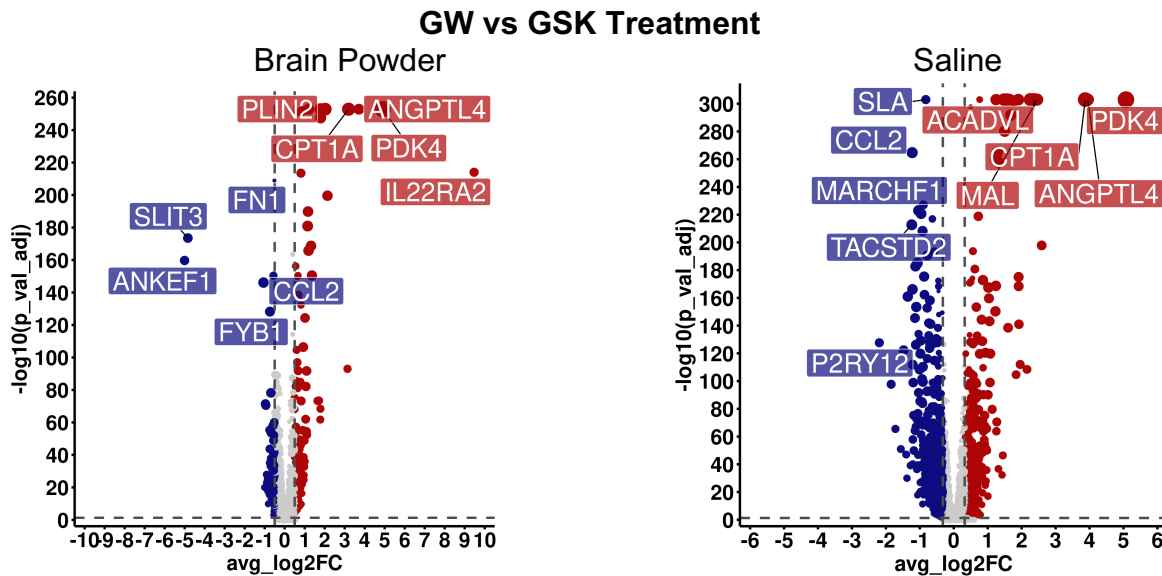


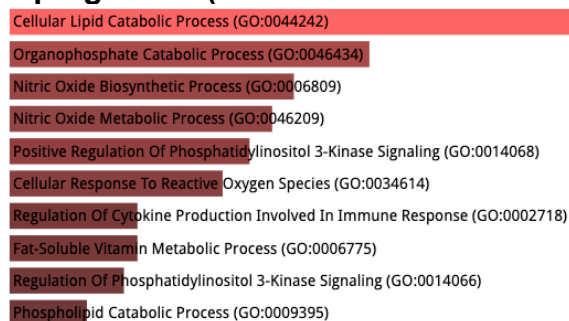
Figure 13: Differential gene expression based on PPAR δ treatment in iTF-Microglia.
 Volcano plots generated from the differential gene expression between PPAR δ agonist and antagonist groups in brain powder (Left) and Saline (Right) treatments.

The changes between agonist and antagonist are similar when looking at the saline condition (without the addition of brain powder). One of the most interesting additional findings from this dataset is the downregulation of *P2RY12* with PPAR δ agonism. It has the largest fold change decrease when looking at PPAR δ agonist compared to the antagonist. This is of interest because *P2RY12* is a key marker of microglial homeostasis. The fact that it is being downregulated with PPAR δ agonism further supports that PPAR δ agonism is capable of activating microglia at baseline.

Lastly, it is interesting to compare what PPAR δ agonism does to the cells in the DAM-like state. I compared the gene expression of the agonist-treated cells in the DAM-

like state with the vehicle-treated cells in the DAM-like state. The top upregulated biological process for these cells is “Cellular Lipid Catabolic Process”, with many other

Upregulated (GW vs Veh in DAM-Like)



Downregulated (GW vs Veh in DAM-Like)

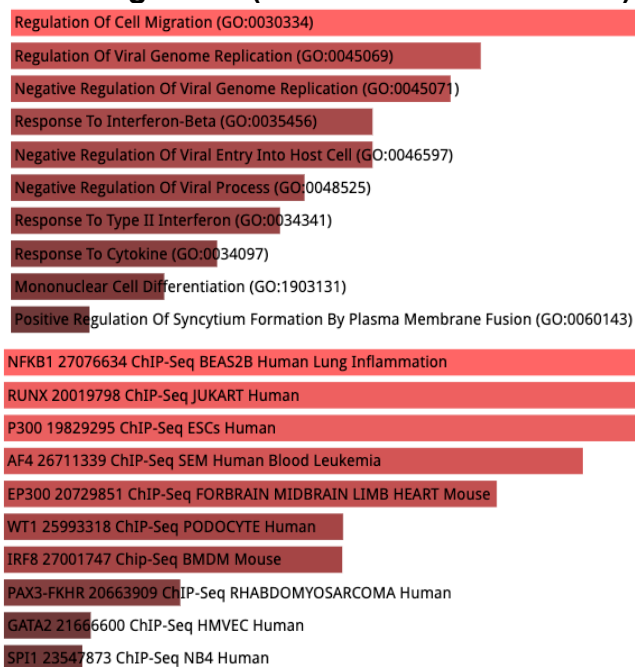


Figure 14: Gene ontology analysis of PPAR δ agonist treated cells compared to vehicle treated cells in the DAM-like cluster.

Enrichr analysis plots showing upregulated biological processes (Top) and downregulated biological processes and ChEA predicted transcription factors (Bottom) for cells that are in the DAM-like cluster when treated with PPAR δ agonist versus vehicle.

metabolism terms popping up (**Figure 14**). The downregulated terms of GW vs vehicle-treated in the DAM-like state have to do with immune system processes, with NF κ B1 activity being seemingly repressed by agonism. Yet again, this supports that PPAR δ

agonism upregulates processes that are beneficial in AD such as lipid metabolism, while mitigating actions that can become detrimental, such as cytokine release. Of note *IL1022RA2* was the most highly upregulated gene between these groups as well, suggesting more anti-inflammatory roles. Conversely, I analyzed what PPAR δ antagonism did to cells in the DAM-Like cluster. Comparing GSK-treated to Vehicle-treated cells in the DAM-like cluster, the biological processes that are downregulated have to do with lipid metabolism (**Figure 15**). Then, comparing the GW treated cells to the GSK treated cells in the DAM-Like cluster further revealed many of these changes. Further confirming we are targeting PPAR δ , the top ChEA hit when looking at these genes was PPARD (**Figure 15**). Additionally, CD36 appeared as a hit for TF-Gene Cooccurrence. CD36 functions as a scavenger receptor for microglia⁶⁵, supporting the role of PPAR δ in upregulating phagocytosis.

Overall, this single-cell experiment has provided us with a framework of how PPAR δ agonism affects microglia. PPAR δ agonism upregulates genes that are associated with phagocytosis and lipid metabolism and downregulates genes that are associated with inflammatory cytokine and chemokine release. The microglia treated with PPAR δ agonism were shifted into a cluster that focused on these beneficial pathways and

Downregulated (GSK vs Veh in DAM-Like)

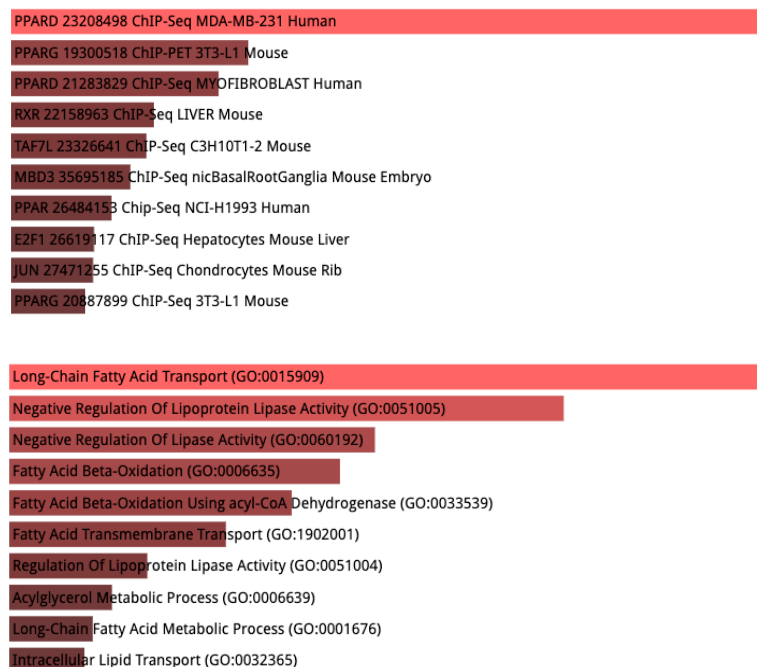


Figure 15: PPAR δ antagonism in the DAM-Like cluster downregulates lipid metabolism.

Enrichr analysis plots showing ChEA predicted transcription factors (Top) and downregulated biological processes (Bottom) for cells that are in the DAM-like cluster when treated with PPAR δ antagonist (GSK) versus vehicle.

differed from the DAM-Like cluster due to the fact that inflammatory genes were repressed, with NF κ B1 activity being particularly repressed. This is still a preliminary analysis and we expect to uncover more pathways with more time and different analyses.

PPAR δ interacts with PU.1

As mentioned previously, the *in vivo* transcriptome data for mice treated with KD3010 revealed a PU.1 signature. Similarly, in the single-cell data, when analyzing the DEGs between GW and GSK treated cells, a significant *SPI1* signature emerges through ChEA. PU.1 is a therapeutic target for AD, with many efforts recently focused on targeting its activity³⁴. Thus, we inquired whether PPAR δ and PU.1 might physically interact. Using

overexpression in HEK293T cells, endogenous protein in BV2 cells, and endogenous protein in iTF-Microglia, PPAR δ and PU.1 interact with each other via co-immunoprecipitation (**Figure 16A, 16B & 16C**). Investigating this interaction further in terms of how PPAR δ agonism affects the interaction, I used NanoBRET to show that as the amount of PPAR δ agonism increases, the interaction between PU.1 and PPAR δ decreases in a dose-dependent manner (**Figure 16D**). Though we discovered this interaction, we still have yet to know what the functional significance is. This work is still underway, but the previous studies described here have helped to hint at the significance, with the idea that PU.1 is important for maintaining a homeostatic signature in microglia and regulating immune processes²⁶.

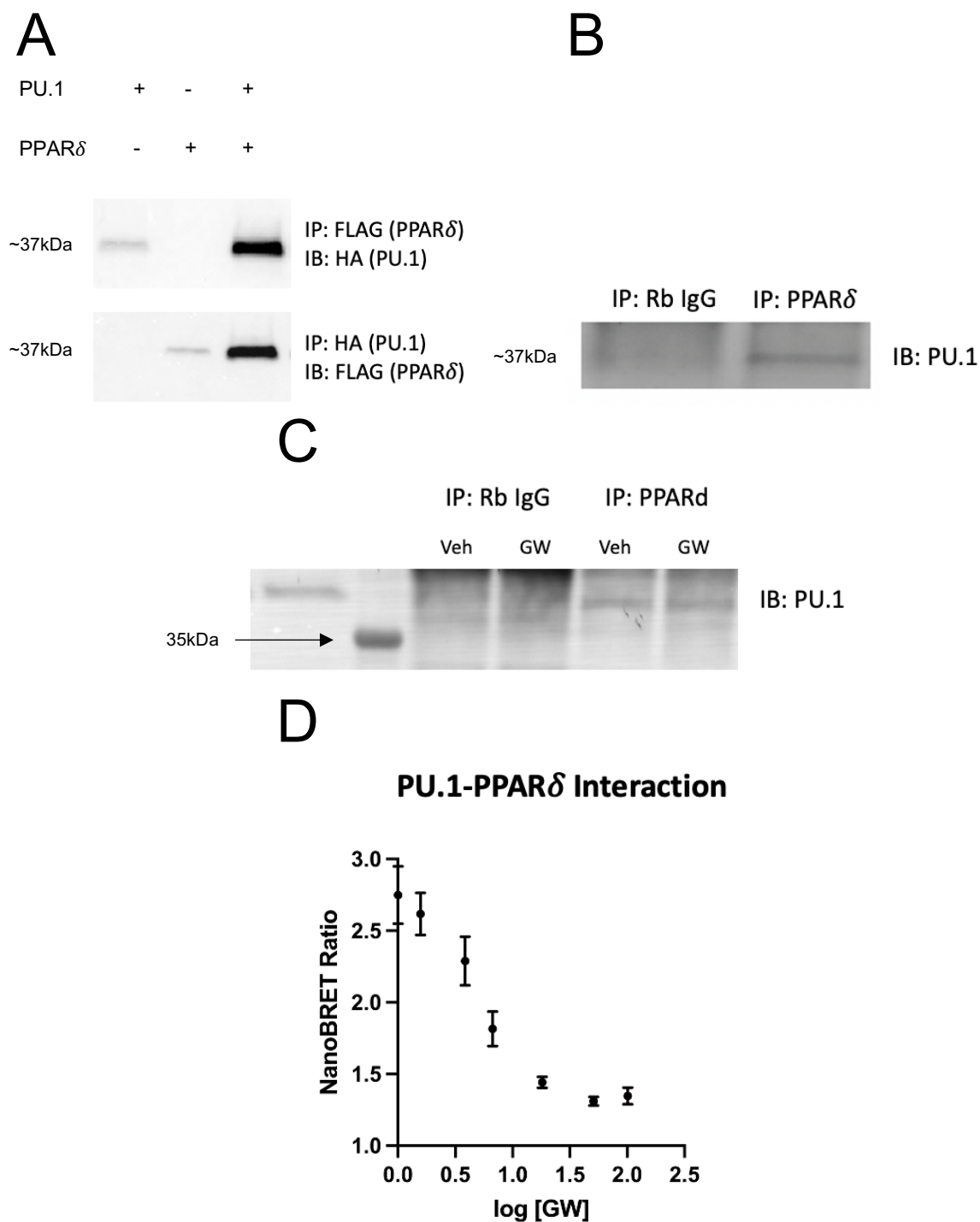


Figure 16: PPAR δ interacts with PU.1.

A) Co-immunoprecipitation experiment in HEK293T cells with overexpressed FLAG-tagged PPAR δ and 3xHA-tagged PU.1. Lane 1 is single transfected with PU.1. Lane 2 is single transfected with PPAR δ . Lane 3 is co-transfected with PPAR δ and PU.1. Pull down and western blot conditions are labeled on the right side of the blot.

B) Co-immunoprecipitation in BV2 cells with endogenous protein.

C) Co-immunoprecipitation in iTF-Microglia with endogenous protein.

D) NanoBRET experiment demonstrating that PPAR δ and PU.1 interact at baseline and that addition of GW501516 (PPAR δ agonism) decreases this interaction. Experiment done in HEK293T cells.

Materials and Methods

Mouse Treatment Protocol

Eight wild-type mice between the ages of 6 and 8 months were divided into two cohorts of 4 each. One group received intraperitoneal (IP) injections of KD3010 5 days per week at a dose of 50 mg/kg while the other received vehicle IP injections of the same frequency. Injections were carried out for a total of six weeks. KD3010 was prepared at a concentration of 10 mg/mL in Corn Oil with 5% DMSO and vortexed immediately prior to administration.

Microglia Isolation

Whole brains from mice were first finely chopped, then dissociated by trituration with Pasteur pipettes in a dissociation solution containing Collagenase A and DNase I. Microglia were then isolated using CD11b microbeads and a magnetic stand. Samples from the whole tissue lysate before isolation and flowthrough samples after isolation were harvested for further analysis. Following subsequent washes, isolated microglia and other samples are stored at -80°C either in Trizol for RNA processing or lysis buffer for protein processing.

Transfection

HEK293T cells were transfected with plasmids utilizing Lipofectamine 3000 according to manufacturer's instructions. Cells were cultured in 10cm plates and transfected with 14µg total of plasmid for 24 hours before media was replaced and treatment was administered.

Co-immunoprecipitation

Cells from the variety of treatment groups were lysed using IP lysis buffer. After lysis, the lysate was spun at 13,000xg for 15 minutes and the concentration of the lysate was calculated by BCA on the supernatant of the spin-down. At this point, the samples were further diluted to a final concentration of 1 µg/mL. For this immunoprecipitation, samples were incubated overnight with FLAG-beads or 5 µg antibody according to manufacturer's instructions. If just incubated with the antibody, the next day, Protein A beads were added and the samples were incubated on a rotator for 1.5 hours. After the incubation, the flow-through was saved and the antigen-antibody-bead complex was transferred to a new tube for elution after a series of washes with IP lysis buffer and water. The samples were eluted by heating samples at 70°C for 10 minutes in a solution composed of IP lysis buffer, 4x LDS Sample Buffer and 10x Reducing Agent. After this, the sample was removed from beads using a magnetic rack and samples were loaded into a gel for western blot analysis.

NanoBRET

HaloTag and NanoLuc tagged plasmids for PPAR δ and PU.1 were prepared using a NanoBRET starter kit from Promega. All combinations of N- vs C- tagged plasmids were tested and the best combination providing dynamic range was utilized. HEK293T cells were plated at a density of 350,000 cells in a 6-well plate and allowed to settle overnight. The following day, cells were transfected with appropriate plasmids using Lipofectamine 3000, with p53 and MDM2 being used as a positive protein interactor control, using

manufacturer's recommendations. The following day, cells were replated in a 96 well plate and treated with predetermined concentrations of GW501516, with concentrations ranging from 1 nM to 10 μ M. The following day, the NanoBRET assay was carried out in the 96-well plate and absorbance readings were taken at 460nm (donor emission) and 618nm (acceptor emission), with a NanoBRET ratio being calculated after removing background absorbance.

Human iPSC Culture

Human iPSCs were cultured in mTeSR Plus Medium on 60 mm TC-treated Culture dishes coated with LDEV-free Corning Matrigel hESC-Qualified Matrix diluted 1:100 in Dulbecco's Modified Eagle's Medium (DMEM), 1X. Media was replaced daily. Cells were ReLeSR passaged when 70-80% confluent. For experiments, cells were dissociated with Accutase, counted using the Countess 3 Automated Cell Counter and plated with Y-27632 dihydrochloride ROCK inhibitor diluted 1:1000 in differentiation media.

To freeze iTF-iPSCs for future use, old media from plates was aspirated. Then, the plate was rinsed with 1x DPBS and treated with ReLeSR. ReLeSR was aspirated within 1 minute, and plates were incubated at 37°C for 10 minutes. Plates were then treated with DMEM, 1X with strong enough force to dislodge iTF-iPSCs. Free floating cells were then transferred to a 15mL conical tube and centrifuged at 50xg for 3 minutes. The resulting supernatant was aspirated, and cells were resuspended in 1mL of CryoStor CS10 before being transferred to CryoPure 1.6mL tubes and placed into a Mr. Frosty Freezing

Container at -80°C overnight. No later than 48 hours later, the cells were transferred to -160°C for long term storage.

iTF-iPSCs that are stored as frozen aliquots were thawed in a 37°C water bath, and diluted 1:15 in mTeSR Basal Medium. Diluted iTF-iPSCs were transferred to a 15mL conical tube and centrifuged at 220xg for 5 minutes before aspirating the supernatant. Cells will then be resuspended in mTeSR Plus Basal Medium supplemented with 10nM Y-27632 dihydrochloride ROCK inhibitor and plated onto Matrigel-coated plates. After one day, media was aspirated and replaced with mTeSR Plus Basal Medium.

Differentiation of iTF-Microglia

iTF-iPSCs will be thawed and plated as described above. Cells will be cultured for at least 24 hours without Y-27632 dihydrochloride ROCK inhibitor before being dissociated with Accutase. Cells were incubated at 37°C for 7 minutes with Accutase, after which time the Accutase was quenched by adding DMEM at a 3:1 ratio. Dissociated cells were transferred to a 15mL conical tube and centrifuged at 220xg for 5 minutes. After aspirating the resulting supernatant, cells were resuspended in Day 0 Differentiation Media: Essential 8 Basal Medium containing 10nM ROCK inhibitor and 2 µg/ml Doxycycline hyclate. iTF-iPSCs were counted and plated (Day 0) onto Poly-D-Lysine Coated plates that are additionally coated with Matrigel. After two days (Day 2), the media was aspirated and changed to Day 2 Media: Advanced DMEM/F12 Medium containing 2 µg/ml doxycycline, 1x GlutaMAX™ Supplement, 100 ng/ml Human IL-34, and 10 ng/ml Human GM-CSF. After two additional days (Day 4), the media was aspirated and

replaced with Day 4 media: Advanced DMEM/F12 containing 1X GlutaMAX, 2 μ g/ml doxycycline, 100 ng/ml Human IL-34 and 10 ng/ml Human GM-CSF, 50 ng/ml Human M-CSF and 50 ng/ml Human TGF- β 1. Four days later (Day 8), cells were fully differentiated (termed iTF-microglia) and ready for use. Media was replaced on Day 8 and every 3-4 days as needed with the same Day 4 media. Differentiation was confirmed by morphology and immunocytochemistry.

LPS Treatment/Cytokine Array

iTF-Microglia were treated with 100 ng/mL LPS diluted in DPBS, as done previously⁵², with or without the addition of PPAR δ agonism. The inflammatory readout was assessed by analyzing inflammatory cytokine production using an Abcam Human Cytokine Array for 42 targets. After 24 hours of treatment with or without LPS and with or without 1 μ M GW501516, the conditioned media from the iTF-Microglia was collected and added to the cytokine array per the manufacturer's instructions. After taking chemiluminescence images of the blots using a Bio-Rad ChemiDoc Imaging System, the images were analyzed using the Protein Array Analyzer tool on FIJI to quantify the amount of protein.

Phagocytosis Assay

To assess phagocytic activity of iMGLs and the ability of a PPAR δ agonist to alter it, we utilized fluorescent fibrillar beta-amyloid (1-42) and pHrodo tagged rat synaptosomes. To prepare fluorescently labeled A β , we followed a previously published protocol⁴⁹. The lyophilized stock was first dissolved in NH₄OH to an initial concentration of 1 mg/mL, then further diluted to 100 μ g/mL with UltraPure™ DNase/RNase free water

and incubated for 7 days at 37°C. To isolate rat synaptosomes, we obtained Innovative Grade US Origin Rat Sprague Dawley Brain. Then, following a previously reported protocol⁵², 10 mL / gram of brain of Syn-PER Synaptic Protein Extraction Reagent was supplemented with protease and phosphatase inhibitor was added to the brain and was used to homogenize using a Dounce homogenizer on ice. The contents were then put into a tube and were centrifuged at 1200xg for 10 minutes at 4°C. Then, the pellet was discarded and this centrifugation was repeated using a new tube. After that repeat spin-down, the supernatant was spun down at 15,000xg for 20 minutes at 4°C. After discarding the supernatant, the wet pellet was weighed and the synaptosome was resuspended at a concentration of 50 mg/mL. Then, in order to conjugate these to the pH-sensitive pHrodo dye, 3uM of pHrodo-Red was added to the synaptosomes and this mixture was incubated for 45 minutes in the dark at room temperature. Then, the synaptosomes were diluted 1:10 in DPBS and spun down at 2500xg for 5 minutes. After two more washes, the synaptosomes were resuspended at 50mg/mL in Advanced DMEM/F12 with 5% DMSO for future use.

Day 9 iTF-Microglia were used for phagocytosis assays. We had conditions where iPSC cultures are combined with 2 µg/mL fluorescent fibrillar beta-amyloid or 50 µg/mL pHrodo tagged human synaptosomes, with or without 1uM GW501516 treatment, as well as a no substrate condition. We incubated these cells for 24 hours in an Incusyte S3 at 37°C to allow for phagocytosis. We analyzed using the Incucyte S3 software.

Migration Assay

The ability of iTF-Microglia to migrate towards various attractants was tested. 20,000 iTF-Microglia were plated onto PDL and ECM coated 8 μm transwells in assay media with GW, DMSO, or DMSO/ADP. The transwells were placed in 24-well plates containing assay media (Advanced DMEM/F12 + 1X GlutaMAX + 2 $\mu\text{g/mL}$ Doxycycline) and GW or DMSO. The cells were incubated for an hour at 37°C. Attractants (1:20 A β , 1:1000 P301S Tau preformed fibrils, 1:50 synaptosomes, 35,000 apoptotic SH-SY5Y / cm^2 , and 1:50 ADP) were added to the bottom of 24-well plates and the transwells were moved onto them. Fibrillar A β and synaptosomes was prepared as described before. Tau preformed fibrils were purchased. Apoptotic SH-SY5Y cells were generated by utilizing UV radiation at 50 J/m^2 with an UV crosslinker, as done previously⁶¹. The cells were incubated with the attractants for 4 hours at 37°C. The cells were then fixed with 4% PFA and stained with Hoechst. The cells on the top of the transwells were wiped away and the remaining migrated cells on the bottom of the transwells were imaged using an Echo Revolution microscope and the total migrated cells were compared.

RNA Extraction and qRT-PCR

RNA was extracted using Qiagen RNeasy Mini RNA extraction kit, converted to cDNA with SuperScript IV VILO Master Mix with ezDNase Enzyme, and genes of interest were tested for expression change using qRT-PCR. Primer sequences were obtained from origene.com and validated using the UCSC Genome Browser website.

Preparation of Mouse Brain Powder

Twelve-month old female wild-type mice were perfused with ice-cold PBS and their brains were isolated. Olfactory bulbs and cerebellum were removed from the brain, and the brain was then cut in half and immediately flash frozen with liquid nitrogen. Frozen brains were made into powder using a tissue pulverizer, all while kept frozen, and were weighed out to predetermined amounts based on experimental needs. On the morning of the experiment, the mouse brain powder was resuspended at a concentration of 3.33 mg / 100 μ L of sterile 1X DPBS. The powder was then homogenized by using a handheld Eppendorf tube homogenizer 10 times. Then, the powder was run through a 70 μ m filter to remove any remaining large clumps.

Single Cell Experiment for iTF-Microglia

Day 8 iTF microglia were replated in 24 well plates at a density of 100,000 cells/well. Brain powder treatment was at 20 μ L of the prepared solution per well. iTF-Microglia were then treated with vehicle, 1 μ M GW501516 or 1 μ M GSK3787. We began with 3 wells per replicate per condition, with a total of four replicates. To collect cells and clean them from the brain powder treatment, we went through a cleanup protocol. Briefly, cells were lifted using TrypLE as described above, replicate wells were pooled, and cells were spun at 200xg for 5 minutes. After aspirating the supernatant, the pellet was resuspended in 1mL of Advanced DMEM/F12. Each pooled sample was then run through a 70 μ m filter into a new tube. An additional 5.2 mL of Advanced DMEM/F12 was added to the cells and the tube was mixed. 1.8 mL of SIP was added to each sample and the tube was mixed by inversion three times. Then, 2 mL of 1X DPBS was gently added on

top of the SIP/cell mixture. The tubes were then centrifuged at 400xg for 20 minutes with acceleration at 0 and brake at 4. The supernatant was aspirated and the pellet was resuspended in 1mL of 0.5% BSA in 1X DPBS, and an additional 2 mL of this solution was added to each sample. The samples were centrifuged again at 200xg for 5 minutes. The supernatant was aspirated and the pellet was resuspended in 105 μ L of the 0.5% BSA solution. Cells were then fixed using the Parse Biosciences Evercode™ Cell Fixation v2 kit using the according to the manufacturer's protocols. Samples were then barcoded using the Evercode™ WT v2 kit according to manufacturer's procedures and then given to the UCI Genomics Core for library prep and sequencing.

From the Parse protocol, 8 identical sublibraries were prepared, each aiming to capture 12,500 cells for a total of 100,000 cells. Samples were sequenced aiming for a target of 50,000 reads per cell. FASTQ files were aligned to the Ensembl GRCh38 transcriptome (filtered to keep protein coding genes). The total number of captured cells was 74,014, with an average of 63,370 reads per cell. Each sample ended up with an average of 3,084 cells, for an average of 12,335 cells / experimental group. Samples were then imported into Seurat v5 for quality control preprocessing, including the removal of doublets with scDbfFinder, and the removal of cells with low genes/UMI, high mitochondrial percentage or high ribosomal percentage. Following this cleanup, 55,802 cells remained.

Samples were then merged, counts were normalized and data scaling was performed while regressing out the "Sublibrary" batch effect. Principal Component

Analysis (PCA) was performed using the top 2,000 variable features across all datasets. Clustering and dimension reduction was performed with the top 31 PCs and a resolution of 0.3. The resulting clusters were identified and evenly distributed across sublibraries. Cells were analyzed for predicted cell type and only microglial/monocytic cell types were left in as part of the experiment. The other clusters were removed and the remaining cells were reclustered. A total of 44,151 cells remained.

Chapter 2: Phase Separation of PPAR δ

Introduction

The mechanistic basis of PPAR δ function remains understudied. PPAR γ undergoes phase separation to regulate gene expression at PPAR Response Elements (PPREs) and breaking apart PPAR γ phase separated condensates decreases the expression of target genes⁴¹. As described earlier, phase separation is the process by which different biomolecules self-assemble into membraneless compartments to carry out specialized functions. PPAR δ also forms condensates *in vitro*⁴¹, but there is no work describing the importance of PPAR δ phase separation for its transactivation functions. This is an important gap in knowledge to fill because it provides insight into how PPAR δ is actually carrying out the roles described in the previous chapter.

Results

PPAR δ is Capable of Phase Separation

To begin this work, we established a collaboration with the lab of Dr. Xavier Salvatella, an expert in phase separation. From this collaboration, we obtained an in-depth analysis of the structure of PPAR δ and determined the likelihood of phase separation based on this analysis. As mentioned previously in the introduction, phase separation is often driven by regions of disorder within the amino acid sequence of a protein. As demonstrated in **Figure 17**, PPAR δ has two main regions of disorder that are high in aliphatic and acidic residues. From this analysis, given that PPAR δ has regions of

disorder, we proceeded with further work attempting to elucidate its ability to phase separate.

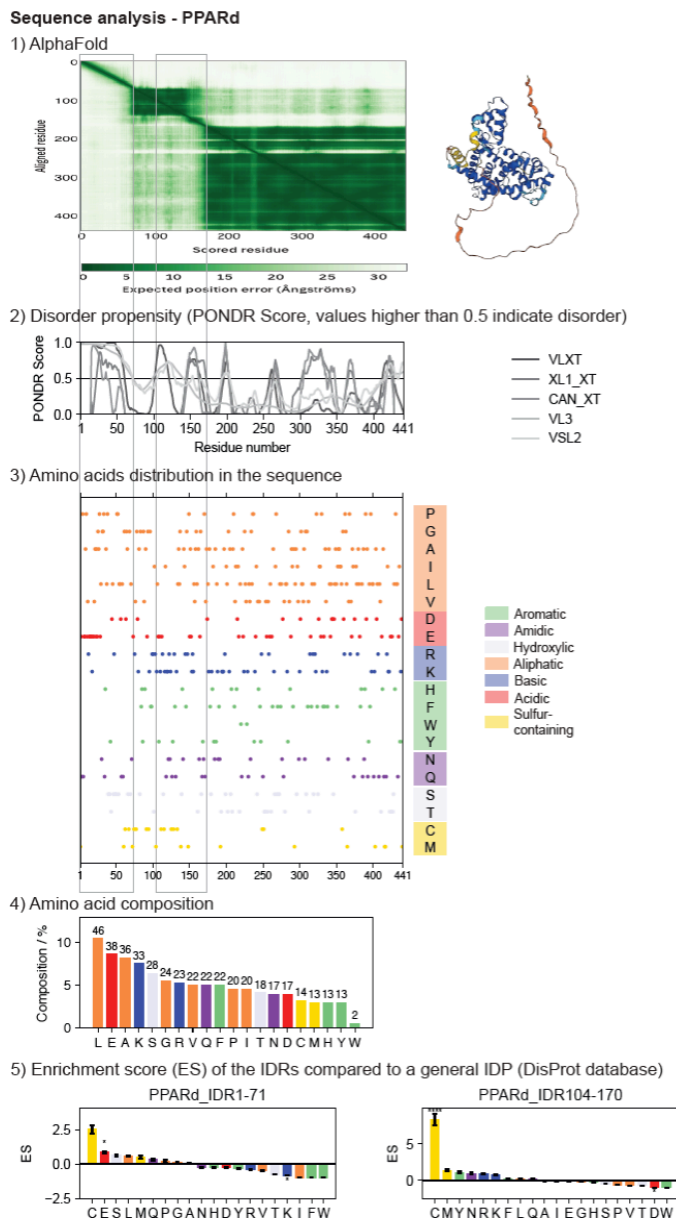
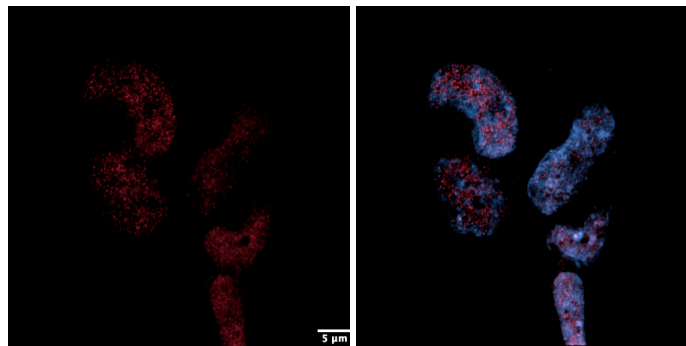


Figure 17: PPAR δ sequence analysis reveals PPAR δ is capable of phase separation.

Analysis of the amino acid structure of PPAR δ including 1) AlphaFold prediction of protein structure, 2) the presence of regions of predicted disorder using various databases, 3) the amino acid families that are present in each of the regions of the protein, 4) the overall amino acid composition of the protein and 5) the enrichment score of the disordered regions when compared to a general intrinsically disordered protein.

Another characteristic of phase separated proteins is that they can exhibit nuclear speckling. In other words, upon staining for the proteins in the nucleus, one should be able to visualize scattered puncta in the nucleus. I stained for PPAR δ in iTF-Microglia and confirmed that PPAR δ shows the nuclear speckling that is typical of phase-separated proteins (**Figure 18A**). I also worked on a protocol for live-cell imaging of PPAR δ . As a first pass for this experiment, I utilized HEK293T cells and overexpressed a HaloTag-PPAR δ construct in the cells. Then, using a fluorescent ligand for the HaloTag protein, I

A



B

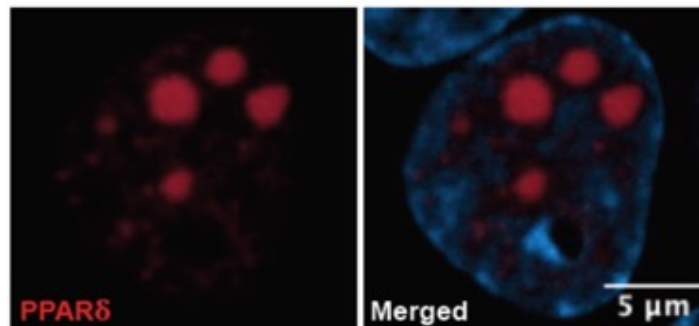


Figure 18: PPAR δ displays typical nuclear speckling of phase-separated proteins.

A) ICC for PPAR δ in iTF-Microglia demonstrating the nuclear localization and appearance of the protein in a microglial model. Red = PPAR δ and Blue = Nuclei

B) Live-cell imaging of HEK293T cells that were transfected with a HaloTag-PPAR δ construct and incubated with a red fluorescent ligand that is specific for HaloTag, showing that PPAR δ also forms condensate-like structures in HEK293T cells.

was able to visualize PPAR δ in real time in live cells using a live-cell setup on our Confocal Microscope. A representative image of these transfected HEK293T cells is shown in **Figure 18B**, and as can be seen, there are large puncta in the nucleus of the cells, likely due to the fact that there is a large quantity of transfected protein.

PPAR δ phase separates for optimal function

PPAR δ is a ligand-activated transcription factor, and transcription factors that phase separate are typically reliant on phase separation for optimal function. Thus, to interrogate whether PPAR δ transactivation of targets is dependent on phase separation, I assessed activation of *ANGPTL4* and *UCP2* by PPAR δ agonism in the presence of 1,6-hexanediol, an aliphatic alcohol that is widely used to disrupt phase separated condensates^{41,42}. I treated iTF-Microglia with or without PPAR δ agonism and with or without 1,6-hexanediol for 30 minutes, after which time I extracted RNA from the cells and proceeded with qRT-PCR experimentation for those targets. Even after 30 minutes, the cells responded to PPAR δ agonism and significantly (though minimally for *UCP2*) upregulated the downstream targets. I found that addition of 1,6-hexanediol significantly reduced expression of *ANGPTL4* and *UCP2* in the setting of PPAR δ agonism in iTF-microglia (**Figure 19**). This further suggests that PPAR δ activity relies upon the biochemistry that results in phase separation for its activity.

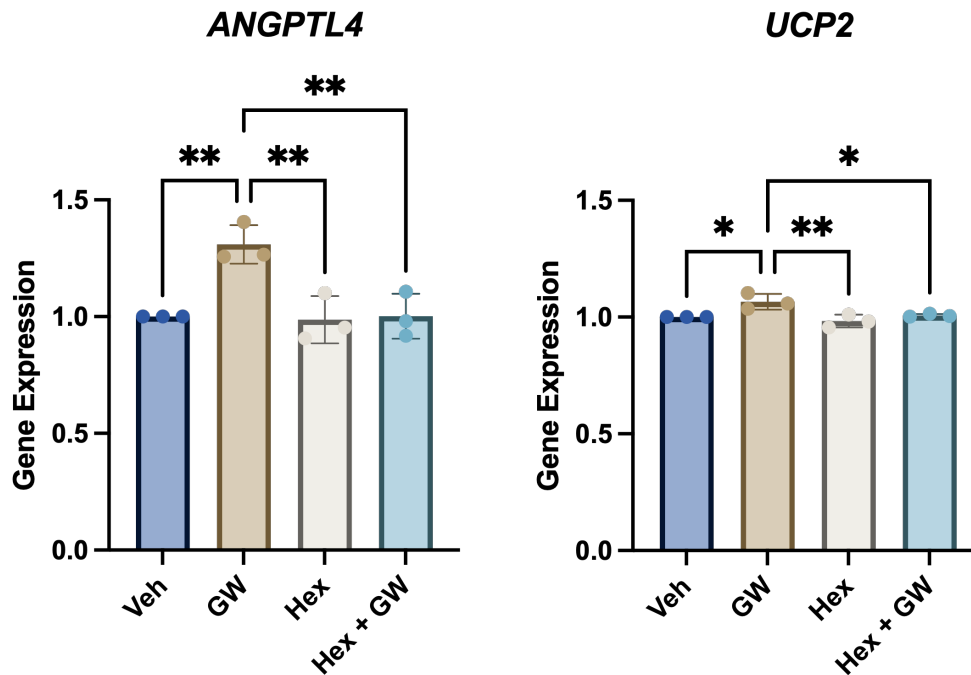


Figure 19: PPAR δ activation of targets is dampened in the presence of 1,6-Hexanediol.

iTF-Microglia were treated with or without 1 μ M of the PPAR δ agonist GW501516 and with or without 1.5% 1,6-hexanediol for 30 minutes. qRT-PCR on established PPAR δ targets *ANGPTL4* and *UCP2* reveal a blunting of PPAR δ activity in the presence of Hexanediol. n=3; *p<0.05; **p<0.01

PPAR δ interacts with and depends on Mediator 1 for proper functioning

After this initial work demonstrating that PPAR δ has the potential to phase separate, I turned to study what other proteins or molecules might be involved in the phase separation of PPAR δ . One of the first findings of this mechanistic work with PPAR δ is that PPAR δ interacts with Med1 of the Mediator complex, as will be shown in a future figure. The Mediator complex is a multi-subunit protein complex that is conserved from yeast to humans⁶⁶. It functions as a signal integrator for transcription, and Med1 in particular has been shown to be important for ligand-dependent interactions with transcription factors⁴². Med1 has been studied as an interactor of PPAR α and PPAR γ ⁶⁷, but not yet with PPAR δ , which is why we interrogated this interaction. As the interaction

between PPAR δ and Med1 increases with PPAR δ agonism with the drug GW501516 (GW), this further supports the hypothesis that Med1 could be important for PPAR δ transactivation of its downstream targets. Med1 is extensively studied in phase separation because it has intrinsically disordered regions (IDR) which are prone to forming phase separated condensates^{42,43}, which could further signify that the interaction between Med1 and PPAR δ is governed by phase separation.

Additionally, I received an immortalized mouse embryonic fibroblast (MEF) line from the lab of Dr. Robert Roeder that was genetically modified to be Med1 KO, along with a control MEF line. As a preliminary experiment to test the effect Med1 might have on PPAR δ mediated transcription, I treated both the Med1 WT and KO MEF cells with the selective PPAR δ agonist GW. While in the WT cells there was significant upregulation of the established PPAR δ targets *ANGPTL4* and *UCP2*, in the Med1 KO cells, the increase was significantly blunted (**Figure 20**). Even at basal levels, the expression of *ANGPTL4*

and *UCP2* was considerably decreased. This further suggests that Med1 is important for PPAR δ activation of downstream targets.

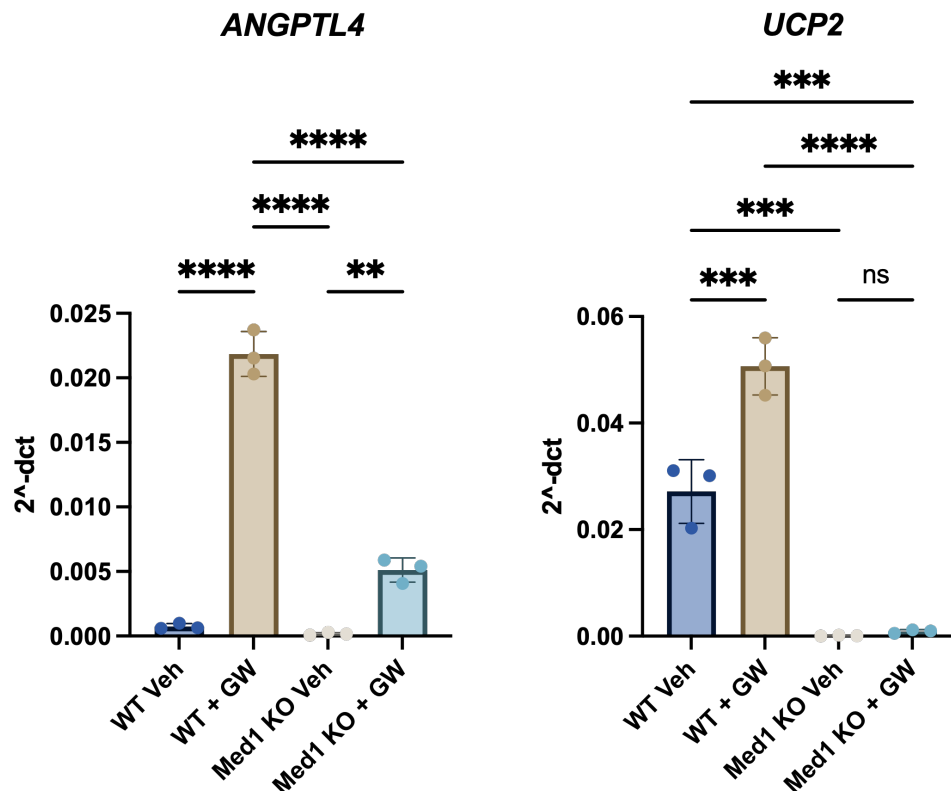


Figure 20: PPAR δ activation of targets is dampened in the absence of Mediator 1. MEF cells (WT and Med1 KO) were treated with either vehicle (DMSO) or GW501516. qRT-PCR was used to measure the expression of the PPAR δ target genes *ANGPTL4* and *UCP2*. n=3. **p<0.01; ***p<0.001

Then, moving to a more physiologically relevant microglial model, we tested the hypothesis of Med1 dependence in iTF-Microglia. iTF-Microglia were nucleofected with siRNA for Med1 to knock down Med1 expression or non-targeting control (NTC) siRNA. Both sets were treated with GW or vehicle and it was seen that iTF-Microglia with Med1 knock-down responded less to PPAR δ agonism than did the NTC transfected cells (**Figure 21**) – further bolstering that Med1 is important for PPAR δ function in microglia.

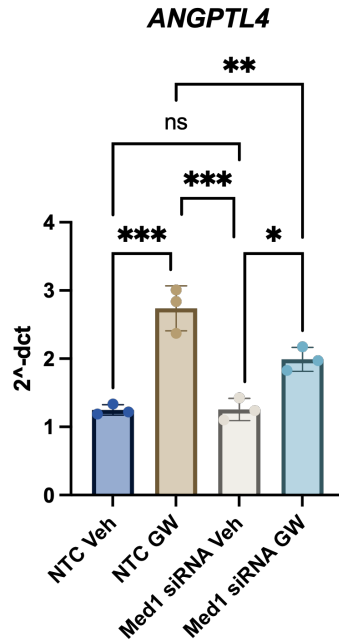


Figure 21: Knockdown of Med1 in iTF-Microglia blunts response to PPAR δ agonism.

qRT-PCR analysis of *ANGPTL4* expression of iTF-Microglia that were nucleofected with either NTC or Med1 siRNA and treated with vehicle (DMSO) or GW501516. n=3; *p<0.05; **p<0.01; ***p<0.001

PU.1 interferes with the PPAR δ -Med1 interaction

I then proceeded to tie in this data with my previous data demonstrating an interaction between PPAR δ and PU.1. As I showed previously, PPAR δ and PU.1 physically interact and addition of the PPAR δ agonist decreases this interaction in a dose-dependent manner. I then overexpressed PU.1 in HEK293T cells and my data revealed that PU.1 may interfere with the interaction between Med1 and PPAR δ (**Figure 22**). In the presence of PU.1 overexpression, the interaction between PPAR δ and Med1 decreased. This introduces the idea of competition in phase separation, which has been a continuously growing field. The concept is such that proteins can compete for phase

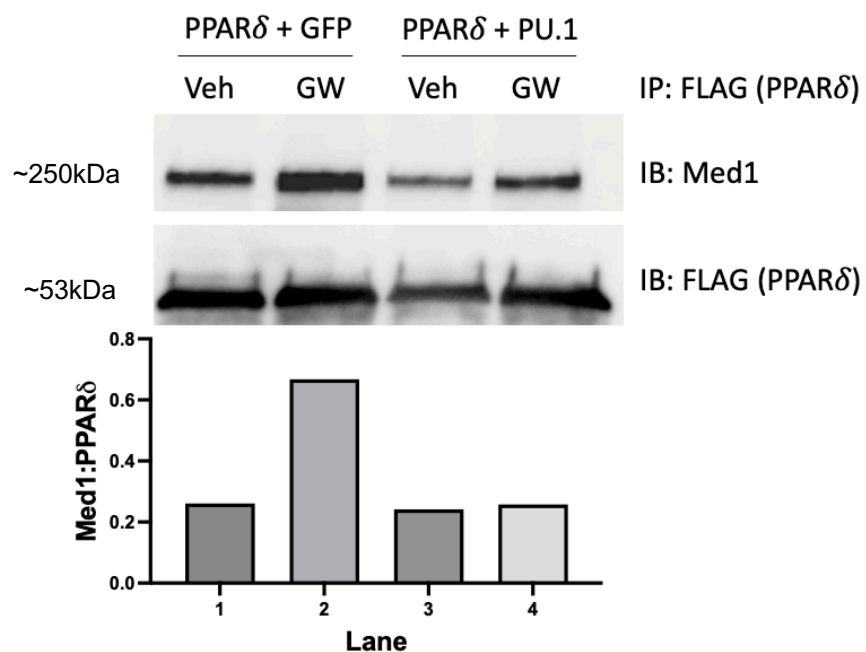
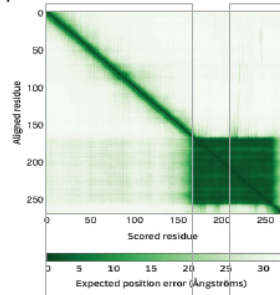


Figure 22: PPAR δ interacts with Mediator 1, and this interaction is disrupted in the presence of PU.1. HEK293T Cells were transfected with either PPAR δ and GFP or PPAR δ and PU.1. Within these conditions, cells were either treated with vehicle or GW501516. While GW501516 increased the interaction between PPAR δ and Med1 under normal conditions, when PU.1 was introduced into the system, the interaction was diminished. Lanes are quantified below the western blot with a ratio of Med1 pulldown to PPAR δ pulldown.

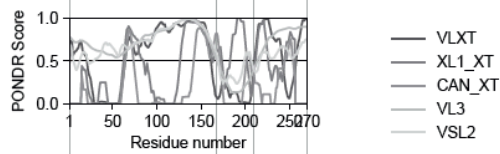
separation with the same binding partner. These two different phase-separated condensates can also be immiscible with each other. Sequence analysis of PU.1 also reveals that it is capable of phase separation, as does previous literature⁶⁸, with two extended regions of disorder that are rich in aliphatic and acidic amino acids (**Figure 23**). This work is being continued with an ongoing collaboration with the Salvatella lab.

Sequence analysis - PU1

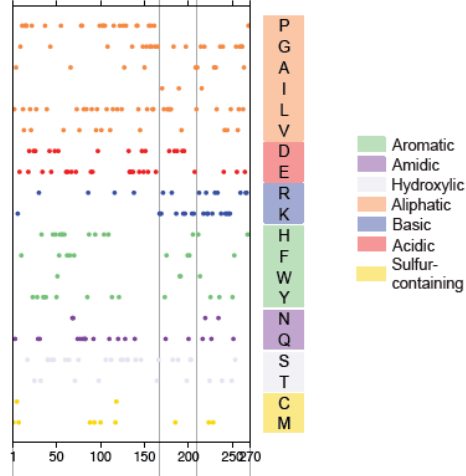
1) AlphaFold



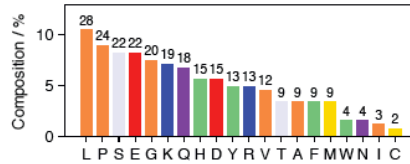
2) Disorder propensity (PONDR Score, values higher than 0.5 indicate disorder)



3) Amino acids distribution in the sequence



4) Amino acid composition



5) Enrichment score (ES) of the IDRs compared to a general IDP (DisProt database)

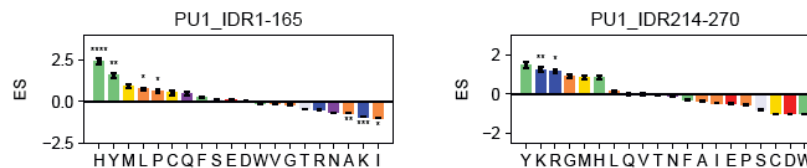


Figure 23: PU.1 sequence analysis reveals PU.1 is capable of phase separation.

Analysis of the amino acid structure of PU.1 including 1) AlphaFold prediction of protein structure, 2) the presence of regions of predicted disorder using various databases, 3) the amino acid families that are present in each of the regions of the protein, 4) the overall amino acid composition of the protein and 5) the enrichment score of the disordered regions when compared to a general intrinsically disordered protein.

Materials and Methods

Hexanediol Treatment

1,6-hexanediol, supplied as a crystalline solid, was dissolved in the corresponding cell culture media for the cells at a final concentration of 1.5%. This media was then supplemented with any other treatments for the experiment (e.g. treatment with GW501516 and DMSO as a vehicle control). This media was added to BV2 cells or iTF-Microglia and cells were placed back in the 37°C incubator for 30 minutes. After this incubation, RNA was isolated from cells and qRT-PCR was used to determine relative gene expression across different treatment conditions.

Live Cell Imaging

HEK293T cells were transfected with appropriate vectors as described in Chapter 1. 24 hours after transfection, a fluorescent HaloTag ligand was added to media and incubated per manufacturer's instructions. After subsequent washes, cells were visualized under the Nikon AR1HD confocal microscope.

Immunofluorescence

After treatment, cells were washed three times with PBS and fixed with 4% paraformaldehyde for 20 minutes. After 3 more washes, the cells were permeabilized using a triton solution and blocked with 5% bovine serum albumin. Cells were incubated with primary antibody overnight at dilutions recommended by the manufacturer, followed by washes and a secondary antibody incubation at 1:1000. Cells were then washed and incubated with Hoechst at 1:10000 for 10 minutes. Cells were washed and a cover slip

was added over Prolong Antifade Glass. Cells were imaged using the confocal microscope.

Nucleofection of iTF-Microglia

Day 8 iTF-Microglia were collected from their original plate by incubation with 1X TrypLE for 10 minutes. After the 10-minute incubation, Advanced DMEM/F12 was added to quench the reaction at a ratio of 3:1. Cells were spun down at 220xg for 5 minutes, resuspended in Day 8 iTF media, and counted using a Countess 3. 2.2×10^5 cells were collected per nucleofection reaction. Nucleofection reagents were prepared as described by the manufacturer's instructions, along with 3 μ M siRNA (either a non-targeting control or Med1 siRNA) and the cells were nucleofected per manufacturer's instructions. Following this, cells were replated in a 12-well plate in Day 8 iTF media and allowed to incubate for 3 days. On day 3 post-nucleofection, iTF-Microglia were treated with 1 μ M GW501516 or DMSO vehicle control for 1 hour, followed by RNA isolation and qRT-PCR for downstream PPAR δ target expression.

Mouse Embryonic Fibroblast Cell Lines

WT and Med1 KO mouse embryonic fibroblast (MEF) cell lines were generously provided by the lab of Dr. Robert Roeder. Information about these cells can be found in the original publication where they were generated⁶⁹.

CHAPTER 3: Selective vulnerability in Huntington's Disease: From excitotoxicity, mitochondrial dysfunction, and transcription dysregulation to therapeutic opportunity

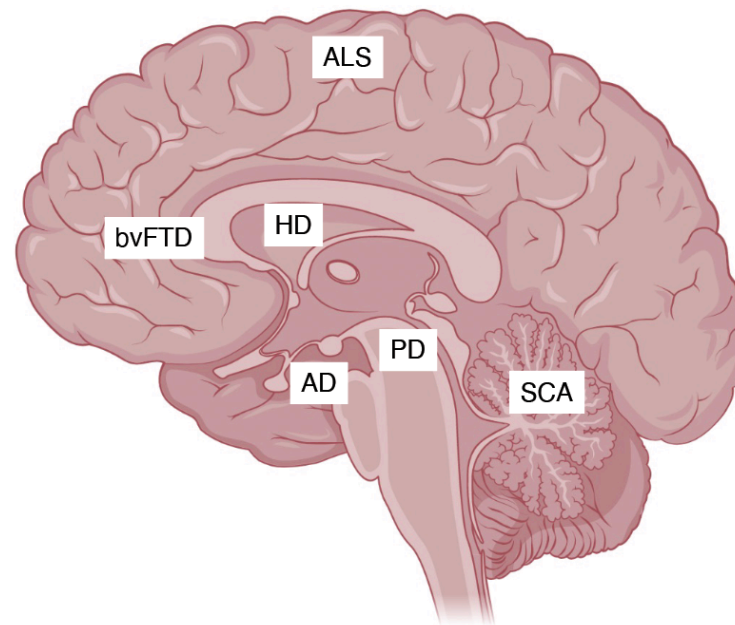
Abstract

Huntington's disease (HD) is an inherited neurodegenerative disorder characterized by selective vulnerability of medium spiny neurons (MSNs) in the striatum. Various lines of investigation have provided insights into possible mechanisms underlying selective vulnerability of corticostriatal circuits in HD. Mitochondrial dysfunction is an important component of the underlying disease process, as it contributes to neurodegeneration and cell death. Explanations for mitochondrial dysfunction include PGC-1 α transcription interference, altered p53 function, and Heat Shock Factor 1 dysregulation. PPAR δ has also emerged as an important target for mitochondrial dysfunction and plays other roles in cellular homeostasis. Studies of these targets have highlighted therapeutic strategies that are effective in HD mouse models and patient iPSC-derived MSNs. Here we review pathways underlying the exquisite vulnerability of MSNs in HD, describe our current understanding of HD mitochondrial dysfunction, highlight key implicated regulatory factors, pathways, and cellular processes, and delineate the therapeutic opportunities emerging from this exciting research.

KEYWORDS: Huntington's disease; medium spiny neuron; selective vulnerability; excitotoxicity; mitochondria; transcription interference; oxidative stress; PPARs; Heat Shock Factor 1; p53

Huntington's Disease (HD) was first described in 1872 by George Huntington⁷⁰. HD is an autosomal dominant disorder that occurs as the result of a CAG trinucleotide repeat expansion in exon 1 of the huntingtin (Htt) gene. HD typically presents in adulthood, although a juvenile form of HD exists. In the non-HD population, normal CAG repeats range in size from 6 to 35, whereas 36-39 CAG repeats may result in a late-onset, less severe HD phenotype or lack of penetrance. When the repeat length of CAG reaches 40 or more, it is highly penetrant. In the case of juvenile HD, signs and symptoms start before the age of 20 years and the number of CAG repeats reported in most cases are 55 or more⁷¹. HD commonly presents in patients in the 4th or 5th decade of life. CAG repeat length is directly related to age of onset of disease symptoms, as the longer the CAG repeat length, the earlier the onset. This phenomenon of worsening disease severity in successive generations is known as anticipation and is seen in HD families especially with paternal inheritance⁷². CAG repeats encode for polyglutamine (PolyQ) tracts and the mutant huntingtin protein contains a long stretch of polyglutamine resulting in toxic aggregates. HD is typically characterized by involuntary movement with cognitive decline including deficits in short term memory, skilled learning, metabolic abnormalities and psychiatric symptoms⁷³. The prevalence of HD varies among geographical locations with a worldwide prevalence of ~2.7 per 100,000⁷⁴.

Selective Vulnerability in Huntington's disease



Disease	Most Affected Neurons	Accumulated Protein
Huntington's disease (HD)	Medium spiny neurons	Huntingtin
Alzheimer's disease (AD)	Pyramidal neurons	Amyloid-beta & tau
Parkinson's disease (PD)	Dopaminergic neurons	α -synuclein
Frontotemporal dementia (behavioral variant) (bvFTD)	Fronto-insular cortex & anterior limbic area neurons	TDP-43
Amyotrophic lateral sclerosis (ALS)	Motor neurons	TDP-43
Spinocerebellar ataxia (SCA)	Purkinje neurons	Various forms

Figure 24. Selective vulnerability in neurodegenerative disease.

Here we see a schematic diagram of the brain showing the preferentially affected CNS regions for six different disorders, specified in a tabular list at the bottom. For each disease, the most affected neurons corresponding to the selectively vulnerable brain region are listed, along with the pathogenic protein which accumulates in each respective disorder. Created with BioRender.com

A hallmark of neurodegenerative diseases is the accumulation of toxic misfolded protein / peptide deposits. However, despite this common feature, the specific protein that accumulates, the cells affected, and the site of protein / peptide accumulation varies by disease. Another distinguishing feature of neurodegenerative diseases is selective vulnerability, which refers to the fact that only certain cell populations in certain regions

of the central nervous system (CNS) are affected in each neurodegenerative disorder (**Figure 24**). This is a hallmark of neurodegenerative disease and has been a puzzling finding. For example, Alzheimer's disease (AD) often affects the entorhinal cortex and hippocampus, while Parkinson's disease (PD) involves the substantia nigra pars compacta (SNpc) dopaminergic neurons. In the case of HD, the cells that are most affected are the medium spiny neurons (MSNs), also known as spiny projection neurons (SPNs) within the striatum, with some damage also occurring in the cerebral cortex. Additionally, prominent atrophy occurs in the neocortex, the main input region to the striatum and later, in advanced stages, other brain regions can also become affected⁷⁵. Cortical neurodegeneration is more pronounced in the motor and premotor areas and mainly affects cortical pyramidal neurons. Changes in neuron function occur before overt cell death, suggesting that alteration in the corticostriatal circuitry precedes onset of disease symptoms. Detailed postmortem analysis of patient brain samples has provided important information about HD pathology. Roughly 40% dopaminergic neuron loss can be observed in the SNpc as well as significant loss of dopaminergic terminals in the striatum⁷⁶. SNpc neurons show impaired oxidative phosphorylation in comparison to dopaminergic neurons in the ventral tegmental area (VTA).

Discovery of Mitochondrial Dysfunction in HD

The energy impairment hypothesis of HD was proposed in the 1980's based upon clinical observations from HD patients. Many studies with glucose metabolism using positron emission tomography (PET) suggest impaired energy metabolism. HD patients exhibit increased lactate production at early symptomatic stages, indicating the possible

reduction of oxidative mitochondrial metabolism and a metabolic shift from oxidative phosphorylation to glycolysis^{77–79}. In HD patients, impaired glucose metabolism in the BG and cerebral cortex has been documented^{80–82}. Neurons in the brain continuously have a high demand for ATP to maintain normal functioning. Mitochondria in neurons are responsible for ATP production and supply energy for ion exchange pumps for maintaining electrochemical gradients across the membrane. Defective energy metabolism could translate into an enhanced susceptibility of mitochondria for depolarization. The progressive impairment of energy metabolism may secondarily result in slow excitotoxic neuronal death⁸³. Various mechanisms have been proposed for energy impairment in the HD brain, including oxidative stress, abnormal mitochondria trafficking, dysregulation of peroxisome proliferator activated receptor gamma co-activator -1 alpha (PGC-1 α), and excitatory corticostriatal glutamatergic inputs.

PGC-1 α

PGC-1 α is a master regulator of mitochondrial biogenesis, adaptive thermogenesis, beta oxidation of fatty acids, and glucose metabolism, and it responds to changing energy demands in the cell (**Figure 25**)⁸⁴. PGC-1 α is also crucial for a cell to appropriately respond to oxidative stress⁸⁵. PGC-1 α is highly expressed in tissues with high energy demand and large numbers of mitochondria, including brown adipose tissue (BAT), heart, skeletal muscle, and brain. PGC-1 α activates a diverse set of metabolic programs in different tissues by forming a heteromeric complex with a variety of transcription factors, including NRF-1, NRF-2, and especially the nuclear hormone

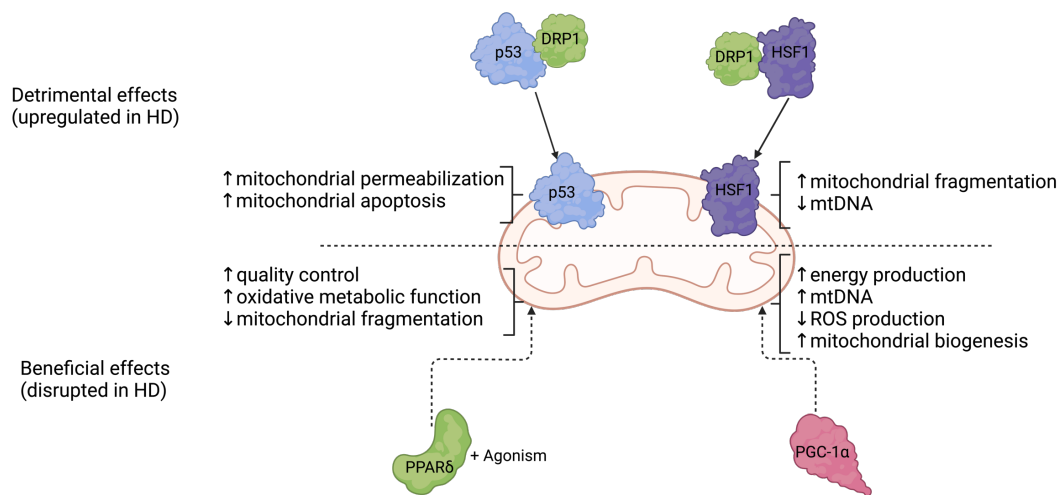


Figure 25. Mitochondrial toxicity in Huntington's disease Mitochondrial toxicity in Huntington's disease. A number of different pathways have been implicated in the mitochondrial dysfunction occurring in Huntington's disease. Here we see a subset of the most predominant pathways involved in the pathogenesis of Huntington's disease. Two pathways (top) that cause detrimental effects are upregulated in their activity, while two pathways (bottom) promoting beneficial effects are disrupted in Huntington's disease. The downstream effects wrought by each of these four pathways, whether detrimental or beneficial, is summarized for each respective pathway. Created with BioRender.com

receptors PPAR α , PPAR δ , PPAR γ , ERR α , and TR⁸⁶. These interactions typically depend on the type of tissue. For example, PGC-1 α interacts with MEF2c in skeletal muscle, while engaging with HNF4a and FOXO1 in the liver. NRF-1 and NRF-2 regulate the expression of many nuclear-encoded mitochondrial genes, including cytochrome c, complexes I-V, and mitochondrial transcription factor A (Tfam)⁸⁷. A role for PGC-1 α in HD was published by three independent groups in 2006. The works published by Cui et al. and Weydt et al. in 2006 separately revealed a specific role for PGC-1 α in mitochondrial dysfunction in HD pathogenesis by studying human post-mortem HD brains, HD mice, and a cell culture model of striatal-like cells from knock-in HD mice (ST-Hdh-Q111)⁸⁸.

We linked PGC-1 α dysregulation to the metabolic abnormalities experienced by the HD N171-82Q mice⁸⁸. These N171-82Q transgenic mice develop behavioral abnormalities, including loss of motor coordination, tremors, and abnormal gait before dying prematurely⁸⁹. While evaluating metabolic parameters, significant changes in body temperature were observed. The transgenic mice were unable to maintain their body temperature and developed progressive hypothermia, accompanied by a progressive decline in motor symptoms and weight loss by 17 weeks of age⁸⁸. HD mice developing hypothermia was a specific phenotype observed only in HD mice, as other neurodegenerative models, such as SOD1-G93A ALS model mice, maintain normal body temperature despite experiencing loss of nearly 30% of their body weight. In rodents, adaptive thermogenesis is mediated by BAT and is different from the white adipose tissue (WAT) in terms of mitochondrial density and vascularization. In mammals, a function of the hypothalamus is to sense a drop in body temperature to eventually lead to thermogenesis and activate the sympathetic nervous system, and BAT is the target for increased sympathetic output.

As PGC-1 α coordinates mitochondrial biogenesis and regulates mitochondrial function, BAT derived from N171-82Q displayed a reduced number of mitochondria and reduced ATP/ADP ratio compared to control mice, similar to the result from PGC-1 α KO mice⁹⁰. Prior to onset of weight loss, N171-82Q mice showed a hypophagic, hypermetabolic phenotype, and they experienced neither the decrease of metabolic rate nor the increase of food intake induced by fasting in wildtype mice. There was no change in glucose level prior to 18 hours of fasting, but both wildtype and HD mice showed a

significant drop in leptin levels, which is a hormone synthesized in response to hunger. BAT UCP-1 levels are normally reduced during fasting⁹¹, but HD mice failed to reduce BAT UCP-1 levels during fasting and showed paradoxical upregulation of PGC-1 α . Taken together, these findings support the role of PGC-1 α transcriptional interference in the degeneration of striatum in HD.

As described thus far, PGC-1 α is an important player in HD as seen by the thermoregulatory issues experienced by HD patients. However, how does this translate into issues in the CNS of HD patients? To gain insight into whether PGC-1 α transcription interference is maintained and has significance in the brains of HD patients, we analyzed microarray expression data from the caudate nuclei of a large cohort of human HD patients and learned that out of 26 known PGC-1 α targets, 24 showed a significant reduction in expression⁸⁸. The microarray data also showed significant upregulation of nuclear hormone receptor and transcription factor genes encoding PPAR α , RXR- α , and NRF-1, which are all known to rely upon PGC-1 α for their target gene transactivation, and could reflect a compensatory response due to the fact that they were not able to carry out their proper function. The question then became how transcriptional interference of PGC-1 α affects the striatum. By measuring the oxygen consumption rate of brain slices from HD and control mice, we found that HD neurons do not properly metabolize the lactate-pyruvate substrate, though the electron transport chain capacity appeared to remain intact⁸⁸. Additionally, and quite remarkably, the ability of PGC-1 α to rescue phenotypes of mitochondrial dysregulation was studied using HD neurons. When insulted with 3-NP, the toxin previously described to inhibit complex II of the electron transport chain, these

cells will normally experience mitochondrial membrane potential defects. However, when PGC-1 α was stably transfected and expressed in these neurons, the mitochondria were able to maintain their membrane potential. This further points to the fact that PGC-1 α is a very important transcriptional regulator of mitochondrial health and is a major player in the pathogenesis of HD.

The assessment of transcriptional interference of PGC-1 α in the pathogenesis of HD continued in an *in vivo* experiment, utilizing an inducible model for induction of PGC-1 α expression in HD mice⁹². We found that expression of PGC-1 α was sufficient to improve performance on a variety of behavioral tests, showing that PGC-1 α could rescue HD neurological phenotypes *in vivo*. PGC-1 α expression was also able to reduce the aggregation of Htt in the striatum of HD N171-82Q mice and preserved striatal volume and neuron number in these mice, when compared to HD littermates lacking PGC-1 α over-expression. Mechanistically, it was found that this reduction of mHtt aggregation is due to PGC-1 α induction of transcription factor EB (TFEB). TFEB is a master regulator of autophagy and lysosomal biogenesis. Its induction by PGC-1 α allows cells to better process aggregates of mHtt. Also relevant to the issue of mitochondrial dysfunction in HD, induction of PGC-1 α expression yielded increased expression of genes that are required for increased mitochondrial biogenesis and energy production. Moreover, the mitochondrial genome number was also increased and the ability of cells to counter oxidative stress was also rescued, which is necessary when there is increased mitochondrial respiration.

Peroxisome-Proliferator Activated Receptors (PPARs)

The effect of PPAR agonism in HD has also been studied using the pan-PPAR agonist bezafibrate⁹³. This group treated a mouse model of HD with drug and observed amelioration of motor dysfunction and neuropathology, which was attributed to activation of the PPAR-PGC-1 α pathway and consequently decreased ROS due to increased antioxidant expression. Structural abnormalities in the skeletal muscle of these HD mice were also corrected with bezafibrate treatment. While it is not possible to discern if a certain PPAR is responsible for the protective actions of bezafibrate, many of these same results were seen by using a selective PPAR δ agonist, so PPAR δ is likely a major contributor to these beneficial effects.

Bexarotene

Since PPAR δ was proven to be an attractive therapeutic target and since PPAR δ is known to heterodimerize with the RXR, an FDA approved drug that is a known RXR agonist was evaluated as a potential therapeutic for HD⁹⁴. This drug is bexarotene, also known as Targretin, and is approved for use in human patients that have T cell cutaneous lymphoma⁹⁵. Bexarotene has also been studied as a potential therapy for AD and was reported to be quite effective, though this result has been disputed in follow-up studies. Importantly for HD, RXR agonists are quite capable of promoting PPAR δ activation, and when used as a treatment in primary cortical neurons from BAC-HD mice, the neurotoxic effects of mHtt protein were ameliorated⁹⁴. Bexarotene was similarly neuroprotective in iPSC-derived medium spiny-like neurons from a HD patient and was found to significantly improve motor function and neurodegeneration *in vivo* in HD N171-82Q mice.

In terms of bioenergetics, bexarotene was able to restore proper oxidative metabolic function in HD mice and improve mitochondrial and protein quality control. Genes involved with oxidative phosphorylation and gluconeogenesis were upregulated in HD mice upon bexarotene treatment when compared to vehicle-treated HD mice. Additionally, mitochondria in HD neurons are fragmented, but bexarotene was able to significantly reduce mitochondrial fragmentation, boosting the ability to regulate energy stores and promote neuroprotection. Regarding proteostasis, PPAR δ activation was found to significantly upregulate autophagy. The exact mechanism of PPAR δ activation of autophagy has yet to be elucidated, but it was found that PPAR δ activates the autophagy pathway in mouse neurons in a TFEB-independent manner, unlike the mechanism of PGC-1 α .

Therapeutic Opportunities

There are a variety of candidate therapies to treat HD based upon these pathogenic processes (**Table 1**). PGC-1 α transcription interference has emerged as a major aspect of HD pathology, leading cells to experience mitochondrial dysregulation and increased oxidative stress. Expression of PGC-1 α was sufficient to reduce disease phenotypes in several models, so a therapeutic agent that would target this transcription interference to allow PGC-1 α to carry out its function would be attractive. Studies have shown that mHtt interferes with PGC-1 α transcription, but the exact mechanism remains elusive. Once understood, this would allow rational targeting of PGC-1 α to enable it to carry out its vital functions of mitochondrial biogenesis and energy production. Along the

same lines as PGC-1 α , TFEB represents another relevant target. Since PGC-1 α can significantly decrease mHtt aggregation in the striatum of mice through TFEB, targeting of TFEB might reduce mHtt aggregation by inducing autophagy, unless autophagic flux is blocked in the context of existing disease. A combinatorial therapy targeting both PGC-1 α and TFEB function would thus be highly desirable.

Table 1. Candidate Therapies for Huntington's Disease Mitochondrial Dysfunction.

Therapeutic	Target / Mechanism of Action	Results of treatment	Citation
PPAR δ agonist therapy (KD3010/T3D-959)	Activation of PPAR δ (while KD3010 is specific for PPAR δ , T3D-959 has 15:1 selectivity for PPAR δ over PPAR γ and Bezafibrate is a pan-PPAR agonist)	-decreased motor dysfunction and neuropathology in model mice -decreased ROS damage -decrease mitochondrial fragmentation -upregulates oxidative metabolic function	Dickey et al., 2016
Bexarotene	RXR α agonist	-restore proper oxidative metabolic function -improve mitochondrial and protein quality control -reduced mitochondrial fragmentation -upregulate autophagy	Dickey et al., 2017
Bezafibrate	Pan-PPAR Agonist	-decreased motor dysfunction and neuropathology in model mice -decreased ROS damage -decrease mitochondrial fragmentation -upregulates oxidative metabolic function	Chandra et al., 2016

PPAR δ agonism also presents a very attractive therapeutic approach for HD. For one, there are already two PPAR δ agonists that are approved by the FDA for use in clinical trials and both (KD3010 and T3D-959) were shown to be safe in human patients in clinical trials. Indeed, T3D-959 is currently being evaluated in Phase 2 clinical trials for mild-to-moderate Alzheimer disease⁹⁶. Not only can PPAR δ be targeted directly, but it can be activated indirectly by activation of its dimerization partner, RXR. RXR is targetable by bexarotene (Targretin), which is already an approved drug for use in humans, though blood brain barrier penetration of bexarotene in the human appears to be suboptimal. PPAR δ agonism has shown exceptional promise in a preclinical trial for HD, and has several established mechanisms for neuroprotection in neurons⁵. This is further corroborated by the fact that loss of PPAR δ function in the MSNs of mice was sufficient to elicit neurodegeneration. By targeting PPAR δ in HD mice, we were able to correct motor phenotypes, restore mitochondrial functioning, and prevent excessive neuronal death. Even when using a pan-PPAR agonist, bezafibrate, there were marked improvements in HD mice⁹³, and many of these drugs are already approved for use in humans to treat Type II diabetes mellitus. This has yet to be shown in humans, but it remains a very promising mode of therapy. Since there are two established mechanisms described here to activate PPAR δ , one could imagine a combinatorial therapy with a PPAR δ agonist and pan-PPAR agonist, which could each likely be used at a lower dose to minimize their side effects while both acting on the same pathway.

All the above-described modes of therapy present promising ways to potentially treat HD patients. However, as one can imagine, one therapeutic agent on its own is

unlikely to be a cure, especially if therapy is instituted after disease onset. Significant slowing of disease progression or even halting of disease progression, however, is a reasonable and theoretically plausible goal. For maximal efficacy, a combination therapy or even a cocktail of pharmacological approaches could offer synergistic effects and should be considered, especially as effective treatments are identified.

Conclusion & Future Directions

HD is a devastating neurodegenerative disorder characterized by motor, psychiatric, metabolic and cognitive abnormalities. In the brain, MSNs appear to be especially vulnerable to the disease process, and though there are potential explanations, a parsimonious conclusion is lacking. Mitochondrial dysfunction is an important component of the underlying disease process in HD, as it contributes to neuron dysfunction and cell death. A key aspect that has emerged as important when discussing mitochondrial dysfunction in HD is PGC-1 α transcription interference. PPAR δ has also emerged as an important target for mitochondrial dysfunction and likely plays other roles in cellular homeostasis. Research on these different targets has highlighted various therapeutic strategies that are effective in HD mouse models and patient iPSC-derived MSNs. However, how these therapies will fare in human HD patients remains an open question.

Moving forward, certain key questions require particular attention for HD therapy development. One enigmatic issue in the neurodegenerative disease field is that of selective vulnerability, which needs to be better understood in order to prioritize the

contribution of the many complex pathways already implicated in the pathogenesis of neurodegenerative diseases. Research should focus on PGC-1 α -TFEB regulation in the context of mitochondrial dysfunction. As crosstalk between regulatory pathways acting on the mitochondria almost assuredly exists, we need to identify the processes by which the different inputs to mitochondria are balanced and weighed against one another to dictate the metabolic and functional status of mitochondria, and this effort must take into account the likelihood that mitochondrial regulatory pathways operate differently in neurons, astrocytes, oligodendrocytes, and microglia. Hence, deconstructing mitochondrial dysfunction at the level of different cell types should be an important goal of future work. Regulatory nodes with significant therapeutic potential deserve special consideration. For example, PPAR δ agonism should be further investigated as a potential therapeutic for HD given how important proper PPAR δ function appears to be for MSNs. While many challenges remain, advances over the last two decades have now set the stage for rational treatment strategies to be tested in HD in an ever growing number of relevant model systems. Hence, it is reasonable to envision that the goal of identifying a series of meaningful, disease-modifying therapies for HD will soon be within our grasp.

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Declarations

The authors have nothing to declare.

Chapter 4: Discussion

Research over the last several years has revealed that microglia are key players in AD, but has also generated conflicting results as to whether microglia are beneficial or detrimental in the diseased brain^{2-4,29}. Microglia clear debris and pathogenic proteins in the brain, but they can also cause excessive neuroinflammation⁴. Their role certainly involves some sort of balance between their different actions and is still contentious. Interestingly, when looking at genetic risk factors for sporadic AD, microglia that harbor those genetic alterations (e.g., *APOE4* variant and *TREM2* mutations) are typically unable to mount a proper response to the accumulating pathology, and some secrete copious amounts of inflammatory cytokines^{17,18,26}. An attractive therapeutic route to pursue is one in which the beneficial aspects of microglia are enhanced, while the detrimental are suppressed. Given this current understanding of microglial function, and my research surrounding PPAR δ agonism in microglia, PPAR δ represents one such therapeutic target.

The culmination of the first chapter of my research suggests that PPAR δ agonism activates microglia into a state focused on phagocytosis and lipid processing, while reducing subsequent inflammation. As described in the introduction, a recent single-nucleus sequencing analysis of microglia across 443 subjects (AD vs non-AD) revealed 12 different microglial clusters²⁰. These microglial states ranged from homeostatic, to a variety of inflammatory states, and even included a lipid processing state. Within the lipid processing state, *PPAR γ* was enriched, along with other lipid processing genes such as *ABCA1*. This lipid processing state is similar to what I have observed in the iTF-Microglia

model when the cells were treated with PPAR δ agonism, as lipid metabolism genes (including *ABCA1*) were among the highest upregulated genes by PPAR δ in my dataset.

Lipid metabolism is crucial for microglia, and appears to be especially important in a disease state^{97–101}. An entire arm of AD research is dedicated to studying lipid dysregulation. This is well warranted, as the single greatest genetic risk factor for sporadic AD is the *APOE4* variant, which is itself involved in lipid transport¹⁰². Lipid metabolism has been shown to be closely tied to inflammation²⁰. Additionally, a class of microglia has been described that are lipid-laden and dysfunctional by way of defective phagocytosis, production of excessive reactive oxygen species and secretion of proinflammatory cytokines^{103,104}. *APOE4* has been linked to this lipid droplet accumulation in microglia as well¹⁰¹. Thus, the ability of PPAR δ agonism to promote lipid metabolism in microglia would likely prove beneficial in disease.

Our data shows PPAR δ as a driver of lipid metabolism in microglia, and the single-nucleus dataset from the Tsai lab demonstrates that PPAR γ is involved in promoting a state of lipid metabolism as well. It will be important to determine which pathways are more affected by PPAR δ versus PPAR γ in microglia, and if there is a large overlap, which PPAR is a more effective target or if a dual agonist approach might be more beneficial. We initially chose to target PPAR δ due to my lab's previous findings that PPAR δ is neuroprotective in HD⁵ and because it is the most highly expressed PPAR in microglia^{6,7}.

Endocytic pathways were also upregulated by PPAR δ agonism in our single-cell data, and PPAR δ agonism increased the phagocytosis of A β and rat synaptosomes through our phagocytosis assays. Supporting our findings, PPAR δ appeared as a positive hit in a CRISPRa screen conducted by the Kampmann lab for modulators of phagocytosis in iTF-Microglia⁵². Separately, in older studies, PPAR γ has also been shown to increase phagocytosis in microglia^{105,106}. Thus, it will again be important to discern the difference in regulation of phagocytosis for PPAR δ versus PPAR γ .

Phagocytosis is a key function of microglia, and it has been shown that microglia that harbor risk genes for AD are not effective phagocytes^{17,18}. Effective phagocytosis is crucial in the AD brain to aid in clearance of protein accumulation and damaged cells, which in turn can help resolve inflammation and aid in tissue repair¹⁰⁷. On the other hand, excessive phagocytosis by microglia is detrimental and correlates with cognitive decline¹⁰⁸. Therefore, it will be especially important to assess the implications of the increased phagocytosis of synapses *in vivo* because it is certainly a concern if microglia would undergo uncontrolled phagocytosis of healthy synapses in the diseased brain⁶⁰. However, many other factors would regulate this process *in vivo*¹⁸, and the synaptosomes used in our experimentation are derived from a rat brain which, while they are often used as a model to study microglial phagocytosis⁵², are not a human model.

Additionally, PPAR δ reduced the inflammatory nature of microglia in our experimentation both *in vivo* and *in vitro*. Proinflammatory cytokine production in AD has many detrimental effects including neuronal damage, neuronal loss, reduced A β

clearance by microglia, and tau hyperphosphorylation^{109,110}. *APOE4* microglia upregulate many immune-related genes and have been described as “immune-prone”¹⁷. Thus, PPAR δ reduction of inflammatory cytokine production and secretion could be beneficial to the diseased brain. These favorable effects have been shown in a mouse model of experimental autoimmune encephalomyelitis (EAE), where PPAR δ agonism reduced inflammation and was neuroprotective in EAE⁴⁰, furthering the rationale that this would be beneficial in other neurodegenerative diseases.

The interaction between PPAR δ and PU.1 represents another potential beneficial aspect to PPAR δ agonism. The PPARs and PU.1 had not previously been studied together in microglia; however, a functional interaction between PPAR γ and PU.1 has been shown in adipocytes, where PU.1 overexpression altered the cistrome of PPAR γ ¹¹¹. Binding of PPAR γ throughout the genome was decreased based on PU.1 overexpression, but the group who studied this did not report a physical interaction between the two proteins¹¹¹. Regardless, the combination of NanoBRET and co-immunoprecipitation experiments I have completed is suggestive of a physical interaction between PPAR δ and PU.1.

PU.1 is an attractive therapeutic target for many reasons. It has been repeatedly identified as an AD risk gene, with greater expression correlating with greater risk^{32,33,112}. PU.1 regulates inflammatory processes as well as many AD risk genes^{33,34}. PU.1 activity was also recently shown to prevent *APOE4* microglia from taking on a DAM-like state²⁶. Lastly, and most recently, repression of PU.1 activity in AD mouse models increased

cognition while decreasing neuroinflammation and neuronal loss³⁴. Thus, if activating PPAR δ can modulate PU.1 activity, it could be part of the reason why we note a decrease in inflammatory processes in microglia upon PPAR δ agonism. Moving forward, the ability of PPAR δ to affect PU.1 function can be tested using the BV2-PU.1 Luciferase reporter cell line that was developed by the Tsai lab³⁴. This immortalized microglia cell line has a plasmid that was stably integrated into the *Rosa26* locus that has a PU.1 binding motif upstream of the luciferase gene. Thus, luminescence will correlate with PU.1 activity. PPAR δ agonism can be tested in this cell line to determine the downstream functional effect of PPAR δ agonism on PU.1 activity.

Additionally, as mentioned above, PU.1 activity was shown to prevent *APOE4* microglia from taking on a DAM-like state, and instead kept microglia in a more homeostatic state that was unable to respond to AD pathology²⁶. My single-cell dataset revealed that PPAR δ agonism shifted microglia into a “primed” or pre-activated state that increased the expression of lipid-processing and phagocytic pathways while reducing inflammatory pathways. With this in mind, and given the NanoBRET data that shows a decrease in PPAR δ -PU.1 interaction with PPAR δ agonism, it is possible that the interaction between PU.1 and PPAR δ is one of the ways in which PU.1 maintains a homeostatic signature in microglia. Release of PPAR δ from PU.1 upon agonism might allow the microglia to take on a more activated and disease responsive phenotype.

The next portion of my research focused on the mechanistic basis of PPAR δ activity in microglia. My data demonstrates that PPAR δ is capable of phase separation

and that PPAR δ is dependent upon the transcriptional coactivator Med1 for optimal function. This was completed with the overall hypothesis that PPAR δ phase separates with Med1 to activate expression of its downstream targets. This body of work began because PPAR γ has been shown to undergo phase separation, and its ability to phase separate was critical to its function⁴¹. This is a characteristic that has continued to be true for many different transcription factors⁴³. We interrogated an interaction between PPAR δ and Med1 because Med1 has been shown to interact with and be crucial for PPAR α and PPAR γ function^{69,113–115}. Thus, there is strong rationale that PPAR δ also undergoes phase separation to activate its targets and that Med1 is part of this complex. Aberrant phase separation has been reported in several different diseases^{116,117}; therefore, understanding the phase separation dynamics of PPAR δ is crucial to see how it functions at baseline and whether this is altered in disease states. This work will be continued through our collaboration with the Salvatella lab to determine whether the interaction between PPAR δ and Med1 is indeed mediated by phase separation.

Overall, microglial PPAR δ has promise as a therapeutic target for AD due to the fact that it increases beneficial functions of microglia while decreasing detrimental functions. Further research is needed to continue characterizing the effects of PPAR δ in microglia in AD mouse models and in systems other than a monoculture of microglia-like cells, as well as the mechanisms by which PPAR δ carries out these functions. These lines of investigation should be pursued to determine if PPAR δ agonism can emerge as a meaningful, disease-modifying therapy for AD and related disorders in the field.

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