The Role of PARP-1/TRPM2 Signalling in Regulating Microglial Phagocytosis in the Context of Alzheimer's Disease

By

Alana Lamont

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba,

in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

Department of Pharmacology and Therapeutics

University of Manitoba

Winnipeg, Canada

ABSTRACT

Chronic neuroinflammation contributes detrimentally to the pathology of Alzheimer's disease (AD), wherein synaptic transmission is diminished, leading to severe cognitive decline. Microglia immune cells drive a number of neuroinflammatory responses, such as release of pro-inflammatory mediators and clearance of beneficial targets (such as pathogens), via phagocytosis. In AD, build-up of excessive amyloid-beta (Aβ) may impact the ability of microglia to efficiently clear such beneficial targets.

Previous work from our labs has shown that the microglial nuclear enzyme poly(ADP-ribose) polymerase-1 (PARP-1), a driver of pro-inflammatory cellular functions, may also be involved in phagocytic regulation. Inhibition of PARP-1 has been shown to have no impact on beneficial phagocytosis of Aβ targets in conditions representing health. However, whether this beneficial phagocytosis is maintained in the context of amyloidopathy (to mimic one component of AD pathology), has not been examined. PARP-1 has also been shown to act in concert with Ca^{2+} permeable TRPM2 (Transient Receptor Potential Melastatin 2) channels, and inhibition of PARP-1 or TRPM2 (individually) suppresses pro-inflammatory nitric oxide (NO) release. The role of TRPM2 alone on phagocytosis has also not been examined.

In this study, we investigate whether amyloidopathy influences microglial phagocytosis of fluorescently labeled Aβ (FAM-Aβ) and if so, the extent to which the pro-inflammatory PARP-1/TRPM2 signalling pathway is involved. We used primary murine microglial cultures harvested at DIV 7-11. In basal conditions, approximately 60% of microglia took up FAM-Aβ within 8 hours, with no significant change in this uptake following inhibition of PARP-1 or TRPM2. Microglia treated with Aβ oligomers (AβO) for 20 hours showed reduced phagocytic uptake of FAM-Aβ per cell. Pharmacological inhibitors for PARP-1 (PJ34, 500 nM), or TRPM2 (JNJ, 1 μM) did not restore the amount of FAM-Aβ engulfed. Additionally, inhibition of other downstream components of the PARP-1/TRPM2 signalling pathway

(inducible nitric oxide synthase (iNOS) and NUDIX hydrolase type 5 (NUDT5)), in amyloidopathy conditions as above, did not restore the amount of FAM-Aβ taken up by cells either. However, pharmacological inhibition of PARP-1, TRPM2, iNOS and NUDT5 prevented amyloidopathic inflammatory signalling through iNOS, resulting in decreased nitric oxide release.

Our major findings show that: inhibition of PARP-1 or TRPM2 did not affect beneficial phagocytic uptake of Aβ in either conditions of health or conditions of amyloidopathy; and that NO produced by this pathway is not a major regulator of beneficial phagocytosis of Aβ.

In conclusion, this data shows that PARP-1 and TRPM2 do not have a role in the uptake of Aβ in homeostatic conditions. Further, although amyloidopathy compromises the ability of microglia to engulf Aβ, inhibition of PARP-1 or TRPM2 does not further compromise Aβ clearance by microglia. Thus, a therapeutic targeting the inhibition of PARP-1/TRPM2 in homeostatic conditions should not have any negative effects such as decreased Aβ phagocytosis. As well, beneficial outcomes that we report from PARP-1/TRPM2 elimination in AD mouse model studies are occurring without any changes in phagocytic Aβ clearance. Thus, PARP-1/TRPM2 inhibition provides favourable outcomes such as inhibiting pro-inflammatory outcomes such as nitric oxide production.

ACKNOWLEDGEMENTS

I would like to start by expressing my deepest appreciation in thanking my advisors Dr. Michael Jackson and Dr. Tiina Kauppinen. This project would not have been possible without them, and I am extremely grateful for their immeasurable guidance, feedback, and support throughout my graduate studies journey. Their mentorship will stay with me throughout my scientific career.

I would also like to thank my committee members, Dr. Jill Stobart and Dr. Vern Dolinsky, for all of their helpful and kind feedback during committee meetings.

I am very grateful to many of my present and former lab members, starting with Natalie Lavine who graciously prepared the AβO used in my experiments, and also provided me with many technical lab tips for culturing, biochemical assays, and more. As well, former lab members Shubhamsingh Tanwar and Matthew Martin for teaching me many important protocols, including dissections and microglial culturing. I would also like to thank Chetan Patil for assistance with setting up the preliminary calcium imaging protocol, as well as Christian Humphreys for additional dissections. As well, Jessica Jarmasz for help with media changes and many questions; Harrison Engen for assistance with NO assays and dissections/culturing; and Oona Kauppinen for assistance with some NO assays and morphology analysis. Thank you as well to my other lab members, Olya, Dali, and Parisa, for our chats and help with any question big or small.

I am also grateful to have received funding support from CIHR and the Rady Faculty of Health Sciences graduate studentship.

Last but not least, I would like to sincerely thank my friends and family for all of their support and encouragement during the completion of my Master's.

TABLE OF CONTENTS

LIST OF FIGURES

ABBREVIATIONS

1400W	N-(3-(Aminomethyl)benzyl)acetamidine
7PA2	CHO cells expressing V717F human amyloid precursor protein
7PA2-CM	CHO cells expressing V717F human amyloid precursor protein conditioned media
μM	micromolar
nM	nanomolar
ml	milliliter
Aβ	Amyloid beta
AβO	Amyloid beta oligomers
AD	Alzheimer's disease
ADP	Adenosine diphosphate
ADPR	Adenosine diphosphate ribose
ANOVA	Analysis of variance
APOE	Apolipoprotein E
APP	Amyloid precursor protein
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BACE1	Beta-site APP cleaving enzyme 1
BBB	Blood-brain barrier
C1q	Complement 1q
C3	Complement 3
Ca^{2+}	Calcium ion
CA1	Cornu ammonis 1
CHO	Chinese hamster ovary
cKO	Conditional knockout
CNS	Central nervous system
COX	Cyclooxygenase
CSF	Cerebrospinal fluid
CTZ	Clotrimazole
CX3CR1	CX3C motif chemokine receptor 1

DIV	Days *in vitro*
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
FAD	Familial AD
FBS	Fetal bovine serum
FC	Fragment crystallizable
FPR	Formyl peptide receptor
GPCR	G protein-coupled receptor
GWAS	Genome-wide association study
HBSS	Hank's balanced salt solution
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
IFN-ϒ	Interferon gamma
IDE	Insulin-degrading enzyme
IGF-1	Insulin-like growth factor 1
IκB	Inhibitor of nuclear factor kappa B
IκK	Inhibitor of nuclear factor kappa B kinase
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
IL-10	Interleukin-10
iNOS	Inducible nitric oxide synthase
JNJ	JNJ-2858311
KO	Knockout
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
MEM	Minimal essential medium
NAD+	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate

NFT	Neurofibrillary tangle
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
NF-κB	Nuclear factor kappa B
NO	Nitric oxide
NSAID	Non-steroidal anti-inflammatory drug
NUDT5	NUDIX hydrolase type 5
NUDT5i	NUDT5 inhibitor, TH5427
PAR	Poly ADP ribose
PARP	Poly ADP ribose polymerase
PARP-1	Poly ADP ribose polymerase 1
PARG	Poly ADP ribose glycohydrolase
PBS	Phosphate buffer solution
PJ34	N-(6-oxo-5,6-dihydrophenanthridin-2-yl)-N, N-dimethylacetamide
PSEN1/2	Presenilin 1/2
P2Y12	P2Y purinoreceptor 12
RAGE	Receptor for advanced glycosylation endproducts
RIPA	Radioimmunoprecipitation
R5P	Ribose-5-phosphate
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SR	Scavenger receptors
SEM	Standard error of the mean
SOCS3	Suppressor of cytokine signalling 3
TAK242	Resatorvid; (*R*)-Ethyl 6-(N-(2-chloro-4-fluorophenyl)sulfamoyl)cyclohex-1-enecarboxylate
TNF-α	Tumour necrosis factor alpha
TREM2	Triggering Receptor Expressed on Myeloid cells 2
TRPM2	Transient receptor melastatin 2
WT	Wild type

CHAPTER 1. INTRODUCTION

1.1 Alzheimer's Disease Hypotheses and Therapeutics

1.1.1. Introduction to Alzheimer's Disease

Alzheimer's disease (AD) is one of the most prevalent neurodegenerative diseases worldwide, displaying a wide range of detrimental effects on cognition and memory, as well as significant emotional burden for caretakers. While a single underlying cause of AD has yet to be proven definitively, there are 3 major prevailing hypotheses, each based on a characteristic pathology observed in AD brains: amyloid beta (Aβ) plaques, tau neurofibrillary tangles, and chronic neuroinflammation (Kinney et al. 2018).

The two major types of AD are classified as sporadic and familial. Sporadic AD accounts for the vast majority of cases, while familial accounts for only an estimated 1-5% of total cases (Liu et al. 2019). Interestingly, although familial and sporadic AD differ based on whether or not disease risk is inherited, the observed pathology of plaques and tangles is consistent across both types (O'Brien and Wong 2011). AD can also be differentiated as early-onset or late-onset, based on the age of a patient (early-onset AD is considered to be when a patient is under 65 years of age). Familial AD (FAD) is more commonly seen in early-onset individuals, and associated genes are inherited as autosomal dominant, meaning that any carrier will develop AD. Two examples are the genes for presenilin 1 and 2 (PSEN1, PSEN2). Presenilins are an enzymatic component of ϒ-secretase, which has a key role in amyloid beta production (discussed below). Interestingly, genetic mutations for PSEN1 were found to be associated with 81% of FAD cases (Ertekin-Taner 2007). Mutations in the gene coding for the amyloid-beta precursor protein, APP, are also linked to familial AD, albeit making up a smaller proportion of total cases in the same study (14% of cases) (Liu et al. 2019).

There have also been multiple AD-risk associated genes identified – however, in contrast to the autosomal dominant genes, individuals who carry risk-associated factors are not guaranteed to develop

AD. The most prominent example of a risk gene is APOE (apolipoprotein E, specifically the ε4 allele), which has a 40% allele frequency in AD individuals (Farrer et al. 1997). This gene codes for an apolipoprotein, which can bind Aβ (O'Brien and Wong 2011). However, the ApoE4 lipoprotein has a detrimental effect on clearance of Aβ, and is thought to both increase the formation of Aβ plaques and affect microglia's ability to phagocytize Aβ plaques (Koutsodendris et al. 2022).

Another risk gene associated with sporadic AD is TREM2 (Triggering Receptor Expressed on Myeloid cells 2), a receptor expressed on microglia cells. When the TREM2 R47H mutation is present, it is associated with an almost 3-fold increased risk of AD, and carriers of this mutation also have worse cognition scores than non-carriers (Jonsson et al. 2013). This is a direct example of how microglial genes/functions can have a direct role in AD, which will be discussed in more detail in later sections.

1.1.2. Amyloid Hypothesis

In 1906, Alois Alzheimer presented the observation of a patient that had died from a cognitive disorder, showcasing the patient's brain containing both plaques and neurofibrillary tangles (O'Brien and Wong 2011). But it was not until 1968 that work by Blessed et al. showed a correlation between the amount of Aβ plaques and dementia risk (Blessed, Tomlinson, and Roth 1968). A later crucial paper identified the amino acid sequence of purified Aβ (Glenner and Wong 1984), which ultimately led to the identification of APP, as the precursor protein from which Aβ is cleaved. The amyloid hypothesis of AD was first presented in 1991 by Dr. John Hardy, who combined previous knowledge regarding genetic mutations for APP with the observation of severe Aβ accumulation – this hypothesis was termed "the amyloid cascade hypothesis" (J. Hardy and Allsop 1991; J. A. Hardy and Higgins 1992).

Amyloid beta plaques accumulate in the brain following cleavage of amyloid precursor protein (APP, 695 amino acid residues long). APP can be processed by two pathways, amyloidogenic or nonamyloidogenic, based on the initial protease that carries out the first cleavage. When APP is processed

by the nonamyloidogenic pathway, it is cleaved by α-secretase and then ϒ-secretase (MacLeod et al. 2015). The α-secretase cleaves APP within the peptide sequence for Aβ – therefore no substantial amount of Aβ is produced. It should be noted that production of a small amount of Aβ peptides still occurs in a healthy state, but these are removed by immune cells such as microglia (Wang et al. 2017). Conversely, when processed by the amyloidogenic pathway, APP is first cleaved by β-secretase (beta-site APP cleaving enzyme 1, or BACE1), followed by ϒ-secretase. Interestingly, the site on APP where BACE1 cleaves is very close to the site where 2 point mutations associated with familial AD were identified (these are termed the 'Swedish mutations', and will be discussed below). This set of cleavages in the beta pathway produces monomers of Aβ peptides that are prone to aggregation, leading to the formation of oligomers and eventually larger Aβ plaques. (Liu et al. 2019).

While plaques were identified as part of the cause in the original amyloid hypothesis, many studies published since have shown that plaques do not necessarily correlate with cognitive decline in AD individuals (Price and Morris 1999). Rather, amyloid beta oligomers (AβO) of varying peptide lengths are now appreciated to be more prone to aggregation, and also more neurotoxic than plaques (Wang et al. 2017). A 2002 study identified that Aβ oligomers, naturally secreted, could inhibit long term potentiation (LTP) *in vivo* in rats (Walsh et al. 2002). Later work building on this finding showed that when Aβ oligomers isolated from AD patients were introduced into mice, mice exhibited numerous outcomes consistent with AD symptoms: inhibition of LTP and decreased density of dendritic spines (reflecting the loss of excitatory synapses in the brain) (Shankar et al. 2008).

Further findings supporting the amyloid hypothesis are related to FAD. Knock-in mice with both APP and PSEN1 mutations have been developed (APP/PS1 model), and these mice exhibit key phenotypes of human AD, such as: loss of dendritic spines (Bittner et al. 2012), increased number of microglia at site of amyloid plaques (Radde et al. 2006), impaired long-term potentiation (LTP) measured in hippocampus (Gengler et al. 2010), and cognitive deficits in memory tests (Radde et al. 2006). As

these mice express human versions of the APP and PSEN1 mutations associated with FAD, and do not accurately model tau pathology (Radde et al. 2006), this is very strong evidence that the amyloid pathway is directly responsible for AD outcomes in humans. Additionally, the fact that all FAD mutations discovered so far have been found in genes with functions related to amyloid precursor processing also support amyloid as the underlying cause of AD. This will be discussed in more detail in section 1.1.5 below. Taken together, these findings provide support for the link between Aβ pathology and the detrimental memory and cognition outcomes seen in AD.

1.1.3 Tau Hypothesis

The tau (tubulin associated unit) hypothesis is more recent than the amyloid hypothesis, having first been formally introduced in 2009 following observations that aggregated tau could become translocated outside of neurons (Frost, Jacks, and Diamond 2009). One piece of even earlier evidence supporting the tau hypothesis is from a 1991 paper (Braak and Braak 1991), where it was shown that neurofibrillary tangles (NFTs), rather than amyloid, were associated with distinctive "characteristic distribution patterns" of neuropathology, in brains from AD individuals. In other words, it was found that the accumulation of NFTs could be matched to discrete stages of neuropathology, while amyloid could not. Tau pathology has since been shown to correlate more closely with poor cognitive outcomes than Aβ.

Tau proteins can exist in 6 isoforms, which range from 352 to 441 amino acids in length. Tau proteins participate in microtubule stabilization, a function that is especially important for neurons because of the high degree of material transport that takes place from cell soma to cell processes, using the cytoskeleton (Wang and Mandelkow 2016). Tau has specific residues that allow it to bind to microtubules, but when these residues become hyperphosphorylated, tau is unable to stabilize microtubules. Tau will instead form a paired helical filament with other hyperphosphorylated tau polymers, and these polymers eventually lead to formation of intracellular neurofibrillary tangles that

facilitate neurotoxicity. These tangles can form in cells individually, but can also spread to adjacent cells in a manner that can be thought of as similar to infectious prions (Wang and Mandelkow 2016). It should also be noted that tau pathology or 'tauopathies' are not unique to AD, and have been observed in other neurodegenerative diseases such as Parkinson's disease and chronic traumatic encephalopathy (CTE) (Kametani and Hasegawa 2018). The presence of tau pathology in other neurological diseases (non-AD), suggests that tau pathology alone is not responsible for the symptoms of AD.

1.1.4 Neuroinflammation Hypothesis

In this text, neuroinflammation will be defined as chronic, unresolved inflammation taking place in the CNS, in response to an insult or perceived insult. Measures of neuroinflammation vary widely in the literature, but one of the most common measures is increased density of glial cells (microglia and astrocytes), and release of pro-inflammatory signals such as cytokines, chemokines, and reactive oxygen/nitrogen species.

While there are other glia cells in the CNS, such as astrocytes, that are also contributors to inflammation, the role of microglia will be focused on here, as the major immune cell and therefore the major modulator of the inflammatory response in the CNS. The inflammatory response in AD was previously considered to be a reactionary response to the loss of neurons that occurred at a later timepoint in disease progression (Kinney et al. 2018). However, the role of immune cells in the brain has begun to be appreciated as a major underlying contributor to AD pathology, taking place well before neuronal damage or death begins (Mrak, Sheng, and Griffin 1995; Walters et al. 2016).

Microglia can act to both alleviate, as well as contribute to, inflammation in the brain. As is the case for all immune cells, microglia respond to an injury or insult (such as detection of a pathogen) by carrying out a wide variety of functions. Microglia can release pro-inflammatory mediators (such as cytokines IL-1, IL-6, and TNF-α) to draw other microglia to the area and alert other cells in the brain there is something wrong, and can also initiate intracellular signalling to phagocytize the source of the

insult (Smith et al. 2012). The purpose of an acute immune response such as this is 'neuroprotective': for local inflammation to increase temporarily, while microglia and other immune cells work to resolve the injury or perceived threat.

However, if acute inflammation is maintained over a long period of time, pro-inflammatory responses can become damaging to CNS cells and tissues, leading to chronic, detrimental neuroinflammation. For example, prolonged exposure to the same pro-inflammatory cytokines that are released by microglia to draw immune cells to the site of injury can also induce synaptotoxicity and neurodegeneration, increase BBB permeability, and even stimulate Aβ synthesis (Smith et al. 2012; Wang et al. 2017). A few specific cytokines (IL-1, TNF-α) released by microglia can drive astrocytes towards a neurotoxic phenotype (Liddelow et al. 2017). Microglia involved in chronic neuroinflammation will also change their expression profiles (transcripts/proteins expressed) to reflect their pro-inflammatory functions. However, not all cells in a microglial population will transform into a pro-inflammatory phenotype. Some microglia present in a state of chronic inflammation can have anti-inflammatory phenotypes, and release anti-inflammatory signals such as IGF-1 and SOCS3 (suppressor of cytokine signalling) (Li et al. 2021).

Some of the strongest evidence linking microglial-driven neuroinflammation to AD comes from GWAS (genome-wide association studies) that have identified specific genes as having a statistically significant association with late-onset AD. The majority of these genes are expressed by microglia or involved in inflammation, such as: TREM2, CD33, PLCG2, MS4A6A, and INPP5D (Hansen, Hanson, and Sheng 2017; Griciuc and Tanzi 2021). Of particular interest to us is that two of these key genes identified, TREM2 and CD33, have a role in microglial phagocytosis. Overall, chronic neuroinflammation sustained by pro-inflammatory microglia can accelerate neurodegeneration, and it is clear to see how this process could have a major contribution to a neurodegenerative disorder such as AD.

1.1.5 Rationale of Research Investigating Amyloid Hypothesis

As the previous text demonstrates, although AD has been identified for over 100 years, there is still some debate over the exact root cause of its pathology, which presents an obvious limitation to AD therapeutic design. The rationale for studying the amyloid hypothesis in my work is based on the strong reported association of Aβ with genetic mutations as seen in familial AD. In particular, a highly influential paper published in 1992 found that when 2 point mutations were added to the structure of APP, mimicking the exact mutations seen in a Swedish family that were carriers for familial AD, cells produced 6-8 fold higher amounts of Aβ (Citron et al. 1992). This demonstrates a direct connection between amyloid and AD.

A major limitation of previous AD therapeutics is the timing of therapy administration – for example, many human trials include participants who are already showing symptoms of cognitive impairment at baseline. However, given the familial genetic evidence linking Aβ and APP, it is clear that Aβ is toxic, and many trials were not designed in a way to prevent this toxicity from building. As AD is a progressive disease, it seems logical that there may be a point at which no therapy would be effective in preventing cognitive decline. As well, the vast majority of clinical trials to date have focused on targeting Aβ levels in the CNS, through either decreased production or increased removal – strategies that generally have not been successful in showing a measurable decrease in cognitive decline (Honig et al. 2018; Cummings et al. 2021).

Neuroinflammation occurs early in disease pathology before significant levels of neuronal or synapse damage occur, and therefore provides an intriguing target as there is a time advantage over amyloid or tau therapies (Kinney et al. 2018). However the inflammatory response must be targeted with extreme care, as a therapeutic that results in a dampened inflammatory response must maintain a balance between helpful and harmful. This work together underlies that the neuroinflammatory response is an extremely important target in the development of AD. As more is understood about neuroinflammation

and microglia functioning (in states of both health and disease), better AD therapies with improved specificity will be able to be designed.

1.2 Microglia

1.2.1 Background on Microglia Cell Lineage

Microglia have an interesting ontogeny in the CNS, as they are the only cells found there that are derived from yolk-sac progenitor cells. Outside of the CNS, other cells from yolk sac progenitors include myeloid cells such as macrophages, dendritic cells, and granulocytes. Neurons and the other major types of glia cells in the CNS (astrocytes, oligodendrocytes), are descended from a different progenitor cell lineage, which is neuroectodermal or neuroepithelial (Ransohoff and Cardona 2010). Early in development before the blood-brain barrier is completely formed, yolk sac progenitor cells enter the CNS and mature into microglial progenitors. Microglia then carry out 'self-renewal' for maintaining their cell numbers in the CNS by growing from local pools of cells (rather than more precursor cells entering the CNS post-development), a process that is dependent on a microglial receptor called colony stimulating factor 1 (CSF-1R) (Elmore et al. 2014). This receptor recognizes colony stimulating factor 1 released from other glia and neurons, and this sustains the microglial self-renewal process. Interestingly, in experiments where microglia are significantly depleted, it has been shown to take 5 days for microglia to repopulate from these self-renewal pools (Bruttger et al. 2015).

1.2.2. Microglial Phenotypes and Current Terminology

While previously the microglia field widely used terminology that suggested that cell morphology was tied directly to function (for example, cells with ramified morphology being labelled as 'resting', and amoeboid cells being labelled as 'activated'), it has begun to be appreciated that there is more nuance to the relationship between morphology and function. Some of the earliest work that showed evidence that 'resting' microglia were not actually resting came from Nimmerjahn et al. in 2005, who demonstrated

that microglia with ramified morphology can extend processes to actively survey their environment (Nimmerjahn, Kirchhoff, and Helmchen 2005).

A recent review by some experts in the field has highlighted the need for an end to the use of simplistic terms 'resting' vs. 'activated', as well as 'M2' (anti-inflammatory) vs. 'M1' (pro-inflammatory) for description of microglia phenotypes (Paolicelli et al. 2022). It is more likely that most brains have a combination of microglial states that are dynamic, not static. Therefore, the authors suggest to focus on describing microglial actions, as well as transcriptomic and proteomic expression profiles, rather than using binary 'resting' vs. 'activated' terminology.

To supplement this shift in focus, there have been a number of recent transcriptomic and single-cell studies attempting to characterize specific sub-types or 'clusters' of microglia. These studies have looked for differences in gene expression in a variety of conditions: across the lifespan of an organism (Hammond et al. 2019), in states of health vs. disease (Masuda et al. 2019), and in states of neuroinflammation (Jordão et al. 2019). These studies detect many genetic markers that are used to identify microglia, such as Tmem119, P2ry12, and Cx3cr1. Cells expressing these markers are often labelled as 'homeostatic' cells (Hansen, Hanson, and Sheng 2017). In comparison, a common subset of 'disease associated microglia' (DAMs) has been identified, in which authors report an increased expression of genes such as Axl, Igf1, Apoe, and Trem2 (Deczkowska et al. 2018). One goal of identifying subsets of microglia such as DAMs would be the potential for developing therapeutics to target only these microglial subsets that are carrying out detrimental or damaging functions. However, this idea is more difficult to implement than it sounds in theory, as genes classified with increased expression in the category of DAMs are not always completely detrimental, and their upregulation may be because they allow for adaptation of microglial responses. Additionally, some DAM gene data may actually include data from brain macrophages, which have a cell lineage that is distinct from microglia.

Another downside of these large-scale `omic studies is that the amount of data generated can be difficult to parse through, and that different studies may give different names or functional descriptions to similar sub-groupings of microglia. Additionally, transcriptome data can be highly variable. Overall, this work has been extremely helpful for the field in elucidating the diversity of microglial phenotypes, but more work is needed to bring results together to be conclusive (Prinz, Jung, and Priller 2019).

1.2.3. Microglial Functions in Health and Development

Microglia are characterized by the variety of crucial functions they carry out in the CNS throughout the lifespan of an organism during development and into adulthood. These functions include surveillance of the environment – microglia express receptors that can bind signals released by neurons, such as neurotransmitters and cytokines (Pocock and Kettenmann 2007). Microglia also provide trophic support of synapses and neurons. For example, microglia aid neuronal growth by production of growth factors such as IGF (insulin growth factor) (Kettenmann et al. 2011). Microglia also have a role in synaptic plasticity – the modulation of synaptic strength that underlies the processes of learning and memory. Microglial-produced BDNF (brain-derived neurotrophic factor) has been shown to be important for synaptic plasticity and learning (Parkhurst et al. 2013). As showcased by Parkhurst et al., microglial depleted (and therefore microglial BDNF depleted) mice had significantly worse outcomes in learning and decreased synaptic plasticity. Another key function carried out by microglia during development is the phagocytic clearance of apoptotic neurons, excess neuronal progenitor cells (NPCs), and cellular debris (Prinz, Jung, and Priller 2019), to maintain a homeostatic environment.

1.2.4. Microglial Functions in AD

As described, the neuroinflammation hypothesis suggests that a chronic inflammatory response itself is a major contributor to AD pathology, and has been linked to AD (Bales et al. 2000; Rao et al. 2012). As the major driver of neuroinflammation in the CNS, microglia play dual roles in AD – protective and detrimental.

In the presence of Aβ, microglia become stimulated and migrate to cluster around amyloid plaques, where they phagocytize Aβ (Bard et al. 2000; Bolmont et al. 2008), an example of a beneficial outcome. However, chronically stimulated microglia become less efficient at phagocytizing Aβ, leading to decreased Aβ clearance and increased growth of plaques (Hickman et al. 2008; Krabbe et al. 2013). Microglia also release pro-inflammatory mediators, the purpose of which in an acute inflammatory response is to draw more glia cells (microglia and astrocytes) to the site of injury. However, when chronically inflamed conditions are maintained, sustained release of pro-inflammatory mediators causes significant damage to surrounding neurons. Thus, microglia contribute to a positive feedback loop of pro-inflammatory signalling, where they are promoting damaging signalling cascades in surrounding CNS cells, while these same cells also release pro-inflammatory signals back to microglia. This phenomenon has also been termed reactive gliosis (Kinney et al. 2018).

For example, Aβ directly triggers microglia to release multiple types of pro-inflammatory signals: cytokines such as TNF-α, IL-6, and IL-1B (Rogers and Lue 2001), reactive nitrogen species (RNS), and reactive oxygen species (ROS) (Prinz, Jung, and Priller 2019). These signals are neurotoxic and damaging to neurons as well as other glial cells. The pro-inflammatory cytokines TNF-α and IL-6 can induce neurodegeneration and synaptotoxicity (Smith et al. 2012), and IL-1B increases neuronal excitability by inhibition of a type of Ca^{2+}-activated K^{+} channel (Zhang et al. 2008). Release of microglial TNF-α and IL-1 can also activate astrocytes to a neurotoxic phenotype (Liddelow et al. 2017). Additionally, these pro-inflammatory cytokines can disrupt synaptic plasticity (via reduction of LTP) that underlies memory and learning (Wu et al. 2015).

In addition to releasing cytokines, Aβ-stimulated microglia can also produce the reactive nitrogen species nitric oxide, another pro-inflammatory mediator. Microglial NO is produced by the enzyme inducible nitric oxide synthase (iNOS) (Yuste et al. 2015). Nitric oxide can be extremely damaging to brain tissue, and there have been a few mechanisms illustrating how this can happen. First, Brown et al.

have shown that NO produced by elevated iNOS inhibits neuronal cellular respiration, by inhibition of neuronal mitochondrial cytochrome oxidase. This leads to neuronal cell depolarization, and subsequent glutamate release that leads to excitotoxicity and can result in neuronal cell death (Brown 2007). Interestingly, microglial NO can also cause astrocytes to release glutamate by a different mechanism, which would further contribute to excitotoxicity (Bal-Price and Brown 2001). NO can also cause oxidative stress, as it can combine with another reactive species called superoxide (produced via NADPH oxidase), to form a new reactive species, peroxynitrate, which can also ultimately also cause neuronal cell death by inhibition of normal mitochondrial functioning (responsible for cell respiration) (Mander et al. 2005).

Elevated NO produced in response to Aβ has been found to worsen the inflammatory environment through other methods as well. For example, NO causes S-nitrosylation of multiple enzymes that have functions in mitochondrial homeostasis and cell energetics. NO-induced S-nitrosylation of an enzyme called IDE (insulin-degrading enzyme, which can degrade Aβ) has been shown to inhibit normal enzymatic functioning in neurons, which results in higher Aβ levels (i.e. due to less Aβ breakdown). Elevated levels of Aβ then contribute to a pro-inflammatory positive feedback loop, shown to directly associate with impaired synaptic transmission and loss of dendritic spines (Cho et al. 2009; Akhtar et al. 2016).

Lastly, while phagocytosis may typically be considered a protective function that combats inflammation via removal of pathogens and other pro-inflammatory stimuli, phagocytosis can turn detrimental in an inflamed and/or disease state. Microglia have been observed to phagocytize aberrant targets that they would not normally eat under homeostatic conditions (for example, healthy functioning synapses or neurons) (Guy C. Brown and Neher 2014; Butler et al. 2021; Neher et al. 2011). This loss of control in an inflamed brain is understandably extremely damaging, and contributes to synaptic dysfunction and memory loss in AD.

It is not clear in the literature on whether nitric oxide is required (or stimulates) an increased level of microglial phagocytosis. It is also not known if NO may have a broad effect on all types of phagocytosis, or only on specific types of cargo. One hypothesis is that microglial nitric oxide, induced in response to pro-inflammatory signals, could act in a feedback loop to have an effect on phagocytosis. For example, Scheiblich et al. published a study where they showed that when microglia were treated with an NO donor, they had significantly increased phagocytosis of neuronal debris, hypothesized to be a result of upregulation of TLR (Scheiblich and Bicker 2015). There are a few methodological details to keep in mind when assessing different phagocytic studies like these. Some of these studies examined phagocytosis of different types of cargo (cell debris, beads, or pathogens), and there is not a standard practice for how phagocytic assays are analyzed across the literature. For example, some groups will count the percentage of cells that took up any cargo, some groups will assess exactly how much cargo each cell took up (for example, if using bead cargo, how many beads were engulfed per cell), and so on.

Thus, this is why understanding potential signalling that regulates phagocytosis is of great interest to neurodegenerative disease researchers, as understanding the signalling that regulates phagocytosis is necessary. As well, an ideal therapeutic targeting the function of phagocytosis would ideally promote beneficial phagocytosis of debris or pathogens, while inhibiting aberrant phagocytosis of targets such as functioning neurons.

1.2.5 Microglial Phagocytic Receptors in AD

Phagocytosis is defined as cell engulfment of an extracellular particle >0.5 microns. In general, the process of phagocytosis is: recognition of a ligand on a molecular target or ‘cargo’ (ex. a pathogen, a cell surface marker, etc.) by a receptor on the plasma membrane of the phagocytic cell, initiation of intracellular signalling to form a phagocytic cup around the cargo in an actin-dependent process, then fusing of the vesicle containing the cargo into the cell, where the vesicle (also called a phagolysosome) breaks down the cargo by acidification (Gordon 2016). As receptors are involved in the crucial first

step of recognizing a phagocytic target, they will be discussed in detail. The following will describe key receptors expressed on microglia that have relevance to both the process of microglial phagocytosis and AD.

1.2.5.1 Receptors that Recognize Aβ

There are a wide variety of microglial receptors that have been shown to bind Aβ. A common theme for these receptors is that they can all bind numerous ligands – there is no receptor that solely binds Aβ alone and nothing else. A selection of receptors that are able to recognize Aβ include: Toll-Like Receptors (TLRs), TREM2 (discussed in 1.2.6.2 below), Scavenger Receptors (SRs), Fc receptors, Formyl Peptide Receptors (FPRs) and RAGE (Receptor for advanced glycosylation endproducts) (Doens and Fernández 2014). All of these receptor types, aside from RAGE, have been implicated in the process of Aβ phagocytosis. A few of these receptors will be discussed in more detail below.

Evidence supporting scavenger receptors (SR) binding to Aβ is as follows. There are 2 types of scavenger receptors, which differ in their structure and the types of cells they are expressed on. SR-A is mainly expressed on microglia and astrocytes, and can recognize diverse ligands like endotoxins and microbes. SR-A has been shown to mediate internalization of Aβ (Yang et al. 2011). The second type of SR's are called SR-B (or CD36), and these are considered a type of pattern recognition receptor that can recognize both exogenous and endogenous ligands. Evidence for the role of CD36 is that this receptor is activated in response to Aβ (Coraci et al. 2002), and cells deficient for CD36 had decreased production of selected pro-inflammatory cytokines and chemokines in response to Aβ (El Khoury et al. 2003). Interestingly, CD36 can act as a co-receptor to assist other receptors in binding Aβ – this includes a role in forming a complex with TLR4 and TLR6 (Stewart et al. 2010).

Additionally, a study in the amyoloidopathy mouse model, APP/PS1, showed that as these mice aged, expression of multiple types of Aβ receptors - SR-A, CD36, and RAGE - were decreased, while other neuroinflammatory markers increased (Hickman et al. 2008). This downregulation of receptors that

can recognize and internalize Aβ (SR-A, CD36) was hypothesized to be one way that Aβ clearance could be decreased in amyloid conditions.

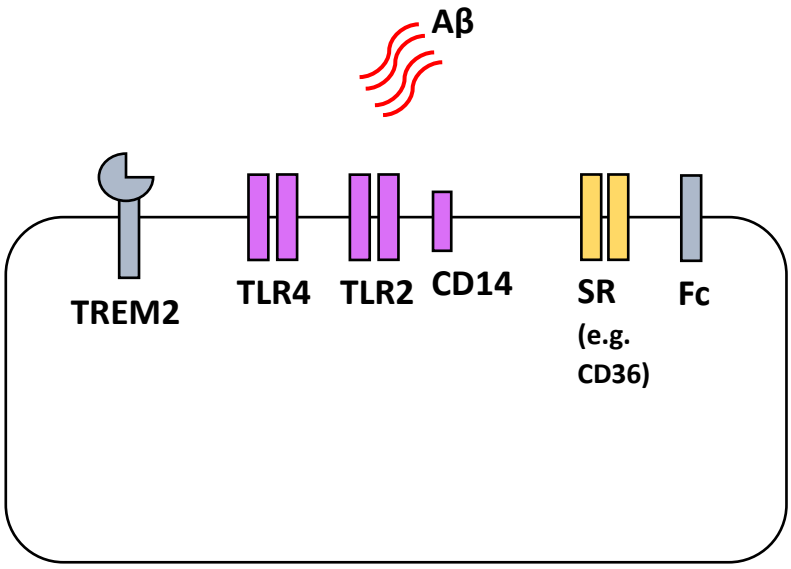

Figure 1: Illustration of selected Aβ receptors expressed on microglia.

A selection of receptors expressed on microglia that can recognize Aβ and initiate a plethora of downstream signalling, including: TREM2 (Triggering Receptor Expressed on Myeloid cells 2), Toll-Like Receptors 2 and 4 (TLR2,4), CD14 (cluster of differentiation), SR (scavenger receptors), and Fc (fragment crystallizable) receptors.

1.2.5.2 Toll-Like Receptors (TLR's)

Toll-like receptors (TLR's) are a family of pattern recognition receptors that are expressed on most cell types in the CNS including microglia, which can express TLR's 1-9 (Fiebich et al. 2018). These different types of TLR's have some structural differences that allow them to recognize specific ligands. TLR activation initiates pro-inflammatory signalling, including release of typical mediators like cytokines and NO (Fiebich et al. 2018). TLR2 and TLR4, along with co-receptor CD14, have been shown to be crucial for microglial activation in response to fibrillar Aβ (Reed-Geaghan et al. 2009). This section will focus on describing the background for TLR4 as this is most relevant for my project.

TLR4 is one of the most-studied TLR receptors in relation to AD. The primary ligand for TLR4 is LPS (lipopolysaccharide, a pro-inflammatory component expressed on Gram-negative bacterial cells),

but it can also recognize endogenously produced ligands such as heat shock proteins and components of dying cells, in addition to recognizing Aβ (Erridge 2010). Activation of TLR4 by Aβ has been shown to set off intracellular signalling that results in pro-inflammatory signal release and uptake of Aβ, through intermediate signalling involving many complexes such as MyD88 (Wu et al. 2022). In particular, it has been shown that TLR4 activation by LPS increases assumed Aβ clearance (in this study, Aβ uptake was measured indirectly, by quantifying Aβ 1-42 concentration left over in cell medium) (Tahara et al. 2006). In a TLR4 mutated version of the Tg AD mouse model (deficient in TLR4), mice had decreased microgliosis, more cognitive deficits, and lower Aβ clearance (Song et al. 2011). These results suggest that TLR4 plays a role in both pro-inflammatory outcomes and removal of Aβ.

Other studies interrogating TLR4 and phagocytosis have found that LPS activation of TLR4 reduced microglial clearance of Aβ42, by decreasing expression of CD36 in primary microglia cultures (Li et al. 2015). As described above, CD36 is a scavenger receptor that phagocytizes Aβ, therefore it is logical that lower expression would correlate with lower Aβ phagocytosis. This study shows that TLR4 activation indirectly (via changes to *another* receptor) had an impact on decreasing Aβ phagocytosis. Interestingly, the results of this study are in contrast to those of Tahara et al., which found that LPS activation of TLR4 increased clearance of Aβ.

A common hypothesis related to neuroinflammation in AD is that different functions will be 'helpful' or 'detrimental' at different times in the course of disease. For example, if TLR4 activation promotes Aβ uptake while also increasing pro-inflammatory mediator release, this would be beneficial early in disease, when overall level of inflammation is low (acute inflammatory response). However, at a later timepoint in AD when chronic inflammation is rampant, TLR4 activation would just contribute to this already high level of inflammation, and Aβ uptake is decreased (Huang et al. 2017). In other words, microglia in chronically inflamed environments may become hyposensitive to signals they would normally react to. In fact, it has been shown that late-disease TgAPP/PS1 mice models were less sensitive

to TLR4 agonist LPS, compared to early-disease TgAPP/PS1 mice (2 months vs. 12 months) (Go et al. 2016). Therefore, it should also be taken into consideration that the stage of disease is important for whether TLR4 activation (and activation of many other neuroinflammatory modulators discussed here) is going to be beneficial or harmful.

1.2.5.3 TREM2

TREM2 (Triggering Receptor Expressed on Myeloid cells 2) is part of the immunoglobulin (Ig) receptor superfamily, which are expressed on myeloid cells, including microglia. This receptor is 230 amino acids long, and the outer domain can recognize and bind a variety of ligands. In addition to Aβ, it can also bind glycoproteins and lipoproteins. On the inner side of the cell membrane, TREM2 needs to be bound to DAP10 or DAP12 proteins in order to facilitate intracellular signalling in response to a ligand (Li and Zhang 2018). TREM2 is involved in a number of cellular functions, of which the regulation of phagocytosis (including synaptic pruning), and microglial migration to targets are most relevant here (Filipello et al. 2018). Notably, TREM2 also promotes anti-inflammatory responses in microglia (Liu et al. 2020).

Some of the strongest evidence for microglial genes having a role in AD comes from studying the TREM2 gene. A significant GWAS study found that a rare TREM2 mutation in the population (called the R47H allele) can increase AD risk by ~3-fold (Jonsson et al. 2013), which makes it one of the strongest genetic predictors of disease risk after ApoE4 (Kinney et al. 2018). This R47H mutation seems to be responsible for a loss of function of TREM2. Animal models have followed up on the GWAS data. For example, many authors have shown that when TREM2 is either fully or half knocked out (haploinsufficiency), fewer microglia are observed to surround amyloid beta plaques (Wang et al. 2016; Mazaheri et al. 2017). In particular, Wang et al. showed that TREM2 deficient mice (5XFAD model) not only had fewer microglia surrounding plaques, but TREM2 deficiency also resulted in less dense plaque structure than in controls. They hypothesized that this difference in plaque structure resulted in increased

damage to neurons in TREM2 deficient mice. Other functions of TREM2 are related to the migration of microglia. For example, Mazaheri et al. found that microglia without TREM2 had impairments in both the growth of microglial processes in response to stimuli and in migration towards neurons that were undergoing cell death (Mazaheri et al. 2017). All of this data together suggests that TREM2 plays an important and protective role in microglial migration towards cargo or damaged cells.

TREM2 also plays a role in phagocytosis - as mentioned, it can bind and recognize a variety of ligands that initiate intracellular signalling for phagocytosis. Support for this role comes from work on TREM2 KO mice, where both decreased phagocytic capacity and decreased production of transcripts for phagocytic genes has been observed (Poliani et al. 2015). One recent finding of note is from Yeh et al. (2016), in which the authors first identified that TREM2 can bind apolipoproteins APOE and clusterin, which are often complexed with Aβ aggregates. In fact, Aβ aggregates complexed to these lipoproteins were phagocytized more effectively than when they were Aβ aggregates alone (Yeh et al. 2016). This demonstrates clearly that a lipoprotein, such as APOE, binding to Aβ increases the phagocytic uptake of Aβ. It has been suggested that this could potentially provide a mechanism for how the loss of functional TREM2 confers increased AD risk: the loss of function may lead to less Aβ taken up by microglia. Another hypothesis is that loss of TREM2 may underlie the fact that microglia are no longer able to move to where Aβ is to eat it, therefore leading to aggregation of Aβ outside cells (Hansen, Hanson, and Sheng 2017). There is still a lot of exciting work to be done examining TREM2, and it is an especially enticing receptor because the data suggests that it may able to promote phagocytic uptake of Aβ but while boosting anti-inflammatory responses.

1.3 Molecular Signalling that Regulates Microglial Functions

All of the complex processes that can be carried out by microglia and contribute to a mixture of protective and destructive functions in AD has led to strong interest in studying the signalling regulation of these processes. As Aβ is one major trigger of neuroinflammation and AD, assessing pro-inflammatory

signalling that occurs downstream of Aβ is a logical avenue. As one example, Aβ can be recognized by TLRs, which initiates signalling within MEK/ERK pathway, and ultimately leads to pro-inflammatory mediator production via NF-κB (nuclear factor-kappa B) (Minter, Taylor, and Crack 2016). Therefore, ERK, NF-κB, and other components associated with these cascades such as calcium, are highly relevant signalling cascades to examine further.

NF-κB is an inducible transcription factor that is found in almost all cell types, and can be activated via signalling initiated by numerous types of receptors such as GPCR's, TLR's, and cytokine receptors (Popiolek-Barczyk and Mika 2016). As a transcription factor, it is responsible for gene expression of a wide variety of genes, such as those for immune response, cell cycle, and of course, inflammatory response. Work done in macrophages has also shown that NF-κB has a role in regulating phagocytosis, as the inhibition or genetic deletion of NF-κB results in significantly decreased phagocytosis (Courtine et al. 2012; Yutian Wang et al. 2020). The exact mechanism for how this regulation may occur is not known, though I would hypothesize it may occur by decreasing cell's ability to migrate towards targets.

In the cytosol of a resting cell, NF-κB is initially sequestered by an interaction with an inhibitor called IκB. After this inhibitor is degraded (a process carried out by κB kinase), NF-κB is free to move into the nucleus (Valerio et al. 2006), where it binds to DNA upstream of target genes. Inhibition of NF-κB can be achieved using pharmacological inhibitor BAY 11-7082, which inhibits the activity of κB kinase so that NF-κB itself remains inhibited (Lee et al. 2012). What are other reported results of the direct inhibition of NF-κB? In relation to inflammation, the inhibition of NF-κB has been shown to reduce expression of pro-inflammatory cytokines such as IL-6, and other pro-inflammatory mediators such as iNOS (inducible nitric oxide synthase), the enzyme responsible for NO production (Meunier et al. 2007; Kauppinen et al. 2011). As discussed, NO is a strong pro-inflammatory signal, and can also contribute to neuroinflammation by damaging neurons and astrocytes (Bal-Price and Brown 2001).

However, NF-κB requires other proteins in order to form a transcription complex and promote gene transcription. PARP-1, which will be discussed in detail below, is an important one of these proteins, and can act as a co-activator or co-repressor of NF-κB (Hassa and Hottiger 2002).

1.3.1 PARP-1

Poly (ADP-ribose) polymerase-1 (PARP-1) is a nuclear enzyme that is part of the PARP protein family, which contains 17 proteins total in humans (Bai 2015). PARP-1 is 1014 amino acids long and consists of 3 domains. As all enzymes within the PARP family, PARP-1 converts NAD+ (nicotinamide adenine dinucleotide) into polymers of ADPR (also called PAR). PARP enzymes produce an estimated 80% of PAR molecules in a cell (Rouleau et al. 2010). These PAR polymers are added onto protein targets as a post-translational modification, as well as added to a domain on the structure of PARP-1 itself, in a process called 'auto-parylation'. PAR can also be converted into ADPR monomers by an enzyme called poly(ADP-ribose) glycohydrolase (PARG) (Bai 2015).

PARP-1 has a few different known functions. It is a crucial part of the transcriptional machinery in a cell, where it acts by controlling other multimolecular transcriptional complexes (such as NF-κB). In this way, PARP-1 regulates expression of certain genes under transcriptional control of these complexes, such as genes associated with inflammation, DNA repair, or cell cycle regulation (Sinha, Katyal, and Kauppinen 2021). PARP-1 itself also has a direct role in the cellular DNA damage response, discussed below. However, too much PARP-1 activation can have fatal consequences for a cell. As activated PARP-1 consumes NAD+ as a substrate, a cell will need to produce increasingly high levels of NAD+ to keep up with the demand. As NAD+ is also a crucial component of the electron transport chain in mitochondria which produces ATP as part of cellular respiration, PARP-1 overactivation can lead to mitochondrial damage and ultimately cell death (Bai 2015).

Role of PARP-1 in DNA Repair

The most well-known role of PARP-1 in the literature is likely its involvement in the cellular response to DNA damage. In this context, PARP-1 is activated in response to DNA damage occurring from cellular insults such as reactive oxidative molecules. PARP-1 takes part in multiple forms of DNA repair, including the single strand and double strand break repair pathways (Chaudhuri and Nussenzweig 2017). PARP-1 activates other proteins for DNA repair by adding PAR molecules to these proteins, as well as adding PAR onto histones themselves. This PARylation causes histones to change the chromatin configuration, allowing for other DNA repair proteins to gain access to the site that needs repair (Kraus and Lis 2003). Other proteins that PAR can be added to are involved in functions such as the cell cycle, DNA replication, and transcription (Gagné et al. 2008). Thus, this functioning has attracted a large amount of focus in the cancer field, as PARP inhibitors can boost the impact of chemotherapeutic agents that cause DNA damage, leading to cancer cell death (Rouleau et al. 2010).

Role of PARP-1 in Inflammation

PARP-1 also has an important role in promoting inflammation, and can be activated in the absence of DNA damage in a Ca^{2+} dependent manner, via direct phosphorylation carried out by ERK1/2 kinases (Kauppinen et al. 2006; Vuong et al. 2015). The role of PARP-1 in regulating the expression of inflammatory genes is due to its PARylation of NF-κB, and other transcription factors in this related transcription complex. Protein-protein interactions that occur after PARylation activates transcription factors, allowing for transcription to take place. Multiple reports have shown that PARP-1 regulates NF-κB in association with microglial changes seen for inflammatory response and morphological changes (Chiarugi and Moskowitz 2003; Ha, Hester, and Snyder 2002; Vuong et al. 2015). As stated, NF-κB is a crucial transcription-level regulator of a variety of pro-inflammatory genes. Some of the best studied products of these genes are the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α. All 3 of these cytokines are known pro-inflammatory cytokines in macrophages (Liu et al. 2017). In relation to AD,

these cytokines have been found at a higher level in the plasma and CSF of AD patients (Zheng, Zhou, and Wang 2016), and it has been shown that microglia treated with Aβ *in vitro* produce these 3 cytokines, in increasing concentration in correspondence with the dose of Aβ (Lue et al. 2001; Rogers and Lue 2001). Another report found that these 3 cytokines were associated with higher levels of Aβ in an AD mouse model (Benzing et al. 1999). Microglial pro-inflammatory cytokines are also detrimental to other cells in the CNS (such as astrocytes and neurons), and disrupt synaptic transmission and synaptic plasticity (Wu et al. 2015).

Role of PARP-1 in AD

Microglial stimulation with Aβ can lead to activation via NF-κB (Chen et al. 2005). PARP-1 was identified as a key player in between these two steps of microglial response to Aβ when Kauppinen et al. showed that PARP-1 was involved in various outcomes following stimulation with Aβ, such as: release of pro-inflammatory signals (NO and cytokines) via NF-κB, the change of microglia to an activated morphology, and neuronal death (Kauppinen et al. 2011). Additionally, experiments using $hAPP_{J20}$/*PARP-1* $^{-/-}$ mice (offspring from an amyloid AD mouse model in which Aβ accumulates with age, and PARP-1 was deleted) found that PARP-1 depletion resulted in fewer activated microglia, and fewer deficits in memory (measured by novel object recognition). Further, multiple studies have found that PARP-1 plays a role in blood-brain barrier (BBB) integrity and permeability. The BBB is made up of multiple cell types, including brain endothelium and astrocytes, and has a very important function of regulating which molecules from the circulation can enter the brain fluid. It has been suggested that in AD, Aβ and pro-inflammatory signals result in increased permeability of the BBB, leading to poorer protection of the brain due to this leaky barrier (Mao and Zhang 2022).

To this end, since microglia can produce molecules that activate astrocytes and other cells in the CNS, it is plausible that microglia may have a role related to changes in BBB permeability during AD. Mehrabadi et al. found that when microglia pre-treated with Aβ were added to brain endothelial cells,

there was decreased expression of two tight junction proteins, which have an important role in formation of tight junctions between adjacent endothelial cells, and therefore a role in maintaining the strict low level of permeability of the BBB. Microglial products NO and TNF-α were linked to decreased tight junction protein expression, as well as to increased paracellular permeability of brain endothelial cells. Microglial PARP-1 also promoted astrogliosis (Mehrabadi et al. 2017). This data together points to a potential mechanism by which microglial PARP-1 in an AD state could decrease the permeability of the BBB and contribute to worsening AD pathology. Another study linking PARP-1 to decreased BBB integrity was completed by Rom et al., in which leukocyte migration into the BBB was examined *in vivo* in an aseptic rodent model, with and without application of PARP-1 inhibitors. It was found that fewer leukocytes migrated to the site of injury when PARP-1 was inhibited (Rom et al. 2015), suggesting that PARP-1 is important for leukocyte migration and therefore contributes to a component of neuroinflammation. Together, these 2 studies highlight an important role for PARP-1 in relation to the BBB, and this no doubt has relevance to AD.

Some previous work has demonstrated that microglial PARP-1 has a role in regulating phagocytosis, especially in the context of AD. This was first shown by Kauppinen (Kauppinen et al. 2011), wherein Aβ-stimulated microglia isolated from PARP-1 KO mice were found to decrease phagocytic uptake of co-cultured WT neurons, while there was no effect on the uptake of fluorescently-tagged Aβ. PARP-1 is a potential therapeutic target for AD due to its important role in pro-inflammatory responses. Whether PARP-1 may regulate microglial phagocytosis of cargo (positively or negatively) is also an interesting therapeutic avenue, and this is one of the major areas of interest of my work.

Additionally, as mentioned previously, PARP-1 can also be activated by increased concentration of intracellular calcium (Kauppinen et al. 2006; Vuong et al. 2015). Therefore, a calcium channel that can be opened and sustain an influx of calcium would be sufficient to activate PARP-1. One such example of a calcium channel highly expressed in microglia is the TRPM2 channel.

1.3.2 TRPM2

Transient receptor potential melastatin 2 (TRPM2) is a Ca^{2+} permeable non-selective cation channel that is part of the TRP family. TRPM2 is 1503 amino acids long, and there are multiple splice variants. The structure is made up of 6 transmembrane domains, and 1 pore loop formed between the 5^{th} and 6^{th} transmembrane domain (Belrose and Jackson 2018). The N terminus has a conserved TRPM subfamily amino acid sequence, while the C terminus has a structural motif called NUDT9-H (nucleoside diphosphate-linked moiety X-type homology motif). This motif is where the intracellular ligand, ADPR, binds, leading to a conformational change that opens the pore for ions to enter the cell. As TRPM2 is a non-selective cation channel, multiple positively charged ions can enter the cell, due to the selectivity filter in the pore: Ca^{2+}, Na^{+}, Mg^{2+} (Belrose and Jackson 2018) – however, the influx of Ca^{2+} is most crucial for downstream signalling that will be described.

TRPM2 expression (measured as mRNA) has been reported in most organs of the body, although the highest expression is in the CNS, where it is expressed by both microglia and neurons (Kraft et al. 2004). It is also expressed on peripheral immune cells such as monocytes and macrophages, where it has been shown to respond to pro-inflammatory stimuli by initiating an influx of calcium (Turlova, Feng, and Sun 2018).

It should also be noted that TRPM2 requires a certain concentration of intracellular calcium in order for the channel to open, and increasing concentrations of intracellular calcium sensitizes the channel to its agonist, ADPR (Du, Xie, and Yue 2009). In fact, intracellular calcium alone in the absence of ADPR is sufficient to open the channel, and is required for ADPR-mediated channel opening. This is achieved via calmodulin-calcium binding to a structural motif for calmodulin, found on the intracellular side of the channel, closer to the N terminus of TRPM2. This was first demonstrated in experiments where the structure for the calmodulin binding motif of TRPM2 was disrupted, and the channel was not able to be opened (Du, Xie, and Yue 2009).

Role of TRPM2 in Inflammation

There is interesting work proving the importance of TRPM2 in maintaining inflammation. TRPM2 can be activated by multiple types of stimuli, such as ROS (e.g. H_2O_2) (Wehage et al. 2002; Fonfria et al. 2004), amyloid beta (Fonfria et al. 2005), and even cytokines like TNF-α (Roberge et al. 2014). This is because these stimuli initiate signalling that leads to the production of ADPR, the agonist for opening the channel. The major way that this ADPR elevation occurs is through activation of PARP-1. For example, a reactive oxygen species like H_2O_2 would cause DNA damage, activating the DNA damage repair activity of PARP-1. As discussed, activated PARP-1 produces PAR polymers, and then PARG cleaves these polymers into monomers, ADPR. Thus, activation of PARP-1 leads to an increased production of the end product of ADPR, leading to channel opening and an influx of calcium (Syed Mortadza et al. 2015; Syed Mortadza et al. 2018). For other stimuli like TNF-α, since it is possible for PARP-1 to be activated independently of DNA damage through ERK (Vuong et al. 2015), there is the same end result of an increased level of ADPR being produced and ultimately opening TRPM2. TRPM2 activation alone has also been shown to have a role in promotion of inflammation, via production of pro-inflammatory cytokines such as IL-6 and TNF-α (Wehrhahn et al. 2010).

Role of TRPM2 in AD

In the APP/PS1 AD mouse model, a global TRPM2 KO showed reduced activation of microglia, and fewer deficits in spatial memory of the mice (Ostapchenko et al. 2015). As well, in microglial cell cultures, Aβ activation of PARP-1 has been shown to be inhibited in cells where TRPM2 is knocked out (Syed Mortadza et al. 2018). Aging brains have decreased glutathione (GSH), an enzyme that normally reduces ROS, therefore there is potential for more oxidative stress in the brains of older individuals such as AD patients (Aoyama et al. 2006). As these oxidative stresses are activators for TRPM2, this is one potential cause of increased channel activation. GSH has also been shown to directly inhibit TRPM2, both in neurons and microglia (Belrose et al. 2012; Yıldızhan and Nazıroğlu 2020). When GSH was

inhibited or depleted in both studies, TRPM2 channel function was significantly increased. Thus, this suggests that the reduction of GSH itself can be a trigger for activation of TRPM2 in older individuals, which would of course contribute to increased inflammation that could underlie AD.

NUDT5

NUDT5 is one enzyme from the NUDIX (nucleoside diphosphate X) hydrolase family, that can break down ADPR monomers into AMP (adenosine monophosphate) and R5P (ribose-5-phosphate) (Formentini et al. 2009). AMP can eventually be converted into NAD+, so this is effectively a way to replenish the NAD+ stock/pools, after they would have been used by PARP-1 activation (Page et al. 2018). Therefore, NUDT5 offers an interesting target due to its direct role acting on the TRPM2 agonist ADPR, and its indirect role in regulation of PARP-1 signalling. A specific NUDT5 inhibitor has been developed by Page et al. (Page et al. 2018), which inhibits ADPR metabolism carried out by NUDT5 specifically.

Therefore, in my work, I was interested in observing if NUDT5i may also affect production of pro-inflammatory mediators like nitric oxide, or affect beneficial phagocytosis of Aβ. Overall, this points to the interest in studying the PARP-1/TRPM2 pro-inflammatory signalling pathway in the context of regulating neuroinflammation.

1.3.3 TRPM2 and PARP-1 Can Work Together to Contribute to Chronic Neuroinflammation

PARP-1 and TRPM2 alone are both important components that can be linked to pro-inflammatory cell functions and to AD. Therefore, a mechanism by which these components could work together to promote acute signalling that can become chronic and sustained, during a neuroinflammatory state like in AD, would be the following: an inflammatory stimuli (such as Aβ) binds to a surface receptor such as TLR, initiating eventual MEK/ERK2 signalling and activating PARP-1. This leads to production of ADPR monomers, the intracellular agonist for the TRPM2 channel. The channel opens and an influx of calcium enters the cell, further maintaining the activation of PARP-1 through MEK/ERK kinases in a

positive feedback loop. Activated PARP-1 positively regulates NF-κB, leading to transcription of genes for pro-inflammatory mediators such as cytokines (TNF-α, IL-1, IL-6) and iNOS. Once activated, this pathway overall consistently keeps a high level of inflammation in the CNS environment that contributes to assorted detrimental outcomes that are seen in AD, such as neurotoxicity.

Support for the mechanism proposed above comes from previous work that has shown a link between TRPM2 channel opening and PARP-1 activation (Fonfria et al. 2004; Syed Mortadza et al. 2018). One recent study showcasing how TRPM2 and PARP-1 work together to maintain chronic inflammation is from 2020 (Raghunatha et al. 2020), where it was found that NMDAR activation led to TRPM2 channel opening, via PARP-1 activation. This is extremely relevant as elevated glutamate (ligand for NMDAR) leads to neuronal excitotoxicity and injury, underlying the impaired LTP and impaired memory outcomes in AD. Therefore, this work provides an example of how high glutamate levels could activate the PARP-1/TRPM2 signalling loop, leading to this sustained chronic inflammatory response in microglia that would further contribute to worsening neurotoxicity (Raghunatha et al. 2020).

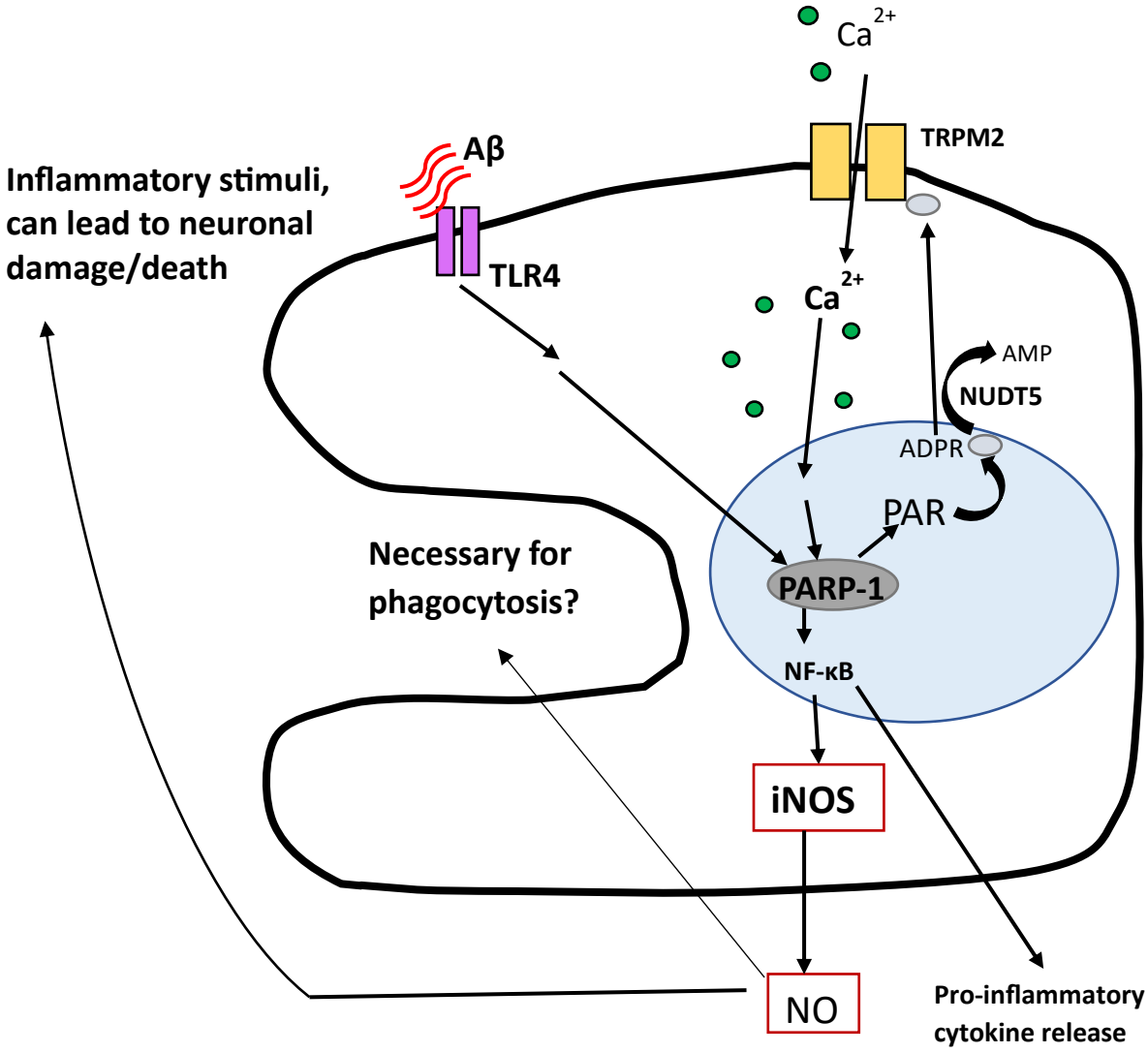

Figure 2: Illustration of PARP-1/TRPM2 signalling pathway in microglia.

Poly(ADP-ribose) polymerase-1 (PARP-1) is centrally involved in promoting inflammation, but it was unknown if it had a central role in dysregulated phagocytosis. Inflammatory stimuli (such as Aβ) induces PARP-1 activation that can be maintained via a positive feedback loop with the calcium permeable cation channel transient receptor potential melastatin type 2 (TRPM2). PARP-1 activation produces PAR (polymers of ADPR), which is hydrolyzed to ADPR (adenosine diphosphate ribose) monomers. ADPR can be broken down by NUDT5, but otherwise acts as an intracellular agonist for TRPM2 channel, thus allowing increased Ca^{2+} influx into the cell, which further promotes PARP-1 activity. Activated PARP-1 is a co-activator of transcription factor NF-κB, promoting inducible nitric oxide synthase (iNOS) expression and synthesis of nitric oxide (NO), a powerful inflammatory signal released by microglia cells. Whether iNOS/NO is required or centrally involved in regulation of phagocytosis was also unknown.

1.4 Rationale, Hypothesis and Objectives

As stated previously, three major pathologies underlying AD have been identified: build up of excessive Aβ plaques, neurofibrillary tangles consisting of hyperphosphorylated tau, and neuroinflammation, which is recognized as a precursor to AD (Kinney et al. 2018). Previous therapeutic strategies targeting accumulation of Aβ or tau deposition (or increasing clearance of these aggregates) have been unsuccessful at preventing AD progression. Given the crucial and multifaceted role of neuroinflammation in AD pathogenesis, microglia offer an intriguing therapeutic target. However, a better understanding is needed of the mechanisms regulating specific microglial functions, such as phagocytosis. This knowledge may allow for design of an ideal AD therapeutic that can suppress specific detrimental functions of microglia (ex. excessive pro-inflammatory mediator release), while maintaining beneficial functions, such as phagocytosis of pathogens and cell debris like Aβ.

Past work in the Kauppinen lab has identified nuclear enzyme poly(ADP-ribose) polymerase-1 (PARP-1) as a key inducer of pro-inflammatory and neurotoxic microglial responses (Kauppinen and Swanson 2005; Kauppinen et al. 2011; Raghunatha et al. 2020). As discussed earlier, PARP-1 acts as a co-activator of NF-κB transcription factor. NF-κB promotes expression of inducible nitric oxide synthase (iNOS), the enzyme responsible for production of nitric oxide (NO) and is also a marker of microglial pro-inflammatory state (Jurga et al. 2020).

PARP-1 can be activated via Ca^{2+} permeable membrane channel TRPM2 (transient receptor potential melastatin type 2). Ca^{2+} entry via TRPM2 activates ERK1/2 kinases, which phosphorylate and stimulate PARP-1. PARP-1 then acts in a positive feedback loop to continually keep the TRPM2 channel open, as PARP-1 produces (poly(ADP-ribose) polymers, or PAR) that are a precursor to ADPR, agonist for TRPM2. PAR is hydrolyzed by an intermediate enzyme into ADPR monomers, which bind to an intracellular domain on TRPM2 channel. This increase in intracellular Ca^{2+} activates ERK1/2 kinases,

which phosphorylate and stimulate PARP-1 in a positive feedback loop (Vuong et al. 2015; Raghunatha et al. 2020).

It has been shown that PARP-1 inhibition or deletion prevents Aβ-induced microglial NO release, but does not prevent Aβ phagocytosis (Kauppinen et al. 2011). This suggests that during beneficial phagocytosis of Aβ, activity of both PARP-1 and NO together may not be required. This is also an important finding as it demonstrates that PARP-1 does not appear to regulate all aspects of microglial functions relevant to AD.

However, targeting PARP-1 in a clinical setting has some limitations. For example, PARP inhibitors such as olaparib and talazoparib can lead to cell death through DNA damage induced via PARP-DNA trapping. This would not be ideal for a drug being used in the CNS, where cell survival is critical (Sinha et al. 2021). PARP inhibitors such as PJ34 have not been found to have this PARP-DNA trapping effect, however, PJ34 does not specifically inhibit PARP-1; it is a broad inhibitor of multiple enzymes in the PARP family (Thorsell et al. 2017). Additionally, PARP inhibitors used clinically have been reported to have numerous undesirable adverse side effects for patients, including severe fatigue, nausea, anemia, and low platelet counts (Bitler et al. 2017). Therefore, inhibiting another component of the PARP-1/TRPM2 signalling pathway, such as TRPM2, may be more clinically relevant.

Past work from the Jackson lab has shown that global TRPM2 KO in an APP/PS1 mouse model showed fewer cognitive outcomes and less microglial activation compared to wild-type controls (Ostapchenko et al. 2015). Additionally, TRPM2 KO was well-tolerated in mice suggesting that inhibition of TRPM2 may be an effective therapeutic approach in AD. However, the role of TRPM2 in other microglial functions, such phagocytosis of Aβ, remains unknown. It is also unknown whether iNOS, activated downstream of PARP-1 and TRPM2, is required for phagocytosis of Aβ. Other authors have reported that NO is required for microglial phagocytosis in conditions mimicking infection or

autoimmune conditions (Kakita et al. 2013; Maksoud et al. 2019). However, this literature did not look at phagocytic uptake of Aβ cargo.

Given the importance of understanding the regulation of microglial phagocytosis of Aβ, my project focused on elucidating the potential role of TRPM2/PARP-1 signalling in regulating phagocytic clearance in conditions of health and conditions representative of AD (amyloidopathy). While it has previously been shown that TRPM2/PARP-1 signalling is involved in modulating microglial inflammatory responses in the context of AD, the specific role of this signalling in relation to phagocytosis has not been shown. This study will provide insight into the individual functional roles of TRPM2, PARP-1, and iNOS during microglial phagocytosis of Aβ - thus providing insight into potential therapeutic development for AD.

Hypothesis:

I hypothesize that inhibition of components of a pro-inflammatory microglial signalling pathway, PARP-1 or TRPM2, will not affect beneficial phagocytic uptake of Aβ. I will address if amyloidopathy impacts the control of microglial phagocytosis ability, through comparison of healthy microglia vs. microglia in amyloidopathy state. Additionally, I hypothesize that NO, produced through this pathway via iNOS, is not required for microglial phagocytosis of Aβ.

Objectives:

1. Assess the role of TRPM2/PARP-1 in regulating microglial phagocytic uptake of Aβ in health vs. conditions associated with elevated AβOs (amyloidopathy).
2. Investigate whether microglial NO is required for phagocytosis of Aβ, in health vs. conditions associated with elevated AβOs (amyloidopathy).

CHAPTER 2. MATERIALS AND METHODS

2.1 Experimental Animals and Cell Culturing

Primary mixed glial cultures were prepared from cortices of newborn (<48 hours) CD1 mice. Mice colonies were maintained at University of Manitoba's Central Animal Care Services facilities, and protocols were approved by the Animal Welfare Committee.

Pups were processed for culturing as described previously (Raghunatha et al. 2020). Briefly, following sterile dissection of pup brains and removal of meninges, cortices were isolated and processed further, all while on ice. Cortices were minced in cold dissection media (Hank's balanced salt solution (ThermoFisher; Cat # 14170161), containing 2 mM HEPES (ThermoFisher; Cat # J16924.AE), 50 U/ml Penicillin and 50 µg/ml Streptomycin (PenStrep; ThermoFisher; Cat # 15070063)).

Cortices were transferred to a tube, supernatant removed, and digested with 0.05% trypsin, in warm dissection media at 37°C for 3 minutes. Trypsin was quenched using an equal volume of ice-cold glial media (minimal essential media (MEM) containing phenol red pH indicator and 5.5 mM glucose (ThermoFisher; Cat # 11090099), 2 mM GlutaMAX (ThermoFisher; Cat #35050-061), 10% fetal bovine serum (FBS, containing low (<0.05%) endotoxin levels (ThermoFisher; Cat #12483-020)), and 0.01% Streptomycin (ThermoFisher, Cat # 11860038)). A low level of endotoxin in culturing media is crucial as microglia are immune cells, and therefore extremely sensitive to reacting to endotoxin. A potential endotoxin such as LPS could promote unwanted production of pro-inflammatory products by microglia in these cultures.

Supernatant volume was removed again, following which small volumes of warm glial media (0.5 mL) were added at a time to triturate tissue into single cells. Then, tissue was plated into T75 (75 cm^2) or T25 (25 cm^2) flasks with a final media volume of 15 mL or 5 mL respectively, and incubated at

37°C with 5% CO2. T75 flasks received tissue originating from 2-3 brains per flask, while T25 flasks received 1 brain per flask.

2.2 Preparation of Amyloid beta oligomers (AβOs) conditioned medium (7PA2-CM)

The preparation of amyloid beta oligomers used in this study was graciously completed by Natalie Lavine of the Jackson lab. The methods used for preparing these oligomers are as follows. Amyloid beta soluble oligomers used were secreted from Chinese hamster ovary (CHO) cells that express hAPP (V717F) – human amyloid precursor protein with familial V717F mutation (Shankar et al. 2011). Briefly, these CHO cells expressing hAPP (called 7PA2), and control CHO cells with no altered expression, were grown on 100 mm culture dishes. Cells were split into fresh dishes at 90% confluency, and maintained for approximately 4 passages before being used further, using MEM (minimal essential media) with 10% FBS and 2 mM GlutaMAX. Once ready for harvesting, cells were washed twice with serum-free MEM and incubated overnight for 18 hours. Media was collected from cell dishes into 50 mL tubes, then centrifuged at 200 x g for 10 minutes to separate conditioned media (CM) supernatant, containing cell secretions. This conditioned media was then aliquoted and stored at -80°C.

To confirm presence of secreted AβOs in conditioned media, an ELISA (enzyme-linked immunosorbent assay) was run to measure both the concentration of Aβ 1-42 and Aβ 1-X (total amyloid beta). Human amyloid beta (1-X) Assay Kit (Immuno-Biological Laboratories, Cat# 27729) and Ultrasensitive kit (Life Technologies, Cat# KHB3544) were used to measure concentrations from 7PA2-CM and CHO-CM (control cell conditioned media). The concentration of Aβ 1-42 in 7PA2 was 57.2 pg/ml, and the concentration of Aβ 1-X (total amyloid beta) was 6357 pg/ml. Consistent final concentration of 1100 pg/mL of Aβ 1-X was used in experiments.

2.3 Harvesting Microglia and Cell Treatments

Media changes for mixed glia cultures were done at DIV 1 and DIV 4. When microglia were visually ready to be harvested (at earliest ~DIV 7), microglia were harvested by gently flicking each flask

manually and collecting the detached microglia cells. Collected microglia were harvested in a 50 mL conical tube and gently mixed before plating equal volumes into cell culture plates (24 or 48-well). Cells were ready to be used approximately 24 hours after harvest. Cells were plated at approximately 5 x 10^5 cells per well.

Cells were treated for chronic experiments for 20 hours, while acute experiment treatment ranged from 1-8 hours. Cell treatments were prepared in media containing MEM, 2 mM GlutaMAX, and 5-10% glial conditioned media from harvested cells. Cells were treated with amyloid beta oligomers (AβOs) at final concentration of 1100 pg/mL (Aβ 1-X). This means that 7PA2-CM (conditioned media containing AβO) made up just under one-sixth of its respective treatment volume. As conditioned media from CHO cells can secrete other factors, it was important to include a CHO-CM vehicle control, in which it was diluted in media to match 7PA2-CM dilution. CHO-CM was thus chosen as the most relevant control condition for assays, unless otherwise specified.

In addition, in experiments with inhibitors, inhibitors were added for the full experimental time, at the following final concentrations: 500 nM PJ34 / 1 μM Clotrimazole / 1 μM JNJ / 1 μM TAK242 / 10 μM 1400W / 3 μM NUDT5i. Final volume of treatments per well in 24-well plates was 250 μL.

2.4 Phagocytic Assays

For chronic phagocytic experiments, cells were treated as above for 20 hours. One hour before the end of cell incubation time, an aliquot of fluorescently-labelled FAM (5,6-carboxyfluorescein) Aβ 1-42 peptide (herein called FAM-Aβ) cargo was prepared. This cargo was diluted in MEM to intermediate concentration of 250 μM, and incubated at 37°C for 1 hour, to allow for a mix of amyloid beta monomers and oligomers to form. Finally, FAM-Aβ was added to cells at final concentration of 0.3 μM, and cells incubated for further 2 hours, while protected from light.

Concentrations of inhibitors were maintained in wells already containing inhibitor treatments (i.e. wells treated with PJ34 at 500 nM were treated with FAM-Aβ + 500 nM PJ34). Cytochalasin D was added to select wells 30 minutes prior to cargo at a final concentration of 10 μM. Cells were incubated with cargo for 2-8 hours at 37°C, 5% CO2, while protected from light.

Following the end of phagocytic cargo incubation, cells were quenched with trypan blue (final concentration 2 mg/ml) for 2 minutes at room temperature. This was done to quench any fluorescent cargo left outside of cells while leaving intracellular fluorescence intact, as trypan blue cannot pass through the membrane of live cells. Then, all volume was removed from wells, and cells were fixed with PLP solution (Periodate-Lysine-Paraformaldehyde, (McLean and Nakane 1974)) for 10 minutes at room temperature. Fixing solution was removed and cells washed twice with PBS (pH 7.4). Final fixed cells were stored at 4°C, protected from light. Phase and fluorescent images were obtained from Evos FL Digital Inverted Fluorescence Microscope. All images within same dataset were obtained using identical imaging settings for brightness and exposure time.

2.5 Analysis for Phagocytic Assays

All assays representing biological replicates were prepared from cells cultured independently. Each assay was completed in a 24 or 48-well plate, and consisted of 2 wells for each unique treatment group. Images were taken from 2-3 fields of view per experimental well, for a minimum count of 100 cells per well. Fluorescence values were obtained from ImageJ software (ImageJ, version 1.53), via the steps below.

Each fluorescent image was converted to 16-bit format, and a duplicate image was produced. The duplicate image was used for thresholding, while the original image was maintained as reference for the program when quantifying raw fluorescence values. The threshold was adjusted equally for each image using Li threshold method, and particle size parameters were set to detect particles larger than 20 μm^2. The ‘Analyze Particles’ function was used to quantify the final output value of Raw Integrated Density

per image. Average of this intensity was reported as 'FAM-Aβ Intensity per Fluorescent Cell'. The number of fluorescent cells per image was also counted by ImageJ, while number of phase cells was counted manually. Final phagocytic uptake fluorescence data will be shown in this thesis as 'FAM-Aβ Intensity per Fluorescent Cell'. This analysis demonstrates the amount of cargo able to be taken in by cells that did phagocytize cargo.

2.6 NO Assays

Following 20 hours of treatment with AβO and inhibitors as described, aliquots of cell supernatant were used to measure nitric oxide production. This was done using the Griess reagent, which is used to measure nitric oxide levels in solution, based on detection of nitrite (NO_2). Nitric oxide has an extremely short half-life, but in aqueous solution is hydrolyzed to form nitrite, which can be more readily detected. In particular, in Griess reagent assays, the sequential reaction of nitrite with sulfanilamide forms an intermediate diazonium salt, and this salt reacts with N-(1-Napthyl)ethylenediamine to produce a compound with an azo dye structure (Ignarro et al. 1987). Azo dyes colour intensity can be measured, and therefore this colour intensity can be linked to nitrite concentration.

Griess reagent used for these assays was prepared as follows: 4 mM N-(1-Napthyl)ethylenediamine, 60 mM sulfanilamide, and 1.95% phosphoric acid (all from Sigma-Aldrich) were prepared in ddH_2O (double distilled water), vortexed vigorously until all components well mixed, then further filtered using 0.22 μm pore filter. Griess reagent was protected from light and stored at 4°C until needed.

Standards for this assay were sodium nitrite ($NaNO_2$, Sigma-Aldrich), and were diluted using the same aliquot of MEM used for cell treatment preparation. Inclusion of this same batch of MEM was necessary for consistency as it is known that media alone can have background absorbance in plate reader assays. Therefore, preparing standards in the same media as samples, and including a media only 'blank' sample, is crucial. 50 μL of each sample or standard was plated in 96-well plate in triplicate, and 50 μL

of Griess reagent added per well. After 10 minutes incubation at room temperature, the absorbance values were read at 540 nm on a VarioSkan Lux plate reader.

In addition, a matched protein assay was completed for the same samples using DC Protein Assay kit (Bio-Rad Cat# 5000111). Protein quantification is necessary for each well of treated cells, because logically, samples with more protein or more cells would be expected to produce more nitric oxide. Therefore, total nitric oxide concentration per sample is normalized to total protein for the same sample. Thus, final data is presented as nitric oxide concentration per μg/ml of protein per sample.

Following removal of supernatant from treated cells, RIPA (Radioimmunoprecipitation) lysis buffer containing protease inhibitor was added to wells to maintain sample quality. Then, wells containing treated cells were scratched with a pipette tip to lyse cells. A standard curve consisting of BSA protein standards was prepared, and 5 μL of sample or standards was plated in 96-well plate in triplicate. Colorimetric reagents from DC Protein Assay kit were added, and plate incubated 15 minutes at room temperature. Absorbance was read at 750 nm on VarioSkan Lux plate reader.

2.7 Preparation of NO Donor for Use in Phagocytic Assays

The NO donor NOC-18 (DETA NONOate, Fisher Scientific) was prepared in sterile H_2O, pH 7.4. Aliquots were prepared fresh prior to each experiment to avoid degradation. NOC-18 was added to cells at final concentration of 25 μM, and incubated with cells at 37°C for 1 hour, to allow for nitric oxide production from the donor to act on cells prior to addition of phagocytic cargo (2 hours).

2.8 Morphological Analysis of Cell Cultures

Morphological analysis of cell cultures post-treatment, along with cell counts, was performed for all assays. Microglial morphology is analyzed as a secondary outcome, as it provides insight to microglial function and state, although there is not always a direct correlation. Images for analysis were taken from 1-2 fields of view, with a minimum total count of ~60 cells per well. Images were taken

using Evos Microscope. Cell morphology was divided into 4 categories: Ramified, Hypertrophic, Classical Amoeboid, and TLR-Stimulated ('Spiky') Amoeboid. The criteria for a cell to fall into each category was as follows:

Cell Morphology Label	Criteria
Ramified	Oval-shaped/small soma Elongated, rod shaped 2 or more thin processes
Hypertrophic	Enlarged soma Thicker and shorter processes May have amoeboid endfeet
Amoeboid (Classical)	Large soma Round, fried egg appearance Often has dark soma and light cytosol
Amoeboid (Spiky)	Large soma High number of processes, spiky appearance Often has dark soma and dark cytosol

Table 1. Criteria used to classify microglial morphology.

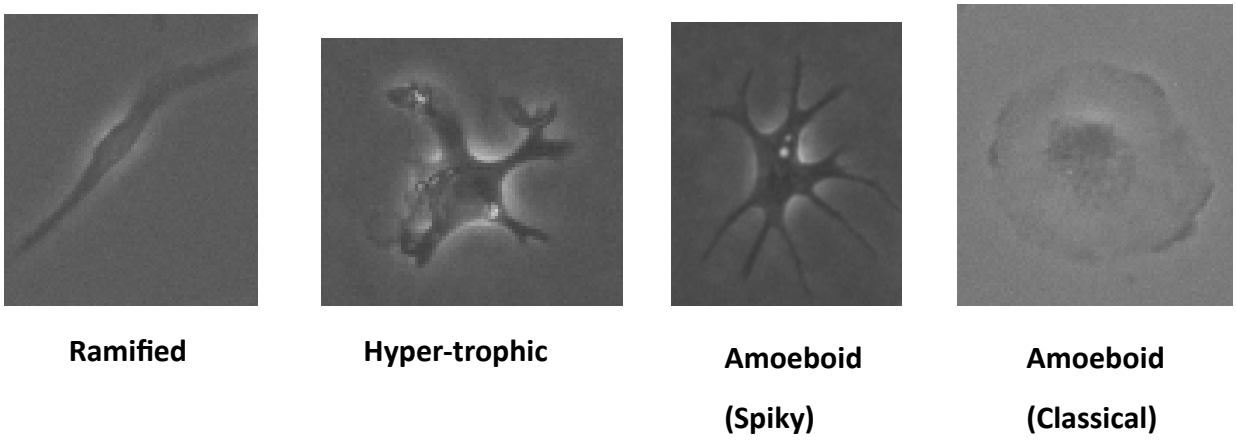

Figure 3: Representative images of microglial morphology classifications.

2.9 Calcium Imaging Methods and Analysis

Calcium imaging experiments methods were as follows: microglia cells were harvested into imaging grade plastic dishes (35 mm diameter, Ibidi) approximately 24 hours before an experiment. On the day of imaging, cells were washed with warm ECF (solution whose contents match that of extracellular fluid *in vivo*) which contained: NaCl (140 mM), KCl (5.4 mM), HEPES (25 mM), Glucose (5.5 mM), and pH adjusted to be between 7.38-7.45. Lastly, osmolality was measured via osmometer, and adjusted to be between 305-312 mmol/kg. In relevant experiments, ECF also contained Ca^{2+} (2 mM) and Mg^{2+} (1 mM). As a note, standard ECF is normally prepared with a higher glucose concentration of 33 mM, but previous work from our lab has shown that many key microglia functions are impacted when glucose levels are >5.5 mM (Vuong et al. 2017). As we didn't want standard ECF alone to induce any crucial changes in microglial function, ensuring final glucose concentration was <5.5 mM was key.

The Ca^{2+} indicator solution (CAL-520 AM dye, 2 μM) was added, and cells incubated for 30 minutes at 37°C, protected from light. Cells were washed again with warm ECF and left in incubator for 15 minutes for de-esterification, and left in final volume of 0.5 mL ECF.

Live cells were imaged on Zeiss LSM 880 Confocal laser microscope for 10 minutes total, using 40X objective lens with oil immersion, and 488 laser for fluorescence excitation. After 3 minutes of baseline recording, a volume of treatment was added to the dish using a micropipette. After 10 minutes, a positive standard for Ca^{2+} was added (Ionomycin, 1 μM final concentration in dish). If cells did not show a minimum 2-fold increase of fluorescence with the addition of standard, they were deemed dead/non-reactive and excluded from analysis. For analysis, a region of interest of each cell was analyzed in ImageJ using 'Raw Integrated Density' output. Values were normalized to baseline (0-180 seconds) for each individual cell.

2.10 Statistical Analysis

The minimum number of biological replicates for any assay was n=3, where each replicate was prepared from an independent culture and each independent culture had 2-3 wells per treatment group. GraphPad Prism (version 9, GraphPad Software, CA) was used for creating figures and completing statistical analysis. Statistical tests used were ANOVA (One-way or Two-way), with Dunnett's multiple corrections comparison. Data values are reported as mean ± SEM, and P values <0.05 are reported as significant.

CHAPTER 3. RESULTS

3.1 Optimization of FAM-Aβ Concentration and Incubation Time

I first undertook experiments to optimize the incubation time and concentration of FAM-Aβ (1-42) cargo to be used in phagocytic assays. Previous work had incubated this cargo with microglia for up to 24 hours (Kauppinen et al. 2011), but we were interested in assessing shorter incubation times in this project, as microglia in later experiments would be pre-stimulated for longer timepoints overnight prior to adding cargo. Additionally, cargo concentration needed to be optimized so that I could avoid using an amount of cargo that would lead to cells taking up the absolute maximum amount of FAM- Aβ possible (100%), which would lead to saturating conditions. The idea is that avoiding cargo saturation allows us to observe more modest changes in phagocytic uptake that may occur in other experimental conditions.

Primary microglia were incubated with 0.1, 0.3, 0.5, or 1 μM of FAM-Aβ, for timepoints of 2.5 hours or 5 hours total (Fig 4A, 4B). Cells were then fixed and imaged. I analyzed this data by counting the number of cells that were fluorescent (indicating that cells had taken in FAM-Aβ), as a percentage of total cell count, per image. The results showed that after 2.5 hours, cells that received the highest concentration of 1 μM FAM-Aβ were close to saturation (94.5% of cells showing uptake). The percentage of total microglia taking up cargo decreased with successive decreases in FAM-Aβ concentration.

The concentration of 0.3 μM FAM-Aβ showed total percentage uptake by cells of 62.2% after 2.5 hours, and 62.9% after 5 hours. Importantly, this concentration was not close to saturation at either timepoint. We wanted baseline/control group phagocytosis to be around 50-60% to be able to reliably observe a possible increase or decrease in uptake with addition of pharmacological inhibitors. Therefore, the final concentration of 0.3 μM FAM-Aβ cargo was chosen to be used in phagocytic experiments going forward.

We also assessed the chosen concentration of 0.3 μM FAM-Aβ at multiple incubation time lengths, from 2.5 to 16 hours (Fig 4C). Ultimately, there was no obvious change in cargo uptake for 2.5-

8 hours, with percentage of uptake ranging from 54.5 - 62.9% (N=1-3). After 16 hours incubation, uptake was greatly diminished down to 13.2% (N=2). It's possible that this observation of decreased fluorescent uptake may be due to diminished fluorescence of the FAM-Aβ cargo after 16 hours incubation, or could even be due to intracellular degradation of this cargo. Regardless, this data showcased the need to limit future experimental cargo incubation times to well under 16 hours.

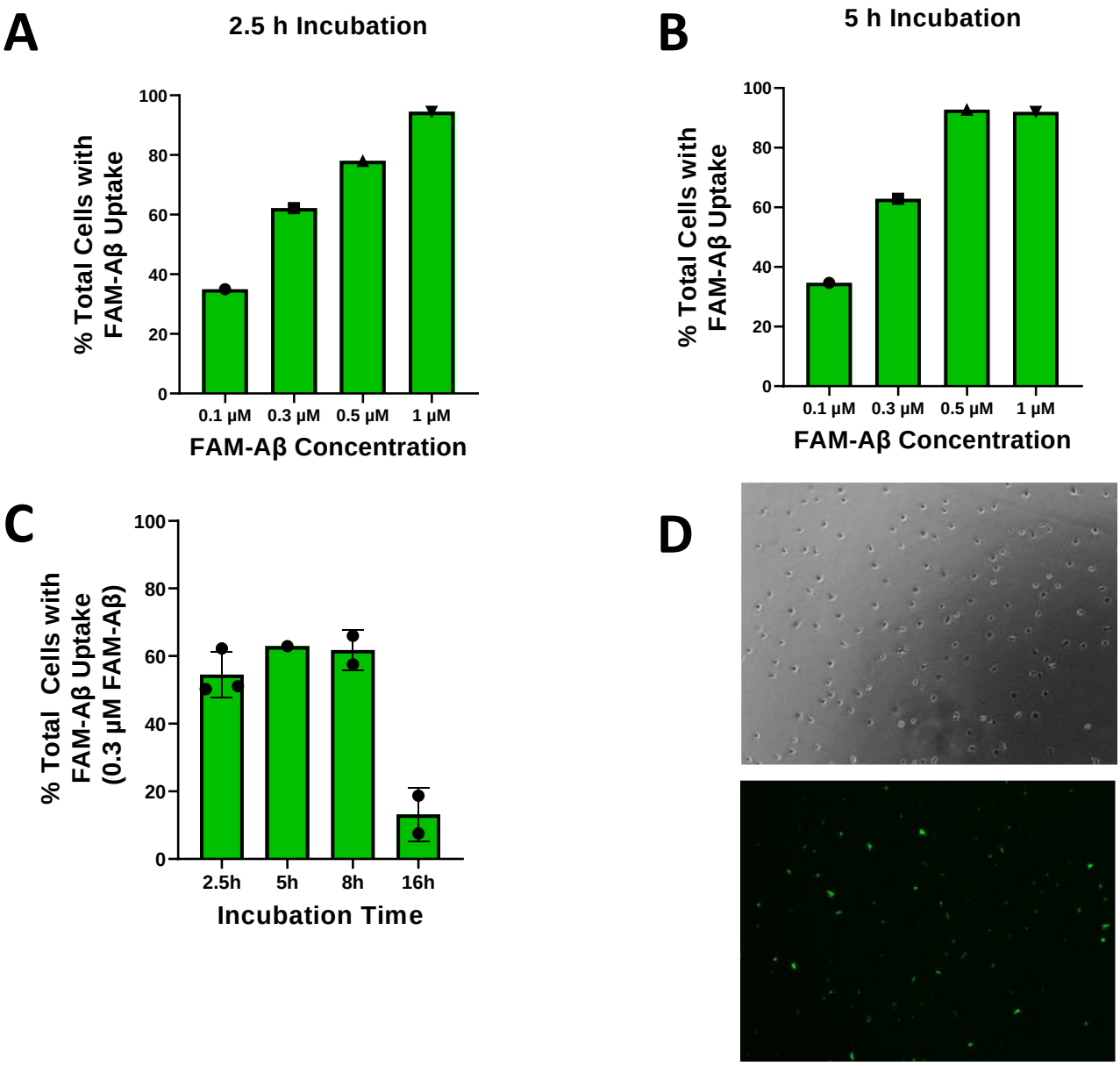

Figure 4: Increasing concentration of FAM-Aβ results in increased uptake by microglia.

FAM-Aβ prepared at concentrations of 0.1, 0.3, 0.5, or 1 µM was incubated with microglia for 2.5 (A) or 5 hours (B). Data counted across 2 treatment wells, 5-8 fields of view per treatment (N = 1). (C) Percentage of total cells showing phagocytosis of FAM-Aβ (0.3 µM) across multiple incubation times (2.5-16h). Data presented as mean, N=1-3. (D) Images show example of phase contrast and fluorescent images of microglia after incubation with 0.3 µM FAM-Aβ for 2.5h. Images from EVOS FL, 10X.

3.2 Inhibition of PARP-1/TRPM2 does not impact microglial phagocytosis of FAM-Aβ in healthy conditions

The purpose of examining microglia's ability to phagocytize Aβ cargo in conditions of 'health' (non-amyloidopathy), was to understand how healthy microglia would be affected by inhibition of the pro-inflammatory PARP-1/TRPM2 signalling pathway. Past work found that PARP-1 pharmacological inhibition or KO, in a state of health, did not have an impact on microglial phagocytosis of FAM-Aβ cargo (incubated with cells for 24 hours) (Kauppinen et al. 2011). I set out to replicate this finding at 8 hours incubation, and to also examine the effects of pharmacological inhibition of other components of the PARP-1/TRPM2 pathway, namely TRPM2, iNOS, or NUDT5i, on phagocytic uptake of Aβ by healthy microglia.

To inhibit PARP-1, I used a PARP inhibitor called PJ34, which is a competitive inhibitor as its structure is similar to that of NAD+, and therefore it blocks the substrate binding site of PARP-1 (Thorsell et al. 2017). To inhibit TRPM2, I used two different drugs – the first being clotrimazole, a well-known antifungal that is a blocker of cation channels such as TRPM2. Previous work in the lab has confirmed that clotrimazole inhibits inward currents in microglia mediated by TRPM2 (Raghunatha et al. 2020). The second drug used to inhibit TRPM2 is JNJ-28583113 (herein called JNJ). This is a relatively new drug that was designed to be more specific for blocking only TRPM2 channels, and is also more potent and therefore can be used at a lower final concentration (Fourgeaud et al. 2019). The drug used to inhibit iNOS is a well-established specific inhibitor 1400W, which is a competitive blocker of iNOS as it binds in the substrate-binding pocket (Garvey et al. 1997). Lastly, the drug used to block NUDT5 (a NUDIX hydrolase that breaks down ADPR in cell), is a proprietary compound provided by Dr. Brent Page at UBC, called TH5427, but will be called NUDT5i from herein (Page et al. 2018).

To test the effect of inhibitors, FAM-Aβ (0.3 μM) was prepared as described in methods and incubated with microglia for 8 hours total, with or without the previously described drugs: PJ34 (0.5

nM), CTZ (1 μM), JNJ (1 μM), 1400W (10 μM), or NUDT5i (3 μM). Figure 5A showcases representative images of fixed cells at the end of experiment. There were no significant changes observed for amount of cargo taken up per cell (measured as average fluorescence intensity per fluorescent cell, Fig 5B).

Thus, the previous finding that PARP-1 inhibition does not significantly impact FAM-Aβ uptake in cellular conditions of health was confirmed (Fig 5B). Additionally, other components of the PARP-1/TRPM2 pro-inflammatory signalling hub that either have a role in activation of PARP-1 (such as TRPM2, NUDT5 domain), or are activated downstream of PARP-1 (such as iNOS), do not regulate Aβ uptake either. Together, this data shows that in a healthy state, PARP-1/TRPM2 signalling does not play a significant role in regulating phagocytosis of Aβ.

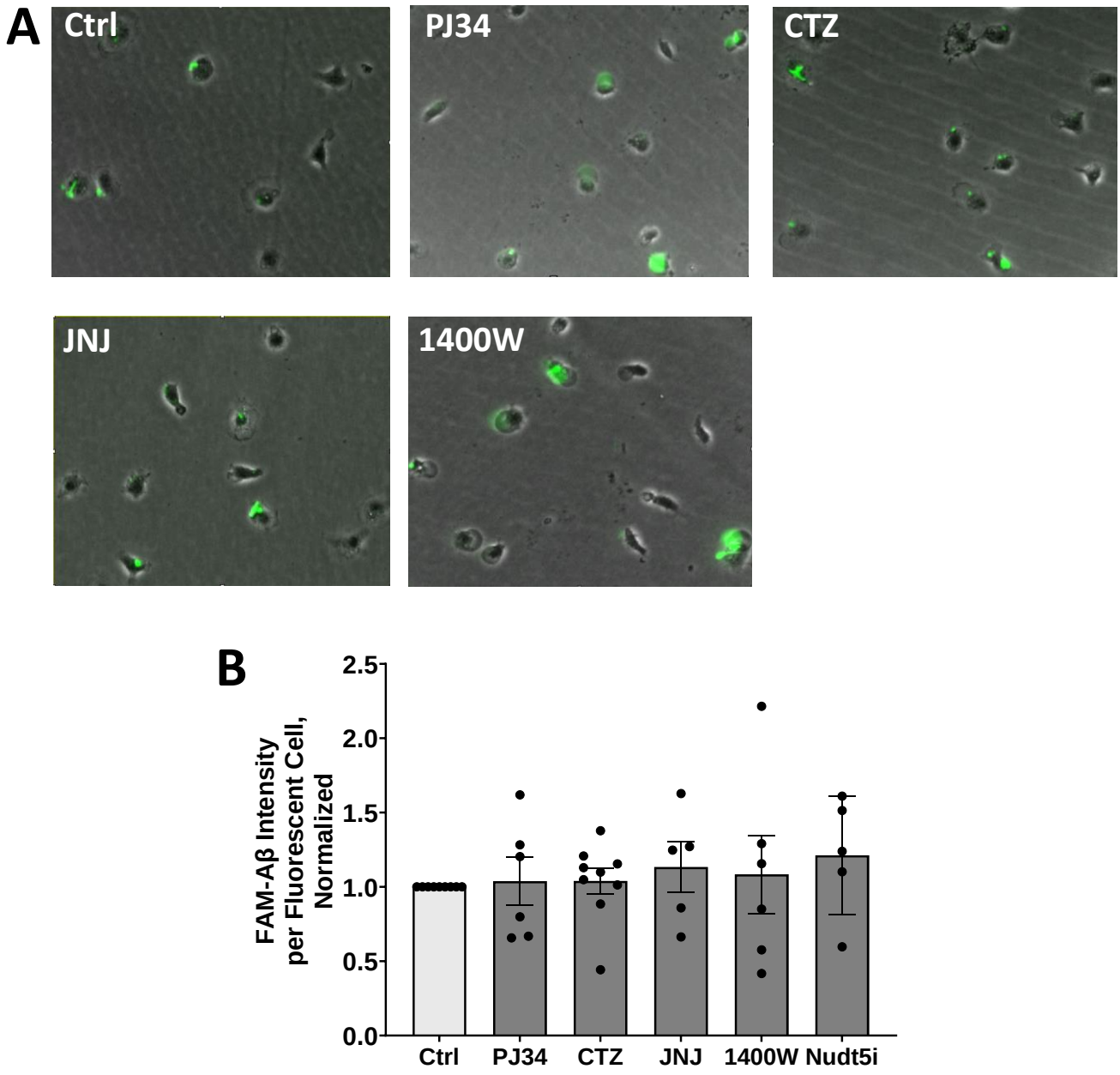

Figure 5: Inhibition of PARP-1/TRPM2 signalling does not alter microglial phagocytosis of FAM-Aβ in healthy conditions.

(A) Representative images (phase and fluorescence overlay) used for analysis. **(B)** Microglial phagocytic uptake of FAM-Aβ (0.3 µM) was assessed after 8 hours incubation with and without inhibitors targeting PARP-1 (PJ34, 500 nM), TRPM2 (CTZ, JNJ; 1 µM), iNOS (1400W, 10 µM), or NUDT5 (NUDT5i, 3 µM). N=5-9, one-way ANOVA , n.s. Data is shown as average fluorescence intensity of FAM-Aβ (0.3 µM) per fluorescent cell.

3.3 AβO treatment compromises microglial phagocytosis of FAM-Aβ independently of PARP-1/TRPM2 signalling

The next question of interest was whether microglial phagocytosis is impacted in assays that model the amyloidopathy conditions of Alzheimer's disease, induced via amyloid beta oligomers (AβOs), and whether the pro-inflammatory signalling of PARP-1/TRPM2 may contribute to this phagocytic uptake. To test this, microglia were treated with AβOs (final concentration Aβ 1-X, 1100 pg/ml) for 20 hours, in presence or absence of inhibitors for PARP-1 (PJ34, 500 nM) or TRPM2 (JNJ, 1 μM), before FAM-Aβ cargo (0.3 μM) was added for 2 hours.

AβO-treated cells were only able to take up just over one-third the amount of cargo compared to cells in control conditions (Fig 6B, Ctrl mean = 1, AβO mean = 0.356, a 64.4% decrease, p=0.007), and the inhibition of PARP-1 or TRPM2 in the presence of AβO-treatment did not have a significant effect on phagocytic uptake (Fig 6B). These results, when taken in addition to results from previous section 3.2, suggest that inhibiting PARP-1/TRPM2 signalling does not show any significant decrease in phagocytic uptake of Aβ in conditions of health or in conditions mimicking AD. The fact that there is no decreased capacity for phagocytic uptake of a pro-inflammatory cargo such as Aβ would be a beneficial outcome for a therapeutic targeting PARP-1/TRPM2. As well, this data shows that the suppression of cargo uptake observed in conditions of amyloidopathy is not dependent on PARP-1/TRPM2 signalling.

In Fig 6C, cells treated with CytD (10 μM) 30 minutes prior to addition of FAM-Aβ cargo show an 86.2% decrease in phagocytic uptake, compared to control. Cytochalasin D (CytD) is a compound that binds the actively growing end (or 'barbed end') of actin filaments, effectively blocking any further actin polymerization from occurring. As phagocytosis is an actin-dependent process, CytD therefore strongly inhibits phagocytosis (Gao and Nakamura 2022). CytD was used as a negative control as it showcases the maximum level to which phagocytic uptake can be inhibited in our assay (i.e. the maximum inhibitory effect that can be seen). In comparison, AβO-treated cells have a 64.4% decrease in

Aβ uptake compared to control – therefore while AβO treatment is significantly decreasing microglial ability to engulf cargo, the phagocytic suppression is not as severe as observed with CytD. This also indicates that even under conditions of amyloidopathy, microglia are still able to ingest FAM-AB in a manner dependent on actin.

Lastly, matched data for these phagocytic experiments can be examined for two other major outcomes: morphological transformation, and cell counts as a measure of cell viability. Morphological changes following AβO treatment were exemplified by a significant increase in proportion of cells with spiky amoeboid morphology, and a decrease in proportion of cells with ramified morphology (Fig 6D). Inhibition of PARP-1 or TRPM2 alongside AβO treatment did not have a major impact on morphological changes. Cell viability was also well maintained in presence of AβO or inhibitors, suggesting that the reduced phagocytosis observed was not due to a reduction in cell viability (Fig 6E).

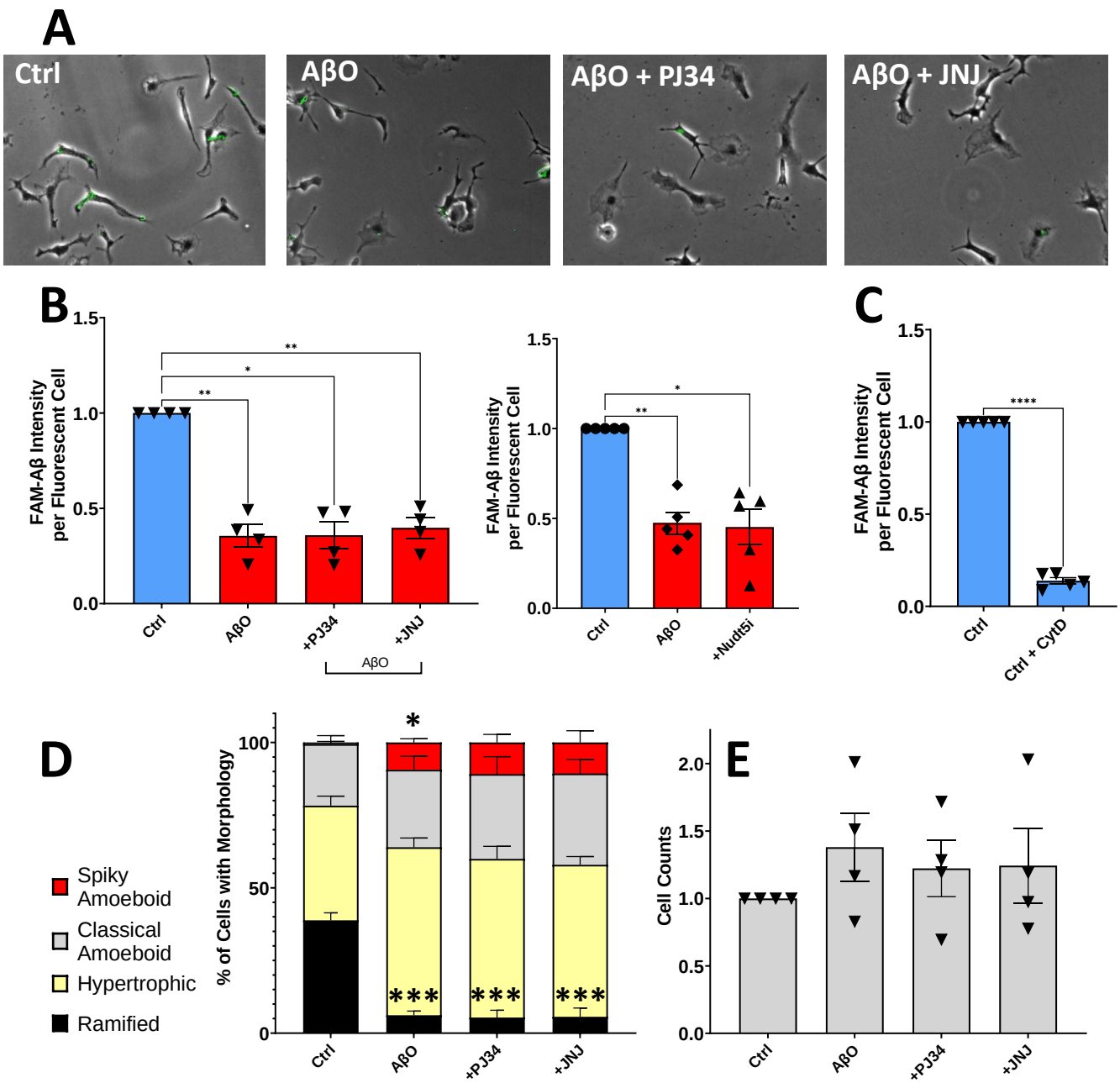

Figure 6: AβO treatment compromises microglial phagocytosis of FAM-Aβ independently of PARP-1/TRPM2 signalling.

Representative phase-contrast images (A) show microglial ability to phagocytize FAM-Aβ in control conditions (Ctrl, received vehicle control media) and after treatment with AβO (1100 pg/ml for Aβ 1-X), in the presence or absence of inhibitors targeting PARP-1 (PJ34, 500 nM) or TRPM2 (JNJ, 1 µM). (B) Phagocytic uptake was decreased following AβO treatment, while inhibitors for PARP-1 or TRPM2 did not increase uptake. N=4, one-way ANOVA, * $p<0.05$, **$p<0.01$. (C) Cells treated with broad phagocytic blocker cytochalasin D (CytD, 10 µM) show strong decrease in phagocytic uptake (N=5, one-way ANOVA, ****$p<0.0001$). (D) Analysis of microglial morphological transformation shows how AβO treatment transforms cell morphology (N=4, two-way ANOVA, *$p<0.05$, ***$p<0.001$, compared to control). (E) Cell counts reflecting cell proliferation and viability (N=4, one-way ANOVA, n.s.).

3.4 Microglial AβO treatment induces changes in microglial NO release and morphology, suppressed by inhibition of PARP-1/TRPM2 signalling pathway.

It has been well-established that PARP-1 contributes to pro-inflammatory signalling through a variety of functions such as cytokine production. However, quantification of another pro-inflammatory marker, nitric oxide, is a more easily accessible assay to perform. I was interested in showing whether other components of the PARP-1/TRPM2 signalling pathway were required for NO production. Additionally, following the phagocytic assay results in sections 3.2 and 3.3 (in which pharmacological inhibitors had no effect), I was interested in confirming that these same pharmacological inhibitors were working as expected. Thus, I carried out measurements of NO production in the presence and absence of these inhibitors, to confirm that inhibition of PARP-1/TRPM2 signalling hub components using my selected inhibitors can prevent Aβ-stimulated NO release.

Microglia were treated with AβO (1100 pg/ml for 20 hours), inducing an increased production of NO (Fig 7A). This also led to a significant shift in cell morphology, with the proportion of spiky amoeboid cells significantly increased post-AβO, and proportion of hyper-trophic cells decreased (Fig 7B). Interestingly, NUDT5 treatment does not show the same morphological transformations seen in AβO-treated cells. This is particularly interesting as NUDT5 is the only drug that has morphological transformations that are significantly altered from AβO-treated cells. As in 3.3, treatments did not lead to significant changes in cell viability. (Fig 7C).

Cells in the presence of AβO treated with inhibitors for PARP-1 (PJ34), TRPM2 (CTZ), NUDT5 (NUDT5i), or iNOS (1400W) for 20 hours had significantly decreased production of NO (Fig 7A). Thus, each of these components in the PARP-1/TRPM2 pathway individually play a role in intracellular signalling responsible for NO release.

However, results for one drug that inhibits TRPM2 (JNJ) did not successfully show a suppression of NO release in cells treated with AβO (Fig 7A). The reason why one TRPM2 inhibitor (CTZ) may

block nitric oxide production, while another (JNJ) does not, is unclear. This JNJ preparation was tested and shown to significantly block TRPM2 current in cells by electrophysiology (unpublished data, Jackson lab), therefore the drug effectiveness is confirmed. One possible explanation is that JNJ was structurally unstable when incubated 20 hours overnight at 37 C. JNJ has been shown to be metabolized *in vivo* (plasma) (Fourgeaud et al. 2019).

Additionally, previous work in the lab has shown that microglia from TRPM2 KO mice do not release increased NO following treatment with either AβO or NMDA (Raghunatha, n.d.) – therefore providing strong evidence that TRPM2 is a contributor to NO release in this context.

It should be noted that in the assays using JNJ to inhibit TRPM2, AβO-treatment increased NO release compared to control, but this was not statistically significant (Fig 7A). Therefore it is also possible that JNJ treatment was reducing the NO release, but that the effect size was too small in assays here.

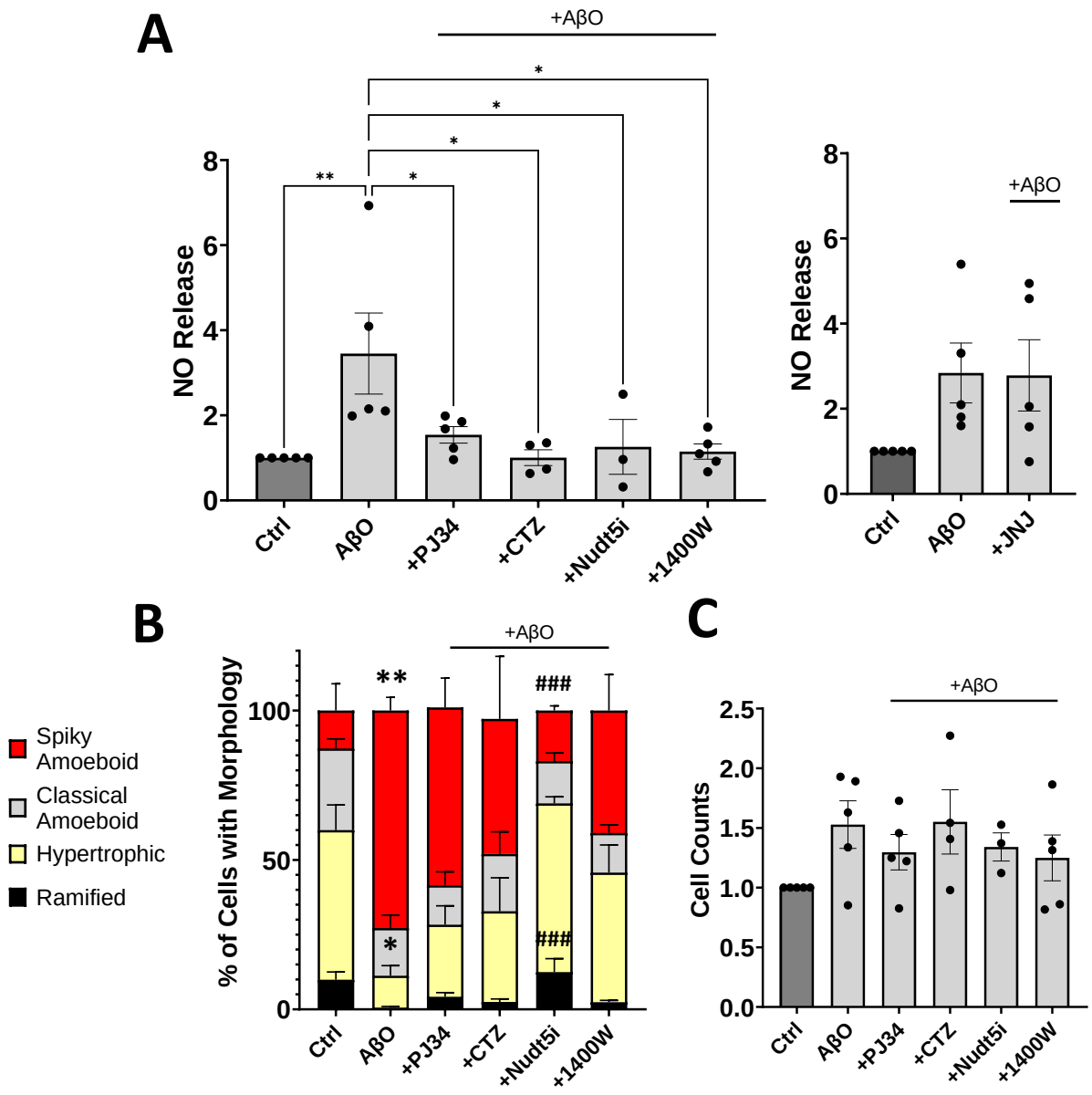

Figure 7: AβO treatment induces changes in microglial NO release and morphology, suppressed by inhibition of PARP-1/TRPM2 signalling pathway.

(A) Microglial NO release after 20 hours of treatment with AβO (Aβ 1-X, 1100 pg/ml), alone and in the presence of inhibitors targeting PARP-1 (PJ34, 500 nM), TRPM2 (CTZ or JNJ, 1 µM), NUDT5i (3 µM), or iNOS (1400W, 10 µM). N=3-5, one-way ANOVA,*p<0.05, **p<0.01. (B) Morphological analysis of microglia used for NO release assays shows that AβO-treated cells have significant changes in 2 categories of cell morphology compared to control, which NUDT5i appears to significantly prevent (N=3-5, two-way ANOVA, *p<0.05, **p<0.01 compared to control; ###p<0.001 compared to AβO-treated). (C) Cell counts for microglia used for NO release assays show that neither treatment with AβO or inhibitors caused a significant change in cell viability or survival (N=3-5, one-way ANOVA, n.s.).

3.5 Neither inhibition of iNOS nor addition of nitric oxide donor significantly impacts microglial phagocytosis of FAM-Aβ in amyloidopathy conditions.

The current literature is unclear regarding the requirement of nitric oxide, produced via iNOS, for microglial phagocytosis. It has been reported that NO/iNOS is necessary for microglial phagocytosis, and this is not due to down regulation of receptors for relevant cargo (Kakita et al. 2013; Scheiblich and Bicker 2015; Maksoud et al. 2019). Meanwhile others report that NO/iNOS is not necessary for phagocytosis (Kopec and Carroll 2000).

Aside from easily identifiable methodological differences between these studies (such as type of microglial cells and cargo used, cargo incubation times, etc.), there is still value in questioning whether NO is required, as it provides clarity on whether the pro-inflammatory signalling of NO/iNOS is required for phagocytosis, which would be an important consideration when designing a therapeutic targeting phagocytosis. Therefore, I completed further experiments to assess the specific role of NO/iNOS in microglial phagocytosis of Aβ cargo.

Microglia were treated with AβOs (final concentration Aβ 1-X, 1100 pg/ml) or vehicle control for 20 hours, in the presence or absence of iNOS inhibitor (1400W, 10 μM, 20 hrs). Alternatively, cells treated with AβOs or vehicle control were also treated with NOC-18 (25 μM, added 1 hour prior to cargo addition). NOC-18 (also called DETA (diethylenetriamine) NONOate due to its molecular structure) is a known slow-release NO donor, and has a half life of 20 hours (Feelisch 1998). The purpose of using an NO donor in these experiments was to reflect an elevated level of extracellular nitric oxide, as would exist in a state of neuroinflammation, and to assess if this stimulates phagocytosis as some authors have suggested (Maksoud et al. 2019), or alternatively if higher extracellular NO mimicking an inflammatory state may instead decrease or have no effect on microglial phagocytic uptake.

In order to determine a suitable incubation time and concentration of NOC-18 to use in phagocytic experiments, I first assessed the amount of NO produced in cell-free media following incubation for 1 or

3 hours with 25 μM NOC-18 (Fig 8B). After 3 hours, approximately 6 μM NO was produced - this was chosen as the total incubation time to be used for 25 μM NOC-18 going forward. As in previous phagocytic assays, FAM-Aβ cargo (0.3 μM) was added for the final 2 hours and fluorescent cargo uptake was assessed. Therefore, NOC-18 was added 1 hour before this cargo addition to be present for a full 3 hours.

AβO-treated cells again showed a significant decrease in FAM-Aβ uptake compared to cells in control conditions (Fig 8A, Ctrl mean = 1, AβO mean = 0.313, $p<0.0001$). AβO-treated cells did not have significant changes in cargo uptake with either inhibition of iNOS or addition of NOC-18. The outcome was the same for cells under control conditions – neither inhibition of iNOS, nor additional NO via NOC-18, showed any significant change in phagocytic uptake of FAM-Aβ, in comparison to control conditions alone (Fig 8A).

Analysis of morphological changes reflects the results seen for phagocytic assays. AβO-treated cells had a significant increase in proportion of cells with amoeboid morphology, and iNOS or NOC-18 treatment under AβO conditions did not change this outcome (Fig 8C). Cell viability measures show that cells tolerated NO inhibitors/donors with and without AβO well (Fig 8D).

In summary, these results show that using our methods, we observed that microglial production of nitric oxide (iNOS) or exogenously supplied nitric oxide (NOC-18) does not play a significant role in the modulation of Aβ phagocytosis. Thus, our work adds to the relatively small number of publications in the microglial field concerning whether nitric oxide alters phagocytosis. To our knowledge, this is the first work to examine the role of nitric oxide on the uptake of Aβ phagocytic cargo (other works have examined other cargo types, the most common being latex beads). Therefore, it is possible that NO may affect uptake of different types of phagocytic cargo differently. This data suggests that microglial phagocytosis of Aβ and nitric oxide production may not be controlled by the same signalling pathway (i.e nitric oxide production is under regulation of PARP-1/TRPM2 signalling, while phagocytosis is not).

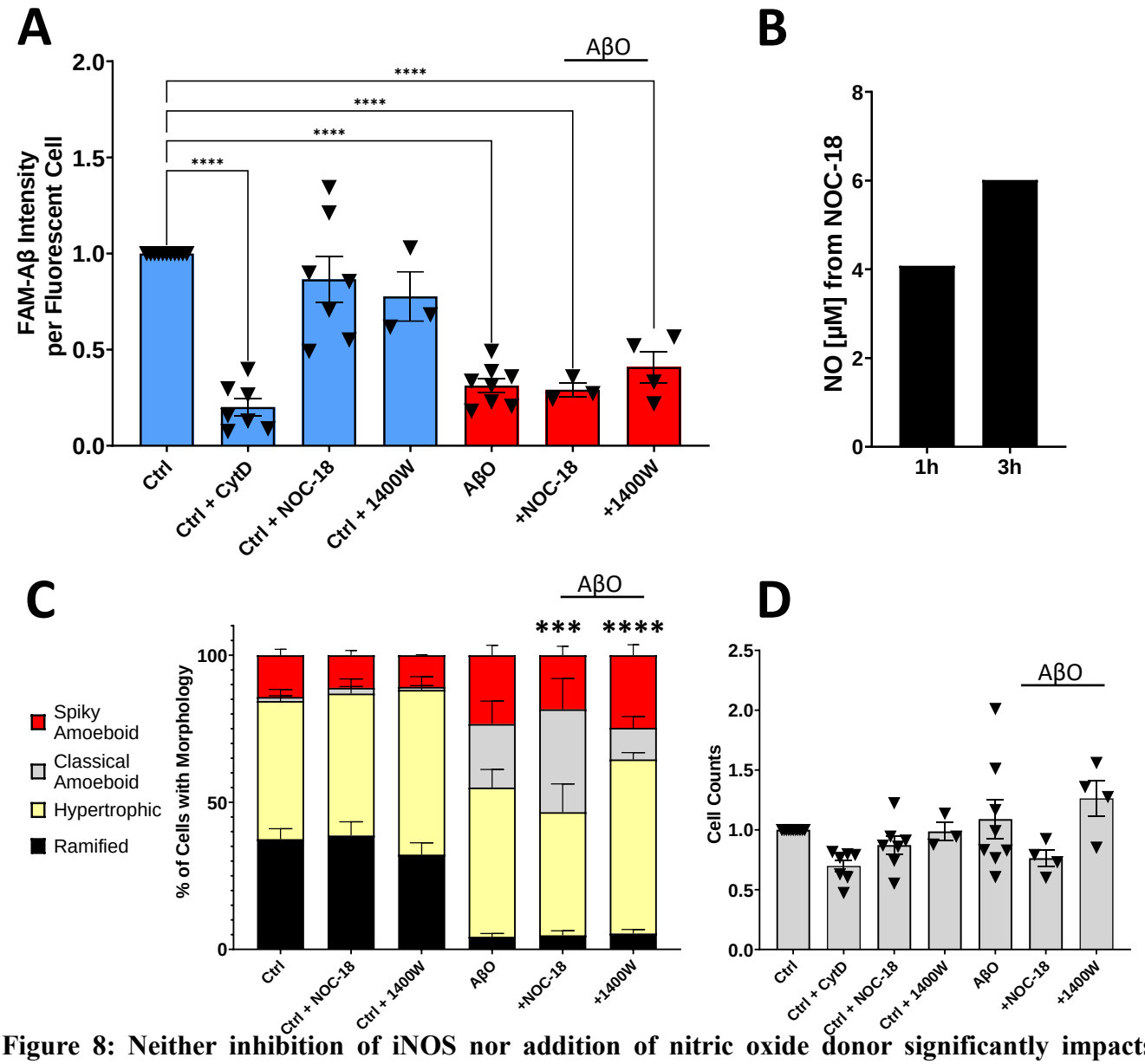

Figure 8: Neither inhibition of iNOS nor addition of nitric oxide donor significantly impacts microglial phagocytosis of FAM-Aβ.

(A) To investigate the necessity of iNOS/NO in phagocytosis of FAM-Aβ, microglia in control conditions (Ctrl, received vehicle control media), or AβO-treated (1100 pg/ml for Aβ 1-X, 20 hrs), were assessed utilizing NO donor (NOC-18, 25 μM addition for 1 hr) or iNOS inhibitor (1400W, 10 μM, 20 hrs). To detect maximal achievable inhibition of phagocytosis, actin polymerization was prevented with Cytochalasin D (CytD, 10 μM). N=3-11, one-way ANOVA, ****p<0.0001 compared to control. (B) NO concentration was measured in cell-free media following addition of NOC-18 (25 μM), following incubation from 1-3 hours at 37 C (N=1). (C) Analysis of microglial morphological transformation showed AβO treatment transforms cell morphology, and NO donor/iNOS inhibition does not prevent this (N=3-11, two-way ANOVA, ***p<0.001, ****p<0.0001, compared to control). (D) Cell counts showed that treatments did not significantly affect cell proliferation and viability (N=3-11, one-way ANOVA, n.s.).

3.6 Inhibition of Toll-Like Receptor 4 (TLR4) does not affect microglial phagocytosis of FAM-Aβ

My results thus far show that neither PARP-1, TRPM2, or iNOS are necessary for phagocytosis of FAM-Aβ, nor are they responsible for amyloidopathy induced inhibition of FAM-Aβ uptake. Therefore, my next question was to investigate alternate signalling pathways that may be regulating phagocytosis instead. One receptor that recognizes Aβ and also enhances signalling within microglia that leads to Aβ uptake is Toll-Like Receptor 4 (TLR4) (Reed-Geaghan et al. 2009). TLR4 is also an interesting target to study as its major ligand is LPS, commonly expressed on cell walls of Gram-negative bacteria. The cellular response of LPS binding TLR4 is relevant in diseases like sepsis (bacterial infection), which is relevant to the CNS in brain infections like pneumonia.

I carried out experiments where TLR4 was inhibited by a drug called TAK242. TAK242 is a small-molecular inhibitor for TLR4 but not other members of the TLR channel family, and can cross the blood-brain barrier (Hua et al. 2015). The mechanism of action is believed to be that TAK242 blocks intracellular signalling of TLR4, but does not directly block the ligand binding to the TLR4 receptor. However, it is not clear at which step this intracellular signalling is blocked (i.e if the first step, which is dimerization of the TLR receptor, is prevented, or so on) (Kawamoto et al. 2008). Of additional interest, LPS is a TLR4 specific ligand.

AβO-treated cells had strongly diminished FAM-Aβ cargo uptake as reported in previous sections, and the inhibition of TLR4 in the presence of AβO-treatment did not alter this. In control conditions (absence of AβO treatment), TLR4 inhibition was also not a determinant of phagocytic uptake (Fig 9A). In a separate set of experiments, I also tested the effect of treating cells with LPS (0.5 ng/ml) for 20 hours prior to the addition of FAM-Aβ phagocytic cargo. Interestingly, LPS-treated cells showed a significant drop in phagocytosis (Fig 9A, Ctrl mean = 1, LPS mean = 0.502, $p=0.033$), showing the same trend as AβO-treated cells. This suggests that AβO and LPS, both TLR4 agonists, can induce a similar effect on phagocytosis in microglia.

This is especially interesting considering the next set of experiments, which showed that AβO and LPS do not induce the same outcome regarding NO production. As seen in Fig 9B, cells treated with LPS (0.5 ng/ml) when TLR4 was inhibited (TAK242, 1 μM) showed a significant decrease in NO release compared to cells treated with the same concentration of LPS alone. However, cells treated with AβO (1100 pg/ml, Aβ 1-X) where TLR4 was inhibited (TAK242, 1 μM) did not show a significant reduction in NO release, in comparison to AβO treatment alone. All treatments were for 20 hours. As LPS is a specific agonist for TLR4, while AβO is not (AβO can bind to many other types of receptors such as Fc receptors, scavenger receptors, TREM2, and more), this suggests that in our experiments, TLR4 is not a major receptor regulating the AβO-induced production of NO. This also adds further interest to the finding in Fig 9A that AβO and LPS have similar effects on phagocytosis – further evidence suggesting that the microglial functions of phagocytosis and nitric oxide production may be under different regulation.

Morphological analysis following AβO treatment showed a significant increase in proportion of cells with spiky amoeboid morphology, both in the presence or absence of TLR4 inhibitor (Fig 9C). Cell viability was also well maintained across treatments, although, in this set of assays, cells treated with the actin cytoskeleton inhibitor CytD (negative control) did show a slight drop in cell number (Fig 9D).

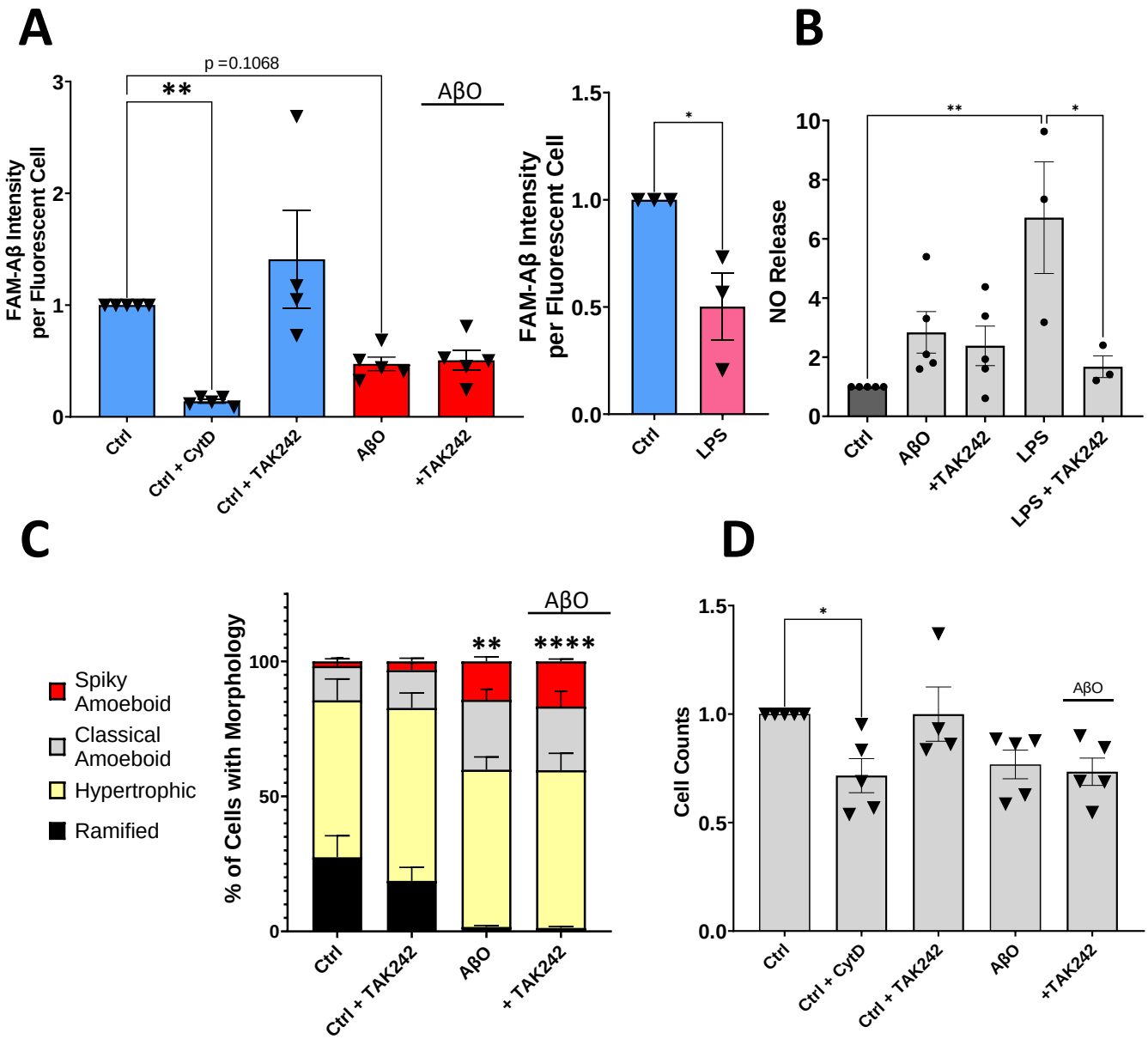

Figure 9: Inhibition of Toll-Like Receptor 4 (TLR4) does not affect microglial phagocytosis of FAM-Aβ.

(A) The role of Toll-Like Receptor 4 (TLR4) in microglial phagocytosis of FAM-Aβ (0.3 µM, 2 hrs) in control conditions (Ctrl, received vehicle control media, 20 hrs) or AβO-treated (1100 pg/ml for Aβ 1-X, 20 hrs) was assessed by blocking TLR4-mediated intracellular signalling with TAK242 (1 µM, 20 hrs). To detect maximal achievable inhibition of phagocytosis, actin polymerization was prevented with Cytochalasin D (CytD, 10 µM). N=4-5, one-way ANOVA, **p<0.001, compared to control. Phagocytosis of FAM-Aβ (0.3 µM, 2 hrs) following treatment with LPS (0.5 ng/ml, 20 hrs) was found to be significantly decreased. N=3, one-way ANOVA, *p<0.05. (B) Nitric oxide release from microglia treated with LPS (0.5 ng/ml, 20 hrs) was suppressed by TLR4 inhibitor (TAK242, 10 µM, 20 hrs),

while cells treated with AβOs (1100 pg/ml for Aβ 1-X, 20 hrs) in addition to TAK242 did not show a significant decrease in NO release. N=3-5, one-way ANOVA, *p<0.05, **p<0.001, compared to control. (C) Quantification of microglial morphological transformation following treatments showed that AβO treatment significantly alters cell morphology, while TAK242 does not induce or prevent significant morphological changes in either control or AβO-treatment conditions. N=4-5, two-way ANOVA, **p<0.001, ****p<0.0001, compared to control. (D) Cell counts showed that treatments did not significantly affect cell proliferation and viability, with the exception of CytD (N=4-5, one-way ANOVA, *p<0.05).

3.7 AβO treated microglia do not exhibit an observable change in basal intracellular calcium levels.

Previous work from my lab has shown that AβOs activates PARP-1/TRPM2 signalling via increasing extracellular glutamate and activating NMDA receptors (NMDARs). However, long-term maintenance of this Ca^{2+} influx through the TRPM2 channel has not been proven (Raghunatha). Therefore, I set out to use confocal microscopy to observe changes in intracellular calcium in microglia in response to AβOs, and to prove definitively that TRPM2 is maintaining the calcium influx that is crucial for downstream PARP-1/TRPM2 signalling.

This was investigated experimentally by treating primary microglia with AβOs for 20 hours. Experiments were also performed looking at shorter time points such as 4-5 hours, but 20 hours was chosen as we wanted cells to have sufficient time for PARP-1/TRPM2 signalling to be activated. Following AβO treatment, microglia were treated with Ca^{2+} indicator dye (CAL-520 AM, 2 μM) prior to live imaging on a Zeiss confocal microscope. CAL-520 AM was chosen in contrast to other common intracellular Ca^{2+} dyes (such as Fluo4), as it has been shown to have a high signal to noise ratio (Tada et al. 2014). All confocal microscope imaging parameters such as gain, laser power, and exposure time were identical when assessing both experimental groups. Intracellular calcium was measured by mean ROI fluorescence using ImageJ.

While the ultimate goal was to use a TRPM2 inhibitor such as clotrimazole or JNJ and observe a block in the influx of Ca^{2+} initiated by AβO treatment, the first step was to see if there was a difference in baseline calcium for cells treated with AβOs (4.41 ng/mL, 20 hours) vs. control cells (received control MEM, 20 hours). As seen in Fig 10, there was no difference in the average fluorescence detected at baseline (0-3 minutes imaging) between the two treatment groups.

I expected to observe AβO-treated cells with a detectably elevated level of intracellular calcium, but this was not the case. Reasons that no difference in Ca^{2+} was observed include the possibility that

control cells were too activated at baseline, perhaps due to processing (cells received multiple sets of washes with ECF prior to imaging, and microglia are extremely easy to perturb). If these experiments were to be tried again and a baseline difference established, I would then expect that in long-term imaging experiments, TRPM2 inhibition in cells treated with AβOs would result in decreased Ca^{2+} influx.

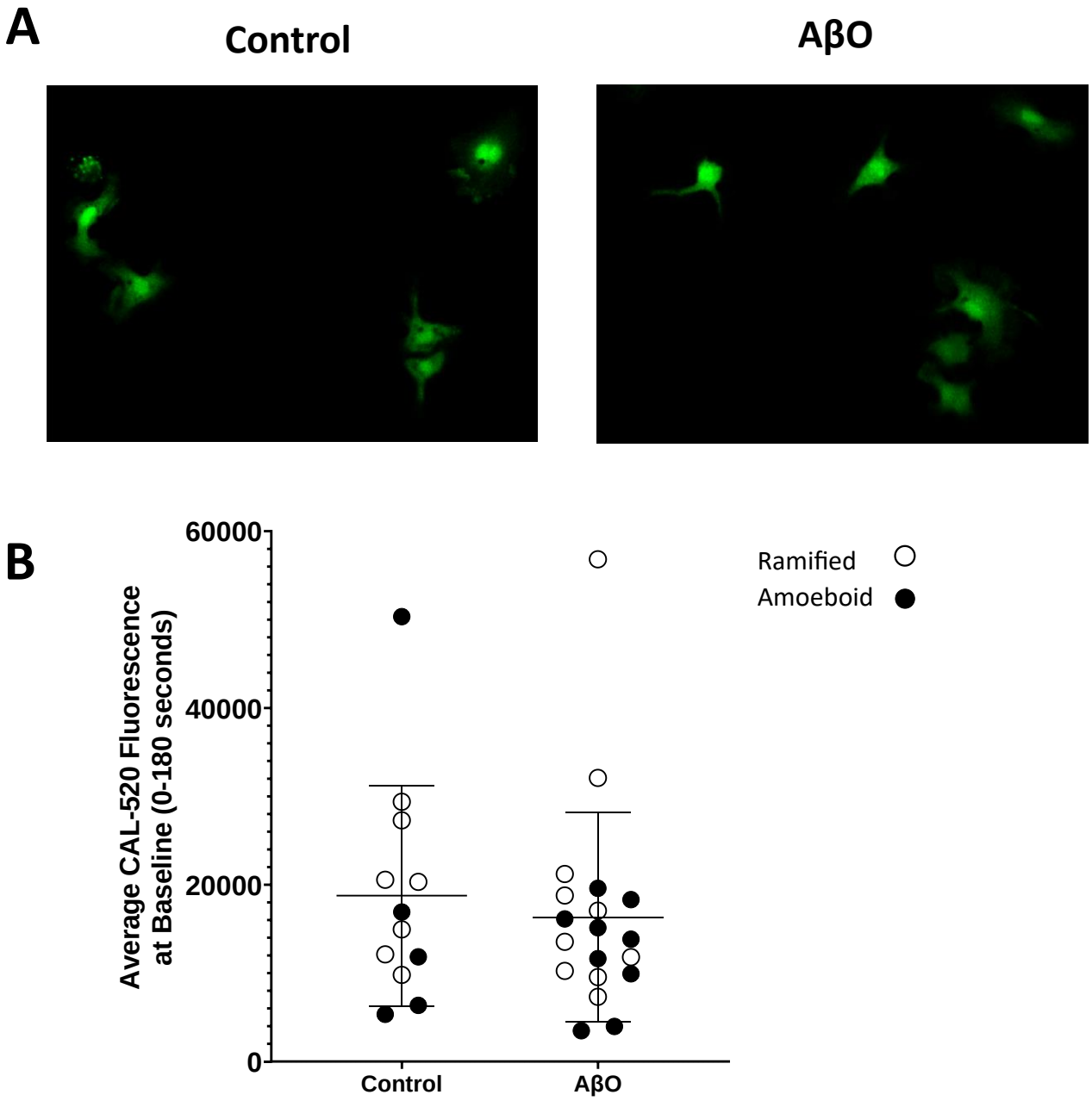

Figure 10: AβO treated microglia do not exhibit an observable change in basal intracellular calcium levels.

(A) Live-cell images of primary microglia cultures loaded with CAL-520 AM intracellular calcium indicator (2 µM). (B) Average calcium level, measured as average ROI per cell, shows no increase in calcium following AβO-treatment (4.4 ng/mL, 20 h**)**. Morphological analysis of cells (ramified, amoeboid) also does not impact calcium measurements. (n=31, unpaired t-test, p=0.5898, n.s.). Imaged at 40X (with immersion oil) using Zeiss Confocal.

3.8 AβO treatment of PARP-1 cKO microglia shows potential sex differences regarding phagocytic uptake of FAM-Aβ.

The relevance of sex differences is beginning to be appreciated across all scientific fields, including animal models of disease. There is evidence supporting the existence of sex differences in AD pathology in humans: women are more likely to be diagnosed with AD, and exhibit quicker cognitive decline and brain atrophy than men (Ferretti et al. 2018).

There have also been reports highlighting sex differences in microglial phagocytosis in animal models, such as those reporting that females have a greater number of phagocytic microglia (Nelson, Warden, and Lenz 2017), and microglia with higher phagocytic activity compared to males (Yanguas-Casás et al. 2020). Additionally, it has been reported that PARP-1 inhibition or deletion may be protective in males but not in females (Liu et al. 2011; Siegel and McCullough 2013). Therefore, investigating potential sex differences in microglial phagocytic capacity was a supplemental area of interest in my study. For all other experiments in my study, microglia were prepared from male pups, up to P2. For the results in this section (3.8), primary microglial cultures were prepared from both male and female CD1 pups (up to P2).

While pharmacological inhibitors used in my study have many advantages, one disadvantage is that they can have multiple targets – for example, PJ34 can block activity of both PARP-1 and PARP-2 enzymes. Thus, there may be some question as to whether results observed with PJ34 are due to PARP-1 activity alone being blocked. This is where knockout (KO) animal models are useful. For these experiments, primary microglial cultures were prepared from PARP-1 conditional knockout mice (cKO, Cre-mediated deletion of PARP-1 in CD11b-cre expressing cells, targeting microglia).

Thus, pups in this experiment were helpful in assessing 2 questions at the same time: do sex differences play a role in microglial phagocytosis of Aβ, and do microglia from PARP-1 cKO mice show the same effect as wild-type microglia when PARP-1 was pharmacologically inhibited?

These phagocytic assays were carried out in an identical manner to the assays in section 3.3 – i.e. cells were treated with either AβOs (final concentration Aβ 1-X, 1100 pg/ml) or Ctrl (vehicle control media) for 20 hours, prior to addition of FAM-Aβ cargo (0.3 μM) for 2 hours. Phagocytic uptake measured via fluorescence intensity per phagocytized cell was assessed. There were two different groups of cells prepared from male and female pups – wild-type (WT) or PARP-1 cKO.

The results show similar findings to the results in section 3.3 from males only, as far as showing that AβO-treated cells were not able to take up as much cargo as control-treatment cells. In Fig 11A, microglia from wild-type males had a severe decrease in phagocytic uptake when treated with AβOs (WT Ctrl mean = 1, WT AβO-treated mean = 0.406, 59.4% decrease), and cells from PARP-1 cKO males showed a similar trend (PARP-1 cKO Ctrl mean = 1.35, PARP-1 cKO AβO-treated mean = 0.380, 71.9% decrease). It should be noted that all raw data values were normalized to the values for WT Ctrl for each sex. Additionally, n values of 2 make it not possible to conduct accurate statistical analysis. These findings that PARP-1 cKO does not change phagocytic uptake in cells treated with AβO matches the results seen for treated cells where PARP-1 was inhibited with PJ34 (see section 3.3, Fig 6B).

In Fig 11A, preliminary data from microglia from females shows a slightly different outcome. AβO-treated cells from wild-type females showed the same strong decrease in phagocytic uptake as seen in microglial cultures from male mice (numerical means from females are WT Ctrl mean = 1, WT AβO-treated mean = 0.463, a decrease of 53.7%). However, AβO-treated microglia from PARP-1 cKO females did not show as drastic of a drop in phagocytic uptake, compared to Ctrl microglia (PARP-1 cKO Ctrl mean = 0.918, PARP-1 cKO AβO-treated mean = 0.719, a decrease of 21.7%).

One hypothesis based on this finding could be that in females, AβOs do not activate PARP-1 pro-inflammatory pathway as severely, or this activation somehow does not decrease phagocytic uptake in the same way that it does in males. In other words, AβO-induced inhibition of FAM-Aβ uptake may be dependent on PARP-1 in females, but not in males. However, the data from cKO females is from two

pups total (n=1 for Ctrl, AβO-treated each), and therefore these experiments would need to be repeated to assess if this finding holds up statistically.

Fig 11B shows the morphological changes seen in WT and cKO cells in both sexes and treatment conditions. Overall, these changes mirror the standard morphological changes seen for wild-type male cells treated with AβOs in previous sections (3.3 and on). In both WT and cKO cells, AβO-treatment results in an increased proportion of cells with spiky amoeboid morphology, and decreased proportion of cells with ramified morphology. There is no obvious difference between WT and cKO or males and females regarding morphological transformations (however, again do not have enough replicates to do statistical analysis).

Fig 11C shows final cell count data for all treatment conditions, normalized to WT control cell counts for males or females in each relevant dataset. This data shows a slight increase in cell proliferation for AβO-treated cells, which is expected and has been observed in WT males previously. PARP-1 cKO cell counts for males show a slight drop compared to WT control males (WT Ctrl mean = 1.0, PARP-1 cKO Ctrl mean = 0.733, 26.7% decrease).

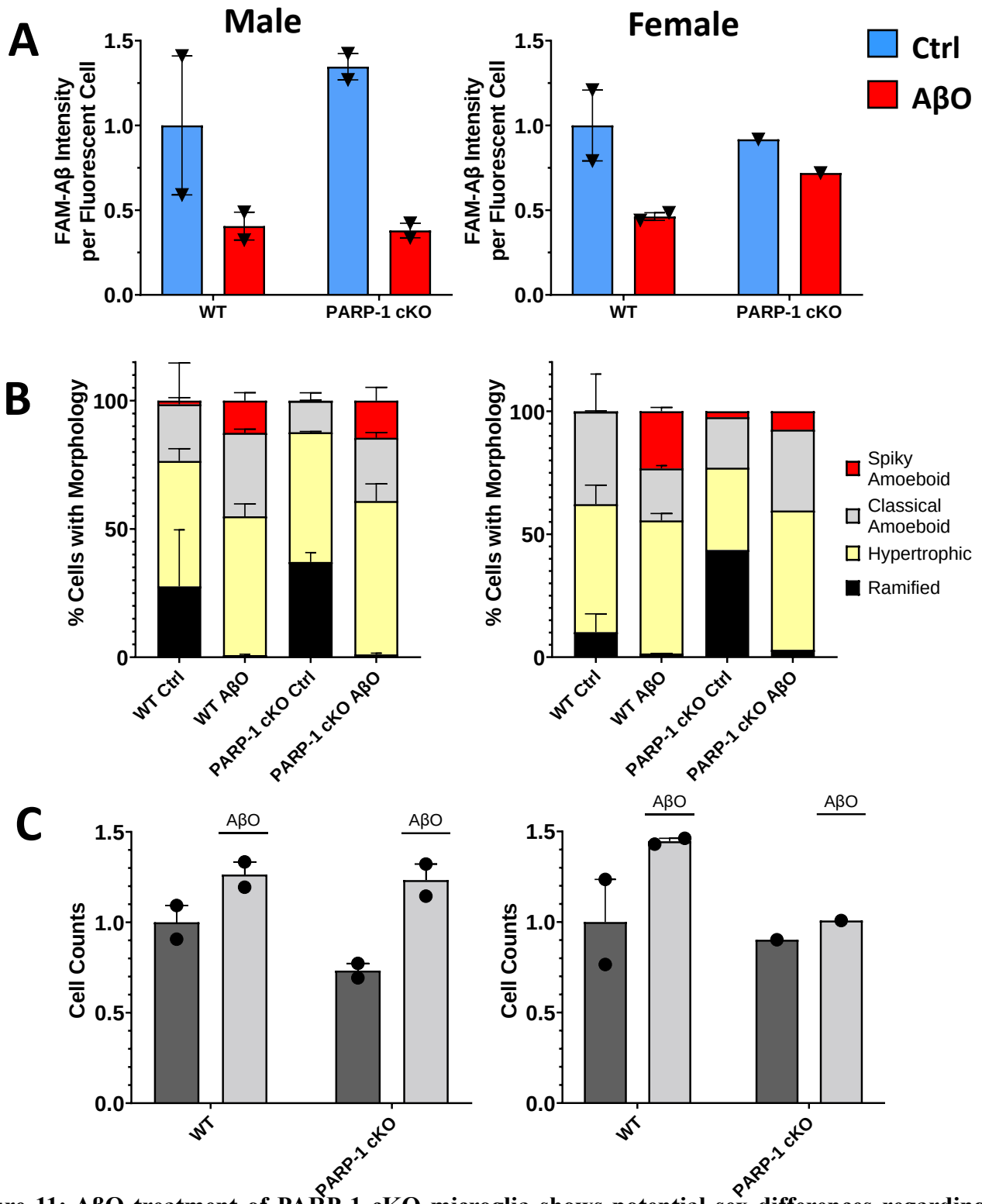

Figure 11: AβO treatment of PARP-1 cKO microglia shows potential sex differences regarding phagocytic uptake of FAM-Aβ.

(A) Males: Phagocytic uptake of FAM-Aβ (0.3 μM) in wild-type males was decreased following AβO treatment (1100 pg/ml for Aβ 1-X, 20 hrs; WT Ctrl mean = 1, WT AβO mean = 0.406). N=2. Phagocytic uptake of FAM-Aβ in PARP-1 cKO males was also decreased following same AβO treatment (PARP-1 cKO Ctrl mean = 1.35, PARP-1 cKO AβO mean = 0.380). N=2. Females: Phagocytic uptake of FAM-

Aβ in wild-type females was decreased in AβO-treated cells (1100 pg/ml for Aβ 1-X, 20 hrs; WT Ctrl mean = 1, WT AβO-treated mean = 0.463). N=2. Phagocytic uptake in microglia from PARP-1 cKO females receiving AβO treatment was not strongly decreased compared to female controls (PARP-1 cKO Ctrl mean = 0.918, PARP-1 cKO AβO-treated mean = 0.719). N=1. (B) Matched analysis of microglial morphological transformation following AβO treatment in WT and PARP-1 cKO cells prepared from males and females. N=2 per treatment group (bar) for males, N=1-2 per treatment group for females. (C) Matched cell counts normalized to relevant WT controls show good cell survival among PARP-1 cKO cells, for both males and females. N=1-2 per treatment group.

CHAPTER 4. DISCUSSION

4.1 Summary of Findings

The major goal of my thesis was to investigate how the PARP-1/TRPM2 pro-inflammatory signalling pathway may be involved in the regulation of microglial phagocytosis, which is extremely relevant to AD pathogenesis. While it has been proven that PARP-1/TRPM2 signalling acting in concert is involved in modulating microglial inflammatory responses in the context of AD (Raghunatha et al. 2020), the specific role of this signalling in relation to phagocytosis in conditions representative of amyloidopathy had not been shown.

Additionally, I investigated the role of nitric oxide signalling on phagocytosis. The release of pro-inflammatory NO is mediated by iNOS which falls under the control of PARP-1 and TRPM2. There is inconclusive evidence regarding whether iNOS/NO is required for microglial phagocytosis, keeping in mind that this requirement may vary depending on the type of phagocytic cargo and therefore the type of phagocytic receptor involved. This is important to understand because nitric oxide production is a crucial component of the microglial pro-inflammatory response, in addition to other functions like phagocytosis. Understanding whether NO under the control of iNOS regulates phagocytosis would be crucial information when designing a therapeutic that may target a portion of the PARP-1/TRPM2 signalling pathway.

I first worked to optimize the conditions that would be used for phagocytic assays going forward. I completed preliminary phagocytic assays using primary microglia incubated with fluorescently-labelled amyloid beta peptide (FAM-Aβ 1-42, herein referred to as 'FAM-Aβ cargo'), to find an appropriate concentration and incubation time. I tried multiple methods to detect this fluorescence, such as seeding cells in microplates and using a fluorescent plate reader to detect total fluorescent output. However, this was unsuccessful as cell density was too low and signal was not easily detectable above background controls. As microglia are immune cells, they are extremely sensitive to being in contact with each other,

and therefore increasing the cell density in wells was not feasible. Therefore, the final method that I ended up using was a microscope able to take images of cells at settings for both phase contrast and green fluorescence (FITC filter, wavelength 488 nm). These two images were then paired for analysis. While taking a high number of images per treatment well was more time-consuming than a plate reader assay would be, this method worked well. As the FAM-Aβ fluorescent cargo was good quality, it was easily detectable by microscopy, and a wide range of fluorescent signal intensities was observable. One obvious drawback to using static images for data is that images show data for one point in time, whereas phagocytosis is a dynamic and motile cellular function. One future direction that could be interesting would be to carry out live imaging of microglia while they are incubated with cargo, and observe the entire process of phagocytosis.

Next, I assessed the effect of inhibiting components of the PARP-1/TRPM2 pro-inflammatory pathway on phagocytic uptake of FAM-Aβ in conditions mimicking health. Previous work had shown that when microglia were incubated with FAM-Aβ cargo for 24 hours, PARP-1 gene deletion or inhibition led to no change in uptake (Kauppinen et al. 2011).

In my experiments, cells were incubated with cargo for 8 hours, during which each pathway component of interest (PARP-1, TRPM2, iNOS, and NUDT5) was pharmacologically inhibited in separate treatment groups. I found that inhibition of PARP-1 at the 8-hour timepoint also did not affect phagocytosis, and neither was there any effect from inhibition of other pathway components in these experiments (Fig 5B). It should be noted that the data in this experiment has wide error bars (standard error of the mean, SEM), representing a large amount of variation between replicates. Nonetheless, we interpreted the results at 8 hours (Fig 5) as showing that the PARP-1/TRPM2 pathway is not a critical modulator of phagocytosis, in the absence of amyloidopathy.

Next, I assessed the effect that inhibiting the PARP-1/TRPM2 pathway has on phagocytosis in conditions more representative of AD pathology. While the pathway didn't seem to have a role in general

phagocytosis of FAM-Aβ in health, we wanted to confirm whether it had a role in an AD disease model. Other studies have reported that microglia exposed to pro-inflammatory stimuli have decreased phagocytic uptake of Aβ (Koenigsknecht-Talboo and Landreth 2005; Yamamoto et al. 2008; Michelucci et al. 2009).

I found that AβO-treated microglia consistently showed a significant decrease in phagocytic uptake compared to controls (Figs 6, 8). However, inhibiting PARP-1, TRPM2, iNOS, or NUDT5, all separately, in cells that received this AβO treatment, did not cause a significant change in phagocytic uptake. If inhibition of one of these pathway components in an AD state had brought phagocytosis levels back up to the level seen in control, this would have been an interesting outcome. This is because those results would have shown a beneficial effect in AD conditions, in combination with results from section 3.2 showing no detrimental effect in healthy conditions. This would mean that a potential therapeutic acting on our pathway component would theoretically boost phagocytosis only in a disease state/amyloidopathy conditions. In other words, the therapeutic should not have detrimental effects on cells in a healthy state.

Additionally, while cells that had iNOS inhibited had no benefit in phagocytic uptake, cells that received an increased level of NO also showed no significant change (section 3.5). This supplements other work in the microglial fields that suggests that NO may be a requirement for microglial phagocytic uptake. However, it should be noted that these other works used different cargo types (most often latex beads) and examined different phagocytic receptors than I did in my work. This is discussed in detail in section 4.4 below.

Separately, I showed that the inhibitors used in phagocytic assays were able to inhibit NO production in AβO-treated cells (Fig 9), with the exception of the inhibitors TAK242 (target: TLR4) and JNJ (target: TRPM2). The fact that the majority of inhibitors inhibited NO release indicates that these

drugs were working as expected, and therefore provides more validity to our phagocytic assay findings, where inhibitors did not cause any changes.

Finally, as blocking the PARP-1/TRPM2 pathway components (including NUDT5, iNOS) had no obvious effect on phagocytic uptake, I decided to assess whether blocking a receptor that recognizes Aβ directly may have an effect. TLR4 has been linked to both pro-inflammatory outcomes in AD (Balducci et al. 2017) and phagocytosis (Zhang et al. 2023). Blocking TLR4 with a specific pharmacological inhibitor did not show a significant change in phagocytic uptake in conditions of health or AD in my assays (section 3.6).

Ultimately, when assessing all of the data from my phagocytic assays in combination, the prevalent finding is that the PARP-1/TRPM2 pro-inflammatory signalling pathway does not play a critical role in regulating microglial phagocytosis, at least for the uptake of Aβ cargo. This finding holds true whether cells are in healthy conditions or in conditions mimicking amyloid pathology as seen in Alzheimer's (AβO-treatment). This suggests that another signalling pathway, or combination of pathways, has a key role in regulating phagocytosis in microglia. However, this is also positive as it means that any potential therapeutics targeting a component of this pathway (such as TRPM2 or NUDT5), would not have any detrimental effects on the beneficial phagocytosis of Aβ by microglia. Additionally, my findings are also consistent with previous work by the Jackson and Kauppinen labs that showed that PARP-1 or TRPM2 KO did not decrease the accumulation of AβOs or amyloid plaques (Kauppinen et al. 2011; Ostapchenko et al. 2015). Thus, suggesting that cognitive benefits seen in KO animals in these studies occurs downstream of AβOs.

4.2 PARP-1 and TRPM2 pathway as therapeutic targets for AD

As described in 1.3, there is a variety of evidence supporting the role of PARP-1 in AD. PARP-1 is responsible for a variety of pro-inflammatory outcomes in AD mouse models, such as: release of pro-inflammatory mediators (NO and cytokines), transformation of microglia to an activated morphology,

death of co-cultured neurons, and memory loss (Kauppinen et al. 2011). This work also crucially showed that when PARP-1 is inhibited or knocked out in microglia, there are still beneficial outcomes maintained: phagocytosis in cells under control conditions and release of anti-inflammatory cytokines. PARP-1 also exhibits pro-inflammatory functions in regards to blood-brain barrier permeability and function, as it promotes damaging events such as astrogliosis and increased vessel permeability (Rom et al. 2015; Mehrabadi et al. 2017).

Thus, PARP-1 clearly has an important role in promoting pro-inflammatory outcomes in the CNS. As neuroinflammation is now recognized as a risk factor for AD, it seems logical to try targeting PARP-1 as a potential therapeutic for neurodegenerative diseases such as AD. However, as stated previously, there are issues with targeting PARP-1 directly. Briefly, these issues include pharmacological inhibitors for PARP-1 that either can induce PARP-DNA trapping, are non-specific to PARP-1, and/or cause severe adverse events in patients (Sinha, Katyal, and Kauppinen 2021; Thorsell et al. 2017; Bitler et al. 2017). This is why my project also included the study of other cellular signalling components that work alongside PARP-1, but may be more readily targeted by a drug, such as TRPM2.

As TRPM2 Ca^{2+} channel can be opened by ADPR monomers produced by PARP-1, TRPM2 exists in a positive feedback loop with PARP-1. However, TRPM2 activation alone has also been shown to increase production of pro-inflammatory cytokines such as IL-6 and TNF-α (Wehrhahn et al. 2010). The action of TRPM2 in neuroinflammation is strongly understood (Fonfria et al. 2005; Alawieyah Syed Mortadza et al. 2018; Malko et al. 2019). In relation to AD specifically, it has been shown that when TRPM2 was knocked out in APP/PS1 mice, numerous amyloid-related sequelae were improved – such as spatial memory improvements, decreased loss of synapses, and reduced microglial activation (Ostapchenko et al. 2015). This suggests that in the APP/PS1 mouse model, TRPM2 is a driver of pro-inflammatory signalling underlying negative outcomes for synaptic loss and memory impairments.

For the majority of time that TRPM2 has been studied, there has been a lack of specific inhibitors for experimental use. One of the most common inhibitors used is clotrimazole, which is non-specific to TRPM2 and also used as an anti-fungal treatment (Belrose and Jackson 2018). In 2019, a new more specific inhibitor for the TRP family of channels was found from a wide screening study, called JNJ-28583113 (Fourgeaud et al. 2019). While this inhibitor is relatively new and more testing needs to be done, its improved specificity is promising. The major downside of this JNJ inhibitor for clinical use is that it has low stability once in the brain, and is quickly metabolized by esterases, after which the compound no longer exhibits its inhibitory properties (Fourgeaud et al. 2019).

Given the importance of the role of PARP-1 and TRPM2 in pro-inflammatory signalling of microglia, there is a lot of potential for exploring this signalling as a neuroinflammation hypothesis-focused therapeutic target for AD. My research is focused on understanding the role of this signalling on regulating phagocytic clearance of phagocytic cargo. To our knowledge, there have been no reports looking at whether microglial phagocytosis may be under the control of TRPM2 in some manner. Thus, while we may hypothesize that inhibition of TRPM2 alone would have a similar result to inhibition of PARP-1 (with phagocytosis being maintained), we set out to prove this experimentally in this study.

4.3 Phagocytosis

As stated, phagocytosis is a crucial microglial function and has received a lot of interest for how it may be improved or made more precise in a disease context such as Alzheimer's, especially as disruptions to microglial ability to clear or degrade Aβ are a huge contributor to disease severity. A number of gene variants significantly associated with AD are in genes involved in phagocytosis (Brown and Neher 2014).

It has been shown that treatment with AβOs or pro-inflammatory signals such as IFN-ϒ or TNF-α, cause decreased phagocytic uptake of cargo (Yamamoto et al. 2008; Michelucci et al. 2009). These 2 cytokines have also been shown to directly decrease enzymatic levels of IDE (insulin-degrading enzyme,

which degrades Aβ) (Yamamoto et al. 2008). Additionally, it has been shown that inhibition or deletion of PARP-1 does not negatively impact phagocytosis of Aβ (Kauppinen et al. 2011).

Therefore, my experiments combined these two concepts and showed that both prior findings held up: when I treated microglia with AβO for 20 hours overnight, phagocytic uptake was severely diminished. But, when PARP-1 was inhibited, there was no change to phagocytic capacity. Inhibition of TRPM2, NUDT5, iNOS, or TLR4, all separately, did not impact phagocytic uptake in amyloidopathy conditions either. This suggests that none of these components are regulating or necessary for phagocytosis of Aβ. Alternatively, it may be possible that the concentration of AβOs used in experiments was high enough that it broadly blocked phagocytosis.

The mechanism for how AβOs may lead to decreased phagocytosis has not yet been proven. One hypothesis is that AβOs (or other pro-inflammatory stimuli, like cytokines), initiates signalling that downregulates phagocytic receptors (Hickman, Allison, and El Khoury 2008; Sanchez-Mico et al. 2021). Therefore, these cells aren't able to recognize as many targets, and would not be able to engulf as much cargo as control cells. It makes sense theoretically that cells that have been stimulated with some type of pro-inflammatory stimuli may shift their cellular energy priorities to focus on other functions (or even just survival), rather than taking in more cargo. This phenomenon was observed in astrocytes: astrocytic phagocytosis of synaptoneurosome cargo was severely decreased in amyloid mice models, which also had decreased expression of two types of phagocytic receptors on astrocytes (Sanchez-Mico et al. 2021).

Another hypothesis is that AβO treatment could impair microglial lysosomes' ability to degrade engulfed cargo, leading to build up of cargo. This again matches some literature evidence, such as Yamamoto et al. showing that IFN-ϒ or TNF-α treated cells inhibited insulin-degrading enzyme levels, which would result in increased Aβ (Yamamoto et al. 2008). A similar effect was seen in an amyloid mouse model, where it was shown that microglia were engulfing Aβ, but not catabolizing it. When authors stained for lysosomes, they found Aβ cargo was still present (Parhizkar et al. 2023).

It should be noted that I was able to measure phagocytosis using 2 different methods: one measurement method was percentage of total cells taking in cargo, and the other was the average fluorescence intensity of the fluorescent cells that did take in cargo.

The percentage measurement was useful in optimizing the time and concentration to be used for FAM- Aβ cargo, which I selected based on conditions in which 60% of cells took up cargo. However, in other experiments, this percentage uptake outcome did not completely capture the results as observed through images, as the percentage uptake measurement does not account for differences in the relative *amount* of cargo being taken up by a cell. Therefore, I also looked at another method of measurement, the average fluorescence intensity per fluorescent cell. This more accurately reflected the data seen in images, and was used to present the data for the remaining phagocytic assays in this work.

Both of these types of phagocytic measurements answer slightly different questions. For example, if a drug treatment significantly decreases the amount of cargo being taken up per cell (fluorescence intensity), but not the percentage of cells taking up any cargo (percentage uptake), this could suggest that the drug is acting downstream of the cellular mechanisms controlling the cargo uptake portion of phagocytosis.

It should also be noted that there are some important differences between our methods and the methods used in other studies. This includes general methods used (primary microglial cultures vs. microglial cell lines or tissues, *in vitro* work vs. *in vivo* work, age of animals microglia were isolated from (pups vs adults)), and phagocytic assessment methods (common methods in literature include flow cytometry, staining for lysosomal marker CD68, staining for cargo). Additionally, a major methodological difference that may not always be considered is how AD is represented in a model. For example, as we are interested in amyloid hypothesis, my experiments repeated in a cell culture model of tauopathy may show different results.

Additionally, amyloid mouse models such as APP/PS1 or 5xFAD are considered more representative of AD in a mammal than cell culture work. Lastly, I did not test or compare different concentrations of AβOs – therefore it cannot be ruled out that a lower concentration of AβOs may not have drastically blunted phagocytosis as much as I observed (and therefore, maybe inhibitors may have been able to help bring phagocytic uptake back up a bit more).

Any future therapy targeting microglial phagocytosis, specifically one promoting an increase of phagocytosis in the CNS, would require precise control. There is currently no drug available for patients that increases microglial Aβ clearance.

4.4 iNOS/NO is not required for phagocytosis of Aβ

As described, microglial NO, produced by inducible nitric oxide synthase (iNOS) is a strong pro-inflammatory signal, and major contributor to both neuroinflammation and AD pathology (Nathan et al. 2005). Microglial nitric oxide can contribute to neuroinflammation and neurotoxicity by damaging neurons and astrocytes. Specifically, NO signalling inhibits mitochondrial cytochrome oxidase in neurons, which ultimately leads to: depolarization of neurons, elevated release of glutamate, impaired synaptic transmission, and eventual excitotoxicity and neuronal death (Brown 2007; Yuste et al. 2015).

Previous work found that inhibition of Aβ-induced NO response could be prevented by inhibiting PARP-1 (Kauppinen et al. 2011), and NMDA-induced NO response could be inhibited by pharmacological inhibition or genetic deletion of PARP-1 or TRPM2 separately (Raghunatha et al. 2020). In my work, I showed that AβO-stimulated NO production was significantly diminished by pharmacological inhibition of PARP-1, TRPM2 (using CTZ), iNOS, or NUDT5 (section 3.4).

Current literature is unclear regarding whether iNOS/NO is a regulator of microglial phagocytosis. It has been reported by a few independent *in vitro* studies that NO increases microglial phagocytosis for multiple cargo types, including latex beads with or without IgG, and apoptotic neurons

(Kakita et al. 2013; Scheiblich and Bicker 2016; Maksoud et al. 2019). For example, Scheiblich et al. showed that microglia treated with an NO donor (NOC-18) had significantly increased phagocytosis of apoptotic neurons, both in conditions mimicking neuroinflammation (microglia pre-treated with LPS) and in controls (Scheiblich and Bicker 2016). In an *in vivo* study, rats treated with LPS to induce an inflammatory state showed that iNOS inhibition decreased phagocytic activity of peritoneal macrophages (Tümer et al. 2007). While this work was in macrophages outside of the CNS, it is still interesting to note. Meanwhile, other authors report that NO negatively regulates phagocytosis – i.e. that NO is inversely correlated to phagocytosis, suggesting NO is not required (Kopec and Carroll 2000).

One hypothesis for how nitric oxide could regulate phagocytosis would be that nitric oxide signals to microglia to either increase or decrease phagocytosis in a positive/negative feedback loop. I would hypothesize that in an acute inflammatory setting (such as one representative of early AD disease state), nitric oxide would be more likely to increase phagocytic uptake. This is because at this point, there is less chronic neuroinflammation relative to later in disease state, and microglia have control of phagocytic targets and are focused on alleviating the cause of acute inflammation. However, in a chronic inflammatory setting (or late in AD disease stage), I would hypothesize that nitric oxide would not have an impact on increasing phagocytosis. This matches what has been seen *in vitro* for other pro-inflammatory signals: LPS, TNF-α, and IFN-ϒ have all separately been shown to decrease phagocytic uptake and/or intracellular enzymatic breakdown of Aβ (Koenigsknecht-Talboo and Landreth 2005; Yamamoto et al. 2008; Michelucci et al. 2009). This may just be representative of a 'loss of control' of Aβ in chronically inflamed environments.

Therefore, I was interested in assessing whether microglia that were treated with AβOs and had either NO production severely diminished (via inhibition of iNOS), or extracellular NO concentration increased (by NO donor), would show any regulation/changes in phagocytic capacity. I did not find that this was the case. There were no significant changes in phagocytic uptake of Aβ cargo whether cells were

present in an environment with high NO levels or low NO levels. Whether the environment was representative of homeostasis (control conditions) or pro-inflammatory (AβO treatment for 20 hours prior to cargo), also had no effect on phagocytic outcomes (section 3.5). This supports the hypothesis that iNOS/NO is not regulating microglial phagocytosis, at least for the cargo of FAM-Aβ.

There are a few methodological details to keep in mind when assessing different phagocytic studies, in general and related to NO. The studies examining phagocytosis related to NO that are referenced here examined phagocytosis of different types of cargo (apoptotic neurons, synaptosomes, pathogens, or less biologically relevant model cargo such as latex beads). As well, there is not a standard practice for how phagocytic assays are analyzed across the field. For example, some groups will count the percentage of cells that took up any cargo, while some groups will assess exactly how much cargo each cell took up (for example, if using bead cargo, how many beads were engulfed per cell). This can make cross-experiment comparisons difficult.

4.5 TLR4 inhibitor TAK242 blocks LPS-induced NO release, but not AβO-induced NO release; TLR4 inhibitor does not prevent TLR4-mediated decreased phagocytic uptake of FAM-Aβ

The two relevant agonists for TLR4 that will be discussed are Aβ and LPS. It has been shown that both of these agonists can individually initiate pro-inflammatory signalling that acts through NF-κB. TLR4 initiates pro-inflammatory signalling by recruiting MyD88, which (without going into extraneous detail) activates downstream signalling to activate NF-κB, allowing NF-κB to be moved into the nucleus and activated where it acts as a transcription factor for inflammatory response genes (L. Wu et al. 2022).

TLR4 activated by LPS in a rat model showed increased microglia and astrocyte density in tissue, in addition to showing that LPS-TLR4 activation caused increased levels of activated, nuclear NF-κB (Zhou et al. 2019). This is evidence that LPS activates NF-κB signalling through TLR4.

TLR4 activated by Aβ(1-42 peptide) was also shown to have an impact on pro-inflammatory outcomes, specifically: increased cytokine levels (TNF-α, IL-1B), and decreased levels of antioxidant

enzymes such as glutathione peroxidase and catalase. But, when microglial Aβ-TLR4 signalling was inhibited by TAK242, cytokines and antioxidant enzyme levels were closer to those observed in non-stimulated control cells (Chen et al. 2021). Aβ-TLR4 signalling was also shown to increase expression of MyD88 and NF-κB. Therefore, this shows that TLR4 has a role in pro-inflammatory signalling initiated by Aβ. My findings in section 3.6 do not match this, as Aβ stimulated cells treated with TAK242 did not show a significant decrease in pro-inflammatory NO production. However, cells treated with AβO in these experiments also did not show a significant increase in NO-production compared to controls – therefore it is possible that a more subtle decrease in NO-production could have been missed.

Multiple studies have reported that treatment of microglia with Aβ or LPS increases TLR4 expression (Zhou et al. 2019; Chen et al. 2021; Zhang et al. 2023). The major hypothesis for why this would occur is that in an inflammatory state, it would be beneficial for cells to be able to respond more effectively to pathogens, and a higher number of receptors allows this to happen. As TLR4 has a role in phagocytosis, it seems logical that increased TLR4 expression could correspond with increased phagocytic uptake.

This has been shown in some work looking at a different type of phagocytic cargo – *E.coli*. Two independent studies found that when cultured microglia were treated with LPS, phagocytic uptake of *E.coli* was increased (Ribes et al. 2009; Sivagnanam et al. 2010). Taken together, these data suggest that TLR4 stimulation by the LPS agonist leads to more uptake of bacterial cargo Regarding other types of agonists and cargo, LPS-treated microglia have shown increased phagocytosis of apoptotic neurons *in vitro*. This would be a beneficial outcome, as microglia in a pro-inflammatory environment should be removing apoptotic neurons that they encounter (Scheiblich and Bicker 2015).

Aside from its role in pro-inflammatory signalling, it has also been demonstrated that TLR4 is important for Aβ uptake in an amyloid context (Tahara et al. 2006; Song et al. 2011). TLR4 has also been shown to be relevant to AD as it has an important role in memory impairment: in C57 mice injected with

AβOs, TLR4 was implicated in both microglial activation (measured by increased glial cell numbers for microglia and astrocytes, and increased pro-inflammatory cytokines), and memory deficits (Balducci et al. 2017). When TLR4 was pharmacologically inhibited, or in TLR4-deficient mice, these outcomes were not observed.

Therefore, in my experiments, I wanted to assess how the inhibition of TLR4 in presence or absence of AβOs would affect phagocytosis. I found that inhibiting TLR4 had no significant effect on FAM-Aβ cargo uptake, whether TLR4 had been stimulated by AβOs or not (section 3.6). This suggests that TLR4 is not mediating the downstream signalling regulating phagocytosis after AβO-stimulation. However, regardless of the role of TLR4, both pro-inflammatory agonists AβO and LPS separately led to a decrease in phagocytic uptake of FAM-Aβ. This is in line with some other literature showing that microglia had decreased phagocytic capacity for microspheres (beads) after treatment with a pro-inflammatory mediator (Koenigsknecht-Talboo and Landreth 2005).

Very interestingly, some work has actually shown that TLR4 activation decreases microglial phagocytic capacity by affecting cells' ability to break down Aβ once engulfed in cellular lysosomes (termed autophagy) (Lee et al. 2018). I did not assess Aβ break down within phagolysosomes, therefore I cannot confirm if the decrease in phagocytosis in our experiments was also occurring due to issues with Aβ break down.

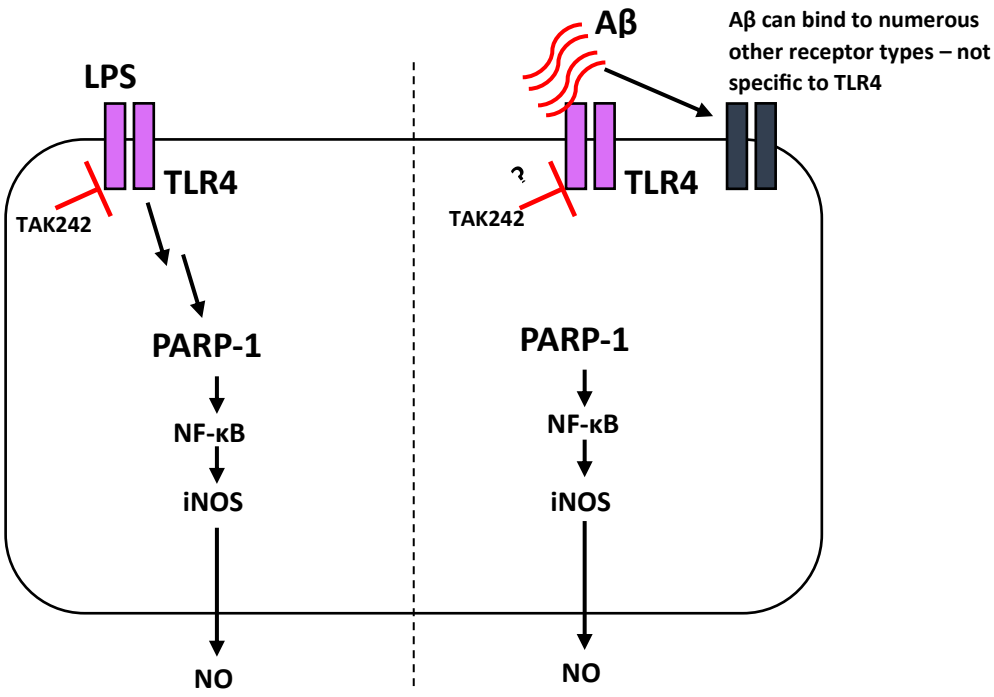

Figure 12: Potential proposed mechanism for TLR4-mediated signalling by agonists LPS vs. Aβ.

4.6 Study Limitations and Future Directions

My results have corroborated some existing published work, as I showed that AβO-treated microglia had decreased phagocytic capacity compared to non-treated controls. This suggests that pro-inflammatory stimulation leads cells to not prioritize the uptake of surrounding cargo, in a way that likely mirrors late stages of AD (Huang et al. 2017).

Inhibition of portions of the PARP-1/TRPM2 pro-inflammatory signalling pathway (namely, PARP-1, TRPM2, or NUDT5) did not improve phagocytic uptake in inflammatory conditions induced by AβO treatment. This suggests that these signalling pathways components are not involved in phagocytic regulation in this context.

Inhibiting the production of pro-inflammatory NO production via iNOS also did not improve phagocytic uptake, and neither did addition of extracellular NO to cell media. This contradicts some

literature reports that NO is required for phagocytosis (Maksoud et al. 2019) or promotes an enhanced level of phagocytosis (Scheiblich and Bicker 2016). However, I showed in separate assays that the majority of pharmacological inhibitors used in phagocytic assays were able to inhibit NO production in AβO-treated (or LPS-treated, when relevant) microglia – therefore suggesting that mechanisms regulating pro-inflammatory responses in microglia seem to be distinct from mechanisms in charge of phagocytosis.

Some limitations to my work shown here are the lack of secondary experiments to confirm my findings. Aside from the nitric oxide related assays mentioned above, I did not carry out other experiments to provide more evidence and confidence in phagocytic assay findings. For example, one method could have been to stain cells for a lysosomal marker such as CD68 or LAMP1, as a way to showcase that phagocytic cargo was in fact inside cellular lysosomes and had been engulfed. Another way to validate my findings could have been to stain for or measure activity of PARP-1/TRPM2 pathway components, to prove that their activity was being inhibited in phagocytic assays. Additionally, the use of cells cultured from KO mice would have been useful in this same regard. While I did have some results for phagocytic assays in both male and female PARP-1 cKO mice (section 3.8), the number of animals per treatment group is too low to be used for statistics or provide definitive results. The use of TRPM2 KO mice for phagocytic assays could have also been used to provide confidence in results achieved using TRPM2 inhibitors.

There are also a wide range of data points and large error bars within treatment groups throughout my results, and sometimes the trend of data points varies within a treatment (i.e. sometimes showing a decrease in phagocytosis in comparison to control, while sometimes showing an increase). A potential reason for this variation may be the methods used, as the range of FAM-Aβ cargo fluorescence detected can vary widely, even when prepared and handled identically.

Future work that could be completed to follow up on the findings of this project include: repeating phagocytic assays with other types of fluorescently-labelled cargo of interest, such as *E.coli*, synaptosomes/neurons, or beads ('neutral cargo'). Synaptosomes in particular would be an interesting cargo, as together with microglia they can represent a model of synaptic pruning, which has direct relevance to AD. These experiments could address the question of whether inhibition of PARP-1 or TRPM2 could prevent or substantially decrease the amount of aberrant synaptic pruning that takes place in AD.

This would be one way to investigate the hypothesis that phagocytosis of different cargo types (or 'modalities') may be regulated differently by PARP-1/TRPM2 signalling. Additionally, as some of my data supports that cells have different responses to 2 types of stimuli, AβO vs LPS (NO data in 3.4, phagocytic data in 3.6), this could be interesting to further explore in phagocytic assays.

Other future experiments investigating microglial phagocytosis could include live cell imaging of the entire time that cells are incubated with cargo using a confocal microscope. This would provide extremely interesting data and videos that could theoretically directly show cells extending their cytoskeleton to take in cargo. However, there would be numerous technical difficulties to work out for this to work, namely how to keep microglia alive and behaving 'normally' in an environment outside of an enclosed incubator, as microglia are immune cells and therefore notoriously sensitive to environmental changes, insults, and stress.

In summary, this work examined whether PARP-1 or TRPM2 signalling has a significant contribution to microglial phagocytosis of Aβ, in the context of health as well as amyloidopathy conditions. The major findings are that chronic AβO-treatment itself significantly decreases phagocytic uptake *in vitro*, while inhibition of PARP-1 or TRPM2 does not have any obvious effect. Additionally, our experiments show that NO produced via the PARP-1/TRPM2 signalling pathway does not act as a major regulator of phagocytosis of Aβ. Therefore, a potential AD therapeutic targeting PARP-1 or

TRPM2 should not have a detrimental effect on phagocytic uptake of Aβ, a beneficial target for clearance. Finally, these results support findings from mouse model studies, and suggest that favourable outcomes from PARP-1 or TRPM2 elimination in these animals is taking place without any significant changes to Aβ clearance.

REFERENCES

Akhtar, Mohd Waseem, Sara Sanz-Blasco, Nima Dolatabadi, James Parker, Kevin Chon, Michelle S. Lee, Walid Soussou, et al. 2016. "Elevated Glucose and Oligomeric β-Amyloid Disrupt Synapses via a Common Pathway of Aberrant Protein S-Nitrosylation." *Nature Communications* 7 (1): 10242. https://doi.org/10.1038/ncomms10242.

Alawieyah Syed Mortadza, Sharifah, Joan A. Sim, Veronika E. Neubrand, and Lin-Hua Jiang. 2018. "A Critical Role of TRPM2 Channel in Aβ42-Induced Microglial Activation and Generation of Tumor Necrosis Factor-α." *Glia* 66 (3): 562–75. https://doi.org/10.1002/glia.23265.

Aoyama, Koji, Sang Won Suh, Aaron M. Hamby, Jialing Liu, Wai Yee Chan, Yongmei Chen, and Raymond A. Swanson. 2006. "Neuronal Glutathione Deficiency and Age-Dependent Neurodegeneration in the EAAC1 Deficient Mouse." *Nature Neuroscience* 9 (1): 119–26. https://doi.org/10.1038/nn1609.

Bai, Peter. 2015. "Biology of Poly(ADP-Ribose) Polymerases: The Factotums of Cell Maintenance." *Molecular Cell* 58 (6): 947–58. https://doi.org/10.1016/j.molcel.2015.01.034.

Balducci, Claudia, Angelisa Frasca, Margherita Zotti, Pietro La Vitola, Emanuela Mhillaj, Emanuele Grigoli, Martina Iacobellis, et al. 2017. "Toll-like Receptor 4-Dependent Glial Cell Activation Mediates the Impairment in Memory Establishment Induced by β-Amyloid Oligomers in an Acute Mouse Model of Alzheimer's Disease." *Brain, Behavior, and Immunity* 60 (February): 188–97. https://doi.org/10.1016/j.bbi.2016.10.012.

Bales, K. R, Y Du, D Holtzman, B Cordell, and S. M Paul. 2000. "Neuroinflammation and Alzheimer's Disease: Critical Roles for Cytokine/Aβ-Induced Glial Activation, NF-KB, and Apolipoprotein E." *Neurobiology of Aging* 21 (3): 427–32. https://doi.org/10.1016/S0197-4580(00)00143-3.

Bal-Price, Anna, and Guy C. Brown. 2001. "Inflammatory Neurodegeneration Mediated by Nitric Oxide from Activated Glia-Inhibiting Neuronal Respiration, Causing Glutamate Release and

Excitotoxicity." *The Journal of Neuroscience* 21 (17): 6480–91. https://doi.org/10.1523/JNEUROSCI.21-17-06480.2001.

Bard, F., C. Cannon, R. Barbour, R. L. Burke, D. Games, H. Grajeda, T. Guido, et al. 2000. "Peripherally Administered Antibodies against Amyloid Beta-Peptide Enter the Central Nervous System and Reduce Pathology in a Mouse Model of Alzheimer Disease." *Nature Medicine* 6 (8): 916–19. https://doi.org/10.1038/78682.

Belrose, Jillian C, Yu-Feng Xie, Lynn J Gierszewski, John F MacDonald, and Michael F Jackson. 2012. "Loss of Glutathione Homeostasis Associated with Neuronal Senescence Facilitates TRPM2 Channel Activation in Cultured Hippocampal Pyramidal Neurons." *Molecular Brain* 5 (April): 11. https://doi.org/10.1186/1756-6606-5-11.

Belrose, Jillian Corinne, and Michael Frederick Jackson. 2018. "TRPM2: A Candidate Therapeutic Target for Treating Neurological Diseases." *Acta Pharmacologica Sinica* 39 (5): 722–32. https://doi.org/10.1038/aps.2018.31.

Benzing, W. C., J. R. Wujek, E. K. Ward, D. Shaffer, K. H. Ashe, S. G. Younkin, and K. R. Brunden. 1999. "Evidence for Glial-Mediated Inflammation in Aged APPSW Transgenic Mice." *Neurobiology of Aging* 20 (6): 581–89. https://doi.org/10.1016/S0197-4580(99)00065-2.

Bitler, Benjamin G., Zachary L. Watson, Lindsay J. Wheeler, and Kian Behbakht. 2017. "PARP Inhibitors: Clinical Utility and Possibilities of Overcoming Resistance." *Gynecologic Oncology* 147 (3): 695–704. https://doi.org/10.1016/j.ygyno.2017.10.003.

Bittner, Tobias, Steffen Burgold, Mario M. Dorostkar, Martin Fuhrmann, Bettina M. Wegenast-Braun, Boris Schmidt, Hans Kretzschmar, and Jochen Herms. 2012. "Amyloid Plaque Formation Precedes Dendritic Spine Loss." *Acta Neuropathologica* 124 (6): 797–807. https://doi.org/10.1007/s00401-012-1047-8.

Blessed, G., B. E. Tomlinson, and Martin Roth. 1968. “The Association Between Quantitative Measures of Dementia and of Senile Change in the Cerebral Grey Matter of Elderly Subjects.” *British Journal of Psychiatry* 114 (512): 797–811. https://doi.org/10.1192/bjp.114.512.797.

Bolmont, Tristan, Florent Haiss, Daniel Eicke, Rebecca Radde, Chester A. Mathis, William E. Klunk, Shinichi Kohsaka, Mathias Jucker, and Michael E. Calhoun. 2008. “Dynamics of the Microglial/Amyloid Interaction Indicate a Role in Plaque Maintenance.” *The Journal of Neuroscience* 28 (16): 4283–92. https://doi.org/10.1523/JNEUROSCI.4814-07.2008.

Braak, H., and E. Braak. 1991. “Neuropathological Stageing of Alzheimer-Related Changes.” *Acta Neuropathologica* 82 (4): 239–59. https://doi.org/10.1007/BF00308809.

Brown, G.C. 2007. “Mechanisms of Inflammatory Neurodegeneration: INOS and NADPH Oxidase.” *Biochemical Society Transactions* 35 (5): 1119–21. https://doi.org/10.1042/BST0351119.

Brown, Guy C., and Jonas J. Neher. 2014. “Microglial Phagocytosis of Live Neurons.” *Nature Reviews Neuroscience* 15 (4): 209–16. https://doi.org/10.1038/nrn3710.

Bruttger, Julia, Khalad Karram, Simone Wörtge, Tommy Regen, Federico Marini, Nicola Hoppmann, Matthias Klein, et al. 2015. “Genetic Cell Ablation Reveals Clusters of Local Self-Renewing Microglia in the Mammalian Central Nervous System.” *Immunity* 43 (1): 92–106. https://doi.org/10.1016/j.immuni.2015.06.012.

Butler, Claire A., Alma S. Popescu, Emily J. A. Kitchener, David H. Allendorf, Mar Puigdellívol, and Guy C. Brown. 2021. “Microglial Phagocytosis of Neurons in Neurodegeneration, and Its Regulation.” *Journal of Neurochemistry* 158 (3): 621–39. https://doi.org/10.1111/jnc.15327.

Chen, Jennifer, Yungui Zhou, Sarah Mueller-Steiner, Lin-Feng Chen, Hakju Kwon, Saili Yi, Lennart Mucke, and Li Gan. 2005. “SIRT1 Protects against Microglia-Dependent Amyloid-β Toxicity through Inhibiting NF-KB Signaling *.” *Journal of Biological Chemistry* 280 (48): 40364–74. https://doi.org/10.1074/jbc.M509329200.

Chen, Rui, Ziyu Wang, Zhongwen Zhi, Jisha Tian, Ying Zhao, and Junshan Sun. 2021. “Targeting the TLR4/NF-KB Pathway in β-Amyloid-Stimulated Microglial Cells: A Possible Mechanism That Oxysophoridine Exerts Anti-Oxidative and Anti-Inflammatory Effects in an in Vitro Model of Alzheimer’s Disease.” *Brain Research Bulletin* 175 (October): 150–57. https://doi.org/10.1016/j.brainresbull.2021.07.019.

Chiarugi, Alberto, and Michael A. Moskowitz. 2003. “Poly(ADP-Ribose) Polymerase-1 Activity Promotes NF-KB-Driven Transcription and Microglial Activation: Implication for Neurodegenerative Disorders.” *Journal of Neurochemistry* 85 (2): 306–17. https://doi.org/10.1046/j.1471-4159.2003.01684.x.

Cho, Dong-Hyung, Tomohiro Nakamura, Jianguo Fang, Piotr Cieplak, Adam Godzik, Zezong Gu, and Stuart A. Lipton. 2009. “S-Nitrosylation of Drp1 Mediates β-Amyloid–Related Mitochondrial Fission and Neuronal Injury.” *Science (New York, N.Y.)* 324 (5923): 102. https://doi.org/10.1126/science.1171091.

Citron, M., T. Oltersdorf, C. Haass, L. McConlogue, A. Y. Hung, P. Seubert, C. Vigo-Pelfrey, I. Lieberburg, and D. J. Selkoe. 1992. “Mutation of the Beta-Amyloid Precursor Protein in Familial Alzheimer’s Disease Increases Beta-Protein Production.” *Nature* 360 (6405): 672–74. https://doi.org/10.1038/360672a0.

Coraci, Indra Sethy, Jens Husemann, Joan W. Berman, Christine Hulette, Jennifer H. Dufour, Gabriele K. Campanella, Andrew D. Luster, Samuel C. Silverstein, and Joseph B. El Khoury. 2002. “CD36, a Class B Scavenger Receptor, Is Expressed on Microglia in Alzheimer’s Disease Brains and Can Mediate Production of Reactive Oxygen Species in Response to β-Amyloid Fibrils.” *The American Journal of Pathology* 160 (1): 101–12.

Courtine, Emilie, Nicolas Cagnard, Julie Mazzolini, Marion Antona, Frédéric Pène, Catherine Fitting, Sébastien Jacques, et al. 2012. “Combined Loss of CRel/P50 Subunits of NF-KB Leads to

Impaired Innate Host Response in Sepsis." *Innate Immunity* 18 (5): 753–63. https://doi.org/10.1177/1753425912440296.

Cummings, Jeffrey, P. Aisen, L. G. Apostolova, A. Atri, S. Salloway, and M. Weiner. 2021. "Aducanumab: Appropriate Use Recommendations." *The Journal of Prevention of Alzheimer's Disease* 8 (4): 398–410. https://doi.org/10.14283/jpad.2021.41.

Deczkowska, Aleksandra, Hadas Keren-Shaul, Assaf Weiner, Marco Colonna, Michal Schwartz, and Ido Amit. 2018. "Disease-Associated Microglia: A Universal Immune Sensor of Neurodegeneration." *Cell* 173 (5): 1073–81. https://doi.org/10.1016/j.cell.2018.05.003.

Doens, Deborah, and Patricia L Fernández. 2014. "Microglia Receptors and Their Implications in the Response to Amyloid β for Alzheimer's Disease Pathogenesis." *Journal of Neuroinflammation* 11 (1): 48. https://doi.org/10.1186/1742-2094-11-48.

Du, Jianyang, Jia Xie, and Lixia Yue. 2009. "Intracellular Calcium Activates TRPM2 and Its Alternative Spliced Isoforms." *Proceedings of the National Academy of Sciences of the United States of America* 106 (17): 7239–44. https://doi.org/10.1073/pnas.0811725106.

El Khoury, Joseph B., Kathryn J. Moore, Terry K. Means, Josephine Leung, Kinya Terada, Michelle Toft, Mason W. Freeman, and Andrew D. Luster. 2003. "CD36 Mediates the Innate Host Response to β-Amyloid." *The Journal of Experimental Medicine* 197 (12): 1657–66. https://doi.org/10.1084/jem.20021546.

Elmore, Monica R. P., Allison R. Najafi, Maya A. Koike, Nabil N. Dagher, Elizabeth E. Spangenberg, Rachel A. Rice, Masashi Kitazawa, et al. 2014. "Colony-Stimulating Factor 1 Receptor Signaling Is Necessary for Microglia Viability, Unmasking a Microglia Progenitor Cell in the Adult Brain." *Neuron* 82 (2): 380–97. https://doi.org/10.1016/j.neuron.2014.02.040.

Erridge, Clett. 2010. "Endogenous Ligands of TLR2 and TLR4: Agonists or Assistants?" *Journal of Leukocyte Biology* 87 (6): 989–99. https://doi.org/10.1189/jlb.1209775.

Ertekin-Taner, Nilüfer. 2007. “Genetics of Alzheimer’s Disease: A Centennial Review.” *Neurologic Clinics* 25 (3): 611–v. https://doi.org/10.1016/j.ncl.2007.03.009.

Farrer, Lindsay A., L. Adrienne Cupples, Jonathan L. Haines, Bradley Hyman, Walter A. Kukull, Richard Mayeux, Richard H. Myers, Margaret A. Pericak-Vance, Neil Risch, and Cornelia M. van Duijn. 1997. “Effects of Age, Sex, and Ethnicity on the Association Between Apolipoprotein E Genotype and Alzheimer Disease: A Meta-Analysis.” *JAMA* 278 (16): 1349–56. https://doi.org/10.1001/jama.1997.03550160069041.

Feelisch, M. 1998. “The Use of Nitric Oxide Donors in Pharmacological Studies.” *Naunyn-Schmiedeberg’s Archives of Pharmacology* 358 (1): 113–22. https://doi.org/10.1007/PL00005231.

Ferretti, Maria Teresa, Maria Florencia Iulita, Enrica Cavedo, Patrizia Andrea Chiesa, Annemarie Schumacher Dimech, Antonella Santuccione Chadha, Francesca Baracchi, et al. 2018. “Sex Differences in Alzheimer Disease — the Gateway to Precision Medicine.” *Nature Reviews Neurology* 14 (8): 457–69. https://doi.org/10.1038/s41582-018-0032-9.

Fiebich, Bernd L., Carla Ribeiro Alvares Batista, Soraya Wilke Saliba, Nizar M. Yousif, and Antonio Carlos Pinheiro de Oliveira. 2018. “Role of Microglia TLRs in Neurodegeneration.” *Frontiers in Cellular Neuroscience* 12. https://www.frontiersin.org/articles/10.3389/fncel.2018.00329.

Filipello, Fabia, Raffaella Morini, Irene Corradini, Valerio Zerbi, Alice Canzi, Bernadeta Michalski, Marco Erreni, et al. 2018. “The Microglial Innate Immune Receptor TREM2 Is Required for Synapse Elimination and Normal Brain Connectivity.” *Immunity* 48 (5): 979-991.e8. https://doi.org/10.1016/j.immuni.2018.04.016.

Fonfria, E., I. C. B. Marshall, I. Boyfield, S. D. Skaper, J. P. Hughes, D. E. Owen, W. Zhang, B. A. Miller, C. D. Benham, and S. McNulty. 2005. “Amyloid Beta-Peptide(1-42) and Hydrogen

Peroxide-Induced Toxicity Are Mediated by TRPM2 in Rat Primary Striatal Cultures." *Journal of Neurochemistry* 95 (3): 715–23. https://doi.org/10.1111/j.1471-4159.2005.03396.x.

Fonfria, Elena, Ian C B Marshall, Christopher D Benham, Izzy Boyfield, Jason D Brown, Kerstin Hill, Jane P Hughes, Stephen D Skaper, and Shaun McNulty. 2004. "TRPM2 Channel Opening in Response to Oxidative Stress Is Dependent on Activation of Poly(ADP-Ribose) Polymerase." *British Journal of Pharmacology* 143 (1): 186–92. https://doi.org/10.1038/sj.bjp.0705914.

Formentini, Laura, Antonio Macchiarulo, Giulia Cipriani, Emidio Camaioni, Elena Rapizzi, Roberto Pellicciari, Flavio Moroni, and Alberto Chiarugi. 2009. "Poly(ADP-Ribose) Catabolism Triggers AMP-Dependent Mitochondrial Energy Failure *." *Journal of Biological Chemistry* 284 (26): 17668–76. https://doi.org/10.1074/jbc.M109.002931.

Fourgeaud, Lawrence, Curt Dvorak, Malika Faouzi, John Starkus, Sunil Sahdeo, Qi Wang, Brian Lord, et al. 2019. "Pharmacology of JNJ-28583113: A Novel TRPM2 Antagonist." *European Journal of Pharmacology* 853 (June): 299–307. https://doi.org/10.1016/j.ejphar.2019.03.043.

Frost, Bess, Rachel L. Jacks, and Marc I. Diamond. 2009. "Propagation of Tau Misfolding from the Outside to the Inside of a Cell." *The Journal of Biological Chemistry* 284 (19): 12845–52. https://doi.org/10.1074/jbc.M808759200.

Gagné, Jean-Philippe, Maxim Isabelle, Ken Sin Lo, Sylvie Bourassa, Michael J. Hendzel, Valina L. Dawson, Ted M. Dawson, and Guy G. Poirier. 2008. "Proteome-Wide Identification of Poly(ADP-Ribose) Binding Proteins and Poly(ADP-Ribose)-Associated Protein Complexes." *Nucleic Acids Research* 36 (22): 6959–76. https://doi.org/10.1093/nar/gkn771.

Gao, Jing, and Fumihiko Nakamura. 2022. "Actin-Associated Proteins and Small Molecules Targeting the Actin Cytoskeleton." *International Journal of Molecular Sciences* 23 (4): 2118. https://doi.org/10.3390/ijms23042118.

Garvey, Edward P., Jeffrey A. Oplinger, Eric S. Furfine, Rachel J. Kiff, Ferenc Laszlo, Brenden J. R. Whittle, and Richard G. Knowles. 1997. "1400W Is a Slow, Tight Binding, and Highly Selective Inhibitor of Inducible Nitric-Oxide Synthase in Vitro and in Vivo*." *Journal of Biological Chemistry* 272 (8): 4959–63. https://doi.org/10.1074/jbc.272.8.4959.

Gengler, Simon, Alison Hamilton, and Christian Hölscher. 2010. "Synaptic Plasticity in the Hippocampus of a APP/PS1 Mouse Model of Alzheimer's Disease Is Impaired in Old but Not Young Mice." *PLOS ONE* 5 (3): e9764. https://doi.org/10.1371/journal.pone.0009764.

Glenner, George G., and Caine W. Wong. 1984. "Alzheimer's Disease: Initial Report of the Purification and Characterization of a Novel Cerebrovascular Amyloid Protein." *Biochemical and Biophysical Research Communications* 120 (3): 885–90. https://doi.org/10.1016/S0006-291X(84)80190-4.

Go, Michelle, Jinghong Kou, Jeong-Eun Lim, Junling Yang, and Ken-ichiro Fukuchi. 2016. "Microglial Response to LPS Increases in Wild-Type Mice during Aging but Diminishes in an Alzheimer's Mouse Model: Implication of TLR4 Signaling in Disease Progression." *Biochemical and Biophysical Research Communications* 479 (2): 331–37. https://doi.org/10.1016/j.bbrc.2016.09.073.

Gordon, Siamon. 2016. "Phagocytosis: An Immunobiologic Process." *Immunity* 44 (3): 463–75. https://doi.org/10.1016/j.immuni.2016.02.026.

Griciuc, Ana, and Rudolph E. Tanzi. 2021. "The Role of Innate Immune Genes in Alzheimer's Disease." *Current Opinion in Neurology* 34 (2): 228–36. https://doi.org/10.1097/WCO.0000000000000911.

Ha, Hyo Chol, Lynda D. Hester, and Solomon H. Snyder. 2002. "Poly(ADP-Ribose) Polymerase-1 Dependence of Stress-Induced Transcription Factors and Associated Gene Expression in Glia."

Proceedings of the National Academy of Sciences of the United States of America 99 (5): 3270–75. https://doi.org/10.1073/pnas.052712399.

Hammond, Timothy R., Connor Dufort, Lasse Dissing-Olesen, Stefanie Giera, Adam Young, Alec Wysoker, Alec J. Walker, et al. 2019. "Single-Cell RNA Sequencing of Microglia throughout the Mouse Lifespan and in the Injured Brain Reveals Complex Cell-State Changes." *Immunity* 50 (1): 253-271.e6. https://doi.org/10.1016/j.immuni.2018.11.004.

Hansen, David V., Jesse E. Hanson, and Morgan Sheng. 2017. "Microglia in Alzheimer's Disease." *Journal of Cell Biology* 217 (2): 459–72. https://doi.org/10.1083/jcb.201709069.

Hardy, J., and D. Allsop. 1991. "Amyloid Deposition as the Central Event in the Aetiology of Alzheimer's Disease." *Trends in Pharmacological Sciences* 12 (10): 383–88. https://doi.org/10.1016/0165-6147(91)90609-v.

Hardy, John A., and Gerald A. Higgins. 1992. "Alzheimer's Disease: The Amyloid Cascade Hypothesis." *Science* 256 (5054): 184–86.

Hassa, P. O., and M. O. Hottiger. 2002. "The Functional Role of Poly(ADP-Ribose)Polymerase 1 as Novel Coactivator of NF-KB in Inflammatory Disorders." *Cellular and Molecular Life Sciences CMLS* 59 (9): 1534–53. https://doi.org/10.1007/s00018-002-8527-2.

Hickman, Suzanne E., Elizabeth K. Allison, and Joseph El Khoury. 2008. "Microglial Dysfunction and Defective β-Amyloid Clearance Pathways in Aging Alzheimer's Disease Mice." *The Journal of Neuroscience* 28 (33): 8354–60. https://doi.org/10.1523/JNEUROSCI.0616-08.2008.

Honig, Lawrence S., Bruno Vellas, Michael Woodward, Mercè Boada, Roger Bullock, Michael Borrie, Klaus Hager, et al. 2018. "Trial of Solanezumab for Mild Dementia Due to Alzheimer's Disease." *New England Journal of Medicine* 378 (4): 321–30. https://doi.org/10.1056/NEJMoa1705971.

Hua, Fang, Huiling Tang, Jun Wang, Megan C Prunty, Xiaodong Hua, Iqbal Sayeed, and Donald G Stein. 2015. “TAK-242, an Antagonist for Toll-like Receptor 4, Protects against Acute Cerebral Ischemia/Reperfusion Injury in Mice.” *Journal of Cerebral Blood Flow & Metabolism* 35 (4): 536–42. https://doi.org/10.1038/jcbfm.2014.240.

Huang, Nan-Qu, Hai Jin, Shao-yu Zhou, Jing-shan Shi, and Feng Jin. 2017. “TLR4 Is a Link between Diabetes and Alzheimer’s Disease.” *Behavioural Brain Research* 316 (January): 234–44. https://doi.org/10.1016/j.bbr.2016.08.047.

Ignarro, L J, G M Buga, K S Wood, R E Byrns, and G Chaudhuri. 1987. “Endothelium-Derived Relaxing Factor Produced and Released from Artery and Vein Is Nitric Oxide.” *Proceedings of the National Academy of Sciences of the United States of America* 84 (24): 9265–69.

Jonsson, Thorlakur, Hreinn Stefansson, Stacy Steinberg, Ingileif Jonsdottir, Palmi V. Jonsson, Jon Snaedal, Sigurbjorn Bjornsson, et al. 2013. “Variant of TREM2 Associated with the Risk of Alzheimer’s Disease.” *New England Journal of Medicine* 368 (2): 107–16. https://doi.org/10.1056/NEJMoa1211103.

Jordão, Marta Joana Costa, Roman Sankowski, Stefanie M. Brendecke, Sagar, Giuseppe Locatelli, Yi-Heng Tai, Tuan Leng Tay, et al. 2019. “Single-Cell Profiling Identifies Myeloid Cell Subsets with Distinct Fates during Neuroinflammation.” *Science* 363 (6425): eaat7554. https://doi.org/10.1126/science.aat7554.

Jurga, Agnieszka M., Martyna Paleczna, and Katarzyna Z. Kuter. 2020. “Overview of General and Discriminating Markers of Differential Microglia Phenotypes.” *Frontiers in Cellular Neuroscience* 14: 198. https://doi.org/10.3389/fncel.2020.00198.

Kakita, Hiroki, Mineyoshi Aoyama, Yoshiaki Nagaya, Hayato Asai, Mohamed Hamed Hussein, Mieko Suzuki, Shin Kato, Shinji Saitoh, and Kiyofumi Asai. 2013. “Diclofenac Enhances Proinflammatory Cytokine-Induced Phagocytosis of Cultured Microglia via Nitric Oxide

Production." *Toxicology and Applied Pharmacology* 268 (2): 99–105. https://doi.org/10.1016/j.taap.2013.01.024.

Kametani, Fuyuki, and Masato Hasegawa. 2018. "Reconsideration of Amyloid Hypothesis and Tau Hypothesis in Alzheimer's Disease." *Frontiers in Neuroscience* 12. https://www.frontiersin.org/articles/10.3389/fnins.2018.00025.

Kauppinen, Tiina M., Wai Y. Chan, Sang Won Suh, Amanda K. Wiggins, Eric J. Huang, and Raymond A. Swanson. 2006. "Direct Phosphorylation and Regulation of Poly(ADP-Ribose) Polymerase-1 by Extracellular Signal-Regulated Kinases 1/2." *Proceedings of the National Academy of Sciences of the United States of America* 103 (18): 7136–41. https://doi.org/10.1073/pnas.0508606103.

Kauppinen, Tiina M., Sang Won Suh, Youichirou Higashi, Ari E. Berman, Carole Escartin, Seok Joon Won, Chao Wang, Seo-Hyun Cho, Li Gan, and Raymond A. Swanson. 2011. "Poly(ADP-Ribose)Polymerase-1 Modulates Microglial Responses to Amyloid β." *Journal of Neuroinflammation* 8 (1): 152. https://doi.org/10.1186/1742-2094-8-152.

Kauppinen, Tiina M., and Raymond A. Swanson. 2005. "Poly(ADP-Ribose) Polymerase-1 Promotes Microglial Activation, Proliferation, and Matrix Metalloproteinase-9-Mediated Neuron Death." *The Journal of Immunology* 174 (4): 2288–96. https://doi.org/10.4049/jimmunol.174.4.2288.

Kawamoto, Tomohiro, Masayuki Ii, Tomoyuki Kitazaki, Yuji Iizawa, and Hiroyuki Kimura. 2008. "TAK-242 Selectively Suppresses Toll-like Receptor 4-Signaling Mediated by the Intracellular Domain." *European Journal of Pharmacology* 584 (1): 40–48. https://doi.org/10.1016/j.ejphar.2008.01.026.

Kettenmann, Helmut, Uwe-Karsten Hanisch, Mami Noda, and Alexei Verkhratsky. 2011. "Physiology of Microglia." *Physiological Reviews* 91 (2): 461–553. https://doi.org/10.1152/physrev.00011.2010.

Kinney, Jefferson W., Shane M. Bemiller, Andrew S. Murtishaw, Amanda M. Leisgang, Arnold M. Salazar, and Bruce T. Lamb. 2018. “Inflammation as a Central Mechanism in Alzheimer’s Disease.” *Alzheimer’s & Dementia (New York, N. Y.)* 4: 575–90. https://doi.org/10.1016/j.trci.2018.06.014.

Koenigsknecht-Talboo, Jessica, and Gary E. Landreth. 2005. “Microglial Phagocytosis Induced by Fibrillar β-Amyloid and IgGs Are Differentially Regulated by Proinflammatory Cytokines.” *The Journal of Neuroscience* 25 (36): 8240–49. https://doi.org/10.1523/JNEUROSCI.1808-05.2005.

Kopec, Karla K., and Richard T. Carroll. 2000. “Phagocytosis Is Regulated by Nitric Oxide in Murine Microglia.” *Nitric Oxide* 4 (2): 103–11. https://doi.org/10.1006/niox.2000.0280.

Koutsodendris, Nicole, Maxine R. Nelson, Antara Rao, and Yadong Huang. 2022. “Apolipoprotein E and Alzheimer’s Disease: Findings, Hypotheses, and Potential Mechanisms.” *Annual Review of Pathology: Mechanisms of Disease* 17 (1): 73–99. https://doi.org/10.1146/annurev-pathmechdis-030421-112756.

Krabbe, Grietje, Annett Halle, Vitali Matyash, Jan L. Rinnenthal, Gina D. Eom, Ulrike Bernhardt, Kelly R. Miller, Stefan Prokop, Helmut Kettenmann, and Frank L. Heppner. 2013. “Functional Impairment of Microglia Coincides with Beta-Amyloid Deposition in Mice with Alzheimer-like Pathology.” *PloS One* 8 (4): e60921. https://doi.org/10.1371/journal.pone.0060921.

Kraus, W. Lee, and John T. Lis. 2003. “PARP Goes Transcription.” *Cell* 113 (6): 677–83. https://doi.org/10.1016/S0092-8674(03)00433-1.

Lee, Jaehwi, Man Hee Rhee, Eunji Kim, and Jae Youl Cho. 2012. “BAY 11-7082 Is a Broad-Spectrum Inhibitor with Anti-Inflammatory Activity against Multiple Targets.” *Mediators of Inflammation* 2012: 416036. https://doi.org/10.1155/2012/416036.

Lee, Ji-Won, Hyeri Nam, Leah Eunjung Kim, Yoonjeong Jeon, Hyunjung Min, Shinwon Ha, Younghwan Lee, et al. 2018. “TLR4 (Toll-like Receptor 4) Activation Suppresses Autophagy through Inhibition of FOXO3 and Impairs Phagocytic Capacity of Microglia.” *Autophagy* 15 (5): 753–70. https://doi.org/10.1080/15548627.2018.1556946.

Li, Jiangtao, and Ying Zhang. 2018. “TREM2 Regulates Innate Immunity in Alzheimer’s Disease.” *Journal of Neuroinflammation* 15 (April). https://doi.org/10.1186/s12974-018-1148-y.

Li, Jiaxin, Xinyu Shui, Ruizheng Sun, Lily Wan, Boxin Zhang, Bo Xiao, and Zhaohui Luo. 2021. “Microglial Phenotypic Transition: Signaling Pathways and Influencing Modulators Involved in Regulation in Central Nervous System Diseases.” *Frontiers in Cellular Neuroscience* 15. https://www.frontiersin.org/articles/10.3389/fncel.2021.736310.

Li, Xianwu, Erica Melief, Nadia Postupna, Kathleen S. Montine, C. Dirk Keene, and Thomas J. Montine. 2015. “Prostaglandin E2 Receptor Subtype 2 Regulation of Scavenger Receptor CD36 Modulates Microglial Aβ42 Phagocytosis.” *The American Journal of Pathology* 185 (1): 230–39. https://doi.org/10.1016/j.ajpath.2014.09.016.

Liddelow, Shane A., Kevin A. Guttenplan, Laura E. Clarke, Frederick C. Bennett, Christopher J. Bohlen, Lucas Schirmer, Mariko L. Bennett, et al. 2017. “Neurotoxic Reactive Astrocytes Are Induced by Activated Microglia.” *Nature* 541 (7638): 481–87. https://doi.org/10.1038/nature21029.

Liu, Fudong, Jesse Lang, Jun Li, Sharon E Benashski, Matthew Siegel, Yan Xu, and Louise D McCullough. 2011. “Sex Differences in the Response to PARP-1 Deletion and Caspase Inhibition after Stroke.” *Stroke; a Journal of Cerebral Circulation* 42 (4): 1090–96. https://doi.org/10.1161/STROKEAHA.110.594861.

Liu, Pei-Pei, Yi Xie, Xiao-Yan Meng, and Jian-Sheng Kang. 2019. “History and Progress of Hypotheses and Clinical Trials for Alzheimer’s Disease.” *Signal Transduction and Targeted Therapy* 4 (1): 1–22. https://doi.org/10.1038/s41392-019-0063-8.

Liu, Ting, Lingyun Zhang, Donghyun Joo, and Shao-Cong Sun. 2017. “NF-KB Signaling in Inflammation.” *Signal Transduction and Targeted Therapy* 2 (July): 17023. https://doi.org/10.1038/sigtrans.2017.23.

Liu, Wenfei, Orjona Taso, Rui Wang, Sevinc Bayram, Andrew C Graham, Pablo Garcia-Reitboeck, Anna Mallach, et al. 2020. “Trem2 Promotes Anti-Inflammatory Responses in Microglia and Is Suppressed under pro-Inflammatory Conditions.” *Human Molecular Genetics* 29 (19): 3224–48. https://doi.org/10.1093/hmg/ddaa209.

Lue, Lih-Fen, Russell Rydel, Elizabeth F. Brigham, Li-Bang Yang, Harald Hampel, Greer M. Murphy Jr., Libuse Brachova, et al. 2001. “Inflammatory Repertoire of Alzheimer’s Disease and Nondemented Elderly Microglia in Vitro.” *Glia* 35 (1): 72–79. https://doi.org/10.1002/glia.1072.

MacLeod, Ruth, Ellin-Kristina Hillert, Ryan T Cameron, and George S Baillie. 2015. “The Role and Therapeutic Targeting of α-, β- and γ-Secretase in Alzheimer’s Disease.” *Future Science OA* 1 (3): FSO11. https://doi.org/10.4155/fso.15.9.

Maksoud, Matthew J. E., Vasiliki Tellios, Dong An, Yun-Yan Xiang, and Wei-Yang Lu. 2019. “Nitric Oxide Upregulates Microglia Phagocytosis and Increases Transient Receptor Potential Vanilloid Type 2 Channel Expression on the Plasma Membrane.” *Glia* 67 (12): 2294–2311. https://doi.org/10.1002/glia.23685.

Malko, Philippa, Sharifah A. Syed Mortadza, Joseph McWilliam, and Lin-Hua Jiang. 2019. “TRPM2 Channel in Microglia as a New Player in Neuroinflammation Associated With a Spectrum of

Central Nervous System Pathologies." *Frontiers in Pharmacology* 10. https://www.frontiersin.org/articles/10.3389/fphar.2019.00239.

Mander, Palwinder, Vilma Borutaite, Salvador Moncada, and Guy C. Brown. 2005. "Nitric Oxide from Inflammatory-Activated Glia Synergizes with Hypoxia to Induce Neuronal Death." *Journal of Neuroscience Research* 79 (1–2): 208–15. https://doi.org/10.1002/jnr.20285.

Mao, Kanmin, and Guo Zhang. 2022. "The Role of PARP1 in Neurodegenerative Diseases and Aging." *The FEBS Journal* 289 (8): 2013–24. https://doi.org/10.1111/febs.15716.

Masuda, Takahiro, Roman Sankowski, Ori Staszewski, Chotima Böttcher, Lukas Amann, Sagar, Christian Scheiwe, et al. 2019. "Spatial and Temporal Heterogeneity of Mouse and Human Microglia at Single-Cell Resolution." *Nature* 566 (7744): 388–92. https://doi.org/10.1038/s41586-019-0924-x.

Mazaheri, Fargol, Nicolas Snaidero, Gernot Kleinberger, Charlotte Madore, Anna Daria, Georg Werner, Susanne Krasemann, et al. 2017. "TREM2 Deficiency Impairs Chemotaxis and Microglial Responses to Neuronal Injury." *EMBO Reports* 18 (7): 1186–98. https://doi.org/10.15252/embr.201743922.

McLean, I. W., and P. K. Nakane. 1974. "Periodate-Lysine-Paraformaldehyde Fixative. A New Fixation for Immunoelectron Microscopy." *The Journal of Histochemistry and Cytochemistry: Official Journal of the Histochemistry Society* 22 (12): 1077–83. https://doi.org/10.1177/22.12.1077.

Mehrabadi, Abbas Rezaeian, Minna A. Korolainen, Gary Odero, Donald W. Miller, and Tiina M. Kauppinen. 2017. "Poly(ADP-Ribose) Polymerase-1 Regulates Microglia Mediated Decrease of Endothelial Tight Junction Integrity." *Neurochemistry International* 108 (September): 266–71. https://doi.org/10.1016/j.neuint.2017.04.014.

Meunier, Alice, Alban Latrémolière, Elisa Dominguez, Annie Mauborgne, Stéphanie Philippe, Michel Hamon, Jacques Mallet, Jean-Jacques Benoliel, and Michel Pohl. 2007. "Lentiviral-Mediated Targeted NF-KB Blockade in Dorsal Spinal Cord Glia Attenuates Sciatic Nerve Injury–Induced Neuropathic Pain in the Rat." *Molecular Therapy* 15 (4): 687–97. https://doi.org/10.1038/sj.mt.6300107.

Michelucci, Alessandro, Tony Heurtaux, Luc Grandbarbe, Eleonora Morga, and Paul Heuschling. 2009. "Characterization of the Microglial Phenotype under Specific Pro-Inflammatory and Anti-Inflammatory Conditions: Effects of Oligomeric and Fibrillar Amyloid-β." *Journal of Neuroimmunology* 210 (1): 3–12. https://doi.org/10.1016/j.jneuroim.2009.02.003.

Minter, Myles R., Juliet M. Taylor, and Peter J. Crack. 2016. "The Contribution of Neuroinflammation to Amyloid Toxicity in Alzheimer's Disease." *Journal of Neurochemistry* 136 (3): 457–74. https://doi.org/10.1111/jnc.13411.

Mrak, Robert E, Jin G Sheng, and W. Sue T Griffin. 1995. "Glial Cytokines in Alzheimer's Disease: Review and Pathogenic Implications." *Human Pathology* 26 (8): 816–23. https://doi.org/10.1016/0046-8177(95)90001-2.

Nathan, Carl, Noel Calingasan, Jon Nezezon, Aihao Ding, M. Scott Lucia, Krista La Perle, Michele Fuortes, et al. 2005. "Protection from Alzheimer's-like Disease in the Mouse by Genetic Ablation of Inducible Nitric Oxide Synthase." *Journal of Experimental Medicine* 202 (9): 1163–69. https://doi.org/10.1084/jem.20051529.

Neher, Jonas J., Urte Neniskyte, Jing-Wei Zhao, Anna Bal-Price, Aviva M. Tolkovsky, and Guy C. Brown. 2011. "Inhibition of Microglial Phagocytosis Is Sufficient To Prevent Inflammatory Neuronal Death." *The Journal of Immunology* 186 (8): 4973–83. https://doi.org/10.4049/jimmunol.1003600.

Nelson, Lars H., Spencer Warden, and Kathryn M. Lenz. 2017. “Sex Differences in Microglial Phagocytosis in the Neonatal Hippocampus.” *Brain, Behavior, and Immunity* 64 (August): 11–22. https://doi.org/10.1016/j.bbi.2017.03.010.

Nimmerjahn, Axel, Frank Kirchhoff, and Fritjof Helmchen. 2005. “Resting Microglial Cells Are Highly Dynamic Surveillants of Brain Parenchyma in Vivo.” *Science* 308 (5726): 1314–18. https://doi.org/10.1126/science.1110647.

O’Brien, Richard J., and Philip C. Wong. 2011. “Amyloid Precursor Protein Processing and Alzheimer’s Disease.” *Annual Review of Neuroscience* 34: 185–204. https://doi.org/10.1146/annurev-neuro-061010-113613.

Ostapchenko, V. G., M. Chen, M. S. Guzman, Y.-F. Xie, N. Lavine, J. Fan, F. H. Beraldo, et al. 2015. “The Transient Receptor Potential Melastatin 2 (TRPM2) Channel Contributes to -Amyloid Oligomer-Related Neurotoxicity and Memory Impairment.” *Journal of Neuroscience* 35 (45): 15157–69. https://doi.org/10.1523/JNEUROSCI.4081-14.2015.

Page, Brent D. G., Nicholas C. K. Valerie, Roni H. G. Wright, Olov Wallner, Rebecka Isaksson, Megan Carter, Sean G. Rudd, et al. 2018. “Targeted NUDT5 Inhibitors Block Hormone Signaling in Breast Cancer Cells.” *Nature Communications* 9 (1): 250. https://doi.org/10.1038/s41467-017-02293-7.

Paolicelli, Rosa C., Amanda Sierra, Beth Stevens, Marie-Eve Tremblay, Adriano Aguzzi, Bahareh Ajami, Ido Amit, et al. 2022. “Microglia States and Nomenclature: A Field at Its Crossroads.” *Neuron* 110 (21): 3458–83. https://doi.org/10.1016/j.neuron.2022.10.020.

Parhizkar, Samira, Grace Gent, Yun Chen, Nicholas Rensing, Maud Gratuze, Gregory Strout, Sanja Sviben, et al. 2023. “Sleep Deprivation Exacerbates Microglial Reactivity and Aβ Deposition in a TREM2-Dependent Manner in Mice.” *Science Translational Medicine* 15 (693): eade6285. https://doi.org/10.1126/scitranslmed.ade6285.

Pocock, Jennifer M, and Helmut Kettenmann. 2007. “Neurotransmitter Receptors on Microglia.” *Trends in Neurosciences* 30 (10): 527–35. https://doi.org/10.1016/j.tins.2007.07.007.

Poliani, Pietro Luigi, Yaming Wang, Elena Fontana, Michelle L. Robinette, Yoshinori Yamanishi, Susan Gilfillan, and Marco Colonna. 2015. “TREM2 Sustains Microglial Expansion during Aging and Response to Demyelination.” *The Journal of Clinical Investigation* 125 (5): 2161–70. https://doi.org/10.1172/JCI77983.

Popiolek-Barczyk, Katarzyna, and Joanna Mika. 2016. “Targeting the Microglial Signaling Pathways: New Insights in the Modulation of Neuropathic Pain.” *Current Medicinal Chemistry* 23 (26): 2908–28. https://doi.org/10.2174/0929867323666160607120124.

Price, Joseph L., and John C. Morris. 1999. “Tangles and Plaques in Nondemented Aging and ‘Preclinical’ Alzheimer’s Disease.” *Annals of Neurology* 45 (3): 358–68. https://doi.org/10.1002/1531-8249(199903)45:3<358::AID-ANA12>3.0.CO;2-X.

Prinz, Marco, Steffen Jung, and Josef Priller. 2019. “Microglia Biology: One Century of Evolving Concepts.” *Cell* 179 (2): 292–311. https://doi.org/10.1016/j.cell.2019.08.053.

Radde, Rebecca, Tristan Bolmont, Stephan A Kaeser, Janaky Coomaraswamy, Dennis Lindau, Lars Stoltze, Michael E Calhoun, et al. 2006. “Aβ42-Driven Cerebral Amyloidosis in Transgenic Mice Reveals Early and Robust Pathology.” *EMBO Reports* 7 (9): 940–46. https://doi.org/10.1038/sj.embor.7400784.

Raghunatha, Prajwal. n.d. “Role of Microglial NMDA Receptor-Initiated PARP-1/TRPM2 Signaling in Driving Chronic Neuroinflammation,” 164.

Raghunatha, Prajwal, Amir Vosoughi, Tiina M. Kauppinen, and Michael F. Jackson. 2020. “Microglial NMDA Receptors Drive Pro-inflammatory Responses via PARP-1/TRMP2 Signaling.” *Glia* 68 (7): 1421–34. https://doi.org/10.1002/glia.23790.

Ransohoff, Richard M., and Astrid E. Cardona. 2010. “The Myeloid Cells of the Central Nervous System Parenchyma.” *Nature* 468 (7321): 253–62. https://doi.org/10.1038/nature09615.

Rao, Jagadeesh S, Matthew Kellom, Hyung-Wook Kim, and Stanley I Rapoport. 2012. “Neuroinflammation and Synaptic Loss.” *Neurochemical Research* 37 (5): 903–10. https://doi.org/10.1007/s11064-012-0708-2.

Ray Chaudhuri, Arnab, and André Nussenzweig. 2017. “The Multifaceted Roles of PARP1 in DNA Repair and Chromatin Remodelling.” *Nature Reviews Molecular Cell Biology* 18 (10): 610–21. https://doi.org/10.1038/nrm.2017.53.

Reed-Geaghan, Erin G., Julie C. Savage, Amy G. Hise, and Gary E. Landreth. 2009. “CD14 and Toll-Like Receptors 2 and 4 Are Required for Fibrillar Aβ-Stimulated Microglial Activation.” *The Journal of Neuroscience* 29 (38): 11982–92. https://doi.org/10.1523/JNEUROSCI.3158-09.2009.

Ribes, Sandra, Sandra Ebert, Dirk Czesnik, Tommy Regen, Andre Zeug, Stephanie Bukowski, Alexander Mildner, et al. 2009. “Toll-Like Receptor Prestimulation Increases Phagocytosis of Escherichia Coli DH5α and Escherichia Coli K1 Strains by Murine Microglial Cells.” *Infection and Immunity* 77 (1): 557–64. https://doi.org/10.1128/IAI.00903-08.

Roberge, Stéphanie, Julien Roussel, Daniel C. Andersson, Albano C. Meli, Bastien Vidal, Florence Blandel, Johanna T. Lanner, et al. 2014. “TNF-α-Mediated Caspase-8 Activation Induces ROS Production and TRPM2 Activation in Adult Ventricular Myocytes.” *Cardiovascular Research* 103 (1): 90–99. https://doi.org/10.1093/cvr/cvu112.

Rogers, Joseph, and Lih-Fen Lue. 2001. “Microglial Chemotaxis, Activation, and Phagocytosis of Amyloid β-Peptide as Linked Phenomena in Alzheimer’s Disease.” *Neurochemistry International* 39 (5): 333–40. https://doi.org/10.1016/S0197-0186(01)00040-7.

Rom, Slava, Viviana Zuluaga-Ramirez, Holly Dykstra, Nancy L Reichenbach, Servio H Ramirez, and Yuri Persidsky. 2015. “Poly(ADP-Ribose) Polymerase-1 Inhibition in Brain Endothelium Protects the Blood—Brain Barrier under Physiologic and Neuroinflammatory Conditions.” *Journal of Cerebral Blood Flow & Metabolism* 35 (1): 28–36. https://doi.org/10.1038/jcbfm.2014.167.

Rouleau, Michèle, Anand Patel, Michael J. Hendzel, Scott H. Kaufmann, and Guy G. Poirier. 2010. “PARP Inhibition: PARP1 and Beyond.” *Nature Reviews. Cancer* 10 (4): 293–301. https://doi.org/10.1038/nrc2812.

Sanchez-Mico, Maria V., Sebastian Jimenez, Angela Gomez-Arboledas, Clara Muñoz-Castro, Carmen Romero-Molina, Victoria Navarro, Elisabeth Sanchez-Mejias, et al. 2021. “Amyloid-β Impairs the Phagocytosis of Dystrophic Synapses by Astrocytes in Alzheimer’s Disease.” *Glia* 69 (4): 997–1011. https://doi.org/10.1002/glia.23943.

Scheiblich, Hannah, and Gerd Bicker. 2015. “Regulation of Microglial Migration, Phagocytosis, and Neurite Outgrowth by HO-1/CO Signaling.” *Developmental Neurobiology* 75 (8): 854–76. https://doi.org/10.1002/dneu.22253.

———. 2016. “Nitric Oxide Regulates Antagonistically Phagocytic and Neurite Outgrowth Inhibiting Capacities of Microglia.” *Developmental Neurobiology* 76 (5): 566–84. https://doi.org/10.1002/dneu.22333.

Shankar, Ganesh M., Shaomin Li, Tapan H. Mehta, Amaya Garcia-Munoz, Nina E. Shepardson, Imelda Smith, Francesca M. Brett, et al. 2008. “Amyloid-β Protein Dimers Isolated Directly from Alzheimer’s Brains Impair Synaptic Plasticity and Memory.” *Nature Medicine* 14 (8): 837–42. https://doi.org/10.1038/nm1782.

Shankar, Ganesh M., Alfred T. Welzel, Jessica M. McDonald, Dennis J. Selkoe, and Dominic M. Walsh. 2011. “Isolation of Low-n Amyloid β-Protein Oligomers from Cultured Cells, CSF, and

Brain." In *Alzheimer's Disease and Frontotemporal Dementia: Methods and Protocols*, edited by Erik D. Roberson, 33–44. Methods in Molecular Biology. Totowa, NJ: Humana Press. https://doi.org/10.1007/978-1-60761-744-0_3.

Siegel, Chad S., and Louise D. McCullough. 2013. "Nicotinamide Adenine Dinucleotide (NAD+) and Nicotinamide: Sex Differences in Cerebral Ischemia." *Neuroscience* 237 (May): 223–31. https://doi.org/10.1016/j.neuroscience.2013.01.068.

Sinha, Asha, Sachin Katyal, and Tiina M. Kauppinen. 2021. "PARP-DNA Trapping Ability of PARP Inhibitors Jeopardizes Astrocyte Viability: Implications for CNS Disease Therapeutics." *Neuropharmacology* 187 (April): 108502. https://doi.org/10.1016/j.neuropharm.2021.108502.

Sivagnanam, Vishanthan, Xiaoping Zhu, and Lyanne C. Schlichter. 2010. "Dominance of E. Coli Phagocytosis over LPS in the Inflammatory Response of Microglia." *Journal of Neuroimmunology* 227 (1–2): 111–19. https://doi.org/10.1016/j.jneuroim.2010.06.021.

Smith, Joshua A., Arabinda Das, Swapan K. Ray, and Naren L. Banik. 2012. "Role of Pro-Inflammatory Cytokines Released from Microglia in Neurodegenerative Diseases." *Brain Research Bulletin* 87 (1): 10–20. https://doi.org/10.1016/j.brainresbull.2011.10.004.

Song, Min, JingJi Jin, Jeong-Eun Lim, Jinghong Kou, Abhinandan Pattanayak, Jamaal A. Rehman, Hong-Duck Kim, Kazuki Tahara, Robert Lalonde, and Ken-ichiro Fukuchi. 2011. "TLR4 Mutation Reduces Microglial Activation, Increases Aβ Deposits and Exacerbates Cognitive Deficits in a Mouse Model of Alzheimer's Disease." *Journal of Neuroinflammation* 8 (1): 92. https://doi.org/10.1186/1742-2094-8-92.

Stewart, Cameron R., Lynda M. Stuart, Kim Wilkinson, Janine M. van Gils, Jiusheng Deng, Annett Halle, Katey J. Rayner, et al. 2010. "CD36 Ligands Promote Sterile Inflammation through Assembly of a Toll-like Receptor 4 and 6 Heterodimer." *Nature Immunology* 11 (2): 155–61. https://doi.org/10.1038/ni.1836.

Syed Mortadza, Sharifah Alawieyah, Lu Wang, Dongliang Li, and Lin-Hua Jiang. 2015. “TRPM2 Channel-Mediated ROS-Sensitive Ca2+ Signaling Mechanisms in Immune Cells.” *Frontiers in Immunology* 6. https://www.frontiersin.org/articles/10.3389/fimmu.2015.00407.

Tada, Mayumi, Atsuya Takeuchi, Miki Hashizume, Kazuo Kitamura, and Masanobu Kano. 2014. “A Highly Sensitive Fluorescent Indicator Dye for Calcium Imaging of Neural Activity in Vitro and in Vivo.” *The European Journal of Neuroscience* 39 (11): 1720–28. https://doi.org/10.1111/ejn.12476.

Tahara, Kazuki, Hong-Duck Kim, Jing-Ji Jin, J. Adam Maxwell, Ling Li, and Ken-ichiro Fukuchi. 2006. “Role of Toll-like Receptor Signalling in Aβ Uptake and Clearance.” *Brain* 129 (11): 3006–19. https://doi.org/10.1093/brain/awl249.

Thorsell, Ann-Gerd, Torun Ekblad, Tobias Karlberg, Mirjam Löw, Ana Filipa Pinto, Lionel Trésaugues, Martin Moche, Michael S. Cohen, and Herwig Schüler. 2017. “Structural Basis for Potency and Promiscuity in Poly(ADP-Ribose) Polymerase (PARP) and Tankyrase Inhibitors.” *Journal of Medicinal Chemistry* 60 (4): 1262–71. https://doi.org/10.1021/acs.jmedchem.6b00990.

Tümer, Cemil, Hakkı Murat Bilgin, Basra Deniz Obay, Hüda Diken, Mukadder Atmaca, and Mustafa Kelle. 2007. “Effect of Nitric Oxide on Phagocytic Activity of Lipopolysaccharide-Induced Macrophages: Possible Role of Exogenous l-Arginine.” *Cell Biology International* 31 (6): 565–69. https://doi.org/10.1016/j.cellbi.2006.11.029.

Turlova, Ekaterina, Zhong-ping Feng, and Hong-shuo Sun. 2018. “The Role of TRPM2 Channels in Neurons, Glial Cells and the Blood-Brain Barrier in Cerebral Ischemia and Hypoxia.” *Acta Pharmacologica Sinica* 39 (5): 713–21. https://doi.org/10.1038/aps.2017.194.

Valerio, Alessandra, Flora Boroni, Marina Benarese, Ilenia Sarnico, Valentina Ghisi, Laura Grazia Bresciani, Marina Ferrario, Giuseppe Borsani, PierFranco Spano, and Marina Pizzi. 2006. “NF-

KB Pathway: A Target for Preventing β-Amyloid (Aβ)-Induced Neuronal Damage and Aβ42 Production." *European Journal of Neuroscience* 23 (7): 1711–20. https://doi.org/10.1111/j.1460-9568.2006.04722.x.

Vuong, Billy, Adam D. J. Hogan-Cann, Conrad C. Alano, Mackenzie Stevenson, Wai Yee Chan, Christopher M. Anderson, Raymond A. Swanson, and Tiina M. Kauppinen. 2015. "NF-KB Transcriptional Activation by TNFα Requires Phospholipase C, Extracellular Signal-Regulated Kinase 2 and Poly(ADP-Ribose) Polymerase-1." *Journal of Neuroinflammation* 12 (1): 229. https://doi.org/10.1186/s12974-015-0448-8.

Vuong, Billy, Gary Odero, Stephanie Rozbacher, Mackenzie Stevenson, Stephanie M. Kereliuk, Troy J. Pereira, Vernon W. Dolinsky, and Tiina M. Kauppinen. 2017. "Exposure to Gestational Diabetes Mellitus Induces Neuroinflammation, Derangement of Hippocampal Neurons, and Cognitive Changes in Rat Offspring." *Journal of Neuroinflammation* 14 (1): 80. https://doi.org/10.1186/s12974-017-0859-9.

Walsh, Dominic M., Igor Klyubin, Julia V. Fadeeva, William K. Cullen, Roger Anwyl, Michael S. Wolfe, Michael J. Rowan, and Dennis J. Selkoe. 2002. "Naturally Secreted Oligomers of Amyloid β Protein Potently Inhibit Hippocampal Long-Term Potentiation in Vivo." *Nature* 416 (6880): 535–39. https://doi.org/10.1038/416535a.

Walters, Anna, Emma Phillips, Rui Zheng, Maya Biju, and Tarun Kuruvilla. 2016. "Evidence for Neuroinflammation in Alzheimer's Disease." *Progress in Neurology and Psychiatry* 20 (5): 25–31. https://doi.org/10.1002/pnp.444.

Wang, Jun, Ben J. Gu, Colin L. Masters, and Yan-Jiang Wang. 2017. "A Systemic View of Alzheimer Disease — Insights from Amyloid-β Metabolism beyond the Brain." *Nature Reviews Neurology* 13 (10): 612–23. https://doi.org/10.1038/nrneurol.2017.111.

Wang, Yaming, Tyler K. Ulland, Jason D. Ulrich, Wilbur Song, John A. Tzaferis, Justin T. Hole, Peng Yuan, et al. 2016. “TREM2-Mediated Early Microglial Response Limits Diffusion and Toxicity of Amyloid Plaques.” *Journal of Experimental Medicine* 213 (5): 667–75. https://doi.org/10.1084/jem.20151948.

Wang, Yipeng, and Eckhard Mandelkow. 2016. “Tau in Physiology and Pathology.” *Nature Reviews Neuroscience* 17 (1): 22–35. https://doi.org/10.1038/nrn.2015.1.

Wang, Yutian, Yihuang Lin, Caiyu Cheng, Pengyu Chen, Ping Zhang, Hangtian Wu, Kaiqun Li, et al. 2020. “NF-KB/TWIST1 Mediates Migration and Phagocytosis of Macrophages in the Mice Model of Implant-Associated Staphylococcus Aureus Osteomyelitis.” *Frontiers in Microbiology* 11. https://www.frontiersin.org/articles/10.3389/fmicb.2020.01301.

Wehage, Edith, Jörg Eisfeld, Inka Heiner, Eberhard Jüngling, Christof Zitt, and Andreas Lückhoff. 2002. “Activation of the Cation Channel Long Transient Receptor Potential Channel 2 (LTRPC2) by Hydrogen Peroxide: A SPLICE VARIANT REVEALS A MODE OF ACTIVATION INDEPENDENT OF ADP-RIBOSE*.” *Journal of Biological Chemistry* 277 (26): 23150–56. https://doi.org/10.1074/jbc.M112096200.

Wehrhahn, Janine, Robert Kraft, Christian Harteneck, and Sunna Hauschildt. 2010. “Transient Receptor Potential Melastatin 2 Is Required for Lipopolysaccharide-Induced Cytokine Production in Human Monocytes.” *The Journal of Immunology* 184 (5): 2386–93. https://doi.org/10.4049/jimmunol.0902474.

Wu, Linyu, Xiaohui Xian, Guangyu Xu, Zixuan Tan, Fang Dong, Min Zhang, and Feng Zhang. 2022. “Toll-Like Receptor 4: A Promising Therapeutic Target for Alzheimer’s Disease.” *Mediators of Inflammation* 2022 (August): 7924199. https://doi.org/10.1155/2022/7924199.

Wu, Yuwen, Lasse Dissing-Olesen, Brian A. MacVicar, and Beth Stevens. 2015. “Microglia: Dynamic Mediators of Synapse Development and Plasticity.” *Trends in Immunology,* Special Issue: Neuroimmunology, 36 (10): 605–13. https://doi.org/10.1016/j.it.2015.08.008.

Yamamoto, Masaru, Tomomi Kiyota, Shannon M. Walsh, Jianuo Liu, Jonathan Kipnis, and Tsuneya Ikezu. 2008. “Cytokine-Mediated Inhibition of Fibrillar Amyloid-β Peptide Degradation by Human Mononuclear Phagocytes1.” *The Journal of Immunology* 181 (6): 3877–86. https://doi.org/10.4049/jimmunol.181.6.3877.

Yang, Cheng-Ning, Young-Ji Shiao, Feng-Shiun Shie, Bo-Shen Guo, Pei-Hao Chen, Chi-Yuan Cho, Yi-Jen Chen, Fong-Lee Huang, and Huey-Jen Tsay. 2011. “Mechanism Mediating Oligomeric Aβ Clearance by Naïve Primary Microglia.” *Neurobiology of Disease* 42 (3): 221–30. https://doi.org/10.1016/j.nbd.2011.01.005.

Yanguas-Casás, Natalia, Andrea Crespo-Castrillo, Maria-Angeles Arevalo, and Luis Miguel Garcia-Segura. 2020. “Aging and Sex: Impact on Microglia Phagocytosis.” *Aging Cell* 19 (8): e13182. https://doi.org/10.1111/acel.13182.

Yeh, Felix L., Yuanyuan Wang, Irene Tom, Lino C. Gonzalez, and Morgan Sheng. 2016. “TREM2 Binds to Apolipoproteins, Including APOE and CLU/APOJ, and Thereby Facilitates Uptake of Amyloid-Beta by Microglia.” *Neuron* 91 (2): 328–40. https://doi.org/10.1016/j.neuron.2016.06.015.

Yıldızhan, Kenan, and Mustafa Nazıroğlu. 2020. “Glutathione Depletion and Parkinsonian Neurotoxin MPP+-Induced TRPM2 Channel Activation Play Central Roles in Oxidative Cytotoxicity and Inflammation in Microglia.” *Molecular Neurobiology* 57 (8): 3508–25. https://doi.org/10.1007/s12035-020-01974-7.

Yuste, Jose Enrique, Ernesto Tarragon, Carmen María Campuzano, and Francisco Ros-Bernal. 2015. "Implications of Glial Nitric Oxide in Neurodegenerative Diseases." *Frontiers in Cellular Neuroscience* 9: 322. https://doi.org/10.3389/fncel.2015.00322.

Zhang, Ruoyu, Jun Yamada, Yoshinori Hayashi, Zhou Wu, Susumu Koyama, and Hiroshi Nakanishi. 2008. "Inhibition of NMDA-Induced Outward Currents by Interleukin-1β in Hippocampal Neurons." *Biochemical and Biophysical Research Communications* 372 (4): 816–20. https://doi.org/10.1016/j.bbrc.2008.05.128.

Zhang, Xiaoyin, Dan Sun, Xin Zhou, Ce Zhang, Qing Yin, Li Chen, Yong Tang, Yonggang Liu, and Ludmilla A Morozova-Roche. 2023. "Proinflammatory S100A9 Stimulates TLR4/NF-KB Signaling Pathways Causing Enhanced Phagocytic Capacity of Microglial Cells." *Immunology Letters* 255 (March): 54–61. https://doi.org/10.1016/j.imlet.2023.02.008.

Zheng, Cong, Xin-Wen Zhou, and Jian-Zhi Wang. 2016. "The Dual Roles of Cytokines in Alzheimer's Disease: Update on Interleukins, TNF-α, TGF-β and IFN-γ." *Translational Neurodegeneration* 5 (1): 7. https://doi.org/10.1186/s40035-016-0054-4.

Zhou, Jiayin, Yuanyuan Deng, Fei Li, Caixia Yin, Jingshan Shi, and Qihai Gong. 2019. "Icariside II Attenuates Lipopolysaccharide-Induced Neuroinflammation through Inhibiting TLR4/MyD88/NF-KB Pathway in Rats." *Biomedicine & Pharmacotherapy* 111 (March): 315–24. https://doi.org/10.1016/j.biopha.2018.10.201.