Role of Microglial NMDA Receptor-Initiated PARP-1/TRPM2 Signaling in Driving Chronic Neuroinflammation

By

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ABSTRACT

An important component of neurodegenerative disorders, like Alzheimer's disease (AD), is the prolonged inflammatory response driven by continuous microglial poly (ADP-ribose) polymerase-1 (PARP-1) activation. However, mechanisms that promote sustained microglial PARP-1 activation and maintaining microglial activity remains elusive. But we know that PARP-1 enzymatic activity requires Ca^{2+} influx, a process that is independent of DNA damage, and this could be through microglial N-methyl D-aspartate receptors (NMDARs). Notably, PARP-1 mediated ADP-ribose production causes activation of Ca^{2+} permeable non-selective cation channel, transient receptor potential melastatin-2 (TRPM2). Hence, we hypothesize that NMDAR activation by amyloid beta oligomers (AβO) or NMDA initiates PARP-1 mediated ADPR production and TRPM2 activation. This in-turn leads to TRPM2-dependent, self-sustaining PARP-1 activation, which promotes pro-inflammatory responses.

Primary microglia treated with AβO and NMDA were assessed for functional TRPM2 currents using whole cell voltage-clamp electrophysiology. Using nitric oxide assay and qRT-PCR, AβO/NMDA treated microglia were also evaluated to identify the contribution of NMDAR/PARP-1/TRPM2 in promoting pro-inflammatory responses.

Our findings, demonstrates that glutamate signaling via NMDARs, promoting detrimental microglial responses, suggests a unifying mechanism linking elevated glutamate levels, associated for example with AD, hypoxic-ischemic injury, or traumatic brain injury, to chronic neuroinflammation.

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DEDICATION

I dedicate this thesis to my parents –

“who let me chase my dreams”

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ABBREVATIONS

aa	amino acids
$[Ca^{2+}]_i$	Intracellular calcium ion concentration
mV	millivolts
mM	millimolar
mL	milliliters
µM	micromolar
ng	nanogram
nM	nanomolar
pg	picogram
pM	picomolar
pS	picoseconds
Aβ	Amyloid beta
AβO	Amyloid beta oligomers
$Aβ_{1-40}$	Amyloid beta (1-40) residues
$Aβ_{1-42}$	Amyloid beta (1-42) residues
ACh	Acetyl choline
AChEI	Inhibitors of Acetyl choline
AD	Alzheimer's disease

ADPR	Adenosine diphosphate ribose
ADPRT	Adenosine diphosphate ribose transferase
AG-1	Arginase-1
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR/AMPARs	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA	Analysis of variance
APP	Amyloid precursor protein
BACE	Beta-secretase 1
BBB	Blood Brain Barrier
$BaCl_2$	Barium Chloride
CA1	cornu ammonis
Ca^{2+}	divalent calcium ion
$CaCl_2$	Calcium chloride
CaM	Calmodulin
CC	coil-coil
CD206	Cluster of Differentiation 206
ChiL3	Chitinase-like protein 3
CHO	Chinese hamster ovary

CHO-CM	Chinese hamster ovary – conditioned media
CNS	Central nervous system
CoA	Co-enzyme A
CsOH	Cesium Hydroxide
CX3CL1	chemokine (C-X3-C motif) ligand 1
CX3CL2	chemokine (C-X3-C motif) ligand 2
CX3CR1	CX3C chemokine receptor 1
Ctz	Clotrimazole
DAM	Disease Associated microglia
DAG	diacyl glycerol
dEGFP	downregulation enhanced green fluorescence protein
DPQ	PARP inhibitor III
DMTs	Disease modifying therapies
DNA	deoxy-ribose nucleic acid
DSB/DSBs	Double strand break/s
DSM-III	Diagnostic and Statistics Manual-III
ECF	Extracellular Fluid
ELISA	Enzyme linked immune sorbent assay

EPSP/EPSPs	Excitatory post-synaptic potential/s
ERK/ERKs	Extracellular regulated kinase/s
EVs	Extracellular vesicles
FFA	Flufernamic Acid
GI	Gastrointestine
GluA1	glutamate ionotropic receptor AMPA type subunit 1
GluA2	glutamate ionotropic receptor AMPA type subunit 2
GluA3	glutamate ionotropic receptor AMPA type subunit 3
GluN1	glutamate ionotropic receptor NMDAR type subunit 1
GluN2A	glutamate ionotropic receptor NMDAR type subunit 2A
GluN2B	glutamate ionotropic receptor NMDAR type subunit 2B
GluN2C	glutamate ionotropic receptor NMDAR type subunit 2C
GluN2D	glutamate ionotropic receptor NMDAR type subunit 2D
GluN3A	glutamate ionotropic receptor NMDAR type subunit 3A
GluN3B	glutamate ionotropic receptor NMDAR type subunit 3B
GM	Glial growth media
GM-CSF	Granulocyte-macrophage colony-stimulating factor
H2AX	histone H2A histone family member X

HBSS	Hank’s Balanced Salt Solution
HEPES	N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid
HPRT	Hypoxanthine Phospho-ribosyl transferase
Iba1	ionized calcium binding adaptor molecule 1
ICF	Intracellular Fluid
IkB	inhibitory I-kappa-B
IKK/IKKs	inhibitory I-kappa-B kinase/s
IL-1α	Interleukin-1 alpha
IL-1β	Interleukin-1 beta
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
IL-13	Interleukin-13
iNOS	inducible nitric oxide synthase
I-V	Current-Voltage
I_{Peak}	Peak current
I_{Res}	Residual current

I_{TRPM2}	TRPM2 current
KCl	Potassium chloride
LTD	Long-term depression
LTP	Long-term potentiation
LTRPC2	long transient receptor potential channel Subfamily C Member 2
MAPs	Microtubule associated proteins
MAPK/MAPKs	mitogen-activated protein kinases
MCAO	Middle cerebral artery occlusion
MCI	mild cognitive impairment
MEM	Minimum essential media
$MgCl_2$	Magnesium chloride
MHD	Multiple homology domain
mRNA	messenger ribonucleic acid
NAD^+	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NAM	nicotinamide

NF-κB	Nuclear factor kappa-B
NFT	Neurofibrillary tangle
NLS	Nuclear localization signal
NMDA	N-methyl-D-aspartate
NMDAR/NMDARs	N-methyl-D-aspartate receptors
NO	Nitric oxide
NOS2	Nitric oxide synthase 2
NR1	NMDA receptor sub-unit 1
NR2A	NMDA receptor sub-unit 2A
NR2B	NMDA receptor sub-unit 2B
NR2C	NMDA receptor sub-unit 2C
NR2D	NMDA receptor sub-unit 2D
NR3A	NMDA receptor sub-unit 3A
NR3B	NMDA receptor sub-unit 3B
NUDT9	Nudix-like domain
NUDT9-H	NUDT9-homology domain
PAR	poly-ADP (Adenosine Diphosphate) ribose
PARylate	poly-ADP (Adenosine Diphosphate) ribosylate

PARP/PARPs	poly-ADP (Adenosine Diphosphate) ribose polymerase/s
PARP-1	poly-ADP (Adenosine Diphosphate) ribose polymerase-1
PARP-2	poly-ADP (Adenosine Diphosphate) ribose polymerase-2
PARG	poly-ADP (adenosine diphosphate) ribose glycohydrolase
PC	Phosphatidyl choline
PC-PLC	Phosphatidyl choline-Phospholipase C
PBS	Phosphate buffered saline
PNS	Peripheral nervous system
PSD95	postsynaptic density protein 95
PSEN	presenilin
PSEN1	presenilin 1
P_2X_7	P2X purinoceptor 7
P_2X_{12}	P2X purinoceptor 12
qRT-PCR	Real-Time Quantitative Reverse Transcription PCR
RNA	ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPLP0	ribosomal protein lateral stalk subunit P0

SEM	standard error of the mean
shRNA	short hairpin RNA
siRNA	small interfering RNA
SSB/SSBs	Single strand break/s
SSBR	Single strand break repair
TGF-β	Tumor growth factor beta
TLR4	Toll-like receptor 4
TM	Transmembrane
TNF-α	Tumor necrosis factor alpha
TNFR	Tumor necrosis factor receptor
TREM2	Triggering receptor expressed on myeloid cells 2
TRP	Transient Receptor Potential
TRPC7	Transient Receptor Potential Channel Subfamily C Member 7
TRPM2	Transient Receptor Potential Melastatin 2
TRPM7	Transient Receptor Potential Melastatin 7
WT	wild-type
7PA2	Amyloid Precursor Protein (APP) V717F-expressing CHO cells
7PA2-CM	Conditioned media from Amyloid Precursor Protein (APP) V717F-expressing CHO cells

CHAPTER 1: INTRODUCTION

1.1 Alzheimer's Disease

1.1.1 Introduction to Alzheimer's Disease and it's prevalence

The German psychiatrist Alois Alzheimer first observed strange behavioral symptoms, including short-term memory loss in his patient Auguste Deter (Hippius and Neundörfer 2003). Upon her death, he carefully studied her brain and found some anomalies, termed as senile plaques and neurofibrillary tangles (NFT), which is now considered pathological hallmarks of Alzheimer's disease (Hippius and Neundörfer 2003). Alzheimer's disease (AD) is a common form of dementia that is associated with progressive decline in memory, cognition and loss of thinking ability. Upon progression of the disease, it can be serious enough to interfere with activities of our daily life (Tagarelli et al. 2006). Patients diagnosed with AD lose their ability to be self-reliant, thereby needing support of their family as a care-taker or from healthcare providers. While providing a caretaker is a financial burden to the family (D. M. Castro et al. 2010), what distresses them most is the attached emotional burden of witnessing the slow deterioration of patients memories.

It is very shocking to know that someone in the world develops dementia every 3 seconds. There were an estimated 46.8 million people worldwide living with dementia in 2015, while it is believed to have touched 50 million in 2017 (Prince et al. 2015). AD accounts for up to 60-80% of total dementia cases. In America alone, there were 5.7 million people diagnosed with Alzheimer's dementia. By 2020, this number is projected to rise to nearly 14 million (Alzheimer Association 2018). The magnitude of the disease can be imagined when we think that between 2000 and 2015, the deaths from heart diseases have reduced by 11%, while the death from Alzheimer's have increased by 123%. Currently, the caregivers provide an estimated 18.4 billion

hours of care valued at over $232 billion. Early and accurate detection of this disease is thought to provide the patient with a better chance of benefiting from available treatments (Alzheimer Association 2018). However, the current treatments available for Alzheimer's type dementia only provide symptomatic relief but does not treat the underlying cause. The therapeutics currently available for treatment of AD and the potential drugs in pre-clinical studies will be discussed in detail in section 1.6.

1.1.2 Alzheimer's Disease Pathology: Beta-Amyloid and Tau tangles

Subsequent studies of brain anomalies that were initially found by Alois Alzheimer demonstrated that the proteins isolated from these senile plaques composed of aggregates of amyloid-β (Aβ) of different molecular weight (Tagarelli et al. 2006). Later, it was also found that the aggregates of hyperphosphorylated tau resulted in the formation of neurofibrillary tangles (NFT). Classical view is that the deposition of amyloid beta plaques occurs in between the neuron/brain cells, whereas the neurofibrillary tangles are observed within the neuronal cell body. However, this distinction is not necessary as it is also shown that intracellular accumulation of amyloid beta contribute to disease progression (Bayer and Wirths 2010; LaFerla, Green, and Oddo 2007).

Historically, AD was considered a "proteinopathy", in which the accumulation of Aβ and hyperphosphorylated tau were thought to contribute to disease progression (Tagarelli et al. 2006). Accumulation of amyloid beta was observed in various brain areas including hippocampus, cortex, amygdala, and basal nucleus (Masters et al. 2015). The first sequence of amyloid-β (Aβ) was obtained in 1984 from the meninges of blood vessels of patients suffering from AD (Lindgren et al. 2010). Amyloid-β, being a small ~4.5 kDa peptide, is produced by cleavage of the membrane

bound amyloid precursor protein (APP). The 2 enzymes that catalyze the successive cleavage of APP to amyloid beta are the β- and γ-secretases. Variation in the C-term cleavage location by γ-secretase causes varying lengths of Aβ peptides to be present in the human brain (MacLeod et al. 2015) (Fig 1). The most common form of Aβ is $A\beta_{1-40}$ and $A\beta_{1-42}$, consisting of 40 and 42 residues respectively (MacLeod et al. 2015). The physiological function of these peptides remains poorly understood. Once these peptides are secreted to the extracellular space in the brain, they can exist in different forms (monomers, oligomers and insoluble fibrils), where amyloid beta oligomers (AβO) are found to be the most toxic form (Jun Wang et al. 2017) (Fig 1). Also, with $A\beta_{1-42}$ species known to be more prone to oligomerization than $A\beta_{1-40}$, a larger ratio of $A\beta_{1-42}$ to $A\beta_{1-40}$ has been used as a biomarker for diagnosis of AD (Mucke and Selkoe 2012). Interestingly, phagocytosis by the brain immune cells, microglia, has been shown to affect the clearance of Aβ from the brain (Jun Wang et al. 2017). Together, a global increase in Aβ production, a decrease in its clearance and an imbalance in the equilibrium of Aβ aggregation are all key factors which contribute to the buildup of Aβ in AD (Jun Wang et al. 2017).

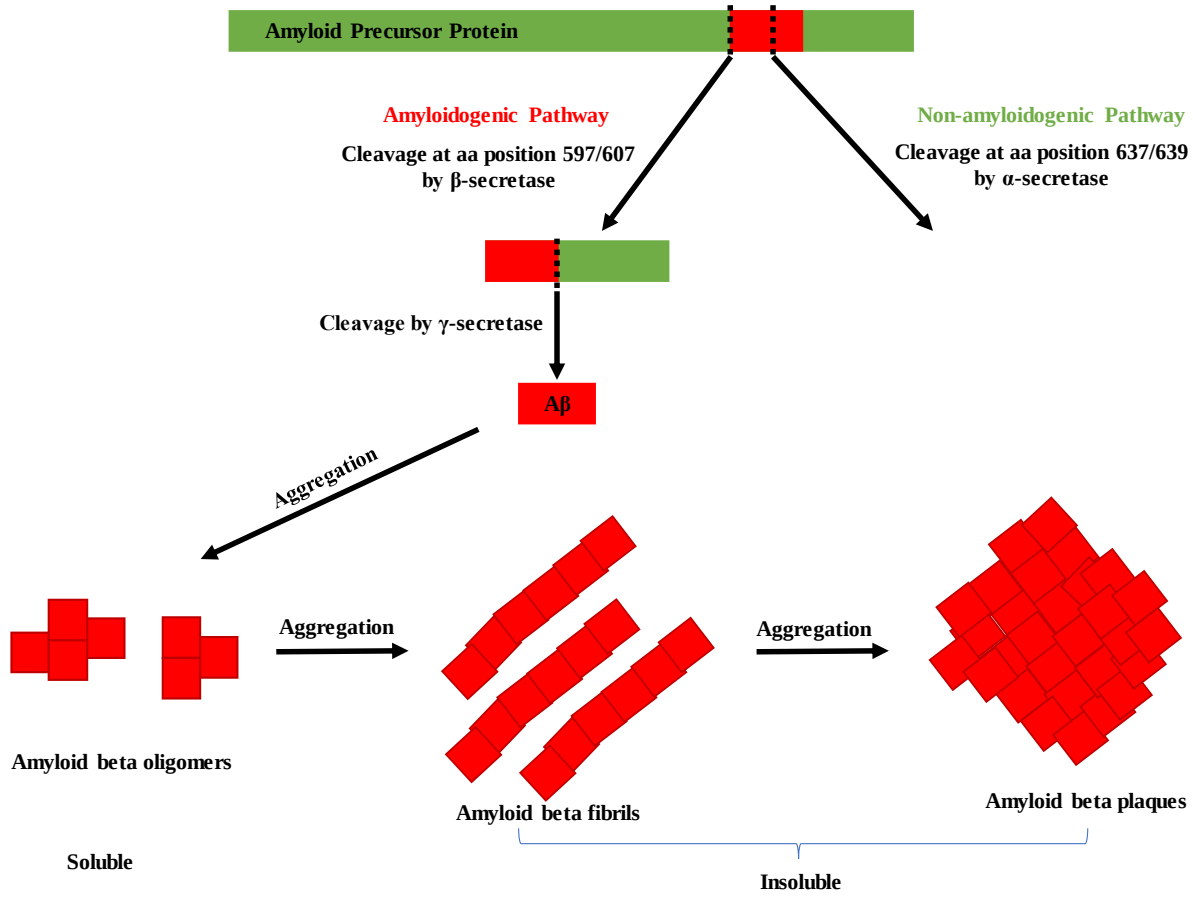

Figure 1: Formation of amyloid beta oligomers (AβO). Aβ is formed after successive cleavage of Amyloid precursor protein (APP) by enzymes beta secretase and gamma secretase. AB_{1-42} monomers are insoluble and can aggregate to form oligomers and senile plaques.

The other brain anomaly, neurofibrillary tangles (NFT) are caused by hyperphosphorylation of Tau protein. Belonging to the family of microtubule-associated proteins (MAPs), Tau proteins are mainly located in neurons and play a central role in assembling and stabilizing neuronal microtubule network under physiological conditions (G. Lee, Neve, and Kosik 1989; Tucker 1990). Six isoforms of tau exist ranging in lengths of between 352 and 441 amino acids. Tau is a phosphoprotein, requiring 3-4 phosphate groups to serve its function (Ferreira et al.

2011). However, under pathological conditions, increased levels of hyperphosphorylated tau proteins detach from microtubules and accumulate in the cytosol. Past studies have also shown increased levels of hyperphosphorylated tau protein accumulated in the neuronal cytosol of AD brain (Ferreira et al. 2011). This soluble hyperphosphorylated tau has been shown to disrupt microtubules by sequestering normal tau and other MAPs. The loss of normal tau protein function affects cellular functions of neurons, such as maintenance of appropriate morphology, axonal transport, synaptic dysfunction and neurodegeneration (Roy et al. 2005). Taken together, alterations to APP and tau proteins can induce its pathological effect on the human brain and lead to progression of Alzheimer's disease (Kametani and Hasegawa 2018).

1.1.3 Amyloid Hypothesis

It is suggested that Alzheimer's disease is triggered by impairment of APP metabolism, and that progresses through tau pathology. The relative ease of metabolism of APP and its rapid turnover makes impairment of APP metabolism a serious effect on cells (Oltersdorf et al. 1990). Increased APP and/or its C-terminal fragments induce axonal and synaptic defects (Rodrigues, Weissmiller, and Goldstein 2012; Rusu et al. 2007), thereby triggering the mis-localization of tau (Hochgräfe, Sydow, and Mandelkow 2013). Mis-localized tau proteins accumulate, form fibril seeds and this pathological tau induce further transport dysfunction (Rusu et al. 2007), creating a vicious circle and leading to tau accumulation. This led to a concept that Aβ and tau serve as respective trigger and bullet for AD pathogenesis (Bloom, George S. (Departments of Biology and Cell Biology, University of Virginia 2014). With Aβ acting as a trigger in AD pathogenesis, some of the key findings of amyloid hypothesis have been listed below.

I. The accumulation of Aβ can precede clinical manifestation of AD by up to 20 years. Biomarker studies of AD patients have demonstrated that a rise in the levels of Aβ are seen before the accumulation of tau, neuroimaging derived structural changes and memory deficits (Clifford R. Jack et.al 2017; Vemuri and Jack 2018).

II. Alteration in Aβ processing, increased total Aβ levels and an imbalance between the oligomerized and fibrillary forms have been detected in patients who possess autosomal mutations in APP and presenilin (PSEN) genes (Ryan, Natalie S 2013).

III. Genetic variants of APOE gene predict the increased risk of AD, where the APOE-ε4 variant is known to be involved in accumulation of Aβ at a significantly lower age (Dumanis et al. 2010; Guojun Bu 2009).

IV. Studies in several animal models have shown that accumulation of Aβ to be sufficient in inducing AD-like memory deficits. Genetic mouse models of AD, which express human APP and presenilin 1 (PSEN1) mutations have been generated to study age dependent increase in amyloid plaque deposition which lead to deficits in hippocampal dependent memory tasks such as spatial working memory (Ittner et al. 2010).

However, high profile clinical studies targeting Aβ and tau have been largely unsuccessful over the past two decades (Mehta et al. 2017). While this necessarily does not rule out the role of these two proteins in disease progression, but it is clear indication of other key players existence of multiple pathway in progression of the disease. These failures in clinical trials motivated scientists to evaluate AD from many different angles and formulate conflicting theories on the mechanisms that cause the onset of this disease. These theories include cholinergic hypothesis, aberrant processing of amyloid precursor protein, ‘traditional’ Aβ cascade hypothesis and tau hypothesis (Davies and Maloney 1976; Hardy and Allsop 1991; Maccioni et al. 2010; Jun Wang

et al. 2017). Although there are findings that provide some support for all these theories, there are also findings that contradict each one of them, creating an ambiguity in the field of AD research. Rather than basing clinical trials on unknown factor or factors causing AD pathology, careful analysis of disease mechanism of all theories of AD suggests neuroinflammation as a single uniting factor (Haneka 2015). Microglia and astrocytes are the two important brain cell types involved in neuroinflammation (Haneka 2015). More about these individual cell types and their role in neuroinflammation will be discussed in detail in the next section.

1.2 Glia and Neuroinflammation

1.2.1 Microglia Ontogeny, Morphology and Functions

The concept of microglia was first introduced by Pio del Rio-Hortega as a cellular component of central nervous system (CNS) in 1932 in a book chapter entitled "Microglia" in Cytology and Cellular Pathology of the Nervous System. Some of his contemporaries also referred to them as Hortega cells. Hortega defined microglia as immune cells having amoeboid morphology which enter the brain during early development, while transforming to a more branched and ramified phenotype in the more mature brain (F. de Castro et al. 2016). He also stated that microglia seem to be evenly distributed in the CNS, often showing no/little variation in morphology and seemingly occupying a defined territory. He further noted that upon any pathological insult to the brain, microglia alter their morphology from ramified to amoeboid morphology (F. de Castro et al. 2016).

Microglial progenitor cells are derived from the embryonic yolk-sac during early development. These precursor microglial cells then enter the brain parenchyma through the bloodstream and colonize the brain (Prinz and Priller 2014). The release of chemotactic cytokines

(chemokines) by neural progenitors and immature astrocytes are likely to mediate this recruitment (Bilbo 2013). Post-infiltration to the brain, microglial precursors reach their intended destination and continue to proliferate. A unique distinction of microglia from other immune cells of the body is that they are no longer replaced from the periphery, but rather rely on their own proliferation and their long lifespan in order to maintain their numbers within the CNS (Ajami et al. 2007; Prinz and Priller 2014).

Microglia contribute to approximately 5-20% of the total cells in the brain. Microglia play key roles in both immune function and in homeostasis in the CNS (Chen and Trapp 2016; Davalos et al. 2005; Nimmerjahn, Kirchhoff, and Helmchen 2012). Microglia, in the present day are usually classified into 3 distinct morphologies – ramified, hypertrophic and amoeboid (Fig 2). Under physiological conditions, microglia are noted to be in their 'ramified' or resting morphology, where they have a small cell soma or cell body, but exhibit thin, elongated and branched processes that extend in all directions (Squire and Berg 2008) (Fig 2). Here, microglia survey the brain microenvironment using their highly branched processes (Q. Li and Barres 2018), but rarely overlap or touch processes of other microglia and forms an organized grid-like network of surveying cells in the brain (Paolicelli and Gross 2012). Apart from surveying the brain microenvironment, these processes are also responsible for maintaining brain homeostasis. Sensing physiological or pathological changes through their cell-surface receptors, microglia respond by releasing various factors and is noticed by their morphological transformation to hypertrophic state (Hellwig, Heinrich, and Biber 2013). In this state, microglia with enlarged cell body/soma, thick processes and fewer processes are observed (Fig 2). This is an intermediate state before they finally transform to amoeboid morphology having few or no processes, but a very

enlarged cell body (Squire and Berg 2008) (Fig 2). However, microglial morphology should not be perceived as an indicator of its functional state.

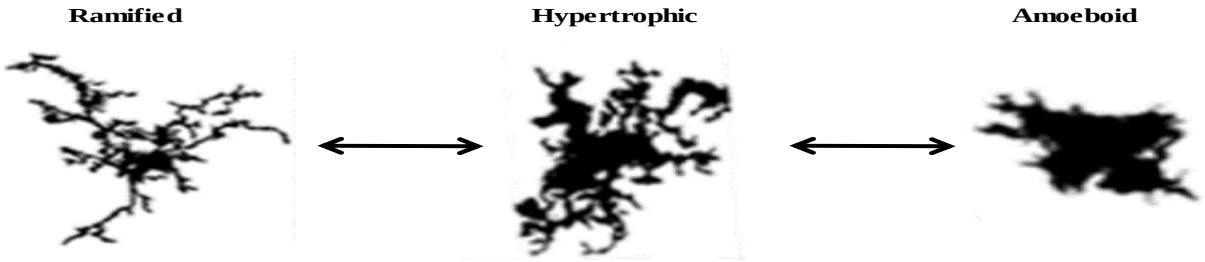

Figure 2: Microglial Morphology. Upon sensing changes in the brain microenvironment through a host of cell-surface receptors, microglia possess the ability to transform from ramified microglia to amoeboid microglia via hypertrophic state (Adapted and modified from Jelinek 2013).

Microglia under any pathological or physiological insult produces pro-inflammatory cytokines, chemokines, proteases and redox factors that help defend the tissue against damage (Kettenmann et al. 2011). Upon resolution of injury, anti-inflammatory cytokines and growth factors promote tissue repair and extracellular matrix reconstruction. Microglia is also known for its ability to phagocytose dead, dying and sometimes healthy live cells in the developing and adult brain (Fu et al. 2014).

Microglia are in close contact with neurons as well as oligodendrocytes and astrocytes. While astrocytes contribution to synapse formation, maintenance and elimination has been well documented, recent research has emphasized the role of microglia in regulating neuronal synaptic connections (Wu et al. 2015). Direct and transient connection of microglial processes with neuronal synapses are observed, where, the frequency of these connections is related to basal neuronal activity (Wu et al. 2015). Emerging evidence for this includes the *in vivo* engulfment assays where presynaptic and postsynaptic elements were found inside microglial lysosomes using

the high-resolution electron microscope (Hong, Dissing-Olesen, and Stevens 2016). Neuronal activity regulated the microglial engulfment of synaptic input, where microglia preferentially phagocytosed fewer active inputs (Hong, Dissing-Olesen, and Stevens 2016; Kano and Hashimoto 2009). This reciprocal neuron-microglia communication is mediated by numerous soluble factors, extracellular vesicles (EVs) as well as contact-dependent mechanisms and is essential for adaptive neuroplasticity and learning (Szepesi et al. 2018).

1.2.2 Microglial Phenotypes

Traditional literature on microglia classifies it into 3 activation phenotypes based on their associated functions – M0, M1 & M2. Upon sensing any pathological insult/physiological through their cell-surface receptors, microglia polarize into M1 phenotype. M1 phenotype is the classical pro-inflammatory phenotype, or the innate immune response associated with the phagocytosis, release of reactive nitrogen species (RNS) and reactive oxygen species (ROS), along with a host of pro-inflammatory cytokines like TNFα, IL-6, and IL-1β (Colton 2009). Microglia then transition towards the M2 activation state, also called the immune resolution state. M2 state is associated with the release of various anti-inflammatory cytokines like tumor growth factor-β (TGF-β), interleukin-4 (IL-4), interleukin 10 (IL-10) and interleukin 13 (IL-13) (Colton 2009). A classical marker for M1 activation phenotype is the expression of inducible nitric oxide synthase (iNOS)/nitric oxide synthase-2 (NOS2) gene, whereas M2 phenotype can be identified by markers such as Arginase1 (AG1), chitinase-like 3 (ChiL3), mannose receptor (Cluster of Differentiation 206, CD206) and/or lack of iNOS (Colton 2009). M2 microglia can be further classified into 2 types based on its specific functions – M2a phenotype and M2b phenotype. M2a phenotype is called alternate activation that is associated with tissue repair and extracellular matrix reconstruction through release of anti-inflammatory cytokines. M2b phenotype is called acquired

activation that is responsible with immune suppression, apoptotic cell uptake and production of TGF-β (Colton 2009; Keren-Shaul et al. 2017).

Activation Phenotype	Inflammatory mediators	Functions
Resting State (M0)	-	Surveying brain microenvironment for changes
Classical Activation State (M1)	TNFα, IL-6, IL-1β	Tissue defense, proinflammatory cytokine production, NO production
Alternate Activation (M2a)	IL4, IL13	Tissue repair, anti-inflammatory cytokine production, ECM reconstruction
Acquired Activation (M2b)	TGF-β, IL10	Immunosuppression and apoptotic cell uptake

Table 1: Microglial Phenotypes and its associated function. Microglial phenotypes namely – resting state (M0), Classical activation state (M1), alternate activation state (M2a) and acquired activation state (M2b) and its associated inflammatory mediators and functions are listed here. Table compiled and modified from (Colton 2009).

This classical grouping of microglia into different phenotypes helps in understanding its various functions in the CNS. However, recent studies have shown that microglial phenotypes are more heterogenous than previously thought, and that microglial phenotype dictates function (McQuade and Blurton-Jones 2019; Weitz and Town 2012). Interestingly, recent studies involving trigger-receptor on myeloid cells 2 (TREM2), which is a receptor highly associated with microglial phagocytosis and inflammatory functions, have shown that microglia can perform M1 like functions (e.g. phagocytosis) yet release anti-inflammatory mediators (Bisht et al. 2016; S. E.

Hickman and El Khoury 2014). This led to transcriptome-based studies in AD-like conditions to elucidate the expression profile of microglia, which resulted in the characterization of two hybrid phenotypes of microglia – "dark microglia" and "disease associated microglia (DAM)". Dark microglia exhibit an activated like morphology, release anti-inflammatory mediators such as TGFβ, and has also been shown to facilitate Aβ plaque removal, and help attenuate AD-like pathologies suggesting a neuroprotective role (Bisht et al. 2016; S. Hickman et al. 2018). Disease associated microglia (DAM) are found in abundance in AD brains where they exhibit dysregulated expression of sensing, housekeeping, and host defense genes (Keren-shaul et al. 2017; Krasemann et al. 2017) (Keren-Shaul et al. 2017, Krasemann et al. 2017, Hickman et al. 2018). These DAM have been associated with enhanced release of pro-inflammatory mediators, which have been shown to disrupt Aβ clearance and facilitate the hyperphosphorylation of tau. Combined, this suggests that while efforts are ongoing to constantly group microglia based on its function/gene expression profile, it is difficult task and is often misleading due to the heterogeneity.

1.2.3 Contribution of glial cells to neuroinflammation

As stated earlier in section 1.1.3, neuroinflammation is the single uniting factor for all theories of AD and glial cells – astrocytes and microglia play an important role in neuroinflammation. Astrocytes are the most abundant glial cells in the human brain involved in gliotransmission, neurotransmitter uptake and production of trophic factors for neuronal growth and survival (Khakh and Sofroniew 2017). In astrocytes, the presence of Aβ activates different cell receptors and intracellular signaling pathways, including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway, responsible for the transcription of pro-inflammatory cytokines and chemokines (Batarseh et al. 2016). The release of these pro-inflammatory agents causes cellular damage and is suggested to even stimulate the production of Aβ in astrocytes (Zhao, O'Connor,

and Vassar 2011). In addition, the presence of Aβ has also been shown to disrupt glutamate uptake and alter calcium signaling in astrocytes (Zhao, O'Connor, and Vassar 2011). On the other hand, microglia, in the presence of Aβ drives neuroinflammation causing release of various neuroinflammatory mediators (pro-inflammatory cytokines, reactive oxygen species, reactive nitrogen species) and promoting neuronal cell death (Cai, Hussain, and Yan 2014).

During normal conditions in the brain, end-feet of astrocytes surround the endothelium and basement membrane of blood brain barrier (BBB), thereby providing physical, biochemical and metabolic support to those cells (M.A. and W.A. 2013). BBB is a highly selective semipermeable structural and chemical barrier which ensures a stable internal environment of the brain and prevents foreign objects invading the brain tissue (M.A. and W.A. 2013). Similarly, microglia interact with BBB by expressing genes that promote microvasculature development and also release trophic factors, important for angiogenesis and the support of vascular structures (da Fonseca et al. 2014).

However, microglia in the presence of Aβ produces matrix metalloproteinases and reactive oxygen species (ROS), which damage the integrity of the blood brain barrier and resulting in BBB breakdown. Moreover, microglial pro-inflammatory responses such as releasing tumor necrosis factor-α (TNFα), interleukin-1β (IL-1β) and interleuikin-6 (IL-6) can affect the endothelial cells, astrocytes and pericytes leading to alteration in BBB permeability and infiltration of peripheral immune cells to the brain (da Fonseca et al. 2014). However, BBB dysfunction in Alzheimer's disease is somewhat still controversial.

On the contrary, neuroinflammation is not just an innate immune response activated by deposition of brain anomalies but contribute immensely to the progression of disease (Haneka 2015). Clinical investigations from patients suffering with mild cognitive impairment, which

precedes dementia stage of Alzheimer's disease, support an early and substantial involvement of neuroinflammation in disease progression (Haneka 2015). The role of microglia in driving neuroinflammation has been well documented in the recent years and explained in detail in the next section.

1.2.4 Microglia drives neuroinflammation in AD

Inflammation is often thought to play a casual role in disease progression. However, in Alzheimer's disease, neuroinflammation is not a passive system activated by emerging senile plaques and neuro-fibrillary tangles, but instead contributes as much (or more) to pathogenesis as do plaques and tangles themselves (Haneka 2015). Hence, microglia are thought to be the drivers of neuroinflammation (X.-H. and Wang T.-L. AO - Wang 2018). In AD, soluble AβO and Aβ fibrils is said to interact with microglia via their cell-surface receptors and initiate an inflammatory signaling cascade culminating in the release of various inflammatory cytokines, proteases, ROS and RNS (Colton 2009). Note that initial release of inflammatory mediators is a protective response, where microglia attempts to combat pathological insults, followed by recruitment of growth factors for immune resolution (Colton 2009).

During AD, both microglia and astrocytes act as the chief sources in production of cytokines (Mrak, Sheng, and Griffin 1995). Interestingly, microglia activation is both characterized by and modulated by cytokines. Cytokines contribute to nearly every aspect of neuroinflammation, including proinflammatory and anti-inflammatory processes, bystander neuronal injury, chemoattraction, and response of microglia to Aβ deposits (Haneka 2015). In fact, ageing TgAPPswe and PSAPP transgenic mice which are mouse models for early onset of AD, shows that increased concentrations of Aβ caused elevations in the level of proinflammatory

cytokines, including TNFα, IL-6, interleukin 1α (IL-1α), and Granulocyte-macrophage colony-stimulating factor (GM-CSF) (Patel et al. 2005). This suggests that Aβ drives the production of pro-inflammatory cytokines in Alzheimer's disease.

Particularly, $A\beta_{1-42}$, has a strong tendency to form soluble oligomers and fibrils, wherein, they interact with ensembles of microglial receptors and macromolecules (Reed-Geaghan et al. 2009). Toll-like receptor 4 (TLR4) act in concert with these immune receptors (Lue et al. 2001), leading to production of pro-inflammatory cytokine like IL-1β and TNFα (Heneka, Kummer, and Latz 2014). In fact, elevated concentrations of the TNFα and decreased concentrations of TGF-β in the CSF is suggested to impair neuronal function even before leading to structural changes, as shown by suppression of long-term potentiation (LTP) of synaptic transmission (Haneka 2015). This impairment in ratio of pro-inflammatory cytokines to anti-inflammatory cytokines has been observed to develop higher risk for conversion from mild cognitive impairment to the dementia stage of Alzheimer's disease patients (Heneka et al. 2013).

A prominent pro-inflammatory mediator observed during neuroinflammation is nitric oxide (NO) through activation of inducible nitric oxide synthase (iNOS)/NOS2 in microglia and astrocytes (Yuste et al. 2015). Nitric oxide (NO) makes for a powerful signaling molecule with far-reaching cellular consequences that can be both protective and maladaptive (Balez and Ooi 2016). At lower concentrations, NO inhibits cytochrome oxidase in competition with oxygen and may play an important role in the physiological regulation of cellular energy metabolism (Brown 2007). On the other hand, higher concentrations of NO inhibit respiratory chain complexes by nitrosylation of critical tyrosine residues or oxidation of protein thiols (D. Butterfield et al. 2007).

In fact, upregulation in level of iNOS has been observed in patients with Alzheimer's disease (Vodovotz 2004). Similarly, Nicotinamide adenine dinucleotide phosphate (NADPH)

oxidase has been shown to be highly expressed enzyme in microglia. This enzyme is rapidly activated by deposition of Aβ, resulting in production of hydrogen peroxide, which further promotes microglia activation (Choi et al. 2012). Combined, increased levels of iNOS induced NO and superoxide from NADPH oxidase results in the production of peroxy nitrate, causing irreversible inhibition of mitochondrial respiration and damage to various mitochondrial components via oxidizing reactions (Mander and Brown 2005). Therefore, NO and its derivative peroxynitrite can inhibit mitochondrial respiration by various mechanisms, inducing neurodegeneration and neuronal cell death. Nitration at key tyrosine residues within Aβ peptide has been shown to increase the propensity of Aβ to aggregate and has been identified in the core of amyloid plaques (Kummer et al. 2011).

In addition to release of these inflammatory mediators, phagocytosis by microglia upon is initially a protective response in response to neuroinflammation. Microglia also start to engulf Aβ fibrils by phagocytosis and these fibrils enter the endo-lysosomal pathway (Ries and Sastre 2016; Weitz and Town 2012). While soluble Aβ is susceptible to degradation by various extracellular secreted proteases, fibrillar Aβ are mostly resistant to enzymatic degradation (Ries and Sastre 2016). Impaired phagocytic clearance of Aβ fibrils leads to accumulation of more Aβ in the brain, thereby enhancing detrimental effects and is suggested to be one of the factors that drive microglia to an uncontrolled inflammatory state or chronic neuroinflammation (Haneka 2015).

Interestingly, studies from our lab have demonstrated that the nuclear enzyme poly (ADP-ribose) polymerase-1 (PARP-1) plays an essential role in mediating microglial pro-inflammatory activation in response to Aβ pathology, and that PARP-1 depletion or inhibition results in microglial anti-inflammatory functions in response to Aβ pathology (T. Kauppinen et al. 2011). With recent research on DAMs, this study becomes all more relevant where PARP-1 may play an

essential role in driving the activation of this pro-inflammatory 'DAM' microglial phenotype, and that depletion of PARP-1 may modulate microglial phenotypes towards anti-inflammatory 'dark microglia' (S. E. Hickman and El Khoury 2014). Also, recently TNFα has been shown to activate PARP-1 by a pathway independent of DNA damage (Vuong et al. 2015). Hence, PARP-1 which plays a key role in neuroinflammation will be explored in detail in the next section.

1.3 PARP-1

Nuclear enzyme poly (ADP-ribose) polymerases (PARPs) are multidomain proteins, where all members share the same catalytic domain with similar structural homology. PARPs are interchangeably used with another term called ADP-ribosyl transferases (ADRTs) (Bai 2015). The PARP enzyme cleaves NAD^+ to nicotinamide (NAM) and Adenosine diphosphate-ribose (ADPR). One or more ADPR units are coupled onto key amino acid residues (glutamate, aspartate or lysine) of acceptor proteins. This is termed as initiation, followed by elongation and branching. These could be either mono, oligo or poly ADP-ribosyl PARylation depending on the number of ADPR units attached. There are 16 classes of PARPs in mice and 17 classes in humans with widespread functions that are vital for homeostasis (Bai 2015).

Poly (ADP-ribose) polymerase-1 (PARP-1) was the first enzyme identified to poly-ADPR ribosylate (PARylate) proteins. PARP-1 consists of three main domains- the N-terminal DNA-binding domain (DBD), the auto-modification domain (AMD) and the C-terminal catalytic domain (Fig 3). It is important to note here that the extent of PARP activity, both basal and stimulated is contributed primarily by PARP-1 (85%-90%) as observed in PARP null mice (Shieh et al. 1998). Poly (ADP-ribose) polymerase-2 (PARP-2) contributes to about (10%-15%), while the rest of the PARPs contribution seem negligible compared to total (Szántó et al. 2012). While the roles of

catalytic domains and associated functions will be more focused in this chapter, it is important to note that PARPs also contain other domains that are involved in protein-protein interactions and protein-nucleic acid interactions (Bai 2015) (Fig 3). With greater extent of PARP activity contributed by PARP-1, the upcoming sections will be focused more on the role of PARP-1 in context of the brain.

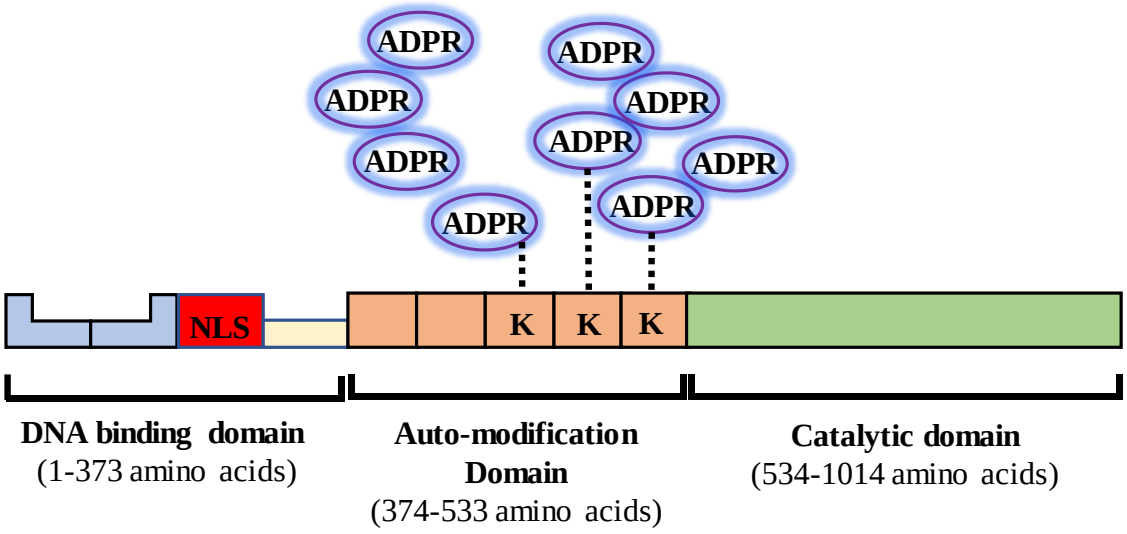

Figure 3: Structure of PARP-1

Structure of a PARP-1 enzyme (~116 kDa) composed of 1014 amino acids. PARP-1 has a DNA binding domain consisting of nuclear localization signal (NLS) domain, followed by the auto-modification domain and enzyme catalytic domain.

1.3.1 PARP-1 in DNA Repair

Various exogenous (Ultraviolet (UV) component of sunlight, genotoxic chemicals, ionizing radiation) and endogenous factors (ROS, RNA) are capable of inducing DNA damage, resulting in activation of PARP-1 enzyme (Ray Chaudhuri and Nussenzweig 2017). The role of PARP-1 in DNA damage response (DDR) is widely studied where PARP-1 is rapidly recruited to sites of DNA damage through its DNA-binding ability (D'Amours et al. 1999). This stimulates the

catalytic activity of PARP-1, resulting in auto-PARylation on itself and other acceptor proteins (Gagné et al. 2008). This long branched PARylation (often more than 200 ADPR units) are used as anchors by proteins involved in DDR and DNA metabolism to bind via non-covalent interactions (Ray Chaudhuri and Nussenzweig 2017). Similarly, DNA lesions formed by spontaneous single strand breaks (SSBs) are rapidly detected and bound by PARP-1 (Ray Chaudhuri and Nussenzweig 2017). PARylation has in fact shown to increase SSB repair mechanism, possibly by promoting the accumulation of single strand break repair (SSBR) components and/or their stabilization at SSBs. Similarly, PARP-1 also acts as a sensor of double strands breaks (DSBs) and involved in the early recruitment of DSB repair (Kim, Zhang, and Kraus 2005).

It is important to note that despite extensive role for PARP-1 in DNA repair, deletion of PARP-1 in several studies have shown non-lethal effects (Bai and Canto 2012). Although PARP-1 is the dominant member of the PARP family of proteins, some of its functions in DNA repair are shared and overlap with those of PARP-2 (Ray Chaudhuri and Nussenzweig 2017). Also note that, PARP-1 merely assists in DNA repair process and is not entirely responsible for DNA repair. While PARP-1 deletion or inhibition did not cause lethal effects, delayed activation of DDR proteins such as phosphorylated histone H2A histone family member X (H2AX) and Tumor protein p53 (p53) were observed (Bai 2015). In fact overexpression of PARP-1 has been observed in various tumor types, such as, brain, lung, breast, ovary, skin where upregulated PARP-1 can enhance anti-apoptotic properties of tumors resulting in resistance to DNA damaging therapeutics (Murnyák et al. 2017). This has led scientists to now explore the role of PARP inhibitors in treating tumors where inhibition of cellular responses to DNA strand breaks in tumor cells do not occur, leading to cell death.

1.3.2 PARP-1 activation in oxidative stress and cell death

Reactive oxygen and reactive nitrogen species can induce damage of nucleic acids, proteins and lipids (Vida et al. 2017). In case of DNA damage, this leads to activation of PARP-1 to facilitate DNA repair machinery. In addition, stress-activated kinase cascade co-operate with DNA damage to activate PARP-1 (Bai 2015). Upon sustained activation of PARP-1, excess levels of Nicotinamide adenine dinucleotide (NAD^+) is consumed leading to requirement of NAD^+ re-synthesis. This depletes cellular energy reserves that then forces cells into a metabolic collapse, leading to mitochondrial damage and cell death.

Specifically, this leads to loss of mitochondrial membrane and dysfunction of electron transport chain (complex 1) (Liaudet et al. 2015). Consequently, there is loss of adenosine tri-phosphate (ATP) production, superoxide production and the destruction of mitochondrial architecture (Liaudet et al. 2015). Combined, this cellular dysfunction acts as a major pathway in pathogenesis, frequently accompanied by PARP-regulated inflammation (Basello and Scovassi 2015). PARP-1 mediated cell death and mitochondrial dysfunction hence affect all major organ systems, in particular – brain, resulting in ischemia-reperfusion injury, inflammatory diseases, and neurodegenerative diseases such as Parkinson's, Huntington's disease and Alzheimer's disease.

1.3.3 Upstream intracellular contributors of PARP-1 activation in AD

The first report of PARP-1's involvement in Alzheimer's disease was in 1999 by Love et al., where they showed high levels of PAR in AD human brains by immunostaining (Love, Barber, and Wilcock 1999). However, the upstream activators of PARP-1 in neurodegenerative disorders were poorly understood at that point. The presynaptic vesicles of a subset of glutamatergic axon terminals in human brain have zinc concentrated in them (Beaulieu, Dyck, and Cynader 1992).

During normal neuronal activity, vesicular zinc is released into the extracellular space in a calcium-dependent manner (Howell, Welch, and Frederickson 1984). However, in pathological conditions like stroke, the release of zinc is exacerbated as protein-bound zinc (Frederickson, Koh, and Bush 2005). Detection of this in microglia leads to activation of NADPH oxidase and superoxide production, culminating in PARP-1 activation (T. M. Kauppinen et al. 2008).

Several studies have shown that neuronal release of fractalkine and ATP during AD activates receptors like CX3C chemokine receptor 1 (CX3CR1) and P2X purinoceptor 7 (P_2X_7) causes further activation of mitogen-activated protein kinases (MAPKs) through a signaling cascade involving p38 MAPKs and the extracellular signal-regulated kinases (ERKs) (Irving and Bamford 2002; Vuong et al. 2015). p38 MAPKs have been shown to upregulate transcripts encoding pro-inflammatory proteins, blocking of which have significantly decreased number of activated microglia in ischemic brain (T. M. Kauppinen et al. 2006). These MAPK/ERK proteins contribute in a much larger way to neuroinflammation through activation of PARP-1 (T. M. Kauppinen et al. 2006; Vuong et al. 2015).

Past studies had shown impaired PARP-1 activation upon lack of phosphorylation at its Ser372 and Thr373 sites (Ba and Garg 2011). These amino acids were replaced with glutamate, thereby mimicking constitutive phosphorylation that increased PARP-1 activity and even eliminated the effect of ERK1/2 inhibitors on PARP-1 activation (T. M. Kauppinen et al. 2006). In fact, Voung et.al. used PARP-$1^{-/-}$ astrocyte cultures were transfected with WT and mutant constructs (at ERK2 phosphorylation sites) and assessed NF-κB transcriptional activation using a lentivirus-delivery of downregulation enhanced green fluorescence protein (dEGFP) reporter gene that is activated by the p65 subunit of NF- κB (Vuong et al. 2015). TNFα induced robust activation was observed in cells transfected with wild-type (WT) PARP-1, but not in non-transfected or

mutant PARP-1 transfected astrocytes, thus confirming a requirement for PARP-1 enzymatic activity in this process.

Another key upstream contributor for activation of PARP-1 is Ca^{2+} entry into microglia. This was shown by Voung et al, where TNF-α activated microglial tumor necrosis factor receptor (TNFR), leading to calcium influx into the cell (Vuong et al. 2015). While previous studies had shown classical PARP-1 activation by DNA damage, this was the first study that showed TNFα induced PARP-1 enzymatic activation to be independent of DNA damage (Vuong et al. 2015). TNFα induced activation of enzyme phosphotidyl choline-specific phospholipase (PC-PLC) leading to cleavage of phosphotidyl choline (PC) to diacyl glycerol (DAG). These studies combine show that MAPK/ERK signaling pathway is crucial for enzymatic activation of PARP-1 and release of inflammatory mediators through NF-κB signaling pathway.

1.3.4 Microglial PARP-1 and NF-κB driven pro-inflammatory response in AD

Prominent PARP-1 activation *in vivo* was demonstrated by Martire et al., where they used a transgenic mouse model with early onset of AD to show increase in PARP-1 activity. Early amyloid deposit occurring in the hippocampus and entorhinal cortex of 3-month-old mice showed significant PARP-1 activation when measured using immunoblot (Martire et al. 2013). Another transgenic mouse model study bearing mutations responsible for Aβ deposition was cross bred with $PARP^{-/-}$ mice, leading to decreased microglial activation and neurotoxicity (T. Kauppinen et al. 2011). *In vitro* poly-ADP-ribose (PAR) accumulation was observed in primary microglial cultures within 1-hour of Aβ stimulation. Co-incubation with PARP-1 inhibitor PJ-34 stopped PAR formation along with impaired morphological transformation in microglia treated with Aβ

(T. Kauppinen et al. 2011). This work presented the therapeutic potential of PARP-1 inhibitor for treatment of Alzheimer's disease.

A chief co-conspirator along with microglial PARP-1 in chronic neuroinflammation is nuclear factor-kappa B (NF-κB) (Valerio et al. 2006). The family of NF-κB factors include five members namely p50, p65/RelA, c-Rel, p52 and RelB (Martire, Mosca, and Erme 2015). In non-stimulated microglia, NF-κB sub-units are retained in the cytoplasm by interaction with the inhibitory I-kappa-B (IkB) proteins. Upon stimulation, IkBs are phosphorylated by main I-kappa-B kinases (IKKs) and degraded to allow NF-κB nuclear translocation (Valerio et al. 2006).

Following nucleolar translocation, it forms transcription complex and binds to DNA. PARP-1 has been shown to be essential for enhancement of NF-κB binding to DNA (Oliver et al. 1999). This increased interaction is followed by increased surface expression of cluster of differentiation 11a (CD11a) and cytokine release. Most notably, NF-κB binding to DNA induces nitric oxide release through upregulation of inducible nitric oxide synthase gene (iNOS/NOS2) (Aktan 2004). Increased acetylation has also been shown to promote PARP-1 mediated NF-κB transcriptional activity (Tiina M. Kauppinen, Li Gan 2013).

However, nucleolar translocation and binding to DNA is necessarily not sufficient for NF-κB-driven gene transcription of pro-inflammatory cytokines, as simultaneous binding of repressors would hamper this process (Tiina M. Kauppinen, Li Gan 2013). Recent reports provide further proof that both genetic and pharmacologic inhibition of PARP-1 allow nuclear translocation of NF-κB, which prevented transcription of pro-inflammatory cytokines, while anti-inflammatory cytokines were not inhibited (Vuong et al. 2015). Further, astrocytes transfected with mutant PARP-1 (mutation at ERK phosphorylation sites) failed to activate NF-κB, confirming that PARP-1 activity is necessary for NF-κB activation and its associated pro-inflammatory responses.

1.3.5 PARG

Poly-ADPR-ribose (PAR/poly-ADPR) formed by PARP-1 activation has a rapid turnover. The hydrolysis of both linear and branched PAR specifically occurs by breaking of ribose-ribose bonds to monomeric ADPRs by poly (ADPribose) glycohydrolase (PARG), an enzyme with exo- and endoglycosidase activities (Krishnakumar and Kraus 2010). ADP-ribose monomers can be re-used as a substrate for the ATP production. PARG is encoded by a single gene in humans producing several isoforms of enzyme with various sub-cellular localizations (Krishnakumar and Kraus 2010). Due to existence of multiple isoforms and embryonic lethality of mice deficient in all of them has made the understanding of this enzyme complicated (Falsig et al. 2004). Thus, PARG is a physiological counterpart of PARP enzymes and is involved in PAR metabolism, thereby serving large number of different biochemical machineries in the cell. Apart from serving as a substrate for ATP production, ADPR monomers produced by PARG activity acts as endogenous agonist to a microglial ion channel called Transient Receptor Potential Melastatin 2 (TRPM2) (Sumoza-Toledo and Penner 2011), and will be discussed in detail in the next chapter.

1.4 TRPM2

1.4.1 Structure of TRPM2

Transient receptor potential (TRP) channels are a wide family of integral membrane ion channel proteins. This protein family was named based on structural homology of its members to the TRP protein of Drosophila melanogaster (Montell and Rubin 1989). To date, 28 TRP channels have been discovered in mammals, which are divided into six subfamilies including the canonical (C), vanilloid (V), polycystin (P), mucolipin (ML), ankyrin (A), and melastatin (M) subfamilies (Nilius and Owsianik 2011). There is substantial diversity in expression patterns, activation

mechanisms, regulatory mechanisms as well as cation permeability in the TRP family (Jiang et al. 2010). Specific ligands, voltage, temperature and/or mechanical force can activate TRP channels (Sumoza-Toledo and Penner 2011). Further, permeability and selectivity of TRP channels to Na^+, K^+, Ca^{2+} and Mg^{2+} vary substantially between family members.

Transient receptor potential melastatin 2 (TRPM2) is a non-selective cation channel of the Melastatin (M) subfamily that is permeable to calcium (Ca^{2+}). TRPM2 was also previously designated Transient Receptor Potential Channel Subfamily C Member 7 (TRPC7) and long transient receptor potential channel Subfamily C Member 2 (LTRPC2). TRPM2 is located on chromosome 21q22.3 in humans (Nagamine et al. 1998). The gene contains 33 exons over 90 kilobase (kb) and produces a 5876 base-pair (bp) full-length transcript which codes for a 1503 amino acid protein. Messenger RNA (mRNA) levels of TRPM2 demonstrates a near ubiquitous expression in all tissues examined, apart from bone and cartilage. However, it is important to note that TRPM2 mRNA is most abundant in the brain (E Fonfria et al. 2008) and is highest in microglia among other brain cells (Y. Zhang et al. 2014). In addition to the full-length form of the protein, several splice variants of TRPM2 have also been identified (Jiang et al. 2010).

TRPM2 channel is a homo-tetramer with each sub-unit possessing 6 transmembrane domains, with a pore-forming re-entry loop between transmembrane domains 5 and 6 (Fig 4). The amino and carboxy terminal regions of the protein are cytoplasmic and contain regulatory features for the channel. The amino-terminus of TRPM2 is approximately 700 amino acids long and contains 4 homologous domains, also called the multiple homology domain (MHD) (Sumoza-Toledo and Penner 2011) (Fig 4). While this domain is largely conserved structural motif across all TRPM family members, significance of the MHD domains in TRPM2 function or expression remains to be established (Sumoza-Toledo and Penner 2011). The homology domain and a

calmodulin (CaM) binding IQ-like motif play a key role in modulating channel activation (Tong et al. 2006). Mutation of the IQ-like CaM binding motif and pharmacological inhibition of CaM has separately been shown to abrogate channel function, demonstrating an important role of Ca^{2+}/CaM in channel activation.

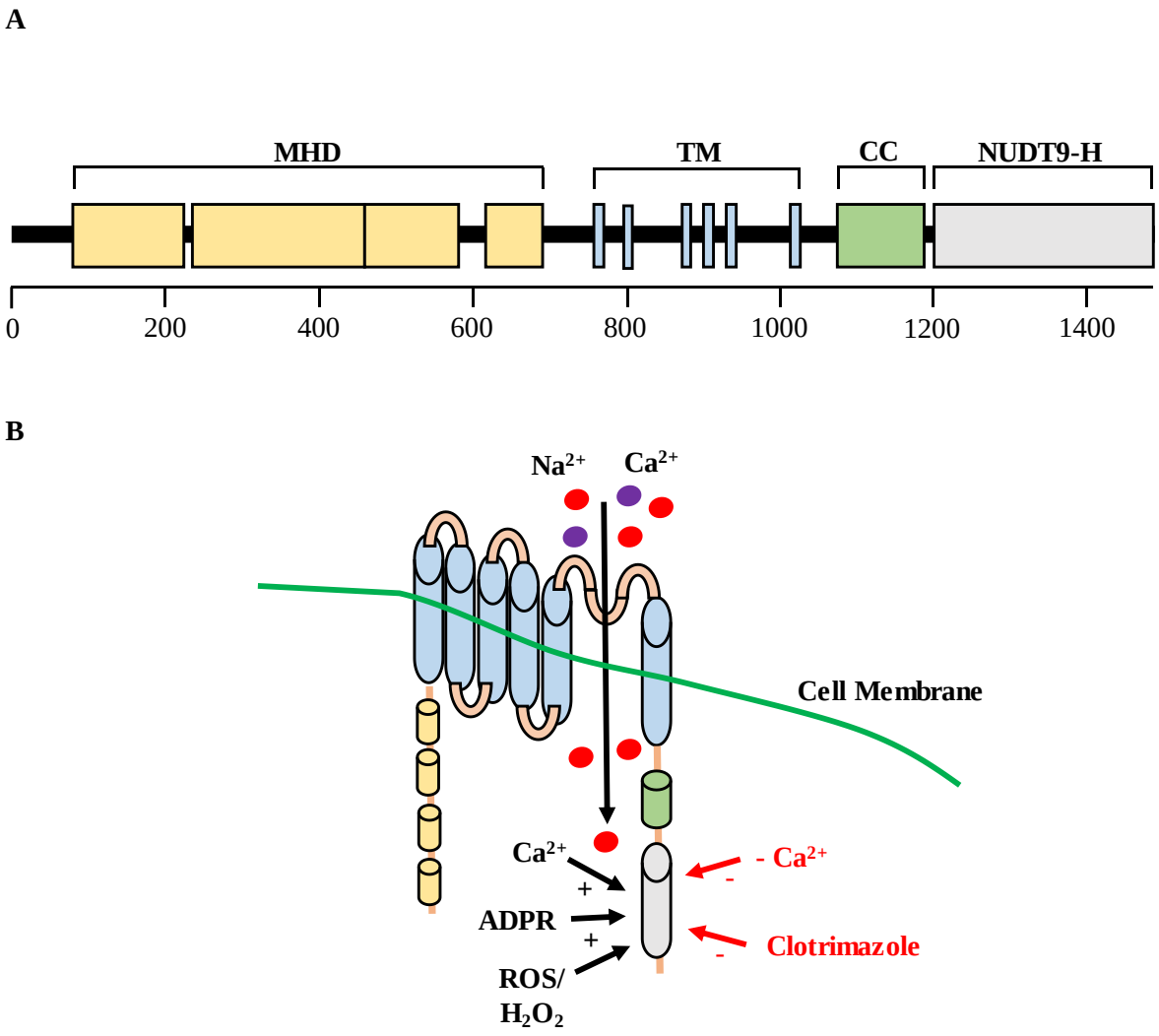

Figure 4: Structure of TRPM2 Channel. (A) Structure of a human TRPM2 channel (~170 kDa) composed of 1503 amino acids. N-terminal is composed of four homologous domains (MHD) of unknown function and a calmodulin (CaM) binding IQ-like motif, followed by six transmembrane (TM) segments. C-terminal contains a TRP box and a coil–coil domain (CC), and a C-terminal

adenosine diphosphate ribose (ADPR) pyrophosphatase domain (Nudix-like domain or NUDT9 homology domain, NUDT9-H). **(B)** Transmembrane topology of TRPM2 channel showing its permeability to Ca^{2+} and Na^{+}.

In the C-terminal portion of TRPM2, the coiled-coiled motif is predicted to participate in interactions between TRPM2 proteins and formation of the functional tetrameric form of the channel (Eisfeld and Luckhoff 2007). Importantly, adenosine diphosphate ribose (ADPR), being an endogenous ligand for TRPM2 binds with high specificity to the C-terminus nucleoside diphosphate linked moiety X-type homology motif (NUDT9-H) domain of TRPM2 (Fig 4). NUDT9-H, involved in gating of TRPM2 channel shares high homology to the mitochondrial NUDT9 responsible for mitochondrial ADPR hydrolase (Sumoza-Toledo and Penner 2011). However, unlike NUDT9, NUDT9-H possess modest enzymatic activity (1%) (Iordanov et al. 2016). Recently, it has been shown to not possess enzymatic activity and the previously reported activity was proposed due to the instability and spontaneous degradation of ADPR (Belrose and Jackson 2018). Binding of ADPR results in opening of the channel and allow the permeation of sodium (Na^{+}), calcium (Ca^{2+}) into the cell with a relative permeability of P_{Ca}:P_{Na} ~0.3–0.9 and movement of potassium (K^{+}) across the cell. TRPM2 currents are characterized by a linear current–voltage (I–V) relationship with a reversal potential of ~0 millivolt (mV) and these characteristics demonstrate that TRPM2 is a non-selective cation channel without voltage-dependent behavior. The single channel conductance is ~60 picosecond (pS) with unusually long open times in the range of several seconds (Sumoza-Toledo and Penner 2011).

At present, there are no specific inhibitors of TRPM2. However, the channel is inhibited by various pharmacological agents such as imidazole antifungal agents (clotrimazole and econazole), 8Br-cADPR, 8Br-ADPR and flufenamic acid (Jiang et al. 2010). Also, external

acidification of 6.5 pH and internal acidification to pH 6 has shown to inhibit TRPM2 channels (W. Yang et al. 2010). Despite the lack of a specific antagonist, studies over the past decade using known inhibitors or genetic approaches have disclosed important physiological roles of TRPM2 and its role in pathology, which is outlined in the next section.

1.4.2 Physiological and pathological role of TRPM2

TRPM2 mRNA expression by real-time quantitative reverse transcription PCR (qRT-PCR) shows that it is ubiquitously expressed in all tissues throughout the body, except for bone and cartilage (Belrose and Jackson 2018). TRPM2 has also been shown to be activated by exposure to warm temperatures and plays an important physiological role in temperature sensation and thermoregulation (Song et al. 2016).

A crucial physical barrier between blood and interstitium is formed by the vascular endothelial cells, where high ROS production is observed during ischemia (Jiang et al. 2010). Endothelial cell barrier dysfunction through ROS induces extracellular Ca^{2+} influx into endothelial cells, promoting inter-endothelial cell gap formation and increase permeability (Jiang et al. 2010). Role of TRPM2 in mediating this increased permeability of endothelial cells during oxidative stress were confirmed by 3 approaches – 1. Overexpressing the TRPM2-S isoform to inhibit normal TRPM2 function, 2. siRNA to knockdown TRPM2 function, 3. By using two PARP inhibitors, 3-aminobenzamide and PARP Inhibitor III (DPQ), to suppress generation of ADPR (Hecquet et al. 2008). These studies showed ROS induced hyper-permeability of endothelial cells were mediated by TRPM2, and a strong potential of the TRPM2 channel as a target to develop novel therapeutic approaches to oxidative-stress-induced vascular barrier dysfunctions. In addition, the role of PARP-1 has been shown in the above study to be crucial in TRPM2 channel

activation. This was supported by recent study in lymphocytes where multiple pathway initiates PARP-1 activation under conditions of oxidative stress. Activation of PARP-1, followed by enzymatic actions of PARG, in turn generates ADP-ribose leading to activation of TRPM2 (Buelow, Song, and Scharenberg 2008). Also, TNF-α induced ROS production activates PARP-1, leading to production of ADPR and TRPM2 activation in adult ventricular cardiomyocytes (Roberge et al. 2014). Combined, these results show that the activity of PARPs and particularly PARP-1 is an essential signaling constituent in the TRPM2 channel activation.

Cation channels sensitive to ROS were long believed to present in insulin secreting cells (Herson and Ashford 1997). These cationic channels were suspected to be TRPM2 due to these 3 reasons - 1. Sensitivity to agonist ADPR and NAD, 2. Linear I-V relationship curve by depletion of extracellular Ca^{2+}, or by TRPM2 inhibitors flufenamic acid (FFA)/clotrimazole (Ctz), 3. Same single channel conductance as TRPM2 (Jiang et al. 2010). TRPM2 KO mice showed higher blood glucose levels and lower levels on incretin hormone when compared to WT mice. This shows that TRPM2 regulates insulin secretion in response to glucose.

Inflammation also causes high ROS production in site of injury or infection leads intracellular Ca^{2+} influx and activate several transcription factors such as NF-κB in macrophages, thereby producing inflammatory cytokines like interleukin-8 (IL-8) and C-X-C Motif Chemokine Ligand 2 (CXCL2) (Jiang et al. 2010). Note that, NF-κB activation requires PARP-1 activity has been well documented. These chemotactic cytokines secreted play a key role in recruiting inflammatory cells such as neutrophils and monocytes/macrophages from the bloodstream to site of injury. However, decreased levels of $[Ca^{2+}]_i$ and the production of these inflammatory cytokines were impaired in TRPM2-KO mice, providing a compelling evidence that the TRPM2 channel has a key role in oxidative-stress-induced Ca^{2+} influx and production of IL8/CXCL2 (Zeng et al. 2003).

Treatment of monocytes with lipopolysaccharide (LPS), a gram-negative bacterial outer membrane component, induced upregulation of TRPM2 transcripts, protein level and showed ADPR induced membrane currents. Additionally, lipopolysaccharide-induced production of cytokines IL-6, IL-8, IL-10 and TNF-α was observed in WT monocytes, while short hairpin RNA (shRNA) targeting TRPM2 downregulation inhibited cytokine production (Wehrhahn et al. 2010). Combined, this shows that TRPM2 activation in response to oxidative stress and inflammatory stimuli causes production of inflammatory cytokines and chemokines.

Ischemic insult resulting in life-long, debilitating and irreversible consequences for survivors is attributed to neuronal cell-death. Hippocampal neurons are highly susceptible to oxidative stress and ischemic injury, causing irreparable damage (Turlova, Feng, and Sun 2018). Glutamate excitotoxicity mediated Ca^{2+} influx has long been linked to ischemic cell death. Recently, Ca^{2+}-permeable non-selective cation channels including large-pore pannexin channels and transient receptor potential member 7 (TRPM7) have been suggested to be recruited by conditions of elevated oxidative stress associated with ischemia. Similarly, several studies using animal models have also shown that TRPM2 contributes to ischemic cell death.

Following hypoxia-ischemia, reduced brain infarct volumes and reduced loss of brain mass were observed in $TRPM2^{-/-}$ mice compared to its WT littermates (Alim et al. 2013). $TRPM2^{-/-}$ mice also showed improved sensorimotor outcomes and reduced expression of inflammatory markers, suggesting neuroprotective function upon blocking TRPM2 in a developmental model of hypoxic-ischemic mice.

Ca^{2+} influx resulting from TRPM2 activation is a key contributor to physiological as well as pathological process. The increase in intracellular concentration of Ca^{2+} by TRPM2 activation for short periods of time is normally buffered to very low resting levels. However, prolonged or

excessive alterations in Ca^{2+} concentrations can lead to cytotoxicity. Oxidative stress mediated cell death was observed in in primary rat neurons. However, the intracellular ca^{2+} influx and neuronal cell death are attenuated by treating them with TRPM2 small interfering RNA (siRNA), proving central role of TRPM2 in cerebral ischemia (Kaneko et al. 2006). Specifically, treatment of TRPM2 inhibitor clotrimazole inhibited oxidative stress induced inward current in cornu ammonis 1 (CA1) hippocampal region of rat hippocampal slices (Olah et al. 2009). Combined, these studies show that TRPM2 activation plays a key role in inflammation, ischemia but is also suggested to play a key role in AD (Jing Wang, Jackson, and Xie 2016)

1.4.3 Role of TRPM2 in AD

Oxidative stress has been widely implicated in widespread pathologies like cancer, atherosclerosis and neurodegenerative disorders including Alzheimer's and stroke (Jiang et al. 2010). Some of the earliest evidence for a role of TRPM2 in AD comes from the study where Aβ and ROS induced cell death in rat striatal cells having endogenous TRPM2 expression. To address the role of TRPM2, two strategies were used; 1. Transfection of cells with a variant of TRPM2 that acts as a dominant negative blocker of TRPM2 function, 2. Knockdown of TRPM2 expression by siRNA providing. Both these strategies inhibited TRPM2 function and expression, providing evidence that TRPM2 activation by Aβ contributes to Ca^{2+} influx and cell death (E. Fonfria et al. 2005).

Aβ-induced oxidative stress and DNA damage has been shown to activate PARP-1 and TRPM2 in endothelial cells, leading to intracellular Ca^{2+} overload and endothelial dysfunction (Park et al. 2014). TRPM2 channel inhibitors were shown to prevent endothelial dysfunction induced by Aβ and reverse such dysfunction in transgenic mouse model of AD, suggesting an important role of TRPM2 in endothelial and neurovascular dysfunction. However, mechanisms by

which TRPM2 channels contribute to neurovascular coupling remains to be established. In fact, deleterious effects of Aβ on the vasculature was suggested to contribute to the cognitive deficits by reducing cerebral perfusion and leading to neuronal cell death.

This was addressed by Ostapchenko et al., in 2015 where they showed that global deletion of TRPM2 is well tolerated and has been shown to prevent cognitive decline in a mouse model of AD (Ostapchenko et al. 2015). Aβ facilitated TRPM2 channel function in neurons and that elimination of TRPM2 globally reversed pathological and behavioral deficits observed in the AD mouse models APP/PS1. Whether this beneficial effect is due to deletion of TRPM2 in neurons or microglia has not been established. However, Alzheimer's disease is characterized by microglial activation. But the authors have shown that increased levels of microglial marker ionized calcium binding adaptor molecule 1 (Iba1) were observed in WT mice, but not in $TRPM2^{-/-}$ * APP/PS1 mice. Taken together, this suggests that global deletion of TRPM2 mitigated inflammatory response providing an additional beneficial outcome via reduced neuroinflammation.

1.4.4 Sex difference upon inhibition of TRPM2 and PARP-1

Alzheimer's disease rate is higher in women when compared to men across most regions in this world. Beyond mere statistical numbers, there is no clear evidence regarding why women are disproportionately affected (Mazure and Swendsen 2016). I would characterize this into 3 possible reasons – 1. Differential expression of genes contributing to amyloid or tau formation (Altmann et al. 2014), 2. Amyloid or tau load in the brain (Barnes et al. 2005), and 3. Potential sex difference of key molecular targets of the signaling pathway downstream of amyloid beta activation (Liu et al. 2011; Nakayama et al. 2013).

A study in 2014 showed increased risk of converting mild cognitive impairment (MCI) to AD in healthy Apolipoprotein E 4 (ApoE4) male carrier compared to non-carriers. However,

female healthy ApoE4 carriers showed twice the risk compared to non-carriers (Altmann et al. 2014). Also, in the same study, CSF biomarker analysis showed increased risk of AD in female APOE4 carriers occurs downstream of Amyloid beta pathology. However, post-mortem studies conducted over the past 15 years from hippocampus and neocortical brain regions do not indicate amyloid plaque burden to be different in male and female brain of patients with AD (Barnes et al. 2005). While there are individual studies claiming higher tau burden in women with AD when compared to men in certain regions of the brain, a clear majority of the post-mortem studies of brain from patients with AD showed no difference in sex on global tau burden (Barnes et al. 2005). These results combined suggest that difference in rate of Alzheimer's disease between men and women is perhaps not due to upstream activities like differential expression of genes contributing to amyloid or tau formation, or amyloid or tau load, but could be due to differential response in key molecular targets of the signaling pathway downstream of amyloid beta activation.

The 2 key players PARP-1 and TRPM2 discussed so far exhibit sex difference upon their inhibition, but from the perspective of stroke pathology. Focal stroke by transient or permanent middle cerebral artery occlusion (MCAO) was induced in WT and PARP-1$^{-/-}$ mice of both sexes, showed smaller infarct size in PARP-1$^{-/-}$ males when compared to WT. Interestingly, PARP-1$^{-/-}$ females had larger stroke infarct volumes compared to WT suggesting that PARP-1 deletion reduces infarct size in male, but exacerbates injury in female (Liu et al. 2011). Whether similar sexual difference upon PARP-1 inhibition is observed in Alzheimer's disease remains to be studied. While PARP-1 can be targeted for neuroprotection, PARP-1 is involved in DNA repair and in production of inflammatory mediators upon injury/infection. Hence, molecular target downstream of PARP-1 activation, like TRPM2, becomes an important therapeutic target for neuroprotection.

Administration of TRPM2 inhibitor clotrimazole (Ctz) 30 minutes after resuscitation from cardiac arrest reduced hippocampal CA1 neuronal injury by inhibiting TRPM2 activity. While neuroprotection was observed in male, no effect on injury was observed in female (Jia et al. 2011; Nakayama et al. 2013). *In vitro* studies were also performed using neurons plated separately from male and female mice, where shRNA targeting TRPM2. Preferential protection of male neurons, but no significant effect on female was observed upon TRPM2 inhibition (Jia et al. 2011). However, single study evaluating sex differences upon specific targeting of microglial PARP-1 and/TRPM2 has not been conducted. Whether targeting key inflammatory players like PARP-1/TRPM2 for treatment of Alzheimer's disease proves beneficial for only one sex over the other remains to be evaluated.

1.5 Role of NMDARs in Alzheimer's Disease

1.5.1 Glutamatergic system in neurons

Neurons are a class of brain cells which are involved in the formation of memory. Neurons communicate with each other through electro-chemical signaling via specialized connections called synapses (Lodish et al 2001). There is a consensus that there are roughly about 100 billion neurons total in the human brain and each of these neurons can have up to 15,000 connections with other neurons via the synapses. A process where synaptic connection between the neurons get strengthened is called synaptic plasticity and a persistent increase in synaptic strength following unique patterns of electrical stimulation is called long term potentiation (LTP) (D. A. Butterfield and Pocernich 2003). However, several studies have shown that loss of synapses precedes actual neuronal death in Alzheimer's disease (Selkoe DJ 2002), which in itself is characterized by gradual loss of cognitive abilities. A key neuronal cell-surface receptor involved in the formation of LTP

is a class of glutamate receptor called the N-methyl-D-aspartate receptor (NMDAR) (Paoletti, Bellone, and Zhou 2013).

Each synapse is composed of a pre-synaptic terminal which comes into close apposition with a dendritic spine, a highly specialized post-synaptic structure present on the dendrites of neurons. Glutamate, a major primary excitatory neurotransmitter is released from the pre-synaptic terminal initiating synaptic transmission (Meldrum 2000). The glutamate in the synaptic cleft binds to the glutamate receptors expressed on the post-synaptic density, an area within the dendritic spine which is enriched with synaptic receptors and scaffolding proteins (Koji Yashiro et. al 2009). The binding of glutamate causes depolarization called an excitatory post-synaptic potential (EPSP). EPSPs are of two type – fast EPSP and slow EPSP (Purves et al. 2001). Fast EPSPs are mediated primarily by a class of glutamate receptors called α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor. AMPA receptors are hetero-tetramers of glutamate ionotropic receptor AMPA type subunit 1 (GluA1)/ glutamate ionotropic receptor AMPA type subunit 2 (GluA2) or glutamate ionotropic receptor AMPA type subunit 2 (GluA2)/ glutamate ionotropic receptor AMPA type subunit 3 (GluA3), and forming a cation selective pore permeable to sodium but not calcium (AMPARs can be permeable to calcium in the absence of GluA2 receptor, but they are rarely expressed) (Man 2012). Binding of glutamate to AMPA receptor causes the opening of cation selective pore leading to influx of sodium ions and leading to rapid depolarization.

The N-methyl-D-aspartate (NMDA) receptor is another type of ionotropic glutamate receptor in the post-synaptic terminal which requires glutamate as the agonist and serine or glycine as co-agonist. Even upon their binding, these receptors contribute very little to the EPSPs (Purves et al. 2001). This is because there is a voltage dependent block of the NMDAR pore by Mg^{2+} at

resting membrane potentials and is relieved only at depolarized state. However, after rapid depolarization by the summation of AMPA receptor mediated EPSPs, the NMDAR pore-channel is relieved of Mg^{2+} block and allows influx of ions, forming the slow EPSPs (Iacobucci and Popescu 2017). However, unlike AMPA receptors which primarily mediate the flux of sodium, NMDA receptors are permeable to both sodium and calcium.

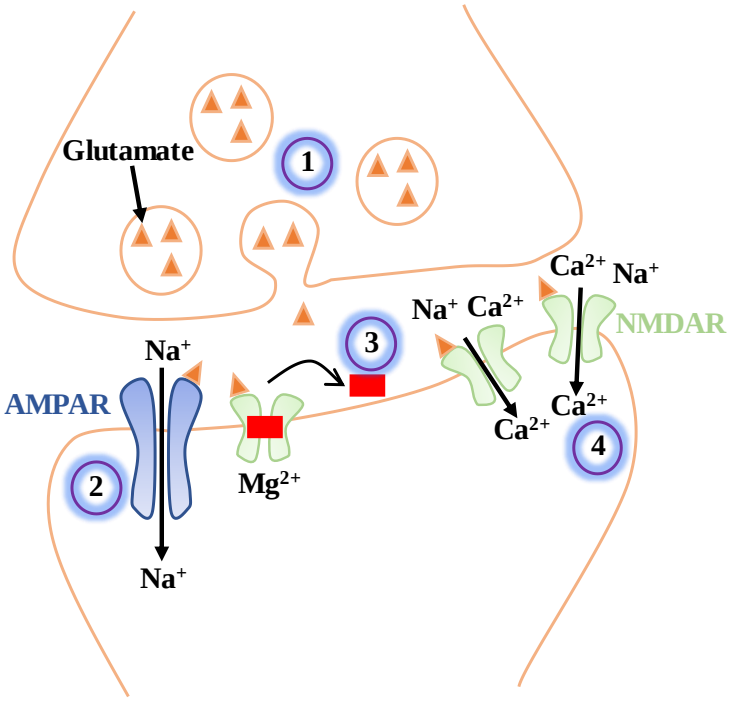

Figure 5: Binding of glutamate to ionotropic NMDA receptors induces excitatory synaptic transmission. (1). Fusion of glutamate containing vesicles to pre-synaptic membrane release causes release of glutamate into synaptic cleft. **(2)** Released glutamate binds to post-synaptic AMPAR causing membrane depolarization. **(3)** Membrane depolarization causes displacement of Mg^{2+} blocking the NMDAR channel pore. **(4)** Displacement of Mg^{2+} in depolarized condition allows influx of Ca^{2+} through NMDARs.

NMDARs are called as co-incident detectors where coincidence of presynaptic glutamate release and strong depolarizing potential in the postsynaptic neuron is required for the opening of

NMDAR channels (Tabone and Ramaswami 2012). These attributes of the NMDA receptor suggested its importance in mechanisms of synaptic plasticity and memory formation in the hippocampus (Paoletti, Bellone, and Zhou 2013). This was shown by genetic ablation models and pharmacological blocking of NMDA receptors causing alteration in anterograde memory formation (Davis, Butcher, & Morris, 1992). In summary, synaptic plasticity plays a key role in memory formation in the hippocampus. However, overstimulation of NMDARs due to excessive accumulation of pre-synaptic glutamate, termed as glutamate excitotoxicity, can render synaptic regulation aberrant, leading to neuronal death and cognitive deficits in neurodegenerative disorders like ischemia and Alzheimer's disease (Yan Zhang et al. 2016).

1.5.2 Role of NMDA receptors in AD

So far, seven NMDAR sub-units have been identified in the brain so far – NR1, NR2(A-D), NR3(A-B) (Yan Zhang et al. 2016). Native NMDARs occur as hetero-tetramers containing two glycine-binding NR1 and two glutamate-binding NR2 subunits. Expression of NMDAR subunits differentially distribute throughout the brain and change strikingly during development. NR2A and NR2B subunits are critical for the induction of LTP and long-term depression (LTD), and are important for cognitive functions (Lau et al. 2009). However, NMDAR dysfunction in the brain leads loss in cognitive decline, as seen with Alzheimer's disease (D. Selkoe and Hardy 2016).

Aβ accumulation causes neuronal NMDAR activation at early stage of the disease (Yan Zhang et al. 2016). Similar to glutamate, Aβ has been shown to evoke an immediately Ca^{2+} rise and impair LTP (CA1-Dentate gyrus region of the brain) through activation of NR2B containing NMDARs (Danysz and Parsons 2012). NR2B-selective antagonist at low concentrations restored impaired LTP, proving that Aβ impair LTP through a NR2B dependent way of NMDARs (Hu et

al. 2009). A shift from α-secretase to β-secretase activity of APP processing occurs upon excessive NMDAR activation, leading to increased Aβ production even with modest deregulation of the glutamatergic transmission (Yan Zhang et al. 2016). However, Aβ-induced LTP impairment in AD was also shown to be ameliorated by decreasing extracellular glutamate levels (D. J. Selkoe et al. 2011).

Excitotoxicity, a pathological condition of increased glutamate concentrations acting on post-synaptic glutamate receptors, has also been shown to be a key pathophysiological component of Alzheimer's disease (AD) (Yan Zhang et al. 2016). Interestingly, Aβ peptide treatment shows enhanced glutamate release and augment NMDAR-mediated transmission in vitro (Brorson et al. 1995). Increase in glutamate concentrations at the synaptic cleft leads to subsequent desensitization of synaptic NMDARs (Rammes et al. 2011). Further increase causes to activation of extra synaptic NR2B-rich NMDA receptors, overactivation of which leads to synaptic impairment (Yan Zhang et al. 2016). Excitotoxicity induced NR2B activation also leads to activation of apoptotic pathways through caspase-8 and caspase-3, causing neuronal cell death (Tackenberg et al. 2013). These studies combined show that both Aβ and excitotoxicity play a key role in synaptic dysfunction and cognitive impairment, as observed in patients with Alzheimer's disease.

Interestingly, neurons are not the only type of brain cells expressing NMDARS. Other brain cells like microglia, astrocytes and endothelial cells that have also reported presence of NMDAR (Hogan-Cann and Anderson 2016). But, a substantial amount of research is focused on effect of glutamate excitotoxicity and Aβ in neurons and effect of β-amyloid on non-neuronal NMDARs are not very well understood (Hogan-Cann and Anderson 2016). In AD, several studies have shown that inflammatory mediators released by microglia play an important role in neuronal cell-

death (Haneka 2015). However, whether microglial activation was initiated by cell-surface expressed NMDARs in response to glutamate excitotoxicity remains unexplored.

1.5.3 Microglial NMDARs: Present or Absent?

Various stimuli initiating microglial PARP-1 activation has been mention in section 1.3.4 and 1.3.5. However, whether these stimuli act through microglial NMDARs were not known. In fact, towards the end of last century, researchers were still uncertain whether microglia expressed NDMARs. However, the first evidence of microglial NMDARs was provided by Gottlieb et.al., in 1997 (Gottlieb and Matute 1997). They showed upregulation of microglial NR1 sub-unit of NMDARs using immunofluorescence in hippocampal CAl region following transient forebrain ischemia (Gottlieb and Matute 1997). Following that, Tikka and Koistinaho in 2001 showed NMDA-induced proliferation of microglia and the increased release of IL-1β and nitric oxide in pure microglia cultures (Tikka and Koistinaho 2001a). The ionotropic function of NMDARs was confirmed here by blocking NMDA induced NO release by using MK-801, a NMDAR pore channel blocker. Further, NMDA treatment alone in mixed spinal cord cultures was sufficient to induce microglial proliferation, which preceded neuronal death (Tikka and Koistinaho 2001a). In addition, administration of extra microglial cells on top of the mixed culture enhanced NMDA neurotoxicity. However, minocycline, an anti-inflammatory compound blocking PARP-1, prevented NMDA induced neurotoxicity, suggesting PARP-1 activation by microglial cell-surface NMDARs (Tikka and Koistinaho 2001a).

Microglial cells under hypoxic conditions induced activation of NMDARs, leading to NF-κB activation and release of nitric oxide and pro-inflammatory cytokines (Murugan et al. 2011). These effects were completely inhibited upon treated with MK-801 suggesting ionotropic action

of NMDARs (Murugan et al. 2011). Interestingly, high levels of NR2D expression has been shown in glial cells, rendering it likely that microglial NMDAR properties differ from their neuronal counterparts (Kaindl et al. 2012). Hallmark feature of ionotropic NMDARs is to show Ca^{2+} influx mediated NMDA-mediated current in response to NMDA. However, only one study until now has reported Ca^{2+} influx and showed NMDAR-mediated current in microglia (Kaindl et al. 2012).

On the contrary, extension of microglial process upon NMDA treatment has been shown to be independent of microglial NMDARs, but it is rather due to the actions of microglial purinergic receptors (Dissing-Olesen et al. 2014; Eyo et al. 2014). Also, they were unable to record NMDAR-mediated currents in microglia that was previously reported (Wendt et al. 2016). These contradicting results by different groups has made researchers in the field have divisive opinions about functional presence of microglial NMDARs.

1.6 Therapeutics for AD

1.6.1 Current Strategy

There are 5 therapies that have been currently approved and in use for treatment of Alzheimer's disease. Four of these, namely – tacrine, donepezil, rivastigmine and galantamine are classified as Cholinesterase inhibitors, while memantine is a N-methyl-D-Aspartate receptor (NMDARs) antagonist.

Acetylcholine (Ach) is a chemical neurotransmitter released into the synaptic space by pre-synaptic terminals of cholinergic neurons. Ach then binds to cholinergic receptors on the post-synaptic neuron and is believed to help in cognition. To regulate this process, there is presence of acetylcholinesterase in the synaptic space, which degrades Ach to acetyl CoA and Choline (Figure 2A). The cholinergic hypothesis of AD states that impairment of cholinergic systems in the basal

forebrain leads to memory loss and deterioration of other cognitive and noncognitive functions (Roy et al. 2005). This is due to the loss of acetylcholine neurons, loss of enzymatic function for acetylcholine synthesis, and degradation by the action of acetyl cholinesterase. Hence, using inhibitors of acetyl cholinesterase (AChEI) would enhance the cholinergic transmission by binding to acetyl cholinesterase in the synaptic space and blocking degradation of acetyl choline. This would lead to increase in the availability of acetyl choline in the synaptic space, thereby helping neuronal transmission (Figure 2B).

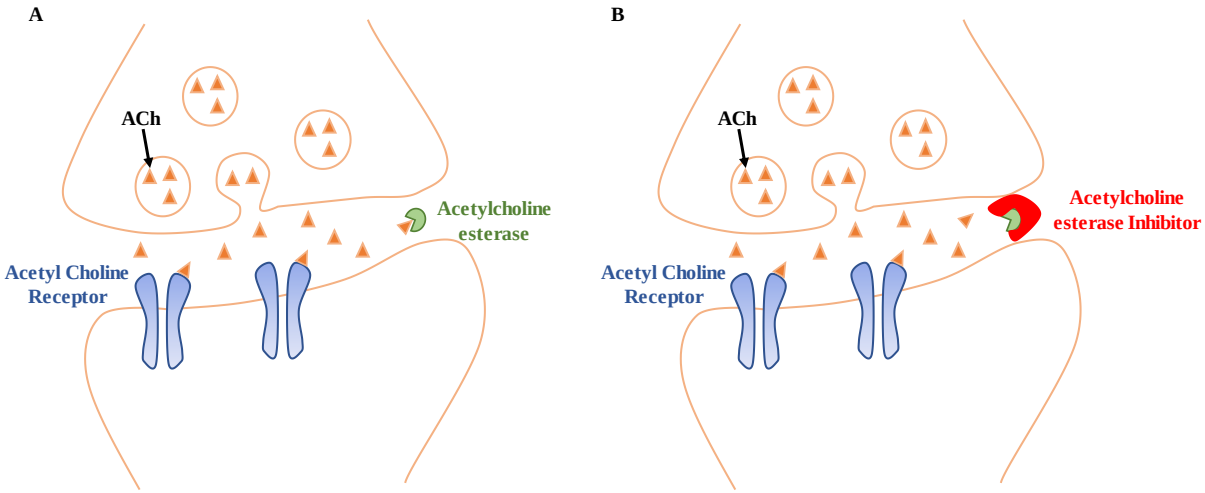

Figure 6: Mechanism of action of Acetyl Cholinesterase. (A) Acetyl Cholinesterase at the pre-synaptic space degrades acetyl choline to Acetyl CoA and Choline. **(B)** Inhibitors of Acetyl Cholinesterase would increase the availability of acetyl choline in pre-synaptic space.

Many clinical trials including 23 for of donepezil (5272 patients randomized), 9 of rivastigmine (3449 patients randomized) and 9 of galantamine (5194 patients randomized) have been done to assess drug safety, efficacy and dosing in humans (J. S. Birks and Harvey 2018; Jacqueline Birks and Craig 2006; Desai and Grossberg 2005). Cognitive function (as measured by psychometric tests), behavioral disturbance and performance of activities of daily living have been

evaluated in these clinical studies. These trials demonstrate beneficial effect of AChEI compared with placebo on cognitive function and measures of global clinical state at 6 months or more (J Birks 2009). While there is nothing to suggest the effects are less for those with severe dementia or mild dementia, the authors found there is very little evidence for use in other than mild to moderate dementia. Similarly, while there were fewer data on measures of behavioral disturbance and activities of daily living, but they showed evidence of benefit of AChEI in these domains (J Birks 2009). These inhibitors have been approved by the food and drug administration (FDA) for mild to moderate AD and have been regarded as standard and first-line treatment for Alzheimer's.

The only available medication for AD that affects the function of NMDA receptor (NMDAR) is memantine. Memantine (1-amino–3, 5-dimethyladamanantate) is an adamantane derivative that blocks the NMDA receptor associated ion channel similar to magnesium by binding to or near the magnesium-binding site. Memantine is an uncompetitive, lower-affinity, open channel blocker with relatively fast off-rate. The relatively fast off-rate helps in lower accumulation of the drug in the channel pore and not impacting normal synaptic transmission. Also, since this drug is uncompetitive blocker, it needs prior binding of glutamate to NDMARs for it to bind, making its antagonistic activity more potent in areas with massive activation of NMDA receptors. Since we know that NMDARs play a key role in regulating synaptic plasticity and memory function (Danysz and Parsons 2012), blocking NMDARs with memantine impair synaptic plasticity, and compromises learning and memory (Danysz and Parsons 2012). Hence, it is important to note that memantine does not block all NMDARs but rather only blocks NMDARs which are in open state or is overstimulated.

Clinical trials accompanying the FDA application for memantine evaluated in long-term care setting with patients recruited through selected via Diagnostics and Statistics manual (DSM-

III) or through diagnostic criteria (Riepe 2015; Winblad and Poritis 1999). These studies were placebo-controlled trial where improvements in behavioral, global and functional aspects were evaluated. These studies showed no behavioral improvements, but their global and functional improvements demonstrated their potential therapeutic effect and FDA approved memantine only for moderate-severe AD (Riepe 2015; Thomas and Grossberg 2009; Winblad and Poritis 1999).

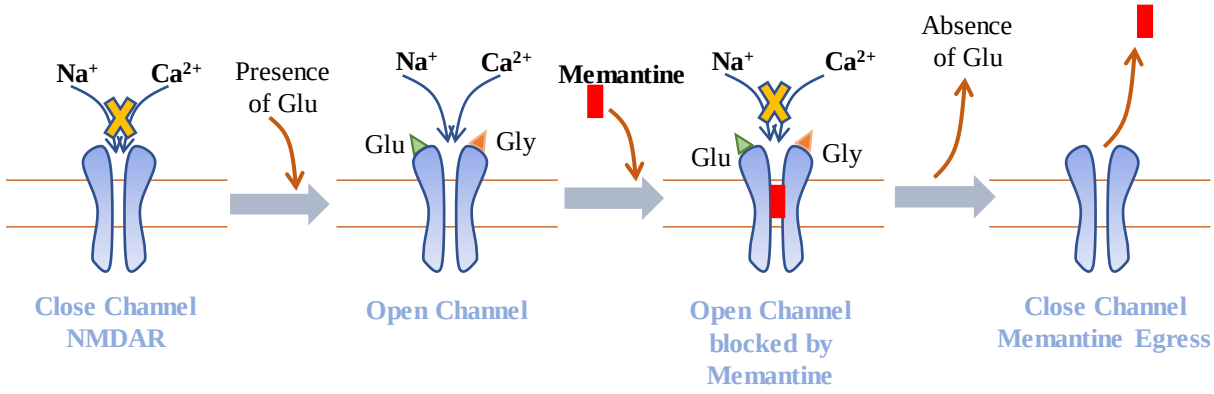

Figure 7: Mechanism of action of Memantine. Memantine binds to the NMDARs in its open channel confirmation at the Mg^{2+} pore and prevents influx of ions.

1.6.2 Limitations

It's often quoted that no drug comes without its own limitations. To start with, these drugs only provide symptomatic relief but does not treat the underlying cause. In addition, using acetylcholinesterase inhibitors increases acetyl choline signaling in CNS. However, note that acetyl choline not just acts on the central nervous system (CNS), but also activates the peripheral nervous system (PNS). These medications can hence cause common side effects like nausea, gastrointestinal (GI) upset, and diarrhea (J. S. Birks 2006; J. 2006). Some of the other less common side effects include muscular weakness, fainting, and significant weight loss on occasion. A drug

with most adverse side effects is rivastigmine, where only a limited number of patients can tolerate the full dose of 6mg twice daily (Casey, Antimisiaris, and O'Brien 2010).

The clinical significance for memantine is only seen in case of moderate-severe AD and the cost is also considerably high (Riepe 2015). Clinical trials evaluating memantine for mild AD have failed so far. No statistical significance was found between memantine and placebo control groups on functional measures (Bakchine and Loft 2008; Peskind et al. 2006). Apart from this, two 24-week studies found no improvement in cognitive abilities (Bakchine and Loft 2008).

Taken together, current treatments modest or sometimes significant effectiveness to patients who can tolerate their side effects. These drugs only have the ability to slow down the progression to about 6 months, but do not alter the course of disease and substantial impact of cognition and memory, activities of daily living have not been observed (Riepe 2015). This has led to researchers to look for other molecular targets mostly altering the course of disease progression.

1.6.3 Drugs currently in AD drug development pipeline

Currently, there are 105 agents in AD drug development pipeline. There are 25 agents in phase I, 52 agents in phase II, and 28 agents in phase III. The current drugs in clinical study can be classified into 3 types namely- Symptom reducing small molecule, Disease-modifying small molecule and Disease-modifying immunotherapy. Seventy percent of drugs in the AD pipeline are disease-modifying therapies (DMTs). Fourteen percent are symptomatic cognitive enhancers, and 13% are symptomatic agents addressing neuropsychiatric and behavioral changes (2% have undisclosed mechanisms). While most trials are sponsored by biopharmaceutical industries, subjects enrolled in trials include patients with (cognitively normal with biomarker evidence of

AD), prodromal AD (mild cognitive symptoms and biomarker evidence of AD), and AD dementia (Cummings et al. 2017).

An enzyme that cleaves Amyloid Precursor Protein (APP) to form beta-amyloid is the beta secretase enzyme (BACE). It's been thought that the therapies targeting the inhibition of this enzyme would prevent accumulation of beta amyloid in the brain. Hence BACE inhibitors are among the most common class of molecules in the drug development pipeline with around 10 Phase II and Phase III trials involved currently. While challenging structure-activity requirements for BACE inhibition had made it difficult to develop molecules targeting their inhibition as well as cross the blood brain barrier. Advances in small-molecule screening approaches have helped overcome these challenges with several BACE inhibitors in the drug development pipeline now (Hamada and Kiso 2016). Favorable PK has been achieved upon oral administration for all small molecule inhibitors currently in Phase II and Phase III trials.

Few BACE inhibitors have bypassed to Phase III testing directly from Phase I trial after bypassing the Phase II trials due to strong biomarker data. Hence, not much is known currently about the long-term safety of the drug and clinical efficacy. The first BACE inhibitor trial to be tested in phase III in an AD dementia population was recently terminated for the lack of efficacy (Hawkes 2017). Similarly, many questions are left unanswered regarding BACE inhibitors including at what point this drug needs to be administered after diagnosis of AD, and whether the degree of amyloid inhibition is required for clinical benefit. At this point, we do not know whether reduction in amyloid synthesis affect clinical progression along with unidentified safety concerns with long-term BACE inhibition as APP and beta-amyloid is known to have physiological function that has not been explored.

CHAPTER 2: RATIONALE OF THE STUDY

Increased Aβ deposition and hyper-phosphorylation of tau, neuronal damage and cognitive impairment in Alzheimer's disease (AD) are all associated with inflammation. Cause or consequence of progression of AD is still not clear. However, microglia, the innate immune cells of the brain play an important role in the response to damage of central nervous system. Clinical work and studies in animal models suggest that prolonged microglial activation contribute to chronic neurodegenerative diseases. Using genome wide association studies (GWAS), recently more than 20 gene variants, many of which are highly encoded in microglia have been shown with an increased risk of late-onset AD (Villegas-Llerena et al. 2016). Further, evaluation of single-nucleotide polymorphisms (SNPs) in the triggering receptor expressed on myeloid cells 2 (TREM2) gene recently showed that the p.R47H variant of this particular gene is associated with late-onset AD in Columbian population (Arboleda-Bustos et al. 2018). This provides a rationale to study gene/proteins that impact inflammation and presents as a risk in developing Alzheimer's disease.

Dr. Kauppinen's findings suggest that nuclear enzyme poly-(ADP-ribose) polymerase-1 (PARP-1) is involved in microglial pro-inflammatory responses in AD (T. Kauppinen et al. 2011). Also shown recently was that PARP-1 can be activated by $[Ca^{2+}]_i$, a process independent of DNA damage. But we do not know here the mechanisms that maintain microglial PARP-1 in an active state provoking sustained pro-inflammatory. One candidate mechanism I propose to investigate is the Ca^{2+} permeable non-selective cation channel, Transient Receptor Protein Melastastin-2 (TRPM2), global deletion of which has been shown by the Jackson lab to mitigate cognitive decline in a mouse model of AD (Ostapchenko et al. 2015).

TRPM2 possesses an adenosine diphosphate ribose (ADPR) pyrophosphatase domain, responsible for initiating TRPM2 channel opening upon binding of ADPR (Sumoza-Toledo and Penner 2011). However, ADPR production requires activation of PARP-1 and poly ADP-ribose glycohydrolase (PARG). This suggests that while TRPM2 could potentially be acting as a part of positive feedback loop maintaining PARP-1 activation, initial PARP-1 activation is likely through another Ca^{2+} entry mechanism in microglia.

Here I propose, Ca^{2+} influx by N-methyl D-aspartate receptors (NMDARs) as the non-oxidative stress mediated pathway involved in initial activation of PARP-1. While the presence of NMDARs in microglia is controversial, Kauppinen et.al., have demonstrated NMDA-stimulation triggers microglial pro-inflammation and neurotoxic responses suggesting direct involvement of NMDARs in microglial activation (Tikka and Koistinaho 2001a). This microglial mediated neurotoxicity was shown to be prevented indirectly by minocycline, an anti-inflammatory compound capable of inhibiting various enzymes including PARP-1 (Alano et al. 2006). NMDA induced nitric oxide release was also blocked by MK-801, a NMDAR inhibitor (Tikka and Koistinaho 2001a). Interestingly, Jackson's findings shows that TRPM2 currents are recruited downstream of NMDARs in neurons (Yu-Feng Xie et al. 2011). We propose that a comparable coupling occurs between NMDARs and TRPM2 occur in microglia. The proposed study will evaluate the role of NMDAR/PARP-1/TRPM2 in Aβ-induced microglial activation outcome and neurotoxicity.

CHAPTER 3: HYPOTHESIS

Aβ initiates extended PARP-1 activation via positive feedback involving NMDAR initiated PARP-1/PARG-mediated ADPR production and TRPM2 activation, leading to TRPM2-dependent, self-sustaining PARP-1 activation.

CHAPTER 4: MATERIALS AND METHODS

4.1 Experimental Animals

Wild-type CD1 mouse colony were primarily used for cell culture experiments. PARP-1 cKO mice (C57BL/6 strain) developed by Dr. Tiina Kauppinen lab (University of Manitoba) was obtained by crossing PARP-$1^{fl/fl}$ with Cre-deleted allele driven by CD-11b promoter elements. PARP-$1^{fl/fl}$ crossed with $Cre^{-/-}$ (Cre^{-}) served as WT control and $Cre^{-/+}$ served as PARP cKO. $Trpm2^{tm1Yamo}$ mice with C57BL/6 strain was obtained from Yasuo Mori's laboratory (Kyoto University). Animals were housed and maintained at the animal care facilities of the University of Manitoba. Experimental protocols were conducted with approval by the Animal Welfare Committee at the University of Manitoba.

4.2 Preparation of Mixed Glial Cultures

0-2-day old WT CD1, PARP-1 KO/WT or TRPM2 KO/WT mice of both sexes was dissected under sterile conditions to obtain mixed glial (astrocytes and microglia) cultures. Mice pups obtained from the central animal care services (CACS) facility of University of Manitoba were transferred to our neuroscience facility where pups were quickly sprayed down with 70% ethanol, and decapitated onto a plate containing cold dissection media (1x Hank's Balanced Salt Solution (HBSS), 2 mM HEPES buffer, 50 U/mL Penicillin-Streptomycin). The brains were removed and then transferred to a new plate containing fresh dissection media kept on ice and Dissections were performed under microscope in a biosafety cabinet to remove midbrain, hippocampus, and meninges, and cortices were collected into a fresh dish containing cold dissection media.

The tissue was then mechanically dissociated into smaller pieces and was trypsinized using 2x trypsin (0.1% w/v) in dissection media at 37°C for 3 minutes. The media containing trypsin

was removed was neutralized by adding glial growth media (GM media: 10% fetal bovine serum at low (< 0.05%) endotoxin content, 2 mM L-glutamine, 0.01% streptomycin, and Minimum Essential Medium (MEM)). Further, this neutralized media was removed, and fresh growth media was added and tissue was further broken up by trituration. The resulting cell suspension was then plated into culture flasks (25cm^2, T25 or 75 cm^2, T75) containing pre-warmed GM media. The cultures were maintained in 5% CO2 at 37°C in a humidified incubator and media was changed twice (the day after dissection and 4 days after dissection) before the cells were ready for harvesting. Microglial cultures were determined to be ready for harvest when the astrocyte monolayer was at 100% confluency with microglia floating or loosely attached and evenly distributed at approximately 250+ cells per field of view at 10x magnification. Pure microglial cultures were harvested from the mixed glia cultures by mild shaking and collecting the floating cells. The cells were then plated at a density of 23×10^5 cells/35mm culture dish or 2.5×10^5 cells/well into 48-well plates or 2.5×10^5 into a 100mm culture dish, and allowed to settle down for 24 hours at 37°C.

4.3 Production and validation of secreted amyloid-β oligomers from 7PA2 CHO-cells

Naturally secreted soluble oligomers of Aβ (AβOs) will be obtained from Amyloid Precursor Protein (APP) V717F-expressing CHO cells (called 7PA2 cells) 21 following established protocols. Control CHO cells and 7PA2 Chinese hamster ovary (CHO) cells were grown briefly and expanded onto 150mm culture dishes. Upon attaining 90% confluency, cells were washed twice with serum free Modified Eagle Media (containing glutamine) and returned to the incubator. After 18 hours, the conditioned media was collected into 50 mL tubes and centrifuged for 10 minutes at 200xg at 4˚C to remove cell debris. The CHO-conditioned media

(CHO-CM) and 7PA2-conditioned media (7PA2-CM) were stored in -80˚C freezer for further experiments.

Enzyme-linked immunosorbent assay (ELISA) kits amyloid-β 42 human ELISA kit, Ultrasensitive (Life tech) and Human Amyloid beta (1-x) Assay Kit (IBL) were used to measure the concentration of $A\beta_{1-42}$ and total amyloid-β in 7PA2-CM and CHO-CM respectively. Procedures provided with the kits were followed, and the concentration of 7PA2-CM was found to be 46.88pg/mL in $A\beta_{1-42}$ and 4.7ng/mL of total amyloid beta. CHO-CM did not show any presence of amyloid beta.

4.4 Materials

All chemicals used to make ICF and ECF solutions for electrophysiology were procured from Sigma Aldrich (Canada). N-methyl-D-Aspartate (NMDA), Dizocilpine (MK-801) and (2R)-amino-5-phosphonovaleric acid (APV) (NMDAR inhibitors) and TRPM2 inhibitor Clotrimazole were obtained from Sigma Aldrich (St. Louis MO). Another NMDAR inhibitor 7-Chlorokynurenic acid (CKA) sodium salt was obtained from Abcam. PARP-1 inhibitor N-(6-oxo-5,6-dihydrophenanthridin-2-yl)-N,N-dimethylacetamide (PJ34) was obtained from Selleckchem. PARG inhibitor PDD00017273 was obtained from Adipogen Life Sciences. AMPA receptor inhibitor CNQX disodium salt was procured from Tocris (Bristol UK).

4.5 Treatment of microglial cultures

Treatments began 24 hours post-plating by removing the growth media and adding treatments prepared in MEM solution. For electrophysiology experiments, microglia were treated with either 10 µM NMDA or 0.25X 7PA2-CM (11.72 pg/mL of $A\beta_{1-42}$). For experiments where inhibitors were used, microglia were treated for 30 mins before agonist treatment (NMDA or AβO) with either 10 µM MK-801/ 50 µM APV/ 20 µM CKA/ 500 nM PJ-34/ 1 µM PDD00017273/ 10

μM clotrimazole/0.05% dimethylsulfoxide (DMSO) control. Also, 20 μM CNQX served as negative control in NMDA treated microglia. Whole-cell voltage clamp recordings were performed 1-7 hours post NMDA/7PA2-CM treatment. Similarly, for immunochemistry experiments, microglia were treated with 10 μM NMDA for 7 hours before fixing the cells for imaging.

Microglia were treated with 10 μM NMDA or 0.25X 7PA2-CM for 24 hours before lysing them for RNA extraction. Similarly, nitric oxide assay was also performed 24 hours after treatment of microglia with 10 μM NMDA or 0.25X 7PA2-CM or inhibitors.

4.6 Whole-cell voltage clamp-electrophysiology

Pure primary microglia (DIV 8-14) harvested on a 35 mm culture dish from mixed glial flask were used to perform whole-cell voltage-clamp recordings. Recordings were done at room temperature on an inverted microscope (Olympus CK-42) using Axopatch-1D amplifier (Axon Instruments). For recording, the intracellular solution (ICF) contained the following (in mM): 142 Cesium Gluconate, 8 NaCl, 10 HEPES, and 2 MgCl2 (pH adjusted to 7.2 using 100 mM CsOH). Standard extracellular solution (ECF) contained the following (in mM): 140 NaCl, 5.4 KCl, 25 HEPES, 33 Glucose, 2 CaCl2, and 1 BaCl2 (pH = 7.4, adjusted using 10 N NaOH). The osmolarity value was confirmed by vapor based osmometer with ICF solution set at around 300 mmol/kg and ECF solution of around 310mmol/kg. Borosilicate glass capillaries (World Precision Instruments, FL) were used to make recording electrodes, having resistance of 3-5 MΩ, using a two-stage pipette puller (PP 83; Narishige, NY). Patch pipettes were freshly pulled before recordings and were filled with freshly prepared ICF.

Voltage-clamp recordings were performed on randomly chose microglia in the 35 mm culture dish by approaching with an ICS filled patch electrode while maintaining a positive

pressure of ~0.05 cc. Once the tip of the electrode made contact with the microglial cell membrane, negative pressure was applied to suction a small fraction of the cell membrane within the patch electrode. A holding potential of -60 mV, near the resting membrane potential, was applied to ultimately achieve the resistance of at least 1 GΩ (Giga-seal). Whole-cell access was achieved by applying a "zap", which is a pulse of 2 kHz for 0.6 ms. On attaining the whole-cell configuration, Once TRPM2 currents are allowed to stabilize, after which a calcium free ECF (total divalent concentration was maintained by replacing CaCl2 with equimolar BaCl2) or 10 µM Clotrimazole containing ECF was perfused into the bath to block TRPM2 channels. TRPM2 currents will be recorded by applying voltage ramps for 500ms every 10 seconds (Clampex 10.2 software; Molecular devices), wherein the voltage is stepped down from -60 mV to -100 mV and ramped up to +100 mV. Data were filtered at 2 kHz, digitized, and acquired using pCLAMP and Axoscope software (Molecular Devices). For experiments using exogenous ADPR, 1 mM ADPR is added to ICF and administered to the cell through patch pipette similar to the protocol above. To record NMDAR-mediated currents, ECF was supplemented by 30 µM serine, which acts as a co-agonist for NMDAR activation. Microglia in ECF bath were subjected to repeated applications of 300 µM NMDA (for 3s, repeated every 60s) using multi-barreled rapid perfusion system followed by wash-out with ECF to record NMDAR-mediated currents.

4.7 RNA Extraction

Pure microglial cultures in 100 mm culture dish were placed in 7 mL of MEM and incubated with 10 µM NMDA. It was washed 24-hours later with ice cold PBS and the cells were scraped with 1 mL of Trizol reagent. The cell suspension in Trizol was homogenized thoroughly by trituration and RNA was extracted using PureLink RNA Mini Kit as per manufacturers

instruction. The yield and quality of RNA was analyzed using Nanodrop. The RNA was stored in -80˚C freezer to avoid degradation and will be used for further experments.

4.8 Real-Time Quantitative Reverse Transcription PCR (qRT-PCR)

cDNA was synthesized by using SuperScript™ IV VILO™ Master Mix with ezDNase™ Enzyme as per manufacturers instruction. To quantify the expression level of genes of interest, cDNA samples were amplified for 40 cycles using primers (Table 1) and SYBRGreen Mastermix with the help of QuantStudio 5 system. HPRT & RPLP0 were used as reference genes since its ubiquitously expressed in microglia. Absence of DNA contaminants was confirmed in reverse transcription-negative samples and by melting curve analysis. Relative quantification of gene expression was done with the delta-delta-Ct method using HPRT and RPLP0 gene expression to normalize the data.

Target Gene	**Primer Sequence**
GluN1	forward, ACACAAGGATGCCCGTAGGA; reverse, TTTTAGGGTCGGGCTCTGCT
GluN2A	forward, TGAAAACTGGGAAGTTGGACG; reverse, TCAATCTGCCTCTTCCAGGG
GluN2D	forward, AACTACATGGCCCGAAAGGA; reverse, TCAATCTCATCGTCCCCCAG
GluN3A	forward, GCTCCTTCCCTACAACCTGT; reverse, AACCCCTTGTACCACTACGG

TRPM2	forward, CTGTCTCCGTCTCATGCACA; reverse, AGACGTCCTTCATCATCCGC
iNOS	forward, CGGAGATCAATGTGGCTGTG; reverse, GGGACAGTCTCCATTCCCAA
AG1	forward, TATGACGTGAGAGACCACGG; reverse, CTTCCAACTGCCAGACTGTG
HPRT	forward, GCTTACCTCACTGCTTTCCG; reverse, TCATCGCTAATCACGACGCT
RPLP0	forward, GACCGCCTGGTTCTCCTATAA; reverse, AGACGATGTCACTCCAACGA

Table 2: Sequences of oligonucleotide primers used for quantitative PCR

4.9 Nitric Oxide Assay

Griess test originally developed in 1879 by Johann Peter Griess to detect the presence of nitrites by forming a red-violet colored azo dye. However, the current assay being used is a modification of original test with higher sensitivity to nitrite with a lower limit of detection, down to approximately 300 nM. The short half-life of nitric oxide (130 secs) in aqueous solution is exploited in this assay, where spontaneous oxidization of nitric oxide with water and subsequent hydrolysis to form the exclusive product nitrite which can be measured spectrophotometrically.

Pure microglial cultures in 48-well plate were placed in 125 µL of MEM and incubated with NMDA alone or with inhibitors, for 20-24 hours. Modified Gries reagent containing 60 mM sulphanilamide, 4 mM N-(1-Napthyl)ethylenediamine and 1.95% phosphoric acid in double distilled water and filtered using 0.4 µm filter was prepared on the day of the assay. This light

sensitive reagent was wrapped with aluminum foil until the experiment. $NaNO_2$ standards were prepared in MEM and 50 μL/well in triplicate on a 96-well plate. Culture medium from each well was plated on the 96-well plate at 50 μL/well alongside the $NaNO_2$ standards and the modified Griess reagent was added at equal volumes and allowed to incubate at room temperature for 10 minutes. The absorbance was then read at 540 nm. A standard curve was plotted, and sample concentrations were calculated. Also, excess culture media from each well was mixed with 2X protease inhibitor and stored in -80˚C for future use to measure the cytokine levels.

The values for nitric oxide release was normalized to the protein content of each well measured. Each well was washed twice with MEM to remove culture medium and the microglia were then lysed by scraping in RIPA lysis buffer on ice. BSA standards and protein extracts were plated into a 96-well plate in triplicate at 5 μL volume per well, and protein concentration of each well was measured using a Bio-Rad DC protein assay kit with absorbance read at 750 nm.

4.10 Immunocytochemistry

Pure cultures of primary microglia on 35 mm imaging-grade plastic-bottom dishes (ibidi, WI) was treated with 10 μM NMDA for 7 hours. The cells were washed in ECF thrice and fixation was done using PLP Fixative (Periodate-Lysine-Paraformaldehyde) for 10 mins at room temperature. To perform immunolabelling, permeabilization of the cell was not performed. The cells were blocked by non-specific protein binding using 5% normal serum in phosphate buffered saline (PBS) for 30 mins. The cells were then incubated with primary antibody for 2 hours (diluted in blocking buffer) at room temperature, followed by washing with PBS. Primary antibody used in my experiment to target NMDAR sub-unit NR1 was obtained from Invitrogen (Cat PA5-34599) at a dilution of 1:1000. Post-washing, microglia were subsequently incubated with appropriate fluorescent secondary antibody in blocking buffer for 90 mins. Excess secondary antibody was

washed with PBS before the dishes were incubated for 20 mins with Actin green to stain the microglial membrane. Excess actin was removed by PBS wash, followed by 5 mins incubation with DAPI to stain the nuclei. After a final three washes with PBS, dishes were preserved in PBS at 4 0C for imaging within two weeks. Images were acquired by 63X objective using super-resolution fluorescence microscopy (Zeiss 880 with AiryScan).

4.11 Statistical Analysis

Raw data from voltage-clamp recordings were acquired & extracted using pCLAMP Clampex 10.2 software (Molecular Devices), and processed & analyzed using pCLAMP Clampfit 10.6 software (Molecular Devices). GraphPad Prism 5 (GraphPad Software, CA) was used for statistical analysis. All the data values reported are expressed as mean ± standard error of the mean (SEM). Means values are compared using Student's t-test for single group comparison, and one-way analysis of variance (ANOVA) with Bonferroni's post-hoc test (compare all pairs of columns) for multiple group comparisons. $P<0.05$ was considered significant for all the tests.

CHAPTER 5: RESULTS

5.1 Functional TRPM2 channels are present in primary microglia

Since the project is focused on investigating the role of TRPM2 channel in microglia, it was important to establish reliable method for the identification of microglial TRPM2 current. Hence, currents were recorded from primary microglia using whole-cell voltage-clamp electrophysiology. Microglia were voltage-clamped at -60 mV, and currents were monitored using voltage ramps by stepping the voltage to -100 mV and ramping to +100 mV over 500 ms. ADP-ribose (ADPR), an agonist of TRPM2, was used to elicit TRPM2 currents. As illustrated in Fig 8A, ADPR (1 mM) was administered exogenously through the patch pipette. Upon gaining access to the cell interior, ADPR freely diffused into the cell and evoked a large current. The current-voltage (I-V) traces showed that currents are linear with reversal potential of 0 mV, consistent with the reported I-V properties of TRPM2 channels (Fig 8B).

Past studies have shown that TRPM2 channels can be inhibited by removal of calcium from extracellular bath (Eisfeld and Luckhoff 2007; Kraft and Harteneck 2005). Hence, to confirm that ADPR-evoked currents in microglia were in fact mediated by TRPM2, calcium was removed from the extracellular bath, which inhibited TRPM2 currents completely (Fig 8 C,D). Similarly, application of 10 µM clotrimazole (Ctz), an inhibitor of TRPM2, completely inhibited TRPM2 currents (Fig 8 C,D). Comparable currents were not observed in the absence of ADPR. Inhibition of currents upon removal of calcium from the extracellular bath/application of Ctz, serve as hallmark for TRPM2 channel. Thus, reliable methods for identification of TRPM2 currents from microglia were established and was deemed qualified for further experimentation.

ADPR-evoked current obtained at +100 mV is termed as peak current (I_{peak}) and the current remaining at +100 mV after removal of calcium from the extracellular bath or application of

TRPM2 inhibitor is termed as residual current (I_{res}). TRPM2 currents represented in my thesis hereon is the difference in current amplitude between I_{peak} and I_{res} as indicated in Fig 8 C,D.

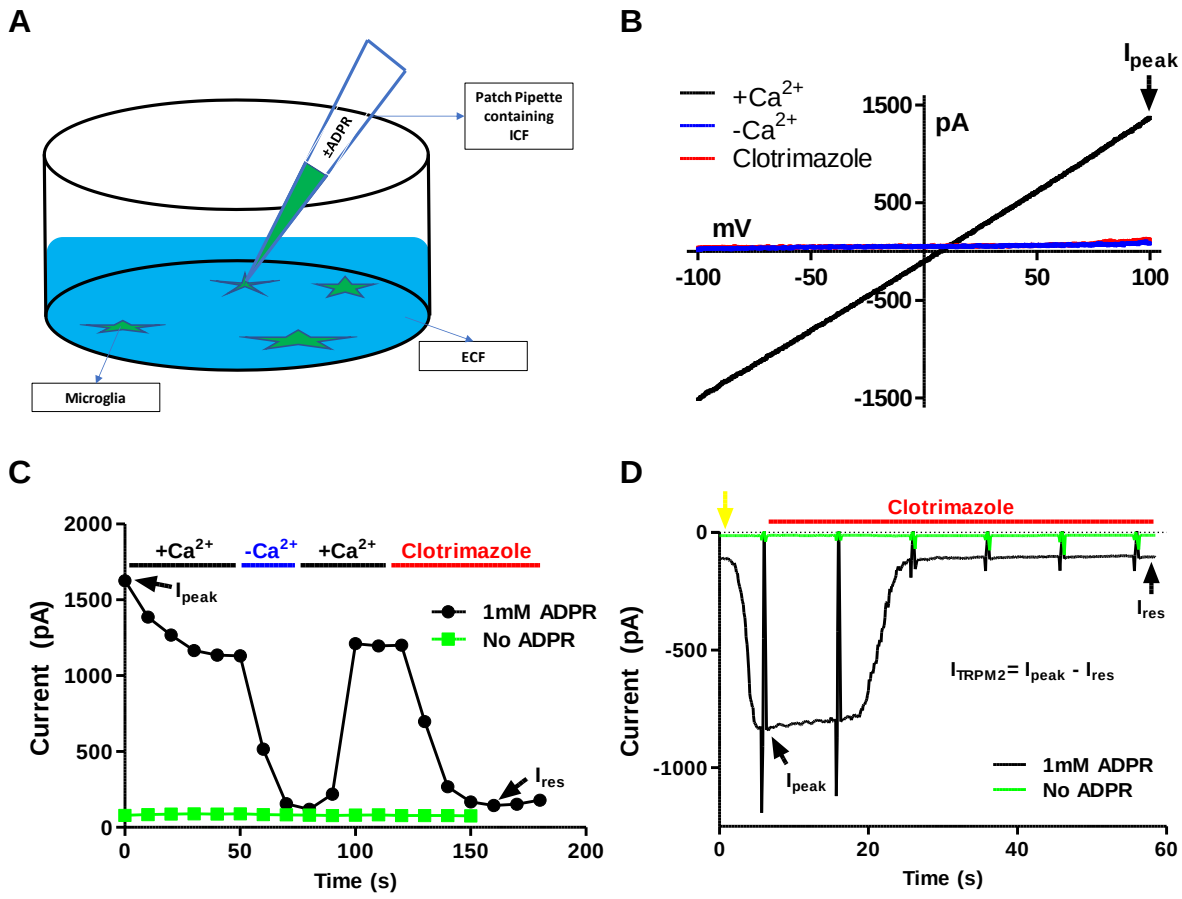

Figure 8: Functional presence of TRPM2 in Microglia

(A) Schematic representation of patch pipette containing ICF being used to administer ADPR intracellularly in microglia (B) Linear current-voltage (I-V) curve with reversal potential at 0 mV confirming ADPR-evoked current as TRPM2 current. Indicated in arrow is the I_{peak} measured at +100 mV. Also shown is the I-V curves after inhibition of TRPM2 current by removal of Ca^{2+} or application of clotrimazole. (C) Illustration of the protocol followed during whole-cell voltage-clamp recordings with current recorded upon exogenous 1mM ADPR stimulation, followed by removal of extracellular calcium from the bath or culminating in application of clotrimazole to the bath, to confirm it as TRPM2 current. Comparable currents were not observed in the absence of ADPR. (D) A representative trace showing ADPR evoked current inhibited by application of

clotrimazole. Marked in yellow arrow is the moment of breakthrough where ADPR enters the cell. Also indicated with arrows is I_{peak} and I_{res} used to calculate TRPM2 current.

5.2 Microglia exhibit sex difference in the amplitude of TRPM2 currents

Past studies have shown that TRPM2 exhibit sex differences in the consequence of their pathological activation. Treatment with TRPM2 antagonist offered neuroprotection in male rodents upon ischemic injury, but no effect on injury was observed in females (Jia et al. 2011; Nakayama et al. 2013). Not known is whether this sex difference is due to differences in TRPM2 function between male and female, and will be assessed by electrophysiological recordings.

To test this, I prepared primary microglial cultures from male and female mice separately. I then performed whole-cell voltage-clamp electrophysiology using the approach described in Fig 8 and recorded TRPM2 currents in these microglia. Upon application of a saturating concentration of ADPR (1mM) intracellularly, I observed that currents were 2.4-fold larger in male compared to female (Fig 9A). This provides evidence of sex difference in the amplitude of ADPR-evoked TRPM2 currents in microglia.

It's been a long-lasting debate whether microglial morphology co-relates to their function. Past studies have also shown increase in microglial morphological state corelate with altered transcription profile, including expression of ion channels. Hence, I wanted to evaluate how TRPM2 current amplitude correlated to each microglial morphology status in both sexes. I observed that increase in TRPM2 current correlating with increase in morphological transformation (Fig 9 B,C). I also observed TRPM2 currents were prominently larger in male compared to female in all morphologies (Fig 9 B,C). Notably, TRPM2 currents in male amoeboid microglia was 2.42-fold larger than female (Fig 9 B,C). This provides evidence of increased TRPM2 channel activity with increase in microglial morphological activity, in both male and female microglia. Furthermore, sex differences are maintained irrespective of cell morphology.

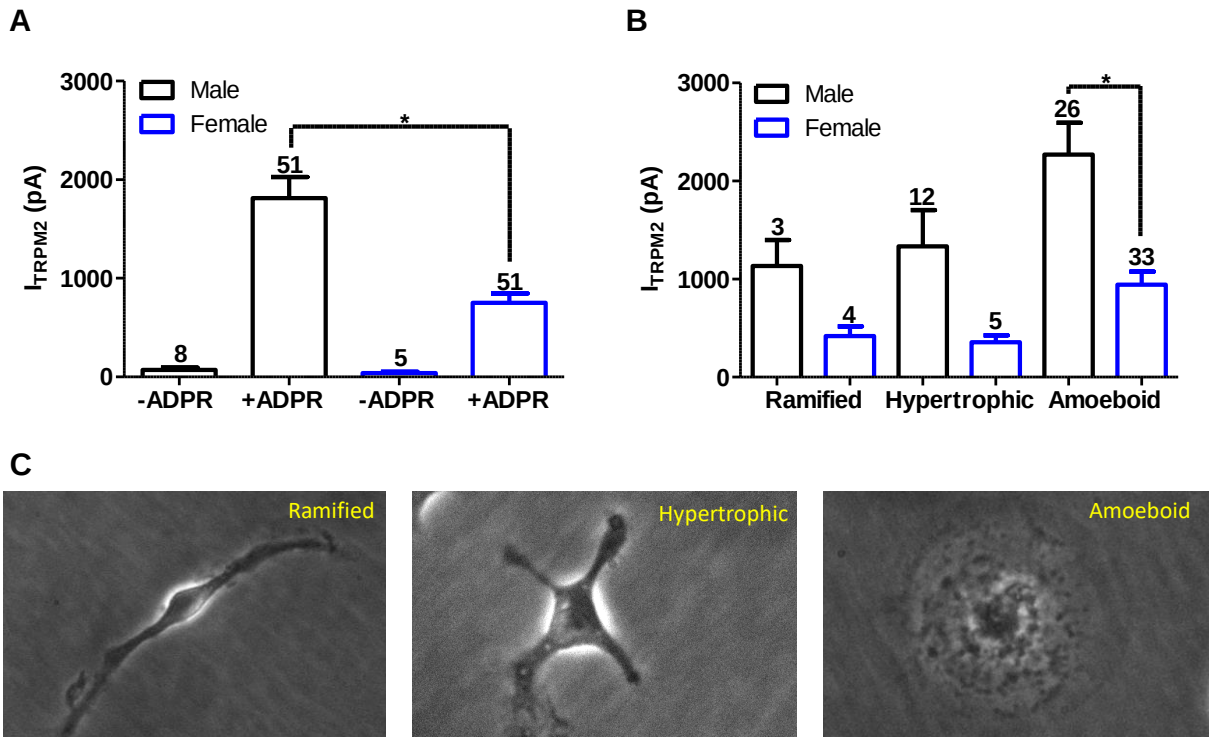

Figure 9: Microglia exhibit sex difference in amplitude of TRPM2 current

(A) Whole-cell voltage-clamp recordings performed separately from male and female microglia upon exogenous ADPR stimulation showed 2.4-fold larger TRPM2 currents in male compared to female. (*p<0.0001). (n indicated over the bars represent number of microglia from 4-8 cell preparations, from 5 male and female mice). (B) Analysis of TRPM2 currents based on microglial morphology from both male and female mice showed increase in TRPM2 currents upon increase in microglial activation status, from ramified to amoeboid morphology. TRPM2 currents in male amoeboid microglia showed 2.42-fold larger than female. (*p<0.0001, n indicated over the bars represent number of microglia from 1-5 cell culture preparations, from 5 male and 5 female mice). (C) Representative images of different microglial morphology used for analysis – ramified, hypertrophic and amoeboid microglia.

5.3 AβO treatment induces activation of TRPM2 channels in a sub-population of microglia

Accumulation of amyloid beta oligomers (AβO) in the brain causes activation of microglia (Dani et al. 2018; Doens and Fernández 2014). Our hypothesis predicts that amyloid beta stimulates PARP-1/PARG endogenous production of ADPR, which leads to TRPM2 channel activation. In this situation, measurable TRPM2 currents should be detected upon gaining whole-cell access using voltage-clamp electrophysiology.

To test this hypothesis, primary microglia treated with AβO (2.57 pM) for 1-7 hours were used for recording TRPM2 currents. Upon gaining whole cell access, large inward currents were visible in AβO amyloid treated microglia, but not in vehicle control (Fig 10A and 10B, where arrow indicates the moment of breakthrough into the cell). These currents were mediated by TRPM2 based on their inhibition by removal of Ca^{2+} from the extracellular bath or by application of clotrimazole, a TRPM2 blocker. Also, the current-voltage (I-V) trace showed that currents recorded were linear with reversal potential of 0 mV, a distinctive feature of TRPM2 channels (Fig 10C). This confirms that AβO treatment of microglia induces TRPM2 channel activation.

Interestingly, I observed that not every microglia treated with AβO recruit TRPM2 current. Using scatter bar graph analysis, I noticed a sub-population where the amplitude of TRPM2 currents in AβO treated microglia that were comparable to vehicle control (Fig 10D). Hence, a criterion was established where AβO treated microglia showing TRPM2 current amplitude 1.5-fold larger than the average TRPM2 current in vehicle control were termed as responders. Further, I performed a series of individual recordings separately from microglia obtained from male or female mice. These microglia were treated with AβO, before recording TRPM2 currents from them. Note that, using the criterion previously established, only amplitude of TRPM2 currents

from responders were reported going further. Firstly, I saw that only 60% of the AβO treated microglia induce TRPM2 channel activation. However, responder rates were comparable in male and female (Fig 10E). I also noticed no difference in amplitude of TRPM2 currents in microglia between male (221.27 ± 38.11 pA) and female (168.50 ± 42.27 pA) upon AβO treatment (Fig 10F). Since I observed no sex difference in the amplitude of TRPM2 currents or responder rates, rest of the data presented here is combined.

Finally, I wanted to evaluate whether AβO treatment changes the number of TRPM2 on microglial cell-surface, leading to altered functional outcomes, i.e., amplitude of TRPM2 current. To evaluate this, I recorded TRPM2 currents in microglial treated with AβO by administering exogenous ADPR (1 mM) though the patch pipette. I observed that amplitude of TRPM2 currents in AβO treated microglia were comparable to control (Fig 11C). This suggests that amyloid beta treatment does not change number of TRPM2 receptors on the cell-surface.

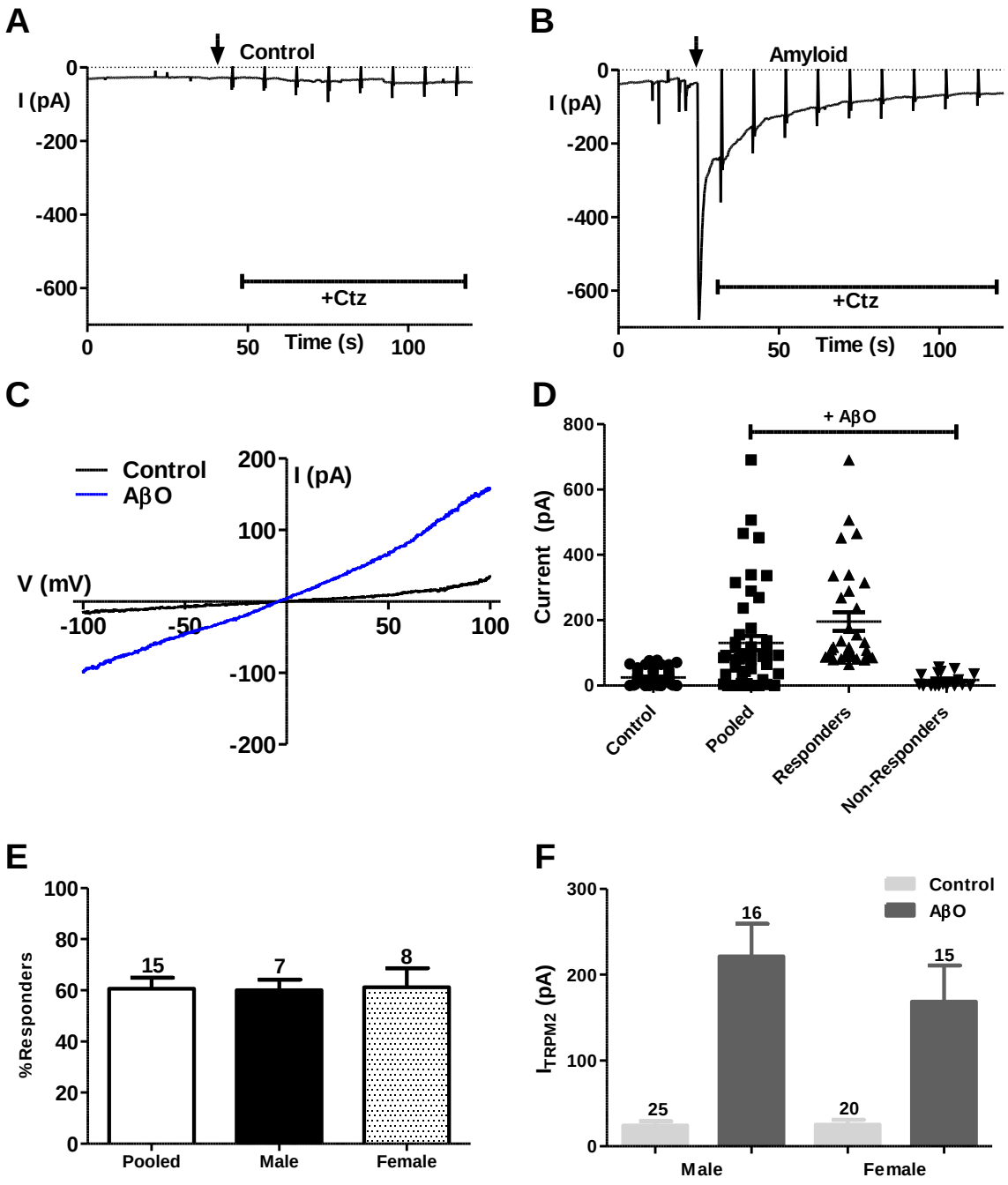

Figure 10: Microglial AβO treatment induces TRPM2 channel activation in a sub-population of microglia.

(A, B) Representative traces showing TRPM2 currents from control and AβO treated microglia showing TRPM2 currents. (C) AβO induced TRPM2 current confirmed by linear I-V curve with reversal potential at 0 mV (D) Sub-population of responders and non-responders were identified in AβO treated microglia based on its ability to activate TRPM2 channel when compared to control. (E) No significant change was observed in responder rate between male and female microglia treated with amyloid beta (n indicated over the bars represent number of different cell preparations from 7 male mice and 8 female mice). (F) The amplitude of TRPM2 current in male (221.27 ± 38.11 pA) and female microglia (168.50 ± 42.27 pA) upon AβO treatment was comparable (n indicated over the bars represent number of microglia prepared from using 3-8 male mice and 2-8 female mice).

5.4 AβO induce activation of TRPM2 through NMDAR/PARP-1/PARG dependent pathway in microglia

Having confirmed that AβO treatment induced microglial TRPM2 channel activation, I next evaluated the role (or contribution) of PARP-1 based signaling in recruitment of TRPM2. Our hypothesis predicts that TRPM2 is recruited downstream of NMDARs, PARP-1 and PARG activation. In order to prove this, I used pharmacologic inhibitors of these targets and genetic ablation models of PARP-1 and TRPM2.

To assess whether AβO induced activation of TRPM2 involves NMDARs, I tested three different inhibitors of NMDA receptors (APV, MK-801 and CKA). APV (2R)-amino-5-phosphonovaleric acid; 50 μM) is a selective NMDA receptor antagonist that competitively inhibits the glutamate binding site of NMDA receptors. MK-801 (10 μM), is an uncompetitive antagonist of the N-Methyl-D-aspartate (NMDA) receptor, that blocks the channel pore. CKA (7-Chlorokynurenic acid; 20 μM) blocks the glycine binding site of NMDA receptors. AβO did not elicit TRPM2 currents in the presence of any of the three NMDAR inhibitors, confirming that amyloid beta stimulated TRPM2 activation requires NMDARs (Fig 11A). In addition, block by MK-801 specifically confirms the requirement of ionotropic function of NMDARs for TRPM2 channel activation in AβO treated microglia.

I next proceeded to evaluate the role of PARP-1 in AβO stimulated TRPM2 channel activation by an experiment using pharmacological blocker of PARP-1 (PJ34). PJ34 competitively blocks binding of NAD^+ to PARP-1 by inserting a flexible moiety into hydrophobic sub-pockets in various ADP-ribosyl transferases (Steffen et al. 2013; Thorsell et al. 2017). TRPM2 currents in AβO treated microglia were completely inhibited by PJ34 (500 nM) (Fig 11A). This proved that AβO stimulated TRPM2 activation is PARP-1 dependent.

My next aim was to evaluate the contribution of PARG to this signaling pathway. I used an inhibitor of PARG, PDD00017273 (PDD), designed as a molecular probe synthesized based on structural knowledge of the PARG binding site. TRPM2 currents in AβO treated microglia were inhibited upon treating with PDD (1 μM) (Fig 11A). This provides a conclusive evidence that AβO stimulated activation of TRPM2 involved a PARG dependent pathway. These results combined support my hypothesis that AβO induce activation of TRPM2 current through a pathway involving NMDARs, PARP-1 and PARG.

However, one caveat to my previous experiments using pharmacological inhibitors was that the inhibitors used (i.e. PJ34 and PDD) could in theory directly inhibit TRPM2. This is because the catalytic site of PARP/PARG accept molecules that are structurally analogous to ADPR (e.g. purine analogs). Also, administration of exogenous ADPR in this experiment is necessary to show the catalytic site of ADPR binding is not inhibited by PARP-1 or PARG blockers. I therefore treated microglia with inhibitors of PARP-1/PARG inhibitors and recording TRPM2 currents by administering exogenous ADPR. I observed that TRPM2 current amplitude in microglia treated with PARP-1/PARG inhibitors did not show significant change when compared to control (Fig 11C).

Further, the lack of selectivity of available inhibitors specific for PARP-1, including PJ-34, provides a strong rationale for using genetic ablation mice models of PARP-1 for validation of my results obtained so far. Hence, primary microglia from PARP-1 KO mice were used to record TRPM2 current upon AβO stimulation. TRPM2 currents were completely absent in microglia recorded from PARP-1 KO mice (Fig 11B). This confirms that PARP-1 is necessary for AβO mediated recruitment of TRPM2 current. However, given that PARP-1 can function as co-activator and repressor of transcription factors, it is possible that PARP-1 depletion could alter the

expression of TRPM2 (Kraus and Lis 2003). Hence, I recorded TRPM2 currents from WT and PARP-1 KO microglia by using exogenous ADPR. However, as shown in Fig 11C, ADPR-evoked TRPM2 currents showed no difference between WT and PARP-1 KO microglia, confirming that TRPM2 expression is not dependent on PARP-1s function as co-activator.

Non-specificity of pharmacological inhibitors is also true for clotrimazole, a non-specific TRPM2 inhibitor. Hence, I performed experiments by recording from TRPM2 KO microglia. AβO treated microglia showed no change in amplitude of TRPM2 compared to control (Fig 11B). Also, I observed no microglial TRPM2 currents upon ADPR stimulation, which confirmed deletion of TRPM2 in TRPM2 KO mice (Fig 11C). Collectively, these results prove that functional TRPM2 channels in microglia remain unaffected by short-term inhibition (PARP-1/PARG) as well as complete deletion of PARP-1.

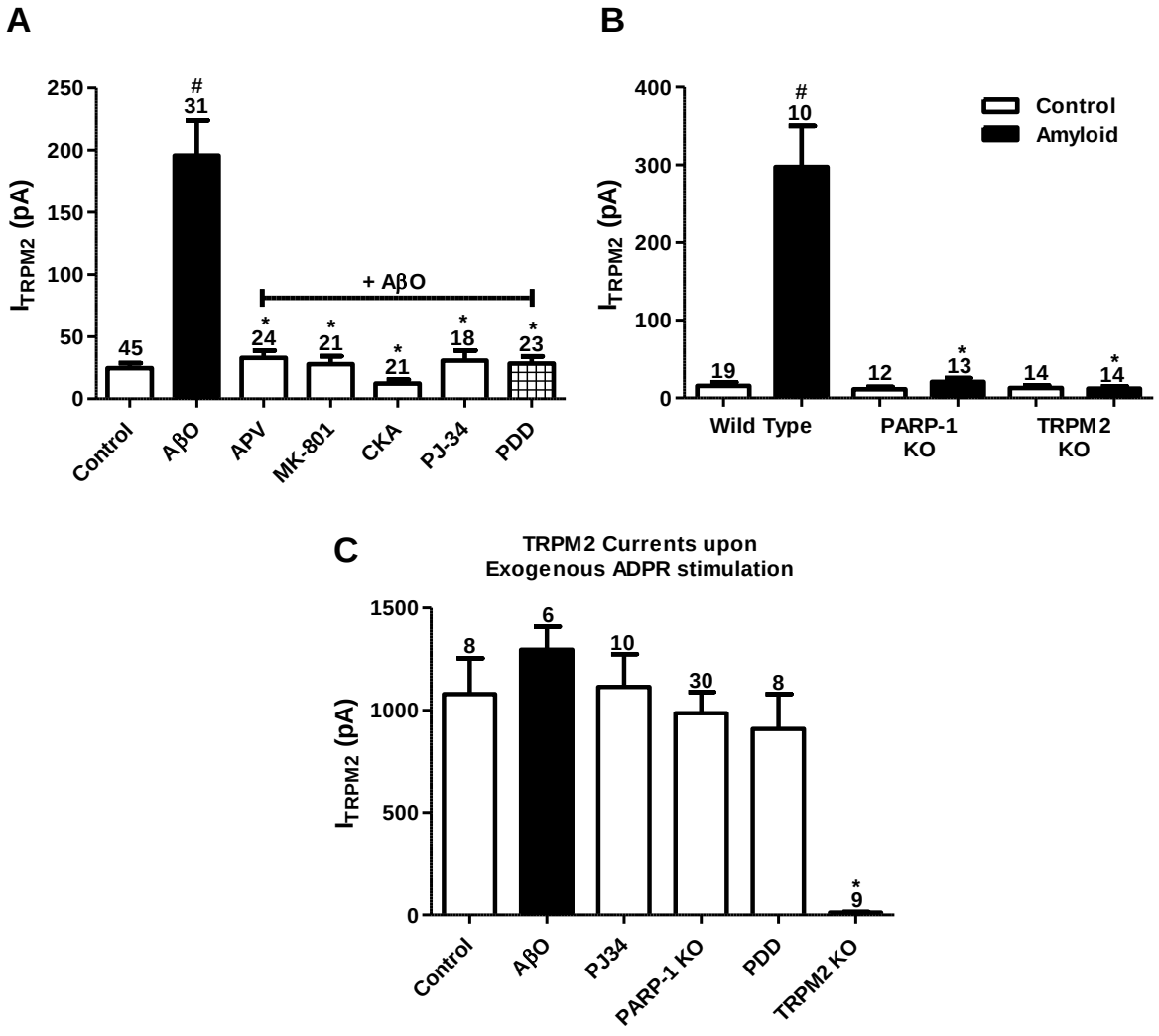

Figure 11: Genetic ablation of PARP-1/TRPM2 inhibits AβO induced TRPM2 currents.

(A) AβO (2.57 pM) treatment recruited TRPM2 currents in microglia were blocked by inhibitors of NMDARs (50 µM APV, 10 µM MK-801, 20 µM CKA), PARP-1 (500 nM PJ-34) and PARG (1 µM PDD). # $p<0.0001$ compared to Control, *$p<0.0001$ compared to AβO (n indicated over the bars represent number of microglia prepared from 5-16 mice). (B) AβO treated microglia showed TRPM2 channel activation in WT microglia but were absent in PARP-1 KO and TRPM2 KO microglia. # $p<0.0001$ compared to WT Control, *$p<0.0001$ compared to WT Amyloid (n indicated over the bars represent number of microglia prepared from 5 WT mice, 4-5 PARP-1 KO

mice, and 3 TRPM2 KO mice). (C) Upon stimulation with exogenous ADPR, no difference in amplitude of TRPM2 currents were observed between microglia treated with vehicle control, AβO, PARP-1 inhibitor and PARG inhibitor. Also, ADPR-evoked TRPM2 currents in PARP-1 KO microglia were comparable to control, but absent in TRPM2 KO. *$p<0.0001$ compared to control (n indicated over the bars represent number of microglia prepared from 2-8 mice).

5.5 NMDA stimulation alone is sufficient to elicit TRPM2 currents in microglia

My results in section 5.4 demonstrates that AβO activates microglial TRPM2 channel through NMDARs. My observation that this development of TRPM2 current is blocked by the NMDAR channel pore blocker MK801, demonstrates ion conduction, presumably of Ca^{2+}. Hence, Ca^{2+} influx through NMDARs is necessary for activation of PARP-1, eventually leading to TRPM2 channel activation. Hence, I wanted to test whether NMDA, an agonist for NMDA receptors is sufficient to activate microglial NMDA receptors leading to recruitment of TRPM2 currents.

To test this hypothesis, I used whole-cell voltage clamp technique to record from primary microglia treated with 10 μM NMDA (10 times the EC_{50} concentration)(Yuan et al. 2009), applied in the presence of glycine (co-agonist) in the media for 1-7 hours. The voltage-clamp data revealed development of inward current upon gaining whole-cell access in NMDA treated microglia (Fig 12B), but not in vehicle control (Fig 12A). These currents were mediated by TRPM2 based on their inhibition by removal of Ca^{2+} from the extracellular bath or by application of clotrimazole, a TRPM2 blocker. Also shown is the current-voltage (I-V) trace, where current developed were linear with reversal potential of 0 mV, a distinctive feature of TRPM2 channels (Fig 12C). This confirms that NMDA treatment is sufficient to induce TRPM2 channel activation in microglia.

Similar to AβO treated microglia, I observed that not every microglia treated with NMDA showed TRPM2 channel activation. Using the previously established criteria for selecting responders, I however observed that only 35% of the NMDA treated microglia induce TRPM2 channel activation compared to 60% with AβO treatment (Fig 12D). I also compared for difference in amplitude of TRPM2 currents recorded from microglia treated separately with NMDA and AβO. No significance difference was observed in terms of TRPM2 current amplitude between

NMDA treated microglia (218.94 ± 40.19 pA) and AβO treated microglia (201.92 ± 29.94 pA) (Fig 12E). Again, no sex difference was observed in the amplitude of TRPM2 currents or responders' rate (data not shown).

The difference in responders' rate between NMDA treated and AβO treated microglia could be due to change in TRPM2 expression upon treatment. To address this, TRPM2 protein levels need to be assessed. However, due to relatively poor availability of specific TRPM2 antibodies so far, measuring TRPM2 protein levels was not feasible. Hence, using qRT-PCR, I evaluated the transcript levels of TRPM2 in microglia treated with NMDA or amyloid beta for 24 hours. RPLP0 and HPRT were used as housekeeping genes for these experiments. I observed 40% increase in level of microglial TRPM2 transcripts upon AβO treatment, but no significant change was observed in TRPM2 levels of NMDA treated microglia (Fig 12F). Increased TRPM2 transcript levels in AβO treated microglia compared to NMDA treatment correlated with the increase in responder rates.

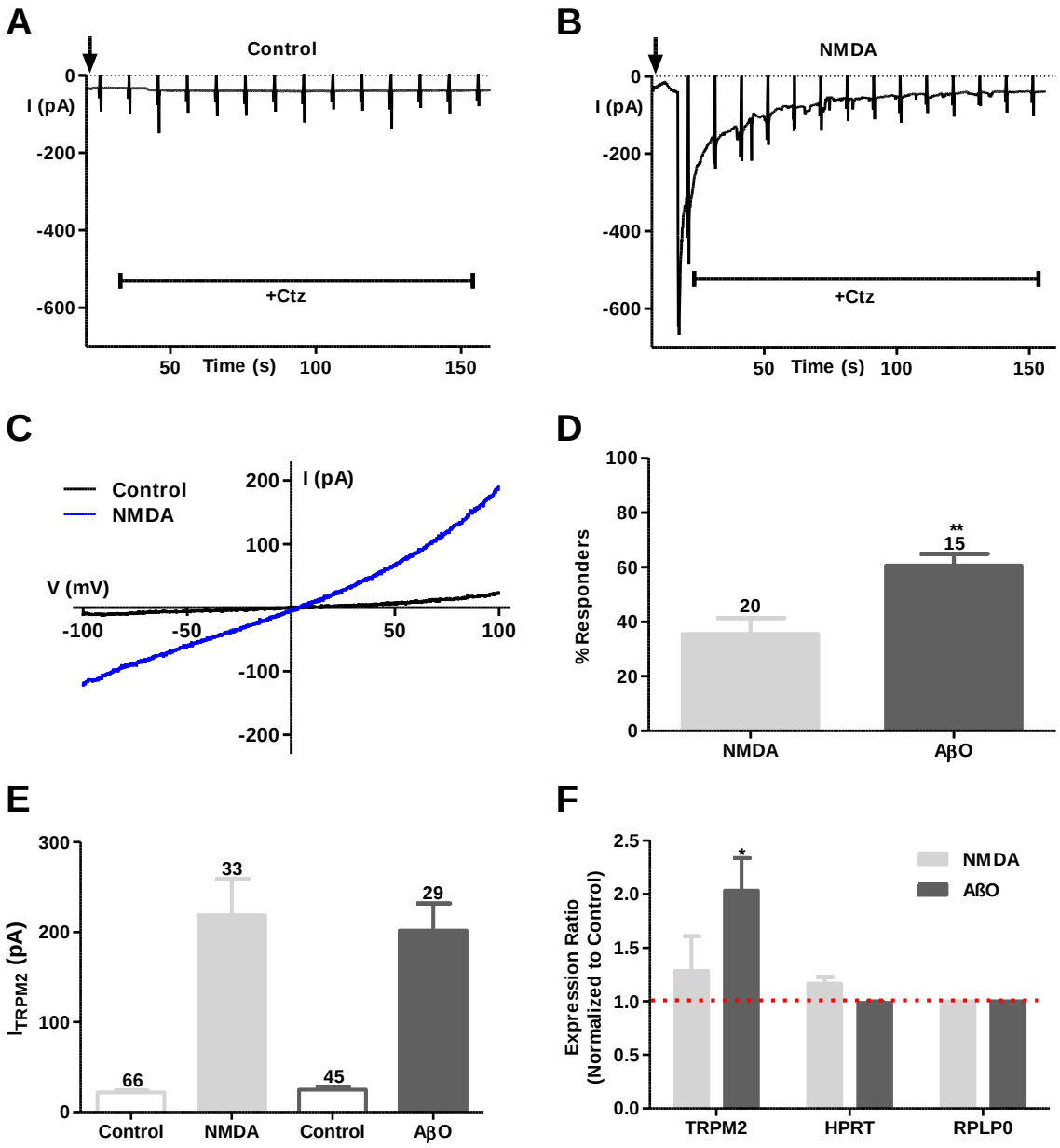

Figure 12: NMDA stimulation is sufficient to induce TRPM2 channel activation in microglia.

(A, B) Representative traces showing TRPM2 currents from control (A) and NMDA (B) treated microglia. (C) NMDA induced TRPM2 current confirmed by linear I-V curve with reversal potential at 0 mV. (D). Larger population of microglia showed TRPM2 currents to AβO treatment

than to NMDA treatment (60% vs 35%) (n indicated over the bars represent number of different cell preparations from 15-16 mice for each group). (E) The amplitude of TRPM2 currents in NMDA (218.94 ± 40.19 pA) and AβO treated microglia (201.92 ± 29.94 pA) was comparable (n indicated over the bars represent number of microglia prepared from 13-16 mice for each group). (F) Differential change in mRNA expression of TRPM2 upon NMDA and AβO treatment in microglia. HPRT and RPLP0 are housekeeping genes (n=2).

5.6 NMDA induced TRPM2 activation also acts through the NMDAR/PARP-1/PARG dependent pathway in microglia

Having confirmed that NMDA treatment is sufficient to induce TRPM2 channel activation in section 5.5, I wanted to evaluate whether PARP-1/PARG mediate recruitment of TRPM2 is involved here. To investigate this, I used 3 pharmacological inhibitors (APV, MK801 and CKA) separately, that block NMDA receptors through different mechanism. To assess NMDARs role in NMDA induced activation of TRPM2, microglia treated with NMDA were co-incubated with these three inhibitors separately. These inhibitors bind to different sites of the NMDARs as explained earlier in section 5.4.

Upon recording from these microglia, I observed no TRPM2 currents in the presence of any of the three NMDAR inhibitors, proving NMDA-induced TRPM2 activation is indeed NDMAR dependent (Fig 13A). This demonstrates that NMDA induced TRPM2 channel activation is indeed via specific binding of the ligand binding sites of NMDARs, and involve its pore-channel opening. Further, PARP-1 and PARG inhibitors namely PJ34 (500 nM) and PDD (1 μM) also completely inhibited NMDA induced TRPM2 activation (Fig 13A). These results in combined support my hypothesis that AβO induces activation of TRPM2 currents through a pathway involving NMDARs, PARP-1 and PARG.

Pharmacological inhibitors PJ-34 and clotrimazole, blocking PARP-1 and TRPM2 respectively, are non-specific and hence further experiments were performed using microglia from genetic ablation mice models of PARP-1 and TRPM2. TRPM2 currents were assessed in primary microglia from WT, PARP-1 KO and TRPM2 KO mice. NMDA treated microglia from WT mice showed recruitment TRPM2 currents, which were completely absent in NMDA treated microglia from PARP-1 and TRPM2-KO mice (Fig 13B). These results combined confirms that NMDA

mediated activation of TRPM2 channel is through the NMDAR/PARP-1/TRPM2 dependent pathway. This further supports the results from experiments with AβO, where TRPM2 was recruited through the same signaling pathway as seen here.

All the utilized pharmacological inhibitors utilized completely blocked the development of TRPM2 currents in response to NMDA or AβO treatment. Serving as a negative control, we used a pharmacological inhibitor blocking a molecular target independent of the proposed signaling pathway. CNQX (20 μM), a blocker of AMPA receptor was not expected to block NMDA induced signaling. Indeed, NMDA treated microglia co-incubated with CNQX did not block NMDA-induced recruitment of TRPM2 current (Fig 13A). This further establishes that microglial NMDA receptor stimulation induces TRPM2 channel activation through a signaling pathway involving NMDAR/PARP-1/PARG.

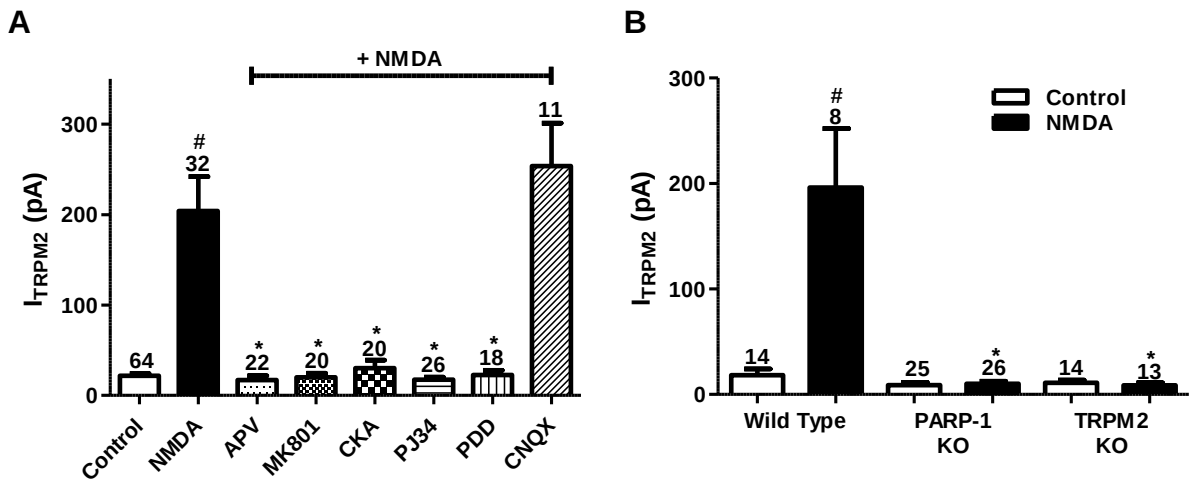

Figure 13: Genetic ablation of PARP-1/TRPM2 inhibits NMDA induced secondary activation of TRPM2

(A) 10 µM NMDA induced TRPM2 currents in microglia, blocked by inhibitors of NMDARs (50 µM APV, 10 µM MK-801, 20 µM CKA), PARP-1 (500 nMPJ34) and PARG (1 µM PDD). CNQX, an AMPA receptor inhibitor used as a negative control, did not prevent NMDA induced TRPM2 current. # $p<0.0001$ compared to Control, *$p<0.0001$ compared to 10 µM NMDA (n indicated over the bars represent number of microglia prepared from using 3-15 mice). (B) TRPM2 currents were recorded from microglia treated with 10 µM NMDA but were completely absent in PARP-1 KO and TRPM2 KO microglia. (# $p<0.0001$ compared to WT Control, *$p<0.0001$ compared to WT NMDA, n indicated over the bars represent number of microglia prepared from 4 WT mice, 4-5 PARP-1 KO mice, and 3 TRPM2 KO mice).

5.7 Presence of NMDAR transcripts and functional protein in microglia is confirmed by qRT-PCR and immunostaining

Using pharmacological inhibitors of NMDARs, I have shown in section 5.6 that TRPM2 channel activation upon NMDA stimulation requires NMDAR. Therefore, I went on to evaluate the level NMDAR transcripts (NR1, NR2A, NR2D, and NR3A) in microglia after treatment with NMDA (10 µM) or amyloid beta (2.57 pM) for 24 hours. NMDA treatment increased expression of NMDAR sub units NR2A and NR2D, whereas NR1 expression was downregulated (Fig 14D). Interestingly, AβO significantly downregulated expression of NR1, NR2A and NR3A, while NR2D expression was upregulated. This provided evidence of presence of NMDAR transcripts in microglia.

However, it is well known that level of transcript does not necessarily corelate to the amount of protein in microglia (Boutej et al. 2017). Hence, I performed immunostaining of NMDA receptor sub-unit NR1, which is necessary for translocation of NMDAR hetero-tetramers to surface of plasma membrane (K. Yang, Jackson, and MacDonald 2014). Primary microglia treated with NMDA (10 µM) for 7 hours were fixed and immunostaining was performed using NR1 antibody, actin green and DAPI. Actin green helps in identifying the microglial cytoskeleton, while DAPI stains the nucleus. I observed NR1 localization in cell body of untreated microglia (Fig 15 A,B), while in NMDA treated microglia, I observed NR1 in the proximal processes (Fig 15 C,D). This provides evidence of NMDAR expression in microglial cell-surface. However, this is a preliminary data and needs to be repeated and quantified in the future.

In addition to showing NMDAR expression on microglia cell-surface, I have also shown in section 5.6 that NMDAR mediated activation of TRPM2 channels requires ionotropic function of NMDARs by using pharmacological inhibitors of NMDARs. A direct way of showing

ionotropic function of microglial NMDARs is by recording NMDAR-mediated currents in microglia. Hence, I proceeded to record NMDAR-mediated currents from primary microglia using whole-cell voltage-clamp electrophysiology.

Repeated applications of 300 µM NMDA (3s, repeated every 60s) using a multi-barreled rapid perfusion system to microglia in extracellular bath (ECF) containing 20 µM D-serine did not evoke measurable NMDAR-mediated current (Fig 14A). Hence, to confirm that the method used for recording NMDAR-mediated currents in microglia was valid, I recorded NMDAR-mediated currents from pyramidal neurons. I observed NMDAR-mediated currents in neurons with repeated applications of 300 µM NMDA (3s, repeated every 60s) (Fig 14B). Upon seeing this, I hypothesized that microglia might need to be primed by AβO for translocation of NMDARs to the surface of the microglial plasma membrane. Hence, microglia were initially treated with AβO for 3 hours. Following AβO treatment, repeated applications of 300 µM NMDA (3s, repeated every 60s) to microglia in ECF containing 20 µM D-serine also did not evoke measurable NMDAR-mediated current (Fig 14C). These results combined showed that the method followed to record NMDAR-mediated currents in microglia were valid, but no measurable currents were evoked in response to NMDA application. However, it is not uncommon to observe no NMDAR-mediated currents in non-neuronal cells (Hogan-Cann and Anderson 2016).

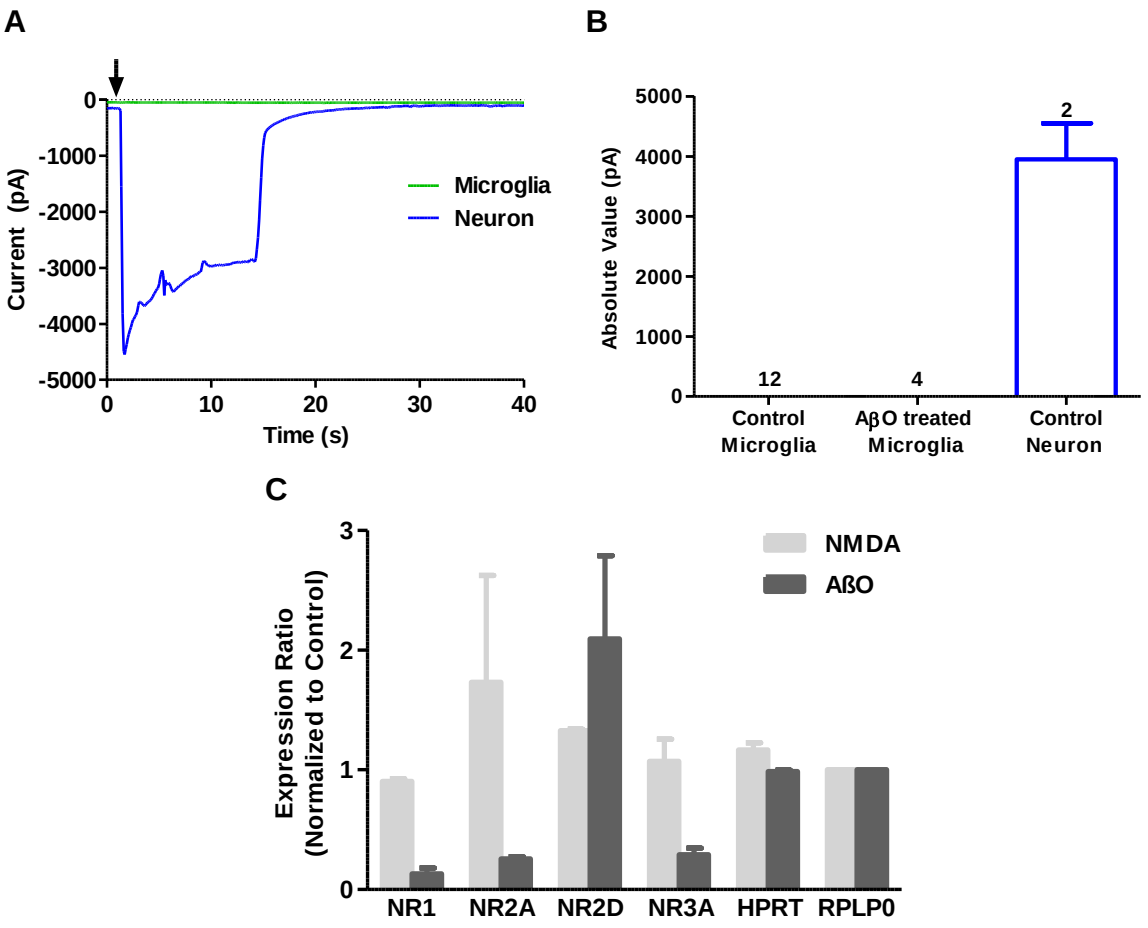

Figure 14: Measurable NMDAR-mediated currents remained elusive in microglia even with the presence of NMDAR transcripts.

(A) Representative trace showing NMDAR-mediated currents upon NMDA stimulation in neurons (positive control), but absent in microglia. Arrow indicates the time when 300 µM NMDA was applied for 3 seconds. (B) Summary bar graph showing NMDAR-mediated currents in neurons upon stimulated with NMDA. But no measurable currents were observed upon NMDA stimulation in control or AβO treated microglia (n indicated over the bars represent individual recordings). (C) Differential change in mRNA expression of NMDAR sub-units upon NMDA and amyloid beta treatment. HPRT and RPLP0 are housekeeping genes (n=2).

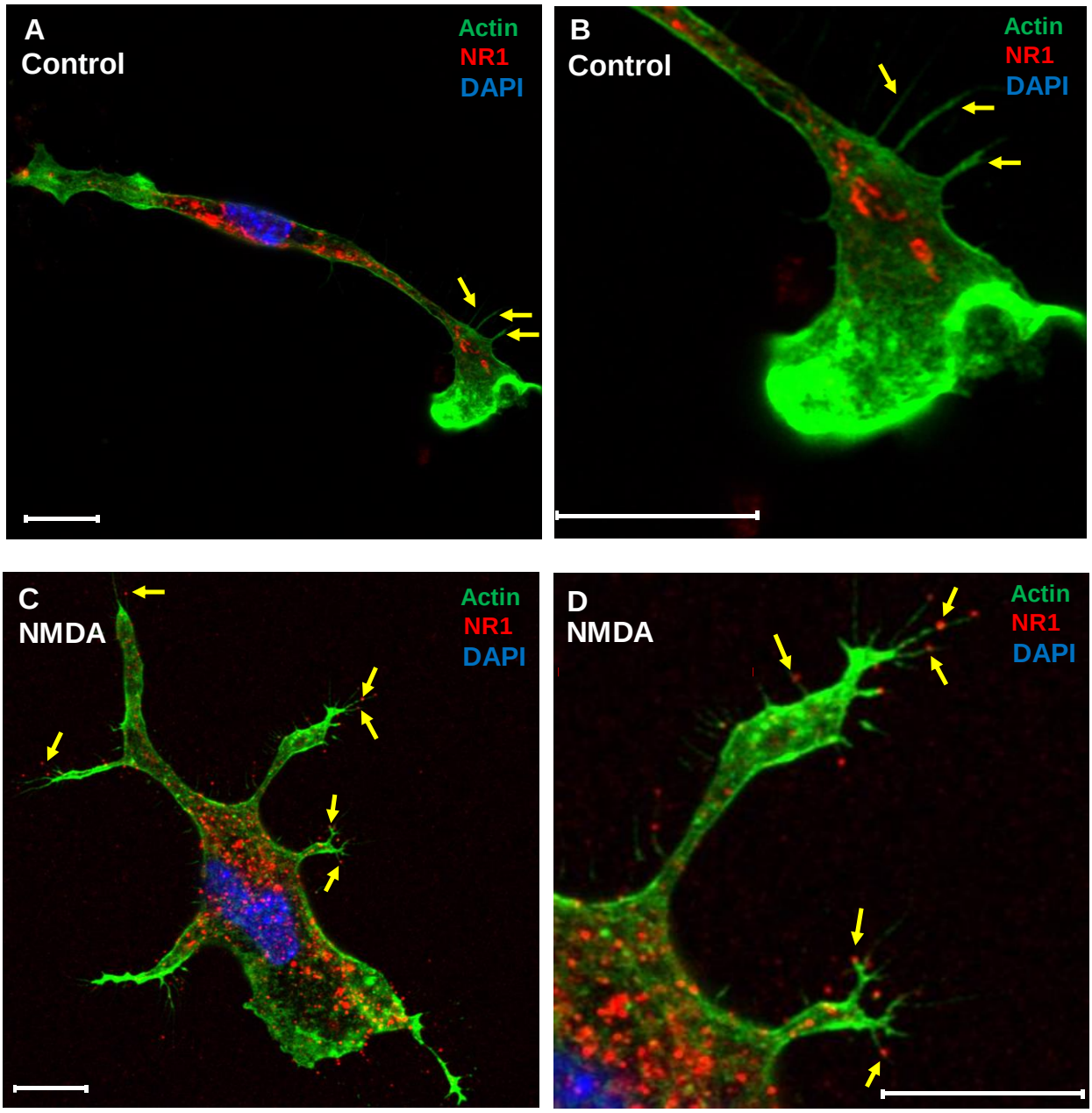

Figure 15: NMDA treatment shows functional NMDAR localization in the proximal process of microglia.

(A, C) Representative images showing presence of NMDA receptor sub-unit NR1 (Red) immunostaining in the cell body of microglia. (B, D) Microglia treated with 10 µM NMDA for 7

hours shows NR1 localization in proximal processes (D), but not in control treated microglia (B) (pointed by yellow arrows). Scale bar indicated above is 10 μm.

5.8 Amyloid beta and NMDA driven pro-inflammatory phenotype of microglia is PARP-1/TRPM2 dependent

Past studies have shown that PARP-1 expression and activity were required for AβO induced microglial activation and neurotoxicity (T. Kauppinen et al. 2011). Effects of PARP-1 are shown to be mediated in part by its interactions with NF-κB in microglia. NF-κB promotes iNOS transcription and expression leading to production of nitric oxide (NO), among several other pro-inflammatory mediators (T. Kauppinen et al. 2011). Note that iNOS/NO is considered a classical pro-inflammatory (M1) marker. To study whether TRPM2 had a role in microglial NO release, I measured NO release from microglia using griess reagent.

Firstly, I showed that microglial NO was stimulated by AβO (2.57 pM). Then, I showed that NO release was blocked with clotrimazole, an inhibitor of TRPM2. Since clotrimazole is a non-specific inhibitor of TRPM2, inhibition of NO release was also confirmed in microglia from TRPM2 KO mice. These results combined show that TRPM2, similar to PARP-1, also plays a key role in microglial NO release (Fig 16 A, B).

To further investigate whether the AβO induced production of NO in microglia follows the proposed signaling pathway of NMDAR/PARP-1/PARG, I used pharmacological inhibitors for these molecular targets. Inhibitors of NMDAR (APV, MK801, PARP-1 (PJ34) and PARG (PDD) blocked amyloid targeted NO release (Fig 16A). These results show that AβO stimulated NO release act through the NMDAR/PARP-1/PARG/TRPM2 dependent pathway.

Past studies by Tikka et.al., have shown that NMDA excitotoxicity induces microglial NO release (Tikka and Koistinaho 2001a). To evaluate whether the same signaling pathway as seen in AβO induced NO release is involved here, I used pharmacological inhibitors of NMDARs (APV, MK801), PARP-1 (PJ34), PARG (PDD) and TRPM2 (clotrimazole) upon NMDA treatment. I

observed all NMDAR, PARP-1, PARG and TRPM2 inhibitors blocked NMDA induced NO release (Fig 16C). I also confirmed the inhibition of NMDA induced NO release in microglia from TRPM2 KO mice (Fig 16B). This confirms that NMDA induced NO release also follows the same signaling pathway as seen with AβO induced NO release, acting through a NMDAR/PARP-1/PARG/TRPM2 dependent pathway. Combined, from the past and current data, I show here that NMDA/AβO induced pro-inflammatory response (i.e., NO release) in a PARP-1/TRPM2 dependent manner in microglia.

I have shown here the functional outcome of microglial activation, i.e., nitric oxide release, a pro-inflammatory response observed upon NMDA as well as amyloid treatment. However, changes in level of anti-inflammatory cytokines was not studied. Further, I assessed for any changes in level of transcripts of AG-1 (anti-inflammatory M2 marker) and iNOS (pro-inflammatory M1 marker) to evaluate prolonged inflammatory response upon NMDA/AβO treatment.

Transcript levels of both iNOS and AG-1 were evaluated in microglia treated with NMDA or amyloid beta for 24 hours. RPLP0 and HPRT were used as housekeeping genes for these experiments, with all changes expressed as ratio of control RPLP0. I observed an 18-fold increase in iNOS expression upon NMDA treatment, whereas no significant changes were observed in level of AG-1 transcripts (Fig 16D). However, AβO treatment induces at 3500-fold increase in pro-inflammatory iNOS levels in microglia. It also induced significant downregulation (by 40%) in transcript levels of anti-inflammatory marker AG-1 (Fig 16D). These results combined suggest that both NMDA as well as AβO treatment of microglia drive its pro-inflammatory phenotype, with AβO also involved in inhibiting the anti-inflammatory mediators.

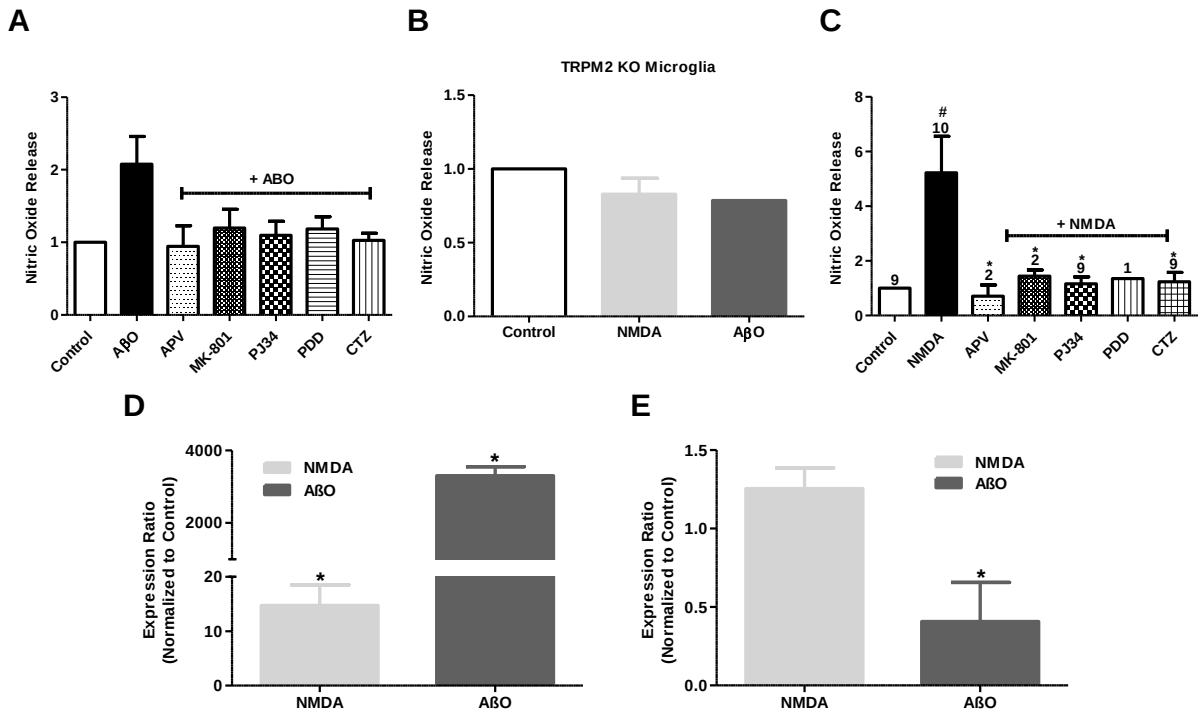

Figure 16: Pro-inflammatory response upon AβO and NMDA stimulation is driven by PARP-1 and TRPM2

(A) AβO exposure (24h) induced nitric oxide (NO) release in microglia cultures, blocked by inhibitors of NMDAR, PARP-1 and TRPM2. (n=2) (B) AβO/NMDA inhibited nitric oxide release in TRPM2 KO mice. (n=1-3) (C) NMDA stimulation induces NO release in WT microglia, blocked by inhibitors of NMDAR, PARP-1, PARG and TRPM2. (#$p<0.0001$ compared to control, *$p<0.0005$ compared to NMDA, where n=1-10) (D) Upregulation in mRNA expression of iNOS, a pro-inflammatory marker (expressed as ratio of control RPLPO level) is observed in microglia after 24h treatment with either NMDA or AβO (*$p<0.0005$ compared to control). (E) No change in mRNA expression of AG-1, an anti-inflammatory marker is observed in microglia treated with NMDA, but AβO treatment downregulates AG-1 transcript level (*$p<0.0005$ compared to control).

CHAPTER 6: DISCUSSION

6.1 Summary

The major goal of my study was to establish the role of glutamate signaling via NMDARs in amyloid beta oligomers (AβO) treated microglia and evaluate its role in the release of reactive nitrogen species, one of the chief contributors of neurotoxicity. It is well established that both PARP-1 and TRPM2 have separately been shown to be involved in Alzheimer's Disease (T. Kauppinen et al. 2011; Ostapchenko et al. 2015). However, global deletion of these genes has failed to address whether actions were due to neuronal, astrocytic or microglia deletion. But our lab has shown the contribution of microglial PARP-1 specifically to neuroinflammation in vitro (T. Kauppinen et al. 2011). Having known that chronic neuroinflammation causes irreversible neuronal damage and contributes to the progression of Alzheimer's disease, my project would be focused on elucidating the signaling mechanism in microglia that contributes to sustained neuroinflammation.

To investigate the proposed signaling mechanism involving TRPM2, using exogenous ADPR, I first validated techniques to record TRPM2 currents in primary microglia using whole-cell voltage-clamp electrophysiology. ADPR-evoked TRPM2 currents were reliably inhibited by TRPM2 inhibitors and showed characteristic linear I-V curve reversing through 0mV. Using this as my criteria to record TRPM2 current in microglia, I investigated whether amyloid beta activates TRPM2 channel. I found that AβO activates TRPM2 channels, but only in a sub-population of microglia. Further, using pharmacological blockers and genetic ablation mice models, I showed that AβO induced TRPM2 channel activation is through the NMDAR/PARP-1/PARG dependent pathway. Given that TRPM2 current was blocked by the NMDAR channel pore blocker MK801, I wanted to test whether NMDA, a specific NMDAR agonist, is sufficient to activate microglial

NMDA receptors. Microglial NMDA treatment alone led to recruitment of TRPM2 currents. I also confirmed that the same signaling pathway as seen in AβO induced recruitment of TRPM2 is involved in NMDA treated microglia. Having said that pharmacological inhibition of NMDAR pore-channel specifically by MK-801 blocked recruitment of TRPM2, proving ionotropic function of NMDARs; no measurable NMDAR-mediated currents were evoked in microglia. However, I found expression of NMDAR transcripts in both NMDA and AβO treated microglia, but also in untreated microglia. Further, NMDAR sub-unit NR1 was visualized using immunofluorescence in proximal process of NMDA treated microglia, but not in control. Finally, I observed nitric oxide release in WT microglia treated with either NMDA or AβO, but not in TRPM2 KO microglia. Further, nitric oxide release in WT microglia treated with NMDA or AβO were blocked by inhibitors of NDMAR, PARP-1, PARG and TRPM2. These results combined confirm that AβO-treated microglia activates TRPM2 through NMDAR/PARP-1/PARG dependent pathway, where sustained PARP-1 and TRPM2 activation plays a key role in chronic neuroinflammation.

6.2 TRPM2 and PARP-1 are emerging molecular targets for development of novel drugs for AD

Aβ induced dysfunction of neuronal glutamate receptors, specifically, ionotropic N-methyl-D-Aspartate receptor (NMDARs) is observed in patients of AD and hence NMDARs have long been a key target for drug development for Alzheimer's disease. However, blockade of NMDARs has failed to provide curative or disease-modifying effects, but rather is a supportive or palliative therapy (Casey, Antimisiaris, and O'Brien 2010). This has led to the focus of recent research on identifying molecular targets positioned further downstream of NMDAR stimulation. Specially, Ca^{2+} influx pathways, which were previously overlooked have recently been suggested to be potential molecular targets (Y-F. Xie, MacDonald, and Jackson 2010).

A cellular target activated downstream of Ca^{2+} influx into the cell is the nuclear enzyme PARP-1. Ca^{2+} influx has been shown to activate PARP-1 through a pathway involving ERK1/MEK2 in microglia, where ERK induced phosphorylation of PARP-1 at Ser372 and Thr373 sites is necessary for its activation (Vuong et al. 2015). PARP-1 is also shown to be an important regulator of neuroinflammation. But, in the past, major theories (cholinergic, Aβ, and tau hypothesis) and some not so widely accepted excitotoxicity theory (in section 6.5) of AD causation contradicted each other. However, a common unifying factor of all these theories is that the respective causative agents initiates a cascade resulting in neuroinflammation and neurodegeneration. Traditionally, inflammation was presumed to be a by-product of neurodegeneration, as inflammation is generally a marker of damage/infection. Neuroinflammation being a driver of neurodegeneration was unheard of in early 2000s (Wyss-Coray 2006). However, a community-based study showed that patients with AD show an increase in pro-inflammatory cytokines and inflammatory proteins in blood plasma levels before the onset of the disease (Engelhart et al. 2004). Also, both human and animal studies have shown that long-term use of Non-steroidal anti-inflammatory drugs (NSAIDs), before onset of AD symptoms, has a sparing effect on AD. Finally, numerous recent studies on mouse models demonstrate that blocking inflammatory responses in the brain attenuates AD pathology (Haneka 2015). These observations suggest that inflammation may precede the accumulation of senile plaques and neurofibrillary tangles and may be necessary for AD progression.

One cell type in the CNS know to promote inflammation is the brain immune cell, microglia. Microglia upon injury, dynamically change their activation state from resting phenotype to a pro-inflammatory phenotype, which leads to the production of pro-inflammatory cytokines, chemokines, proteases and redox factors that help defend the tissue against damage (Colton 2009).

More recently, injection of Aβ into mouse brain has shown morphological transformation into activated microglia. In addition, $hAPP_{J20}$ mice, a widely used model to study amyloid deposition and pathogenesis in AD, has shown robust production of pro-inflammatory mediators (T. Kauppinen et al. 2011). This pro-inflammatory response was completely absent in mice depleted of PARP-1, proving that PARP-1 is essential for microglial activation in response to Aβ. Importantly, PARP-1 depletion is well tolerated, where production of anti-inflammatory mediators like VEGF, and TGFβ has shown to be maintained, along with it retaining its phagocytic activity (T. Kauppinen et al. 2011). This suggests that global PARP-1 deletion is beneficial in Alzheimer's disease.

Interestingly, another glial cell type, astrocytes, mediate release pro-inflammatory cytokines, chemokines, proteases and redox factors in response to injury/pathology (Pekny et al. 2016). However, a past study has shown that astrocytic production of pro-inflammatory mediators like NO, interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) isn't necessarily abolished by global PARP-1 depletion, but rather involves a co-operative role of other PARP isoforms (Phulwani and Kielian 2008). However in microglia, PARP-1 deletion is not compensated by other PARPs (T. Kauppinen et al. 2011). Hence, use of PARP-1 inhibitors, known to inhibit inflammation caused by microglia, have been suggested as clinically relevant therapeutic drugs that can be explored for diseases like AD. However, pharmacological blockers targeting PARP-1 would cause side effects like increased risk of infection, bleeding problems, tiredness among others due (Bitler et al. 2017). In addition, PARP-1 inhibitors have been shown to exert genotoxic effects *in vitro* due to its key role in DNA repair (Ray Chaudhuri and Nussenzweig 2017). Moreover, whether the beneficial effects observed in global PARP-1 deletion were due to neuronal, astrocytic or microglia deletion has not been explored. This suggests that more studies

need to be aimed to determine the contribution of individual glial cell types upon PARP-1 deletion, but also an alternate approach would be to find a molecular target downstream of PARP-1 in glial cells would be ideal for development of novel drugs for AD.

One such downstream target commonly implicated in CNS related diseases is the transient receptor potential melastatin 2 (TRPM2) channel (Malko et al. 2019). TRPM2 is a very relevant molecular target to be investigated because poly-ADPR, an end-product of PARP-1 activation is a precursor for TRPM2 agonist ADPR. TRPM2 has also been shown to be implicated in neurodegenerative diseases (E. Fonfria et al. 2005; Ostapchenko et al. 2015; Y-F. Xie, MacDonald, and Jackson 2010), and highlights the important role of TRPM2 in CNS. 12 to 15-month aged APP/PS1 mice (associated with early-onset model of AD) depleted of TRPM2 channels were shown to have enhanced spatial learning and memory when compared to APP/PS1 mice which showed deficits (Ostapchenko et al. 2015). Further, histological analysis of brains from APP/PS1 mice showed increased level of Iba1 and decreased level of synaptophysin, suggesting enhanced microglial activation coupled with synaptic loss. However, APP/PS1 mice lacking TRPM2 channels did not show these deficits, showing that TRPM2 modulates synaptic loss and inflammation in mouse model of AD (Ostapchenko et al. 2015). Combined, TRPM2 global deletion has been shown to be well tolerated and able to prevent cognitive decline in a mouse model of AD.

Similar to studies involving global PARP-1 deletion, not explored here was whether these beneficial actions were due to neuronal, astrocytic or microglia deletion of TRPM2. However, both studies have independently shown a precedence suggesting PARP-1 and TRPM2 to be relevant players in neuroinflammation. Past studies have shown that microglial morphological activation is commonly observed in Alzheimer's disease (Dani et al. 2018). Interestingly, microglial

morphological activation has been shown to be mitigated by genetic depletion of TRPM2 and PARP-1 separately (T. Kauppinen et al. 2011; Ostapchenko et al. 2015), thereby causing reduced neuroinflammation. Hence, evaluating the functional role of PARP-1 and TRPM2 using pure microglial cultures will help understand their role in amyloid beta induced microglial activation.

6.3 Microglia exhibit robust TRPM2 currents and show sex difference in ADPR-evoked TRPM2 current amplitude

TRPM2 channels are widely expressed in several cell types in the CNS, with a high level in microglial cells (Elena Fonfria et al. 2006; Jeong et al. 2017; Kraft et al. 2004; M. Lee et al. 2010; Mortadza et al. 2017). Activation of TRPM2 channels occurs by binding of ADPR to its C-terminal intracellular ADPR pyrophosphatase domain (Sumoza-Toledo and Penner 2011). However, only 2 studies so far have reported ADPR-evoked currents in primary microglia using whole-cell patch-clamp technique (E Fonfria et al. 2008; Kraft et al. 2004). While one study has shown inhibition of TRPM2 current by flufenamic acid (E Fonfria et al. 2008), a pH-dependent antagonist of TRPM2 channel (Hill et al. 2004), the other study has substituted Na^+ with NMDG (Kraft et al. 2004), which reversibly suppressed by the introduction of divalent ions. Yet, I wanted to establish and validate more reliable methods for the identification of TRPM2-mediated currents in microglia.

The first step towards this was to identify inhibitors which can block TRPM2 channels. Here, I chose clotrimazole, which is a commonly used TRPM2 inhibitor, and removal of Ca^{2+} from the extracellular bath, as 2 approaches to inhibit TRPM2 currents. Using primary microglia, ADPR-evoked currents were recorded using whole-cell voltage-clamp electrophysiology. The inhibition of currents by both clotrimazole and by removal of Ca^{2+} from extracellular bath, coupled with linear I-V curve with reversal at 0 mV confirmed these as TRPM2 current. Comparable

ADPR-evoked currents could not be recorded in microglia derived from TRPM2 KO mice. These experiments helped in identifying reliable methods to record TRPM2 currents in my studies to definitively detect TRPM2 current in my future experiments.

Interestingly, past studies have shown that TRPM2 exhibit sex differences in the consequence of their pathological activation. Treatment with TRPM2 antagonist offered neuroprotection in male rodents upon ischemic injury, but no effect on injury was observed in females (Jia et al. 2011; Nakayama et al. 2013). Not known here was whether microglial TRPM2 had specific role in sex difference. Whether this sex difference was due to differences in TRPM2 function between male and female was also unexplored. Using ADPR to evoke TRPM2 current from microglia prepared from male and female mice separately, I showed TRPM2 currents were 2.4-fold larger in male compared to female. Our study is the first study till date to show sex difference in the amplitude of ADPR-evoked TRPM2 currents in primary microglia. These sex difference could be due to changes in TRPM2 at the level of transcription, protein expression and trafficking or post-translational modifications.

While the level of TRPM2 mRNA expression in primary murine and rat microglial have been evaluated in the past by RT-PCR (Jeong et al. 2017; Kraft et al. 2004), no studies so far have compared the transcript levels of microglia from male and female mice. If TRPM2 mRNA levels are comparable between male and female, it would then suggest that observed sex differences could be due to altered protein expression or trafficking of TRPM2 to the surface of microglia. Surface-labelling of TRPM2 in microglia separately from male and female, and their subsequent quantification could help us understand this. Biotinylation techniques can also be performed to probe for change in surface expression of proteins (Bennett et al. 2016; Toyomitsu and Tsuda 2012). Microglial phagocytic activity could be observed in case of surface labeling and leads to its

morphological as well as functional activation. In addition, lack of TRPM2 specific antibody will be the limitations in using these techniques.

Assuming that the level of surface TRPM2 also remains unaltered, past studies have shown that post-translational modifications (PTM's) of proteins can augment its cellular function (Trepanier, Jackson, and MacDonald 2012). A very common example of post-translational modification is phosphorylation of serine/threonine/tyrosine residues of proteins, thereby regulating its function (K. Yang et al. 2011). Our lab has previously shown that Fyn kinase, a member of the Src family of kinases, plays a key role in regulation of TRPM2 function. Fyn kinase, when applied intracellularly with ADPR, has shown increased TRPM2 current amplitude in neurons (Harish-unpublished). There could be a similar mechanism where microglia from male and female mice are regulated differently by post-translational modifications leading to sex difference in amplitude of TRPM2 currents.

6.4 AβO treatment stimulates TRPM2 channel activation in a sub-population of microglia

Having established conditions where TRPM2 currents can be reliably identified in microglia, I wanted to test the effect of amyloid beta oligomers on microglial TRPM2 channels in culture. This is because we know that Alzheimer's disease has been characterized by the presence AβO forming plaques in the brain, often associated with morphologically activated microglia (Dani et al. 2018; Dhawan, Floden, and Combs 2012; Doens and Fernández 2014). Also, synaptic loss and increased levels of Iba1 immunoreactivity observed in APP/PS1 mice was absent in TRPM2 $^{-/-}$ AD mice (Ostapchenko et al. 2015), suggesting involvement of TRPM2 in Alzheimer's disease. It is already shown by Kauppinen lab that AβO causes PARP-1 activation, and we know that end-product of PARP-1 activation leads to formation of poly-ADPR. Rapid turnover of poly-

ADPR by enzyme PAR glycohydrolase (PARG) causes production of ADPR monomers needed for TRPM2 activation. These combined led to our hypothesis that AβO stimulates PARP-1/PARG endogenous production of ADPR, which leads to TRPM2 channel activation. Naturally secreted soluble oligomers of Aβ (AβOs) enriched from conditioned media (CM) called 7PA2 cells were used to treat primary microglia at physiological concentrations ($A\beta_{1\text{-}42}$ = 2.57pM) for 1-7 hours before recording TRPM2 currents. Conditioned media from CHO cells served as experimental control.

Note that, my initial experiments were conducted using exogenous ADPR to establish reliable methods for recording TRPM2 current. However, upon AβO treatment, TRPM2 currents are predicted to be constitutively active upon breakthrough due to elevated intracellular ADPR levels. It is evident from the I-V traces and inhibition of current by clotrimazole that microglia treated with amyloid beta induce TRPM2 channel activation, but not in experimental control. While we previously observed sex differences in the amplitude of ADPR-evoked TRPM2 currents, TRPM2 current amplitude recorded from AβO microglia were comparable in male and female. One possible reason being while previous studies have reported that half maximum concentration of ADPR required for activating TRPM2 channels (in other cell types) is 90 µM (Gasser and Guse 2005), we had used 1 mM ADPR exogenously to evoke TRPM2 currents saturating all surface expressed TRPM2. This suggests that the endogenous ADPR formed by AβO treatment might not have been sufficient to activate all surface-expressed TRPM2 and hence sex difference was not observed.

AβO treated microglia were chosen systematically to record from microglia with varied morphology, from which TRPM2 currents were recorded. I showed that only 60% of the amyloid treated microglia recorded showed TRPM2 channel activation, but also that morphological status

of microglia had no co-relation in identifying responder population. This is likely due to heterogeneity of microglia showing functional diversity (Hanisch 2013). Past studies have also stated that microglial activation is neither an "all or none state", nor is it a linear path with fixed functional outcome (Arcuri et al. 2017). Studies also suggests that microglia likely exhibit distinct molecular signatures as they correlate with distinct activities and functions (Hanisch 2013). These combined with my experiment showing 60% responders in AβO treated microglia suggests that microglial morphological phenotype is not a sign of its functional state. Furthermore, by recording TRPM2 currents through systematical selection of AβO treated microglia to include varied morphology, I avoided any perception of bias in my experiments.

Now that I have showed TRPM2 channel is activated upon AβO stimulation, the pathway that leads to recruitment of TRPM2 still remains unknown. However, we know that TRPM2 is activated by endogenous ligand ADPR, which an end-product of PARP-1/PARG activation. Previous studies from our lab have shown that PARP-1 is activated by rise in intracellular Ca^{2+} (Vuong et al. 2015). In addition, our lab has also shown NMDAR dependent activation of TRPM2 channel in neurons (Olah et al. 2009). Hence, we predicted that amyloid beta stimulated activation of TRPM2 channels in microglia is through NMDARs and PARP-1.

6.5 NMDARs are required for TRPM2 channel activation in AβO treated microglia

It's well documented that gradual loss of cognitive function is the main symptom of Alzheimer's disease (Masters et al. 2015). This loss of learning and memory in patients with AD happens through amyloid beta deposition and disruption of glutamatergic neurotransmission (Greenamyre et al. 1988). N-methyl-D-aspartate receptors (NMDARs), an important class of ionotropic glutamate receptors play a key role in synaptic transmission, associated with learning and memory (Hunt and Castillo 2012). It is well known through several histological studies that

the brain of patients suffering from AD show presence of amyloid plaques (Penke, Bogár, and Fülöp 2017). However, the disruption in normal function of the brain happens much before the plaque deposition and amyloid beta oligomers are thought to be much potent in their actions (Forloni and Balducci 2018). The loss of cognitive function in AD can be explained as a 2-stage model (Olney, Wozniak, and Farber 1997). Firstly, it is thought that glutamate excitotoxicity together with overproduction of $A\beta_{(1-42)}$ and its interaction with neuronal NMDARs, results in hyper stimulated neuronal activity. This is followed by loss of synaptic NMDARs resulting in hypoactivity, and this coincides with a stage where subtle changes in cognitive function become evident. However, in native conditions in the brain, astrocytes and microglia co-exist with neurons and play a significant and unique role themselves (Fakhoury 2017).

While much research is focused on effect of amyloid beta and glutamate excitotoxicity on neurons, less spoken are the effects of these stimuli on glial cells (astrocytes and microglia) and their subsequent contribution to neuroinflammation, leading to neuronal loss. Astrocytes are important for maintenance of synaptic glutamate concentrations. However, $A\beta_{(1-42)}$ treatment of astrocytes in culture downregulated the astrocytic glutamate uptake capacity, leading to increased concentration of glutamate secreted into the culture (Matos et al. 2008). In fact, past studies show microglial morphological activation upon Aβ deposition (Caldeira et al. 2017), and subsequent release of nitric oxide and pro-inflammatory cytokine like TNF-α (T. Kauppinen et al. 2011). Combined, neuroinflammation by glial cells likely fit in between the hyperactivity of neuronal NMDARs and loss of synaptic function due to hypoactivity, leading to neuronal loss and cognitive decline in patients suffering from AD.

Since I am using pure microglial cultures in my experiments, we focused on establishing the pathway involved in the recruitment of TRPM2 channel upon AβO treatment, and its

contribution to neuroinflammation. Microglia is suggested to respond to Aβ by interaction with variety of cell-surface pattern recognition receptors including class A1 scavenger receptors (SCARA1), and Toll-like receptors (TLRs) 2, 4, and 6, and their co-receptor CD14 among others (Malm, Jay, and Landreth 2015). However, the contribution of NMDARs to amyloid beta stimulated microglial activation has not been studied before. While there is enough evidence of presence of NMDARs in microglia shown by mRNA levels (Kaindl et al. 2012) and immunostaining (Gottlieb and Matute 1997; Kaindl et al. 2012), their functional presence is often debated. Earliest evidence of functional NMDARs in microglia was shown by Dr. Kauppinen, where NMDA induced NO release in microglia was shown to be inhibited by MK-801, a pore-blocker of NMDARs (Tikka and Koistinaho 2001a). Similarly, other groups have shown ionotropic NMDAR mediated Ca^{2+} influx to be blocked by MK-801 (Kaindl et al. 2012; Murugan et al. 2011). In fact, only 1 group until now have reported NMDAR-mediated current in microglia (Kaindl et al. 2012). Extension of microglial process upon NMDA treatment has been shown to be independent of microglial NMDARs, but it is rather due to the actions of microglial purinergic receptors. (Dissing-Olesen et al. 2014; Eyo et al. 2014). Also, Wendt et al., were unable to record NMDAR-mediated currents in microglia (Wendt et al. 2016).

Hence, to identify whether functional NMDARs are involved in amyloid beta stimulated activation of TRPM2, 3 different pharmacological inhibitors of NMDARs (AP5, CKA and MK801) were tested in my thesis (Fig 1). All 3 NMDARs showed inhibition of TRPM2 current in AβO treated microglia. Pharmacological blockade of NMDARs by these inhibitors provided further insights on their sub-unit composition and role in microglia. AP5 is a competitive inhibitor blocking the glutamate binding site in NR2 sub units of NDMA receptors. CKA is a non-competitive inhibitor binding to the glycine binding site of NR1. NR3 sub-unit of NDMARs.

Inhibition of TRPM2 currents by these 2 inhibitors confirm the presence of NR1/NR2 or NR1/NR3 sub-units in a hetero tetramer fashion. In AD, dysfunction of neuronal NR2(B) sub-unit, is observed as impaired LTP. On a similar note, NR2 sub-unit block by AP5 results in TRPM2 channel inhibition suggests an important role of microglial NR2 sub-unit in eliciting NMDA mediated response. NR1 sub-unit is important for translocation of NR2 sub-units to the membrane and contains the glycine binding site. Our data shows that blocking of NR1 sub-unit also prevented TRPM2 activation in microglia. More importantly, inhibition by MK-801, a pore channel blocker of NMDAR, specifically confirms the requirement of ionotropic function of NMDARs for TRPM2 channel activation in AβO treated microglia.

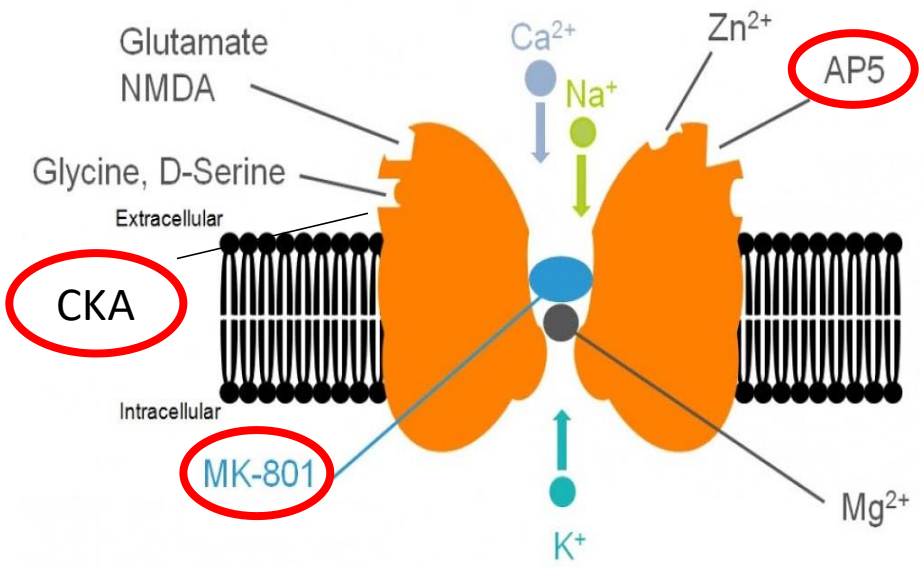

Figure 17: Mechanism of action and binding sites of inhibitors (AP5, MK801, CKA) to NMDA receptors. (Image adapted from http://www.neuroservice.com/solutions-for-cognition/)

Although AβO treatment of microglia induced TRPM2 channel activation via NMDARs, not known was the source of origin for glutamate causing NMDAR activation. it is important to

note was the source of glutamate acting here since my treatment media did not contain glutamate. However, past studies in microglia treated with AβO have shown their ability to secrete glutamate through the Na^+-dependent glutamate transporter (Noda, Nakanishi, and Akaike 1999). Hence, this released glutamate, also known to potentiate the AβO toxicity could be activating microglial NMDAR in autocrine fashion, leading to TRPM2 activation.

The development of TRPM2 currents in AβO treated microglia blocked by the NMDAR channel pore blocker MK801, demonstrates that ion conduction, presumably of Ca^{2+}. In turn, we know that Ca^{2+} causes PARP-1 activation leading to ADPR production and TRPM2 activation. However, whether NMDA, an agonist for NMDA receptors was sufficient to induce TRPM2 channel activation had not been evaluated before. I showed using electrophysiology recordings that microglial NMDA treatment induced TRPM2 channel activation, where NMDA induced inward currents were inhibited by TRPM2 blockers.

However, I observed that only 35% of the NMDA treated microglia induce TRPM2 channel activation compared to 60% with amyloid treatment. The difference in responders' rate between NMDA treated and AβO treated microglia could be due to change in TRPM2 expression upon treatment. Rightly so, evaluation of TRPM2 transcripts using qRT-PCR showed 40% increase in level of microglial TRPM2 transcripts upon AβO treatment, but not in NMDA treated microglia. Increased TRPM2 transcript levels in AβO treated microglia compared to NMDA treatment co-related with the increase in responder rates.

I then evaluated the different NMDAR sub-units present in primary microglial culture by measuring transcript levels of NMDAR sub units (Kaindl et al. 2012). NR1, NR2A, NR2D, NR3A sub-units were present in both NMDA and AβO treated microglia. Past studies have shown varied

sub-unit composition of NMDAR sub-units in neurons depending on their location in the brain (Ewald and Cline 2009).

It is well known that microglial mRNA and protein expression can be divergent (Boutej et al. 2017). For this reason, I probed the microglial cell-surface for presence of NR1 sub-unit, which is needed for trafficking of NR2 sub-units on to the microglial cell-surface (Wenthold et al. 2003; K. Yang, Jackson, and MacDonald 2014). My preliminary data from immune staining experiments show NR1 sub-unit to be present uniformly in the microglial cell body, but more predominant in in the intracellular compartments. But upon NMDA treatment, we showed presence of punctate NR1 staining in the proximal processes of microglia. This data suggests that NMDA treatment is necessary for translocation of NMDARs from intracellular compartments to the cell-membrane.

Combined, the presence of NMDARs on microglial cell-surface as well as them being ionotropic in function is confirmed. If so, then NMDAR-mediated currents can be recorded from microglia, thereby confirming ionotropic function of microglial NMDARs. Using whole-cell patch-clamp technique, NMDA stimulation (with serine as co-agonist) did not evoke measurable NMDAR-mediated current in microglia. In fact, microglia were pre-treated with Aβ to prime the microglial cell surface (J.-W. Li et al. 2018). Post-treatment whole-cell recordings by stimulation with NMDA failed to elicit any NMDAR-mediated current. But, if microglial NMDARs are a hetero tetramer complex of NR1/NR3 sub units, NMDAR-mediated currents will be evoked by glycine stimulation and not by glutamate/NMDA (Neyton 2006; C. T. Smothers and Woodward 2007). Further, zinc is also known to potentiate NMDA-mediated currents in NR1/NR3 hetero-tetramer composed NMDARs (Laube et al. 1995; C Thetford Smothers and Woodward 2009). Hence, we stimulated microglia with glycine and zinc, and this too did not evoke measurable NMDAR-mediated currents.

Taken together, it is not uncommon to not observe NMDAR-mediated currents in non-neuronal cells (Hogan-Cann and Anderson 2016). Distinct structural and functional properties, including weak susceptibility to Mg^{2+} blockade and less Ca^{2+} permeability has been shown in astrocytes (Hogan-Cann and Anderson 2016; Palygin, Lalo, and Pankratov 2011). In my experiments, although we did not observe NMDAR-mediated currents, I showed 3 distinct pharmacological inhibitors binding to different sites on the NMDA receptors inhibiting NMDA/AβO induced TRPM2 channel activation. In addition, MK-801 blockade of NMDA induced TRPM2 channel activation confirms its ionotropic function. Taken together, these results beyond doubt prove the functional presence of NMDARs in NMDA/AβO microglia.

6.6 AβO and NMDA treatment stimulated pro-inflammatory phenotype of microglia is PARP-1/TRPM2 dependent

My results have shown that activation of TRPM2 channels by either NMDA or AβO is through a NMDAR dependent pathway. Specifically, using pharmacological inhibitors, I've shown that ionotropic function of NMDARs which causes Ca^{2+} influx into the cell is involved in NMDA/AβO stimulated activation of TRPM2 channel. Interestingly, previous studies from our lab has shown Ca^{2+} influx in microglia causes activation of PARP-1 and resulting in NF-κB activation (Vuong et al. 2015). Poly (ADP-ribose) polymerase-1 (PARP-1) has been shown to be a key regulator of microglial responses due to its ability to co-activate transcription factor nuclear factor kappa B (NF-κB). One consequence of PARP-1 mediated NF-κB activation is production of NO by inducible nitric oxide synthase (iNOS) (T. Kauppinen et al. 2011). The resulting nitrosative stress contributes to neuronal injury. Neuronal injury and amyloid beta plaques are hallmarks of AD pathology. On the other hand, it is already shown by Kauppinen lab that AβO induced PARP-1 activation leads to poly-ADPR formation. Rapid turnover of poly-ADPR by

enzyme PAR glycohydrolase (PARG) causes production of ADPR monomers needed for TRPM2 activation. However, whether NMDA/AβO stimulates activation of TRPM2 through a PARP-1/PARG dependent pathway has not been established. Also, the contribution of TRPM2 in the NMDA/AβO stimulated release of NO by microglia remains to be evaluated.

NMDA/AβO induced TRPM2 channel activation in microglia was blocked upon treatment with the PARP-1 inhibitor PJ-34, confirming the role of PARP-1 in the pathway. However, PJ-34 along with other commercially available PARP-1 inhibitors like olaparib and minocycline are not specific for PARP-1. Hence, microglia from genetic mouse models depleted of PARP-1 were used in the experiment to confirm the role of PARP-1. TRPM2 currents were found to be completely inhibited in microglia treated with NMDA/AβO obtained from PARP-1 depleted mice. More importantly, it was also a testament that no compensatory effects were observed in PARP-1 depleted microglia.

Subsequently, I focused on establishing the role of PARG in the proposed signaling pathway by using a pharmacological inhibitor. Previously, non-specific PARG inhibitors like gallotannin and ellagitannins, which belonged to a class of hydrolysable tannins were being used (Formentini et al. 2008; Ying and Swanson 2000). Recently, PDD00017273 (IC_{50} = 26nM) was identified as a first-in-class chemical probes against poly (ADP-ribose) glycohydrolase based on its crystal structure (James et al. 2016). Also, since the functions of PARP-1 and PARG are inter-twined, the pharmacology of a PDD00017273 was shown to be different from that observed with the more thoroughly studied PARP inhibitor olaparib (Gravells et al. 2018). This provided us confidence to use PARG inhibitor PDD00017273 in our experiments. TRPM2 channel activation from NMDA/AβO treated microglia was blocked completely with by treatment with

PDD00017273. These results combined confirmed that NMDA/AβO stimulated microglia showed TRPM2 channel activation through a pathway involving NMDAR/PARP-1/PARG.

However, modest structural similarity of inhibitors of PARP-1 and PARG to ADPR binding site could've prevented the endogenous ADPR binding to TRPM2 channel and subsequent activation. To rule this out, I showed that exogenous ADPR administered to microglia in the presence of PARP-1 and PARG inhibitors was still able to activate TRPM2 channel. This proves that the ADPR binding site remain unhindered by PARP-1 and PARG inhibitors. By comparing the amplitude of TRPM2 current upon exogenous ADPR administration between WT and PARP-1 KO microglia, I confirmed that long term PARP-1 depletion had no effect on TRPM2 expression, suggesting PARP-1 might not act as a co-activator for regulation of TRPM2 expression.

Previous study from Dr. Jackson's lab has shown that TRPM2 global deletion has been shown to mitigate cognitive decline in mouse model of AD (Ostapchenko et al. 2015). But whether microglial TRPM2 had any role in neuroinflammation in the context of *in vivo* chronic neuroinflammation in AD was a question that remained unanswered. PARP-1 is upstream of TRPM2 and in addition, PARP-1/PARG end-product is an agonist of TRPM2. With TRPM2 in itself a Ca^{2+} permeable channel, its activation can lead to a positive feedback loop and sustained PARP-1 activation and pro-inflammatory responses. This made TRPM2 an exciting target to study for its role in neuroinflammation.

I observed NO release from TRPM2 WT microglia treated with NMDA/AβO, but not in TRPM2 KO proving that microglial NO release is TRPM2 dependent. In addition, clotrimazole, a non-specific blocker of TRPM2 also blocked NMDA/AβO induced NO release in WT microglia. Previously NMDA induced NO release in microglia was shown to be inhibited by minocycline, an anti-inflammatory compound that potentially blocks PARP-1, suggesting the involvement of

PARP-1 in NO release (Tikka and Koistinaho 2001a). More recently, AβO treated microglia had also shown PARP-1 dependent NO release in microglia (T. Kauppinen et al. 2011). I show here that NMDA/AβO induced microglial NO release is PARP-1/TRPM2 dependent. While past data from Kauppinen lab shows that NMDA/AβO stimulation of PARP-1 activation is necessary in recruiting NF-κB induced NO release, I show here that TRPM2 activation also seems necessary for NO release.

Since we observed NO release, a key pro-inflammatory response of microglia by NMDA/AβO treatment, I also assessed the transcript levels of iNOS, which induces nitric oxide production in microglia. Inducible nitric oxide (iNOS), considered a classical M1 (pro-inflammatory) marker of microglia are expressed at low levels in resting microglia in the brain, but unstimulated microglia in culture may express modest levels of iNOS. In addition, since iNOS is strictly regulated at the transcription level by NF-κB, its expression induced by PARP-1 activation will result in generation of NO. We observed the microglial pro-inflammatory phenotype in both NMDA/AβO treated microglia shown by their upregulation in level of iNOS. With increase in iNOS, there should be balance by production of anti-inflammatory mediators. AG-1 is commonly used M2 (anti-inflammatory marker), reflecting its phenotype. We observed no change in expression of AG-1 in NMDA treated microglia. Interestingly, AβO treatment clearly reduced expression of AG-1 in microglia. Taken together, no change/reduction in M2 marker but increase in M1 marker suggests that NMDA/AβO treatment is driving sustained pro-inflammatory phenotype in microglia (Fig 2).

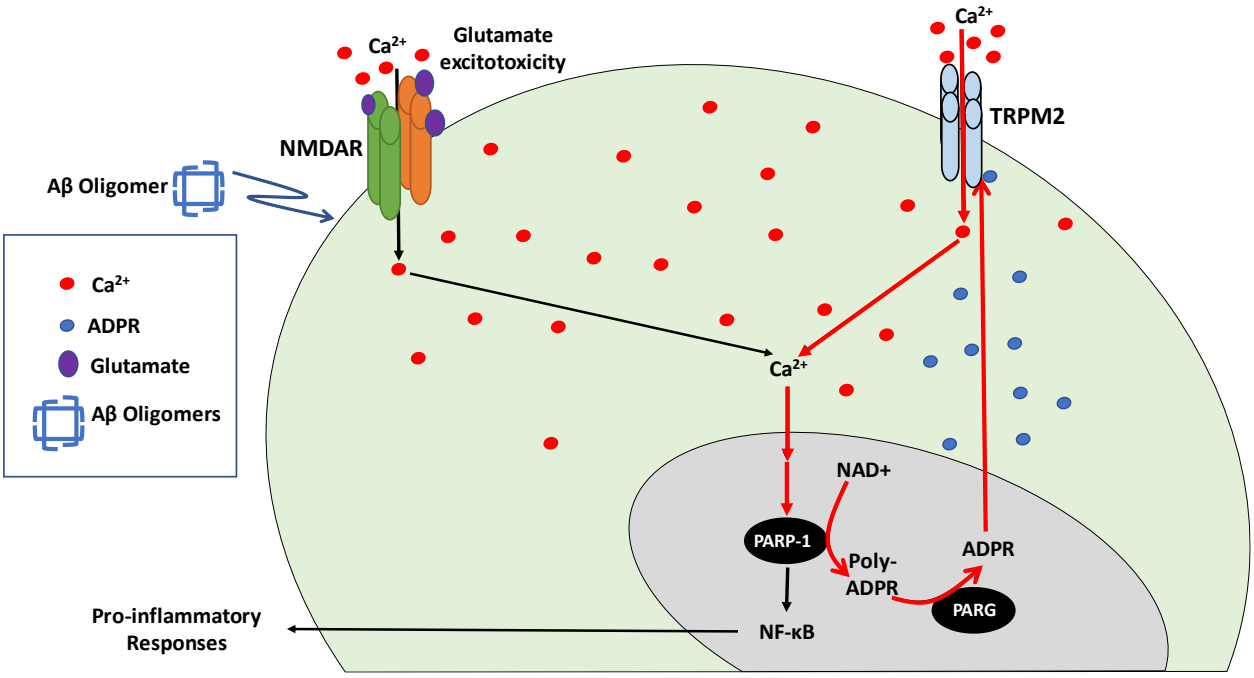

Figure 18: Illustration of positive feedback signaling leading to extended microglial pro-inflammation in response to NMDA/AβOs

6.7 Experiment for the future studies

From the experiments that I have performed in the present study, I have established that NMDA/AβO stimulated microglia activate TRPM2 channel through a pathway involving NMDAR/PARP-1/PARG. By using pharmacological blockers of NMDAR channel-pore, we established that NMDA/AβO induce activation of ionotropic function of NMDARs. However, since we were unable to evoke measurable NMDAR-mediated currents in microglia, it is important to show rise in $[Ca^{2+}]_i$ levels by using calcium imaging. I've already performed preliminary experiments to establish the conditions and method to detect increase in intracellular Ca^{2+} upon stimulation. I have optimized phenol-free imaging media that can be buffered with CO_2, making it a perfect extracellular solution for live/time-lapse imaging. Fura 2 AM and Fluo 4 AM are the most commonly used Ca^{2+} dyes, but recently identified Cal520 (2 µM) has a higher signal to noise

ratio than Fura 2 AM and Fluo 4 AM (Tada et al. 2014). Microglia stimulated with NMDA could show a rise in fluorescence intensity, suggesting a rise in Ca^{2+} influx through NMDAR by applying its inhibitor MK801.

It is to be noted that only 35% of microglia showed recruitment of TRPM2 channel when stimulated with NMDA in my experiments. Hence, it is important to have a good density of microglia, so that there are enough number of microglia in the imaging frame to capture the change in fluorescence intensity due to rise in Ca^{2+} levels. Upon observing rise in Ca^{2+} influx in these NMDA treated microglia, we can extract microglial responders and non-responders separately using laser capture microdissection (LCM). Evaluation of NMDAR transcripts in these microglia by TaqMan qRT-PCR method will help to identify subtle difference in NMDAR sub-unit composition which might differ between the responders and non-responders.

However, we know that that the level of transcripts does not correlate to the level of protein in the cell (Boutej et al. 2017). Once the sub-unit composition is identified, antibodies probing for those sub-units can be used to detect and visualize the cell-surface expression of these sub-units in microglia. Since neuronal NMDARs are important in synaptic transmission, a similar NMDAR composition in microglia could trigger continuous activation even in the presence of basal glutamate required for synaptic activity. Hence, it is possible that the microglial NMDAR sub-unit composition will be different from its neuronal counterparts.

To demonstrate the requirement of NR1 sub-unit in NMDA induced TRPM2 channel activation in microglia is by genetic deletion of NR1 sub-unit specifically in microglia. NR1 sub-unit of NMDAR can be deleted in microglia by crossing floxed GluN1 mice ($grin1^{fl/fl}$, JAX005246) (Tsien, Huerta, and Tonegawa 1996) with Cre-deleted allele driven by CD-11b promoter elements (Ferron and Vacher 2005). $grin1^{fl/fl} \cdot Cre^{+/-}$ (Cre+) mice would show microglial

NMDAR loss of function, while grin1$^{fl/fl}$ · Cre$^{-/-}$ (Cre-) littermates can be used as wild-type controls. The finding that microglia obtained from grin1$^{fl/fl}$ · Cre$^{+/-}$ mice fails to show TRPM2-mediated currents upon NMDA treatment would confirm that microglia requires ionotropic function of NMDARs for TRPM2 channel activation. An alternative approach would be to transfect glial cultures from grin1$^{fl/fl}$ mouse with cre-deleted allele driven by CD-11b promoter elements. These microglia would be lacking NMDAR sub-unit NR1.

Once NMDAR mediated Ca^{2+} influx is established, these NMDA treated microglia should be imaged for longer time periods to show prolonged rise in fluorescence intensity due to activation of PARP-1/TRPM2. TRPM2 activation would allow more Ca^{2+} into the cell and the rise in fluorescence intensity in microglia will be sustained. To show that TRPM2 is involved in sustained Ca^{2+} influx into the cell, TRPM2 inhibitor clotrimazole should be pre-treated as well as present in the bath, which will then show no change in fluorescence intensity. Since we know that clotrimazole is a non-specific inhibitor for TRPM2, we can use TRPM2 KO microglia, which will show initial rise in fluorescence intensity due to Ca^{2+} influx by NMDARs, but it won't be sustained due to the absence of TRPM2 in these cells. These experiments will help in establishing sustained PARP-1/TRPM2 activation observed in NMDA treated microglia.

After microglial Ca^{2+} influx through NMDAR is established, its further downstream effects should be confirmed. I had reported previously here that Ca^{2+} influx into the microglia causes activation of MEK1/2 and ERK2, leading to PARP-1 activation. This can be confirmed by recording TRPM2 current in NMDA/AβO stimulated microglia upon treatment with inhibitor of PD98059 (Vuong et al. 2015). TRPM2 currents will be inhibited due to ERK2 being necessary for PARP-1 activation. This will help in confirming the role of MEK1/2 and ERK2 in Ca^{2+} influx mediated activation of PARP-1 upon microglial NMDA/AβO stimulation.

Next, microglia treated with Aβ have also shown their ability to secrete glutamate through the Na^+-dependent glutamate transporter (Noda, Nakanishi, and Akaike 1999). Studies have also reported that Aβ treated microglial conditioned media has shown activation of neuronal NMDA receptors (Floden 2005). However, the concentration of glutamate release into the media has never been quantified. In my preliminary experiment, I have observed that Aβ treated microglia for 1-7 hours secretes sufficient glutamate into media for it to activate neuronal NMDAR-mediated currents (data not shown). Hence, the secreted glutamate in culture should be quantified by using High Performance Liquid Chromatography LC-18 reverse-phase (RP) column. With our knowledge that both neurons and astrocytes release glutamate in the brain, evaluating the amount of glutamate released by microglia will help in understanding the contribution of AβO stimulated microglia to glutamate dysfunction in context of AD. Also, since we know that NMDA/AβO activates TRPM2 channel, there should be detectable amount of endogenous ADPR produced in microglia (Gasser and Guse 2005). Using HPLC, we should also measure the intracellular ADPR concentration in NMDA/AβO treated microglia.

NMDA/AβO simulated microglial NO release has been shown to be PARP-1/TRPM2 dependent. Previously PARP-1 depletion had abrogated the release of pro-inflammatory cytokines (TNF-α, IL-6 among others), but the level of anti-inflammatory cytokines and growth factors remain unaffected. Hence, the cytokine profile upon NMDA/AβO treatment in the presence and absence of TRPM2 will now need to be evaluated. Milliplex mouse immunoassay bead system (Millipore), where fluorescent signals corresponding to each cytokine/trophic factor from samples will be measured (Vuong et al. 2015). Concentrations of each cytokine will be calculated by using a standard curve and then normalized to protein content should be used (Vuong et al. 2015). TRPM2 acting as a positive feedback for activation of PARP-1 should likely be exhibiting a similar

cytokine profile as observed in PARP-1 depletion and should be evaluated. Also, phagocytosis of Aβ by microglia remain unaffected on PARP-1 depletion (T. Kauppinen et al. 2011). TRPM2 depleted microglia should be incubated with 5 μM of carboxy-fluorescein-labeled amyloid-β_{1-42} (FAM-Ab). While phagocytosis is shown to be independent of PARP-1 deficiency or inhibition, TRPM2 activation activates PARP-1 in an autocrine fashion, and hence TRPM2 depletion or inhibition should not affects microglial phagocytic ability.

All these proposed experiments above were using pure microglial cultures. Microglia-neuron co-culture experiments should be done to assess the neurotoxicity and/neuronal survival rate, which can be quantified using neuronal cell counts. Treatment groups in co-cultured experiments can be limited to 1) untreated and 2) AβO. Additional stimulation with NMDA is unnecessary in these experiments given the presence of glutamate released from neurons. To assess the specific role of microglial PARP-1 and TRPM2 in formation of synapses, microglia will be derived from WT, PARP-1 or TRPM2 KO mice and plated onto neurons derived from WT mice. Moreover, given that AβOs are known to disrupt glutamate synapses, we should also examine the expression of glutamatergic synaptic markers by ICC including, postsynaptic density protein 95 (PSD-95), NR1, and pre-synaptic membrane protein synaptophysin.

Most intriguing question that remains unanswered from the *in vitro* studies is whether the pathway seen in microglia obtained from P0-P2 mice suitable model for a disease prevalent in aged population. Note that the microglia from young animals were used only to establish the role of proposed signaling pathway. Microglial TRPM2 is shown to be a key neuroinflammatory target in *in vitro* studies as presented in my thesis. APP/PS1 mice are considered a less aggressive model of Aβ toxicity compared to TgCRND8 mice harboring 3 mutations in APP gene, revealing early and robust pathology of AD. Significant amounts of soluble and plaque associated Aβ has been

shown as early as 6 months of age in these mice. This transgenic AD mouse model exhibit deficits in spatial learning and memory. These mice can be cross bred with CD11b promoter driven TRPM2$^{-/-}$ mice to achieve deletion of TRPM2 specifically in microglia. The experimental groups of mice can include 1) WT 2) APP/PS1 3) APP/PS1* TRPM2$^{-/-}$ 4) APP/PS1*CD11b driven TRPM2$^{-/-}$.

A battery of behavioral tests like Morris Water Maze or Barnes maze, and novel object recognition tests should be performed to assess improvement of cognitive functions, learning and memory abilities in TRPM2 depleted mice. A battery of biochemical and histological analysis to show improvement of synaptophysin protein levels coupled with reduced morphological activation of microglia in TRPM2 depleted mice compared to APP/PS1 mice. Also, if no difference is observed between APP/PS1* TRPM2-/- and APP/PS1*CD11b driven TRPM2$^{-/-}$ mice that infers that the protection offered in TRPM2$^{-/-}$ mice was specifically due to the contribution of microglial TRPM2 depletion. If no deleterious side effects were observed in TRPM2$^{-/-}$ mice compared to APP/PS1*CD11b driven TRPM2$^{-/-}$, that also provides a reason for not designing a drug to target microglial TRPM2 specifically

Now that we have seen key role of microglial TRPM2 in neuroinflammation, question arises whether the previously shown mitigation of cognitive decline in AD was specifically by microglia (Ostapchenko et al. 2015). If yes, is specifically targeting microglial TRPM2 in microglia feasible? While the in vitro and in vivo studies can be designed to delete microglial TRPM2 specifically, the more important question being whether we can achieve the same with human patients in further clinical trials. While this seems challenging and difficult, we need to evaluate whether targeting microglial TRPM2 specifically is necessary depending on the in vivo

studies. Since global deletion of TRPM2 has previously shown no deleterious side effects (Ostapchenko et al. 2015), there arises no need to specifically target TRPM2.

However, to progress towards the clinical studies, what needs to be performed is synthesis of a specific TRPM2 inhibitor much like how the PARG inhibitor PDD00017273 was designed (James et al. 2016). Recently a novel inhibitor, JNJ-28583113, targeting TRPM2 specifically was synthesized and tested for in vitro studies has been found to be effective (Fourgeaud et al. 2019). Screening of these inhibitors needs to be carefully monitored for their clinical safety and efficacy, since these could be potential drugs for AD in human patients. Main concerns being whether the oral TRPM2 inhibitor could cross the blood brain barrier and whether the drug concentration after crossing the blood brain barrier remains as dosed. There are chances that the structure might tbe altered once it crosses the blood brain barrier and can bind to other proteins. However, drug delivery using exosomes and using nanoparticles have recently been successful and should be explored (EL Andaloussi et al. 2013; Bunggulawa et al. 2018; Wohlfart et al. 2011). Overall, TRPM2 looks to be a promising therapeutic target for treatment of Alzheimer's disease.

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