Role of TRPM2 channels and ADPR signalling in mediating the synaptotoxic effects of Aβ oligomers

By

Olha Myhalatyuk

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Department of Pharmacology and Therapeutics
University of Manitoba
Winnipeg, Canada

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ABSTRACT

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterised by memory loss and cognitive decline due to accumulation of amyloid-beta oligomers (AβOs). AβOs are known to disrupt synaptic transmission and plasticity in the form of long-term potentiation (LTP). AβOs also trigger chronic microglial pro-inflammatory responses that contribute to impaired synaptic transmission and plasticity. Our past work shows that microglial NMDA receptors are coupled to a signalling cascade, which involves activation of PARP-1, previously recognized, as a central signalling hub that drives inflammation through NF-κB. Moreover, our recent work suggests that sustained microglial PARP-1 activation requires Ca^{2+} influx initiated as a result of transient receptor potential melastatin-2 channel (TRPM2) activation, a Ca^{2+} permeable channel most highly expressed in microglia. A by-product of PARP-1 activation, ADP-ribose is required for TRPM2 activation. Thus, NMDAR/PARP-1/TRPM2/ADPR activity is coupled through positive feedback. Our unpublished findings suggest PARP-1 to be crucial in mediating the synaptotoxic effects of AβOs on synaptic plasticity. In my study I aim to establish the role of microglial NMDARs, TRPM2 and ADPR signalling on synaptic plasticity and whether pharmacological inhibition can prevent the synaptotoxic effects of AβOs on LTP.

Acute hippocampal slices were treated with AβO, TRPM2 and NUDT5 inhibitors using field electrophysiology to assess synaptic plasticity. Using microglia specific NR1-iKO mice as well as pharmacological inhibitors for TRPM2 and NUDT5, an enzyme linked to ADPR metabolism, we evaluated the contribution of microglial NMDARs/TRPM2/ADPR signalling of AβO effect on synaptic plasticity.

Our findings, demonstrate that 1) synaptotoxic effects of AβOs are mediated through TRPM2 channels and ADPR signalling and 2) microglial NMDA receptors may be responsible for initiating the TRPM2/ADPR mediated signalling axis.

To conclude, TRPM2/ADPR signalling axis has been shown to be important in mediating the synaptotoxic effects of AβOs on synaptic plasticity. Pharmacological inhibition of TRPM2 with two structurally different agents was effective in both sexes. Thus, TRPM2 channels could potentially make a promising target for the development of therapeutic agents for the effective treatment of AD.

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ABBREVIATIONS

μM	micromolar
nM	nanomolar
ml	milliliter
Aβ	Amyloid beta
AβO	Amyloid beta oligomers
$A\beta_{1-40}$	Amyloid beta (1-40) residues
$A\beta_{1-42}$	Amyloid beta (1-42) residues
aCSF	Artificial cerebrospinal fluid
AD	Alzheimer's disease
ADPR	Adenosine diphosphate ribose
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
APOE	Apolipoprotein E
APO ε4	Apolipoprotein E epsilon 4 isoform
APP	Amyloid precursor protein
ATP	Adenosine tri-phosphate
BBB	Blood Brain Barrier
CA1	Cornu ammonis 1
CA2	Cornu ammonis 2
CA3	Cornu ammonis 3
Ca^{2+}	Divalent Ca ion
CHO	Chinese hamster ovary
CHO-CM	Chinese hamster ovary conditioned media
cKO	Conditional knockout
CNS	Central nervous system
CX3CR1	CX3C chemokine receptor 1

CTZ	Clotrimazole
C1q	Complement 1q
C3	Complement 3
DNA	Deoxy-ribose nucleic acid
ELISA	Enzyme-linked immunosorbent assay
EOAD	Early-onset Alzheimer's disease
EPSP/s	Excitatory post-synaptic potential/s
fEPSP /s	Field excitatory post-synaptic potential/s
ERK/ERKs	Extracellular receptor kinase/s
EV/s	Extracellular vesicle/s
HFS	High-frequency stimulation
IL-1β	Interleukin-1beta
IL-6	Interleukin-6
IκB	Inhibitory I-kappa B
iKO	inducible knockout
iNOS	Inducible nitric oxide synthase
KO	Knockout
LOAD	Late-onset Alzheimer's disease
LTD	Long-term depression
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinases
MCAO	Middle cerebral artery occlusion
MCI	Mild cognitive impairment
MF	Mossy fiber
mGluR	Metabotropic glutamate receptor
NAD^+	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NFT	Neurofibrillary tangles

NF-κB	Nuclear factor κ-B
NMDA	N-methyl-D-aspartate
NMDAR/Rs	N-methyl-D-aspartate receptor/s
NO	Nitric oxide
NR1	NMDA receptor subunit 1
NUDT5	NUDix-like domain
NUDT5i	NUDT5 inhibitor
OGD	Oxygen glucose deprivation
OHT	Hydroxy tamoxifen
PAR	Poly-ADP (Adenosine Diphosphate) ribose
PARylate	Poly-ADP (Adenosine Diphosphate) ribosylate
PARP	Poly-ADP ribose polymerase
PARP-1	Poly-ADP ribose polymerase 1
PARG	Poly-ADP (Adenosine Diphosphate) ribose glycohydrolase
PBS	Phosphate buffer solution
PPF	Paired-pulse facilitation
PSEN	Presenilin
PSEN 1	Presenilin 1
PSEN 2	Presenilin 2
P2X4	P2X purinoreceptor 4
P2Y6	P2Y purinoreceptor 6
P2Y12	P2Y purinoreceptor 12
ROS	Reactive oxygen species

SEM	Standard error of the mean
SNP/s	Single nucleotide polymorphism/s
TAM	Tamoxifen
TRPM	Transient receptors melastatin
TRPM2	Transient receptor melastatin 2
TNFα	Tumor necrosis factor alpha
WT	Wild type
7PA2	Amyloid precursor protein (APP) expressing V717F-expressing CHO cells
7PA2-CM	Conditioned media from Amyloid precursor protein (APP) expressing V717F-expressing CHO cells

CHAPTER 1. INTRODUCTION

1.1. Alzheimer's Disease

1.1.1. Introduction to Alzheimer's Disease

Alzheimer's disease (AD) is a progressive neurodegenerative disease that primarily affects older adults, with first appearance of symptoms in the age of mid-60s. AD is not a normal part of aging. It is the leading cause of dementia accounting for over 50% of all cases. More than 50 million cases of AD worldwide were reported in 2019 (Balsinha et al. 2019) and this number will keep increasing with life expectancy. Evidence shows that every 3 seconds someone in the world develops dementia (Balsinha et al. 2019).

The earliest and most essential symptom of AD is memory impairment, which is the hallmark of the disease. As the disease advances, other clinical manifestations arise, such as anxiety, depression, disturbances in language, and impaired executive function (Stokholm 2006; Thompson et al. 2005; Ryu et al. 2017). As a person's condition declines, they often withdraw from friends and society. AD patients gradually lose their independence and become fully dependent on family members or caregivers. This situation brings financial burden and is emotionally and physically difficult for both patients and caregivers. In Canada alone more than $10.4 billion is spent each year on care for those with dementia ("Alzheimer Society of Canada," n.d.). Thus, identifying AD in its early stages is crucial and will provide a better possibility of benefiting from available treatments. Currently the available treatment is only alleviating the symptoms which may maximize patient's function and maintain independence for a time but does not reverse or slow down the progression of AD.

1.1.2. Sex differences in Alzheimer's disease

Heterogeneity of AD manifestation and progression among affected individuals is an important issue pertaining to diagnosis and development of new therapeutics. About two thirds of all individuals who are diagnosed with Alzheimer's are women (Alzheimer's Association 2022). Besides the disease being more prevalent in women, it also appears that females tend to show slightly different symptomatic profile, than males and a faster cognitive decline after the diagnosis of mild cognitive impairment (MCI) or AD dementia (Gamberger et al. 2017; Lin et al. 2015). Five and a half months old female transgenic APP mouse models of AD had more severe cognitive decline and increased neurodegeneration compared to the same age male mice with equal amyloid burden (Granger et al. 2016; Melnikova et al. 2016).

The risk factors for AD affect females and males differently. For example, the apolipoprotein E (*APOE*) *ε4* allele is an established genetic risk factor for AD. *APOE ε4* allele is one of the three allele types of *APOE* gene in humans. Individuals who carry *APOE ε4* allele have an increased risk for cerebral amyloid angiopathy and age-related cognitive decline in normal aging (Liu et al 2013; Husain, Laurent, and Plourde 2021). Approximately 50% of individuals who are diagnosed with AD carry an *APOE ε4* allele. Case-control studies suggest a stronger link of *APOE ε4* in females, who will have a higher risk for transition from a healthy aging to MCI or AD compared to males (Altmann et al. 2014). Animal studies have also demonstrated that female mice that express human *APOE ε4* allele exhibit cognitive impairments and show faster cognitive decline than males (Bour et al. 2008; Reverte et al. 2012). A large meta-analysis suggested that among older individuals carrying a single copy of *APOE ε4* allele women exhibit higher risk for AD (Neu et al. 2017). In addition, they showed that *APOE ε2/ε3* genotypes have a selective protective effect on women.

One interesting theory of a greater decline in women is that women have smaller cognitive reserve. Cognitive reserve has been linked to the ability to tolerate a greater brain injury prior to showing symptoms or clinical impairments. Therefore, men can tolerate more extensive neuronal damage before clinical impairment (Stern 2002). Males and females may have different coping mechanisms in response to neurodegeneration. In addition, males show more age-related reduction of brain metabolism in frontal medial regions than females (Malpetti et al. 2017).

Cumulative evidence suggests that sex is an important factor in phenotypic variability in AD and should be considered in clinical management and research. Future preclinical studies and therapeutic development should focus on exploring these sex-related differences.

1.1.3. Pathogenesis of Alzheimer's Disease

Alzheimer's disease (AD) is characterized by accumulation of senile plaques (Aβ plaques), neurofibrillary tangles (NFT) and neuroinflammation in the brain, which lead to excitotoxicity, synaptotoxicity and eventually neuronal loss. Neuronal loss is seen at the later stages of the disease progression when intervention is futile. These pathology features have been seen in hippocampus, cortex amygdala and cholinergic basal nucleus, the areas of brain responsible for memory and cognitive functions. Atrophy in hippocampus as well as in the posterior cingulate gyrus can be seen in the earliest stages of the disease, often prior to confirmed diagnosis and clinical impairment (Beart and Robinson 2013).

1.1.3.1. AD types and manifestation

Alzheimer's disease typically manifests in individuals over the age of 65, but cases can be found in younger populations as well. AD in the younger populations is more likely to have a

genetic origin and will have an early onset (EOAD) (Reitz, Brayne, and Mayeux 2011). EOAD is characterized by autosomal dominant transmission accounting for 1-2 % of all AD cases. Mutations in three genes identified are associated with EOAD: *APP* (amyloid precursor protein) found on Chromosome 21 (Goate 1991), *PSEN1* (presenilin1) found on Chr 14 (Sherrington 1995) and *PSEN2* (presenilin2) found on Chr 1 (Levy-lahad et al. 1995). Mutations in all three genes result in an imbalance between Aβ production and clearance. *PSEN1* mutations have also been shown to increase tau deposition (Shepherd et al. 2004). Risk of developing EOAD increases in patients with Down syndrome, likely due to an extra copy of chromosome 21 which carries the *APP* gene.

The majority of AD cases are late onset AD (LOAD) with onset of symptoms after age 65, and sporadic, the causes of which are still unknown. LOAD seems to be multifactorial: age, environmental and genetic factors influence the disease risk, manifestation, and progression. LOAD is highly polygenic with heritability estimated between 40-80 percent, where GWAS studies only explain only nearly 8% of phenotypic variance. SNPs explain approximately 30% of the genetic variance, with the majority of risk attributed to *APOE* (Adams et al. 2016). *APOE ε4* allele, located on Chr 21, has been discovered as the first genetic factor that predisposes to LOAD (Liu et al 2013). Each *APOE ε4* allele reduces the average onset of symptoms by approximately a decade. *APOE ε4* contributes to AD risk by various mechanisms, including increased aggregation and decreased clearance of Aβ, increased tau phosphorylation, reduced vascular and mitochondrial metabolism (Liu et al 2013). The impact of sex and hormonal differences may also play a role as impact of menopause status in females increases the risk of developing AD particularly in *APOE ε4* carriers (Pontifex et al. 2021). Despite a significant difference in the prevalence between early

and late onset AD, they share common pathogenetic features such as Aβ pathology, hyper-phosphorylation of tau protein and inflammation.

1.1.3.2. Aβ - the major component of senile plaques

The major protein component of senile plaques is 40-42 amino-acid polypeptide termed Aβ. Aβ is a small ~4.5 kDa peptide that derives from sequential cleavage of Aβ precursor protein (APP) by α- or γ-secretase that initiate two different pathways: amyloidogenic and non-amyloidogenic. When APP is cleaved by α -secretase, a soluble extracellular fragment and carboxyl-terminal fragment (CTF83) is formed, which is further cleaved by enzymes called γ-secretase (Kametani & Hasegawa, 2018). Catalytic cores of γ-secretase are formed by presenilin proteins (*PSEN1* and *PSEN2*). This pathway can be called a non-amyloidogenic, and is a physiologic one, as the formation of Aβ40 and Aβ42 is prevented. Loss-of-function mutations in *PSEN1* and *PSEN2* genes shift the γ-secretase cleavage to an increase in Aβ 42 production, which increases the risk of developing AD (De Strooper 2007).

Alternatively, APP can be cleaved by β-secretase (amyloidogenic pathway), which will result in a soluble extracellular fragment and a carboxyl-terminal fragment (CTF99). CTF99 will be further cut by γ-secretase leading to formation of Aβ40 and Aβ42. Different cleavage sites at the carboxyl (C) or amino (N) terminus of Aβ lead to fragments of different sizes, for example, 1-x and x-42. The length of final Aβ is critical because it dictates their toxicity (Beart & Robinson, 2013). Therefore, the formation of Aβ requires sequential cleavage by β- and γ-secretase. Being excised from APP by β- and γ-secretase, Aβ is released in the extracellular space and rapidly removed or degraded.

Aβ1-42 is more hydrophobic due to having two additional amino acids, thus has a greater ability to form insoluble aggregates (Zhao et al., 2015). In addition, Aβ1-42 species are known to be more prone to oligomerization, than Aβ1-40. Imbalance between Aβ production and its clearance is a key factor that contributes to Aβ buildup in AD. Accumulation of Aβ can be viewed as an initiator of the disease leading to a pathogenic cascade, eventually resulting in tau pathology, memory deficits and neuronal loss. In aged individuals the ability to clear Aβ is decreased, and Aβ peptides may accumulate.

1.1.3.3. Plaques vs. soluble oligomers

Previously, the main focus of researchers was primarily on Aβ plaque being the key pathogen in the pathogenesis of AD. However, later on it has been discovered that plaques or insoluble amyloid-β deposits correlate poorly with memory impairment in mice (Westerman et al. 2002) and cognitive decline in AD patients (Aizenstein et al. 2008). Plaques are now viewed as a reservoir for Aβ peptides as opposed to inherently toxic molecules. Rather, small aqueously soluble oligomeric forms of Aβ (AβOs), ranging from dimers to higher-order oligomers, are thought to play a more important role in AD than plaques. Studies have shown that there is a strong evidence for the role of natural oligomers of amyloid-β protein in disruption of cognitive function (Cleary et al. 2005; Santos et al. 2012). Because of their small size and solubility, AβOs are able to diffuse into a synapse (Ferreira and Klein 2011; Teresa et al. 2012). AβOs have been shown to be synaptotoxic and cause impairment of synaptic plasticity – by inhibiting long-term potentiation (LTP) (Welzel et al. 2014; Shankar et al. 2008) and facilitating long-term depression (LTD) (Cerpa et al. 2010; H. W. Wang et al. 2002; Origlia et al. 2010). LTP is widely viewed as a correlate of the cellular mechanisms of learning and memory. LTP is a way to change the strength of the synapses, to increase synaptic efficacy by high-frequency stimulation. As memory impairment is

usually the first symptom in AD, studying LTP can help characterize the potential mediators of synaptotoxic effects of AβOs on synaptic plasticity. Besides directly affecting memory-related processes (LTP and LTD), AβOs contribute to other aspects of AD neuropathology (i.e., tau hyperphosphorylation, synapse loss), that supports the idea that AβOs start the mechanisms for initiation of AD pathogenesis.

1.1.3.4. Brain response to Aβ accumulation

The surrounding glial cells in the brain, such as astrocytes and microglia, can respond to Aβ accumulation itself, as well as to its toxic effects, that can cause neuronal and synaptic dysfunctions by different ways. Aβ is considered to be a molecular trigger for reactive gliosis, which will lead to glial cells becoming functionally reactive (David R. Canning, Robert J. McKeon 1993). Reactive astrocytes undergo morphological, molecular and functional changes and can contribute to neuropathology. Moreover, microglia may shift astrocyte signalling from physiological to pathological by increasing the production of TNFα (Habbas et al. 2015). Presence of Aβ peptides induces oxidative stress through generation of superoxide radicals and nitrosative stress (Drake, Link, and Butterfield 2003; Choi et al. 2012; Colton 2009b; Nathan et al. 2005).

Aβ also affects glutamatergic pathways and disrupts calcium homeostasis (Lei et al. 2016). Chronic activation of glutamatergic system and formation of cation channels by Aβ leads to increase in Ca^{2+} uptake and disrupts its balance causing neuronal damage (Querfurth and Laferla 2010). Aβ has been known to inhibit glutamate uptake, which is the main excitatory neurotransmitter in the brain (Lei et al. 2016; Li et al. 2009). Excessive accumulation of glutamate is excitotoxic and disrupts homeostasis of the surrounding cells. Glutamate binds to and activates NMDA receptors. Thus, Aβ drives stimulation of NMDA receptors, which are expressed on neurons and also other CNS cells (L. Lu et al. 2019; Conti et al. 1996), including microglia (Kaindl

et al. 2012). We can speculate, that if AβOs triggers microglial morphological transformation indirectly through NMDA receptors, it will lead to activation of a chain of molecular events that trigger an inflammatory signalling cascade and subsequent microglial release of pro-inflammatory cytokines, chemokines and NO. In addition, Aβ can stimulate other receptors expressed by microglia, which will be discussed in more details further.

1.1.3.5. Protein tau as the main component of neurofibrillary tangles

Neurofibrillary tangles (NFTs), composed of abnormally hyperphosphorylated tau, are another hallmark of AD. Hyper-phosphorylated tau is more resistant to proteolysis and accumulate in AD brain (Beart and Robinson 2013). Aberrant phosphorylation of tau protein disrupts its function in regulating axonal transport and leads to accumulation into tangles. Similarly to Aβ oligomers, aggregates of abnormal tau are cytotoxic and contribute to impairment of cognitive function (Querfurth and Laferla 2010). The burden of NFTs is shown to be a good representation of the degree of cognitive impairment (Braak et al. 2006; Yin et al. 2017; Beart and Robinson 2013). Changes in tau, though important, occur as a consequence or downstream of present AβOs. Toxic Aβ species stimulate formation of pathological tau, by modulating protein kinases and phosphatases that regulate tau phosphorylation and by inducing tau misfolding (Bloom 2014). Moreover, molecular modulators of neuroinflammation have also been linked with tau-mediated neurodegeneration (Jaworski et al. 2011).

1.1.3.6. Defining neuroinflammation

Neuroinflammation plays a significant role in the underlying pathogenesis of Alzheimer's disease (Dheen, Kaur, and Ling 2007; Akiyama et al. 2000). Over the years, genome wide association studies (GWAS) have identified over 20 genetic loci that can contribute to the risk of developing LOAD and implicate immune cells and microglia (Beart and Robinson 2013;

Wightman et al. 2020). Several of those genetic risk factors encode for proteins involved in microglial function and inflammation including TREM2, CD33, CR1, ABCA7 and SHIP1 (Malik et al. 2015; Lambert et al. 2013). A significant proportion of genes with single nucleotide polymorphisms (SNPs) directly relate to innate function. Thus, groups of these variants might potentially drive neuroinflammation and further exacerbate LOAD disease progression.

Neuroinflammation is considered an inflammatory response in the CNS, which is mediated by the glial cell production of cytokines, chemokines and reactive oxygen/nitrogen species (ROS/RNS) (DiSabato, Quan, and Godbout 2016). Instead of being described as a secondary factor caused by accumulation of senile plaques and NFT, it is now suggested that inflammation in the brain contributes as much or even more to the pathogenesis of AD (Cameron and Landreth 2010; Ferretti et al. 2012; Malik et al. 2015). While active inflammation aims in restoring homeostasis and repair, chronic pro-inflammatory responses driven by microglia and astrocytes significantly impair their beneficial functions and further exacerbate neuronal and synaptic dysfunctions (Newcombe et al. 2018). In addition, inflammation can decrease the integrity of blood-brain barrier (BBB) and therefore interfere with the clearance of Aβ. AβOs can trigger inflammatory processes in glia, that can drive neurodegeneration through activation of detrimental inflammatory signalling cascades which contribute to cognitive decline (Ferretti et al. 2012; Medeiros et al. 2010; Rojo et al. 2008; Yin et al. 2017; Heneka et al. 2018). Aβ activated microglia, act as a main driver of inflammation (Kaindl et al. 2012; Teresa et al. 2012). Pro-inflammatory responses such as release of TNFα, IL-1β, IL-6 can affect BBB integrity and lead to alterations in its permeability (Zhao et al., 2015). More importantly, elevated levels of pro-inflammatory cytokines have been shown to affect synaptic plasticity by inhibiting long-term potentiation (Yirmiya and Goshen 2011; Patterson 2015; W. Y. Wang et al. 2015). Release of pro-inflammatory cytokines, such as Il-1, Il-

6 and TNFα and nitric oxide (NO) by microglia is directly synaptotoxic. Therefore, it is important to understand how crucial microglia and their functions are in driving the progression of AD.

1.2. Microglia – beyond innate immune functions

Microglia is an abundant cell in the brain. They constitute about 5-12% of all glial cells in the rodent brain and 0.5-16% in humans (Ma et al. 2017). Such range in distribution in human brain could be explained that microglial density is specific for different brain areas (Mittelbronn et al. 2001). Microglia originate from embryonic yolk sac and migrate to the brain during prenatal development (Sominsky, De Luca, and Spencer 2018). Microglia are the major and the primary resident immune cell in the brain (Colton 2009a). They are known to generate an innate immune response to injury as well as to different exogenous and endogenous stimuli. However, immune surveillance is just one of the many important roles that microglia possess. They take part in phagocytosis (Schafer and Stevens 2013; Z. Liu et al. 2010), neurogenesis (Sierra et al. 2010), axonal growth (Squarzoni et al. 2014), synapse pruning, modification or elimination (R. C. Paolicelli et al. 2011). To maintain CNS homeostasis, microglia express a wide range of receptors that they use to sense the environment and communicate with the surrounding brain cells.

More about the roles of microglia in the CNS and their importance during health and disease will be discussed later in the following chapter. Using correct terminology to describe different microglial states – "activated" vs "rested" or "M1" vs "M2" has been a high topic of discussion as the field is rapidly evolving (R. Paolicelli et al. 2022). In my chapters I will refer to microglia as surveillant – as they are in the healthy brain in their surveillance mode, and as activated-response microglia – as they are in a diseased brain, responding to a stressful stimulus.

1.2.1. Microglial functions

Since its colonization between 4-24th weeks of gestation, microglia remain an important player throughout the development of the adult human CNS (Menassa et al. 2018). Microglia exert their roles in a healthy brain as well as in a diseased. Those roles will differ, between beneficial or detrimental, depending on the environment, location, interactions with nearby cells and structures.

In a healthy brain microglial cells never rest; they are highly dynamic structures. They are performing the surveillance function by continuously sampling the environment with their highly motile protrusions, constantly guarding the surrounding (Davalos et al. 2005; Felten and Shetty 2014). These processes and protrusions directly contact astrocytes, neurons and blood vessels, further implicating that in the healthy brain microglia dynamically interact with other cortical elements (Nimmerjahn, Kirchhoff, and Helmchen 2005). Surveying microglia exhibit a ramified morphology; they have a small oval soma and numerous thin branched processes. Upon detection of an insult or stressful stimulus, microglia respond by changing their molecular profile, motility and function (Kreutzberg 1996). They also undergo morphological transformation to an amoeboid type; acquire slightly larger soma, and less than two unramified processes. Microglial morphological transformation perhaps can be called as one of their unique functions.

Similar to microglial morphology and ultrastructure, their molecular profile is extremely dynamic and plastic. Recent single-cell sequencing studies are showing evidence that there is a lot of microglial diversity during development (Hammond 2019). By about one month of age in a mouse, microglia are largely homogenous, as one big population. However, in the context of aging, there is a shift in some of these states. For example, one particular state or cluster 4 of microglia that appears only during development and expresses genes like *Spp1*, *Igf1*, *Lpl* and others, are also

found in some disease-associated states. The functions of these genes are suggested to be in immune recognition, lysosomal activity, phagocytosis and metabolic activity. Microglia have specific signatures and markers that reveal their location and function (Jurga, Paleczna, and Kuter 2020). Combining single-cell and spatial mapping with functional studies will enable the field to answer some of the very important questions about specific functions of different microglia subpopulations. For example, can various gene mutations influence microglial states and can we target specific subpopulations in disease, and other.

Microglia in the healthy brain can phagocytose cellular debris and facilitate clearance of invading pathogens from the neural environment. It is thought that phagocytosis involves the three main steps: “find-me”, “eat-me” and “digest-me” signals and involves membrane receptors, that recognize the target to engulf (Sierra et al. 2013; Janda, Boi, and Carta 2018). Some of the phagocytic receptors are TREM2, MerTK, Axl, that are expressed on microglia and also on myeloid cells (Savage et al. 2015). Microglia in AD recognize and engulf Aβ via scavenger receptors and promote its clearance (Paresce, Ghosh, and Maxfield 1996). It has been shown that cultured microglia are capable of phagocytosing the fibrillar Aβ (fAβ) when introduced into the media (Koenigsknecht and Landreth 2004). Microglial phagocytosis of microorganisms is not abundant but is thought to help fight infections. Phagocytosis of bacteria has been shown to activate the pro-inflammatory cascade in microglia with production and release of pro-inflammatory cytokines (TNFα), TLRs, scavenger receptors, complement receptors (CR3) (Sivagnanam, Zhu, and Schlichter 2010).

However, during pathological conditions microglial phagocytosis may not always be beneficial and as efficient. For example, there is evidence that after MCAO microglia poorly phagocytose apoptotic neurons, compared to the contralateral brain hemisphere (Faustino et al.

2011). Even though, the reasons behind this are poorly understood, it is speculated that high levels of pro-inflammatory cytokines can be the cause. Chronic disease state would lead to compromised phagocytotic function (loss of controlled phagocytosis by microglia) and may even contribute to neuronal damage.

Microglia are also involved in synaptic pruning; the process of elimination of synapses which is performed via microglial phagocytic properties. During brain development, immature synapses are formed in an excessive amount and are afterwards refined to ensure proper connectivity and wiring. This refinement process is an essential step in the emergence of functional circuits and is regulated, at least in part, by activity-dependent elimination ('pruning') of 'weak' synapses. (Paolicelli et al., 2011). C1q and C3 of classical complement cascade have been shown to facilitate both phagocytosis and synaptic pruning. They "mark" the weaker synapses that have to be eliminated. Furthermore, studies in the hippocampus revealed that microglia regulate the developmental phagocytosis of synapses via the activity of CX3CR1, fractalkine receptor specifically expressed by microglia (Thion & Garel, 2018). In addition, microglia can modify dendritic spine morphology; have influence on perisynaptic environment and can induce spine formation (Miyamoto et al., 2016; M.-ève Tremblay & Majewska, 2011). Sipe and colleagues have shown that microglia lacking purinergic receptor P2Y12, which is responsible for motility, disrupts plasticity in the visual system. Their results have demonstrated that altering purinergic signalling can have severe impact on ocular dependent plasticity in the visual cortex which gives microglia a critical role in late development, when they have adopted their adult ramified morphology (Kelly et al., 2016). This suggests that microglia play a role in modifying synaptic plasticity.

Microglia release a variety of pro- and anti-inflammatory cytokines, as well as trophic factors, such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), that

are used as signalling molecules in communication between cells (Colton 2009a). During development they regulate cell growth and differentiation. During physiological conditions they help to maintain homeostasis, modulate neuronal function and exert some neuroprotective effects.

Understanding of the pleiotropic physiological roles of microglia in the developing as well as in the adult CNS suggests that alterations in these normal homeostatic functions can contribute to disease development and progression. Some of the known triggers for microglial activation and change in functions are cell death (apoptotic or necrotic), excess aberrant protein, ischemia, glutamate, bacterial or viral pathogens, zinc (Carson, Thrash, and Walter 2006; T. M. Kauppinen et al. 2008). Activated microglia will retract their processes, migrate to the site of lesion and release matrix metalloproteinases, reactive oxygen species and pro-inflammatory cytokines, such as IL-1, IL-6 and TNFα (Chao et al. 1992). Such response increases host defenses and helps in restoring CNS homeostasis, but if persists - can contribute to neurotoxicity via oversecreting of superoxide ions, glutamate release, pro-inflammatory cytokines in various neurodegenerative disorders (Kreutzberg 1996). Aberrant levels of pro-inflammatory cytokines lead to alterations in tissue homeostasis and further aggravation of pro-inflammatory response by microglia (Marinelli et al. 2019). Activated microglia can phagocytose viable neurons in the process known as "phagoptosis", which may contribute to neuronal loss, inflammation, and neurodegeneration (Brown and Neher 2014).

Depending on the age, sex and environment, microglia are extremely heterogenous in their transcriptome (Colton 2009a; R. Paolicelli et al. 2022). The nature of the CNS stimulus, its duration and amplitude determine the type of response, which may have detrimental or beneficial effects on surrounding neurons and cells. When microglia lose the ability to return to their homeostatic or surveying state, their beneficial functions are compromised, which may cause

significant damage to the surrounding structures such as neuronal cells and synapses. Therefore, proper balancing of microglial phenotypes, modulating their responses, correct balance between production of pro- and anti-inflammatory cytokines, trophic factors and controlled phagocytosis is a key approach in targeting microglia therapeutically. When thinking about a therapeutic approach we need to acknowledge that microglia functions are highly diverse and complex; their states are defined by multiple layers of complexity such as genetic background, age, spatial location, environment. Therefore, considering sex differences when conducting research and developing therapeutics is highly relevant.

1.2.2. Sex differences in microglia

Male and female microglia have been reported to have different morphological and functional properties. Male microglia are characterized by having enlarged soma and are more reactive in physiological conditions than female microglia. In addition, in males the cells have higher pro-inflammatory response and higher migration capacity. On the other hand, female microglia have higher phagocytic capacity and express more genes that are responsible for repair and control of inflammation (Hanamsagar et al 2017; Yanguas-Casás 2020).

It is thought that sex-specific differences in microglia arise from the early development stages. Male and female microglia follow different trajectories during development (Villa et al. 2018). For example, transcriptomic data have shown that male microglia are developmentally delayed compared to female (Hanamsagar et al 2017). Moreover, some researchers state that sex-differences may arise as early as hematopoiesis (Hanamsagar and Bilbo 2016). In addition, another study showed that microglia can influence the process of brain masculinization, which is induced

by male gonadal activity and are essential for estradiol activity (Lenz et al. 2013). In physiological conditions microglia is known to express steroid hormone receptors and are sensitive to the effects of estrogen and testosterone (Sierra et al. 2008). Once they are differentiated, microglia are able to retain their sex-specific features even after transplantation into the brain of opposite sex during adulthood (Villa et al. 2018). Interestingly, even after surgical removal of gonads (ovariectomy), in the absence of hormones, adult microglia can maintain their sex differences.

Further studies are needed to learn more about microglial sex differences as it is highly relevant and may reveal important functional consequences for disease progression. There is a high probability for different mechanisms and signalling pathways in male and female microglia cells that would require a more targeted therapeutic intervention for a greater benefit. Understanding the complexity of microglial sex differences may reveal potential mechanisms of communication of male and female microglia cells with the surrounding cells and the ways to manipulate their interaction.

1.2.3. Microglia-neuron crosstalk

For microglia to be able to exert their functions, they have to communicate with other brain cells, such as neurons and astrocytes. Communication with neighbouring cells is possible through cell-to-cell contact, release of soluble factors and by exchanging biomolecules via secreted vesicles. For example, cell-to-cell communication is possible via a glycoprotein CD200 found on membranes of neurons, astrocytes and oligodendrocytes (Koning et al. 2009; G. J. Wright et al. 2001; Gavin J. Wright et al. 2003), a chemokine called fractalkine (CX3CL1), expressed on neurons and the equivalent receptors (CD200R and CX3CR1) on microglia. Some studies reported

extracellular vesicles of microglial origin that can vary in shape and modulate the activity of the surrounding cells as well as microglia themselves (Prada et al. 2013; Verderio et al. 2012). In addition, ATP, produced by neurons, can attract microglia to the location of an injury. Laser injury to the brain tissue causes extending of microglia processes and their movement to the injured area (Davalos et al. 2005). Any deviation from a healthy state to a chronically stressed state can lead to hyper-ramification of microglia and more frequent interactions of microglia with the neighbouring cells.

The ability of microglia to sense any change in the environment and communicate with the surrounding cells, such as neurons and other glial cells, requires a presence of variety of receptors and production of trophic factors, cytokines and chemokines (Madry and Attwell 2015). For example the increased accumulation of Aβ, excess levels of glutamate or presence of ATP (Davalos et al. 2005) activate specific receptors expressed in microglia, which initiate a corresponding response. Aβ can be recognized by microglial receptors, such as scavenger receptors, Fc receptors, TLRs, TREM2 and complement receptors (Doens and Fernández 2014). Fc receptors were shown to mediate the phagocytosis of Aβ in the presence of antibodies (Wilcock et al. 2003). There is also evidence that Ig bound to neuronal antigens can trigger microglial inflammatory reaction via FcRs, which were suggested to contribute to neurodegeneration in AD (Engelhardt et al. 2000). In AD human brains and animal models there is seen an increased expression of CD14, TLR2 and TLR4 (Reed-Geaghan et al. 2009). Stimulation of microglial TLR4 with ligands (e.g.LPS), increases Aβ uptake *in vitro* (Tahara et al. 2006). TLR2 deficient microglia showed reduction in the expression of TNFα, IL-6, iNOS, IL-1β in response to Aβ (Jana 2009). TREM2 deficient microglia in AD demonstrated decreased uptake of Aβ-lipoprotein complexes *in vitro* (Yeh et al. 2016) and *in vivo* (Y. Wang et al. 2015).

The presence of ATP released by neurons or astrocytes is sensed by microglia via purinergic receptors. Microglia express variety of purinergic receptors that control motility of their fine processes, release of cytokines, migration and phagocytosis (Pocock and Kettenmann 2007). They are activated by extracellular nucleotides (ATP, UDP, UTP), which are released after the tissue damage (Marinelli et al., 2019). Purinergic receptors can also control the production of cytokines (Pocock and Kettenmann 2007). It has been shown that activation of P2YR inhibits LPS-induced release of TNFα, IL-1β, IL-6 and IL-12 (Harry 2013), whereas blocking P2X7 receptor reduced pro-inflammatory cytokines IL-1β, TNFα, and IL-6 release, neuronal death and neurological deficit after cerebral ischemia (Ma et al., 2017). P2Y6 is upregulated after neuronal injury and mediates microglial phagocytosis (Pocock and Kettenmann 2007). Overall, purinergic signalling is one of key systems in the dynamic communication between microglia and neurons and plays an important role in shaping microglial behaviour.

Fractalkine, a membrane-bound chemokine produced by neurons and expressed on their surface (CX3CL1), signals directly to microglia by interacting with its receptor (CX3CR1) which is specifically expressed by microglia. CX3CL1/CX3CR1 signalling is assigned to the control of sensing and surveying function of microglia (Marinelli et al. 2019). Microglia use fractalkine signalling to recognise distressed and damaged neurons (Zabel and Kirsch 2013). In addition, there is evidence that neurons in distress can "call" microglia for help. Glutamate stressed neurons can increase microglial ability to clear neuronal debris via secreting soluble fractalkine (Ma et al. 2017). Recent studies suggested that fractalkine receptor signalling is also involved in synapse elimination process via phagocytic engulfment mechanism. Absence of CX3CR1 in postnatal mice showed increased numbers of immature synapses in CA1 hippocampal subfield (R. C. Paolicelli et al. 2011). It has also been suggested that fractalkine signalling is important for limiting microglia

toxicity (Cardona et al. 2006). Lack of fractalkine signalling increases neuronal loss in models of PD and ALS (Marinelli et al., 2019). Levels of both fractalkine receptors and ligands were decreased in AD brain. Deletion of CX3CR1 exacerbated microglial activation and increased expression of pro-inflammatory cytokines, such as TNFα and IL-6 in hAPP mice, and exacerbated neuronal dysfunction and cognitive deficits (S. H. Cho et al. 2011). To conclude, fractalkine signalling is a pathway that mediates communication between microglia and neurons, which is deficient in AD brains and down-regulated by amyloid-β.

Another neuron-microglia communication is possible via the interaction between neuronal CD200 glycoprotein and microglial receptor CD200R. CD200Rs are a group of receptors expressed by microglia that are involved in the regulation of inflammatory response and phagocytic activity (Gavin J. Wright et al. 2003; Marinelli et al. 2019). The role of CD200-CD200R interaction is to inhibit the production of pro-inflammatory mediators by microglia. This type of interaction is relevant in the pathogenesis of AD, because levels of CD200 and its receptors are reduced in the hippocampus of AD patients (Cameron and Landreth 2010).

Triggering receptor expressed on myeloid cells-2 (TREM2) is abundantly expressed on microglia (Y. Zhang et al. 2014). Microglial TREM2 and neuronal TREM2-L form a receptor-ligand pair connecting microglia with apoptotic neurons, directing removal of damaged cells to allow repair (Hsieh et al. 2009). It has been suggested that Aβ oligomers can bind to TREM2 with high affinity and activate TREM2-dependent signalling pathways in microglia. TREM2 deficiency impairs microglial migration and clustering around Aβ plaques (Zhong et al. 2018), microglial phagocytosis of Aβ plaques (Sarlus and Heneka 2017). Mice that lack TREM2 showed greater plaque diffusion and damage to neighboring neuronal structures by modulating microglial response (Spangenberg and Green 2017). Which indicates that TREM2 signalling is important for

the buildup of Aβ plaques. Moreover, mutations in TREM2 mutations in TREM2 were identified amongst the 38 genetic loci to increase the risk of AD (Jin et al. 2014; Wightman et al. 2020).

Complement cascade is playing a role in mediating communication between microglia and neuronal synapses. Complement-mediated mechanisms have been shown to be involved in synaptic remodeling. For example, C1q activation results in downstream activation of C3b, which then deposits on neurites "marking" them for elimination (Stevens et al. 2007). CR3 or CD11b can trigger apoptosis by mediating release of superoxide ions which is dependent on Dap12 immunoreceptor (Wakselman et al. 2008). However, this normal developmental synaptic pruning pathway can get activated early in AD and mediate synapse loss. Microglia was demonstrated to be the dominant source of C1q in the brain (Fonseca et al. 2017). Hong and colleagues also demonstrated upregulated microglial C1q expression, implicating microglia as a major source of C1q before formation of plaques (Hong et al. 2016). They injected AβOs into lateral ventricles of WT mice and saw that C1q immunoreactivity was increased particularly in hippocampus. They also used C1qa KO mice in which AβOs failed to induce synapse loss, suggesting that C1q is required for AβO-induced synapse loss in vivo. Importantly, pre-treating hippocampal slices with C1q-antibody significantly prevented inhibition of LTP induced by AβOs.

To sense glutamate levels, microglia express a full range of glutamate receptors, including ionotropic and metabotropic AMPA and NMDA receptors (Kaindl et al. 2012), and release diverse substances that are able to activate neuronal GluRs, outlining a pathway for the possible mutual regulation (Murugan, Ling, and Kaur 2013; H. Liu, Leak, and Hu 2016). Expression of GluR2/3 subunits of AMPA receptors was found in microglia (Guo et al. 2006) and their expression was upregulated following hypoxia (Sivakumar et al. 2010). Until recently functional NMDA receptors in microglia remained doubtful, although the expression of NR1 subunit in microglia have been

reported following transient ischemia in 1997 (Gottlieb and Matute 1997). Studies in rats and in mice are also indicative of functional NMDA receptors present in microglia (Streit, Morioka, and Kalehua 1992; Castellano and Castro 1996; Thomas and Kuhn 2005). However, the exact function of NMDARs in microglia are still under investigation. So far, it is viewed that microglial NMDARs are involved in microglial response, altering their morphology and motility, glutamate release, Ca^{2+} signalling and release of pro-inflammatory cytokines (Kaindl et al. 2012; Raghunatha et al. 2020). This leads to increased expression of surface molecules in microglia and various signalling cascades get activated, leading to an increased expression and/or activation of pro-inflammatory proteins/enzymes and mediating transcription factors (Acarin, González, and Castellano 2000; Murugan et al. 2011). Among those are COX, iNOS, MAPK, NADPH oxidase, PARP-1 and nuclear factor kappa B (NF-κB), an important mediator of inflammatory responses. Note, Aβ is known to trigger an inflammatory response by microglia via NF-κB (R. T. Liu et al. 2014), and unpublished work from the Jackson and Kauppinen labs suggests that this response is initiated through NMDAR-dependent activations of TRPM2/PARP-1 signalling.

1.2.4. Microglia drives neuroinflammation in AD

Microglia has emerged as one of the main players in the pathogenesis of many neurodegenerative diseases (Salter and Stevens 2017). In AD microglia induce different functions with neuroprotective or detrimental outcomes. Microglial cells express several receptors that help in recognition, internalization and clearance of Aβ. Those receptors are scavenger receptors (SR-AI/II), CD36, RAGE (receptor for advanced glycation), Fc receptors and TLRs and complement receptors (Doens and Fernández 2014). Generally, microglia are thought to facilitate clearance of

Aβ at early stages of AD. With the disease progression, the levels of Aβ-degrading enzymes decrease. For example, IFNγ and TNFα in addition to being neurotoxic, also reduce levels of insulin degrading enzyme, a key Aβ degrading protease (Rojo et al. 2008). Aβ binding to receptors such as RAGE and TLRs can trigger microglial inflammatory responses (Origlia et al. 2010). Aβ induces NF-κB transcription factor expression in neurons, microglia and endothelial cells (Barbara Kaltschmidt et al. 1997). In microglia, activation of NF-κB promotes inflammatory response by release of pro-inflammatory molecules via interaction with RAGE. Upregulation of TLRs expression has been observed in pathological conditions associated with microglial activation and production of pro-inflammatory cytokines (Fiebich et al. 2018). When microglia are in chronic pro-inflammatory state, protective phagocytosis (i.e. amyloid clearance) is impaired and cytokine production is increased, which is thought to contribute to neurodegeneration and cognitive decline (Colton 2009a).

There is extensive research documenting a microglial-related pro-inflammatory response (Krause and Müller 2010; Correani et al. 2019; Fiebich et al. 2018; Kaindl et al. 2012). Cytokine production by glia cells shapes the inflammatory response in AD. Microglial cell-surface receptors by interacting with AβO initiate a pro-inflammatory signalling cascade which results in release of various pro-inflammatory cytokines, proteases and free radicals such as ROS. By secreting Il-1α, TNFα and C1q activated microglia will affect the surrounding astrocytes. Reactive astrocytes will lose the ability to promote neuronal survival and gain neurotoxic function (Liddelow et al. 2017) which will further exacerbate neuroinflammation and neurodegeneration. In addition, microglia can release multiple complement components (i.e. C1q) that will help drive synapse degeneration (Stevens et al. 2007). Patients with AD have increased levels of microgliosis and astrocytosis that surround Aβ plaques (Pekny et al. 2016; Heneka, Kummer, and Latz 2014).

Past studies in animal models of AD hinted on presence of early inflammatory processes by reporting an upregulation of pro-inflammatory markers such as Il-1, Il-6 and TNFα in Pd/APP mice (Heneka et al. 2005) and 3xTg mice (Janelsins et al. 2005). Several studies reported a correlation between diminished cognitive status and microglial pro-inflammatory state in a subset of patients with mild cognitive impairment (Okello et al. 2009). Specifically, IL-1 and TNFa have been associated with reduced synaptic plasticity and synaptotoxicity (Qian et al. 2012; John, Lee, and Brosnan 2003; DiSabato, Quan, and Godbout 2016).

Nitric oxide (NO) is a prominent pro-inflammatory marker, levels of which rise through the activity of inducible nitric oxide synthase (iNOS) enzyme in microglia and astrocytes (Aktan 2004; Cherry, Olschowka, and O'Banion 2014). It has been suggested that nitrosative stress is one of the key factors contributing to the pathogenesis in AD. Use of iNOS inhibitors or removal of iNOS in AD mouse model has been shown to be protective against Aβ induced neurotoxicity (Nathan et al. 2005). *In vivo* studies showed increased inflammatory response in rat hippocampus after injection of synthetic preparation of AβOs, indicating that microglia respond to the extracellular Aβ fibrils by increasing levels of iNOS (Bruno et al. 2009). Moreover, upregulation of iNOS was observed in patients diagnosed with AD (Nathan et al. 2005). This increased expression of iNOS in microglia generates increased levels of NO and peroxynitrite via NO/superoxide pathway. Recent studies revealed that excessive Aβ-induced NO production can result in aberrant protein S-nitrosylation which would lead to decrease in insulin-degrading enzyme in AD and impact synaptic function (Akhtar et al. 2016; D.-H. Cho et al. 2009; Nakamura and Lipton 2016).

Microglia are the critical cell type to contribute to neuroinflammatory-related pathology (Sarlus and Heneka 2017). Excessive production of pro-inflammatory mediators by activated glia is indirectly neurotoxic and plays a role in pathogenesis of neurodegenerative disorders (Correani

et al. 2019). Importantly, when microglia are in an activated state chronically the balance between production of pro-inflammatory and anti-inflammatory cytokines are vanished and this leads to the disruption of homeostasis. Finding central regulatory pathways of microglial responses either pro- or anti-inflammatory could provide a novel therapeutic approach to Alzheimer's disease or other neurodegenerative disorders. Recent studies have shown a role of poly (ADP-ribose) polymerase-1 (PARP-1) in NF-κB driven production of inflammatory mediators in the inflammatory response (T. M. Kauppinen, Gan, and Swanson 2013). The next section will address the important role of PARP-1 as pro-inflammatory hub driving detrimental responses by microglia in AD.

1.3. PARP-1 nuclear enzyme

Nuclear enzyme poly (ADP-ribose) polymerase-1 (PARP-1) is the most abundant isoform among PARP enzyme family. PARP-1 is involved in many physiological processes such as differentiation, proliferation and tumor transformation (Muvarak et al. 2016; Bi, Li, and Yang 2013), DNA damage repair (Pascal 2018), induction of inflammation (Mangerich and Bürkle 2012; Yarbro, Russel, and Pence 2020). Recent studies by others and in our lab have shown that PARP-1 is a central signalling hub that drives pro-inflammatory responses in microglia via activation of NF-κB, a transcription factor associated with a variety of pro-inflammatory proteins (Ullrich et al. 2001; T. M. Kauppinen et al. 2011; Vuong et al. 2015). One mechanism that could link these signalling molecules is Ca^{2+}. Indeed, Ca^{2+} has been shown to activate ERK1/2 that is necessary for PARP-1 activation (Vuong et al. 2015). What would be the possible sources of Ca^{2+}? Likewise, what is the role of Ca^{2+} for calcium-permeable channel called TRPM2 and how are they

involved in the signalling hub that keeps microglia chronically activated? More about the role of PARP-1 in microglia, mechanisms of activation and pro-inflammatory signalling cascade will be discussed further.

1.3.1. General overview, mechanisms of activation

Poly (ADP-ribose) polymerases (PARPs) are a family of 18 enzymes that are involved in a number of cellular processes including gene regulation, chromatic remodeling, DNA repair and apoptosis (Jubin et al. 2016). Some of them share homologous domains and have catalytic functions. PARP-1 is a nuclear enzyme that has multiple roles in many cellular processes. Some of these functions include maintaining of cellular homeostasis, DNA repair, role in inflammation and cell death pathways (Luo and Kraus 2012). PARP-1 consists of three main domains: N-terminal DNA-binding domain (DBD), auto-modification domain (AMD) and C-terminal catalytic domain. The DBD domain recognizes DNA breaks and stimulate PARP-1 activity. The AMD domain is involved in PARP-1 automodification. The C-terminal region is involved in catalytic synthesis of poly (ADP-ribose) polymers (PARs) by using nicotinamide adenine dinucleotide (NAD+) as a substrate (Martire, Mosca, and d'Erme 2015). PARP-1 can be activated by DNA strand breaks, changes in the levels of metabolites and some ions (Ca^{2+}, Zn, Mg^{2+}), and post-transcriptional modification (Luo and Lee Kraus 2012; T. M. Kauppinen et al. 2008). PARP-1 synthesises polymers of poly (ADP-ribose) (PAR) from NAD+ to promote DNA repair. When PARP-1 enzyme is activated, it is responsible for three enzymatic reactions during PAR synthesis:1) attachment of the first (ADP) ribose monomer to amino acid residue in an acceptor protein (initiation); 2) formation of (2'-1") ribose-ribose glycosydic bond (elongation) and 3)

formation of ribose-ribose (2”-1’) bond between ADP-ribose units (branching) (Rolli et al. 1997). PAR polymers are also used for PARP-1 automodification – addition of PAR to PARP-1 enzyme (auto-PARylation).

PARP-1 can be activated during ischemia, hypoglycemia, excitotoxicity, and other conditions that include oxidative stress and inflammation. Activation of PARP-1 in neurons will subsequently lead to the death of neurons (Alano et al. 2010), when prolonged PARP-1 activation can be lethal for astrocytes and potentially other brain cells (Alano, Ying, and Swanson 2004). This happens due to robust PARylation in the expense of NAD+, resulting in impaired glycolysis and energy metabolism (Alano et al. 2010). PARP-1 has important role in bioenergetics metabolism, PARP-1 silenced cells exert elevated cellular metabolic phenotype at baseline and under oxidative stress conditions (Martire, Mosca, and d’Erme 2015; Katalin Módis 2012). Studies have shown that PARP inhibitors can improve neuronal survival in ischemia and other states (Luo and Kraus 2012). However, studies have shown that PARP-1 can be activated independently from DNA damage in microglia and astrocyte cultures (Vuong et al. 2015). DNA-independent activation of PARP-1 in microglia leads to increase in pro-inflammatory response and production of pro-inflammatory mediators (Raghunatha et al. 2020). Many toxic stimuli (i.e. amyloid β) have been shown to induce excessive activation of PARP-1 in microglia, where activation of PARP-1 does not lead to cell death (T. M. Kauppinen and Swanson 2005; T. M. Kauppinen et al. 2011; Correani et al. 2019; Raghunatha et al. 2020; Park et al. 2020; T. M. Kauppinen et al. 2009). In addition, PARP-1 is activated in glia treated with LPS, Aβ 1-40 (Chiarugi and Moskowitz 2003) and Aβ 1-42 (T. M. Kauppinen et al. 2011). PARP-1 serves as a co-activator of NF-κB, which is known to play a crucial role in the regulation of genes that are involved in immune and inflammatory responses (Kameoka et al. 2000).

1.3.2. PARP-1 coactivator of NF-κB transcription factor

The transcription factor nuclear factor kappa B (NF-κB) encompasses a family of inducible transcription factors, which are required for regulation of many genes, and has traditionally been linked to cellular immune and inflammatory response. Recent reports provided strong evidence that PARP-1 is required for NF-κB- dependent gene expression in a stimuli dependent manner and acts as a transcriptional coactivator of NF-κB. NF-κB plays a crucial role in the regulation of gene expression of many genes involved in mammalian immune and inflammatory responses, including cytokines, chemokines, cell adhesion molecules, complement factors, acute phase proteins and a variety of immune receptors. It regulates the expression of many genes including cytokines (such as IL-1β, IL-6, TNFα), iNOS and some other enzymes (B. Kaltschmidt and Kaltschmidt 2009). The NF-κB family consists of five members: p65, RelB, c-Rel, p105/p50 and p 100/p52 which form homo- or heterodimers. NF-B activity at sites of inflammation is associated with RelA or c-Rel containing complexes. In most cell types, the most abundant heterodimer consists of p65 and p50 (Hayden and Ghosh 2012). The regulation of NF-κB is complex and requires multiple steps. First step is the release of the dimers from IκB subunit and translocation from the cytosol to the nucleus. The second step of the process occurs in the nucleus and involves the formation of an active DNA-bound transcription complex (Ghosh, May, and Kopp 1998). However, just translocation of NF-κB to the nucleus is not equal to its transcriptional activation (T. M. Kauppinen, Gan, and Swanson 2013). For example, p65 subunit requires acetylation for full transcriptional activity. PARP-1 can also bind to the p65 NF-κB subunit (Zerfaoui et al 2008). Poly (ADP-ribosyl)ation of PARP-1 enhances the formation of the NF-κB transcription complex by dissociating NF-κB p50 from PARP-1 and thereby allowing NF-κB to bind to its DNA binding

sites (Nakajima et al. 2004). In addition, PARP-1 may regulate some NF-κB-dependent gene expression not only by regulating the association of PARP-1 with NF-κB, but also by cooperating with other transcriptional mediators such as p300 (Paul O. Hassa et al. 2003). During oxidative stress or excitotoxicity extensive activation of PARP-1 can promote transcriptional activity of NF-κB by a mechanism that involves p65 acetylation (T. M. Kauppinen, Gan, and Swanson 2013). PARP-1 consumption of NAD^+ prevents deacetylation of p65 that is NAD^+ dependent and therefore promotes NF-κB activation (Chen et al. 2005). PARP-1 depletion in astrocytes did not prevent translocation of NF-κB (p65) from cytosol to nucleus, but NF-κB transcriptional activity was prevented (T. Kauppinen, Gan, and Swanson 2013). Enzymatic inhibitors of PARP-1 can suppress inflammatory responses in microglia (Chiarugi and Moskowitz 2003; T. M. Kauppinen and Swanson 2005) and other cell types (Paul O. Hassa et al. 2003; Nakajima et al. 2004; T. M. Kauppinen et al. 2009). However, it has also been reported that enzymatically inactive PARP-1 can facilitate NF-κB transcriptional activation (Zerfaoui et al 2008; T. M. Kauppinen and Swanson 2005; Paul O. Hassa et al. 2001). This suggests that the anti-inflammatory effects of PARP-1 inhibitors can occur through a different mechanism.

NF-κB activation can be induced by Aβ and signals the priming of NLRP3 inflammasome and the expression of pro- inflammatory cytokines including the inflammasome substrates IL-1β and IL-18 (R. T. Liu et al. 2014). Activation of NF-κB followed by elevation of cytokines such as IL-1, IL-6, TNFα has been shown in tau-stimulated microglia (C. Wang et al. 2022). In Aβ-stimulated microglial cultures release of NO and TNFα was blocked by PARP-1 inhibitor (T. M. Kauppinen et al. 2011). Moreover, NF-κB-dependent pro-inflammatory cytokines were decreased in PARP-$1^{-/-}$ microglia and WT microglia upon PARP-1 inhibition (Vuong et al. 2015). NF-κB-

driven transcription of pro-inflammatory cytokines is impaired in PARP-1 knockout animals (Haskó et al. 2002). In addition, NF-κB-dependent transcription of some inflammatory mediators in response to endotoxin or pro-inflammatory cytokines, such as TNFα and interleukin-1β (IL-1β) is almost completely abrogated in PARP-1 KO mice (Paul O. Hassa et al. 2001). Studies have shown that pharmacological inhibition of PARP-1 reduces both NF-κB activity and the synthesis of inflammatory mediators during glial activation (Chiarugi and Moskowitz 2003). Given that NF-κB regulation is stimuli and cell type dependent, the requirements of PARP-1 interaction with NF-κB might not always require enzymatic activity (Paul O. Hassa et al. 2003; Ha, Hester, and Snyder 2002). Besides NF-κB PARP-1 plays role in coregulating in a number of other transcriptional regulators such as B-Myb, Oct-1, HTLV Tax-1, Sp1, NFAT, Elk1, and others (P O Hassa and Hottiger 2002; Cozzi et al. 2006; Kraus and Lis 2003; Olabisi et al. 2008; Ju et al. 2004). More details about PARP-1, its roles and mechanisms of activation will be described further.

1.3.3. Regulation of PARP-1 activation

PARP-1 is classically triggered by DNA strand breaks and initiate DNA repairs. However, PARP-1 can be activated by the mechanisms independent of DNA damage, for example upon inflammatory stimuli. PARP-1 enzymatic activity and production of PAR polymers cause PARylation of PARP-1 and other proteins (post-translational modification). PARP-1 activation has been shown to occur due to Ca^{2+} dependent PARP-1 phosphorylation via extracellular receptor kinases (ERK1/2), which are one of the major subgroups of mitogen-activated protein kinases (MAPKs). While both ERK1 and ERK2 were found to phosphorylate PARP-1, evidence suggests that enzymatic activity of PARP-1 by measuring PAR formation was blocked by ERK2 inhibition, but not ERK1 (T. M. Kauppinen et al. 2006). ERK1/2 are activated when phosphorylated by the

upstream MAPK/ERK kinases 1/2 (MEK1/2) for which they require Ca^{2+} (Castro-Obregón et al. 2002). Using MEK1/2 inhibitor blocked both ERK1/2 phosphorylation and PARP-1 (T. M. Kauppinen et al. 2006). PARP-1 activation from the extracellular space is possible independently of DNA damage via TNFα. By inducing increase in intracellular Ca^{2+} levels was crucial in PARP-1 activation, as TNFα-induced PARP-1 activation was blocked in Ca^{2+}-free medium (Vuong et al. 2015; T. M. Kauppinen et al. 2006).

Ca^{2+} has a central role in creating a feedback loop of continuous PARP-1 activation. As PARP-1 can be activated in response to increase in intracellular Ca^{2+} levels, the end product of its activation, ADPR, will bind to ADPR pyrophosphatase domain on the intracellular c-terminal region of Ca^{2+} permeable transient receptor potential melastatin 2 (TRPM2) channels (Sumoza-Toledo and Penner 2011) and will allow more Ca^{2+} into the cell (**Figure 1**). Source of ADPR monomers is provided from activity of poly(ADPR) glycohydrolases (PARG enzymes) via hydrolysis of poly-ADPR (Fauzee, Pan, and Wang 2010; Esposito 2009; D'Amours et al. 1999) . This allows PARP-1 activation to be coupled to elevations in intracellular Ca^{2+} levels. Importantly, NUDT5 enzyme interacts with PARP-1/TRPM2 signalling via hydrolyzing ADPR to AMP and ribose 5'-phosphate and thus can control the intracellular levels of ADPR able to activate TRPM2 channel (**Figure 1**) (A.-L. Perraud et al. 2001). More about TRPM2 channel activation and their role in PARP-1-mediated microglial response is discussed in the next section.

1.4. TRPM2 channels

Transient receptor potential melastatin 2 (TRPM2), is a calcium-permeable, ADP-ribose (ADPR) gated channel that is encoded by the TRPM2 gene. It is widely expressed by immune

cells and is important in maintaining proper immune responses (Alawieyah Syed Mortadza et al., 2018). Expression of TRPM2 channels in the CNS is high in microglia and its activation has been shown to stimulate inflammatory responses (Miyake et al. 2014; Jeong et al. 2017; Hecquet et al. 2014). Evidence shows that TRPM2 channels may have a significant role in the pathogenesis of AD as their inhibition attenuated Aβ-induced cell death (E. Fonfria et al. 2005). More importantly, findings from the Jackson lab showed that TRPM2 KO supressed microglial activation in APP/PS1 mice and prevented cognitive decline thus revealing the importance of TRPM2 in AD (Ostapchenko et al., 2015). Activation of TRPM2 channels has been shown to be downstream of PARP-1 activation. Moreover, PARP-1 and TRPM2 activation is needed for microglial transformation to a pro-inflammatory state and production and release of pro-inflammatory mediators in response to NMDAR stimulation (Raghunatha et al. 2020). The structure of TRPM2 and their role in contributing to sustained inflammatory response in AβO-induced microglia is discussed further.

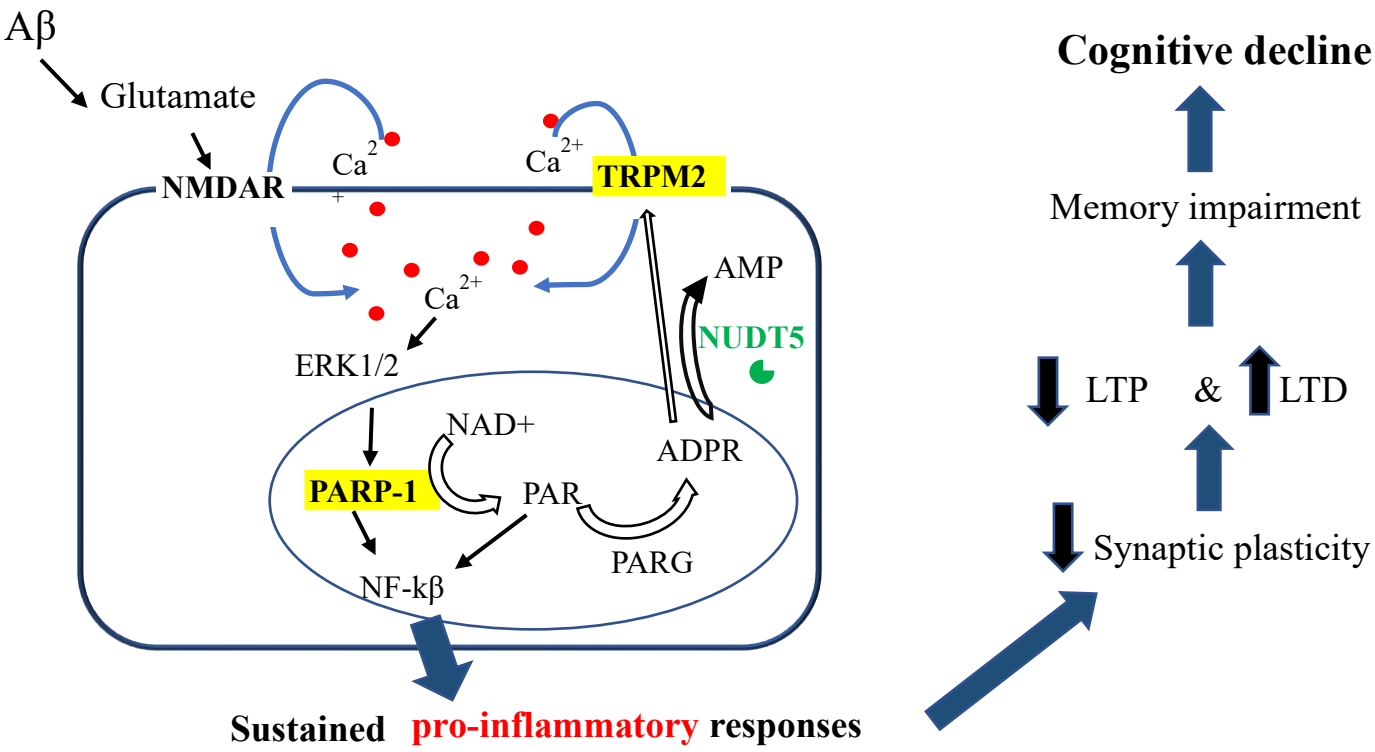

Figure 1: Illustration of the proposed positive pro-inflammatory signalling cascade in microglia in Alzheimer's disease.

1.4.1. Structure of TRPM2 receptors

Transient receptor potential (TRP) proteins represent a large family of six-transmembrane ion channels that were named after Drosophila melanogaster TRP protein (Montell and Rubin 1989). The first TRP protein was isolated in 1989. Nowadays, TRP superfamily consists if 28 members that are diverse in their mechanisms of activation, selectivity, and permeability to cations such as Na^+, Ca^{2+}, Mg^{2+}, and K^+ (Ramsey, Delling, and Clapham 2006; Sumoza-Toledo and Penner 2011). TRPM2 is expressed in all tissues throughout the body, except for the cartilage and bone (Belrose and Jackson 2018). The expression of TRPM2 is highest in the brain (Elena Fonfria et al. 2006), especially in microglia (Kraft et al. 2004). Brain RNA sequencing reports in mice and in humans reported the highest fragments per kilobase of exon per million mapped segments (FPKM) of TRPM2 in microglia (Y. Zhang et al. 2014; 2016).

TRPM2 channel is a multifunctional Ca^{2+} permeable non selective cation channel located on chromosome 21q22.3 in humans and consists of 32 exons (Nagamine et al. 1998). TRPM2 channel is a homo-tetramer, and each subunit has 6 transmembrane domains (S1-S6) with a re-entrant pore forming loop located between 5 and 6 transmembrane domains. Additionally, the intracellular N-terminus has four homologous domains and a calmodulin binding motif (CaM) and C-terminus that contains TRP box and coil-coil domain (L. H. Jiang 2007). N-terminus is thought to play a role in modulating channel activation and C-terminus may be critical for the homo-tetrameric assembly of the channel (Mei et al. 2006). Moreover, an endogenous ligand ADPR binds with high specificity to a C-terminus nucleoside diphosphate linked moiety X-type homology (NUDT9-H) domain(A.-L. Perraud et al. 2001; Kühn and Lückhoff 2004). Upon binding of ADPR, TRPM2 channels open and allow influx of Na^{+} and critically, Ca^{2+} into the cell (McHugh et al. 2003). TRPM2 channel has unusually long open times in the range of several seconds (Fleig and Penner 2004).

Pharmacological inhibition of TRPM2 channel is possible with various agents such as clotrimazole, econazole, flufenamic acid, 8-Br-cADPR and others (Lin Hua Jiang et al. 2010). However, these agents lack specificity. Novel TRPM2 antagonist JNJ-28583113 was shown to be highly specific and CNS penetrant, albeit with limited stability when administered *in vivo*. *In vitro* use of JNJ-28583113 blunted cytokine release in response to pro-inflammatory stimuli in microglia (Fourgeaud et al. 2019). Studies using known TRPM2 inhibitors deepened our understanding on TRPM2 biology and have revealed important information about the roles of TRPM2 in health and pathology which will be outlined in the next section.

1.4.2. Role of TRPM2 in health and disease

Over the past few years, studies using the known inhibitors and TRPM2 knockout mice have disclosed several important roles for TRPM2 in oxidative stress signalling, production of cytokines by immune cells, increased endothelial permeability and cell death in response to oxidative stress. The channel has also been shown to have a role in temperature regulation (Song et al. 2016).

Accumulating evidence suggests that TRPM2 has a crucial role in producing inflammatory cytokines in response to oxidative stress and inflammatory stimuli in the body. In the area of an injury or infection there is an increased production of ROS. Chemotactic cytokines will recruit inflammatory cells such as neutrophils and macrophages to come to such inflammatory sites from the bloodstream. H_2O_2 is generated together with other ROS, such as superoxide and hydroxyl radicals. H_2O_2 has been shown to activate TRPM2 via production of ADPR. This increased the channel's probability to be involved in many processes like inflammation, ischemia-perfusion injury, increased oxidative microenvironment. Hara and colleagues showed that activation of TRPM2 in the cells that highly express the channel can cause cell death due to increased Ca^{2+} concentrations (Hara et al. 2002). A group of researchers showed the significance of TRPM2 in chemokine production during oxidative stress in mice model of human inflammatory bowel disease. The TRPM2 KO mice showed less recruitment of the inflammatory cells to the site of inflammation (Yamamoto 2008).

Vascular endothelium, being the physical barrier between the blood and interstitium, is an important source of ROS production especially during ischemia and stroke. The association between TRPM2 and endothelial plasmalemma has been observed in mouse endothelial cells

(Hecquet et al. 2014). This study revealed a mechanism by which activation of TRPM2 induces apoptosis of endothelial cells, which involves ROS-induced protein kinase C activation and subsequent phosphorylation and activation of TRPM2. Study using TRPM2 KO mice showed that oxidative-stress induced activation of TRPM2 leads to vascular barrier dysfunction via Ca^{2+} influx into endothelial cells (Mittal 2017). These results combined show that TRPM2 have role in response to oxidative stress, vascular permeability, inflammatory processes and plays role in production of pro-inflammatory cytokines and chemokines in the body.

Since TRPM2 is expressed in both neurons and glia, it is not unexpected that they have several roles in the physiological processes in the brain. TRPM2 is involved in neuronal development and has inhibitory role in neurite outgrowth. Axonal outgrowth was significantly increased after inhibition of TRPM2 (Jang et al. 2014). In addition TRPM2 has been shown to contribute to synaptic plasticity (; Xie et al. 2011). A study using TRPM2 KO mice showed that LTD was impaired in those mice compared to WT (Xie et al. 2011). TRPM2 was also shown to interact with NMDA receptors, whereby stimulation of NMDARs was shown to facilitate TRPM2 activation (Olah et al. 2009).

Many neurodegenerative disorders share common features with most prominent being Ca^{2+} dyshomeostasis triggered by oxidative stress. Those include ischemic stroke, Alzheimer's disease, Parkinson disease, bipolar disorder. Patients with bipolar disorder type I have high basal Ca^{2+} levels, and chromosome region 21q22.3 contains genes susceptible to this disease, including TRPM2 (Xu et al. 2009). TRPM2 has also been linked to the development of amyotrophic lateral sclerosis and parkinsonism-dementia (Hermosuraa and Garruto 2007). Studies using TRPM2 KO mice showed reduced brain infarct volume and reduced loss of brain mass after induction of hypoxia-ischemia (Alim et al. 2013). These results tell us that $TRPM2^{-/-}$ is neuroprotective after

ischemia-reperfusion injury *in vivo* (Alim et al. 2013). In addition, these mice showed decreased levels of pro-inflammatory markers, which would mean that TRPM2 has indirect neuroprotective effect. A model of focal ischemia of middle cerebral artery occlusion (MACO) in rat showed upregulation of TRPM2 in microglia in addition to microglia being in a pro-inflammatory state (E. Fonfria et al. 2005). TRPM2 seems to be responsible for the activation of microglia that is mediated by production of ROS, LPS and NO (Yamamoto 2008; Haraguchi et al. 2012), Aβ (E. Fonfria et al. 2005) and activation of NMDARs (Raghunatha et al. 2020). A study using TRPM2$^{-/-}$ mice suggested that TRPM2's sensitivity to H_2O_2 is dependent on the activation state of microglia (Kraft et al. 2004). In addition, application of PARP inhibitors impaired TRPM2 activation (Elena Fonfria et al. 2004). These results suggest that TRPM2 in microglia have an important role in CNS responses to brain damage such as ischemic injury and oxidative stress. TRPM2 receptors are also suggested to play a key role in AD (J. Wang, Jackson, and Xie 2016).

1.4.3. Role of TRPM2 in AD

Besides deposition of senile plaques and tau, pathogenesis of AD includes oxidative neuronal damage. One of the proposed mechanisms of Aβ-induced neuronal injury is thought to be generation of oxidative and nitrosative stress (Drake, Link, and Butterfield 2003). Following oxidative stress TRPM2 is activated and causes sustained increase in Ca^{2+} levels which leads to induction of neuronal cell death. Importantly, AβOs alone were shown to facilitate TRPM2 function and elimination of the channel prevented the pathological and behavioral deficits in the APP/PS1 AD mouse model (Ostapchenko et al. 2015).This mouse model of AD carries the genes of human APP-Swedish mutation and the E9 mutation of presenilin 1 and at 6 months of age develops significant amounts of soluble and plaque-associated AβOs. This is the first study that looked whether loss of TRPM2 had any effect on cognitive deficits in APP/PS1 mice. Two spatial

memory tests were used, Barnes maze and Morris water maze where TRPM2 elimination ameliorated the age-dependent spatial deficits. By measuring synaptophysin the authors also noted that synapse loss was prevented in the brains of TRPM2$^{-/-}$ APP/PS1. Moreover, the study also examined microglial activation by measuring Iba1 immunostaining, a marker of microglia. APP/PS1 mice lacking TRPM2 showed decreased activation of microglia, which suggested a connection of TRPM2 to activation of microglia in AD. Another study looked at potential mechanisms for TRPM2 activation in cultured mouse microglia cells that were exposed to Aβ42 (Alawieyah Syed Mortadza et al. 2018). Aβ42-induced change in morphology of microglia cells was prevented by treatment with TRPM2 inhibitor (2-APB) and PARP-1 inhibitor (PJ34). By using PJ34, they also showed that PARP-1 activity is necessary for TRPM2 activation. Moreover, in TRPM2 KO Aβ42-induced PARP-1 activation was attenuated. Taken together, these results show evidence of TRPM2 channel having not only a role in pathogenesis of AD but also being important mediating microglial inflammatory responses to Aβ.

In addition, studies in our lab suggest that TRPM2 is an important player in PARP-1 centered microglial pro-inflammatory signalling pathway (Raghunatha et al. 2020). Raghunatha and colleagues have identified a mechanism for microglia sustaining chronic inflammatory responses through involvement of TRPM2 Ca^{2+}-mediated positive feedback. The proposed mechanism is as follows: 1) activation of microglial NMDARs allow Ca^{2+} influx into the cell which in turns stimulates ERK1/2-dependent PARP-1 activation; 2) PARP-1 utilizes NAD+ to generate polymers of ADPRs, which bind to TRPM2 channels ligand binding domain and open the channel; 3) activation of TRPM2 channels allows more influx of Ca^{2+} into the cell. This process creates a feedback loop of chronic PARP-1 activation that leads to microglia sustained pro-

inflammatory responses, overproduction of pro-inflammatory cytokines, decrease anti-inflammatory cytokines and increased NO release.

1.5. Rationale and Objectives

The three hallmarks of AD are accumulation of amyloid β (Aβ), neurofibrillary tangles and neuroinflammation (Cameron and Landreth 2010; Hirokawa and Takemura 1986; Shankar et al. 2008). Aβ oligomers are potent synaptotoxins, that disrupt the function of excitatory glutamatergic transmission and are a major cause of memory loss and cognitive decline in patients with AD (Shankar et al. 2008). Importantly, evidence suggests that aberrant inflammatory processes may play a causal role in promoting cognitive decline as a consequence of their detrimental impact on glutamatergic transmission and synaptic plasticity (Riazi et al. 2015). Microglia are suggested to drive neuroinflammation via elevated production of pro-inflammatory cytokines, such as IL-1, IL-6, TNFα, compromised BBB and infiltration of circulating immune cells and transform themselves and astrocytes into a pro-inflammatory state (Liddelow et al. 2017; Vainchtein and Molofsky 2020; Carson, Thrash, and Walter 2006; Heneka, Kummer, and Latz 2014; Heneka et al. 2018). Moreover, microglia are also suggested to impact glutamatergic transmission (Roumier et al. 2008; Riazi et al. 2015) and synaptic plasticity (Hong, Dissing-Olesen, and Stevens 2016; Sominsky, De Luca, and Spencer 2018). Recent findings supported by human genetic evidence from GWAS studies highlight microglia and immune cells as highly relevant to late-onset AD (Wightman et al. 2020). The evidence above provides a rationale to study microglial regulatory signalling proteins involved in inflammation, and by manipulating their activity, we could potentially lower the risk or slow the progression of AD.

Studies from my lab identified PARP-1 as an important modulator of Aβ-induced microglia pro-inflammatory responses. Inhibition of PARP-1 enzyme in microglial cultures reduced Aβ-induced release of NO and TNFα and prevented neurotoxicity (T. M. Kauppinen et al. 2011). Our unpublished data suggests that microglial PARP-1 mediates synaptotoxic effects of Aβ on synaptic plasticity. In addition, Jackson's lab showed that global deletion of TRPM2 can diminish cognitive decline in mouse model of AD (Ostapchenko et al. 2015). In addition, my lab demonstrates that stimulation of microglial NMDARs can initiate pro-inflammatory responses through a cascade that promotes Ca^{2+}-dependent, self-sustaining activation of the nuclear enzyme PARP-1 (Raghunatha et al. 2020). An important component in this signalling cascade is Ca^{2+} permeable TRPM2 that is activated downstream of PARP-1 by binding of ADPR to C-terminal intracellular ADPR-binding domain on microglial TRPM2 channels (Sumoza-Toledo and Penner 2011). This interaction allows Ca^{2+} influx into the cell. Extracellular influx of Ca^{2+} ions is required for PARP-1 activity (Vuong et al. 2015), which creates a feedback loop for continuous PARP-1 activation. TRPM2 is crucial in maintaining sustained pro-inflammatory responses in Aβ-induced microglia. Thus, it is important to investigate the role of manipulating TRPM2 function on LTP.

Given that inflammation is considered a driving force behind the progression of AD, it is logical to consider inhibition of sustaining PARP-1/TRPM2-induced microglial chronic activation as a potential therapeutic target. My lab has previously shown that stimulation of NMDARs in microglia *in vitro* (e.g. cell culture) induces microglia to adopt a pro-inflammatory phenotype, including morphological transformation and release of pro-inflammatory cytokines through PARP-1 centered signalling cascade (Raghunatha et al 2020). However, the role of microglial NMDARs *in vivo* (e.g. live mice, brain slices) is still unknown. Thus, at first, we wanted to establish the role of microglial NMDARs in synaptic plasticity by using brain hippocampal slices.

For that we can use genetic approach using cre recombination system, which would allow us to specifically delete lox P flanked alleles of NR1 subunit of NMDARs from microglia, that would abolish expression of the receptor. This would allow us to conceptualize how big is the role of microglial NMDARs in mediating synaptotoxicity.

However, for future clinical studies a more feasible approach would be to use pharmacological agents to inhibit the key components of the pathway. Inhibition of PARP-1 excessive activation by using known PARP-1 inhibitors would seem like a good way to start. Studies in stroke show that global deletion of PARP-1 does not have detrimental effects (Eliasson et al. 1997), therefore targeting this enzyme specifically in microglia might not be necessary. However, this approach would not be possible in human population for AD treatment, as PARP-1 specific inhibitors have unwanted detrimental side effects. The brain-permeable PARP-1 inhibitor form a PARP-DNA complexes and become trapped onto the DNA, leading to DNA replication stress and inducing double-stranded brakes (Sinha 2021), and subsequent cell death, particularly in dividing cells.

Modulation of TRPM2 channel function may be a better therapeutic interventions option as it's been shown by the Jackson lab that global deletion of TRPM2 can mitigate cognitive decline, is well-tolerated and does not appear to alter behaviour (Ostapchenko et al. 2015). Adenosine diphosphate ribose (ADPR) is responsible for initiating TRPM2 channel opening upon binding of ADPR to the corresponding domain on TRPM2 itself. ADPR production requires activation of PARP-1 which suggests that TRPM2 can be acting as a part of a positive feedback loop maintaining PARP-1 activation.

Here I propose to investigate the role of microglial NMDARs having a role of initiating the PARP-1/TRPM2 centered signalling cascade in mediating the synaptotoxic effects of Aβ. The proposed

study will define the role of microglial NMDARs and interaction of TRPM2 channels and ADPR metabolism as being a part of a pro-inflammatory feedback loop that is crucial in mediating synaptic plasticity in mice.

1. Hypothesis: *Inhibition of PARP-1/TRPM2 signalling with pharmacological agents or by microglia targeted genetic approach will break the positive pro-inflammatory feedback loop in microglia and prevent the synaptotoxic effects of AβO on synaptic plasticity.*

2. Objectives:

1. To establish the role of microglial NMDARs in mediating the synaptotoxic effects of AβOs on synaptic plasticity using cre lox recombination system (NR1-iKO).
2. To establish the role of TRPM2 channels and ADPR signalling in mediating the synaptotoxic effects of AβOs on synaptic plasticity by using two structurally different inhibitors.

CHAPTER 2: MATERIALS AND METHODS

2.1 Experimental animals

Wild-type CD1 mouse colony male and female were primarily used for electrophysiology experiments. Cre-lox recombination system was used to obtain the NR1-iKO mouse line from JAX stock #005246 (C57BL/6 strain males only) (The Jackson Laboratory 2022b). Microglia specific deletion was obtained by using Fractalkine Cx3r1 Cre Ert2 mouse line (The Jackson Laboratory 2022a). NR1 fl/fl was crossed with $Cx3cr1^{CreERT2(+/-)}$. Cre-deleted allele driven by Cx3cr1 promoter elements. NR1 fl/fl crossed with Cre-/- (Cre-) served as WT control and Cre-/+ served as NR1-iKO. A CreER recombinase consists of Cre fused to a mutated ligand-binding domain (LBD) of the estrogen receptor (ER). In the absence of tamoxifen, CreER is retained in the cytoplasm. Binding of tamoxifen (TAM) or hydroxytamoxifen (OHT) to the LBD results in the translocation of the recombinase into the nucleus, where it can essentially cut out its loxP-tagged (flanked) DNA substrate. Cx3cr1 is expressed also in monocytes (Nishiyori et al. 1998; Jung et al. 2000), so we waited 4 weeks prior to performing TAM injections to allow monocytes to renew and lose the cre recombinase. Microglia on the other hand express Cx3cr1 in progenitor cells, and after their renewal will the presence of cre recombinase will remain. TAM (2mg/mouse) injections were given intraperitoneally for 5 consecutive days with 24h window. NR1-iKO mice were 4-5 months old.

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.

2.2 Production and validation of amyloid-beta oligomers from 7PA2 CHO cell line

Amyloid Precursor Protein (APP) V717-expressing CHO cells (7PA2 cell line) were used to obtain naturally secreted amyloid beta soluble oligomers (AβOs) following previously established protocols. Chinese hamster ovary (CHO) cells and control CHO cells were grown and expanded onto 150 mm culture dishes. When 90% confluency was obtained, cells were harvested 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 min at 200xg at 4°C to remove cell debris. After control CHO conditioned media (CHO-CM) and 7PA2 CHO conditioned media (7PA2-CM) were concentrated, they were frozen and stored at -80°C until needed.

Enzyme-linked immunosorbent assay (ELISA) using the Amyloid-β-42 human ELISA, Ultrasensitive (Life tech) and Human Amyloid-β (1-x) kits were used to measure concentration of Aβ1-42 and total amyloid-β in 7PA2-CM and CHO-CM respectively. Procedures provided with the kits were followed and three dilutions of concentrated 7PA2-CM were tested per kit. CHO-CM did not show any presence of amyloid β.

2.3 Brain slice electrophysiology

2.3.1 Acute brain slice preparation

Hippocampal slices were prepared from CD1 mice with ages ranging from 4-6 weeks, NR1-iKO 5 months old. Following isoflurane anesthesia and decapitation, brains were extracted

and quickly submerged into ice-cold oxygenated (5% CO_2, 95% O_2) standard artificial cerebrospinal fluid (aCSF) (in mmol/L: 124 NaCl, 3 KCl, 1.3 MgCl2, 2.6 CaCl2, 1.25 NaH2PO4, 26 NaHCO3, and 10 D-glucose; osmolarity adjusted to 300-310 mOsM). Hippocampi were then dissected, and transverse sections (350 μm) were prepared using a vibratome.

Slices were allowed to recover at room temperature for 1.5 hours in a submersion holding chamber with well-oxygenated aCSF before placing into a recording chamber. For amyloid-β pre-treatment, as well as pre-treatment with inhibitors, slices were transferred to a 5 ml mini submersion holding chamber containing 7PA2-CM diluted in a well-oxygenated aCSF for 2-3.5 hours and/or the inhibitor.

2.3.2 Field EPSP recordings

Field recordings were performed in a submersion recording chamber continuously super-perfused with oxygenated aCSF at a flow rate of 3.5 ml/min and maintained at room temperature. Field excitatory post synaptic potentials (fEPSPs) were evoked by giving stimuli (100 μs duration) with a concentric bipolar stimulating electrode positioned at the Schaffer collateral pathway and recorded with a glass pipette filled with aCSF (pipette resistance between 2-5mOhms) positioned in the CA1 stratum radiatum (**Figure 2**). Responses were evoked at either 0.05 or 0.1 Hz. To determine the input-output relationship, stimulus intensity was increased gradually starting at a level evoking a minimal field response until either a maximal field response was reached, or a population spike was elicited. Stimulus intensity was then set to a level which elicited 30-40% of the maximum response for subsequent tests. Paired-pulse facilitation (PPF) was measured by giving paired stimuli at intervals of 10, 20, 40, 100, 200, 500, and 1000 ms. PPF was expressed as

the ratio of the peak amplitude of the second response (P2) to that of the first response (P1). For long-term potentiation experiments (LTP), baseline fEPSPs were recorded over 20 minutes before induction by high-frequency stimulation (HFS) consisting of two consecutive 100Hz stimulation trains lasting 1 second, with an inter-train interval of 20 seconds. Responses were then recorded for 60 minutes. Slopes of fEPSPs were analyzed and normalized to baseline.

The field potentials were amplified 100 using Molecular Devices 200B amplifier and digitized with Digidata 1322A. The data were sampled at 10 kHz and filtered at 2 kHz. Traces were obtained by pClamp 9.2 and analyzed using Clampfit 10.7.

The representative schematic cross-section diagram of a hippocampus and a viable hippocampal slice image of fEPSP recording are depicted in figure 2 and figure 3 respectively.

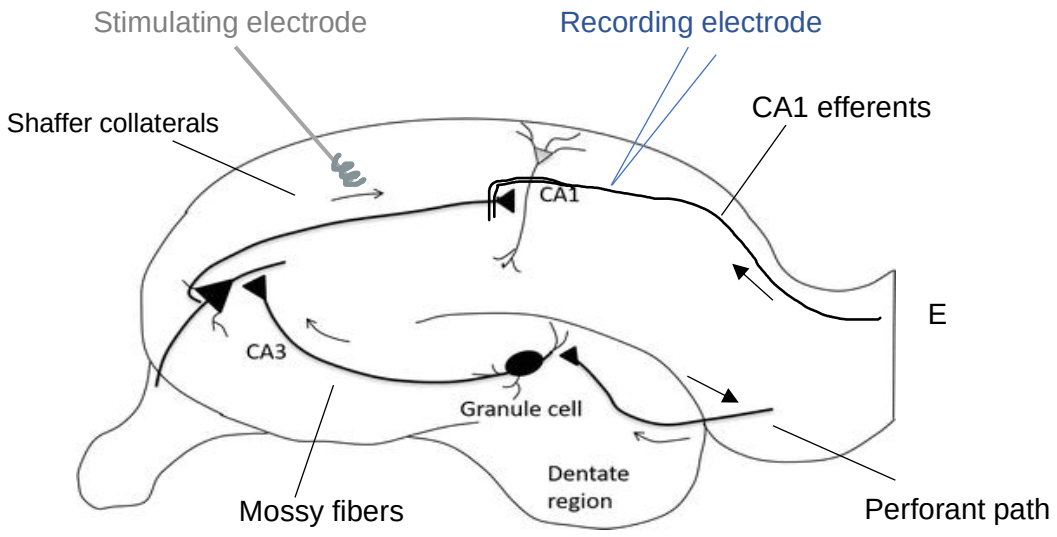

Figure 2: The simplified cross-sectional diagram depicting the tri-synaptic circuit of the hippocampus.

Afferent fibers from layer II neurons of the entorhinal cortex (EC) travel through the perforant path and synapse with the granule cells of the dentate region. Mossy fibers from the dentate region

then project onto the CA3 region of the hippocampus and synapse at the proximal dendrites of the pyramidal neurons. CA3 pyramidal neurons synapse with the CA1 dendritic layer and then CA1 efferents project back onto the entorhinal cortex.

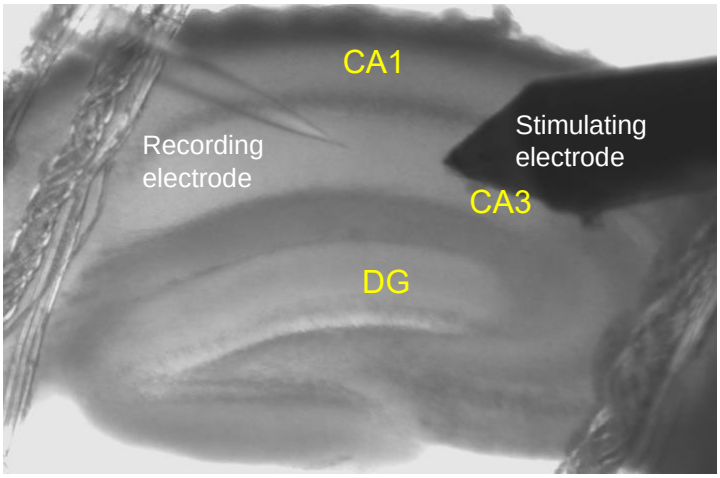

Figure 3: Field EPSP recordings in acute hippocampal slice.

For CA3-CA1 field recordings, the stimulating electrode was positioned at the junction between the CA3 and CA1 regions of the hippocampus to stimulate the Schaffer collateral pathway. The recording electrode was positioned approximately 300 μm away in the stratum radiatum layer of the CA1 region.

2.4 Treatment of hippocampal slices

For electrophysiology experiments, treatments began after slices incubation/recovery period (1.5 h after dissection) by transferring the slices into a 5ml mini holding chamber for 2-3.5h. For experiments where inhibitors were used either 1 μM clotrimazole, 10 μM NUDT5i (TH5427) or 10 μM JNJ-28583113. Slices were treated throughout the incubation period (after dissection) and throughout treatment with AβO and time of recording. For the "inhibitor control" slices, inhibitors were added after the dissection and maintained throughout the incubation period and time of recording.

Throughout this project different batches of AβOs were used with different stock concentrations, ranging from Aβ1-42=1,189.1pg/ml - 891.2pg/ml; Aβ1-x=58.3ng/ml - 89.5ng/ml. Concentrations of total Aβ1-x that were used in most of the experiments for pre-treatment of hippocampal slices to elicit inhibition of long-term potentiation in males were ~ 4.41 ng/ml; Aβ1-42 ~135 pg/ml. The variation can be explained by the nature of the experiment (eg. male vs. female mice) and due to different stock concentration of $A\beta_{1\text{-}x}$ and $A\beta_{1\text{-}42}$.

2.5 Materials

All chemicals used for preparation of aCSF solution used for electrophysiology experiments were purchased from Sigma-Aldrich (Canada). Stock solutions of the drugs used for the treatments in electrophysiology experiments were made in DMSO. Clotrimazole ($C_{22}H_{17}ClN_2$, MW=344.84, catalog number #245-764-8) purchased from Sigma-Aldrich. JNJ-28583113 compound (MW=366.38) was obtained from Dr. Frank Lee, Krembil Research Institute, University of Toronto, Canada; NUDT5 inhibitor (TH5427, MW=550.31) (Page et al. 2018) was obtained from Dr. Brent Page, Faculty of Pharmaceutical Sciences, University of British Columbia, Canada.

2.6 Statistical analysis

Raw data from field recordings were acquired & extracted using pCLAMP Clampex 10.2 software (Molecular Devices), processed and analyzed using pCLAMP Clampfit 10.6 software (Molecular Devices). Statistical analyses were performed using Graphpad Prism 8 (Graphapd

software, CA). Student t-tests were used for comparisons between two groups, with data presented as mean ± standard error of mean. For multiple group comparisons, one-way ANOVA with Turkey's post-hoc test was performed. A p-value of less than 0.05 was considered significant.

2.7 Contributions

Natalie Lavine performed production and validation of amyloid beta oligomers from 7PA2 CHO cell line.

Dali Zhang supervised and helped me during the tamoxifen preparations and IP injections for the NR1 iKO mice.

CHAPTER 3: RESULTS

3.1 7PA2-CM contains amyloid β oligomers (AβOs)

To study the neurotoxic effects of amyloid-β oligomers (AβOs), some groups are using synthetically derived $A\beta_{1-42}$ or $A\beta_{1-40}$ peptides. To facilitate their oligomerization, the peptides are dissolved in non-physiological solvents and administered in micromolar ranges. However, many groups are using "naturally secreted" AβOs from cell lines that mimics the human complement (Selkoe 2008). The concentrations that are used for the administration of naturally secreted AβOs is at picomolar, which is close to physiological concentrations. They have been shown to disrupt hippocampal long-term potentiation (LTP) and induce memory impairment and behavioral deficits (Selkoe 2008). The cell line that is used is called 7PA2 CHO cell line, that expresses human APP variant V717F and secretes a mix of amyloid- β that oligomerize in the culture media (Shankar et al. 2011).

In our lab, media from control CHO cells (CHO-CM) and 7PA2 CHO cells (7PA2-CM) is collected and concentrated down to ~1/30 of the original volume. The concentrated media is stored as frozen aliquots in -80°C until needed for experiments. The presence of AβOs in 7PA2-CM is confirmed using ELISA. As expected, AβOs were not detected in CHO-CM. Detection of AβOs in 7PA2-CM was confirmed by western blot (**Figure 4**). In addition to $A\beta_{1-42,}$ highly prevalent c-terminally truncated forms of Aβ or varying length ($A\beta_{1-x}$) can be detected using a separate ELISA.

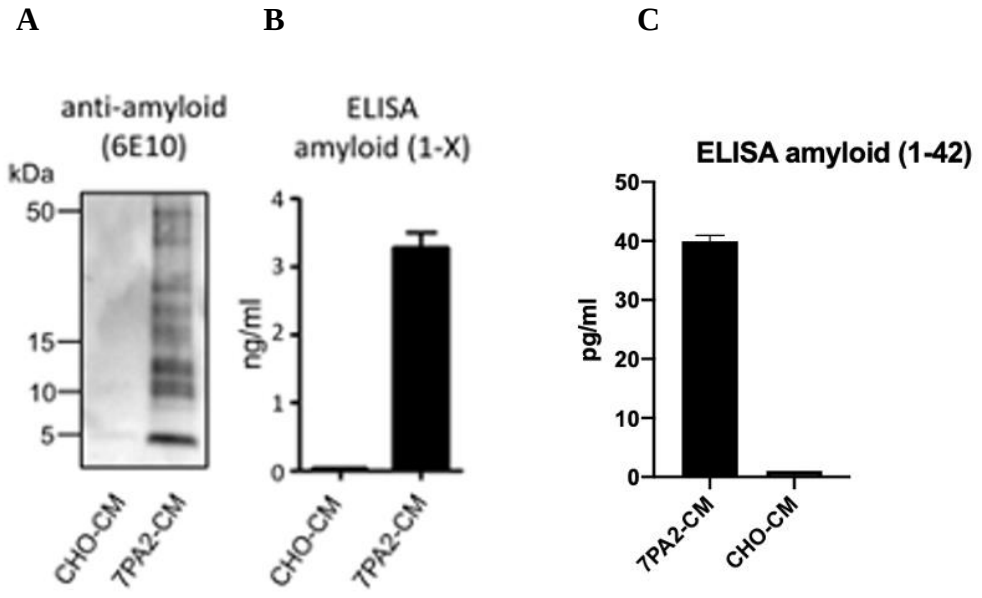

Figure 4: 7PA2 conditioned medium (CM), but not CHO-CM, contains AβOs with molecular weights ~8-50 kDa. AβOs (1-X) and (1-42) are detected in 7PA2-CM, but not CHO-CM.

7PA2 cells: cell line from Chinese hamster ovary (CHO) cells that stably express human APP 751 bearing the V717F familial AD mutation, secrete significant amounts of Aβ proteins into their medium. **(A)** – Representative immunoblot is used to detect AβOs using pan anti-Aβ antibody (6E10 antibody - binds to amino acid residues 1-16 of beta amyloid). Monomers, low-n oligomers, and higher order oligomers could be detected. **(B, C)** – Separate ELISA assays are used to detect amyloid 1-x (**B**) or amyloid 1-42 (**C**) in conditioned medium of 7PA2 cells but not in CHO-CM (Welzel et al. 2014).

3.2. The role of microglial NMDA receptors in mediating the synaptotoxic effects of AβOs

To establish the role of microglia in synaptic plasticity we used mice that were lacking microglial NMDA receptors. I used both WT and NR1-iKO male mice in my experiments. We first wanted to confirm the synaptotoxic effects of 7PA2-CM AβOs purified by us in hippocampal slices in these mice. AβOs are known to cause deficits of synaptic plasticity at CA3-CA1 synapses of hippocampal slices by inhibiting long-term potentiation (LTP) (Selkoe 2008) while facilitating

long-term depression (LTD) (Li et al. 2009). LTP is a process by which synaptic connections between neurons become stronger following frequent stimulation. In response to experience our brain changes and LTP is thought to be the mechanism underlying learning and memory (McNaughton and Morris 1987). Since accumulation of AβOs inhibits LTP (Shankar et al. 2008), AβO synaptotoxicity can be viewed as a direct link impacting learning and memory.

To study molecular processes underlying learning and memory the model of tri-synaptic hippocampal circuit is used (**Figure 2**). Major inputs from the entorhinal cortex first synapse onto neurons in the dental gyrus via the perforant fiber pathway and provide a critical input to CA3 mossy fiber pathway, then to CA1 by means of the Schaffer collateral pathway. The major output is via CA1 region to the subiculum (which is the most inferior part of the hippocampal formation) (C. Lu and Malenka 2012). LTP can be evoked by repetitive high-frequency stimulations (HFS) at the Schaffer collateral pathway at the CA3-CA1 synapse and leads to long-lasting potentiation of synaptic transmission at CA1 neurons. (Levy & Steward, 1979).

For my experiments, field excitatory synaptic potentials (fEPSPs) were evoked at a frequency of 0.05Hz by electrical stimulation delivered to the Schaffer-collateral pathway using a concentric bipolar stimulating electrode (25 μm exposed tip) and recorded using glass microelectrodes (3–5 MΩ filled with aCSF) positioned in the stratum radiatum layer of the CA1 subfield. Peak amplitudes and slopes were recorded and measured from acute hippocampal slices (350 μm) prepared from 5 months old WT and NR1-iKO. Electrode depth was varied until a maximal response was elicited (approximately 175 μm from surface). Measures of excitability, short-term facilitation and LTP from control slices were compared with the slices pre-treated with AβOs (Aβ1-42=83.8 pg/ml; Aβ1-x=4.41 ng/ml) for 2-3.5 hours.

To start, I pre-treated the slices from both WT and NR1-iKO mice with AβO and assessed if there was an effect on baseline transmission, short-term plasticity and LTP. The results from these experiments are below.

3.2.1. Baseline excitability is unchanged with AβO treatment in NR1 WT and NR1-iKO hippocampal slices from males

We began our experiments by examining whether pre-treatment with AβO would cause changes in the baseline excitability by analyzing the input/output relation at the CA3-CA1 synapse in NR1-iKO vs. NR1-WT (**Figure 5**). To do so, I measured the fEPSP amplitude (output) vs fiber volley amplitude (input) and plotted this relation in a scatter plot. From each separate fEPSP amplitude relation vs fiber volley amplitude, linear regression was used to derive a corresponding slope value (representative example shown in **Figure 5 (B)**, used as a measure of synaptic transmission efficacy. Pre-treatment with AβO did not cause any changes in input-output in NR1-WT (Ctrl: 2.278 ± 0.3910 n = 2 vs. AβO: 1.886 ± 0.3709 n = 4, p=0.9934) or NR1-iKO (Ctrl: 2.544 ± 0.6428 n = 7 vs. AβO: 3.187 ± 0.9492 n = 6, p=0.9063) suggesting that deletion of NR1 subunit of NMDAR does not influence basal excitability.

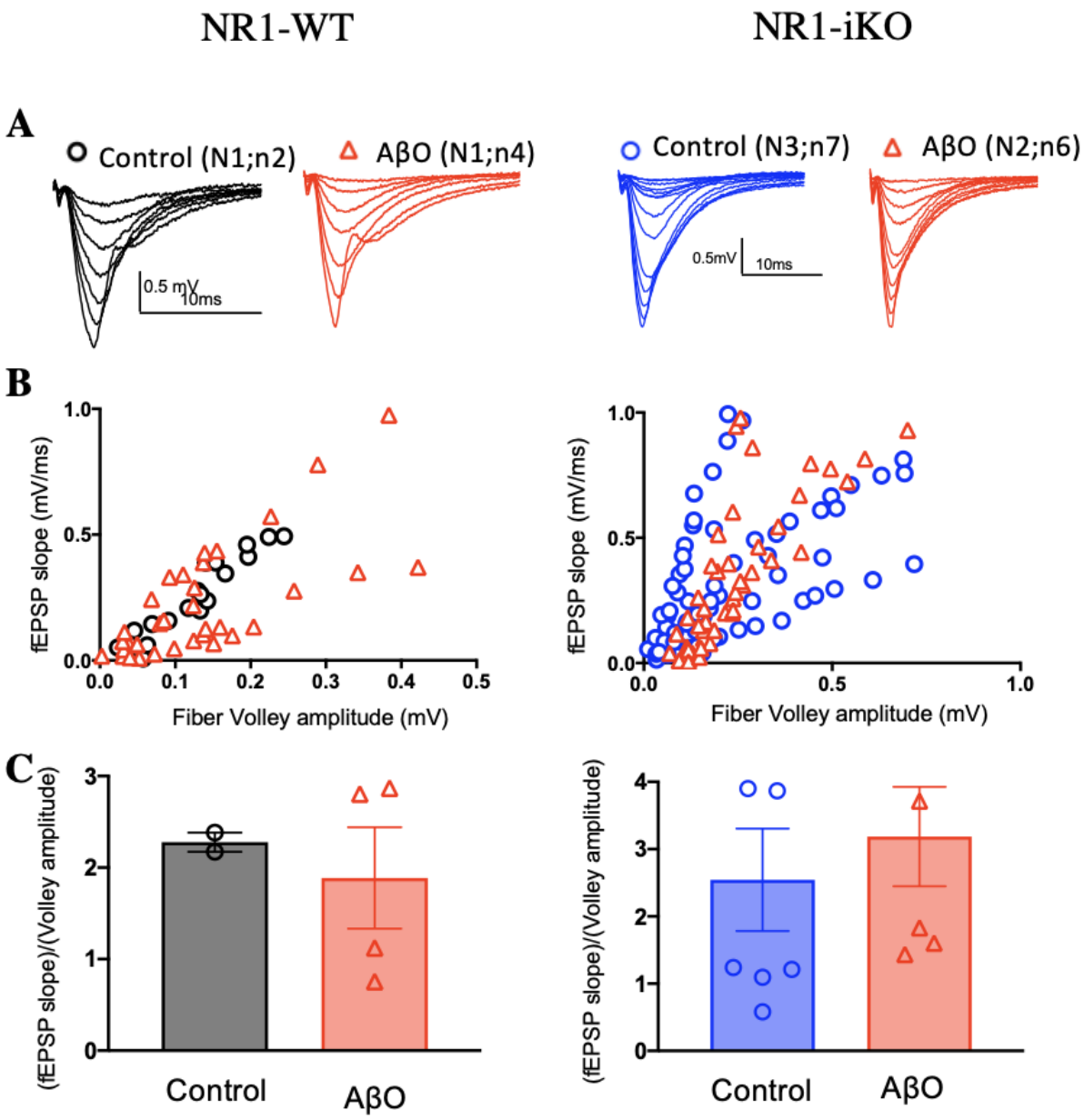

Figure 5:Analysis of input/output relationship in NR1- WT and iKO ctrl vs. AβO-treated male slices.

Representative traces of fEPSPs (output) evoked with increasing stimulus intensities (input) in male NR1-WT and iKO slices Ctrl vs. AβO-treated **(A). (B)** – fEPSP amplitude is plotted against fiber volley amplitude scatter plot summarizing the input-output relationship for all slices recorded from both animal groups. From each separate fEPSP amplitude vs fiber volley amplitude relation, linear regression was used to derive a corresponding slope value, used as a measure of synaptic transmission efficacy in each slice. The mean ± SEM of these values is plotted in **Figure 5 (C)**.

There was no difference found between control and AβO-treated from NR1 WT and iKO male slices (p=0.6969) (ns, two-way ANOVA).

3.2.2. Paired-pulse facilitation is unchanged with AβO pre-treatment of hippocampal slices from NR1- WT and iKO male mice

Next, we wanted to assess PPF and determine if NR1 iKO would have an effect in Ctrl slices and in response to pre-treatment with AβO (**Figure 6**). Paired stimuli applied with short intervals (10-1000ms) caused a temporary facilitation of the second fEPSP response. PPF value reported at 20 ms ISI. PPF was not different between NR1-WT (Ctrl: 1.107 ± 0.7168 n = 2 vs. AβO: 1.179 ± 0.1103 n = 4) and iKO (Ctrl: 1.248 ± 0.1284 n = 7 vs. AβO: 1.376 ± 0.1269 n = 6) male hippocampal slices $p>0.05$, $p=0.5302$ as analysed by two-way ANOVA.

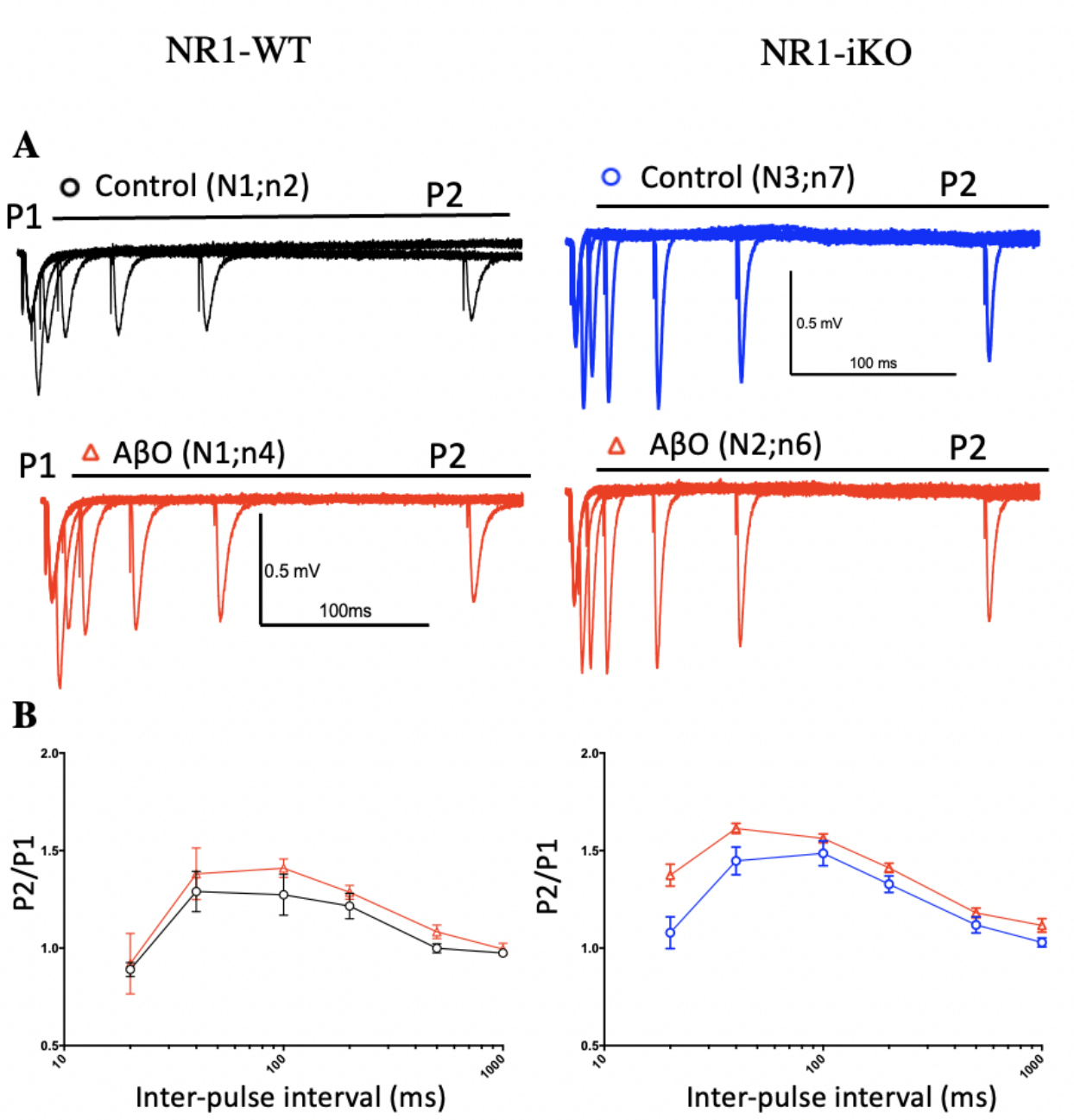

Figure 6: Paired-pulse facilitation is not affected in NR1-WT and iKO mice from Ctrl vs. AβO-treated slices.

(**A**) – Representative traces of paired fEPSPs evoked with stimulus intervals ranging from 10 ms to 1000 ms in male slices, untreated (control) or pre-treated with AβO from NR1-WT or NR1-iKO mice. Compared to the first evoked fEPSP (P1), there is a facilitation of the second evoked fEPSP (P2) in each paired pulse, dependent on their inter-stimulus interval. (**B**) – Paired-pulse facilitation represented as a ratio of the amplitude of the second fEPSP and the first fEPSP (P2/P1) across each inter-stimulus interval tested. No difference in paired-pulse ratio between control and AβO

pre-treatment was detected in male slices from NR1-WT and NR1-iKO mice (ns, $p>0.05$, two-way ANOVA).

3.2.3. Pre-treatment with AβOs did not inhibit LTP in hippocampal slices from NR1-iKO male mice

Treatment with AβOs was performed as described previously (section). We wanted to see if in NR1-iKO mice pre-treatment with AβOs would prevent inhibition of LTP in CA3-CA1 region in hippocampus (**Figure 7**). I observed significant difference in baseline and LTP in hippocampal slices from NR1 WT (Ctrl: 182.90 ± 16.10 n = 2 vs AβO: 129.86 ± 2.70 n = 4, $p<0.05$, $p=0.0076$) vs. NR1-iKO (Ctrl: 176.01 ± 23.98 n = 7 vs. AβO: 142.61 ± 32.73 n = 6), $p>0.05$, $p=0.897$) that showed no difference between Ctrl vs. AβO-treated slices.

These results solidify our hypothesis of that microglial NMDAR may be the initiator of the PARP-1/TRPM2/ADPR pro-inflammatory pathway that is important in mediating the synaptotoxic effects of AβO on synaptic plasticity.

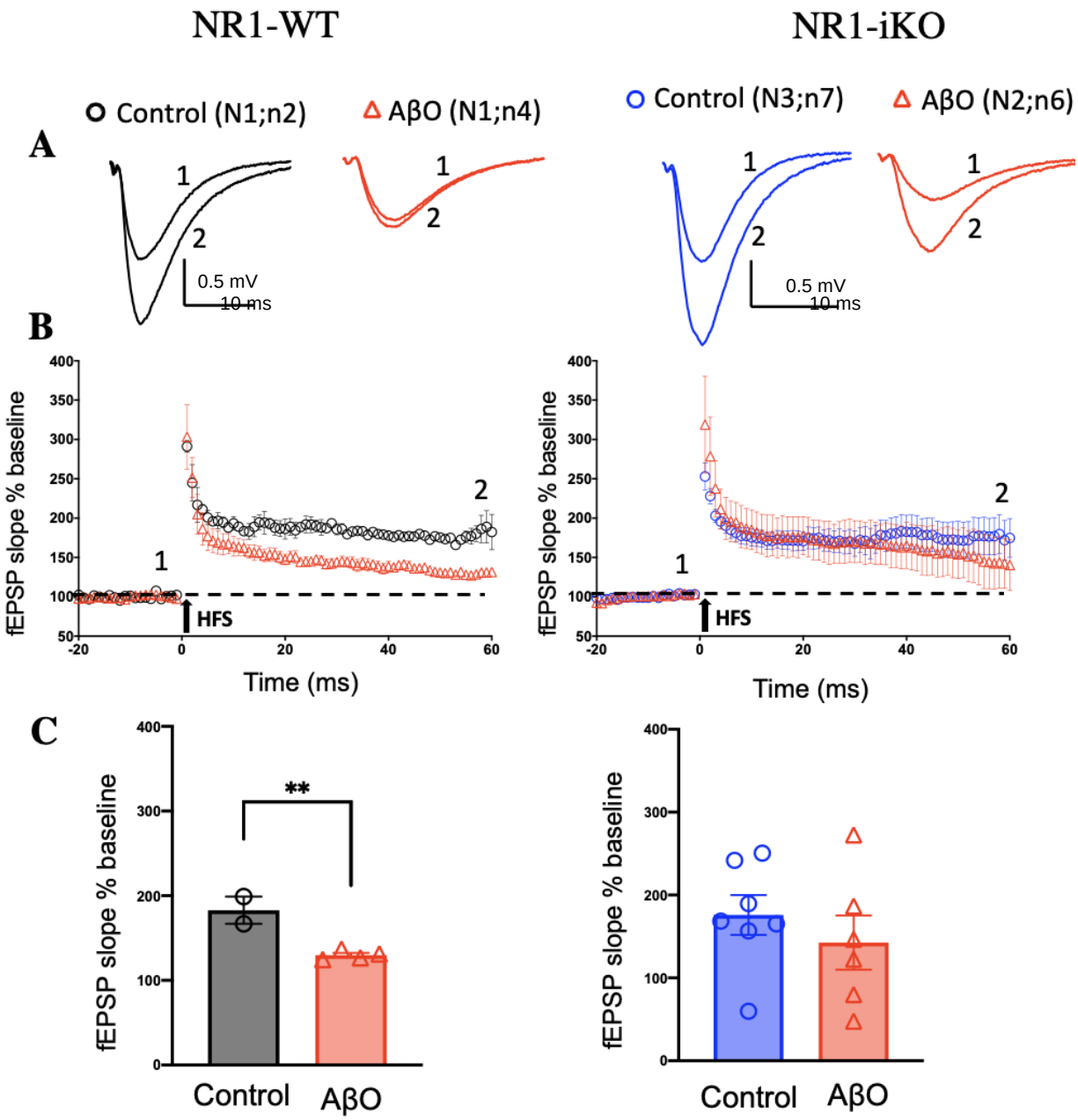

Figure 7: NR1-iKO prevents the inhibition of LTP by AβO in hippocampal slices from male mice.

(A) – Representative traces of fEPSPs at baseline before tetanus (1) and 60 minutes post-tetanus (2) in male slices Ctrl vs. treated with AβO. **(B)** – Plot of averaged fEPSP slopes as a percentage of baseline during each minute of recording with representative traces of fEPSPs at baseline before tetanus (1) and 60 minutes post-tetanus (2) in NR1-WT and iKO male untreated (control) slices or pre-treated with AβO. **(C) –** Averaged fEPSP slopes from the last 5 minutes of each recording. There was a significant reduction in LTP in NR1-WT (Ctrl: 182.90 ± 16.10 n = 2 vs Aβ: 129.86 ±

2.70 n = 4, p<0.05, p=0.0076) slices. However, there was no reduction in fEPSP slope in AβO-treated slices from NR1-iKO compared to Ctrl slices (ctrl: 176.01 ± 23.98 n = 7 vs. Aβ: 142.61 ± 32.73 n = 6) (ns, p>0.05, one-way ANOVA).

3.3. Synaptic plasticity deficits at CA3-CA1 excitatory synapses induced by AβO pre-treatment of CD1 male hippocampal slices

After successfully validating the importance of microglial NMDA receptors in synaptic plasticity after AβO challenge in males, we wanted to assess the role of TRPM2 channels in maintaining the pro-inflammatory feedback loop resulting in continuous microglia activation. Targeting microglial NMDARs in a clinical setting would not be feasible, thus it is crucial to find a molecular agent, that we could target therapeutically. Inhibition of TRPM2 channels have been shown to be beneficial in stroke studies preferentially in males (Jia et al. 2011); as well as their global deletion ameliorates cognitive decline in male mice with AD (Ostapchenko et al. 2015). TRPM2 channels have a critical role in microglial activation and production of pro-inflammatory cytokines (Alawieyah et al. 2018). Thus, we decided to test if inhibition of TRPM2 channels would prevent the effect of AβO pre-treatment on LTP.

As with NR1-iKO and WT mice I used the same protocol explained in section 3.2 and started my experiments with AβO pre-treatment in 4–6-week-old CD1 males, followed by using clotrimazole – TRPM2 inhibitor.

3.3.1. Baseline excitability (input/output) is unchanged with AβO pre-treatment of hippocampal slices from male mice

We began our experiments by examining whether pre-treatment with AβOs would cause any changes in the baseline excitability by analyzing the input/output relation at the CA3-CA1

synapse (**Figure 8**). To do so, I measured the fEPSP amplitude (output) vs fiber volley amplitude (input) and plotted this relation in a scatter plot. From each separate fEPSP amplitude vs fiber volley amplitude relation, linear regression was used to derive a corresponding slope value (representative example shown in **Figure 8C**), used as a measure of synaptic transmission efficacy. Pre-treatment with AβOs did not cause any change in input-output when compared to Ctrl slices (1.450 ± 0.189 n=20 ctrl vs. 1.692 ± 0.239 n=18 Aβ, p=0.4376), suggesting that pre-treatment with AβOs does not cause changes in basal excitability.

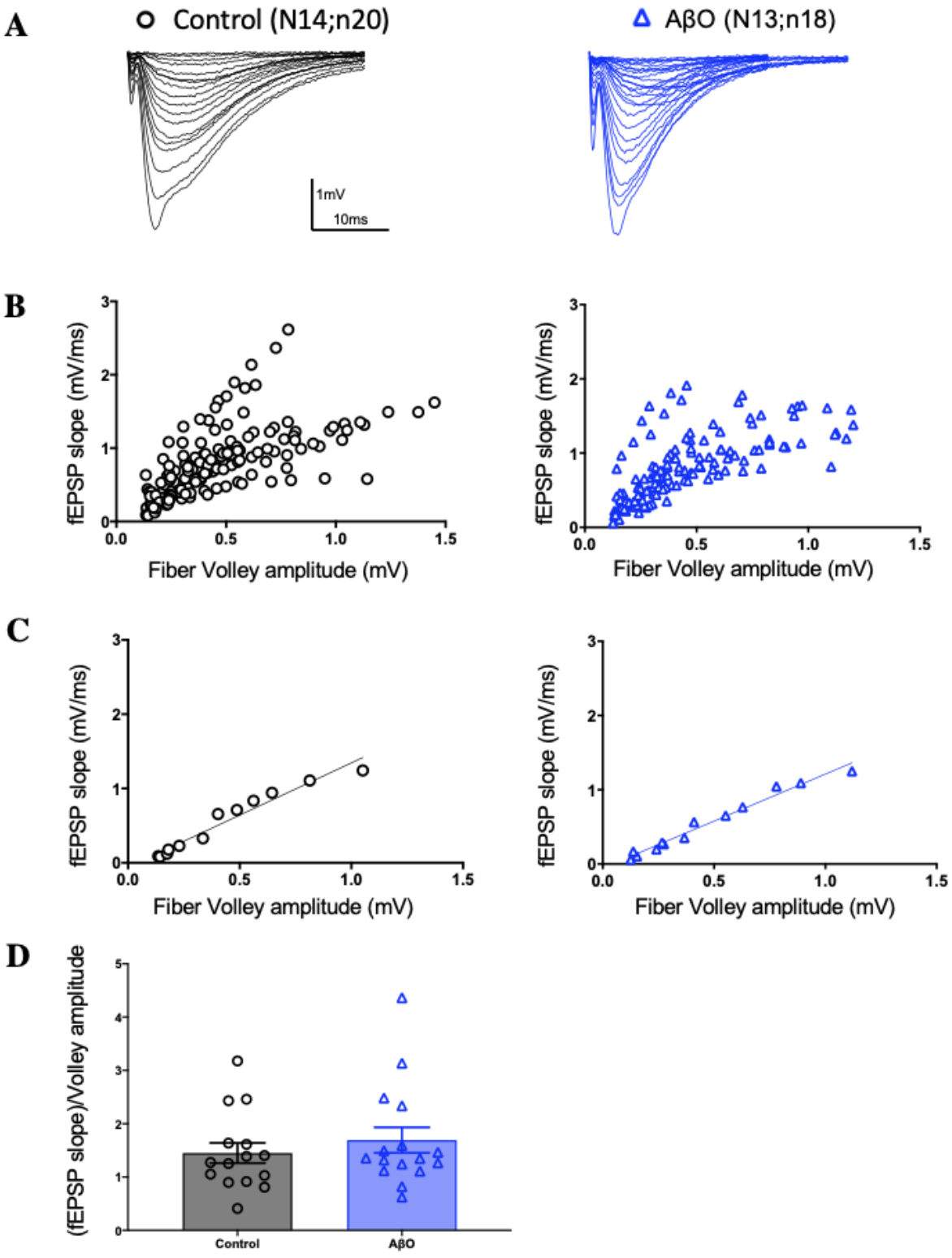

Figure 8: Analysis of input/output relationship in ctrl vs. AβO-treated hippocampal slices derived from males.

(A) – Representative traces of fEPSPs (output) evoked with increasing stimulus intensities (input) in ctrl slices or slices pre-treated with AβOs. **(B)** – fEPSP amplitude is plotted against fiber volley amplitude as a scatter plot summarizing the input-output relationship for all slices recorded from both animal groups. From each separate fEPSP amplitude vs fiber volley amplitude relation, linear regression was used to derive a corresponding slope value – **(C)**, used as a measure of synaptic transmission efficacy in each slice. The mean ± SEM of these values is plotted in **Figure 8 (D)**. There was no significant difference found between Ctrl (N=14; n=20) and AβO-treated (N=13; n=18) slices. Numbers in brackets denote (N - # of animals, n - # of slices) (ns, unpaired two-tailed student t-test).

3.3.2. Paired-pulse facilitation is unchanged with AβO pre-treatment of hippocampal slices from male mice

Next, we assessed a form of short-term plasticity called paired-pulse facilitation (PPF) to determine if pre-treatment with AβOs would cause any change (**Figure 9**). Paired stimuli applied with short intervals (10-1000 ms) leads to a temporary facilitation in the second fEPSP response. The reason for this is the accumulation of residual pre-synaptic calcium that would result in higher amounts of excitatory neurotransmitter release. PPF value reported at 20 ms ISI. PPF was not different between control and AβO-treated hippocampal slices in males ($p=0.2169$) as analyzed by unpaired two-tailed t-test. This suggests that pre-treatment with AβOs does not alter short-term plasticity and is in line with previous studies that have also demonstrated the lack of an effect of acute AβO treatment on slice excitability and PPF (Townsend et al. 2006).

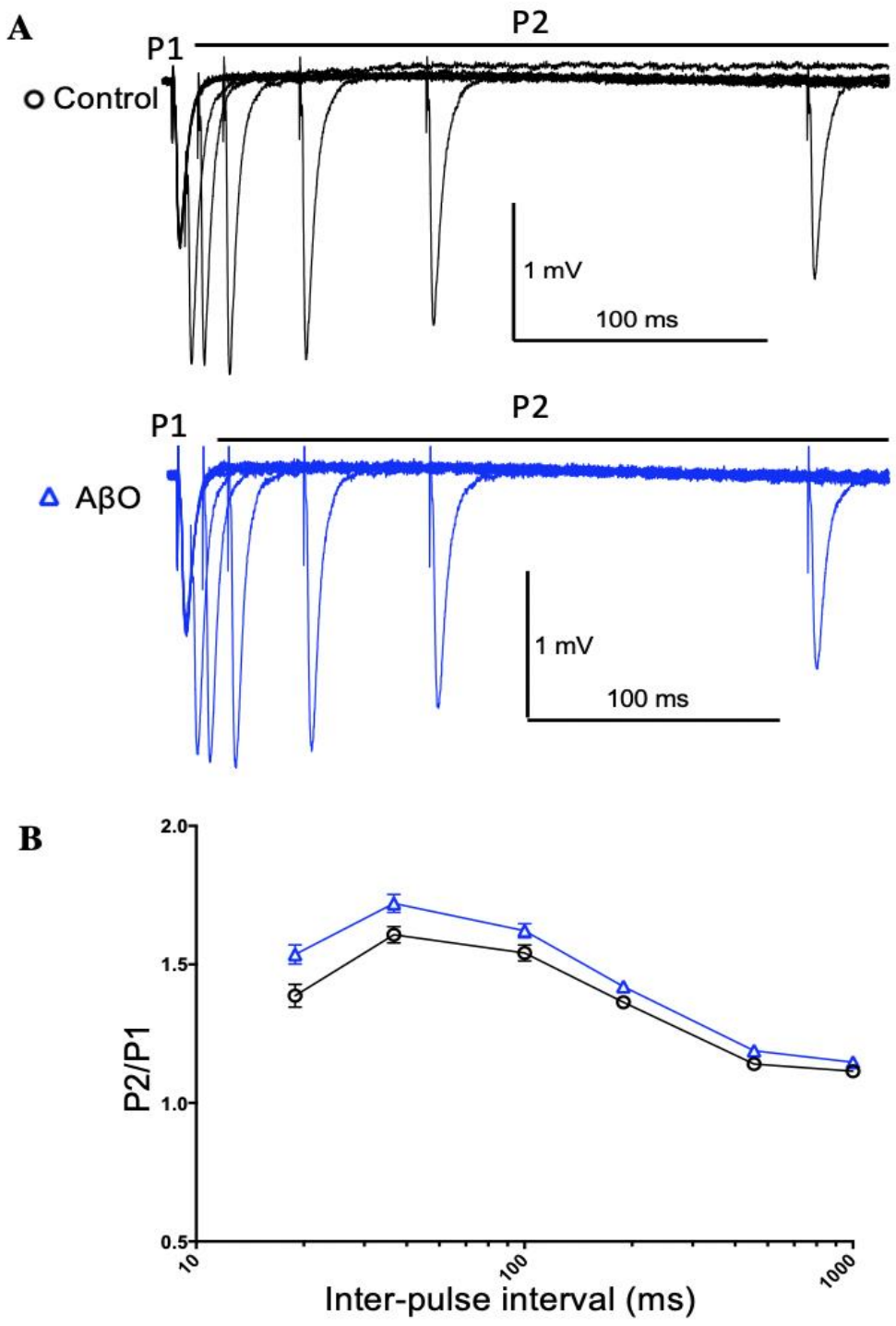

Figure 9: Paired-pulse facilitation is unchanged by AβO pre-treatment in hippocampal slices from male mice.

(A) – Representative traces of paired fEPSPs evoked with multiple stimulus intervals ranging from 10 ms to 1000 ms in untreated (control) slices (n=17) or pre-treated with AβOs (n=21). Compared

to first evoked fEPSP (P1), there is a facilitation of the second evoked fEPSP (P2) in each paired pulse dependent on their inter-stimulus interval. **(B)** – Paired-pulse facilitation represented as a ratio between the amplitude of the second fEPSP and the first fEPSP (P2/P1) across each inter-stimulus interval tested. No difference was detected in paired-pulse ratio between control (n=17) and AβO treated (n=21) slices (ns, p=0.5390, unpaired two-tailed student t-test).

3.3.3. Pre-treatment of hippocampal slices with AβOs reduced LTP in male mice

We next wanted to test whether pre-treatment with AβOs would cause any deficits in LTP induced by high-frequency stimulation. First, baseline fEPSPs were recorded for 20 min before induction of LTP by HFS (2 x 100Hz train lasting 1 sec, separated by 20 sec). Then fEPSPs were recorded for an additional 60 min (**Figure 10**). In control slices application of HFS caused the stable potentiation of fEPSP responses over 60 minutes; the average fEPSP slope from the last 5 minutes of these recordings was roughly 200% of baseline. As expected, pre-treatment of slices with AβOs had no effect on baseline field potential recordings in the absence of a HFS, demonstrating that basal synaptic transmission is unaffected by AβOs. However, pre-treatment of slices with AβOs caused a deficit in LTP (60% reduction). While there was an initial increase in fEPSP responses, the potentiation reduced throughout the 60 minutes of recording and the extent of potentiation over the final 5 minutes of recording was reduced compared to Ctrl (Ctrl: 203.41 ± 10.31 n=20 vs AβO: 141.95 ± 8.28 n=18, $p < 0.05$).

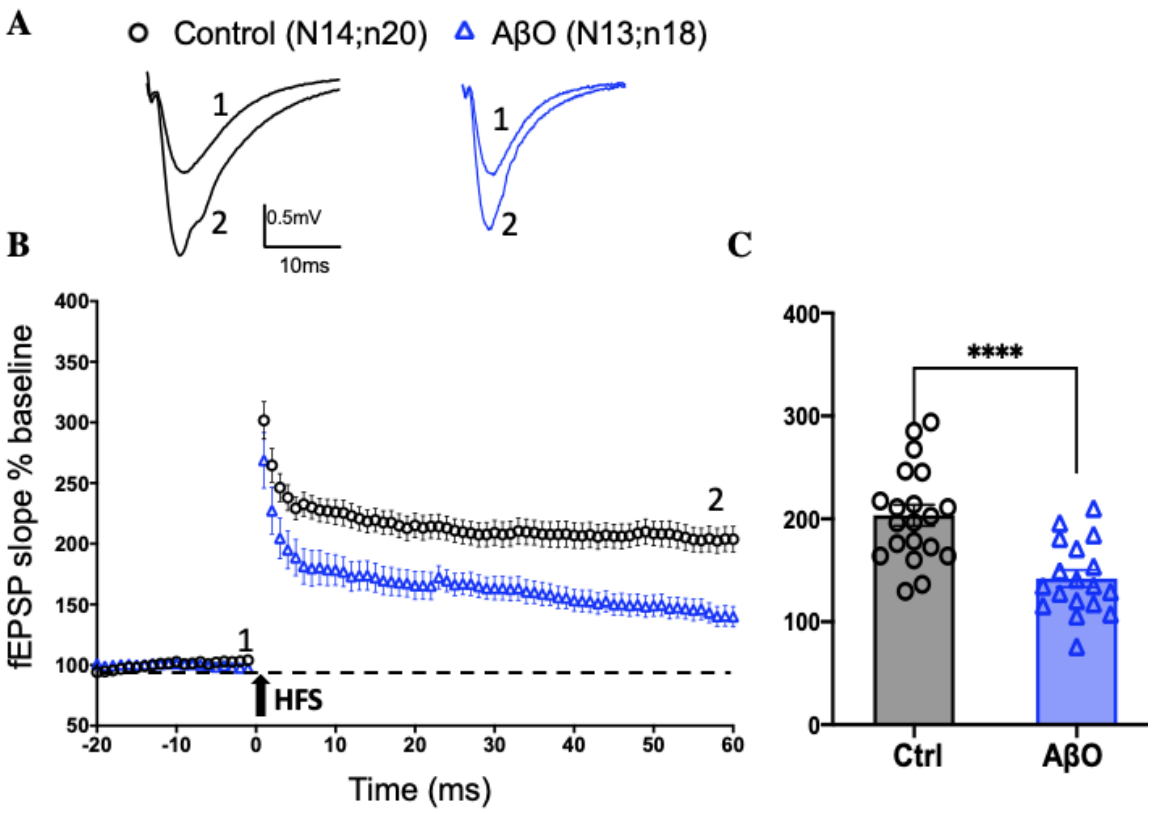

Figure 10: Pre-treatment of hippocampal slices with AβOs causes LTP deficits in male slices.

(A) – Representative traces of fEPSP at baseline before tetanus (1) and 60 minutes post-tetanus (2) in untreated (control) slices (n = 20) or pre-treated with AβOs (n = 18). **(B) –** Plot of averaged fEPSP slopes as a percentage of baseline during each minute of recording. **(C) –** Averaged fEPSP slopes from the last 5 minutes of each recording. There was a 60% reduction in fEPSP slope in AβO-treated slices compared to control. (****$p < 0.0001$, unpaired two-tailed student t-test).

3.3.4. Clotrimazole prevents inhibition of LTP induced by AβO in hippocampal slices from male mice

We wanted to test whether TRPM2 channels play a role in mediating the synaptotoxic effects of AβOs. To test this, we utilized a TRPM2 channel inhibitor, 1 μM clotrimazole (CTZ), hypothesizing that it could prevent the synaptotoxic effects of AβOs on synaptic plasticity. Clotrimazole (1μM) was added right after dissection of the brain and remained in the holding chamber for the duration of slices recovery (1.5h), then together with added AβO in a 5ml chamber (2-3.5h), as well as in the perfusion solution throughout the time of recordings. The concentration of AβO used was Aβ 1-x= 4.41 ng/ml for males and Aβ1-x= 6.61 ng/ml for females.

In slices from each treatment condition, baseline fEPSPs were recorded for 20 min before induction of LTP by HFS. fEPSPs were then recorded for an additional 60 min (**Figure 11**). In control slices from males, application of HFS reliably induced LTP which was maintained for 60 minutes. The average fEPSP slopes from the last 5 minutes of these recordings was roughly 165% in males (161.84 ± 14.9 n = 5). Pre-treatment with AβOs of male slices caused deficits in LTP compared to control (**Figure 11**) (Aβ 129.13 ± 5.33 n = 10 vs. Ctrl 161.84 ± 14.9 n = 16, p=0.0312). In contrast, pre-treatment of slices with CTZ completely prevented AβO-induced deficits in long-term potentiation in males (Aβ 161.84 ± 14.9 n = 13 vs. Ctrl 171.45 8.86 n = 5, p>0.05). This result demonstrates that CTZ can prevent the synaptotoxic effects of AβOs in males.

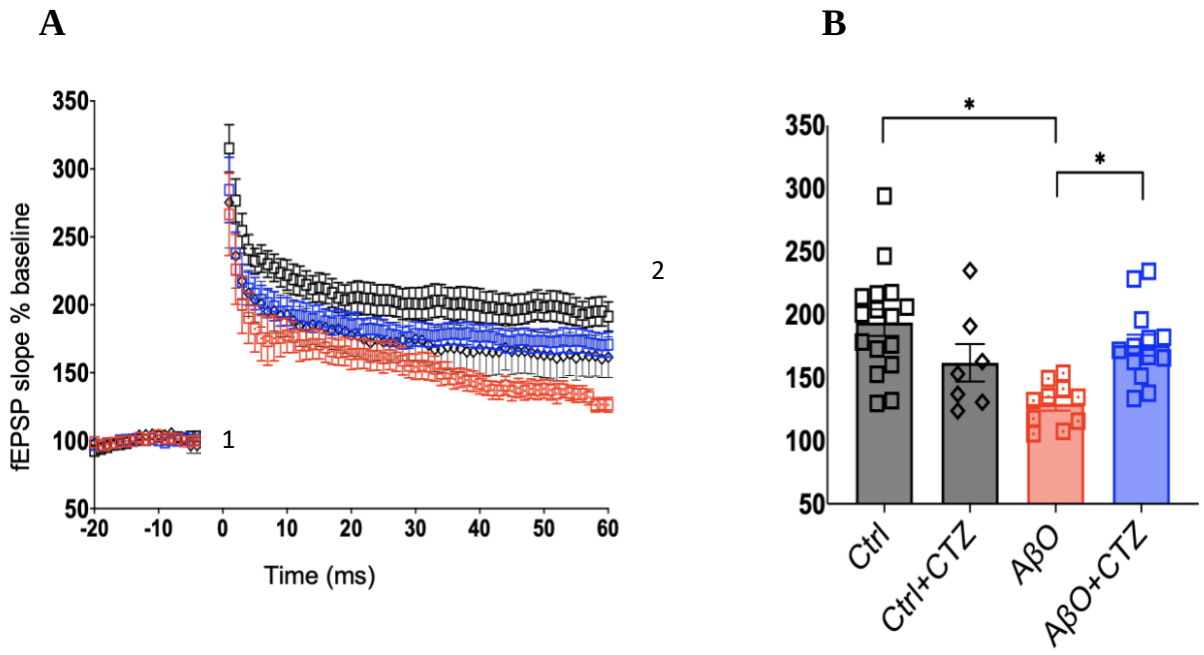

Figure 11: Clotrimazole prevents the inhibition of LTP by AβOs in hippocampal slices from male mice.

(A) – Plot of averaged fEPSP slopes as a percentage of baseline during each minute of recording of fEPSPs at baseline before tetanus (1) and 60 minutes post-tetanus (2) in male slices control, control with CTZ (n = 5), pre-treated with AβO (n = 10) or with CTZ+AβOs (n = 13). **(B) –** Averaged fEPSP slopes from the last 5 minutes of each recording. There was no reduction in fEPSP slope in CTZ+AβOs treated slices vs. ctrl+CTZ from male slices (ns, p>0.05, one-way ANOVA).

3.4. Synaptic plasticity deficits at the CA3-CA1 synapse induced by AβO pre-treatment in female hippocampal slices

As outlined previously (intro section 1.1.2.), research shows that there are sex differences in AD incidence and disease progression. This suggests that we might need to consider different therapeutical approaches to halt the disease progression in males vs. females (Ferretti et al. 2018). Of note, past work suggests that treatment outcomes when targeting PARP-1 or TRPM2 to achieve neuroprotection are sex-dependent. Specifically, pharmacological inhibition or genetic deletion of

PARP-1 (Siegel and McCullough 2013; F. Liu et al. 2011; McCullough et al. 2005) or TRPM2 (Alim et al. 2013; Jia et al. 2011) are reported to be beneficial in males only. This provides a strong rationale for studying the sex-dependent actions of PARP-1 or TRPM2 inhibition in AD models. Therefore, after conducting experiments in males, and seeing promising results from inhibition of TRPM2 we next wanted to assess how would females respond. As with the male cohort we started by assessing the effect of AβO pre-treatment starting with the same concentrations used in males (Aβ1-42=83.8 pg/ml; Aβ1-x=4.41 ng/ml) on baseline transmission, short-term facilitation, and LTP.

3.4.1. Baseline excitability (input/output) is unchanged with AβO pre-treatment of hippocampal slices from female mice

As with the male cohort (section 3.3.1), I started by examining whether pre-treatment with AβOs would affect the baseline excitability by analyzing the input/output relation at the CA3-CA1 synapse (**Figure 12**). Pre-treatment with AβOs did not cause any change in input-output relation in females (1.657 ± 0.3472 n = 12 ctrl vs. 1.346 ± 0.3110 n = 9 AβO, p=0.3816).

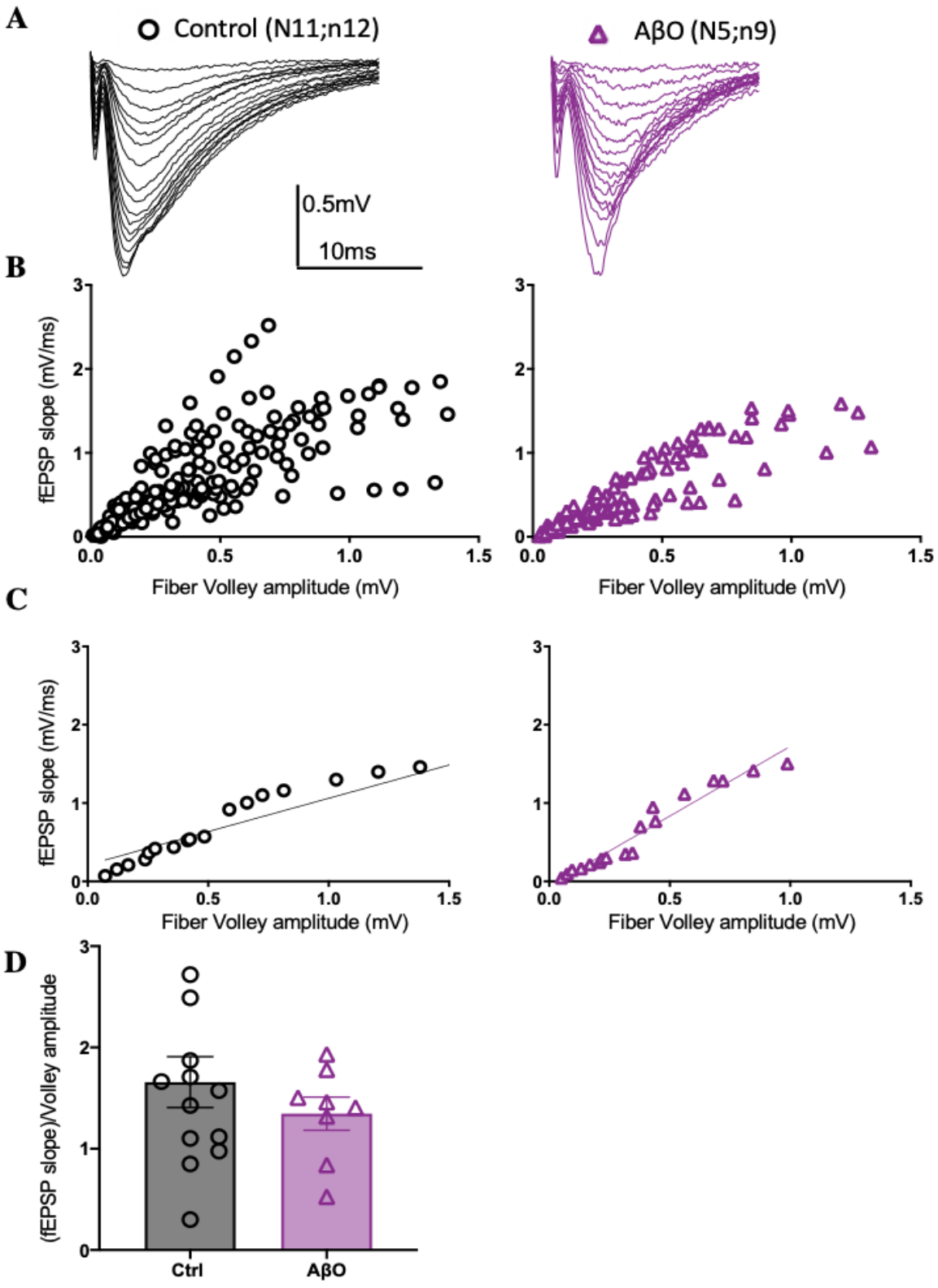

Figure 12: Analysis of input/output relationship in ctrl vs AβO-treated slices derived from females.

(**A) –** Representative traces of fEPSPs (output) evoked with increasing stimulus intensities (input) in slices pre-treated with or without AβOs. (**B) –** fEPSP amplitude is plotted against fiber volley amplitude as a scatter plot summarizing the input-output relationship for all slices recorded from both animal groups. From each separate fEPSP slope vs fiber volley amplitude relation, linear regression was used to derive a corresponding slope value (representative example shown in **Figure 12 (C)**, used as a measure of synaptic transmission efficacy in each slice. The mean ± SEM of these values is plotted in **Figure 12 (D)**. There was no difference in input-output slope between ctrl (n = 12) and AβOs-treated (n = 9) slices (p=03816, ns, unpaired two-tailed student t-test).

3.4.2. Paired-pulse facilitation is unchanged with AβO pre-treatment of hippocampal slices from females

As with males, we wanted to assess PPF and determine if pre-treatment with AβOs would have an effect on short-term plasticity in females (**Figure 13**). As in males, paired stimuli applied with short intervals (10-1000ms) caused a temporary facilitation of the second fEPSP response. PPF value reported at 20 ms ISI. PPF was not different between control and AβO-treated hippocampal slices in females (1.372 ± 0.085 n = 12 ctrl vs 1.467 ± 0.105 n = 9 AβO, p=0.4978) as analysed by student t-test.

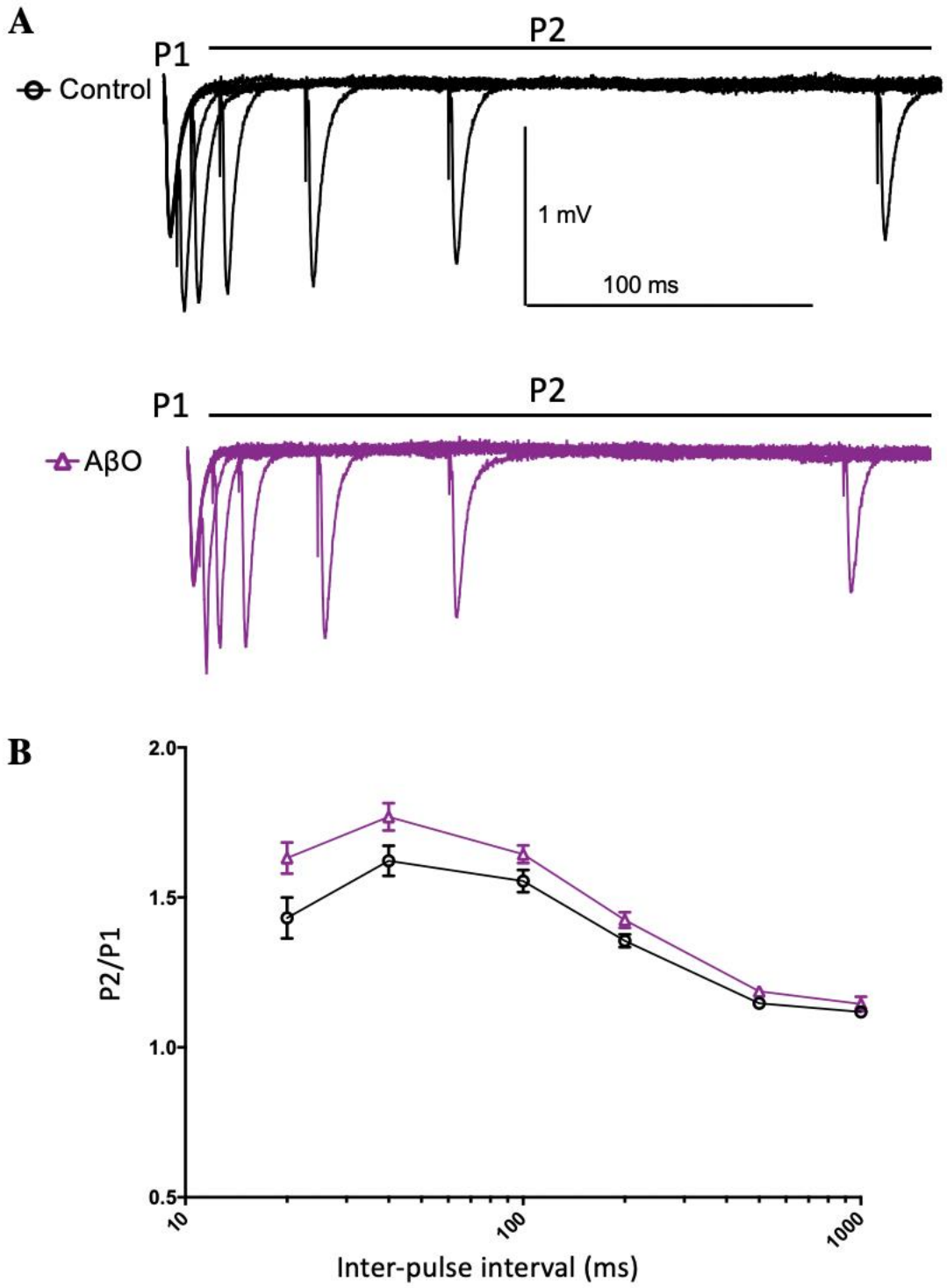

Figure 13: Paired-pulse facilitation is not affected by AβO pre-treatment of female hippocampal slices.

(A) – Representative traces of paired fEPSPs evoked with stimulus intervals ranging from 10 ms to 1000 ms in female slices, untreated (control) or pre-treated with AβOs. Compared to the first

evoked fEPSP (P1), there is a facilitation of the second evoked fEPSP (P2) in each paired pulse, dependent on their inter-stimulus interval. (**B**) **–** Paired-pulse facilitation represented as a ratio of the amplitude of the second fEPSP and the first fEPSP (P2/P1) across each inter-stimulus interval tested. No difference in paired-pulse ratio between control and AβO pre-treatment was detected in female slices (ns, unpaired two-tailed student t-test).

3.4.3. Increased concentration of AβOs is required to reduce LTP in hippocampal slices from female mice

Next, we wanted to determine if treatment with the same AβO concentration that was used in males (Aβ1-42=83.8 pg/ml; Aβ1-x=4.41 ng/ml) would likewise inhibit LTP in female slices. The treatment conditions were otherwise identical for both groups. Surprisingly, when female slices were pre-treated with the same concentration of AβOs, no significant reduction in LTP was observed (**Figure 14**). As it is known that AD is more commonly affects females and cognitive decline is steeper in females compared to males. Given my results, we wanted to test whether a higher concentration of AβOs could inhibit LTP in female slices. For that, we increased the concentration of AβOs by 50% (Aβ 1-x=6.61 ng/ml) from the prior concentration that was enough to inhibit LTP in males (Aβ1-x=4.41 ng/ml).

As described previously, baseline fEPSPs were recorded for 20 min before induction of LTP by HFS (2 x 100Hz train lasting 1 sec, separated by 20 sec); fEPSPs were then recorded for an additional 60 min (**Figure 14**). In control slices the average fEPSP slope from the last 5 minutes of these recordings was roughly 200% of baseline (Ctrl: 214.98 ± 9.53 n = 7). In female slices pre-treated the 50% higher concentration of AβOs (Aβ 1-x=6.61 ng/ml) LTP was now inhibited (AβO: 143.39 ± 7.52 n = 5, $p < 0.05$ compared to Ctrl). Note, that treatment with the higher concentration of AβOs (Aβ 1-x=6.61 ng/ml) had no effect on baseline field potential recordings in the absence of HFS (**Figure 14**) and did not affect input/output or PPF (not shown).

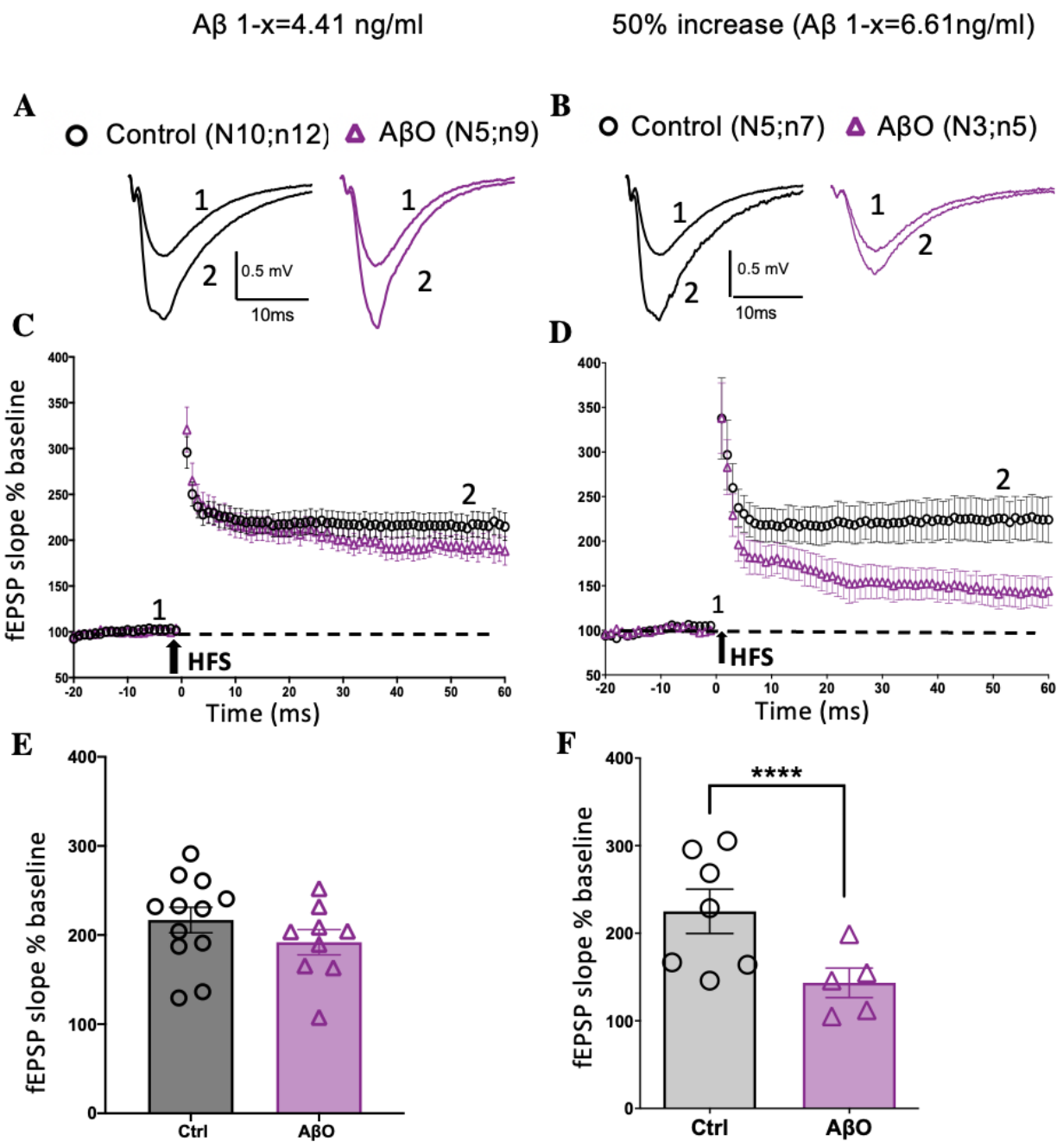

Figure 14: Higher concentration of AβOs is required to inhibit LTP in hippocampal slices from female mice.

(A) – Representative traces of fEPSPs at baseline before tetanus (1) and 60 minutes post-tetanus (2) in untreated (control) slices (n = 12) or slices pre-treated with AβOs (n = 9) (Aβ 1-x=4.41 ng/ml). **(B) –** Representative traces of fEPSPs at baseline before tetanus (1) and 60 minutes post-tetanus (2) in untreated (control) slices (n = 7) or slices pre-treated with 50% increased concentration of AβOs (n = 5). **(C), (D) –** Plots of averaged fEPSP slopes as a percentage of baseline during each minute of recording. **(E), (F) –** Averaged fEPSP slopes from the last 5 minutes

of each recording. There was no significant reduction in fEPSP slope in female slices pre-treated with AβOs with concentration Aβ1-x=4.41 ng/ml (ns, $p > 0.05$, p=0.2406 unpaired two-tailed student t-test). However, inhibition of LTP in female slices was observed with the higher concentration of AβOs). (****$p < 0.0001$, unpaired two-tailed student t-test).

3.4.4. Clotrimazole prevents inhibition of LTP induced by AβOs in hippocampal slices from female mice

Seeing such promising results from clotrimazole treatment in males by rescuing LTP inhibition caused by AβO pre-treatment we were interested in finding out if there would be a similar effect seen in females. The concentration of AβO used was Aβ1-x= 6.61 ng/ml. Similarly, as with males, slices from each treatment condition, baseline fEPSPs were recorded for 20 min before induction of LTP by HFS. fEPSPs were then recorded for an additional 60 min (**Figure 15**). In control slices from females, application of HFS reliably induced LTP which was maintained for 60 minutes. The average fEPSP slopes from the last 5 minutes of these recordings was roughly 185% of baseline (185.31 ± 13.93 n = 12) in females. Pre-treatment with AβOs in female slices caused a deficit in LTP compared to control (**Figure 15**) (Female: Aβ 143.39 ± 16.81 n = 5 vs. Ctrl 224.98 ± 25.22 n = 7, p=0.0244). In contrast, pre-treatment of slices with CTZ completely prevented AβO-induced deficits in long-term potentiation in females: Aβ 185.31 ± 13.93 n = 8 vs. Ctrl 177.51 ± 3.31 n =11, $p>0.05$.

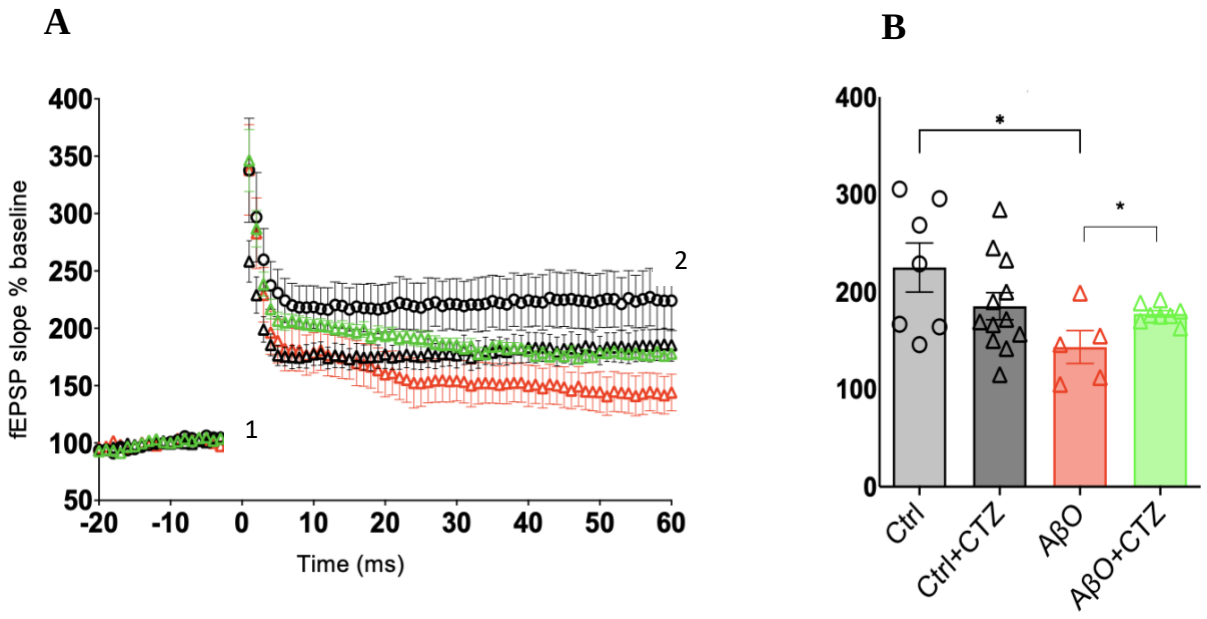

Figure 15: Clotrimazole prevents the inhibition of LTP by AβOs in hippocampal slices from female mice.

(A) – Plot of averaged fEPSP slopes as a percentage of baseline during each minute of recording with representative traces of fEPSPs at baseline before tetanus (1) and 60 minutes post-tetanus (2) in female untreated (control) slices with CTZ (n = 11), pre-treated with AβO (n = 5) or with CTZ+AβO (n = 8). **(B) –** Averaged fEPSP slopes from the last 5 minutes of each recording. There was no reduction in fEPSP slope in CTZ +AβOs treated slices vs. ctrl+CTZ slices (ns, p>0.05, one-way ANOVA).

3.5. Evaluating the effect of clotrimazole pre-treatment on baseline transmission, short-term plasticity and LTP in healthy hippocampal slices

3.5.1. Clotrimazole did not affect baseline excitability (input/output) in both female and male mice

At first, we measured baseline excitability (input/output) to see if there was any change induced by clotrimazole treatment (**Figure 16**). Excitability was assessed as described previously (section 3.2.1). Pre-treatment of hippocampal slices with 1 μM CTZ did not cause a change in input-output

slope in both females (0.8912 ± 0.1833, n = 7 ctrl vs. 0.7078 ± 0.2611, n = 11 ctrl+CTZ) and males (1.650 ± 0.3120, n = 15 ctrl vs. 1.962 ± 0.1828, n = 5 ctrl+CTZ).

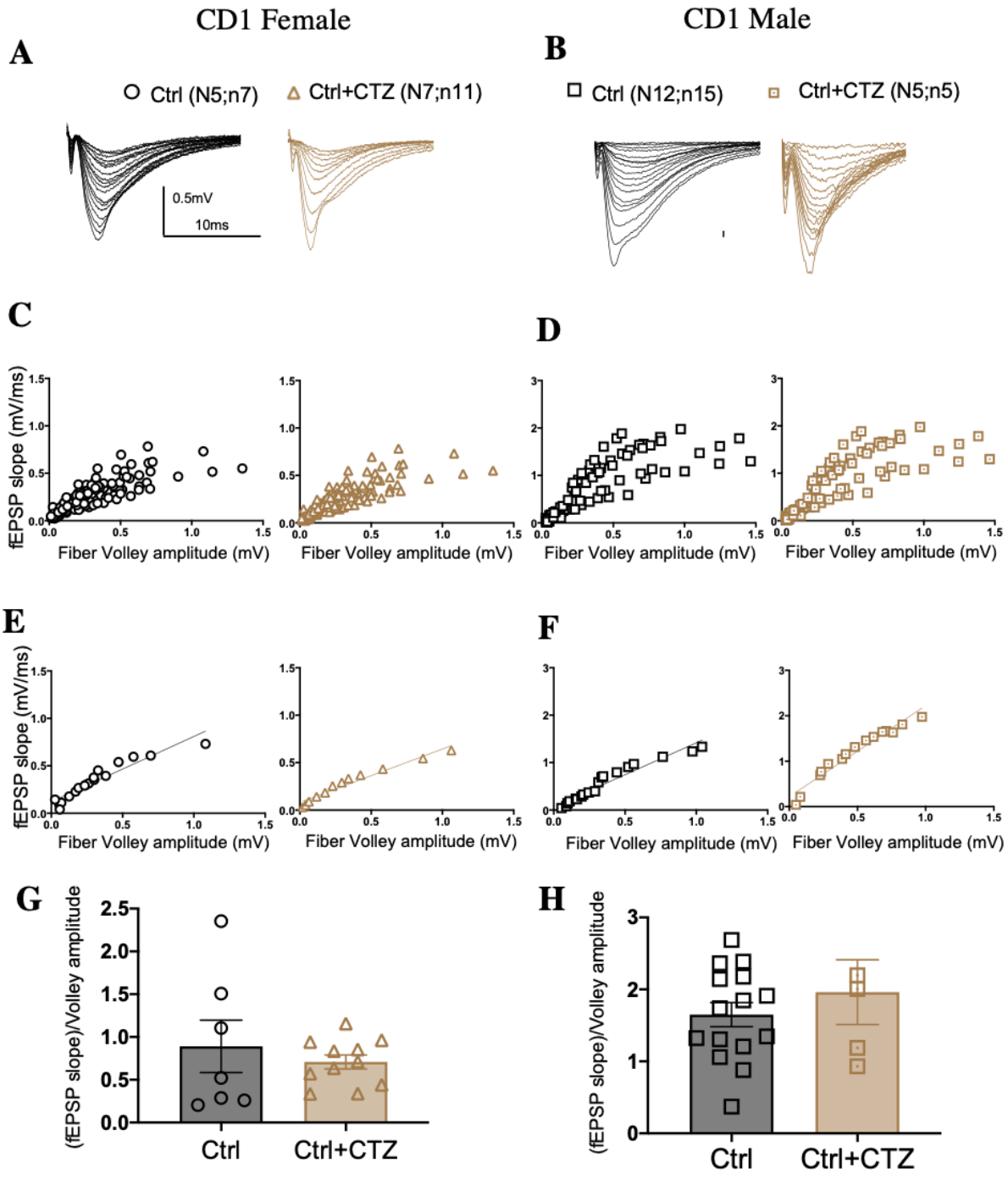

Figure 16: Analysis of input/output relationship in female and male hippocampal slices pre-treated with clotrimazole.

Representative traces of fEPSPs (output) evoked with increasing stimulus intensities (input) in slices from female **(A)** and male **(B)** mice treated with CTZ (ctrl+CTZ) or without (Ctrl). **(C), (D) –** fEPSP amplitude is plotted against fiber volley amplitude as a scatter plot summarizing the input-output relationship for all slices recorded from both groups. From each separate fEPSP amplitude vs fiber volley amplitude relation, linear regression was used to derive a corresponding slope value, used as a measure of synaptic transmission efficacy in each slice, (representative example shown in **Figure 16 (E), (F)**). The mean ± SEM of these values is plotted in **Figure 14 (G), (H)**. There was no difference found between control and CTZ treated hippocampal slices in females ($p=0.1438$) and males ($p=0.7564$) (ns, two-way ANOVA).

3.5.2. Clotrimazole did not affect paired-pulse facilitation in both female and male mice

We tested short-term, pre-synaptic plasticity using the PPF protocol defined earlier (section 3.2.2). We wanted to see if pre-treatment with 1 μM CTZ would cause any changes compared to control slices (**Figure 17**). PPF value reported at 20 ms ISI. PPF was not different between control and CTZ-treated hippocampal slices in females (ctrl: 1.263 ± 0.1210 n = 7 vs CTZ: 1.267 ± 0.105 n = 11, $p=0.9998$) and in males (ctrl: 1.238 ± 0.1433 n = 15 vs CTZ: 1.373 ± 0.1354 n = 5, $p=0.0064$) ($p>0.05$, $p=0.2737$) as analysed by two-way ANOVA.

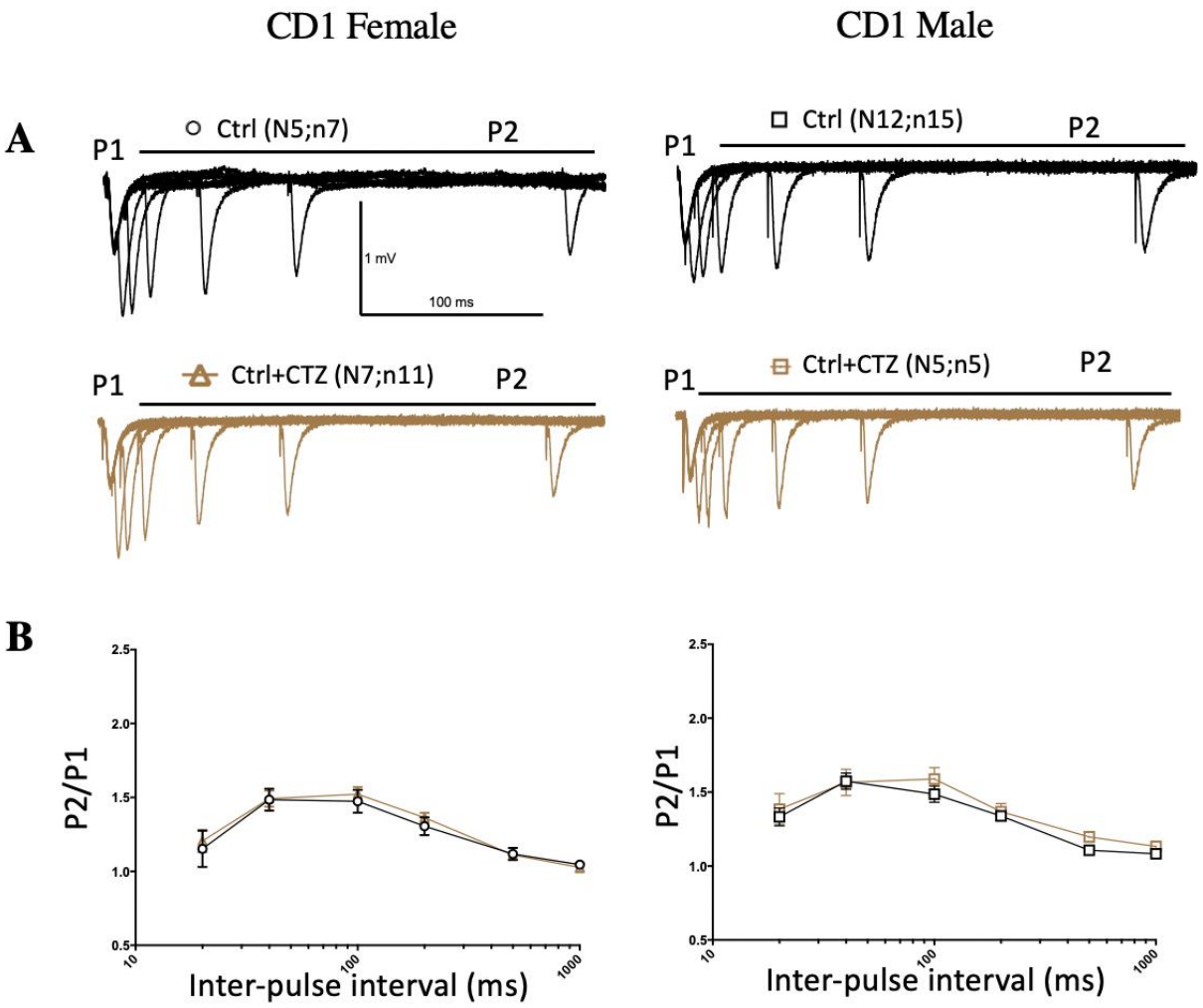

Figure 17: Paired-pulse facilitation is unaffected by CTZ pre-treatment in female and male hippocampal slices.

(**A**) – Representative traces of paired fEPSPs evoked with stimulus intervals ranging from 10 ms to 1000 ms in female and male slices untreated (Ctrl) or pre-treated with CTZ (Ctrl+CTZ). Compared to first evoked fEPSP (P1), there is a facilitation of the second evoked fEPSP (P2), the extent of which is dependent on their inter-stimulus interval. (**B**) – Paired-pulse facilitation represented as a ratio between the amplitude of the second fEPSP and the first fEPSP (P2/P1) across each inter-stimulus interval tested. There was no difference detected in paired-pulse ratio between control slices and pre-treated with CTZ in female ($p=0.1236$, two-way ANOVA) and male slices ($p=0.2737$, two-way ANOVA).

3.5.3. Clotrimazole did not affect LTP in both female and male mice

It is important to determine if pre-treatment with clotrimazole has an effect on LTP in CA3-CA1 region in hippocampus (**Figure 18**). This is crucial as treatment with high doses of CTZ can have an impact on the function of NMDAR and therefore induction of LTP would be compromised. Jacksons lab has addressed this in their past studies, however only in male slices (Sun et al. 2009). Thus, we wanted to evaluate the response in female slices. We saw that the dose of 1 μM is sufficient to exert the effect without impacting the function of neuronal NMDARs.

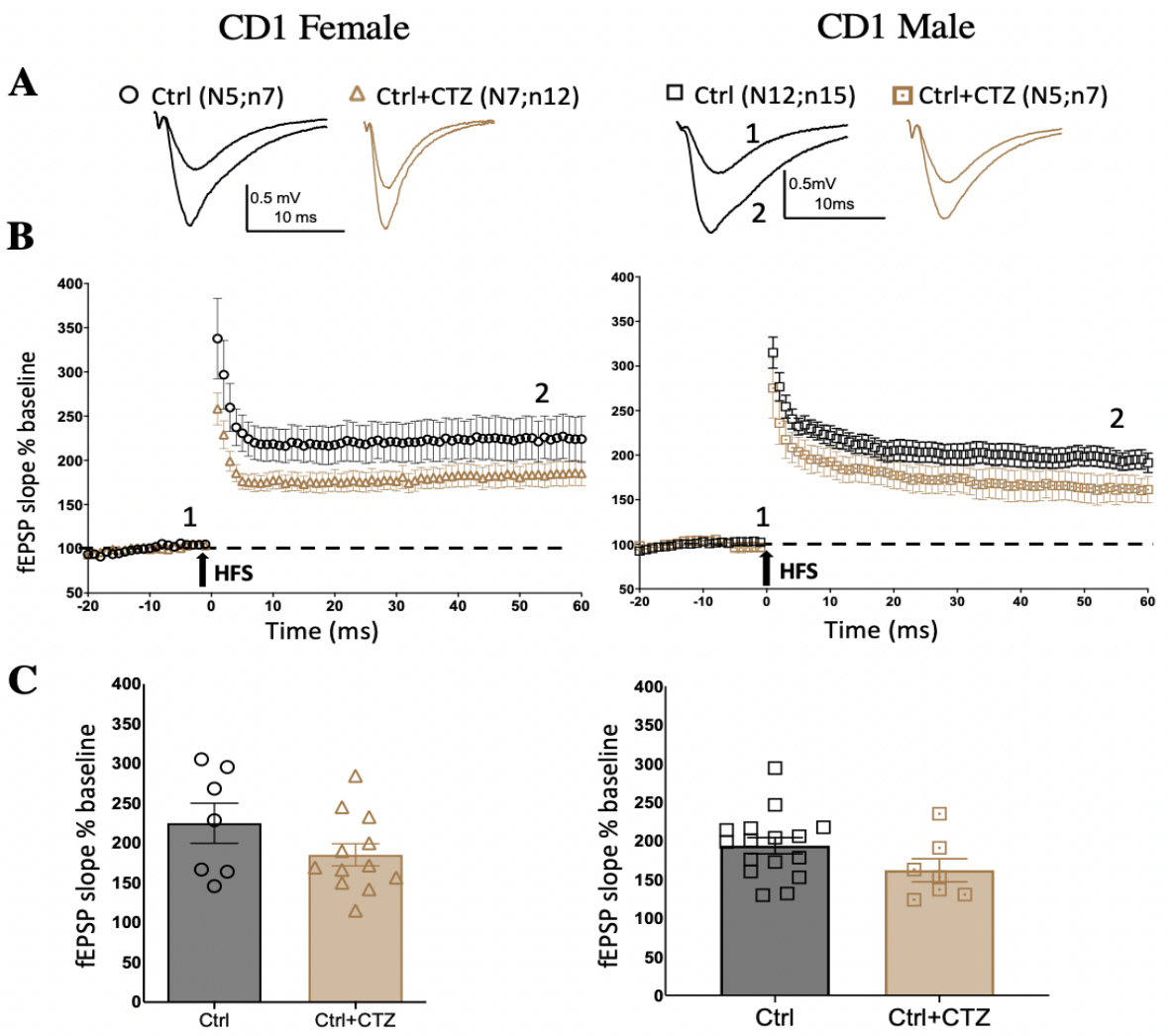

Figure 18: Pre-treatment of hippocampal slices with clotrimazole did not affect LTP in male and female mice.

(A) – Representative traces of fEPSPs at baseline before tetanus (1) and 60 minutes post-tetanus (2) in untreated (Ctrl) slices incubated with clotrimazole (ctrl+CTZ) in both sexes. **(B) –** Plot of averaged fEPSP slopes as a percentage of baseline during each minute of recording with representative traces of fEPSP at baseline before tetanus (1) and 60 minutes post-tetanus (2) in control alone vs incubated with clotrimazole from female hippocampal slices (225.0 ± 26.39 n = 7 ctrl vs. 185.3 ± 16.0 n = 12 ctrl+CTZ, p>0.05) as well as in male control slices vs incubated with clotrimazole (193.7 ± 10.48 n = 16 ctrl vs. 161.84 ± 14.90 n = 7 ctrl+CTZ, p>0.05). **(C) –** Averaged fEPSP slopes from the last 5 minutes of each recording in female and mal**e**. There was no difference between fEPSP slope in control slices vs ctrl+CTZ females (ns, p>0.05, p=0.1512, two-tailed unpaired student t-test) and control slices vs ctrl+CTZ CD1 WT males (p>0.05, p=0.1031, ns, two-tailed unpaired student t-test).

3.6. The selective TRPM2 inhibitor JNJ-28583113 prevents the synaptotoxic effects of AβO on LTP in males

Since clotrimazole is not quite selective/specific to TRPM2, we wanted to solidify our hypothesis and test a more selective and potent TRPM2 inhibitor. Some of the currently available TRPM2 inhibitors lack selectivity, have low potency, which limits their usefulness. CTZ, for example, is reported to inhibit both small and intermediate Ca^{2+} -activated K^+ channels and ATP-gated K^+ channels in pancreatic cells (Jäger et al. 2004), as well as NMDAR-mediated currents (Isaev et al. 2002) albeit at relatively high concentrations (10-50 μM). That is why it is important to test whether CTZ alone inhibits or even blocks LTP. The concentration of CTZ I used in my experiments was lower (1 μM) which did not affect the NMDAR function and I confirmed that CTZ treatment did not affect LTP. JNJ-28583113 is novel potent TRPM2 antagonist that is more selective and brain penetrant. Even though, JNJ-28583113 is rapidly cleaved in the plasma leading to very transient exposure to the brain, it is a good option for acute drug challenge in an experimental setting. With an IC_{50} of 126 nM for hTRPM2, JNJ-28583113 is potent compared to the most recently identified TRPM2 antagonists (Fourgeaud et al. 2019). For these experiments we focused on working with slices from male mice, as I saw that application of CTZ had equal effect in both females and males.

3.6.1. Pre-treatment of hippocampal slices with JNJ-28583113 had no effect on baseline excitability (input/output), short-term plasticity, and LTP in males

JNJ-28583113 (10μM) was added after dissection of the brain slices and remained in the holding chamber for the duration of slices recovery (1.5h). We wanted to see if pre-treatment with JNJ-28583113 had any effect on LTP in the hippocampal CA3-CA1 region (**Figure 19**). We

observed no effect on input/output relation, paired-pulse facilitation, baseline transmission and LTP induction after pre-treatment of hippocampal slices with JNJ-28583113.

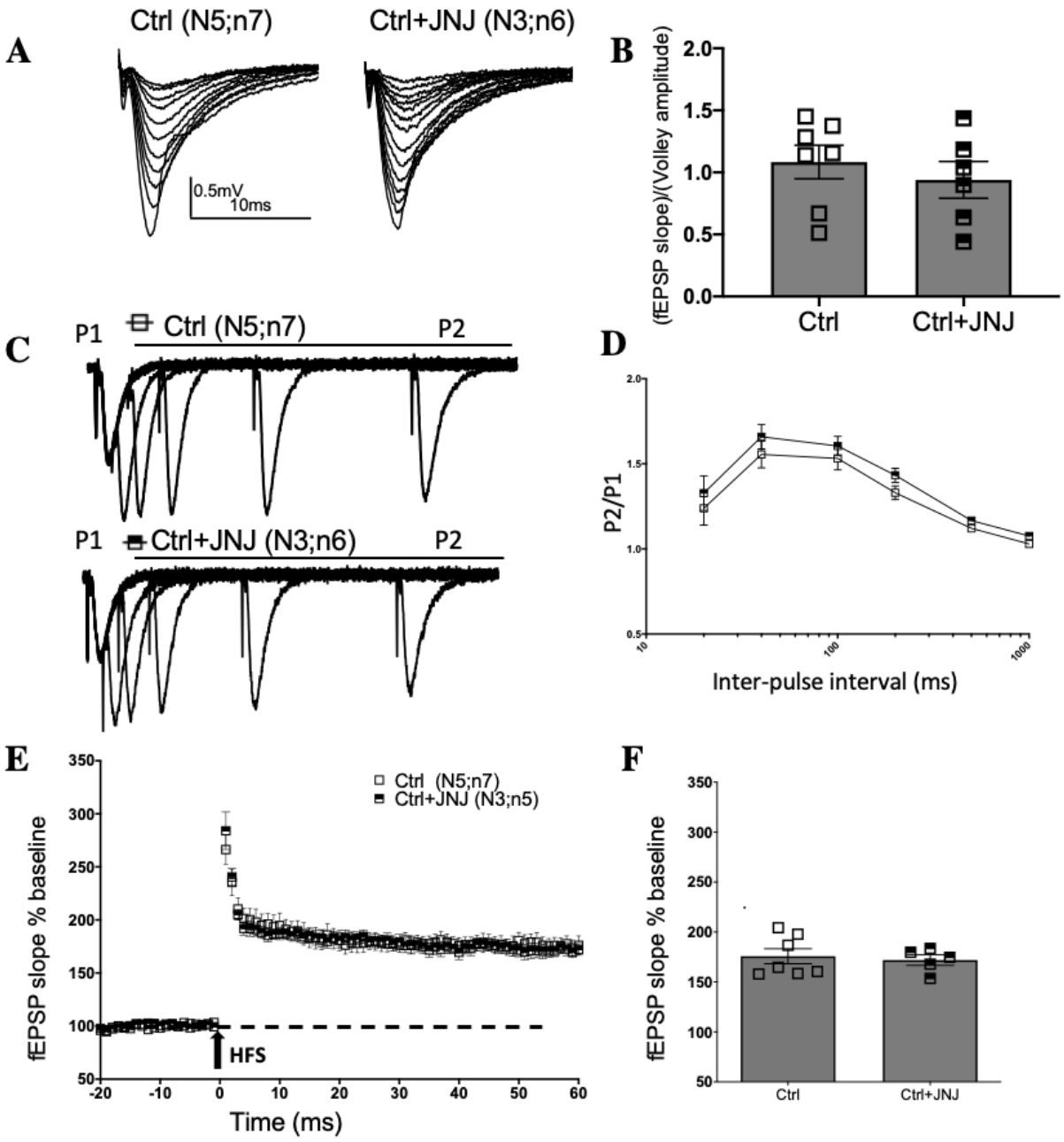

Figure 19: Pre-treatment of hippocampal slices with JNJ-28583113 had no effect on baseline excitability (input/output), short-term plasticity and LTP in males.

(A) – Representative traces of fEPSPs (output) evoked with increasing stimulus intensities (input) in ctrl slices or slices pre-treated with JNJ-28583113. From each separate fEPSP amplitude vs fiber

volley amplitude relation, linear regression was used to derive a corresponding slope value, used as a measure of synaptic transmission efficacy. The mean ± SEM of these values is plotted in **Figure 19** (**B**) (ns, p>0.05, p=0.9018, student t- test). (**C**) **–** Representative traces of paired fEPSPs evoked with stimulus intervals ranging from 10 ms to 1000 ms in male slices untreated (control) or pre-treated with JNJ-28583113. Compared to first evoked fEPSP (P1), there is a facilitation of the second evoked fEPSP (P2)), the extent of which is dependent on their inter-stimulus interval. (**D**) **–** Paired-pulse facilitation presented as a ratio between the amplitude of the second fEPSP and the first fEPSP (P2/P1) across each inter-stimulus interval tested. No difference in paired-pulse ratio between control and JNJ-28583113 treatment was detected (p>0.05, p=0.9328, student t-test). **(E) –** Plot of averaged fEPSP slopes as a percentage of baseline during each minute of recording. **(F) –** Averaged fEPSP slopes from the last 5 minutes of each recording. There was no difference in fEPSP slope between control (175.77 ± 7.55, n = 7) and JNJ-28583113 treated (171.85 ± 5.29, n = 6) slices (p=0.8534, student t-test).

3.6.2. Pre-treatment of hippocampal slices with JNJ-28583113 prevents the synaptotoxic effects of AβO on LTP in male mice

JNJ-28583113 (10μM) was added after dissection of the brain slices and remained in the holding chamber for the duration of slices recovery (1.5h) and was maintained during pre-treatment with AβO (2-3.5h) (Aβ1-x= 4.41 ng/ml). We wanted to test whether pre-treatment with JNJ-28583113 would prevent inhibition of LTP caused by synaptotoxic effects of AβO in CA3-CA1 region in hippocampus (**Figure 20**). I observed no difference in baseline and LTP in Ctrl slices (175.77 ± 7.55 n = 7) vs. Ctrl+JNJ (171.85 ± 5.29 n = 5) vs. AβO+JNJ (183.82 ± 7.63 n = 7), p>0.05, p=0.8971. Slices treated with AβO alone (129.70 ± 3.10 n = 5) had significant reduction of LTP compared to all three groups (p<0.05). These results demonstrate that the observed result is indeed due to inhibition of TRPM2 receptors as JNJ-28583113 is more potent and specific compared to clotrimazole.

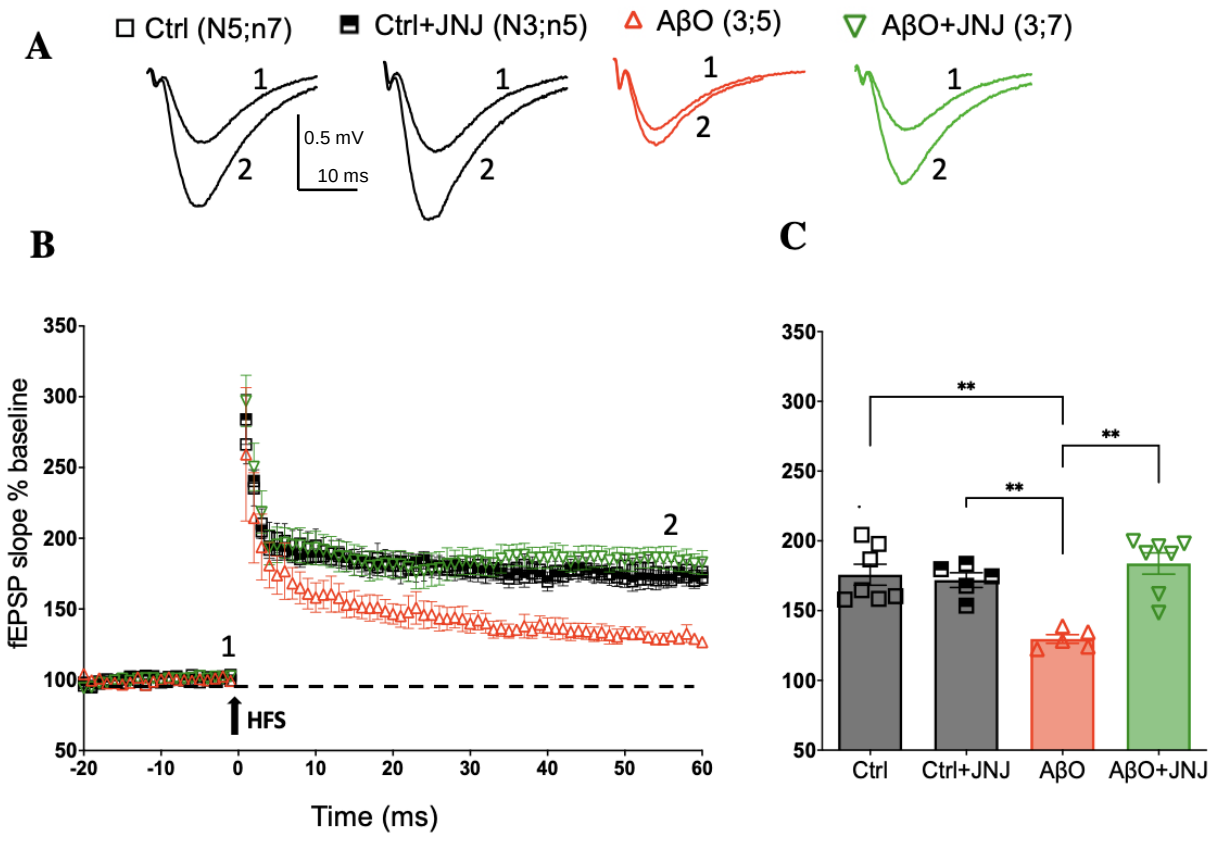

Figure 20: JNJ-28583113 prevents inhibition of LTP in male hippocampal slices.

(A) – Representative traces of fEPSPs at baseline before tetanus (1) and 60 minutes post-tetanus (2) in untreated (control) slices (n = 7), and in slices pre-treated with JNJ (n = 5), AβOs (n = 5) or AβO+ JNJ (n = 7). **(B) –** Plot of averaged fEPSP slopes as a percentage of baseline during each minute of recording. **(C) –** Averaged fEPSP slopes from the last 5 minutes of each recording. There was a significant reduction in fEPSP slope in AβO-treated slices (n = 5) (175.77 ± 7.55 n7 vs. 129.71±3.11 n = 5) (**p < 0.05, p=0.0082, one-way ANOVA with Tukey post hoc). Slices treated with JNJ (n = 7) did not show any inhibition of LTP after pre-treatment with AβOs. Difference between slices treated with AβO+JNJ (183.82 ± 7.63 n = 7) and AβO alone (129.71 ± 3.11 n = 5) was significant (**p<0.05, p=0.0019, one-way ANOVA with Tukey post hoc). However, there was no difference between slices treated with AβO+JNJ (183.82 ± 7.63 n = 7) vs. control slices (175.77 ± 7.55 n = 7), (ns, p>0.05, p=0.8971, one-way ANOVA with Tukey post hoc).

3.7. Inhibition of NUDT5 prevents deficits in LTP induced by AβO pre-treatment at the CA3-CA1 synapse in slices from male mice

A member of nucleotide-metabolizing enzymes - NUDT5 cleaves ADP-ribose (ADPR) in the cytosol (Kulikova and Nikiforov 2020). As ADPR serves as a TRPM2 agonist, NUDT5 activity is thus expected to limit activation of Ca2+ influx from the extracellular space through the plasma membrane TRPM2 channels. Unexpectedly however, inhibition of NUDT5 by a selective inhibitor developed by a collaborator (Dr. Brent Page, UBC) (Page et al. 2018) was shown to limit Ca2+ entry and cell death induced by hydrogen peroxide (unpublished). One possible explanation for this observation is that by inhibiting NUDT5 function we prevent ADPR cleavage and the abundance of ADPR (substrate saturation) feedbacks to inhibit upstream signalling by PARP-1/PARG. Thus, we hypothesized that NUDT5 inhibition might likewise inhibit AβO-induced inhibition of LTP.

3.7.1. Pre-treatment of hippocampal slices with a NUDT5 inhibitor (NUDT5i) has no effect on baseline excitability (input/output), short-term plasticity and long-term potentiation in males

To start, we wanted to see if pre-treatment of hippocampal slices with 10μM NUDT5 inhibitor (NUDT5i) would affect baseline excitability (input/output), short-term plasticity and LTP in the CA3-CA1 region of the hippocampus. NUDT5i (10μM) was added after dissection of the brain slices and remained in the holding chamber for the duration of slices recovery (1.5h). We

saw that pre-treatment of hippocampal slices with 10µM NUDT5i did not cause a change in input-output slope, PPF or LTP in males (**Figure 21**).

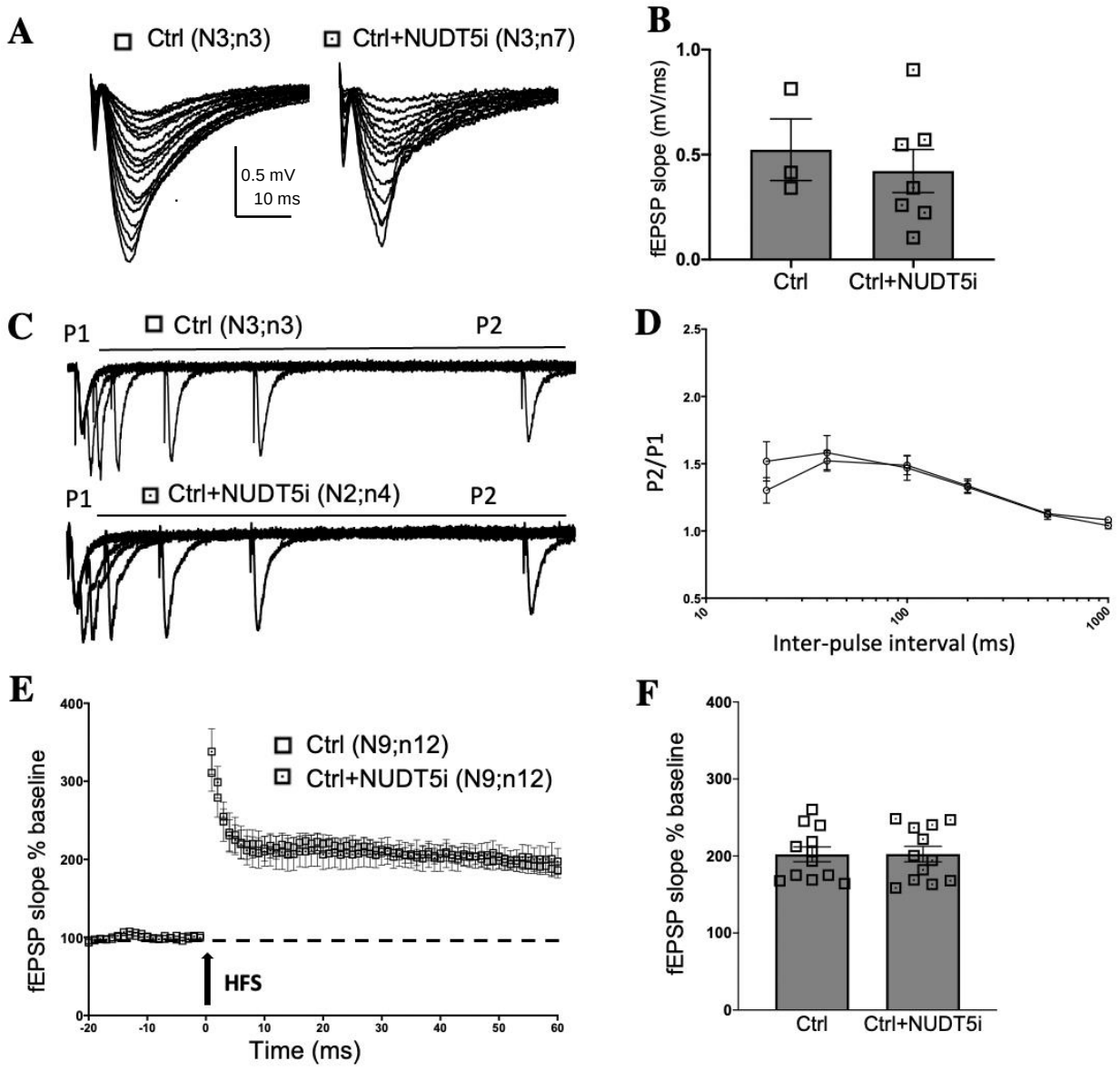

Figure 21: NUDT5i does not affect baseline excitability (input/output), short-term plasticity and LTP.

(A) – Representative traces of fEPSPs (output) evoked with increasing stimulus intensities (input) in male ctrl slices (n = 3) or slices pre-treated with NUDT5i (n = 7). From each separate fEPSP amplitude vs fiber volley amplitude relation, linear regression was used to derive a corresponding

slope value, used as a measure of synaptic transmission efficacy in each slice. The mean ± SEM of these values is plotted in **Figure 21** (**B**). There was no difference between ctrl vs NUDT5i-treated slices (ns, p>0.05, student t-test). (**C**) **–** Representative traces of paired fEPSPs evoked with stimulus intervals ranging from 10 ms to 1000 ms in untreated (control) slices or pre-treated with NUDT5i. Compared to first evoked fEPSP (P1), there is a facilitation of the second evoked fEPSP (P2) in each paired pulse dependent on their inter-stimulus interval. (**D**) **–** Paired-pulse facilitation presented as a ratio between the amplitude of the second fEPSP and the first fEPSP (P2/P1) across each inter-stimulus interval tested. No difference in paired-pulse ratio between control and NUDT5i-treated slices was detected (p=0.999, student t-test). **(E) –** Plot of averaged fEPSP slopes as a percentage of baseline during each minute of recording. **(F) –** Averaged fEPSP slopes from the last 5 minutes of each recording. There was no significant reduction in fEPSP slope in control (202.18 ± 9.64, n = 12) vs. NUDT5i-pretreated slices (202.50 ± 10.05, n = 12), (p>0.9999, student t-test).

3.7.2. Pre-treatment of hippocampal slices with NUDT5 inhibitor prevents the synaptotoxic effects of AβOs on LTP in males

NUDT5i (10µM) was added after dissection of the brain slices and remained in the holding chamber for the duration of slices recovery (1.5h) and was then maintained during treatment with AβOs (Aβ1-x= 4.41ng/ml). We wanted to see if pre-treatment with NUDT5i would prevent the inhibition of LTP caused by the synaptotoxic effects of AβOs in CA3-CA1 region in hippocampus (**Figure 22**). We observed that by inhibiting NUDT5 LTP was preserved in hippocampal slices treated with AβOs.

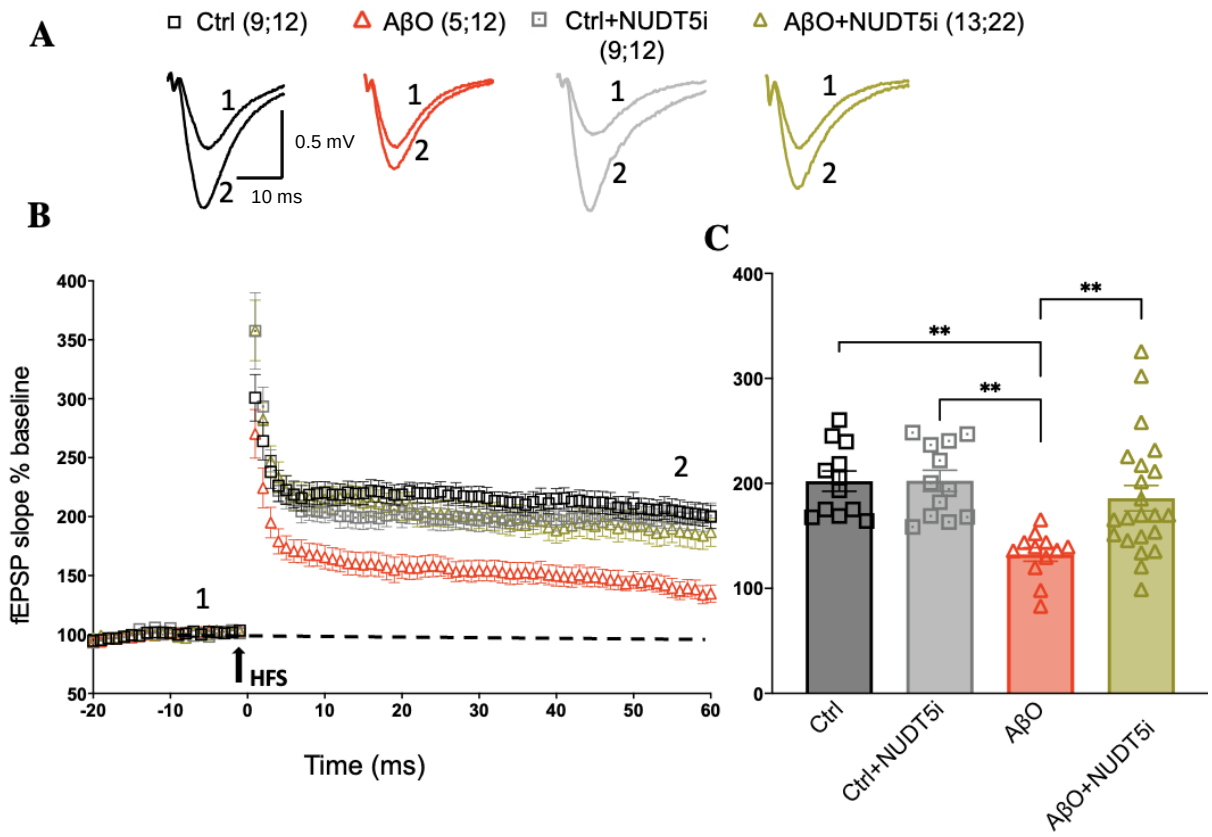

Figure 22: NUDT5i prevents the inhibition of LTP in males induced by AβOs.

(A) – Representative traces of fEPSP at baseline before tetanus (1) and 60 minutes post-tetanus (2) in untreated (control) slices (n = 12), or in slices treated with NUDT5i (ctrl+ NUDT5i, n = 12), AβOs (n = 12) or AβO+NUDT5i (n = 22). **(B) –** Plot of averaged fEPSP slopes as a percentage of baseline during each minute of recording. **(C) –** Averaged fEPSP slopes from the last 5 minutes of each recording. There was a significant reduction in fEPSP slope between control (202.50 ± 10.05 n = 12) vs. AβO-treated slices (132.34 ± 6.54 n = 12), (**p < 0.05, two-way ANOVA). Slices pre-treated with NUDT5i and AβOs (n = 22) did not show any deficits in LTP. There was a difference between slices treated with AβO+NUDT5i (185.94 ± 12.10 n = 22) and AβO alone (132.34 ± 6.54 n = 12), (**p<0.05, p=0.0052, one-way ANOVA). There was no difference between the slices treated with AβO+NUDT5i (185.94 ± 12.10 n = 22) and control slices (202.50 ± 10.05 n = 12), (ns, p>0.05, p=0.7168, one-way ANOVA).

CHAPTER 4. DISCUSSION

The goal for my thesis was to look at the role of NMDAR/PARP-1/TRPM2 pro-inflammatory signalling axis in mediating the synaptotoxic effects of AβOs on hippocampal synapses, with a specific focus on the role of TRPM2 and ADPR in maintaining the feedback loop of continuous activation of PARP-1. Previous work from Jackson and Kauppinen labs has shown separately that both TRPM2 and PARP-1 are involved in the Alzheimer's disease (T. M. Kauppinen et al. 2011; Ostapchenko et al. 2015). A recent study from our lab also has shown that microglial NMDARs have a role of being an extracellular trigger by activating the PARP-1/TRPM2 pro-inflammatory signalling cascade (Raghunatha et al. 2020). It is known that microglia can communicate with astrocytes and neurons as well as play role in modifying synaptic plasticity. Unpublished data from our lab using PARP-1 cKO suggests that microglia specific PARP-1 deletion prevents inhibition of long-term potentiation (LTP) induced by AβO treatment of hippocampal slices. Previously, the Jackson lab has shown that genetic deletion of TRPM2 normalized synaptic deficits and improved age-depended spatial memory in APP/PS1 mouse model of AD (Ostapchenko et al. 2015). However, there are no studies to date that have shown the effect of inhibiting TRPM2 pharmacologically on the synaptotoxic, synaptic plasticity impairing actions of AβOs.

We first utilized microglia specific NR1-iKO mice to evaluate the role of microglial NMDARs mediating the effects of AβO on synaptic plasticity. Next, we decided to focus on the approach of pharmacological inhibition. I employed two pharmacological inhibitors for TRPM2 channels (CTZ and a more specific and potent - JNJ) and one inhibitor for NUDT5 enzyme. CTZ and JNJ act by inhibiting the influx of Ca^{2+} into the cell through TRPM2 channels. Novel NUDT5 inhibitor acts by preventing the breakdown of ADPR monomers. Even though we are most

interested in microglia and its pro-inflammatory signalling pathway involving TRPM2 mediated feedback loop, I recognize that CTZ and JNJ did not target microglia exclusively. In addition, they would affect TRPM2 channels expressed in neurons (Chung, Freestone, and Lipski 2011) and in other cells, such as vascular endothelial cells (Mittal 2017). As much as we would like to think that the observed effect of TRPM2 inhibition is mostly because of microglial TRPM2 function and PARP-1 centered microglial pro-inflammatory signalling, we did not confirm it. In the future the validity of our proposed mechanism would have to be tested. This could be done by deleting TRPM2 from microglia specifically and performing electrophysiology experiments.

Past stroke studies reported sex-dependent neuroprotective outcomes upon TRPM2 and PARP-1 inhibition. Use of TRPM2 inhibitors in male mice significantly decreased neuronal cell death following oxygen-glucose deprivation in male cortical neurons, and decreased total infarct volumes compared to female (Jia et al. 2011). Another study observed that TRPM2 KO males is neuroprotective after *in vivo* ischemia-reperfusion injury and absence of TRPM2 promotes synaptic excitability in hippocampal CA1 neurons (Alim et al. 2013). PARP-1 gene deletion and pharmacological inhibition in the model of focal ischemia decreases infarction volume in the male brain, but increases the damage in the female KO brain (F. Liu et al. 2011; Siegel and McCullough 2013), (McCullough et al. 2005). To date it is not known if similar sex differences would be observed upon PARP-1 or TRPM2 inhibition in AD.

Therefore, to investigate the proposed role of TRPM2 and microglial NMDAR in mediating synaptic plasticity, I first wanted to assess sex-dependent effects of AβOs alone on LTP in CD1 female and male mice. By measuring field EPSPs at the Shaffer collateral CA3-CA1 pathway in acute hippocampal slices treated with AβOs for 2-3.5 h, I found that male slices respond by exhibiting inhibition of LTP compared with control slices, consistent with past reports (H. W.

Wang et al. 2002; Li et al. 2009; Lei et al. 2016). However, I saw that females require a 50% increase in the concentration of AβOs to exhibit a similar degree of inhibition of LTP. Further, using pharmacological blockers for TRPM2 as well as NUDT5 enzyme inhibitor, I showed that TRPM2 and NUDT5 play an important role in mediating the effects of AβOs on synaptic plasticity. I observed that treatment with clotrimazole, JNJ compound and NUDT5 inhibitor did not influence the baseline excitability, short-term plasticity or induction of LTP in hippocampal slices in both sexes. Moreover, inhibition of TRPM2 and NUDT5 enzyme was shown to be effective in preventing the inhibition of LTP in both female and male mice.

4.1 Current therapeutics for AD

Over the years our understanding of the clinical presentations and underlying pathology of AD has increased dramatically. However, we are yet to find a cure for AD. Tacrine was the first drug approved by U.S. Food and Drug Administration in 1993, which increases the levels of acetylcholine in the brain by slowing down its breakdown acetylcholine (Crismon 1994). Only four drugs have been approved since then; three are cholinesterase inhibitors and one is an NMDAR receptor antagonist (memantine). Despite tremendous research efforts and investments into potential therapeutics, all Aβ-directed therapies have failed in clinical trials (Cummings, Morstorf, and Zhong 2014). The US National Institutes of Health invested an estimated £2.9 billion into dementia research in 2017 alone (Society 2018). In 2018 pharmaceutical company Pfizer stopped funding AD research, citing that money can be better spent elsewhere (News 2018). In June 2021, a new drug was approved by the U.S. Food and Drug Administration called aducanumab (Aduhelm), which is an amyloid beta-directed monoclonal antibody that targets aggregated forms of amyloid beta in the brain (FDA 2021). However, the efficacy of this newly approved drug is controversial, because two large trials produced contradictory results as to

whether it slows Alzheimer's cognitive decline (Knopman, Jones, and Greicius 2021). Some of FDA's scientific advisers resigned in protest after Aduhelm's approval. In addition, it has severe side effects, such as brain edema, hemorrhage, headaches, and seizures. The study found that about 19% of patients who received the dose had brain bleeding and swelling that showed up on imaging. A total of 41% of patients had either brain swelling, bleeding or both, that required hospitalization. Besides the controversy about its efficacy, aducanumab has a price tag of $56,000 per patient per year which makes it not an affordable option.

Most available pharmacological interventions are symptomatic treatments only. Those include antidepressants, mood stabilising, antianxiety or/and antipsychotics medications, that are used primarily to treat psychotic and behavioral symptoms. Non-pharmacological treatments also exist and mostly aim to manage the cognitive and behavioral changes (Beart and Robinson 2013).

As life expectancy increases and mortality rates decline, we are facing the global aging population, which would mean substantial increase in dementia numbers across the world. Knowledge about underlying pathophysiological mechanisms, clinical presentations and genetics of AD have increased tremendously since the first known case over a century ago. Regulation of microglial inflammatory responses has been a promising target of many researchers, for reasons outlined in the following section.

4.2 PARP-1 and TRPM2 as potential molecular targets for AD treatment

In addition to being potent synaptotoxins, AβOs have been shown to trigger inflammatory processes in glial cells, early in the progression of AD (Ferretti et al. 2012; Rojo et al. 2008; Yin et al. 2017). Interestingly, a population-based study discovered that patients with AD have increased levels of pro-inflammatory mediators in their blood even before the onset of the disease

(Engelhart et al. 2004). Moreover, genome-wide association studies identified more than 38 genetic loci that implicate immune cells and microglia that increase risk of developing LOAD (Wightman et al. 2020). Long-term use of non-steroidal anti-inflammatory drugs (NSAIDs) has also shown the ability to decrease incidence of AD if taken prior to developing symptoms (McGeer, Schulzer, and McGeer 1996). However, the effectiveness of NSAIDs use remains controversial (P. S. Aisen, Schmeidler, and Pasinetti 2002; Paul S. Aisen et al. 2003; Imbimbo, Solfrizzi, and Panza 2010). Inhibiting pro-inflammatory responses, for example by suppressing astrocytic reaction and interfering with activated T-cells signalling pathway was shown to decrease the AD pathology (Furman et al. 2012), as well as deficiency of NLRP3 inflammasome skewed microglia cells to a neuroprotective phenotype which resulted in decreased deposition of Aβ in APP/PS1 model of AD (Heneka et al. 2013).

Studies from our lab demonstrate that the nuclear enzyme PARP-1 is responsible for mediating pro-inflammatory responses in microglia in mouse models of AD (T. M. Kauppinen et al. 2011). Importantly, PARP-1 inhibition or deletion does not prevent all microglial functions. Beneficial functions of microglia, such as ability to release anti-inflammatory mediators, cytokines, and trophic factors, as well as their proper phagocytic activity are not affected. Thus, use of PARP-1 inhibitors can be suggested to be a therapeutic choice for AD treatment. However, pharmacological PARP-1 inhibitors exert extensive toxic side effects profile, and their ability to trap onto DNA cause DNA damage which jeopardizes cell viability (Sinha 2021). This make them not the best option for Alzheimer's. In addition, stroke studies suggest that there are sex differences when targeting PARP-1 in males vs. females. PARP-1 deletion leads to different effects depending on the animal's sex, with neuroprotection in males and increased infarct size in females (F. Liu et

al. 2011). Thus, an alternate approach, targeting downstream signalling molecules would perhaps be a more suitable option.

An end product of PARP-1 activation, ADPR, is required for activation of TRPM2 in microglia. The role of TRPM2 receptors has been shown to be implicated in neurological diseases (E. Fonfria et al. 2005; Ostapchenko et al. 2015; J. Wang, Jackson, and Xie 2016). Further, studies from our lab showed that in global deletion of TRPM2 in APP/PS1 mice reduced cognitive decline compared to WT (Ostapchenko et al. 2015). Moreover, brains from APP/PS1 mice showed increased activation of microglia, inferred from increased levels of Iba1 (marker of microglia) and decreased synaptophysin levels, reflecting synapse loss. However, mice lacking TRPM2 did not exhibit the abovementioned deficits, which suggests that TRPM2 mediates microglial activation and synaptic loss in AD. Global deletion of TRPM2 was shown to be well tolerated, which makes an option of pharmacological inhibition a possible therapeutic intervention for treating AD. To date there are no studies that explored the role of TRPM2 in AD in both sexes. The data available from stroke studies having TRPM2 deleted or pharmacologically inhibited suggests that TRPM2 involvement in ischemic outcome is male specific. The use of TRPM2 inhibitors significantly decreased neuronal death in male cortical neurons but not in female (Jia et al. 2011). Therefore, when examining whether TRPM2 inhibition can provide protection from the synaptotoxic effects of AβOs it is crucial that this question will be addressed in both sexes. This is further supported by the fact that the prevalence and progression of AD in humans is highly sex-dependent.

4.3. Sex differences in mediating the synaptotoxic effects of AβOs

It is well-known that women have a higher risk of developing AD than men. Meta analysis showed that women also have a steeper decline in memory and more severe cognitive symptoms

in AD (Irvine et al. 2012). Sex differences are also reported in hippocampal plasticity, adult neurogenesis and cognitive function. Besides morphological differences, there are sex differences in LTP in the hippocampus. For example, early and late-LTP is larger in dental gyrus, CA3 and CA1 regions in male rats compared to females (Monfort et al. 2015; Yang et al. 2004).

Many AD animal models display similar sex-dependent pattern, where female mice show greater cognitive decline and more severe neuropathology than males. Moreover, in some AD models APP processing and levels of accumulated Aβ peptides is higher in females (Callahan et al. 2001). Another study showed that in models where APP is driven by prion protein (PrP), females express APP in higher levels than males. Females also showed greater degree of atrophy in the dentate gyrus in hippocampus (Melnikova et al. 2016).

It has been shown that AβOs inhibit synaptic plasticity in the form of LTP (Townsend et al. 2006). To date, no study showed any differences in the LTP between male and female response to AβO pre-treatment. Thus, I wanted to first establish if there are any sex differences in the response to AβOs and I found that while males respond to AβOs by exhibiting a deficit in LTP, females did not show significant inhibition of LTP when treated with the same concentration of AβOs. These findings go against the general knowledge that females are more susceptible to AD and show greater cognitive decline. Whether this sex difference can be explained by structural differences in the hippocampus, hormonal activity or by differences in the inflammatory response to AβOs is yet to be discovered.

The influence of sex hormones could provide some explanations. Hippocampus contains sex hormone receptors such as androgen receptors (AR), estrogen receptors (ER- α and β) and G-protein coupled receptors (GPCRs – e.g. GPR30). Manipulations of sex steroid hormones has been shown to reveal differences in hippocampus morphology (E. Gould, C.S. Woolley, M. Frankfurt

1990). This is an early study that showed that estrous cycle and estradiol supplementation can prevent the observed decrease in spine density in the dentate gyrus region of the hippocampus in females. The loss of neuroprotective influence of estrogen could explain why women in menopause have increased sensitivity to inflammatory events and are more likely to develop AD (Mosconi et al. 2018). The female mice that are used for my experiments were 4-6 weeks old, that have not yet reached menopause, which would make the neuroprotective effects of estrogen a possible explanation for the observed outcome.

Another possible explanation could be the difference in promoting inflammatory responses between two sexes (Sabeh et al. 2021; Zhao and Eghbali-Webb 2002). In physiological conditions microglia is known to express steroid hormone receptors and are sensitive to the effects of estrogen and testosterone (Sierra et al. 2008). The study suggested that down-regulation of steroid receptors serves as a pre-requisite the anti-inflammatory action on the immune system. Neuroimmune cells derived from cortical tissue together with astrocytes in males had significantly larger response to inflammatory insults (LPS) compared to female-derived cells (Santos-Galindo et al. 2011). In addition, there are sex-specific responses have been observed in NF-κB-p65 mediated neuroprotection in human glutamatergic neurons (Ruiz-Perera et al. 2018). NF-κB mediated inflammatory responses can also regulate male vs female responses and contribute to the observed differences to AβOs. It has been suggested that estrogen can modulate NF-κB signalling by promoting new synthesis of IκBα, thus inhibiting binding of NF-κB to the promoters of inflammatory genes (Xing et al. 2012). These results support the protective role of estrogens in the inflammatory events, which would confirm the notion why females become more susceptible to AD during menopause.

More studies are needed to investigate the responsible mechanisms that are orchestrating the inflammatory responses in both females and males. Understanding of underlying pro-inflammatory pathways would help in developing more targeted therapies for AD and other neurodegenerative disorders.

4.4 TRPM2 inhibitors clotrimazole and JNJ-28583113 prevent the synaptotoxic effect of AβOs on LTP in mice of both sexes

It is known that AβOs cause glutamatergic excitotoxicity, which preferentially affects neurons (Talantova et al. 2013). Less is known and spoken about their effects on glial cells (astrocytes and microglia) and their contribution to neuroinflammation which can indirectly or directly impact neuronal connections (synapses) and consequently inhibit synaptic plasticity. Synaptic plasticity is an ability of synapses to strengthen or weaken over time. In AD patients synaptic plasticity decreases, which manifests as the main symptom of the disease - memory loss (Ferreira and Klein 2011). LTP is the measurement of synaptic function and glial cells do not have any direct roles in its induction, which is dependent on the activation of neuronal NMDARs (Bear and Malenka 1994; C. Lu and Malenka 2012). However, glial cells, such as microglia can indirectly affect neurons by secreting various cytokines such as IL-1β and even trigger LTD through complement involvement and activation of NADPH oxidase (J. Zhang et al. 2014; York et al. 2021). Some studies discovered that neuronal dysfunction and inflammation found in AD are linked to microglia triggered by Aβ through MAPKs and extracellular signal-regulated kinases (ERKs) (Doens and Fernández 2014), (Querfurth and Laferla 2010).

Past studies from our lab showed that Aβ-mediated microglial activation leads to activation of nuclear enzyme PARP-1, followed by the release of nitric oxide and pro-

inflammatory mediators, such as TNF-α (T. M. Kauppinen et al. 2011). Moreover, the Kauppinen and Jackson labs have also demonstrated that activation of microglial NMDARs triggers microglia pro-inflammatory responses and cytokine release (Raghunatha et al. 2020). Application of NMDAR inhibitors (APV, 7-CK and MK-801) significantly reduced microglial morphological proliferation and release of pro-inflammatory mediators, while levels of anti-inflammatory mediators were maintained. Raghunatha and authors went on to demonstrate that TRPM2 signalled in concert with PARP-1 through a positive feedback scheme via Ca^{2+} in which NMDAR stimulation resulted in increased proliferation, morphological activation and release of pro-inflammatory mediators.

These results, combined with those from Jackson (Ostapchenko et al. 2015; Belrose and Jackson 2018) and Kauppinen (T. M. Kauppinen et al. 2011; 2009; T. M. Kauppinen and Swanson 2005) showing that inhibition or deletion of PARP-1 or TRPM2 affords protection against AD pathology and cognitive decline, paired with our more recent unpublished findings that microglia-target PARP-1 conditional deletion likewise can reduce AD pathology and cognitive decline, led to our hypothesis that inhibition of TRPM2 may represent an ideal target for reducing microglial sustained pro-inflammatory responses, which would limit the effect of AβOs on synaptic plasticity by preventing inhibition of LTP.

Clotrimazole (CTZ) is a commonly used inhibitor for TRPM2. We decided to start with this particular TRPM2 inhibitor for its economical properties, accessibility and common usage in research. However, it has some limitations: it is not very selective. CTZ can also block Na^+ and K^+ channels and in high doses – NMDARs (Rittenhouse et al. 1997; Jäger et al. 2004; Isaev et al. 2002). The dose of CTZ that was used in my experiments (1 μM) did not affect the function of NMDARs, as we saw the induction of NMDAR-dependent LTP. Indeed, hippocampal slices of

both females and males did not show any deficit in LTP when pre-treated with CTZ alone. Moreover, in slices treated with CTZ we saw no effect of AβO treatment on LTP. Importantly, CTZ was equally effective in slices from both male and female mice. The reason why we did not observe any sex differences when treating with CTZ is yet to be discovered. However, observed results from this experiment make inhibition of TRPM2 a prominent future therapeutic target for treating both female and male AD.

Next, we tested a novel TRPM2 inhibitor JNJ-28583113, application of which was shown to decrease cytokine release in response to pro-inflammatory stimuli in microglia (Fourgeaud et al. 2019). JNJ-28583113 is potent and more selective than clotrimazole. Application of JNJ-28583113 to male hippocampal slices had no effect on baseline excitability and short-term plasticity but prevented the Aβ-induced inhibition of LTP. We only used JNJ in male slices due to observing the same effect of CTZ in both sexes. Nevertheless, testing the drug effect on female slices should also be done in future to confirm that it is equally effective in females.

Even though TRPM2 is highly expressed in microglia, electrophysiology experiments cannot confirm that CTZ or JNJ-28583113 inhibit TRPM2 receptors preferentially in microglia. When pharmacological inhibitors are used, they work on all TRPM2 receptors that are present in the brain. To check the microglia involvement specifically, immunostaining could be done using microglia specific markers, such as CD11b or Iba1. Another approach would be to look at the microglia pro-inflammatory responses by measuring iNOS expression levels in brain slices or pro-inflammatory cytokine profile. However, that would be difficult-to-impossible to measure, as it takes longer for iNOS levels to rise than the slices incubation period during an electrophysiology experiment. Using two structurally different inhibitors (CTZ and JNJ-28583113) against the same target (TRPM2 channels) makes the outcome to be less likely related to the off-target effects.

4.5 NUDT5 inhibitor prevented the AβOs synaptotoxic effects on LTP in males

NUDT5 inhibitor (TH5427) is a novel inhibitor that is used to study NUDT5 enzyme activity and ADP-ribose metabolism. NUDT5 catalyzes the breakdown of ADPR to ribose-5-phosphate (R5P) and adenosine 5'-monophosphate (AMP) (Gasmi, Cartwright, and Lennan 1999; Formentini et al. 2009), which allows recovery of NAD^+ pools after PARP-1 activation or DNA damage (A. L. Perraud et al. 2003). Research suggests that TN5427 is selective inhibitor of NUDT5 and has a promising safety profile (Page et al. 2018). Since NUDT5 is involved in ADPR metabolism we were interested in its role in modulating PARP-1 and TRPM2 activity. Logically, inhibition of NUDT5 should prevent the breakdown of ADPR (**Figure 1**) and therefore create more ADPR available in the cell to activate TRPM2. If this was the case, the outcome would have been a more deleterious inhibition of LTP caused by AβO treatment compared to the observed continuous potentiation and no difference between control and AβO-treated slices. However, application of NUDT5 inhibitor prior to and during treatment with AβO prevented the expected deficit in LTP in male hippocampal slices.

The clear explanation for the observed effect is not exactly known. However, we believe that the observed outcome could be due to the alteration of PARP1/PARG/NUDT5 activity via substrate feedback inhibition. The exact mechanism of action of NUDT5 inhibitor would have to be evaluated in the future studies as well as its effect in female mice. However, one possible explanation could be that alteration in PARP-1/PARG/ADPR metabolism. Inhibition of NUDT5 likely causes increase in levels of ADPR, which acts as a negative feedback loop by inhibiting PARG activity. Reduced PARG activity would accumulate PARs, including PARP-1 auto-PARylation, known to suppress PARP-1 activity (T. M. Kauppinen 2007; Burns et al. 2009; Kulikova and Nikiforov 2020). It has been shown that the use of PARG inhibitors did not affect

NAD^+ depletion and counteracted ATP loss (Formentini et al. 2009). Silencing or inhibiting PARG impaired PAR degradation and reduced AMP formation and therefore prevented the activation of PARP-1 by negative feedback inhibition. Supporting our proposed mechanism, another study has shown that depleting or inhibiting PARG, the main enzyme in PAR metabolism prevented activation of NF-kB and activator protein-1 (AP-1), which significantly supressed inflammatory gene expression (Erdèlyi et al. 2005). Thus, altering the mechanism of action of NUDT5 contributes to disruption of energetic metabolism which consequently would slow down the pro-inflammatory reaction, by preventing PARP-1 activation and consequently TRPM2.

Future experiments are needed to evaluate the activation of TRPM2 channels, which could be done by doing electrophysiology experiments by measuring the current activity or by Ca^{2+} imaging. In addition to evaluating the activity of PARP-1 by measuring PAR formation.

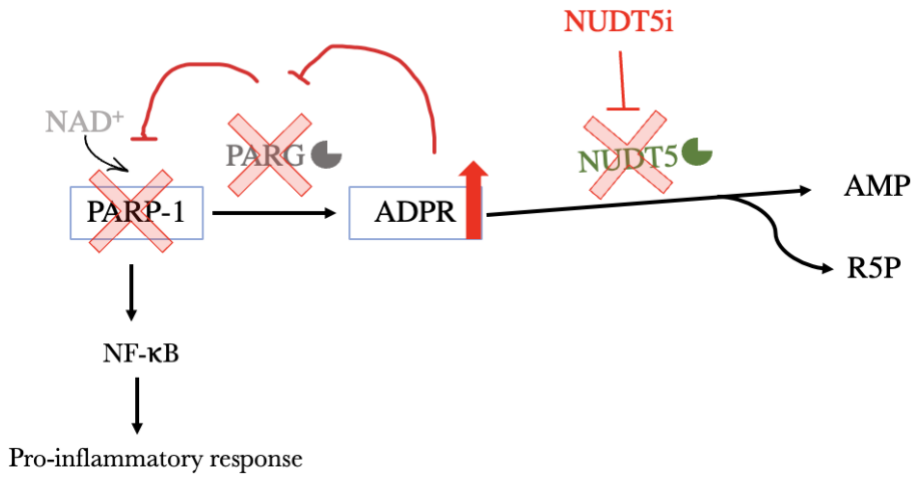

Figure 22: Proposed crosstalk of PARG, PARP-1 and NUDT5 enzyme in microglial regulation. r s catalyzing the breakdown of ADPR to AMP and R5P. When NUDT5 is inhibited, this could lead reduction in PARP-1/PARG activity via substrate feedback inhibition. Indeed, pharmacological inhibition of PARG did not affect NAD^+ depletion, but counteracted ATP loss. Pharmacological inhibition NUDIX activity or genetic suppression of NUDT5 reduced PARP-1 dependent ATP loss (Formentini et al. 2009). In our case this would mean prevention of PARP-1 activation and suppression of the whole the TRPM2/ADPR pro-inflammatory signalling pathway.

4.6 Study limitations

My results have shown that TRPM2 mediate the synaptotoxic effects of AβOs on synaptic plasticity. Specifically, using pharmacological inhibitors of TRPM2 (such as CTZ and JNJ-28583113), I have shown that treatment with AβO did not elicit any deficit in LTP in male/female hippocampal slices. I have also shown that by manipulating ADPR/TRPM2 interaction via NUDT5 inhibition also prevents the inhibition of LTP caused by AβO. Whether it is microglial TRPM2 that is responsible for the observed effect, have to be investigated further by future studies. In the brain TRPM2 are present in neurons, endothelial cells, astrocytes and microglia (Kraft et al. 2004; Elena Fonfria et al. 2006; Olah et al. 2009; Turlova, Feng, and Sun 2018). Evidence suggests that inhibition or deletion of TRPM2 in different cells in the brain is neuroprotective (Alim et al. 2013; Ostapchenko et al. 2015; Yamamoto 2008; Belrose and Jackson 2018), that could have affected the outcome of my experiment by combining of the protective roles of TRPM2 inhibition expressed in other cells. This makes TRPM2 inhibition an interesting target for its role in neuroinflammation.

To prove the concept of microglial involvement based on our proposed pro-inflammatory pathway we would have to create a Cx3Cr1-CreERT mouse line, that will allow microglia specific TRPM2 deletion in a time specific manner. Since our lab's previous *in vitro* work suggests that stimulation of microglial NMDARs leads to recruitment of TRPM2 through the same signalling pathway (Raghunatha et al. 2020), I started my project by examining the role of microglial NMDARs as a possible way to disrupt the NMDAR/PARP-1/TRPM2 signalling axis. By utilizing field electrophysiology technique, we wanted to investigate whether the effects of AβO treatment on hippocampal slices would be abrogated in the microglial NMDAR-iKO mice. We used cre-recombinase system (Cre-ERT2) to specifically and inducibly delete NMDARs from microglia.

However, I have encountered some difficulties, such as slices viability and survival and the effectiveness of cre-targeted deletion (see limitation section). Among 14 WT and iKO mice only 5 (3 iKO and 2 WT) were suitable to use for my experiments. The remaining mice after dissection exhibited severe hypoxia in the hippocampal slices, did not have any or had weak synaptic responses and could not be used further for electrophysiology. We are not exactly aware what the problem was, however, viability was not related to either TAM injections or genotype (**Table 1**). In addition, we were not able to confirm the effectiveness of Cre by measurements of transcripts or protein by western blot or IHC. However, I did observe the LTP in NR1 fl/fl Cre+/- injected with TAM which argues that there was no deletion of NR1 in neurons. The available data from the mice that were viable for the electrophysiology experiments showed that deletion of microglial NMDAR prevents the inhibition of LTP in AβO-treated slices.

Future studies have to be done to evaluate the effect of JNJ-28583113 and NUDT5 inhibitor in females. Future studies using microglial TRPM2 KO can be used for electrophysiology experiments to evaluate the role of microglial TRPM2 on synaptic plasticity as well as cognition. This is important to establish if targeting microglial TRMP2 specifically is necessary. However, the question arises if it would be possible to target those in human studies. Since global deletion of TRPM2 has previously been shown by Jacksons lab to not elicit any harmful side effects, perhaps there would be no need to specifically target TRPM2 in microglia.

When moving to clinical studies in humans, specific TRPM2 inhibitor has to be developed. The main question would be bioavailability and brain penetrance. Overall, TRPM2 seems to be a promising therapeutic target for treatment of Alzheimer's disease.

Another limitation to this project is regarding the NR1-iKO mice, as during conducting the experiments I have faced some challenges. Altogether there were 14 NR1-iKO and WT mice 5

months of age that received 5 intraperitoneal (IP) TAM (2mg/mice) or vehicle (sunflower oil) injections for 5 consecutive days. Further I provide a table describing the genotype, slices viability and whether the mouse was injected with TAM or a vehicle. Out of 14, 5 were suitable to be used in my experiments. The reason for strikingly low number of mice available to be used for the experiments are not exactly known. However, we can exclude the effect of genotype and TAM from the possible explanation, as we extracted healthy viable slices from mice with both genotypes (WT and NR1-iKO) as well those injected with TAM and not.

Table 1: Description of mice genotype, type of injection and slice viability

Date	Genotype	Tamoxifen	Viability
2021-10-25	WT	Yes	Good
2021-10-26	Knockout	Yes	Good
2021-10-27	Knockout	Yes	Dead
2021-10-28	WT	Yes	Dead
2021-11-01	Knockout	Yes	Good
2021-11-02	Knockout	Yes	Dead
2021-11-03	WT	Yes	Dead
2021-11-04	Knockout	Yes	Dead
2021-11-08	Knockout	Yes	Dead
2021-11-09	WT	Yes	Dead
2021-11-24	Knockout	Yes	Good
2021-11-25	WT	No	Good
2021-11-26	Knockout	No	Dead
2021-11-30	WT	No	Dead

In addition, unhealthy hippocampal slices had a different appearance (having stratum radiatum region hyperintense) when placed into the recording chamber after the incubation period (1.5h) compared to the healthy slices (**Figure 23**).

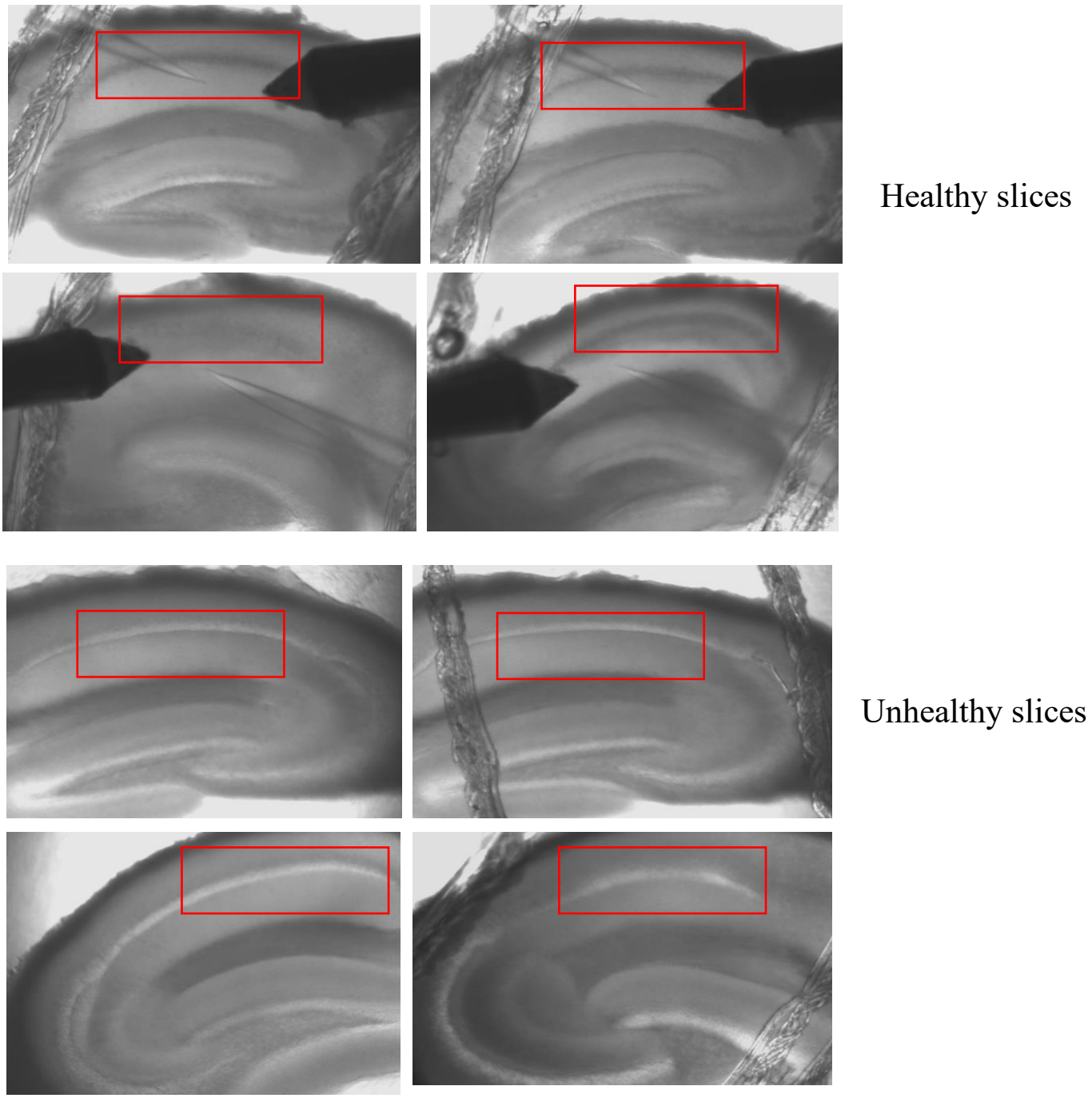

Figure 23: Viability of NR1-iKO male hippocampal slices.

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