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Microglial Phenotype in Neuroinflammation: The Role of the CB2 Receptor in Anti-Inflammatory Signaling

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Mikroglijos fenotipo reguliacija neuroūždegimo metu: CB2 receptoriaus reikšmė priešuždegiminiam atsakui

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ABBREVIATIONS

2-AG	–	2-arachidonoylglycerol
AC	–	Adenylate cyclase
AEA	–	Anandamide
AMPA	–	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Arg1	–	Arginase 1
BBB	–	Blood-brain barrier
BDNF	–	Brain-derived neurotrophic factor
BHB	–	β-hydroxybutyrate
cAMP	–	Cyclic adenosine monophosphate
CB1R	–	Cannabinoid receptor type 1
CB2R	–	Cannabinoid receptor type 2
CBD	–	Cannabidiol
COX	–	Cyclooxygenase
CREB	–	cAMP response element-binding protein
CSF1R	–	Colony-stimulating factor 1 receptor
CX3CR1	–	C-X3-C motif chemokine receptor 1
DAGL	–	diacylglycerol lipase
DAMP	–	Damage-associated molecular pattern
DIO	–	Diet-induced obesity
DMEM/F-12	–	Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12
E	–	Embryonic day
eCBS	–	Endocannabinoids
ECS	–	Endocannabinoid system
EMP	–	Erythromyeloid progenitors
ERK	–	Extracellular signal-regulated kinase
FAAH	–	Fatty acid amide hydrolase
GAPDH	–	Glyceraldehyde-3-phosphate dehydrogenase
GPCR	–	G protein-coupled receptors
HBSS	–	Hank's Balanced Salt Solution
Iba-1	–	Ionized calcium-binding adapter molecule 1
IFNγ	–	Interferon γ
IKK	–	IκB kinase
IL	–	Interleukin
iNOS	–	Nitric oxide synthase
JAK/STAT	–	Janus kinase/signal transducer and activator of transcription

JNK	–	c-Jun NH2-terminal kinase
LPS	–	Lipopolysaccharide
MAGL	–	Monoacylglycerol lipase
MAPK	–	Mitogen-activated protein kinase
mTOR	–	Mammalian target of rapamycin
NAPE-PLD	–	N-acyl-phosphatidylethanolamine-specific phospholipase D
NF-κB	–	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLRP3	–	NOD-like receptor family pyrin domain containing 3
NO	–	Nitric oxide
NOX2	–	NADPH oxidase 2
PAMP	–	Pathogen-associated molecular pattern
PBS	–	Phosphate-buffered saline
PI3K/AKT	–	Phosphatidylinositol 3-kinase/Protein kinase B pathway
PP2A	–	Protein phosphatase 2A
PRR	–	Pattern recognition receptor
PSD95	–	Postsynaptic density protein 95
ROS	–	Reactive oxygen species
TGF-β	–	Transforming growth factor β
THC	–	Δ9-tetrahydrocannabinol
TLR	–	Toll-like receptor
TNF-α	–	Tumor necrosis factor-alpha
VEGF	–	Vascular endothelial growth factor
YS	–	Yolk sac

INTRODUCTION

The endocannabinoid system (ECS) is a complex regulatory system that modulates numerous physiological processes across the nervous, immune, and gastrointestinal systems (Șerban M et al., 2025). It comprises cannabinoid receptors, endogenous cannabinoids (eCBS), and the enzymes responsible for their synthesis and metabolism (Rathod SS et al., 2023). Among its receptors, cannabinoid receptor type 2 (CB2R) is predominantly expressed in immune cells and is also found in microglia, where it contributes to the regulation of inflammatory responses and microglial activation (Sobue A et al., 2024). CB2R has gained increasing attention as a potential regulator of neuroinflammation, as cannabinoids have been shown to reduce microglial pro-inflammatory responses and promote a shift toward an anti-inflammatory phenotype (Saito VM et al., 2012; Young AP & Denovan-Wright EM, 2022). As the resident immune cells of the CNS, microglia are key regulators of brain homeostasis and neuropathology (Le Thuc O et al., 2015). However, under prolonged stimulation, microglia can shift from a surveillant state toward a reactive phenotype, characterized by amoeboid morphology and increased production of pro-inflammatory cytokines. This sustained activation may contribute to chronic neuroinflammation, neuronal damage, and neurodegeneration (Levtova N et al., 2017; Wang WY et al., 2015; Tong L et al., 2017). Neuroinflammation itself is a complex response of the CNS to stimuli such as trauma, infection, or neurodegenerative disease (Adamu A et al., 2024). Although initially protective, prolonged or dysregulated neuroinflammation can damage neural tissue (Le Thuc O et al., 2015). Chronic neuroinflammation is characterized by sustained microglial activation, increased oxidative stress, and the release of inflammatory mediators (Leszek J et al., 2016). Because effective therapeutic strategies for chronic neuroinflammatory conditions remain limited, increasing attention has been given to lifestyle-based approaches, including dietary interventions (Thompson B et al., 2016; Kempermann G, 2019; Ornish D et al., 2024).

β-hydroxybutyrate (BHB), a ketone body produced during ketosis and intermittent fasting, has attracted interest due to its anti-inflammatory potential and beneficial effects in neuroinflammatory conditions (Newman JC and Verdin E, 2017; Yamanashi T et al., 2017; Polito R et al., 2023; Jiang Z et al., 2022; Jin LW et al., 2023). Although BHB has been shown to modulate microglial activity, and pathways such as the NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling have been implicated, the receptor-dependent mechanisms linking BHB to microglial inflammatory

regulation remain insufficiently understood (Fu SP et al., 2015; Youm YH et al., 2015). In this context, cannabinoid signaling, particularly through CB2R, is a relevant pathway given its established role in regulating neuroimmune responses. Although synthetic cannabinoid receptor agonists are available (Schimrigk S et al., 2017; Abu-Sawwa R et al., 2020; Patti F et al., 2022), their use is often limited by adverse effects (Prieto González JM and Vila Silván C, 2021). Therefore, it remains important to identify compounds that modulate anti-inflammatory cannabinoid-related pathways without directly activating cannabinoid receptors, and BHB may be one such candidate.

In this study, we hypothesized that BHB exerts anti-inflammatory effects through CB2R signaling in microglia. Using a mouse model of diet-induced obesity (DIO), which is considered a model of low-grade chronic inflammation (Avtanski D et al., 2019; Kiran S et al., 2021; Mandal M et al., 2025), we assessed microglial function, as well as CB2R and monoacylglycerol lipase (MAGL) expression, and pro-inflammatory cytokine expression in the mouse brain. To further investigate the role of CB2R, we developed a primary microglia isolation protocol from mice and performed *in vitro* experiments using stimulated cells with lipopolysaccharide (LPS) and evaluated the effects of BHB and CB2R antagonism on phagocytosis, reactive oxygen species (ROS) production, pro- and anti-inflammatory gene expression, as well as NF-κB signaling. Finally, phospho-antibody microarray analysis was performed to identify signaling pathways involved in the effects of BHB and CB2R modulation in microglia. These findings provide insight into the interaction between BHB and CB2R signaling in regulating microglial function and highlight its relevance to targeting neuroinflammation.

The **aim** of this study was to assess the role of CB2R signaling in mediating the anti-inflammatory effects of BHB in microglia.

To achieve this aim, the following **objectives** were set:

1. To comprehensively characterize the effect of BHB on reactive microglial phenotype at both cellular and molecular levels in a DIO mouse model, including the assessment of CB2R and MAGL expression profiles.
2. To optimize and rigorously validate a reliable protocol for the isolation of primary microglia for downstream *in vitro* applications.
3. To investigate the effects of BHB on microglial functional activity and gene expression in an *in vitro* model of LPS-induced inflammation, with particular emphasis on CB2R-mediated mechanisms.
4. To delineate the signaling pathways underlying BHB action and to determine how CB2R antagonism modulates BHB-induced signaling responses.

SCIENTIFIC NOVELTY

Microglial dysregulation is increasingly recognized as an important contributor to neuroinflammation and neurodegenerative disease. Although BHB has been shown to exert anti-inflammatory effects in microglial cells, the precise molecular mechanisms underlying these effects remain only partially understood (Fu SP et al., 2014; Polito R et al., 2023). At the same time, CB2R is considered an important regulator of microglial activation and a promising target in neuroinflammatory conditions (Komorowska-Müller JA & Schmöle JC, 2020; Reusch N et al., 2022).

This thesis investigated the interaction between BHB and CB2R as a potential mechanism regulating microglial phenotype under inflammatory conditions. A combined evaluation of BHB-mediated metabolic signaling and CB2R-related regulation of microglial responses remains underexplored in the current literature (Komorowska-Müller JA & Schmöle JC, 2020; Polito R et al., 2023). In this work, a mouse model of DIO and primary microglial cultures were used to evaluate both *in vivo* and *in vitro* effects of BHB on microglial state and function. Using both approaches, this thesis provides a novel characterization of the molecular mechanisms underlying BHB's anti-inflammatory effects in microglia, with particular emphasis on the involvement of CB2R-related signaling. In addition, it broadens the current understanding of BHB action by identifying intracellular signaling pathways associated with its effects using a phosphorylation-related protein array analysis. This part of the work represents a particularly novel aspect of the study, as the signaling pathways underlying BHB effects in inflammatory microglia, especially in relation to CB2R blockade, have not been comprehensively examined. These data expanded the mechanistic understanding of BHB action beyond functional readouts, revealing pathway-level changes related to inflammatory signaling, survival responses, and metabolic regulation – processes relevant to microglial activation.

Taken together, the results of this thesis expand current knowledge of the links between metabolic regulation and endocannabinoid signaling in microglia. The practical value of this study lies in its relevance to future research aimed at developing metabolic and cannabinoid-related strategies to modulate neuroinflammation.

STATEMENTS TO DEFEND

1. BHB supplementation induces beneficial phenotypic and functional changes in microglial cells in the DIO model, accompanied by modulation of key components of the endocannabinoid system.
2. Neonatal primary microglia represent a robust and reliable *in vitro* model for elucidating the molecular mechanisms underlying microglial activation and anti-inflammatory modulation.
3. BHB treatment exerts pronounced anti-inflammatory effects in microglial cell cultures, indicating its modulatory potential on neuroinflammatory responses.
4. Pharmacological antagonism of the CB2 receptor abrogates the beneficial effects of BHB in primary microglial cultures, highlighting a CB2-dependent mechanism of action.

1. LITERATURE OVERVIEW

1.1. The endocannabinoid system

1.1.1. ECS structure

The ECS was identified through studies of phytocannabinoids such as Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD) and has attracted considerable scientific interest due to its modulatory role in the CNS. It consists of three major components: the eCBs, the cannabinoid receptors to which they bind, and the enzymes involved in their synthesis and degradation (Figure 1.1).

ECS includes two main G protein-coupled receptors (GPCRs), CB1R and CB2R, as well as orphan receptors such as GPR3, GPR6, GPR12, GPR55, and GPR18. eCBs are lipid-soluble molecules secreted on demand in response to increased intracellular Ca^{2+} concentration (Castillo PE et al., 2012). The most common eCBs are anandamide (AEA) and 2-arachidonoylglycerol (2-AG) (Mechoulam R et al., 1995; Sugiura T et al., 1995; Stella N et al., 1997). AEA is a high-affinity, partial agonist of CB1R, and almost inactive at CB2R; whereas 2-AG acts as a full agonist at both cannabinoid receptors with moderate-to-low affinity (Di Marzo V and De Petrocellis L, 2012). In addition to eCBs, exogenous cannabinoids, such as the plant-derived compounds THC and CBD, can interact with cannabinoid receptors and elicit psychoactive effects (Crocq MA, 2020).

In addition, the ECS comprises biosynthetic and degradative enzymes that tightly regulate eCB's levels. AEA is synthesized from *N*-acyl-phosphatidylethanolamine (NAPE) by NAPE-specific phospholipase D (NAPE-PLD) and degraded by fatty acid amide hydrolase (FAAH) into free arachidonic acid and ethanolamine. 2-AG is synthesized from diacylglycerol (DAG) by either DAG lipase (DAGL) α or β. However, most, if not all, 2-AG in the adult brain is generated by DAGLα (Murataeva N et al., 2014) and degraded by monoacylglycerol lipase (MAGL) to arachidonic acid and glycerol (Lu HC & Mackie K, 2016; Young AP & Denovan-Wright EM, 2022) (Figure 1.1).

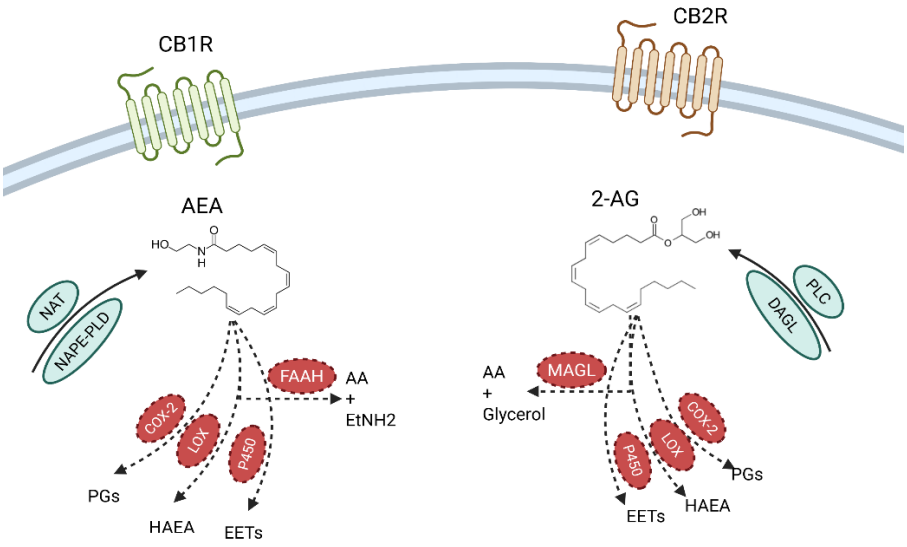


Figure 1.1. The components of ECS. The two principal bioactive lipids (AEA, 2-AG), the main receptors (CB1R, CB2R), and the biosynthetic (NAPE-PLD or DAGL) and catabolic enzymes (FAAH, MAGL, or alternative oxidizing enzymes) are shown. Created with BioRender according to Iozzo M et al., 2021.

1.1.2. Cannabinoid receptors

As mentioned before, CB1R and CB2R are the most characterized cannabinoid receptors, and both are class A seven-transmembrane GPCRs that primarily couple to inhibitory G proteins (Reggio PH, 2010). These receptors are encoded by the *CNR1* and *CNR2* genes, respectively. However, several orphan receptors (see Table 1.1 for details) have been proposed as potential cannabinoid receptors primarily based on phylogenetic arguments and reactivity towards known eCBs and phytocannabinoids (Biringer RG, 2021) (Table 1.1).

- CB1R: These are multifunctional receptors that present different functions depending on their location. They are particularly enriched in the nervous system but are also present in diverse organs, including the liver, adipose tissue, and skin (Lu HC and Mackie K, 2021):

I. Liver: CB1R activation promotes *de novo* lipogenesis and contributes to hepatic steatosis and insulin resistance, while also being associated with chronic inflammation, fibrosis, and cirrhosis (Osei-Hyiaman D et al., 2005; Cooper ME & Regnell SE, 2013).

II. Adipose tissue: CB1R is expressed in both mature adipocytes and preadipocytes from visceral and subcutaneous depots, where it regulates energy homeostasis, lipolysis, adipocyte differentiation, and inflammatory processes (Engeli et al., 2005; Roche et al., 2006; Sidibeh et al., 2017)

III. Skin: CB1R is present in cutaneous sensory nerve fibers, hair follicles, sebaceous glands, and sweat glands, suggesting roles in epidermal homeostasis, hair growth regulation, and sebaceous gland function (Ständer et al., 2005; Bíró et al., 2009; Telek et al., 2007).

IV. CNS: CB1R is localized primarily in areas controlling higher-order functioning, such as the hippocampus (memory and learning), the basal ganglia (motor control), the cerebellum (coordination), the prefrontal cortex (decision making) and in the hypothalamus (regulation of food intake) (Maroto IB et al., 2023; Aguiar AFL et al., 2023; Pucci M et al., 2021). CB1R is predominantly expressed at presynaptic neuronal terminals (Nyíri G et al., 2005), reflecting its major role in modulating synaptic transmission; however, they are also expressed at functionally important levels on neuronal soma and dendrites (Bacci A et al., 2004; Kreitzer AC et al., 2002; Maroso M et al., 2016) as well as on mitochondria (Bénard G et al., 2012). At presynaptic terminals, CB1R suppresses neurotransmitter release at both gamma-aminobutyric acid -ergic neurons and glutamatergic synapses (Howlett AC et al., 2002). However, CB1Rs are found on a wide range of other neurons, including cholinergic, glycinergic, and serotonergic neurons, throughout the brain (Hu SS and Mackie K, 2015). In addition to neurons, CB1R is expressed in astrocytes, oligodendrocytes, and microglia. It has been shown to mediate synaptic transmission (Zou S & Kumar U, 2018) and inflammation by suppressing the expression of IL-1β and tumor necrosis factor (TNF)-α (Șerban M et al., 2025).

- CB2R was initially characterized as a peripheral cannabinoid receptor, with high expression in immune tissues and cells. Early studies reported abundant CB2R expression in the spleen, tonsils, thymus, and peripheral blood mononuclear cells, while expression in most non-immune tissues was low or undetectable. At the cellular level, CB2R was associated with immune cell populations including B lymphocytes, T lymphocytes, natural killer cells, dendritic cells, and neutrophils (Munro S et al., 1993; Galiègue S et al., 1995). This distribution pattern led to the early view of CB2R as an immune-associated receptor. Additionally, subsequent studies showed that CB2R is not restricted to peripheral immune compartments. Distinct CB2R isoforms, including CB2A and CB2B, have been described, with tissue-specific expression patterns: CB2A is reported to be higher in the testis and

CNS, and CB2B is more abundant in the spleen and other peripheral tissues (Liu QR et al., 2009).

Table 1.1 ECS-associated receptors, their main locations, and functions

Receptor name	Location	Function	References
GPR55	Bone marrow Lymphoid tissues Blood Gastrointestinal tract	Inflammation Insulin secretion Metabolic regulation Gut-brain signaling	Uhlén M et al., 2015; Saliba SW et al., 2018; Tudurí E et al., 2017; Balenga NA et al., 2011; Farah SI et al., 2022; Simard M et al., 2022
GPR18	Blood Lymphoid tissues T cells B cells Natural killer (NK) cells Neutrophils Macrophages Brain Gastrointestinal tract	Immune regulation Resolution of inflammation Metabolic regulation Gut-brain signaling	Uhlén M et al., 2015; Kohno M et al., 2006; Console-Bram L et al., 2014; Zhang L et al., 2019; Lopategi A et al., 2019; Burstein SH et al., 2011; Recchiuti A and Serhan CN, 2012; Takenouchi R et al., 2012; Farah SI et al., 2022; Simard M et al., 2022
GPR3	Brain Oocytes	Neuronal differentiation Neurite outgrowth Neuronal survival Axonal regeneration Maintenance of meiotic arrest	Tanaka S et al., 2007; Laun AS et al., 2019
GPR6	Brain	Neuronal function Neuroprotection	Benoit ME et al., 2013; Laun et al., 2019
GPR12	Brain	Neurite outgrowth Cell proliferation, Cell survival	Hinckley M et al., 2005; Lu X et al., 2012; Laun et al., 2019

Although CB2R expression in the CNS is markedly lower than that of CB1R, its presence in certain CNS cell populations has been reported. Studies have

shown that CB2R is expressed in microglia, astrocytes, and some neuronal populations, particularly under pathological conditions, although lower basal expression has also been reported (Quintero JM et al., 2024; Gong JP et al., 2006; Benito C et al., 2003; Lanciego JL et al., 2011; Li Y and Kim J, 2015; Stempel AV et al., 2016). Notably, CB2R is considered a highly inducible receptor, as its mRNA expression increases significantly following nerve injury and during inflammatory conditions (Maresz K et al., 2005; Hsieh et al., 2011). In the CNS, CB2R is predominantly expressed in microglia, the resident macrophage-like immune cells of the brain, where it mediates immunosuppressive and anti-inflammatory effects (Rakotoarivelo V et al., 2024; Howlett AC et al., 2002; Stella N, 2010). CB2R expression appears to increase in response to inflammatory and neurodegenerative conditions, supporting its role in neuroimmune regulation rather than classical neurotransmission (García-Gutiérrez MS et al., 2018; Navarrete F et al., 2018; Komorowska-Müller JA et al., 2021; Vijaya AK et al., 2025). Thus, CB2R is now recognized as a receptor expressed in both the periphery and the CNS, with particular relevance to immune and neuroinflammatory processes.

1.1.3. CB2R signaling

CB2R is involved in activating signaling cascades that include adenylate cyclase (AC) and cyclic adenosine monophosphate (cAMP), mitogen-activated protein kinase (MAPK), and modulation of intracellular calcium levels (Demuth DG & Molleman A, 2006; Zoratti C et al., 2003). CB2R ligands, including the eCBs 2-AG and AEA, as well as synthetic CB2R-selective agonists, such as JWH-133 (Malfitano AM et al., 2014), bind to CB2R and induce a conformational change in the receptor. This promotes the exchange of GDP for GTP on the receptor-coupled heterotrimeric G protein, leading to dissociation of the G protein into Gα and Gβγ subunits (Zhang M et al. 2024). CB2R signaling is primarily mediated by the Gαi subunit (Demuth DG & Molleman A, 2006). While both Gαi and Gβγ can alter intracellular signaling pathways, the βγ subunits are thought to participate in signaling pathways distinct from those of the α subunit, such as regulation of phospholipase C isoforms and activation of the MAPK signaling network (Howlett AC and Mukhopadhyay S, 2000). CB2R partly exerts its effects through initiation of phospholipase C and inositol 1,4,5-trisphosphate signaling pathways that result in increased levels of intracellular calcium (Zoratti C et al., 2003)

The relationship between the Gαi subunit's AC and CB2R receptor activation is of interest (Bala K et al., 2024). AC is inhibited by CB2R activation, thereby

decreasing cAMP levels (Turcotte C et al., 2016). This, in turn, inhibits protein kinase A and prevents the induction of inflammatory genes, such as TNF-α, inducible nitric oxide synthase (iNOS), interleukin (IL)-6, IL-1β, and cyclooxygenase (COX)-2 (Wang F et al., 2019; Amenta PS et al., 2014; Javed H et al., 2016).

CB2R modulates additional signaling pathways, including phosphoinositide 3-kinase/protein kinase B (PI3K/AKT), NF-κB, janus kinase/signal transducer and activator of transcription (JAK/STAT), and cAMP response element-binding protein (CREB)-related signaling, as well as calcium and potassium ion channel activity; these pathways are involved in the regulation of cell survival, inflammation, and immune response (Haspula D and Clark MA, 2020; Rakotoarivelo V et al., 2024). However, the signal depends strongly on both the cell type and the stimulus (Figure 1.2).

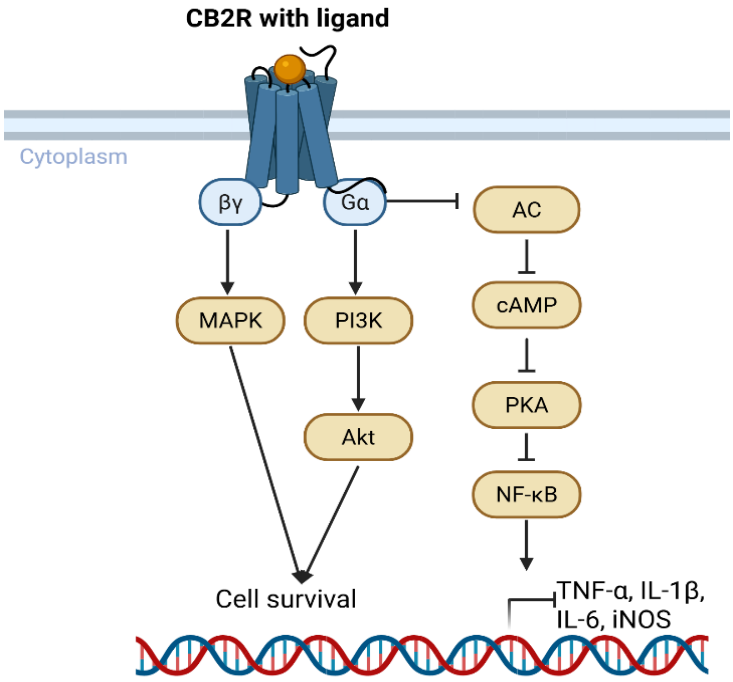


Figure 1.2. Schematic summary of CB2 receptor signaling. Activation of CB2R is coupled to several cellular pathways, including AC, cAMP, protein kinase A, MAPK, and AKT. Stimulatory effects are shown by the (→) sign and inhibitory effects by the (⊥) sign.

1.1.4. Anti-inflammatory effects of CB2R

In the CNS, CB2R is known to hyperpolarize neuronal membrane potential, inhibit postsynaptic neuronal function, and reduce neuronal excitability. These effects have been shown to modulate reward neuronal networks involved in food and drug-seeking behaviors, which finally affect body weight, depression, anxiety, and schizophrenia-like behavior (Chen DJ et al., 2017). Most importantly, CB2R is expressed in the CNS across various inflammatory states. This expression has been localized primarily to microglia and is detected upon their activation by various insults and stimuli (Cabral GA and Griffin-Thomas L, 2009). CB2R is best known for its anti-inflammatory and immunomodulatory effects, where its activation is commonly associated with suppression of neuroinflammatory responses (Komorowska-Müller JA & Schmöle AC, 2020). This effect is thought to occur through the suppression of immune cell activation and reduced production of pro-inflammatory cytokines and chemokines, which together limit the amplification of inflammatory cascades and the recruitment of additional immune cells to the site of injury (Rakotoarivelo V et al., 2024). In parallel, CB2R signaling promotes a more resolving environment by enhancing anti-inflammatory mediators such as IL-10 and by shifting immune responses away from pro-inflammatory profiles (Cabral GA and Griffin-Thomas L, 2009; Turcotte C et al., 2016). Accordingly, studies generally show that loss of CB2R worsens inflammatory outcomes, whereas pharmacological activation of CB2R tends to alleviate inflammatory damage, supporting its role as an endogenous regulator that restrains excessive inflammation (Turcotte C et al., 2016).

Although CB2R activation has shown beneficial effects in a wide range of inflammatory disorders, including rheumatoid arthritis, atherosclerosis, ischemia-reperfusion injury, colitis, and neuroinflammatory conditions, these observations collectively point to a broader principle rather than to isolated disease-specific effects: CB2R signaling generally acts to restrain excessive immune activation and promote a more balanced inflammatory response (Turcotte C et al., 2016; Rakotoarivelo V et al., 2024). This is consistent with evidence showing that cannabinoids can suppress antibody production, reduce cytotoxic immune responses, and shift cytokine profiles away from pro-inflammatory mediators such as interferon γ (IFNγ) and IL-12 toward a more regulatory environment enriched in IL-10 and transforming growth factor β (TGF-β) (Cabral GA & Griffin-Thomas, 2009; Massi P et al., 2000; Klein TW et al., 2000; Zhu LX et al., 2000). At the same time, CB2R signaling cannot be regarded as uniformly suppressive, since endogenous ligands such as 2-AG have also been linked to immune cell migration, chemokine production, and

proliferation, suggesting that CB2R not only limits inflammation but also helps coordinate immune-cell positioning and functional adaptation when required (Jorda MA et al., 2002; Reyman N et al., 2004; Kishimoto S et al., 2003; Kishimoto S et al., 2004; Carrier EJ et al., 2004). Thus, rather than functioning as a purely anti-inflammatory receptor, CB2R is better understood as a context-dependent immunomodulatory regulator that shapes the intensity, quality, and resolution of immune responses. In neuroinflammatory settings, this dual role is particularly important, as CB2R may both attenuate damaging inflammatory cascades and influence the cellular processes that determine how tissue injury evolves or resolves.

1.2. Microglia

Microglia are the resident macrophages of the CNS, serving as the brain's primary innate immune cells that continuously surveil the microenvironment and respond to injury, infection, and cellular debris through phagocytosis and inflammatory signaling (Perry VH and Teeling J, 2013). They account for approximately 0.5% to 16% of all CNS cells, depending on the anatomical region (Franco-Bocanegra et al., 2019), and use their specific receptor repertoire to dynamically monitor the microenvironment (Arcuri C et al., 2017). Microglia are highly motile, phagocytic cells that patrol the CNS parenchyma using lamellipodia, a process driven by actin cytoskeletal remodeling (Franco-Bocanegra DK et al., 2019). Their primary function is to continuously survey the microenvironment and detect perturbations, which may arise from microbial invasion, cellular injury, accumulation of neurotoxic proteins such as β-amyloid, or changes in the levels of inflammatory cytokines and chemokines (Hickman SE & El Khoury J, 2019; Smolders SM et al., 2019). Microglia phagocytose misfolded proteins, cellular debris, and dying cells to maintain homeostasis (Sarlus H and Heneka MT, 2017). Finally, they monitor and protect neuronal function through microglia-neuron crosstalk (Cserép C et al., 2020).

1.2.1. Origin and development of microglia

Microglia were long thought to be of neuroectodermal origin, like other glial cells and neurons. Nevertheless, microglia are a unique lineage of tissue macrophages, and it is now well established that they derive from yolk sac (YS) erythromyeloid precursors (EMPs). These EMPs give rise to YS macrophages, which serve as precursors that inhabit the embryonic brain (Ginhoux F et al., 2010; Kierdorf K et al., 2013). In rodents, hematopoiesis

contains at least three waves (Li Q and Barres BA, 2018). The first wave, termed “primitive” hematopoiesis, begins in the YS blood islands at approximately embryonic day (E) 7.0. Between E7.0 and E8.0, this wave generates primary EMP cells. These primary EMPs in the YS generate YS macrophages, which differentiate into microglia or non-parenchymal macrophages in the CNS and tissue macrophages in the peripheral tissues (Lee E et al., 2021). Primary EMPs express the macrophage colony-stimulating factor 1 receptor (CSF1R) and depend on it for survival and differentiation (Ginhoux F et al., 2010). The second “transient definitive” hematopoietic wave begins in the YS at E8.25, producing c-Myb-dependent, CSF1R-negative EMPs (Hoeffel G et al., 2015). The third “definitive” wave starts at E8.5 in the embryo proper, generating hematopoietic stem cells from hemogenic endothelium in the para-aortic region, which later develops into the aorta, gonads, and mesonephros (Hoeffel G et al., 2015; Ginhoux F and Guilliams M, 2016). Thus, primary EMPs, secondary EMP-derived fetal liver monocytes, and hematopoietic stem cell-derived fetal liver monocytes contribute to all tissue macrophages, with microglia arising solely from primary EMPs (Li Q and Barres BA, 2018).

Then, YS-derived precursors begin to colonize the CNS. At E9.5, microglia precursors infiltrate the brain rudiment, entering the leptomeninges and the lateral ventricles to spread throughout the cortex at varying speeds depending on the region and developmental stage (Ginhoux F et al., 2010; Arnoux I et al., 2013). Blood circulation is required for YS macrophage seeding of the CNS, and between E8.0 and E10.0, blood vessels form and remodel in the mouse embryo, coinciding with the appearance of YS precursors in the embryo (Walls JR et al., 2008). YS EMPs develop into immature precursor cells (A1 stage) and then mature to macrophages (A2 stage). This transition is marked by the loss of c-Kit, a marker of early erythromyeloid/hematopoietic progenitors, and the acquisition of C-X3-C motif chemokine receptor 1 (CX3CR1), which is associated with maturation toward the macrophage/microglial lineage (Kierdorf K et al., 2013). These A2 macrophages enter the developing brain via the pial surface at E9.5 and migrate along the abluminal surface through the vasculature to become microglia (Kierdorf K et al., 2013; Stremmel C et al., 2018; Prinz M et al., 2021).

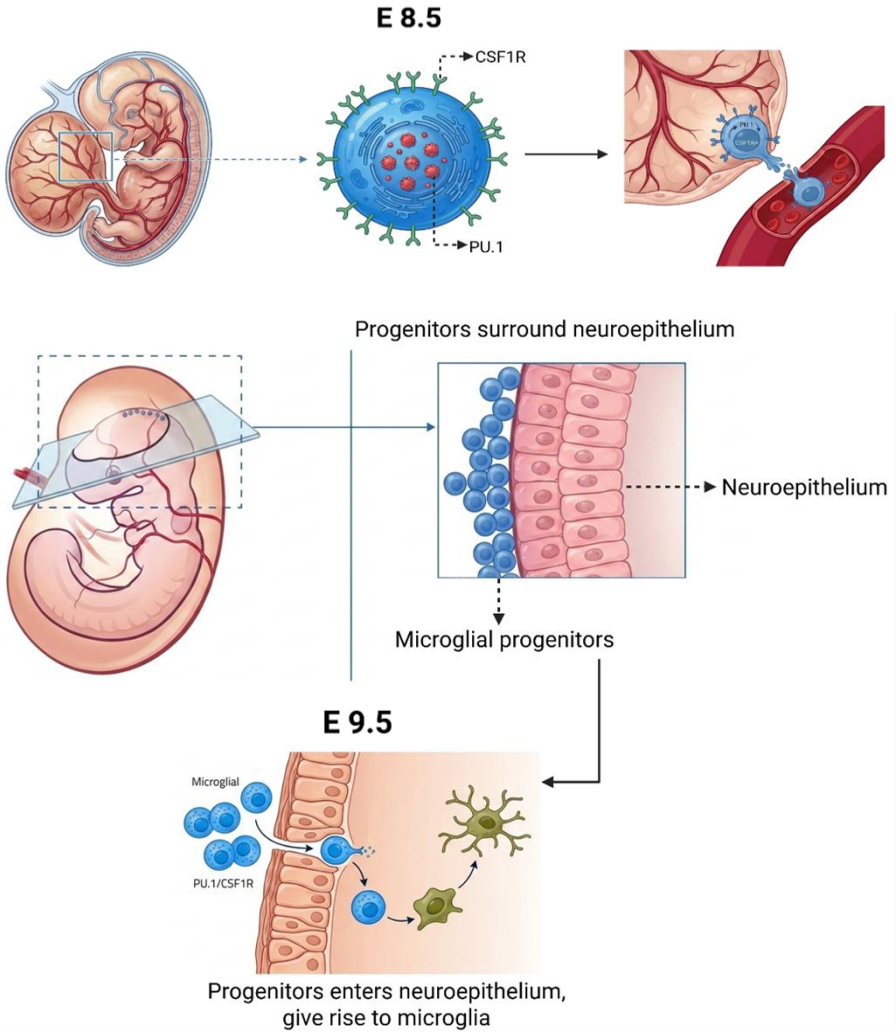


Figure 1.3 Microglial origin and development. Microglia are ontologically distinct from bone marrow-derived monocytes/macrophages and arise from primitive YS-derived macrophages. These progenitors surround the neuroepithelium of the developing brain at E9.5 and, one day later, enter the neuroepithelium, beginning to colonize the CNS parenchyma. Early microglia display an amoeboid morphology and become completely ramified throughout the brain by postnatal day 28. Development and survival of microglia are critically dependent on several factors, including the transcription factor PU.1 and the CSF1R receptor. Created with Google NotebookLM and BioRender according to Nayak D et al., 2014.

Once within the brain parenchyma, microglial precursors receive instructive signals from the CNS environment that aid their differentiation (Swinnen N et

al., 2013). Amoeboid microglial precursors eventually acquire a ramified morphology and cover more of the CNS between E14.5 and the first postnatal week (Li Q and Barres BA, 2018) (Figure 1.3).
During development, microglial numbers fluctuate as they proliferate and populate the brain. Although the number of proliferating microglia decreases between E14.5 and E15.5, a significant increase in the total number of microglial cells is observed during this period, suggesting the possible existence of an additional source of microglial cells that contributes to the resident microglial population (Swinnen N et al., 2013). At least two progenitor pools for microglia have been demonstrated: canonical non-homeobox B8 microglia and homeobox B8 microglia. Homeobox B8 microglia progenitors appear to arise during the second wave of YS hematopoiesis and then enter the aorta, gonads, and mesonephros region and fetal liver, where they expand before migrating into the developing brain at E12.5. Non-homeobox B8 microglia accounts for approximately 70 % of all adult microglia, significantly outnumbering homeobox B8 microglia (De S et al., 2018). After microglial populations are established in the adult brain, resident microglia maintain their cell density primarily through a balance between proliferation and apoptosis. Microglia exhibit strong self-renewal capacity, and evidence indicates they can repopulate from CNS-endogenous cells following global depletion of microglia (Bruttger J et al., 2015; Parkhurst CN et al., 2013).

1.2.2. Functions of microglia during homeostasis

In the healthy brain, microglia maintain a homeostatic state defined by highly motile processes that continuously survey the microenvironment and enable rapid responses to subtle changes. Their functions are multifaceted and essential, encompassing: (1) neurogenesis; (2) synaptic pruning and refinement of neural circuits; (3) regulation of synaptic plasticity; (4) immune surveillance; (5) modulation of inflammation and coordination of tissue repair. Together, these homeostatic activities are fundamental for CNS development, synaptic plasticity, and long-term brain function (Figure 1.4).

- *Neurogenesis:* Microglia are necessary for the proper assembly of neuronal networks. After they migrate to the CNS, microglia guide neurons and axons in the formation of prenatal circuits (Squarzoni P et al., 2015). Augmentation of microglial activity *in utero* demonstrates that microglial deactivation increases the number of neural precursor cells, whereas activation decreases it in the developing cerebral cortex. This indicates that microglia are fundamental in balancing neural precursor proliferation and ensuring proper

cortical development. Additionally, in the developing cerebral cortex, microglia help regulate the size of the neural precursor pool by phagocytosing excess precursor cells as neurogenesis nears completion (Cunningham CL et al., 2013). In the adult brain, microglia also secrete a variety of cytokines and growth factors, such as brain-derived neurotrophic factor (BDNF) and TNF-α, which can either promote or inhibit neurogenesis depending on the physiological or pathological context (Araki T et al., 2021). During aging, progressive microglial activation and increased secretion of pro-inflammatory cytokines create an anti-neurogenic microenvironment, while activated microglia phagocytose apoptotic neural stem cells (Fourgeaud L et al., 2016) and attenuate neural stem cell proliferation (Solano Donseca R et al., 2016).

- *Synaptic pruning:* During postnatal development, microglia eliminate redundant neurons that do not establish functional circuits. Importantly, microglia shape neuronal synapses by phagocytosing dendritic spines that are not receiving synaptic input (Kettenmann H et al., 2013; Tremblay ME et al., 2011). This elimination process is vital for the formation of mature and efficient neuronal circuits during normal brain development (Paolicelli RC et al., 2011). This synaptic striping is achieved through several mechanisms. On the one hand, two complement cascade proteins, C1q and C3, tag unused synapses for microglial recognition via complement receptor 3 or for direct lysis via the complement cascade (Schafer DP et al., 2012; Stevens B et al., 2007). On the other hand, CX3CR1 in microglial cells, which interacts with C-X3-C motif chemokine ligand 1, a transmembrane glycoprotein expressed on the neuronal surface that is also released as a soluble molecule after proteolytic cleavage, could mediate synaptic pruning (Paolicelli RC et al., 2011).
- *Synaptic plasticity*: Microglia impact synapse strength and plasticity through the release of proinflammatory cytokines, ROS, and nitric oxide (NO), as well as neurotrophic factors (Vezzani A and Viviani B, 2015). One type of synaptic plasticity includes long-term potentiation and long-term depression, which promote rapid adjustment in the strength of individual synapses in response to specific temporal patterns of synaptic activity (Paulsen O and Sejnowski TJ, 2000). In that context, microglia enhance glutamate-induced long-term depression (Collingridge GL, Peineau S, 2014) through microglial N-methyl-D-aspartate receptors. Neuronal glutamate release activates those receptors, triggering reduced nicotinamide adenine dinucleotide phosphate oxidase-dependent ROS production, which ultimately induces α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor internalization and weakens excitatory synaptic transmission via activation of serine/threonine protein phosphatase 2A (PP2A) in neurons.

Likewise, hypoxia and inflammatory stimuli such as LPS synergistically activate microglial complement receptor 3, inducing the same ROS-PP2A-AMPA receptor endocytosis cascade and thereby reducing synaptic efficacy (Zhang J et al., 2014). In contrast, microglia can also promote synaptic plasticity through the PI3K/BDNF pathway, as microglia-derived BDNF enhances dendritic spine plasticity in the adult cortex (Rauti R et al., 2020; Saw G et al., 2020). Thus, microglia play an indispensable role in regulating the formation, plasticity, and elimination of synapses throughout development and adulthood. However, under pathological conditions, excessive or aberrant synaptic pruning by maladaptively activated microglia may further exacerbate tissue damage.

- *Immune surveillance*: In the adult brain, microglia maintain tissue homeostasis through continuous surveillance of brain parenchyma. Under physiological conditions, adult microglia display a highly ramified morphology and form an evenly spaced network in which the soma remains relatively immotile, while processes and filopodia constantly extend and retract to sample the surrounding environment (Davalos D et al., 2005; Bernier LP et al., 2019). This dynamic behavior, once mischaracterized as a "resting" state, is now recognized as active surveillance, enabling microglia to monitor neighboring neurons, astrocytes, oligodendrocytes, synapses, and blood vessels (Nimmerjahn A et al., 2005; Davalos D et al., 2005). Motility is tightly regulated by distinct signaling mechanisms, including cAMP-dependent filopodia extension and purinergic receptor P2Y12 (P2RY12)-mediated regulation of larger process dynamics (Bernier LP et al., 2019; Pozner A et al., 2015; Haynes SE et al., 2006). Through these mechanisms, microglia can rapidly detect subtle local perturbations and maintain tissue stability. Microglia also contact synapses, nodes of Ranvier, neuronal soma, and capillaries, reflecting their diverse homeostatic roles (Bisht K et al., 2021; Hristovska I et al., 2022; Cserép C et al., 2020). Importantly, efficient tissue surveillance is required for normal brain function, and defects in the signaling pathways underlying this process have been linked to neurodevelopmental and neurodegenerative disorders (Petry P et al., 2023).

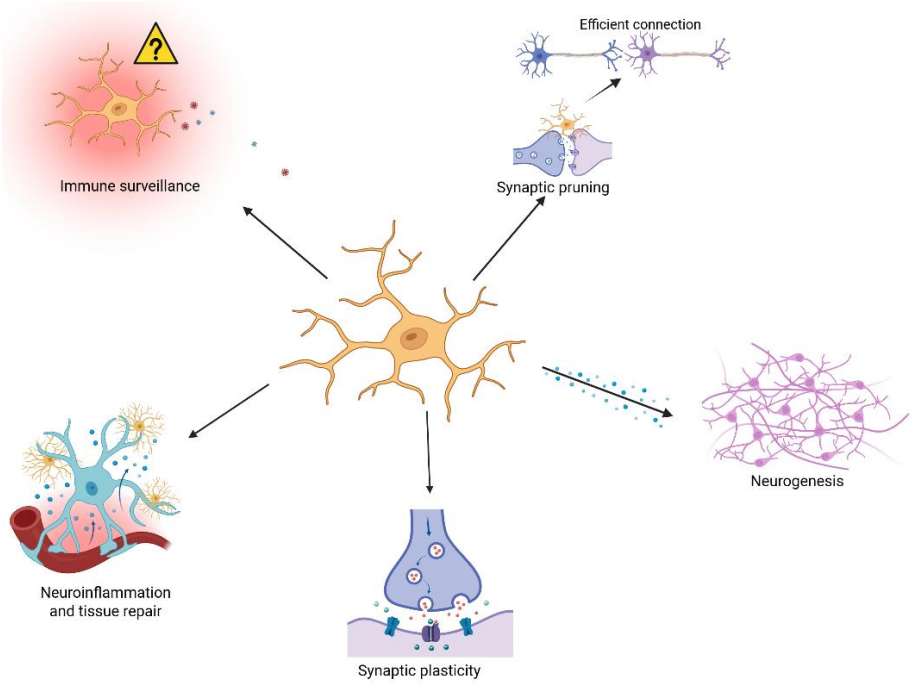


Figure 1.4. Schematic representation of the principal roles of microglia in the CNS. Microglia continuously survey the neural environment to detect disturbances and maintain homeostasis. They also contribute to synaptic pruning, neuronal circuit refinement, synaptic plasticity, neurogenesis, neuroinflammatory regulation, and tissue repair after injury. Through these functions, microglia act as key modulators of CNS development, homeostasis, and response to damage. Created with BioRender.com based on the literature cited in this section.

- *Inflammation and tissue repair:* Additionally, microglia actively modulate inflammation by producing soluble mediators, including cytokines, chemoattractants, and neurotrophic factors, which shape immune responses and contribute to tissue repair within the CNS (Colonna M & Butovsky O, 2017). Their specific functions in neuroinflammation, however, will be discussed in the following chapters. Under conditions that promote the resolution of inflammation and tissue repair following inflammatory insult, microglia can adopt a phenotype characterized by the release of anti-inflammatory cytokines, such as IL-10 and TGF-β, as well as growth and neurotrophic factors, including insulin-like growth factor 1, fibroblast growth factor, colony-stimulating factor 1, nerve growth factor, BDNF, neurotrophin-4/5, and glial cell line-derived neurotrophic factor. By producing these mediators, microglia help establish a microenvironment that is more supportive of tissue recovery and the restoration of CNS homeostasis

(Colonna & Butovsky, 2017). Microglia also coordinate tissue repair by clearing apoptotic cells and cellular debris, thereby facilitating tissue reorganization and initiating regenerative responses after CNS injury (Neumann H et al., 2009). Importantly, defective or insufficient microglial clearance of debris has been linked to impaired regeneration, and experimental evidence shows that reduced microglial phagocytosis of myelin debris disrupts proper remyelination, indicating that the removal of damaged material is required for effective repair (Neumann H et al., 2009; Lampron A et al., 2015). In addition, microglia contribute to recovery after CNS injury by coordinating interactions with other glial cells and infiltrating leukocytes, and depletion studies in spinal cord injury models have shown that the absence of microglia exacerbates tissue damage and worsens functional recovery (Brennan FH et al., 2022).

Overall, microglia are essential regulators of CNS homeostasis. Through their roles in neurogenesis, synaptic refinement and plasticity, tissue surveillance, and clearance of cellular material, microglia help maintain the structural and functional integrity of the brain. Therefore, proper microglial homeostatic activity is critical for normal CNS development, maintenance, and long-term brain function.

1.3. Neuroinflammation

Inflammation is a biological process that occurs in response to injury, infection, or cellular stress. A successful inflammatory response eliminates invading pathogens and initiates tissue repair and angiogenesis (Carson MJ et al., 2006). In the past, CNS was believed to be immune-privileged – not prone to undergo strong inflammation and lacking lymphatic drainage. However, emerging evidence on neuroimmune interactions has challenged the historical notion of the immune-privileged brain, isolated by the blood-brain barrier (BBB) (Gimsa U et al., 2013). It is known that CNS cells are reactive to inflammatory factors, and under certain circumstances, peripheral immune cells can infiltrate the brain, leading to *neuroinflammation*. That term generally refers to the inflammatory response of the CNS to stimuli such as trauma, infection, or neurodegenerative pathology.

Neuroinflammation contributes to normal brain development (Mottahedin A et al., 2017) and homeostasis and is necessary for brain plasticity during critical developmental periods (Chagas LDS et al., 2019). While transient neuroinflammation protects the brain from pathogens and facilitates repair, perpetuating inflammatory processes leads to progressive chronic

inflammatory conditions, primarily stemming from autoimmune or neurodegenerative disorders (Kolliker-Frers R et al., 2021).

Persistent neuroinflammatory activity is a hallmark of multiple neurodegenerative and neurodevelopmental diseases, including multiple sclerosis, Alzheimer's disease, Parkinson's disease, narcolepsy, and autism spectrum disorders (Carson MJ et al., 2006; Kunevičius A et al., 2025).

As the principal innate immune cells of the CNS, microglia play a central role in detecting, containing, and helping resolve neuroinflammatory events in cooperation with other glial cells. In neurodegenerative disorders such as Alzheimer's and Parkinson's disease, microglia initially exert protective functions by recognizing and attempting to clear pathogenic protein aggregates, including β-amyloid and α-synuclein (Clayton KA et al., 2017; Lull ME and Block ML, 2010). However, when these stimuli persist, microglia can remain chronically activated, gradually lose their homeostatic and protective functions, and shift toward a maladaptive state that promotes sustained production of pro-inflammatory cytokines and neurotoxic mediators (McQuade A and Blurton-Jones M, 2019). In this way, microglia may transition from limiting tissue damage to contributing to the progression of neurodegeneration (Stefanis L, 2012).

However, neuroinflammation manifests not only in neurodegenerative diseases but also in low-grade chronic inflammatory states, such as those that occur during aging and in metabolic pathologies, including obesity (Youm YH et al., 2013). Aging is accompanied by a persistent low-grade inflammatory state, known as *inflammaging*, which contributes to functional decline and increased susceptibility to age-related pathology (Baechle JJ et al., 2023; Vijaya AK et al., 2024). In the CNS, this process is closely linked to microglia, which are regarded as key mediators of neuroinflammaging. With age, microglia acquire a primed or dysregulated phenotype characterized by exaggerated inflammatory responses, impaired homeostatic functions, and reduced capacity to resolve inflammatory challenges (Spittau B, 2017). As a result, aged microglia contribute to the maintenance of chronic neuroinflammation (Soraci L et al., 2024). Additionally, changes during aging lead to increases in circulating C-reactive protein and IL-6 (Onyango IG et al., 2021; Bradburn S et al., 2018) and in the expression of TNF-α and its receptors (Marcos-Pérez D et al., 2020). The neuroimmune interactions between the CNS and the periphery are bidirectional. IL-6, for example, can cross the BBB or gain access to the CNS via regions of increased permeability in the circulating milieu (Harré EM et al., 2003).

Interestingly, obesity is accompanied by low-grade inflammation due to increased infiltration and activation of innate and adaptive immune cells

within peripheral tissues, such as adipose tissue (O'Brien PD et al., 2017; Zatterale F et al., 2020), which can contribute to neuroinflammation (Cai D, 2013; Kempuraj D et al., 2016). This peripheral inflammatory state can contribute to neuroinflammation by increasing circulating cytokines and other inflammatory mediators, disrupting BBB integrity, and promoting microglial and astrocytic activation within the CNS (Guillemot-Legris O et al., 2017)

Under obesity conditions, an increase in free fatty acids results in hypertrophic adipocytes and overproduction of adipokines and cytokines such as TNF-α, INF-γ, IL-1β, IL-6, IL-17, and IL-18, triggering systemic inflammation (Neto A et al., 2023; Wen X et al., 2022; Chemini M et al., 2017). Circulating inflammatory factors can further propagate peripheral inflammation and activate macrophages in other organs such as the liver and pancreas, as well as trigger brain inflammation by disrupting BBB integrity, activating endothelial cells and glial cells, and facilitating the transmission of peripheral inflammatory signals into the CNS (Neto A et al., 2023; Hong YP et al., 2020; Sun Y et al., 2022). Obesity can further increase BBB permeability by promoting systemic inflammation and elevated circulating free fatty acids, which induce endothelial dysfunction and disrupt tight junction proteins that normally maintain barrier integrity, making the brain more vulnerable to inflammation (Feng Z et al., 2024). Additionally, obesity-induced gut dysbiosis can increase intestinal permeability, leading to a “leaky gut” and increased LPS secretion, which can further trigger inflammation and increase BBB leakage (An L et al., 2022; Megur A et al., 2022; Ortiz-Samur NS et al., 2025). These factors collectively propagate inflammation throughout the body, including the brain (Loh JS et al., 2024).

Brain cell response, infiltrating immune cells, and oxidative stress generation. Upon stimulation, microglia release pro- and anti-inflammatory mediators and generate ROS and reactive nitrogen species that influence neuronal and glial function. Several factors, including the nature of the initiating insult, genetic background, environmental influences, age, sex, and prior immune experiences, interact to modulate microglial function and shape the neuroinflammatory response (Bradl M et al., 2003; Carson MJ et al., 2006; Müller L et al., 2025). Thus, neuroinflammation is not a binary phenomenon that can be described as simply present or absent. Rather, it is a highly dynamic and context-dependent process that varies in intensity, duration, and outcome depending on the nature of the stimulus and the CNS environment (DiSabato DJ et al., 2016).

1.3.1. Microglia in neuroinflammatory context

Microglial activation, as the key player in brain inflammation and inflammatory neurodegenerative diseases, is the earliest indicator of neuroinflammation (Harry GJ et al., 2012). Under acute stimulation, they may kill and/or remove pathogens in a neuroprotective role. An efficient, active process for the phagocytosis and clearance of aberrant or excess proteins is necessary to maintain the homeostatic balance of protein burden in the brain and prevent neurodegeneration. Many pro-apoptotic pathways are mediated by signaling molecules overproduced during neuroinflammation, suggesting that neuroinflammation could directly influence neuronal apoptosis (Harry GJ et al., 2008).

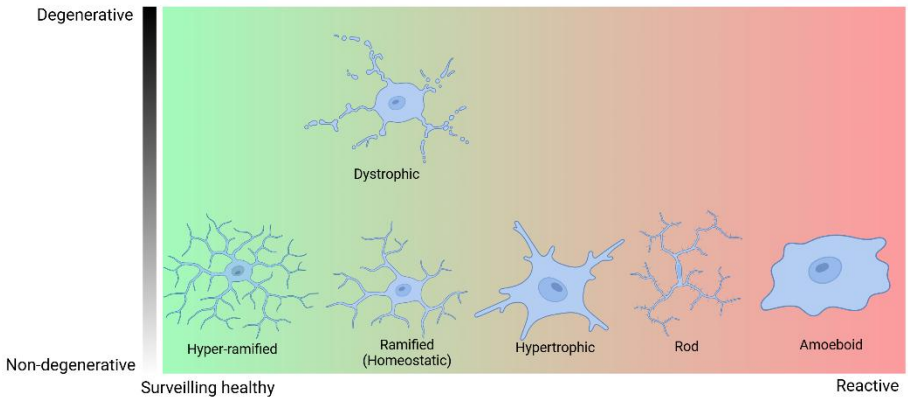


Figure 1.5. Microglial phenotypic states across non-degenerative and degenerative conditions. Schematic representation of microglial phenotypic states distributed along a continuum from non-degenerative to degenerative conditions. On the non-degenerative side, microglia are shown in a surveilling, highly ramified morphology associated with homeostatic function. Moving across the continuum, microglia adopt progressively altered morphologies, including less ramified and reactive-like states, reflecting functional changes in response to environmental stress or pathology. In degenerative conditions, microglia exhibit dystrophic/fragmented and amoeboid-like morphologies, consistent with impaired homeostatic support, enhanced reactivity, and neurodegeneration-associated changes. This schematic illustrates that microglial phenotypes exist along a dynamic spectrum rather than as strictly discrete states. Created with BioRender, according to Murray CJ et al., 2025.

However, after continuous stimulation (i.e., pathogens, tissue damage, neurotoxins, infection, or injury), microglia become overreactive. Reactive microglia are characterized by a round shape, with maladaptive functions and

releasing a variety of oxidants and activating various genes and proteins, including iNOS, pro-inflammatory cytokines like IL-1β, TNF-α, COX-1, -2, as well as ROS synthesis and potentially neurotoxic compounds which cause neuronal dysfunction and cell death (Park SE et al., 2011) (Figure 1.5). They can attack healthy neurons either by phagocytosis or via secreted apoptosis factors. When chronic neuroinflammation is established, these cells can remain reactive for extended periods, releasing cytokines and neurotoxic molecules that contribute to long-term neurodegeneration (Lyman M et al., 2012).

Microglia protect the CNS against injurious stimuli by recognizing pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) through cellular receptors (Glass CK et al., 2010; Stephenson J et al., 2018). This surveillance relies on a diverse repertoire of receptors collectively referred to as the *sensome*. Transcriptomic profiling has revealed the expression of pattern recognition receptors (PRRs), fragment crystallizable receptors, purinergic receptors, extracellular matrix receptors, chemokine and cytokine receptors, and cell-cell interaction receptors (Hickman et al., 2013). Prominent sensome components, including triggering receptor expressed on myeloid cells 2 (TREM2), CX3CR1, and purinergic receptor P2Y12, are highly expressed under homeostatic conditions, enabling microglia to sense environmental cues and transition to a reactive state to mitigate detected threats. Notably, the expression of many of these sensome receptors is downregulated once microglia adopt an over-reactive phenotype (Lively S and Schlichter LC, 2018; Vijaya AK et al., 2024).

Among the most critical receptors driving microglial activation are PRRs, which detect both PAMPs and DAMPs. PAMPs are structural motifs of microbial origin. For example, toll-like receptor (TLR) 4, one of the best-characterized PRRs, recognizes LPS, a hallmark of Gram-negative bacterial infection in the CNS. Conversely, DAMPs are endogenous molecules released during cellular injury. The purinergic receptor P2X7, which extracellular ATP activates, exemplifies this mechanism. Because ATP and related nucleotides are normally intracellular, their presence in the extracellular milieu is interpreted as a danger signal.

In addition to classical microglial receptors, which primarily sense environmental changes, detect danger-associated or pathogen-associated signals, and initiate immune responses, microglia also express CB2R. Unlike many pattern-recognition and immune-activating receptors that promote inflammatory signaling, CB2R is primarily associated with immunomodulatory effects. In microglia, CB2R activation has been shown to shift the phenotype from a pro-inflammatory state toward a more pro-

resolving profile, as evidenced by a reduction of IL-1β and IL-6 expression and iNOS activity, and an increase in the anti-inflammatory marker Arg1 and IL-10 expression (Rodrigues FDS et al., 2024; Ma L et al., 2015; Jiang F et al., 2022). Functionally, CB2R activation can constrain microglial overreactivity by attenuating excessive phagocytosis or maladaptive synaptic pruning (Han QW et al., 2022), reducing the symptomatology of Alzheimer's disease and α-Syn pathology by modulating amyloid β phagocytosis (Ruiz de Martín Esteban S et al., 2022) and the pCREB-c-FOS signaling pathway (Feng L et al., 2023), respectively.
In summary, CB2R has been identified as a promising therapeutic target for conditions characterized by chronic neuroinflammation, as agonists can temper microglial pro-inflammatory responses and may promote a switch to an anti-inflammatory phenotype.

1.3.2. Strategies to mitigate neuroinflammation

Strategies to mitigate neuroinflammation in pathological conditions greatly depend on the nature of the disease. Pharmacological strategies to mitigate neuroinflammation include both approved immunomodulators and experimental anti-inflammatory agents. These include agents that suppress microglial activation and attenuate neuroinflammatory changes (Fan R et al., 2007), modulators of sphingosine-1-phosphate receptor signaling that reduce immune-cell trafficking to the CNS and can also dampen microglial activation and pro-inflammatory cytokine production (Jackson SJ et al., 2011; Hunter SF et al., 2016), inhibitors of IL-1 signaling that target cytokine-driven inflammation and have been explored in neurological disorders with prominent inflammatory components (Yamanaka G et al., 2021; Lindblad RC et al., 2023), and selective inhibitors of NLRP3 inflammasome activation that block inflammasome-dependent IL-1β release (Coll RC et al., 2015). Although these pharmacological approaches can provide important therapeutic benefits, their broader or long-term application may be limited by adverse effects, variable treatment responses, and the complexity of targeting neuroinflammation across different diseases (Chun J et al., 2021). Therefore, increasing attention has been directed toward non-pharmacological strategies, particularly lifestyle-related interventions, as complementary or preventive approaches to reduce neuroinflammatory burden (Kip E and Parr-Brownlie LC, 2023). For example, physical activity is increasingly recognized as an important lifestyle factor influencing neuroinflammatory processes. Studies have shown that exercise can suppress microglial activation by reducing expression of pro-inflammatory mediators such as IL-1β, TNF-α, C-C motif

chemokine ligand 2, and C-X-C motif chemokine ligand 10, and enhancing anti-inflammatory and neuroprotective factors, including CD200-CD200R signaling, TREM2, heat-shock proteins, metabolic regulators, antioxidants, and neurotrophic factors such as BDNF and tropomyosin receptor kinase B (TrkB) (Real CC et al., 2017; Kim TW et al., 2020; Kim TW et al., 2016; Spencer JS et al., 2017). Although physical activity is a well-supported strategy to reduce neuroinflammatory tone, it is not always feasible or sufficient due to limitations such as disability, chronic illness, or advanced age (Wildekamp M et al., 2024; Meredith SJ et al., 2023). Therefore, complementary lifestyle approaches are gaining attention, particularly dietary interventions that can be implemented more consistently.

Caloric restriction is associated with slower memory decline (Mela V et al., 2025; Gomes Gonçalves N et al., 2024) and attenuates neuroinflammatory responses by reducing microglial activation (Yin Z et al., 2018; Wang R et al., 2021). Intermittent fasting, as a form of caloric restriction, reduces oxidative stress markers (Ebrahim HA et al., 2021), astrocytic apoptosis and microglial activation, and reduces memory deficits (Azam AA and Sharif R, 2020; Lee N et al., 2024). Importantly, both caloric restriction and intermittent fasting promote a metabolic shift from glucose utilization to fatty acid oxidation, leading to increased ketone body production, including BHB, which may contribute to their neuroprotective and anti-inflammatory effects (Mela V et al., 2025; Mattson MP et al., 2020). Moreover, ketosis may benefit the brain by providing ketone bodies as an efficient alternative fuel that supports neuronal function and brain energy metabolism (Griss EC et al., 2019). Thus, long-term caloric restriction confers greater systemic metabolic benefits, including improved synaptic function, neuronal insulin signaling, neurogenesis, and increased mitochondrial and autophagic function, compared to exercise (Pantiya P et al., 2023).

Ketone bodies are small, lipid-derived molecules synthesized primarily in the liver from fats that circulate through the bloodstream and that provide energy to tissues when glucose is scarce. During fasting, after muscle and liver glycogen stores are depleted, fatty acids are mobilized from adipocytes and transported to the liver for conversion into ketone bodies. Ketone bodies are then distributed via the bloodstream to metabolically active tissues, such as muscle and brain, where they are metabolized into acetyl-CoA and, ultimately, ATP (Newman JC & Verdin E, 2017). Among the ketone bodies, BHB is the most abundant in circulation during ketosis (Yao A et al., 2021; Pali DV et al., 2025). In addition to its role as an energy substrate, BHB has been shown to act as a signaling molecule that modulates cellular stress and inflammatory responses. Emerging evidence shows that BHB has a variety of signaling

functions that can affect gene expression, lipid metabolism, neuronal function, and metabolic rate. Some of these effects are direct actions of BHB itself, and some are indirect effects governed by downstream metabolites into which BHB is converted, such as acetyl-CoA. (Newman and Verdin, 2017). Beyond its role as an alternative energy substrate, BHB is increasingly recognized as a signaling metabolite with important immunomodulatory functions.

BHB may regulate inflammatory responses through several complementary mechanisms, including direct inhibition of class I histone deacetylases, β-hydroxybutyrylation-mediated regulation of gene expression, activation of the GPCR hydroxycarboxylic acid receptor 2, suppression of NF-κB signaling, and inhibition of NLRP3 inflammasome activation. Through these pathways, BHB has been associated with reduced expression or release of pro-inflammatory mediators and, in some contexts, with the induction of anti-inflammatory or reparative programs (Fu SP et al., 2015; Fu SP et al., 2014; Youm YH et al., 2015). Collectively, these findings support the view that BHB is not merely a metabolic by-product of ketosis but a bioactive immunometabolic regulator capable of modulating inflammatory tone across different cell types and tissues. Although BHB is widely regarded as having anti-inflammatory potential, its ultimate effects may depend on the target cell type and whether BHB elevation is endogenous or exogenous (Qi J et al., 2022).

Beyond its metabolic role, BHB has emerged as a potential regulator of neuroinflammatory processes. In microglia, BHB appears to favor a less reactive and more neuroprotective phenotype, as evidenced by the attenuation of inflammatory morphological changes, the suppression of pro-inflammatory cytokine expression, and the enhancement of anti-inflammatory signaling (Polito R et al., 2023; Garcia C et al., 2025). Beyond its relevance to neuroinflammatory conditions, BHB has also been reported to reduce pro-inflammatory responses in experimental models of Parkinson's disease (Fu SP et al., 2015). Studies have shown that BHB can reduce the production of pro-inflammatory cytokines by inhibiting the NLRP3 inflammasome and the NF-κB signaling pathway (Fu SP et al., 2015; Youm YH et al., 2015). Several molecular pathways have been proposed to mediate the effects of BHB on microglial activity, including MAPK, PI3K/Akt, histone deacetylase-related, and cytoskeleton-associated signaling pathways (Shimazu T et al., 2013; Huang C et al., 2016; Neubrand VE et al., 2014; Garcia C et al., 2025) (Fig. 1.6). Since these pathways regulate inflammatory signaling, morphology, and phagocytosis, they may contribute to BHB's immunomodulatory effects in microglia. However, the precise mechanisms through which BHB affects microglial activity remain unclear.

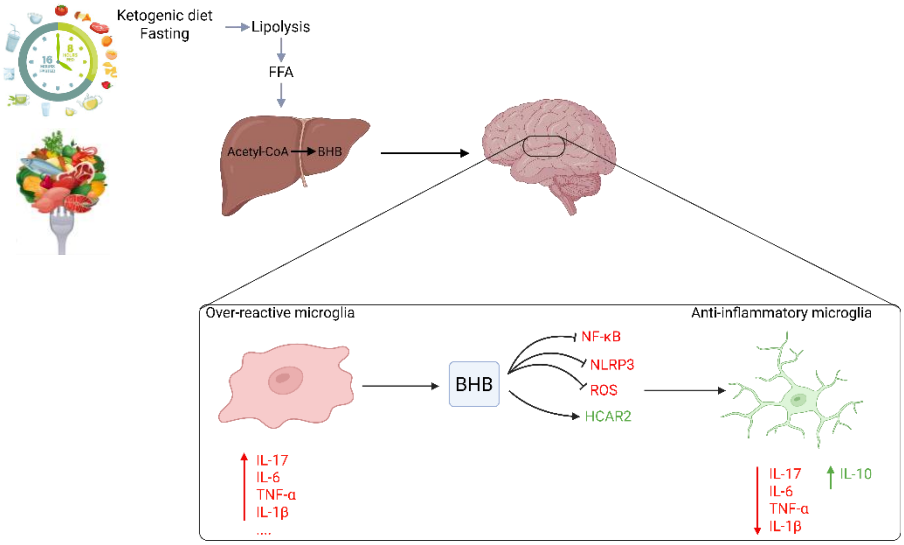


Figure 1.6. Overview of BHB production and its anti-inflammatory effects in microglia. Ketogenic diet and fasting promote a metabolic shift from glucose utilization toward fatty acid oxidation. Increased lipolysis releases free fatty acids, which are converted in the liver into acetyl-CoA and subsequently into BHB. BHB can reach the brain and modulate microglial inflammatory responses by attenuating these responses through suppression of NF-κB activation, NLRP3 inflammasome signaling, and ROS production, as well as via receptor-mediated mechanisms involving hydroxycarboxylic acid receptor 2. These effects may contribute to a shift toward a less inflammatory microglial phenotype, characterized by reduced pro-inflammatory cytokine production and increased anti-inflammatory signaling, including IL-10.

1.3.3. Molecular pathways driving neuroinflammation

Neuroinflammation is orchestrated by a variety of molecular signaling pathways that regulate the activation and function of immune cells in the CNS. Key pathways include the NF-κB signaling cascade, the JAK-STAT pathway, and mitogen-activated protein kinase MAPK signaling. Each of these pathways can be activated by different stimuli, including infections, misfolded proteins, and cellular stress (Kim ME & Lee JS, 2024).

NF-κB signaling pathway

The NF-κB transcription factor family regulates multiple cellular signaling pathways and mediates cellular responses to a broad range of inflammatory stimuli (Liu T et al., 2017). The canonical NF-κB pathway is activated in response to ligands of various cytokine receptors, PRRs, and members of the

TNF receptor superfamily (Zhang H and Sun SC, 2015). Upon exposure to stimuli (PAMPs such as LPS or pro-inflammatory cytokines including TNF-α and IL-1β), intracellular signaling cascades are triggered (Hayden MS and Ghosh S, 2011). Once activated, NF-κB binds to κB sites in the promoters of target genes, initiating transcription of a broad array of pro-inflammatory genes, including cytokines, chemokines, and adhesion molecules, thereby recruiting immune cells and amplifying and sustaining the inflammatory response (Sivamaruthi BS et al., 2023).

In microglia, NF-κB activation is primarily associated with a pro-inflammatory state (Anilkumar S and Wright-Jin E, 2024). This activation results in the robust production and secretion of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, as well as chemokines, iNOS, COX-2, and ROS. NF-κB activation influences microglial proliferation, migration, and phagocytic activity, as well as the expression of genes involved in cell survival and apoptosis. Released mediators (i.e., TNF-α and IL-1β) can also contribute to neuronal injury and the progression of neurodegenerative diseases if their production is excessive or sustained by positive feedback loops in microglia, thereby perpetuating inflammation and neurotoxicity. Dysregulation of this pathway contributes to chronic inflammation in neurodegenerative diseases, including Alzheimer's disease, where it is associated with the production of β-amyloid plaques and tau pathology (Gou Q et al., 2024).

JAK-STAT signaling pathway

The JAK/STAT pathway is the predominant signaling pathway activated by cytokines and is critical for initiating innate immunity, orchestrating adaptive immune responses, and ultimately constraining inflammatory responses (Benveniste EN et al., 2014; O'Shea JJ et al., 2015). JAK has four isoforms (JAK1, JAK2, JAK3, and TYK2) and STAT has seven isoforms (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6) (Nicolas CS et al., 2013), where the cytokine or growth factor initiating the cascade determines JAK and STAT protein activation (Sarapultsev A, 2023). The classic JAK/STAT signaling pathway starts with ligand binding to its receptor. These connections induce JAK transphosphorylation. Activated JAK causes tyrosine phosphorylation of the bound receptor, forming a docking site for STATs. At this docking site, JAK phosphorylates STAT, and then STAT dissociates from the receptor and forms homodimers or heterodimers through SH2-domain–phosphotyrosine interactions. These dimers translocate to target gene promoters, regulating the transcription of the target genes (Durham GA et al., 2019; O'Shea JJ et al., 2015). STAT usually regulates transcription through

the following mechanisms: (1) STAT binds to its DNA target site to drive transcription activation. (2) STAT protein may form a transcription complex with non-STAT transcription factors to trigger the transcription mediated by STAT; (3) STAT associates with non-STAT DNA-binding elements to promote STAT-dependent transcription; (4) STAT and non-STAT transcription factors can synergistically activate transcription by binding to clusters of independent DNA-binding sites (Hu X et al., 2021). In microglia, activation of this pathway is highly context-dependent. During neuroinflammation, these JAK/STAT signaling cascades typically lead microglia to adopt an inflammatory phenotype (see Table 1.2 for details). JAK/STAT signaling in microglia should not be interpreted as a uniformly anti-inflammatory or pro-inflammatory pathway, but rather as a cytokine-specific regulatory system that integrates inflammatory and anti-inflammatory signals to shape microglial phenotype, cytokine production, inflammasome activity, and the progression or resolution of neuroinflammatory responses.

Table 1.2. JAK/STAT signaling in microglia and its functional outcomes

Stimulus	STAT protein	Microglial response	Main effects	References
IFN-γ	STAT1	Pro-inflammatory	Increased expression of TNF-α, IL-1β, iNOS, and other inflammatory genes	Jain M et al., 2021
IL-6	STAT3	Pro-inflammatory	Promotion of inflammatory signaling in neurodegenerative and injury-related conditions	Jain M et al., 2021
IL-10	STAT3	Anti-inflammatory	Suppression of IL-1β, TNF-α, IL-6, and NLRP3 expression; reduced NF-κB-dependent transcriptional activity	Liu J et al., 2022
IL-4	STAT6	Anti-inflammatory	Repolarization of microglia toward an anti-inflammatory phenotype	Sarapultsev A et al., 2023; He Y et al., 2020

MAPK signaling pathway

MAP kinases are serine/threonine protein kinases with widespread expression and are essential for several important functions, including cell proliferation, differentiation, and apoptosis (Zhang W and Liu HT, 2002). Additionally, they regulate cellular responses to various stressors, including inflammation, oxidative stress, and infection (Cargnello M and Roux PP, 2011). In the CNS, the MAPK pathway plays a critical role in modulating neuroinflammation by influencing the production of pro-inflammatory cytokines, chemokines, and other immune mediators (Ramesh G et al., 2013). Its activation is particularly relevant during microglial and astrocytic responses, which contribute significantly to the progression of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and multiple sclerosis (Gao C et al., 2023).

Three primary branches are present in the MAPK signaling pathway, namely p38 MAPK, c-Jun NH2-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK1/2). Distinct stimuli activate each of them, mediating specific cellular outcomes. ERK1/2 is associated with proliferation and survival, whereas p38 MAPK and JNK are involved in apoptosis (Subhramanyam CS et al., 2019).

The ERKs were the first mammalian MAPKs to be identified. In many cases, ERKs can be activated by inflammatory cytokines, including members of the TNF family. These mechanisms of ERK activation play important roles in inflammation and innate immunity (Kyriakis JM et al., 2012)

The JNKs are multifunctional signaling molecules activated by a wide range of inflammatory mediators and cellular stresses. JNKs regulate various processes, such as neuroinflammation, neuronal death, memory formation, brain development, and repair (Orejana L et al., 2013; Sclip A et al., 2011; Shoji M et al., 2000).

The p38 MAPK pathway has been extensively studied in relation to neuroinflammation, as it is directly activated by inflammatory cytokines such as IL-1β and TNF-α, as well as by oxidative stress and misfolded proteins. Upon activation, the p38 MAPK pathway promotes the expression of pro-inflammatory cytokines by phosphorylating transcription factors and enhancing mRNA translation. These mechanisms contribute to increased production of inflammatory mediators, including IL-6 and TNF-α. In addition, p38 MAPK signaling can enhance inflammasome activity, including activation of the NLRP3 inflammasome, thereby further amplifying the inflammatory response (Schieven GL, 2000; Chen J et al., 2021). The MAPK pathway heavily influences microglial activation during neuroinflammation. In particular, p38 MAPK activation in microglia promotes the production of ROS and pro-inflammatory cytokines, which exacerbate neuronal damage

(Lee YB et al., 2000; Kim SH et al., 2004). In neurodegenerative diseases, aberrant MAPK signaling contributes to chronic neuroinflammation and neuronal dysfunction (Bachstetter AD et al., 2011). In Alzheimer's disease, β-amyloid- and tau-associated pathology has been linked to activation of p38 MAPK signaling in microglia, astrocytes, and neurons, thereby promoting the production of inflammatory mediators and Alzheimer's disease-related cellular dysfunction (Perea JR et al., 2022; Zhang F and Jiang L, 2015). In particular, p38 MAPK has been shown to regulate β-amyloid-induced IL-6 production in human microglia, while microglial activation and IL-1/p38 MAPK signaling have been associated with tau phosphorylation and aggregation (Lin H et al., 2022; Bhaskar K et al., 2010). Sustained activation of these inflammatory pathways may contribute to synaptic dysfunction, oxidative stress, and neuronal loss (Zhang F and Jiang L, 2015).

2. MATERIALS AND METHODS

2.1. Animals

Eight-week-old male C57BL/6 mice were used for the DIO model and BHB supplementation procedures. Animal protocols were approved by the local Ethics Committee and conducted in compliance with Royal Decree 1201/2005 (BOE No. 252) on animal welfare. All procedures adhered to the regulations outlined in Royal Decree 53/2012 for the care and use of animals in scientific research. To reduce experimental bias, mice were randomly assigned to treatment groups. Animal experiments were performed at IBIMA Plataforma BIONAND, Málaga, Spain, in accordance with the animal experimentation ethics approval issued by the relevant Spanish authority.
For microglia isolation from adult mice, six-month-old C57BL/6J mice were used. For microglia cell isolation from neonatal mice, C57BL/6J P0–P3 mice were used. Cell isolation experiments were conducted with the permission of the Lithuanian State Food and Veterinary Service (No. G2–104), and all procedures complied with the requirements of the 2010/63/EU Directive.
All animals were housed under controlled conditions (22 °C ± 1, 40 % humidity, 12-hour light/dark cycle), with food and water supplied *ad libitum* and under veterinary supervision.

2.2. DIO mouse model and BHB supplementation

A total of 20 male mice were fed a 60% high-fat diet (Brogaarden) for 6 weeks to induce obesity. Following this period, animals were switched to a standard chow diet to examine the isolated effects of ketone body supplementation. BHB supplement (KetoForce food supplement) was provided in the drinking water at a concentration of 0.08 g/mL (corresponding to a final 4.2 % BHB salt) for 6 weeks (Figure 2.1). Half of the mice received BHB-supplemented water (BHB group), while the remaining mice received plain drinking water and served as controls (Con group). The selected BHB concentration was based on previous studies reporting effective doses within the physiological range of 1–5 mM, achieved during fasting or ketogenic conditions, which are known to modulate metabolic and immune functions. This level of BHB has been shown to enhance mitochondrial activity, suppress inflammation by inhibiting the NLRP3 inflammasome, and act as a signaling metabolite via histone deacetylase inhibition and GPCR activation (Ferrere G et al., 2021; Caminhotto R.O. et al., 2017). Fresh BHB solution was prepared daily to

prevent precipitation and ensure compound stability. Vanesa Jimenez and Dr. Virginia Mela conducted experiments.

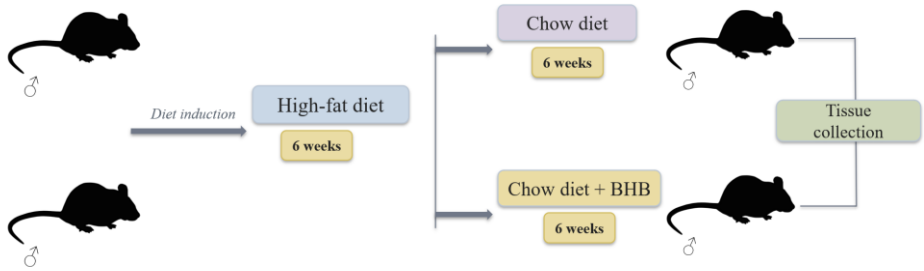


Figure 2.1. Experimental plan of the DIO mice model and BHB supplementation

2.3. Mouse sample collection

At the end of the experiment period, mice were anesthetized with sodium pentobarbital (Euthanimal) and transcardially perfused with phosphate-buffered saline (PBS). Blood samples were collected by cardiac puncture prior to perfusion. The brains were dissected and used for immunohistochemical analysis and protein and RNA expression studies.

2.4. Metabolite quantification by ^{1}H NMR spectroscopy

All spectra were acquired on a Bruker AVANCE 600 MHz Spectrometer (Bruker BioSpin, Ettlingen, Germany) equipped with an *Advance III* console and either a 4 mm TXI ^{1}H high-resolution magic angle spinning probe for intact tissue analysis or a nitrogen-cooled TCI Prodigy cryoprobe for plasma samples analysis.
Water-suppressed HR-MAS NMR spectra of intact tissue samples were acquired using a Carr-Purcell-Meiboom-Gill (CPMG) sequence with the following parameters: 12 kHz spectral width, 64 000 data points, 64 scans, 1 ms echo time (2τ), a total echo time of 130 ms, and a 5 s relaxation delay. Water presaturation was applied during the relaxation delay. Metabolite quantification was performed using the LCModel software (Provencher SW, 1993), as described elsewhere (Righi V et al., 2009), with creatine as the internal reference.
For plasma samples, the following acquisition sequences were used: one-dimensional Nuclear Overhauser Effect Spectroscopy, 1D CPMG for T_2 editing of macromolecular signals, and 2D J-resolved (JRES) spectroscopy to aid metabolite identification. Small metabolites were quantified from the CPMG spectra using Chenomx software (V.10.0, Chenomx, Edmonton,

Canada), with the Electronic Reference To Access *In Vivo* Concentrations, as implemented in TopSpin 3.5.pl7, serving as the concentration reference.
NMR spectra acquisition was carried out at the U28 Unit of the ICTS 'NANBIOSIS' at IBIMA Plataforma BIONAND by Dr. María Luisa García-Martín.

2.5. Microglia morphological analysis

Immunostaining of the microglial marker Iba-1 was used to evaluate whether morphological alterations occur in microglia from the Con and BHB-fed mice. Image stacks (0.5 µm step size; 33 steps) were taken from Iba-1-stained hippocampal tissue sections (20 µm) using a Leica SP8 Stellaris scanning confocal microscope. Image analysis was performed using ImageJ. Images were converted to 8-bit greyscale, a threshold was set, and binarised images were filtered by pixel size to reduce background and enhance contrast. A skeleton analysis was used to assess changes in features relevant to microglia ramification. In contrast, fractal analysis was used to evaluate characteristics associated with cell surface, soma thickness, cell size, the complexity of their ramifications, and the heterogeneity of their shape.

2.6. Microglia isolation from adult mice

For this study, microglia were isolated using three different protocols: protocol 1 (modified in our laboratory), protocol 2 (Woolf Z et al., 2021), and protocol 3 (Lee JK & Tansey MG, 2013). Protocols 2 and 3 were performed exactly as described in the original publications and are therefore not detailed here.

1. Animals were euthanized by cervical dislocation, and the heads were removed using sterile scissors. Brains were dissected and transferred to ice-cold transfer solution (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) supplemented with 3 % penicillin–streptomycin solution (10,000 units/mL penicillin and 10,000 µg/mL streptomycin)) for further processing.
2. Brains were minced with a sterile scalpel into small fragments.
3. Tissue was transferred to 5 mL of digestion mix containing 10 mg Collagenase A (0.197 U/mg; Roche), 70 µL DNase I (1000 U/mL; Roche), 50 µL of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; 1 M; ThermoFisher Scientific), 250 µL FBS (ThermoFisher Scientific), and Hank's Balanced Salt Solution (HBSS; without Ca^{2+}/Mg^{2+}; ThermoFisher Scientific) to a final volume of 5 mL. The tissue was incubated at 37 °C for

15 minutes, with gentle shaking every 5 minutes. It was then dissociated into single cells by pipetting 20 times with flame-polished Pasteur pipettes of decreasing diameter (7 mm, 5 mm, and 3 mm). After the first and second pipetting rounds, the sample was re-incubated at 37 °C for 15 minutes before proceeding. The resulting cell suspension was filtered through a sterile 70 µm nylon mesh, and the filtrate was brought to a final volume of 14 mL with HBSS (with Ca^{2+}/Mg^{2+}; ThermoFisher Scientific). Samples were centrifuged at 300 × g for 10 minutes at 4 °C (maximum acceleration, half brake).

4. The supernatant was discarded, and the cell pellet was resuspended in 3,1 mL of PBS. Then, 900 µL of Debris Removal Solution (Miltenyi Biotec) was added, and the suspension was gently pipetted up and down ten times. 4 mL of PBS was slowly layered on top. Samples were centrifuged for 10 minutes at 3400 × g at 4 °C. The top two layers with cell debris were removed, and the remaining suspension was brought up to 14 mL with PBS and centrifuged again for 10 minutes at 1000 × g at 4 °C. The supernatant was discarded, and the pellet was resuspended in 300 µL of Red Blood Cell Lysis Buffer (Sigma) and incubated at room temperature for 5 minutes. Then, 5 mL of cell medium was added, followed by centrifugation at 200 × g for 5 minutes at 20 °C.
5. The supernatant was discarded, and the pellet was resuspended in 5 mL of complete medium (DMEM/F-12 supplemented with 10 % FBS and 1 % penicillin–streptomycin). Cells were seeded into T25 flasks and incubated at 37 °C with 5 % CO_2 in a water-saturated incubator. After 3 hours, the medium containing unattached cells was discarded and replaced with fresh medium. On day 2, the culture medium was supplemented with macrophage colony-stimulating factor (100 ng/mL; R&D Systems) and granulocyte–macrophage colony-stimulating factor (100 ng/mL; R&D Systems).
6. Microglia were maintained for 7 days, with medium changes every 3 days. Upon reaching appropriate morphology and confluency, cells were detached using 0,05 % trypsin-0.5 mM EDTA, counted using a trypan blue exclusion assay with a hemocytometer, and seeded at an appropriate density for experiments.

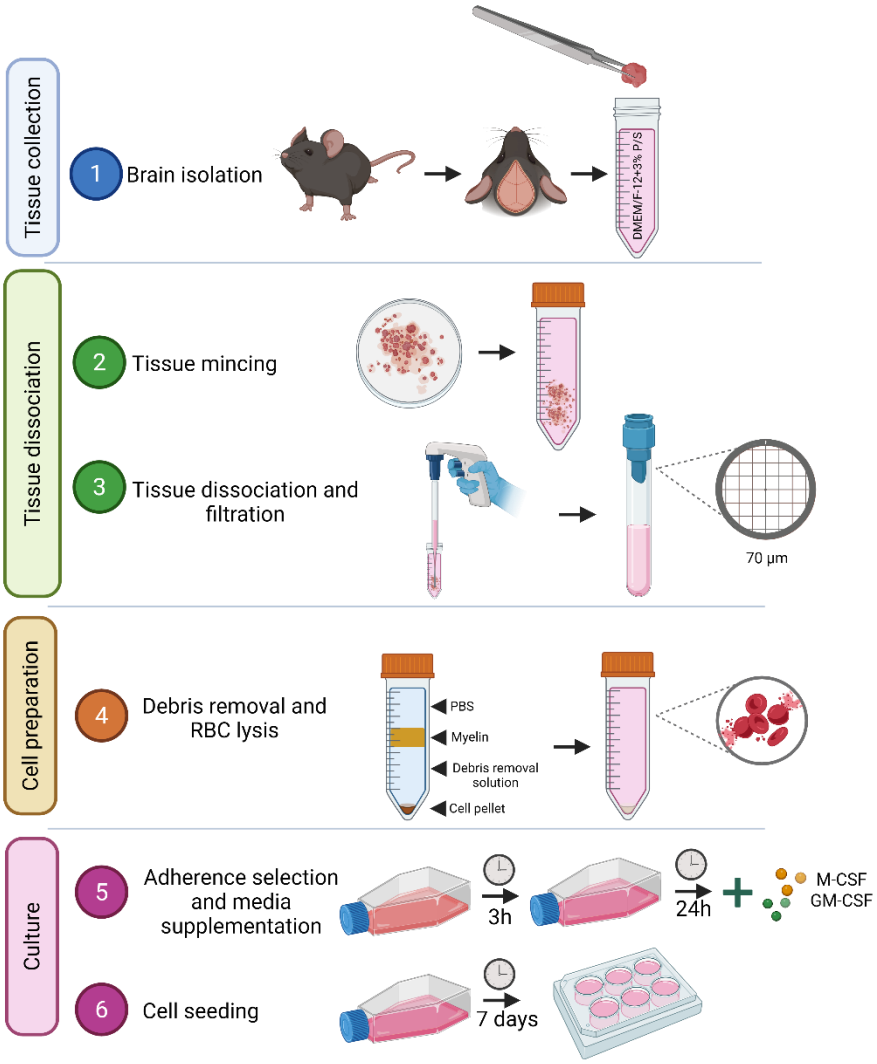


Figure 2.2. Schematic overview of the modified protocol used for the isolation and culture of primary mouse microglia.

2.7. Preparation of L929 conditioned media

The L929 cell line (ATCC, CCL-1™) was cultured in DMEM/F12 medium supplemented with 10 % heat-inactivated FBS and 1 % penicillin–streptomycin at 37 °C in a humidified atmosphere of 5 % CO_2. The L929 cells were grown in T-75 cell culture flasks and allowed to reach 90 % confluency. The culture medium was then discarded and replaced with 30 mL of fresh medium, and the conditioned medium was collected 14 days later. The

supernatant was centrifuged at 300 × g for 10 minutes, filtered using 0.22 µm syringe filters, aliquoted into sterile tubes, and stored at −80 °C until use.

2.8. Microglia isolation and culture from neonatal mice

Microglia were isolated as described by Scott N et al. (2022) with minor modifications. Briefly, C57BL/6J neonatal (P0-P3) mice were culled, brains were extracted, and meninges were removed. The brain tissue was digested in 30 ml of dissection media (HBSS with Ca^{2+}/Mg^{2+}, 4.5 g/L glucose, 10 mM HEPES, and 1× Antibiotic-Antimycotic solution) supplemented with 2.5 % trypsin/EDTA solution at 37 °C for 15 minutes. Afterward, 1.5 ml of trypsin inhibitor (1 mg/ml, Gibco) was added, followed by 750 µl of DNase I (1 mg/ml), to stop the reaction. The mixture was centrifuged at 400 × g for 5 minutes at 20 °C. The cell pellet was resuspended in 5 ml of warm culture medium (DMEM/F-12 supplemented with 10 % HI FBS and 1 % penicillin–streptomycin solution), centrifuged again under the same conditions, and the pellet was resuspended in 10 ml of fresh media and transferred to T-75 flasks pre-coated with 0.1 mg/mL poly-L-lysine (Sigma-Aldrich).
The next day, the medium was replaced with fresh culture medium supplemented with 10% L929-conditioned medium, and the flasks were incubated for an additional 13 days. Microglia-enriched cultures were obtained from mixed glial cultures by manually agitating the culture flask approximately 40 times to detach loosely adherent microglial cells. Detachment was confirmed under a microscope. The culture medium containing detached microglia was then collected and centrifuged at 800 × g for 5 minutes to pellet the cells. The cell pellet was resuspended in fresh culture medium, counted, and seeded for further experiments. For confocal microscopy, 50,000 cells were seeded onto 13-mm glass coverslips in 24-well plates. For RNA and protein extraction, 500,000 microglia cells were seeded into 6-well plates.

2.9. Cell treatments

On the first day, cells were seeded at an appropriate density as mentioned above. The following day, cells were treated under the following conditions: LPS alone (100 ng/mL; Sigma Aldrich) for 24 hours; BHB (10 mM; Sigma Aldrich) for 1 hour, followed by LPS (100 ng/mL) for 24 hours or AM-630 (1 µM; Sigma Aldrich) for 1 hour, followed by BHB (10 mM) for 1 hour, and then LPS (100 ng/mL) for 24 hours (Fig. 2.3).

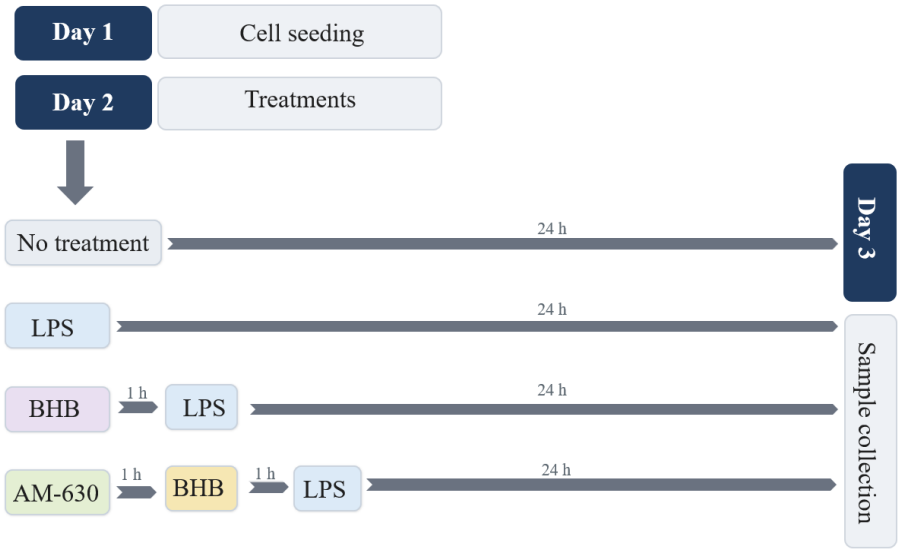


Figure 2.3. Schematic representation of the *in vitro* treatment protocol.

2.10. Immunocytochemistry

Microglia cells were washed twice with PBS for 5 min and fixed with 4% PFA for 15 min. After that, the cells were washed again and blocked in a blocking solution containing 3% bovine serum albumin and 10% FBS in PBS for 1 hour. Following blocking, cells were incubated with an anti-CD11b antibody for 1 hour (Supplementary Table S1) and washed again. Finally, nuclei were counterstained with DAPI (1 µg/mL; ThermoFisher Scientific) for 5 min and washed again. Then, the glasses were prepared for image acquisition, and micrographs were taken as described in Chapter 2.18

2.11. Phagocytosis assay

Following treatment, 0.025% latex beads (1 μm^2, carboxylate-modified polystyrene, fluorescent yellow-green, Sigma-Aldrich) were added to the culture medium, and the cells were incubated for 4 hours at 37 °C and 5% CO_2. After incubation, immunocytochemistry was performed as described in Chapter 2.10. Image acquisition was performed as described in Chapter 2.18.

2.12. Assessment of ROS production

ROS generation was assessed using the CellROX™ Green reagent (ThermoFisher Scientific) according to the manufacturer's instructions. Briefly, CellROX solution was added to the culture medium at a final

concentration of 5 µM after treatment, and cells were incubated in the dark at 37 °C and 5 % CO_2 for 30 min. After incubation, cells were fixed with 4 % PFA. Nuclei were counterstained with DAPI (1 µg/mL) for 5 min. After image acquisition (Chapter 2.18) in ImageJ, background fluorescence was subtracted, and the fluorescence intensity of individual cells was measured using the Integrated Density function.

2.13. NF-κB localization analysis

Cells were fixed and permeabilized as previously described. Immunolabeling was performed using a primary antibody against NF-κB, followed by incubation with a secondary antibody (Supplementary Table S1). Nuclei were counterstained with DAPI (1 µg/mL) (ThermoFisher Scientific) for 5 min. After image acquisition (Chapter 2.18) in ImageJ, whole-cell regions of interest were selected, and both mean fluorescence intensity and area were measured. Subsequently, nuclear ROIs were defined using the DAPI channel and the corresponding NF-κB signal, and the mean intensity and area of these ROIs were calculated. Cytoplasmic NF-κB intensity (cyt.int.) was determined using the following formula (1):

$$Cyt.int. = (cell\ mean\ int. * cell\ area) - (nuclear\ mean\ int. * nuclear\ area) \quad (1)$$

Then, the cytoplasmic area was calculated using formula (2):

$$Cytoplasmic\ area = whole\ cell\ area - nuclear\ area \quad (2)$$

Cytoplasmic mean intensity was calculated following formula (3):

$$Cytoplasmic\ mean\ int. = \frac{cytoplasmic\ intensity}{cytoplasmic\ area} \quad (3)$$

Finally, nuclear/cytoplasmic NF-κB intensity was calculated as the nuclear mean intensity divided by the cytoplasmic mean intensity, and the data were expressed in arbitrary units.

2.14. Real-time qPCR analysis

Total RNA was isolated using TRIzol™ Reagent according to the manufacturer's protocol. The RNA yield and quality were quantified using a

Nanodrop spectrophotometer (NanoPhotometer N60/N50). cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer's instructions and using the maximum quantity of RNA per sample.
qPCR was performed using PowerUp™ SYBR™ Green Master Mix according to the manufacturer's protocol. Each reaction was run in duplicate. The primers used for gene expression analysis are listed in Supplementary Table S1. qPCR was carried out in the QuantStudio™ 5 Real-Time PCR System (ThermoFisher Scientific). Gene expression data were analyzed using the ΔΔCt method and normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which served as an endogenous control for quantification assays.

2.15. Western blot analysis

Total protein from cells was extracted from the organic phase of the RNA isolation using TRIzol™ reagent (ThermoFisher Scientific) according to the manufacturer's protocol. From mouse hippocampus tissue, proteins were isolated using the column flow-through of the NucleoSpin RNAII kit (Macherey- Nagel) and incubated in lysis buffer (Tris-HCl 1 M, thiourea 2 M, urea 70 mM, 4% 3-[(3- Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS); Sigma, UK). The protein concentration in the supernatant was determined using a PierceTM BCA protein assay (Thermo Fisher Scientific; Cat# 23225). Proteins were denatured (95 °C for 5 min) in 4x Laemmli buffer containing 5 % 2-mercaptoethanol. Protein sample was loaded onto 12% acrylamide gels (20 µg of hippocampal protein and 10 µg of protein from cell culture) and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Proteins were then transferred to a polyvinylidene fluoride membrane and blocked for 1 h in 3% BSA in Tris-Tween-buffered saline (TTBS). Primary antibodies were diluted in TTBS with 3% BSA, as specified in Supplementary Table S1. Membranes were incubated with antibodies overnight at 4 °C at constant rotation. After washing in TTBS (3 × 10 min), the membranes were incubated at room temperature for 2 h with secondary antibodies diluted in TTBS containing 3% BSA (see Supplementary Table S1). After washing (3 × 10 min), membranes were treated for 5 min with Clarity™ Western ECL Substrate (Bio-Rad, #170-5061). Protein band intensities were captured using the ChemiDoc™ MP digital imager (Bio-Rad Laboratories). Image Lab software (Bio-Rad Laboratories) was used to analyze images and determine the signal intensities

of the identified bands. Bands of interest were then normalized to the tubulin intensity of a given lane.

2.16. Immunohistochemistry

The brain was dissected, and the tissue was fixed in 4 % PFA for 24 hours. Following fixation, the tissue was incubated in 30% sucrose for 48 hours to cryoprotect it, then frozen in isopentane. The samples were embedded in optimal cutting temperature compound, and 20 µm coronal sections were prepared using a cryostat. Sections were stored at −20 °C in a freezing solution (30% ethylene glycol, 30% sucrose in PBS) until immunohistochemical processing. Immunohistochemistry was performed on brain slices containing the medial hippocampus. Sections were washed, permeabilized in PBS with Triton X-100 (PBS + 0.3 % Triton X-100), and blocked for one hour in blocking solution (PBS + 0.3 % Triton X-100 + 3 % BSA). The samples were then incubated overnight at 4 °C with primary antibodies diluted in blocking solution under continuous rocking. Specific antibody product codes and dilutions are listed in Supplementary Table S1. After washing, sections were incubated for two hours at room temperature with secondary antibodies (Supplementary Table S1). Nuclei were counterstained with DAPI (1 µg/mL, ThermoFisher) for 10 minutes at room temperature. Brain sections were mounted in ProLong® Glass Antifade Mountant (P36984, Thermo Scientific). Imaging was performed using a Leica SP8 Stellaris scanning confocal microscope (3 fields per section at 40X; 63X for PSD95 phagocytosis), and images were processed in ImageJ. To assess (postsynaptic density protein 95) PSD95 engulfment by (ionized calcium-binding adapter molecule 1) Iba-1$^+$ cells, 3D reconstruction and analysis were conducted using IMARIS software (Bitplane, Oxford Instruments).

2.17. Enzyme-linked immunosorbent assay (ELISA)

The brain cortex was mechanically homogenized in radioimmunoprecipitation assay (RIPA) buffer supplemented with 1× Protease Inhibitor Cocktail (Thermo Fisher). A total of 3 µL of RIPA buffer was used per 1 mg of tissue. The homogenates were then centrifuged at 12,000 × g for 10 min at 4 °C, and the supernatants were collected. Protein concentration was determined using the Bradford assay. For subsequent ELISA analysis, samples from the two treatment groups were diluted 1:3 and analyzed using a mouse IL-6 ELISA kit (DuoSet, R&D Systems) according to the manufacturer's instructions. Briefly, 96-well plates were coated with goat anti-mouse IL-6 capture antibody (R&D

Systems), incubated overnight at room temperature, and blocked with 1% BSA in PBS for 1 hour at room temperature. Duplicate samples and standards were added and incubated for 2 hours at room temperature. Plates were washed and incubated with biotinylated goat anti-mouse IL-6 detection antibody for 2 hours at room temperature. Plates were incubated with horseradish peroxidase-conjugated streptavidin for 20 minutes, then washed again. Substrate solution (1:1 H_2O_2:tetramethylbenzidine; Sigma-Aldrich) was applied and incubated in the dark at room temperature for 20 minutes. The reaction was stopped with 1 M H_2SO_4, and absorbance was measured at 450 nm using a BioTek Agilent microplate reader (Agilent Technologies). The concentrations were calculated from the calibrated standard curve.

2.18. Image acquisition and analysis in cell samples

After staining with appropriate dyes, coverslips were mounted on microscopy slides using Moviol (Roth) mounting medium, and the samples were stored at room temperature in the dark for at least 24 hours. Cell visualization was conducted using a Leica SP8 Stellaris scanning confocal microscope (3 fields, 63X magnification (phagocytosis, NF-κB localization) or 3 fields, 20X magnification for ROS and CD11b visualization). To quantify phagocytosis, the ImageJ Z-Project function was used to process the images, and the number of beads per cell was manually counted. To assess the purity of the microglial cultures, the number of CD11b-positive cells was quantified and compared with the total number of DAPI-stained nuclei. Culture purity was calculated as the percentage of CD11b-positive cells relative to the total number of cells.

2.19. Antibody microarray experiment

Cells were seeded into 6-well plates at a density of 250,000 cells per well. For each treatment group, six wells were used, yielding a total of 1.5×10^6 cells per group. Primary microglia were isolated from 20 pups to ensure sufficient cell numbers and biological diversity and then mixed across groups. Treatments were performed as described in Section 2.12. After 24 h of treatment, protein samples were prepared using the Antibody Array Assay Kit (Full Moon BioSystems, Sunnyvale, CA, USA; #KAS02) according to the manufacturer's instructions. Briefly, the culture medium was removed, and cells were washed three times with ice-cold 1× PBS. Residual PBS was carefully aspirated, and cells were lysed in 100 µL of Extraction Buffer per treatment group (six wells were pooled for each group). The lysates were transferred to clean microcentrifuge tubes, and Lysis Beads were added to

each sample. Samples were vortexed vigorously for 30 s to 1 min, then incubated on ice for 10 min; this cycle was repeated 5 times. Lysates were then centrifuged at 10,000 × g for 5 min at 4 °C. The supernatants were transferred to fresh tubes and centrifuged again at 16,000 × g for 25 min at 4 °C. The final supernatants were collected for further processing. Lysates were purified by buffer exchange using spin columns. Columns were hydrated with Labeling Buffer, equilibrated, and centrifuged; 100 µL of protein extract was then loaded onto each column and eluted at 1500 × g for 2 min. Protein concentration was determined by measuring absorbance at 280 nm. Purified lysates were then biotinylated and used within the recommended input range for array analysis.

Protein expression profiling was performed using the Full Moon Biosystems Cell Signaling Phospho-Antibody Array (Full Moon Biosystems, Sunnyvale, CA, USA; #PCS300) according to the manufacturer's instructions. The array contains 304 site-specific and phospho-specific antibodies targeting proteins involved in 16 major cell signaling pathways, including PI3K/AKT, AMPK, apoptosis, autophagy, cell cycle, ErbB, focal adhesion, insulin, JAK/STAT, MAPK, mTOR, NF-κB, p53, Ras, TGF-β, and VEGF signaling. Each antibody is printed in six replicates. Shortly, antibody array slides were brought to room temperature, dried, and blocked in Blocking Solution for 45 min on an orbital shaker. After extensive water rinsing, biotinylated protein samples were mixed with the coupling solution and applied to the array slides in the coupling chamber for 2 h at room temperature with gentle shaking. Slides were then washed three times with 1× Wash Solution and rinsed thoroughly with water. For detection, slides were incubated for 20 min at room temperature with Cy3-streptavidin (Invitrogen) prepared in Detection Buffer and protected from light. Three additional wash steps and extensive water rinsing followed this. Finally, slides were dried by centrifugation and scanned using an InnoScan 710 microarray scanner. The resulting TIFF images were used for downstream quantitative analysis.

2.20. Antibody microarray result analysis

Raw spot intensities were first corrected for background and then normalized to the mean signal intensities of the housekeeping proteins GAPDH and tubulin. The normalized phosphoprotein signal for each replicate spot was divided by the average normalized total protein signal of the corresponding target. Because six technical replicate spots represented each protein, this yielded six phospho/total ratio values per protein for each condition. To evaluate signal consistency, the coefficient of variation (CV) was calculated

for each protein as the standard deviation of the six phospho/total ratio values divided by their mean. The inverse coefficient of variation (1/CV) was then used as a metric of variability, with higher values indicating lower relative variability and therefore greater consistency among replicate spots. Differential regulation between groups was expressed as log2 fold change (log2FC). The processed data were subsequently visualized using scatter plots and pathway–protein interaction network graphs to identify the principal signaling pathways associated with the regulated proteins.

To visualize the relationship between regulated proteins and associated signaling pathways, a pathway–protein interaction network was generated in R using the clusterProfiler, enrichplot, ggplot2, and dplyr packages. A ranked protein list was generated using log2 fold-change values. Overrepresentation analysis was performed using the enricher function in the clusterProfiler package, with the custom pathway–protein reference table. To visualize all mapped associations, inclusive cutoffs were used (pvalueCutoff = 1, qvalueCutoff = 1, minGSSize = 1). The resulting enrichment object was visualized as a category-gene network using the cnetplot function, in which pathways and their associated proteins were connected based on shared membership. Protein node color reflected log2 fold change values, allowing simultaneous visualization of pathway membership and direction/magnitude of regulation.

2.21. Statistical analysis

All statistical analyses were performed using GraphPad Prism 10. Data are presented as mean ± SEM, with the number of biological replicates or experimental repeats indicated in each figure. Normality and homogeneity of variances were assessed using the Shapiro–Wilk and Levene's tests, respectively. For comparisons between two independent groups, Student's t-test was applied when data were normally distributed with equal variances; Welch's t-test was used when normality was met, but variances were unequal; and the Mann–Whitney U test was used when data were not normally distributed. For comparisons involving more than two groups, one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used when assumptions of normality and equal variances were met; Welch's ANOVA followed by the Games–Howell post hoc test was used when variances were unequal; and the Kruskal–Wallis test followed by Dunn's test (with Bonferroni correction) was applied for non-parametric data. The significance level was set at $p < 0.05$.

3. RESULTS

3.1. Analysis of BHB Anti-Inflammatory Effects in the Brains of Obese Mice

In this study, we first investigated the effects of BHB supplementation alone in a DIO mouse model, aiming to isolate its impact from other intermediate effects associated with the dietary intervention. Our previous data demonstrated that ketone body supplementation significantly reduced body weight in DIO mice during the experiment, while no significant changes were observed in cognitive performance (Mela V. et al., 2025). Building on these findings, we examined whether BHB supplementation modulates neuroinflammatory processes at the cellular and molecular levels, providing a more comprehensive understanding of its effects in this model. To confirm the effectiveness of BHB supplementation, we measured the levels of various ketone bodies in mouse serum. Next, we assessed microglial morphology and phenotype at the functional and molecular levels to determine how BHB affected microglial state.

3.1.1. Serum Ketone Body Changes Following BHB Supplementation

To confirm the supplement's effectiveness, various ketone bodies were analyzed in the serum of obese mice with or without BHB supplementation. In the supplemented group, a significant increase in 3-hydroxyisobutyrate (Fig. 3.1 A; $p < 0.05$) and acetate (Fig. 3.1 C; $p < 0.05$) was observed, along with a trend towards increased 3-hydroxybutyrate (Fig. 3.1 D; $p = 0.0528$) and a light increase in acetone (Fig. 3.1 E) in the supplemented group. Additionally, a decrease in 1,3-dihydroxyacetone was observed (Fig. 3.1 B; $p < 0.001$). Overall, these findings indicate that BHB supplementation altered systemic ketone-related metabolic profiles, supporting the supplement's biological activity in this model.

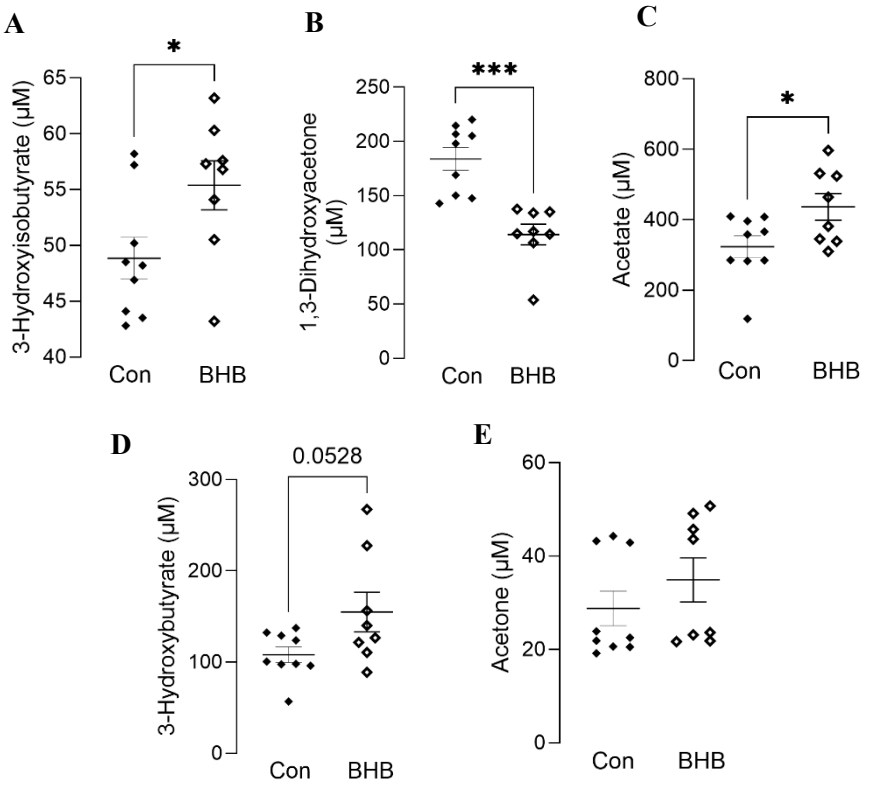


Figure 3.1. Serum Ketone Body Changes in the BHB Group Compared to the Untreated Group. (**A**) 3-Hydroxyisobutyrate. (**B**) 3-Hydroxybutyrate. (**C**) 1,3-Dihydroxyacetone. (**D**) Acetate. (**E**) Acetone. Con – control group, BHB – BHB treatment group. n = 8-9. Data are presented as mean ± SEM; *$p < 0.05$; ***$p < 0.001$.

3.1.2. Microglia Morphological Changes in Response to BHB Treatment

Then, we aimed to assess changes in microglial complexity and branching patterns to investigate the potential effects of BHB treatment on microglial function. To accomplish this, we quantified morphological parameters of Iba-1-labeled hippocampal microglia. The analysis revealed significant differences in key structural parameters of microglia. Specifically, BHB-treated mice exhibited a notable increase in end-point voxels (Fig. 3.2 A, B; $p < 0.01$), number of branches (Fig. 3.2 A, C; $p < 0.05$), number of junctions (Fig. 3.2 A, D; $p < 0.05$), and junction voxels (Fig. 3.2 A, E; $p < 0.05$) compared to obese controls. Although the soma parameter did not reach statistical significance, there was a trend toward increased soma size (Fig. 3.2 A, G; $p = 0.068$) in BHB-treated mice.

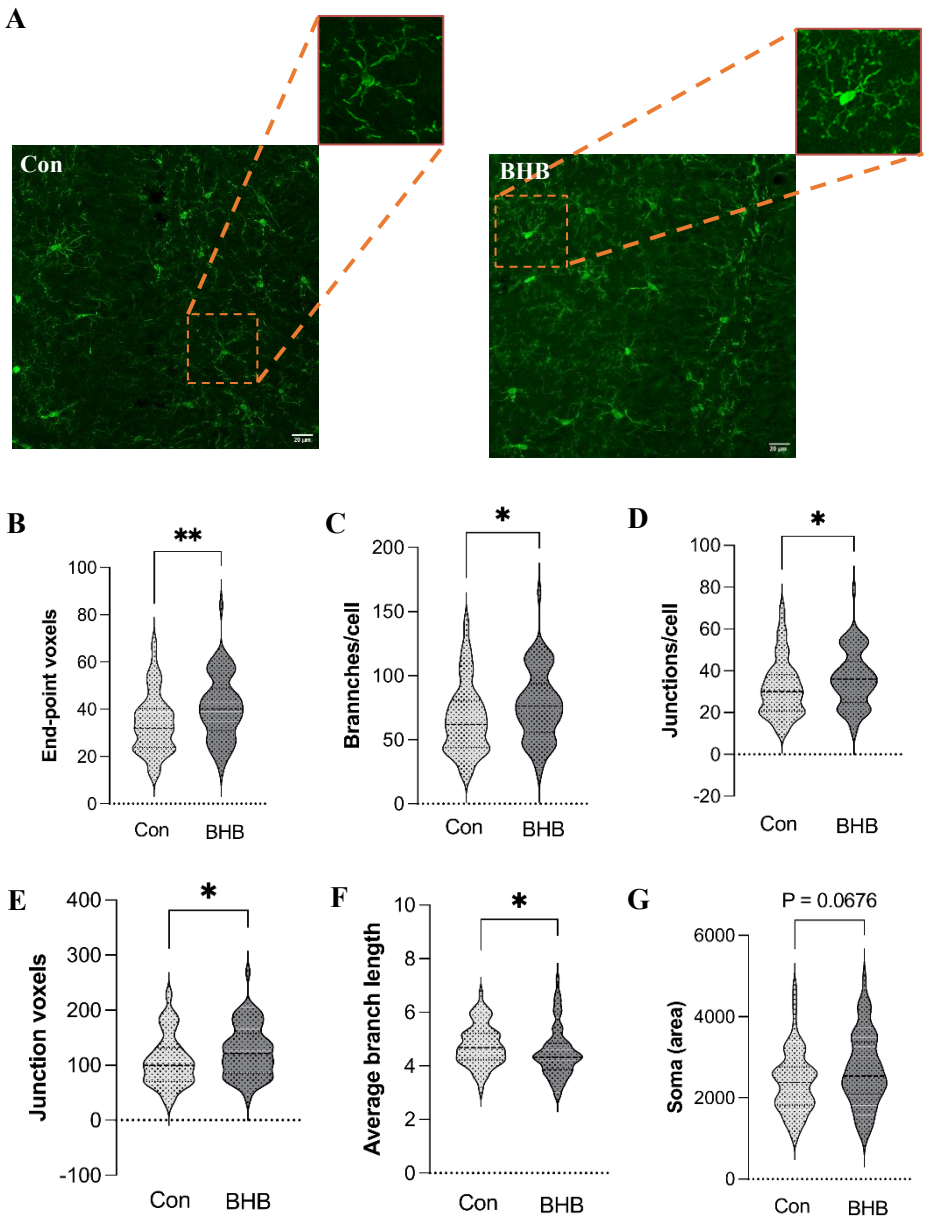


Figure 3.2. BHB Treatment Modified Microglial Morphology in the Hippocampus of Obese Mice. (**A**) Confocal microscopy images of microglia stained with Iba-1 antibody, showing microglia morphology; (**B**) #End-point voxels; (**C**) #Branches/cell; (**D**) #Junctions/cell; (**E**) #Junction voxels; (**F**) Average branch length; (**G**) Soma size. Con – control group, BHB – BHB treatment group. n = 6 mice. For each animal, 10 – 15 microglial cells were analyzed. Data are presented as violin plots showing the distribution of individual values; the central line represents the mean; *$p < 0.05$; **$p < 0.01$.

These changes reflect increased microglial complexity and arborization, suggesting that BHB modulates microglial morphology in obese mice. Although increased arborization may be associated with a less reactive microglial state, morphology alone cannot be used to define a neuroprotective or anti-inflammatory phenotype. It should be interpreted together with molecular and functional indicators of neuroinflammation.

3.1.3. BHB Influences Microglial Phagocytic Activity and Reduces Microglia-Synapse Association

To further characterize the effects of BHB on microglial state, we performed immunofluorescent colocalization analysis using CD68/Iba1 and PSD95/Iba1 labeling to evaluate microglial phagocytic capacity and synaptic engagement.

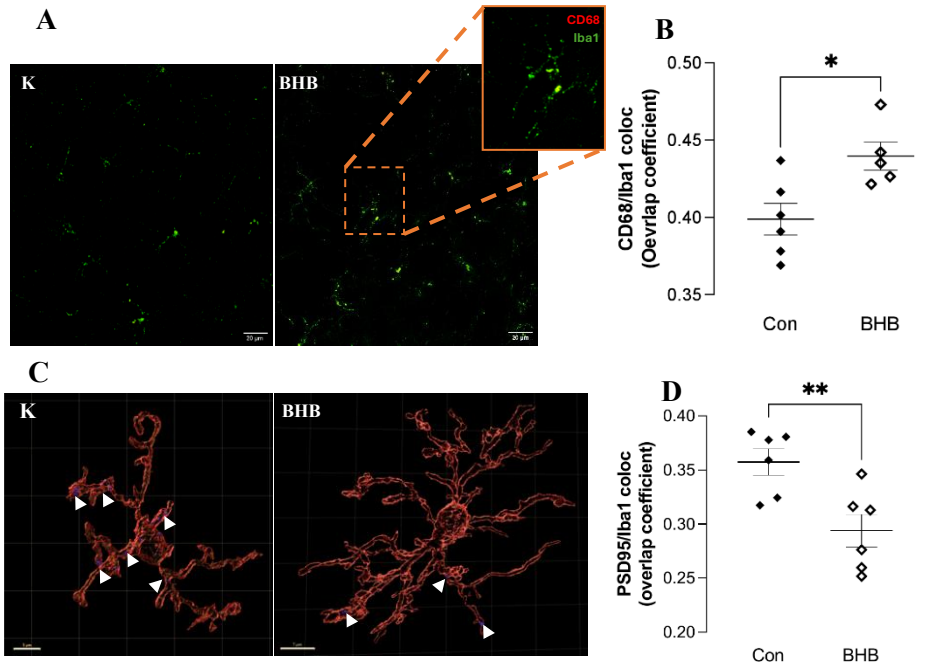


Figure 3.3. BHB Treatment Modulates Microglial Phagocytic Properties (**A**) Representative confocal image showing CD68/Iba1 colocalization in the hippocampus; (**B**) Quantification of CD68/Iba1 overlap coefficient, showing increased colocalization in BHB-treated mice; (**C**) Representative IMARIS 3D reconstruction image showing PSD95 localization in microglia; (**D**) Quantification of PSD95/Iba1 overlap coefficient, showing decreased colocalization in BHB-treated mice. Con – control group, BHB – BHB treatment group. n = 6. For each animal, 10–15 microglial cells were analyzed. Data are presented as mean ± SEM; *$p < 0.05$; **$p < 0.01$.

Quantitative analysis of the overlap coefficient revealed a significant increase in CD68/Iba1 colocalization in BHB-treated mice compared to controls (Fig. 3.3 A-B; $p < 0.05$), indicating enhanced microglial phagocytic activity. In contrast, PSD95/Iba1 colocalization was significantly reduced in the BHB group (Fig. 3.3 C-D; $p < 0.01$), suggesting decreased microglial engulfment of postsynaptic elements

3.1.4. BHB Downregulates IL-1ß and IL-6 Expression in Mice Hippocampus and Cortex

To assess the anti-inflammatory effect of BHB in obese mice, we measured IL-1β and IL-6 mRNA expression in the hippocampus. This region was a primary focus of this study and is particularly relevant to obesity-associated neuroinflammatory and microglial alterations. These cytokines are commonly associated with neuroinflammatory responses and can be produced by activated microglia. BHB treatment significantly reduced IL-1β and IL-6 expression compared with untreated obese controls (Fig. 3.4 A, B; $p < 0.05$). These results suggest that BHB administration attenuates obesity-associated neuroinflammatory responses in the hippocampus.

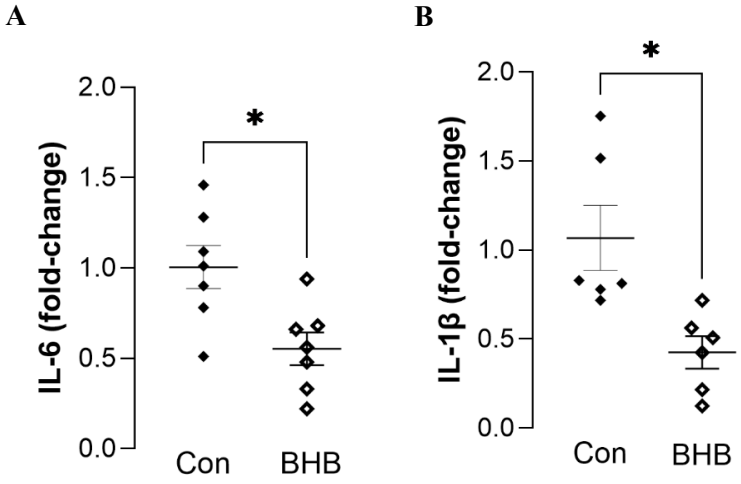


Figure 3.4. BHB Treatment Reduces Pro-inflammatory Cytokine mRNA Levels in the Hippocampus of Obese Mice. (**A**) *IL-1ß* RNA expression fold-change compared to untreated control, n = 7 (**B**) *IL-6* RNA expression fold-change compared to untreated control, n = 6. Con – control group, BHB – BHB treatment group. Data are presented as mean ± SEM; *$p < 0.05$.

To further evaluate the anti-inflammatory effects of BHB in the brain of obese mice, we measured IL-1β protein expression by Western blot and IL-6 levels by ELISA in cortical tissue. BHB treatment significantly reduced IL-1β expression (Fig. 3.5 A, B; $p < 0.0001$) and decreased IL-6 levels compared with untreated obese controls (Fig. 3.5 C; $p < 0.01$). These results indicate that BHB administration attenuates obesity-associated neuroinflammatory responses in the cortex.

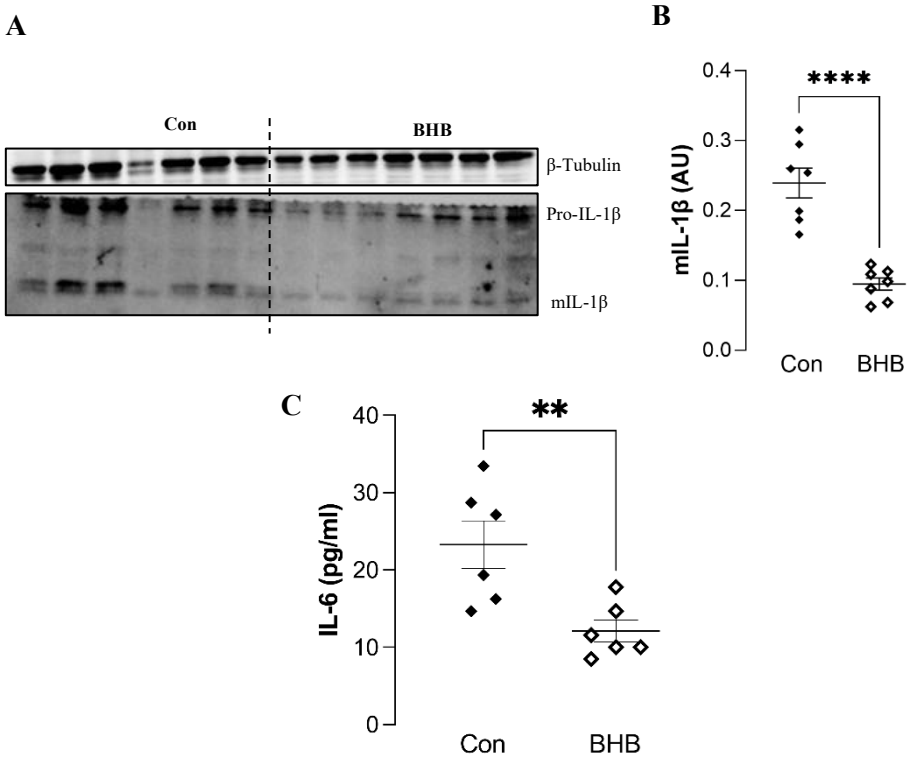


Figure 3.5. BHB Treatment Reduces Pro-inflammatory Cytokine Levels in the Brains of Obese Mice. (**A**) Representative Western blot data of cortex tissue from control and BHB-treated group showing pro-IL-1ß, mature IL-1ß (mIL-1ß), and ß-tubulin expression. (**B**) Quantification of mIL-1ß expression by densitometric analysis normalized to ß-tubulin, n = 7 (**C**) IL-6 expression measured by ELISA, n = 6. Con – control group, BHB – BHB treatment group. AU – arbitrary units. Data are presented as mean ± SEM; $^{**}p < 0.01$; $^{****}p < 0.0001$.

3.2. BHB Treatment Induces CB2R and Reduces MAGL Expression in the Mouse Hippocampus

Because the drivers of BHB's anti-inflammatory effects remain unknown, we hypothesized that BHB acts by modulating the ECS, particularly CB2R. For

this purpose, we assessed the expression of CB2R and MAGL, the primary enzyme responsible for eCBs degradation, in both experimental groups.

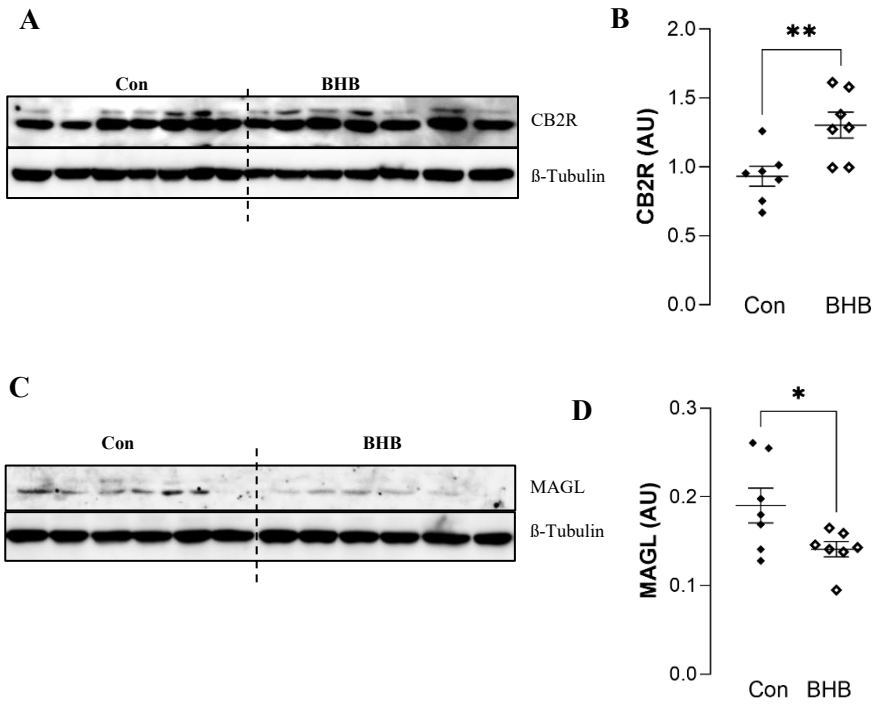


Figure 3.6. BHB Modulates CB2R and MAGL Expression in the Hippocampus of Obese Mice. (A) Representative Western blot data of hippocampal tissue from control and BHB-treated groups showing CB2R and α-tubulin expression. (**B**) Quantification of CB2R expression by densitometric analysis normalized to α-tubulin. (**C**) Representative Western blot data of hippocampal tissue from control and BHB-treated groups showing MAGL and α-tubulin expression. (**D**) Quantification of MAGL expression by densitometric analysis normalized to α-tubulin. Con – control group, BHB – BHB treatment group. n = 7. AU – arbitrary units. Data are presented as mean ± SEM. *$p < 0.05$; **$p < 0.01$.

Notably, CB2R expression was significantly higher in the BHB-treated group compared to controls (Fig. 3.6 A, B; $p < 0.01$). Since CB2R is expressed in microglia and involved in regulating neuroimmune responses, this finding may be relevant to microglial modulation by BHB treatment. In line with our hypothesis, MAGL expression was significantly reduced in BHB-treated mice (Fig. 3.6 A, C; $p < 0.05$), suggesting a possible shift in eCBs signaling associated with BHB administration.

3.3. Development of Primary Mouse Microglia Cell Isolation Protocol

To further assess the effects of BHB on microglia and to explore possible underlying mechanisms, we moved to *in vitro* cell culture models. This approach allowed us to study microglial responses in a more controlled setting and to investigate mechanistic links more directly than was possible *in vivo*. As a first step, we selected primary microglia isolated from adult mice. Because neuroinflammation and neurodegeneration are closely associated with aging, adult microglia were considered a more relevant model for these studies. Therefore, this model was chosen to reflect better the inflammatory context in which BHB-mediated modulation of microglial function may be biologically relevant. However, microglia isolation from adult mice remains constrained by low cell yield and limited culture purity, and no sufficiently efficient protocol is currently available. To address this, we systematically evaluated existing isolation methods and optimized a protocol to enhance cell yield. We subsequently compared the optimized adult isolation approach with neonatal (pup-derived) microglia isolation in terms of both yield and purity.

3.3.1. Microglia Isolation from Adult Mice

To optimize the microglial isolation protocol, we first focused on isolating cells from adult mice, as this model better reflects the pathophysiological context of neuroinflammatory diseases, which typically manifest with aging. We evaluated three isolation strategies: protocol 1 (originally described by Bordt et al., 2020, and subsequently modified in our laboratory), protocol 2 (Woolf et al., 2021), and protocol 3 (Lee JK & Tansey MG, 2013). These methods were compared in terms of cell yield and purity. A comparison of the three isolation strategies revealed that our optimized protocol yielded the highest microglial yield. Specifically, it resulted in significantly more cells than protocol 2 (Fig. 3.5A, B; $p < 0.001$) and protocol 3 (Fig. 3.5A, B; $p < 0.01$). In terms of cultural purity, no statistically significant differences were detected among the three isolation methods (Fig. 3.5C, D). The highest purity was obtained with protocol 3, followed by protocol 2, whereas our optimized protocol yielded the lowest purity; however, no statistically significant differences were detected between groups. These findings indicated that, although microglia could be isolated from the adult brain, both yield and purity remained suboptimal. To improve culture quality and reduce the number of animals required for mechanistic studies, we therefore continued with microglia isolation from neonatal mice.

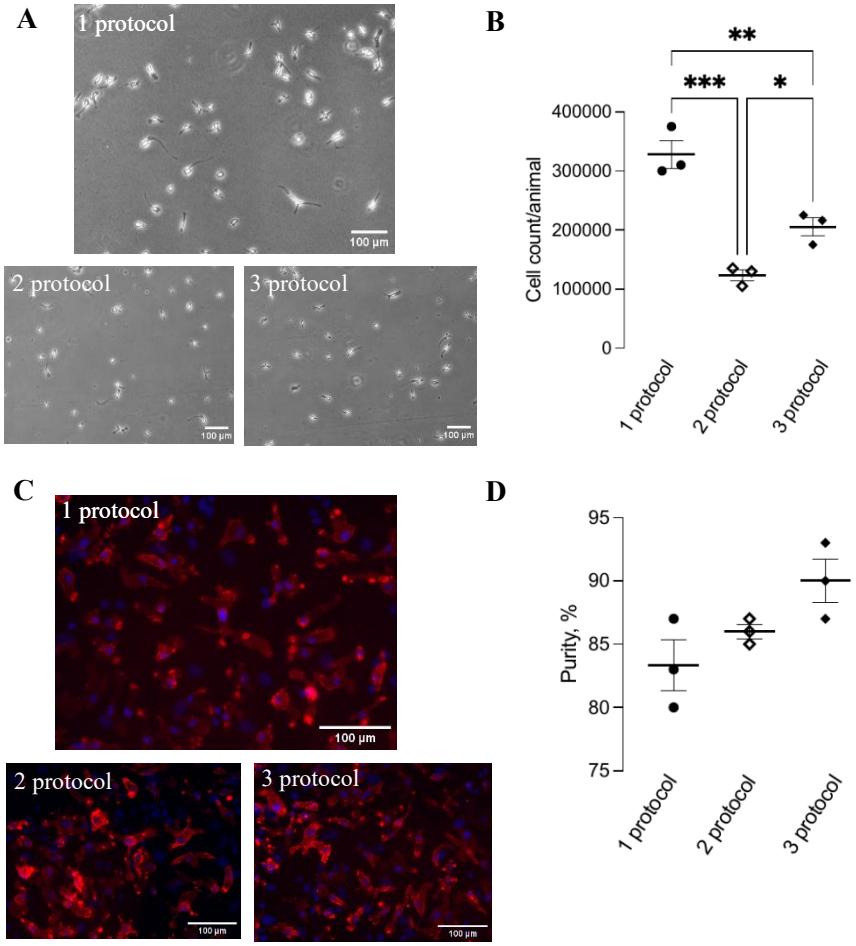


Figure 3.7. Assessment of Microglia Isolation from Adult Mouse Brain. (**A**) Representative light microscopy image of isolated primary microglia, illustrating cell density and morphology. (**B**) Cell yield obtained using three different isolation protocols. (**C**) Representative immunostaining image of isolated microglia, CD11b (red), nuclei (blue). (**D**) Purity of isolated primary microglia from pup and adult mice. n = 3. Data are presented as mean ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

3.3.2. Microglia Isolation from Neonatal Mice

To compare microglia isolation efficiency between pups and adult mice, we evaluated total cell yield and culture purity between our optimized protocol for microglia isolation from adult mice (protocol 1) and the protocol for isolating microglia from pups.

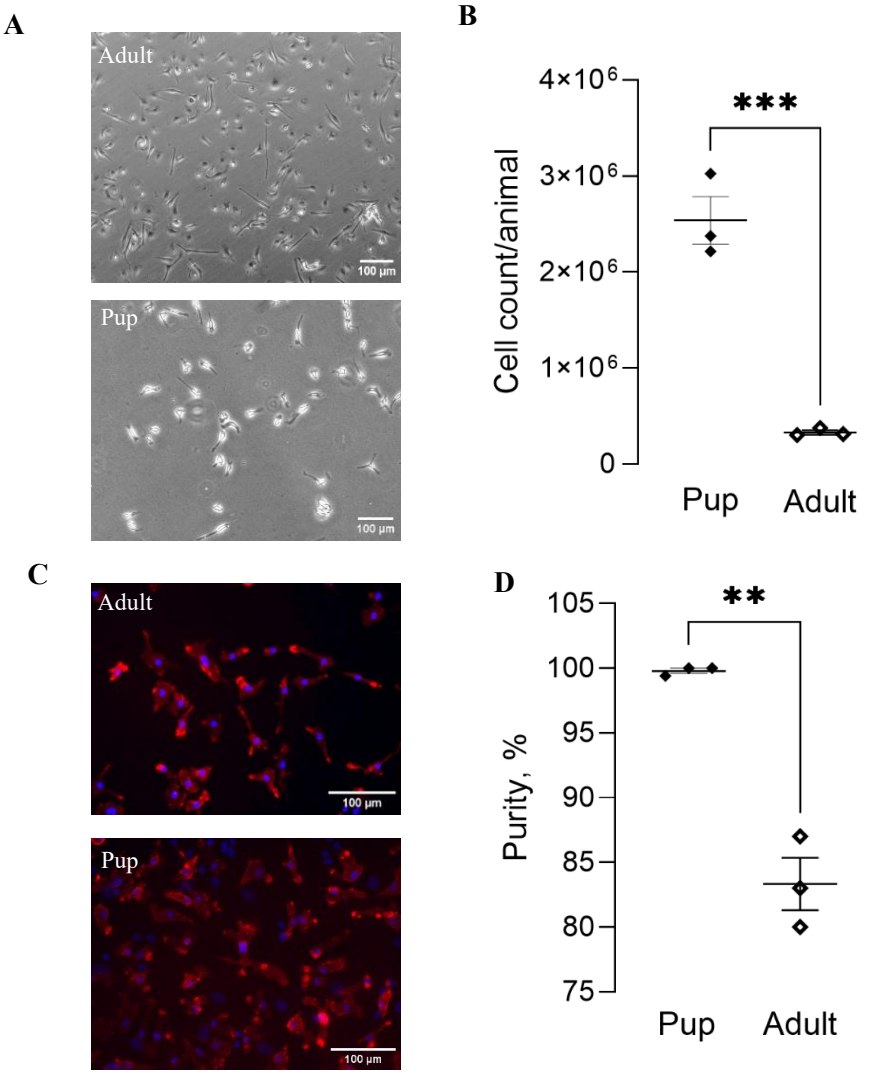


Figure 3.8. Cell Isolation Characteristics in Pup and Adult Mice (previously protocol 1) (**A**) Representative light microscopy image of isolated primary microglia, illustrating cell density and morphology. (**B**) Cell yield per animal following microglia isolation from pup and adult mice. (**C**) Representative immunostaining image of isolated microglia, CD11b (red), nuclei (blue). (**D**) Purity of isolated primary microglia from pup and adult mice. n = 3. Data are presented as mean ± SEM. *$p <$ 0.05; **$p < 0.01$.

Cell isolation yield was significantly higher in pups than in adult mice. Pup-derived samples yielded almost ten times more cells per animal, with counts

ranging from ~2.2-3×10^6 cells/animal, whereas adult-derived samples showed substantially lower yields, clustering more narrowly around ~3.0–3.8 × 10^5 cells/animal (Fig. 3.8 A, B; $p < 0.001$).
Immunostaining with the microglial marker CD11b revealed differences in culture purity between age groups. Quantitative analysis showed that microglial purity was significantly higher in pup-derived isolates, reaching nearly 100 %, whereas adult-derived isolates showed a substantially lower purity of approximately 80-87% (Fig. 3.8 C, D; $p < 0.01$). In light of these findings, primary microglia isolated from pups were used in subsequent experiments, as this approach provided more reliable results and reduced animal use.

3.4. BHB Anti-Inflammatory Effects are CB2R Dependent in Primary Mouse Microglia

After optimizing the primary microglia isolation procedure, we further investigated the involvement of CB2 receptor signaling in the actions of BHB. For this purpose, cells were stimulated with LPS, a strong pro-inflammatory stimulus. To assess the effect of BHB, microglia were pretreated with BHB prior to LPS exposure. To evaluate the contribution of CB2R to the anti-inflammatory effects of BHB, cells were pretreated with the CB2R inverse agonist AM630 before BHB treatment. We first examined microglial functional responses, including phagocytosis and ROS production, and then analyzed the expression of pro- and anti-inflammatory genes. Finally, we assessed changes in NF-κB signaling to determine whether the effects of BHB and CB2R involvement are mediated through this pathway.

3.4.1. CB2 Receptor Blockade Reverses BHB-induced Functional Changes in Primary Microglia

To better understand the direct effect of BHB on microglial function, we performed *in vitro* analysis, focusing on phagocytic activity and ROS production, and examined whether CB2R modulates these effects.

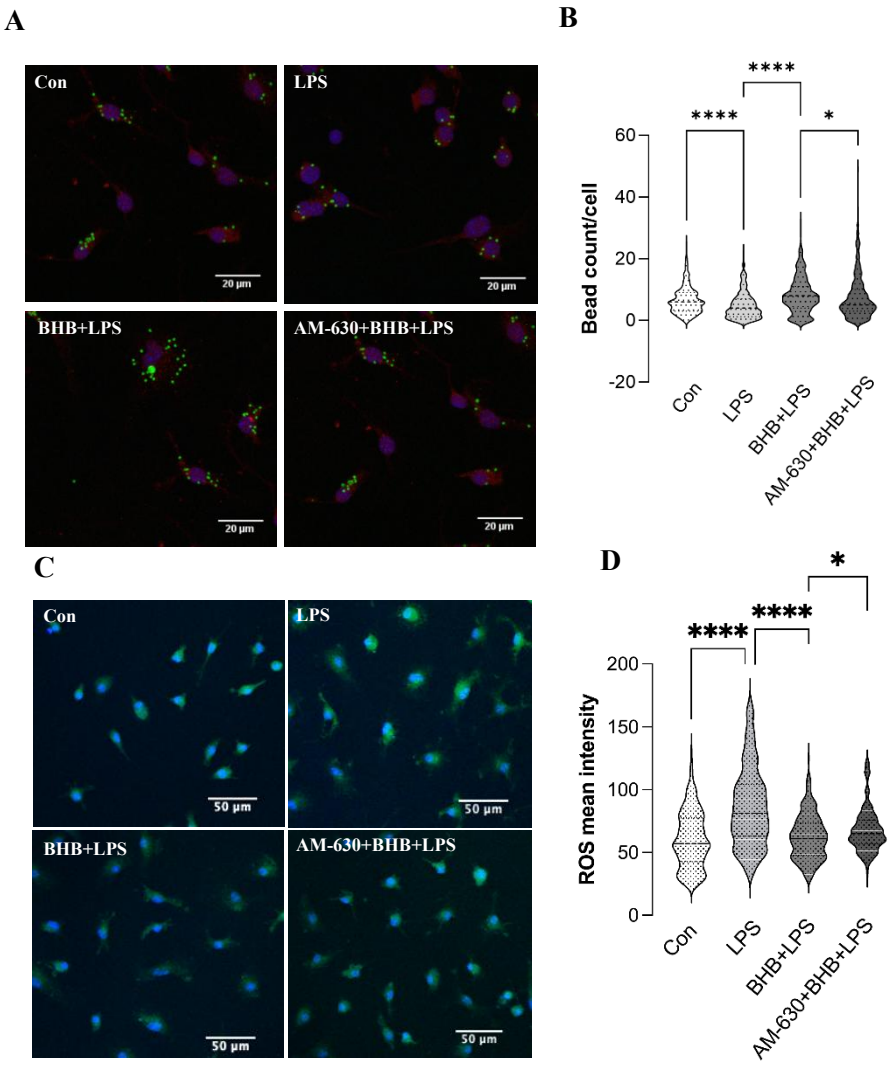


Figure 3.5. Effects of BHB and CB2R Blockade on Microglial Phagocytosis and ROS Production Following Inflammatory Stimulation. (**A**) Representative fluorescence images showing phagocytosis of primary mouse microglia in different experimental groups. Green: internalized fluorescent latex beads; red: microglial marker CD11b; blue: nuclei stained with DAPI. (**B**) Violin plot depicting microglial phagocytosis of latex beads, n = 260-320 cells analyzed. (**C**) Representative fluorescence images showing ROS production of primary mouse microglia in different experimental groups. Green: ROS signal; blue: nuclei stained with DAPI. (**D**) Violin plot depicting ROS synthesis, n = 200 cells (three independent biological replicates). Con – Control group; LPS – LPS-treated group (100 ng/mL); BHB+LPS – 1 h pretreatment with BHB before LPS; AM-630+BHB+LPS – 1 h pretreatment with AM630 (1 µM) before BHB and LPS. Data are presented as violin plots showing

the distribution of individual values; the central line represents the mean. *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$.

LPS treatment significantly impaired microglial phagocytosis, reducing the mean number of internalized latex beads compared with the control group (Fig. 3.9 A, B; $p < 0.0001$). Co-treatment with BHB and LPS significantly enhanced phagocytic activity compared with LPS alone (Fig. 3.9 A, B; $p < 0.0001$), suggesting that BHB promotes microglial functional recovery under inflammatory conditions. Co-treatment with the CB2R antagonist AM-630, in the presence of BHB and LPS, resulted in a modest but statistically significant decrease in phagocytosis compared to the BHB+LPS group (Fig. 3.9 A, B; $p < 0.05$). These results indicate that CB2R signaling contributes, at least in part, to the pro-phagocytic effects of BHB in microglia under inflammatory stress. ROS levels were significantly elevated following LPS treatment (Fig. 3.9 C, D; $p < 0.0001$), consistent with an activated pro-inflammatory microglial state. Pre-treatment with BHB significantly reduced ROS production compared with the LPS group (Fig. 3.9 C, D; $p < 0.0001$), supporting its role in mitigating oxidative stress and exerting anti-inflammatory effects. Importantly, CB2R inhibition with AM-630 partially reversed the ROS-lowering effect of BHB, increasing ROS production relative to the BHB+LPS group (Fig. 3.9 C, D; $p < 0.05$). These findings are consistent with the phagocytosis data and suggest that CB2R blockade may influence microglial inflammatory responses under the experimental conditions used. Collectively, the results indicate that CB2R signaling may contribute to, but may not fully account for, BHB-associated anti-inflammatory and homeostatic effects in microglia.

3.4.2. Effects of BHB and CB2R signaling on inflammatory gene expression in primary mouse microglia

Next, we investigated whether BHB can downregulate pro-inflammatory gene expression in response to LPS stimulation, and whether this effect is mediated via the CB2R. To this end, we measured the mRNA expression levels of *TNF-α*, *NOX2*, and *iNOS* using qPCR.

As expected, *TNF-α* mRNA expression was strongly upregulated in the LPS-treated group compared with control cells (Fig. 3.10 A; $p < 0.0001$). Pre-treatment with BHB significantly reduced *TNF-α* mRNA expression compared with the LPS-only group (Fig. 3.10 A; $p < 0.001$). Importantly, pre-treatment with the CB2R antagonist AM-630 before BHB and LPS exposure significantly increased *TNF-α* mRNA expression compared with the BHB+LPS group (Fig. 3.10 A; $p < 0.01$). These findings suggest that BHB

suppresses LPS-induced *TNF-α* mRNA expression and that this effect is at least partly dependent on CB2R signaling.

NOX2 mRNA expression remained unchanged following LPS treatment compared to the control group (Fig. 3.10 B). However, in the group pre-treated with BHB prior to LPS stimulation, *NOX2* mRNA expression was significantly reduced compared to the LPS-only group (Fig. 3.10 B; $p < 0.05$). Furthermore, when cells were pre-treated with the CB2R antagonist AM-630 before BHB and LPS exposure, *NOX2* mRNA expression increased relative to the BHB+LPS group (Fig. 3.10 B; $p < 0.05$). These findings are consistent with the previously observed reduction in ROS levels and suggest that BHB may influence oxidative stress-related pathways in microglia. Since NOX2 is a major enzymatic source of ROS, the observed decrease in NOX2 mRNA expression may indicate reduced NOX2-associated oxidative activity. However, as NOX2 activity was not directly measured, these results should be interpreted as indirect evidence. The data further suggest that this effect may be partially associated with CB2R signaling.

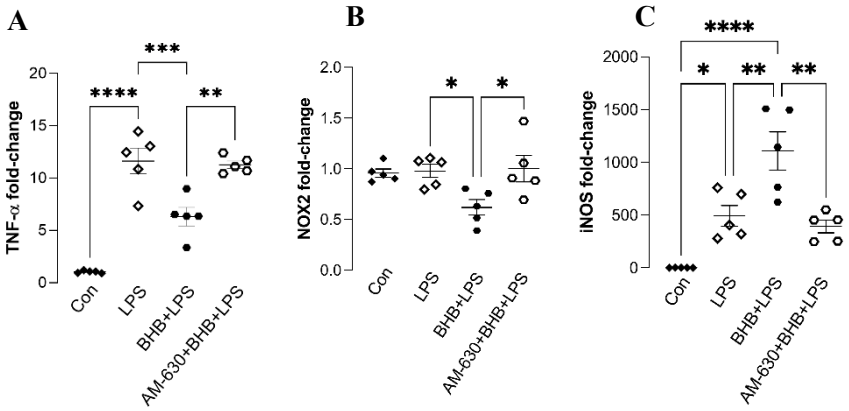


Figure 3.10. Modulation of Pro-inflammatory Cytokine Expression in Primary Mouse Microglial Cells Under LPS, LPS+BHB, and LPS+BHB+AM-630 treatments. (**A**) TNF-α, (**B**) NOX2, (**C**) iNOS. Data are presented as $2^{-\Delta\Delta Ct}$ with individual points, n = 5 biological replicates per experimental group. Targets are normalized to the geometric mean of the reference gene *GAPDH*. Con – Control group; LPS – LPS-treated group (100 ng/mL); BHB+LPS – 1 h pretreatment with BHB before LPS; AM-630+BHB+LPS – 1 h pretreatment with AM630 (1 μM) before BHB and LPS. Data are presented as mean ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

iNOS mRNA expression was markedly upregulated following LPS treatment, showing a statistically significant increase compared to the control group (Fig.

6C; $p < 0.05$). Interestingly, contrary to the expected anti-inflammatory effect of BHB, pre-treatment with BHB further enhanced *iNOS* mRNA expression compared to the LPS-only group (Fig. 3.10 C; $p < 0.01$). Moreover, pre-treatment with the CB2R antagonist AM-630 prior to BHB and LPS exposure significantly reduced *iNOS* mRNA expression compared to the BHB + LPS group (Fig. 3.10 C; $p < 0.01$). While CB2R blockade has generally been associated with increased pro-inflammatory marker levels, in this context it appears to attenuate *iNOS* expression.

3.4.3. Regulation of Anti-Inflammatory Gene Expression in Primary Mouse Microglia by BHB and the Role of CB2R

Next, we aimed to determine whether BHB upregulates anti-inflammatory gene expression and whether these effects are mediated via CB2R. To investigate this, we assessed the expression levels of *IL-10*, *TGF-β*, and *Arg1* using quantitative PCR.

After cells were treated with LPS, *IL-10* mRNA expression increased, but the difference was not statistically significant. BHB treatment did not significantly affect *IL-10* mRNA expression following LPS stimulation. Interestingly, co-treatment with the CB2R antagonist AM-630 increased IL-10 mRNA expression compared with the BHB + LPS group (Fig. 3.11 A; $p < 0.01$).

No significant changes in *TGF-β* mRNA expression were observed across any treatment group, except for a significant decrease after LPS treatment. These results suggest that, under the experimental conditions used, modulation of TGF-β mRNA expression is unlikely to be a major component of the anti-inflammatory effects associated with BHB treatment or CB2R signaling. (Fig. 3.11 B).

Regarding *Arg1* mRNA expression, a marker of alternatively activated microglia, was modestly elevated following LPS treatment, though the increase was not statistically significant. However, pre-treatment with BHB significantly increased Arg1 mRNA expression compared with the LPS-only group (Fig. 3.11 C; $p < 0.01$). This BHB-induced upregulation was completely reversed by CB2R blockade, with AM-630 reducing *Arg1* mRNA relative to the BHB + LPS group (Fig. 7 C; $p < 0.001$).

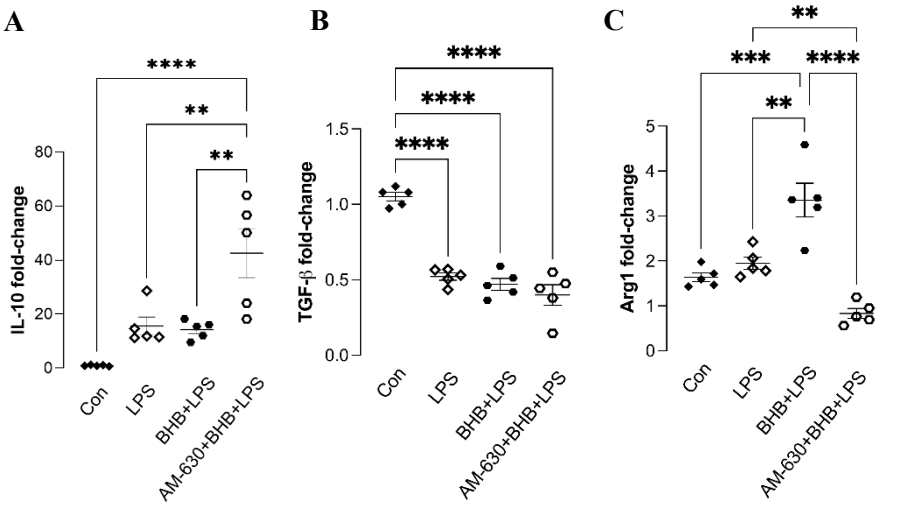


Figure 3.11. Modulation of Anti-inflammatory Cytokine Expression in Primary Mouse Microglial Cells Under LPS, LPS+BHB, and LPS+BHB+AM-630 treatments. (**A**) IL-10, (**B**) TGF-β, (**C**) Arg1. Data are presented as $2^{-\Delta\Delta Ct}$ with individual points. Targets are normalized to the geometric mean of the reference gene GAPDH. Con – Control group; LPS – LPS-treated group (100 ng/mL); BHB+LPS – 1 h pretreatment with BHB before LPS; AM-630+BHB+LPS – 1 h pretreatment with AM630 (1 μM) before BHB and LPS. n = 5. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

3.4.4. BHB Enhances Expression of iNOS and Arg1 Enzymes in a CB2R-Dependent Manner

mRNA expression levels do not always correlate directly with protein abundance. Therefore, we next aimed to determine whether Arg1 and iNOS protein levels reflected the transcriptional changes observed in response to treatment.

iNOS protein expression showed a modest, non-significant increase following LPS treatment alone. However, pre-treatment with BHB prior to LPS stimulation significantly increased iNOS expression (Fig. 3 .12A; $p < 0.001$). This BHB-induced upregulation was attenuated by co-treatment with AM-630 (Fig. 3.12 A; $p < 0.01$), suggesting involvement of the CB2 receptor.

Similarly, Arg1 protein expression followed a pattern consistent with its known role as an anti-inflammatory marker. LPS treatment reduced Arg1 levels, although the difference was not statistically significant (Fig. 3.12B). Notably, co-treatment with BHB and LPS resulted in an increase of Arg1 protein expression compared to LPS alone (Fig. 3.12 B; $p < 0.001$). AM

significantly reversed this effect-630 ($p < 0.01$), further supporting a CB2-mediated mechanism.

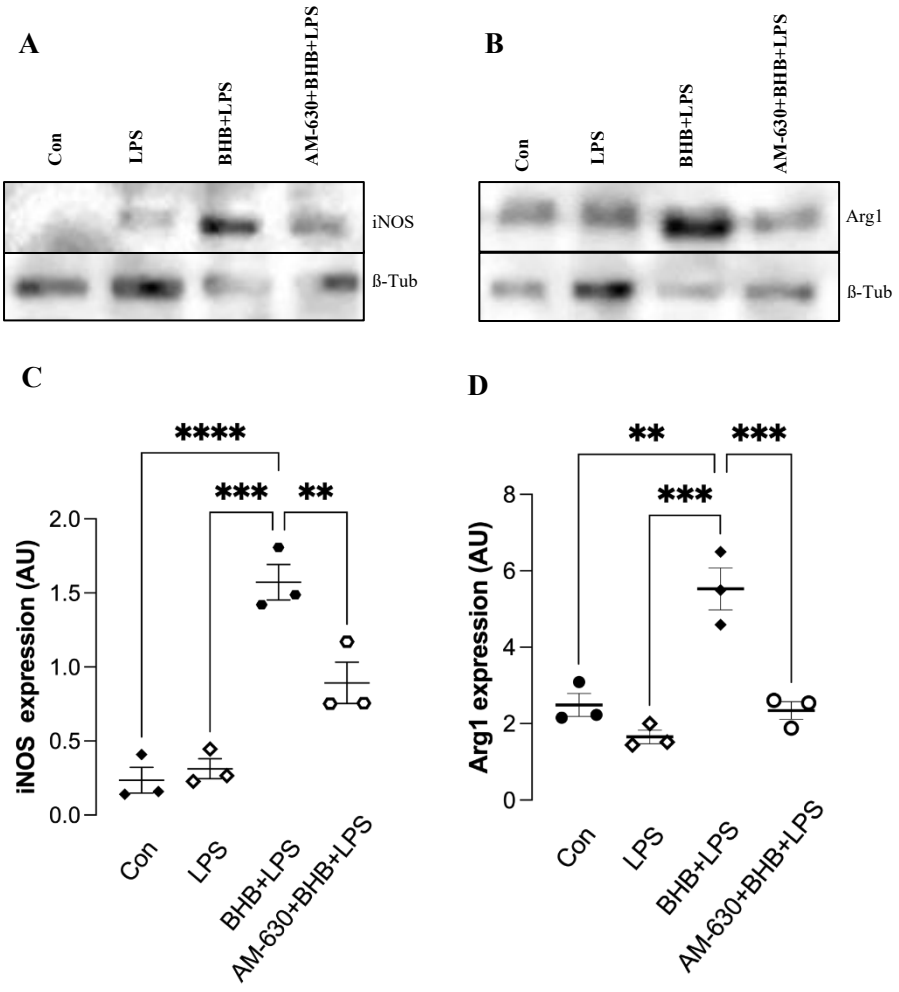


Figure 3.12. BHB induces iNOS and Arg1 protein expression in a CB2R-dependent manner. (**A**) Representative Western blot bands showing iNOS and α-tubulin expression in primary microglia cells (**B**). Representative Western blot bands showing Arg1 and α-tubulin expression in primary microglia cells. (**C**) Quantification of iNOS expression normalized to α-tubulin. (**D**) Quantification of Arg1 expression normalized to α-tubulin. Con – Control group; LPS – LPS-treated group (100 ng/mL); BHB+LPS – 1 h pretreatment with BHB before LPS; AM-630+BHB+LPS – 1 h pretreatment with AM630 (1 μM) before BHB and LPS. n = 3. Data are presented as mean ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

3.4.5 BHB Attenuates Inflammatory Signaling Through CB2R-dependent Suppression of the NF-κB Pathway

Next, we investigated whether BHB inhibits the NF-κB signaling pathway, a central regulator of microglial immune function that drives the production of pro-inflammatory cytokines and chemokines, thereby amplifying neuroinflammation. We also aimed to determine whether CB2R blockade could reverse BHB's inhibitory effects on this pathway. To assess NF-κB activity, we performed a nuclear translocation assay.

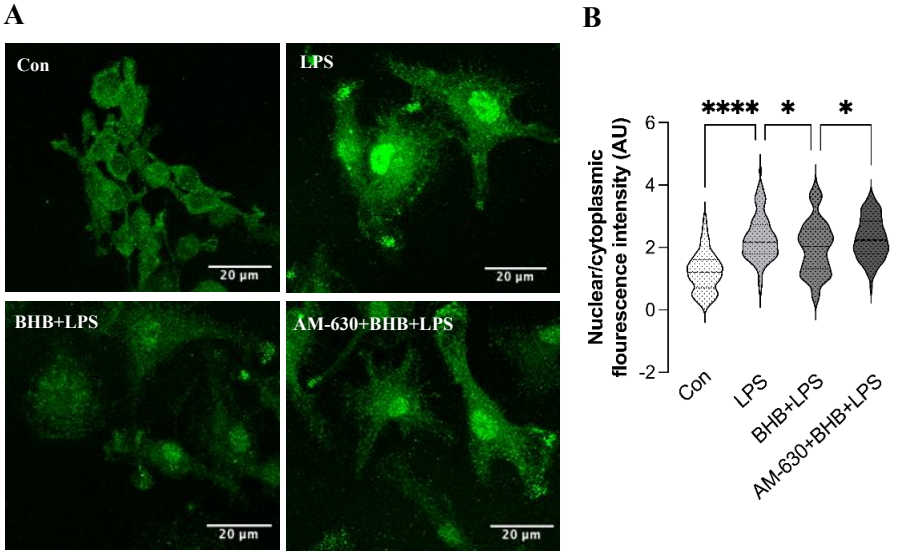


Figure 3.13. CB2R Blockade Partially reverses inhibition of NF-κB Nuclear Translocation by BHB. (**A**) Representative fluorescence images of primary mouse microglia stained with an antibody against NF-κB p65 (green). (**B**) Violin plot showing the nuclear-to-cytoplasmic intensity ratio of NF-κB p65 across treatment groups. Con – Control group; LPS – LPS-treated group (100 ng/mL); BHB+LPS – 1 h pretreatment with BHB before LPS; AM-630+BHB+LPS – 1 h pretreatment with AM630 (1 µM) before BHB and LPS. n = 90 cells. Data are presented as violin plots showing the distribution of individual values; the central line represents the mean. $^{**}p < 0.01$; $^{**}p < 0.0001$.

As expected, LPS stimulation significantly enhanced nuclear localization of NF-κB, increasing its translocation compared to control (Fig. 3.13 A, B; $p < 0.0001$), confirming engagement of the inflammatory pathway. Pre-treatment with BHB prior to LPS exposure significantly attenuated this effect (Fig. 3.13 A, B; $p < 0.05$), consistent with BHB's reported anti-inflammatory properties. Notably, co-treatment with the CB2R antagonist AM-630 partially reversed

the effect of BHB, increasing nuclear NF-κB localization compared to the BHB + LPS group (Fig. 3.13A, B; $p < 0.05$).
These findings suggest that BHB exerts its anti-inflammatory effects, at least in part, by suppressing NF-κB activation, a key transcription factor in microglial-mediated neuroinflammation.

3.5. Effects of BHB and CB2R Antagonism on Intracellular Signaling Pathways Under Inflammatory Conditions in Primary Microglia

Lastly, to investigate the molecular mechanisms underlying BHB action and the potential involvement of CB2R signaling, we assessed changes in the phosphorylation status of proteins associated with major intracellular signaling pathways in primary microglia under inflammatory conditions. The analyzed proteins were related to pathways involved in inflammatory signaling, metabolic regulation, cell survival and death, proliferation, and stress responses (PI3K/AKT, AMPK, apoptosis, autophagy, cell cycle, ErbB, focal adhesion, insulin, JAK/STAT, MAPK, mTOR, NF-κB, p53, Ras, TGF-β, and VEGF signaling). Comparisons were performed between the control and LPS-treated groups to assess inflammation-induced changes, between the LPS and BHB + LPS groups to evaluate the effect of BHB under inflammatory conditions, and between the BHB + LPS and AM630 + BHB + LPS groups to assess the potential involvement of CB2R signaling.

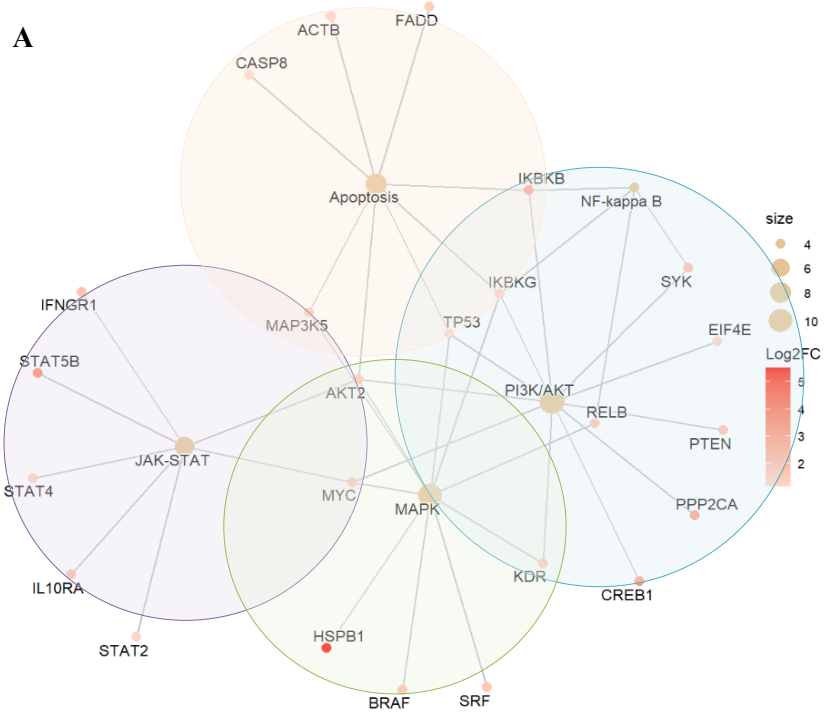
3.5.1. Effects of LPS Stimulation on Phosphorylation-associated Signaling Pathways in Primary Microglia

To characterize the effects of inflammatory stimulation on intracellular signaling, phosphorylation profiles were compared between control and LPS-treated primary microglia. LPS induced broad, bidirectional changes across interconnected pathways associated with inflammatory responses, cellular stress, survival, apoptosis, growth, and metabolism. Among proteins with increased phosphorylation after LPS treatment, the most prominent network clusters were associated with inflammatory signaling, stress-response pathways, apoptosis/survival regulation, and immune-related signal transduction. These interconnected clusters suggest that LPS strongly activated signaling modules involved in the microglial response to inflammatory stimulation. Pathways such as JAK/STAT, MAPK, PI3K/AKT, and apoptosis-related signaling appeared as major hubs within the network, indicating coordinated activation of pathways involved in cytokine signaling,

transcriptional regulation, cellular stress responses, and inflammatory mediator production (Fig 3.14 A).

In contrast, proteins with decreased phosphorylation after LPS exposure clustered mainly within the PI3K/AKT, JAK/STAT, ErbB, and autophagy (Fig. 3.14 B). This indicates that LPS stimulation was accompanied not only by increased phosphorylation of inflammatory and stress-related signaling proteins, but also by reduced phosphorylation within selected pathways related to growth factor signaling, cellular survival, metabolic adaptation, and homeostatic regulation. The presence of both increased and decreased phosphorylation within pathways such as PI3K/AKT and JAK/STAT suggests that LPS did not uniformly activate or suppress these signaling cascades, but rather induced pathway-specific remodeling of individual signaling components.

Together, these findings indicate that LPS induced extensive, bidirectional remodeling of phosphorylation-dependent signaling in primary microglia. Rather than uniformly increasing inflammatory pathway activity, LPS altered distinct signaling nodes across inflammatory, survival, metabolic, and growth factor-associated networks. This pattern suggests that LPS-induced microglial activation involves a coordinated reorganization of intracellular signaling, with simultaneous enhancement and attenuation of phosphorylation eve



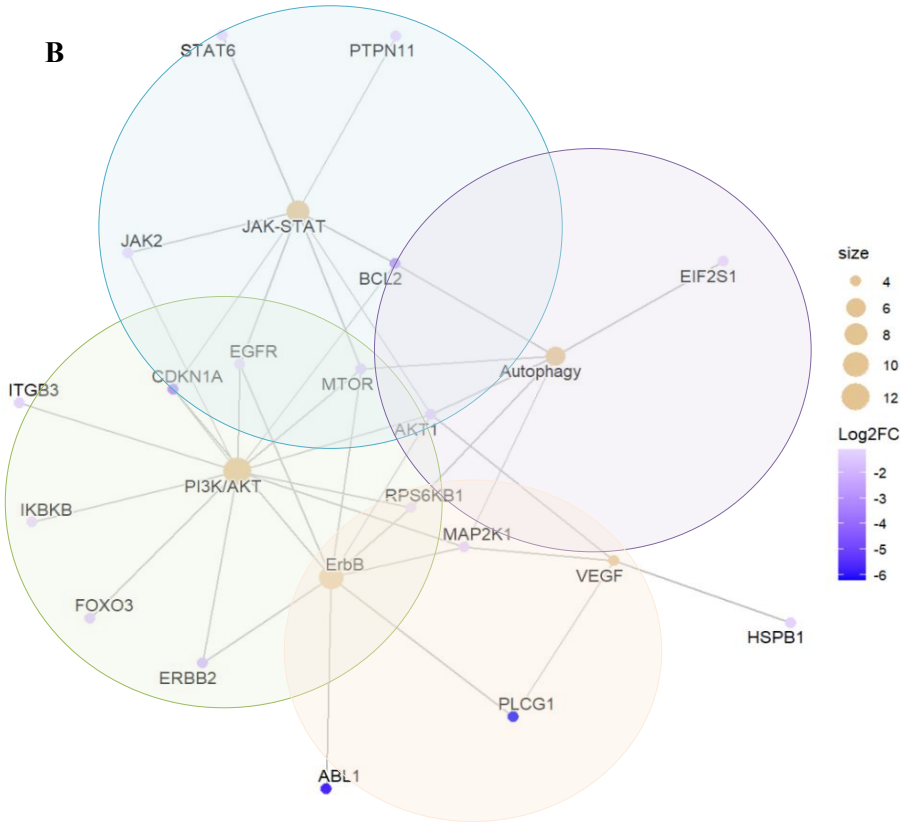


Figure 3.14. Pathway Network Analysis of Proteins Regulated in LPS-treated Primary Microglia (**A**) Network analysis of proteins with increased phosphorylation in LPS-treated primary microglia. (**B**) Network analysis of decreased phosphorylation signals in LPS-treated primary microglia. Nodes represent signaling pathways or individual proteins associated with reduced phosphorylation-related signals following LPS stimulation compared with control cells. Edges indicate associations between proteins and their corresponding pathways. Pathway node size reflects the number of connected proteins, whereas node color intensity represents the magnitude of log2 fold change, with darker color indicating stronger downregulation. Red indicates proteins or pathways with increased phosphorylation signal, while blue indicates proteins or pathways with decreased phosphorylation signal; darker colors indicate a greater magnitude of change.

3.5.2.BHB Modulates Phosphorylation-associated Signaling Pathways in LPS-stimulated Primary Microglia

Pathway network analysis was performed to characterize further phosphorylation changes associated with BHB treatment in LPS-activated primary microglia. Proteins with increased phosphorylation in the BHB+LPS

group clustered mainly within PI3K/AKT/mTOR, JAK/STAT, autophagy, and cell-cycle-related networks. The PI3K/AKT/mTOR-associated cluster was among the most prominent signaling groups, indicating that BHB treatment was associated with phosphorylation changes in pathways involved in cellular survival, metabolic adaptation, and growth-associated signaling. This cluster was closely associated with autophagy-related signaling, suggesting that BHB affected adaptive cellular response pathways under inflammatory conditions. Increased phosphorylation within JAK/STAT-associated components further indicates that BHB influenced cytokine-responsive signaling pathways in LPS-activated microglia. Notably, the presence of STAT1 among the proteins with increased phosphorylation suggests that BHB treatment was associated not only with anti-inflammatory signaling but also with activation of selected pro-inflammatory or interferon-associated signaling components.

In contrast, proteins with decreased phosphorylation after BHB treatment were primarily associated with TGF-β, AMPK, insulin signaling, apoptosis, and NF-κB-associated networks. Reduced phosphorylation within AMPK- and insulin-related pathways indicates that BHB altered metabolic signaling responses in LPS-activated microglia. The association with TGF-β-related signaling suggests changes in pathways involved in immune regulation and cellular homeostasis, while reduced phosphorylation within this network may reflect suppression of selected regulatory or anti-inflammatory signaling components. Decreased phosphorylation within apoptosis-associated networks indicates that BHB treatment also affected signaling components related to cell fate and stress-response regulation. In parallel, the presence of NF-κB-associated components among proteins with reduced phosphorylation suggests attenuation of selected inflammatory signaling nodes under LPS-stimulated conditions.

Together, these data indicate that BHB induced selective remodeling of phosphorylation-dependent signaling in LPS-activated primary microglia. The coexistence of reduced phosphorylation in NF-κB-associated pathways and increased phosphorylation of cytokine-responsive components, such as STAT1, suggests that BHB does not act as a simple global anti-inflammatory regulator in this model. Instead, BHB appears to reshape the inflammatory signaling state of activated microglia by simultaneously attenuating some inflammatory pathways while maintaining or enhancing selected pro-inflammatory, metabolic, and adaptive signaling components.

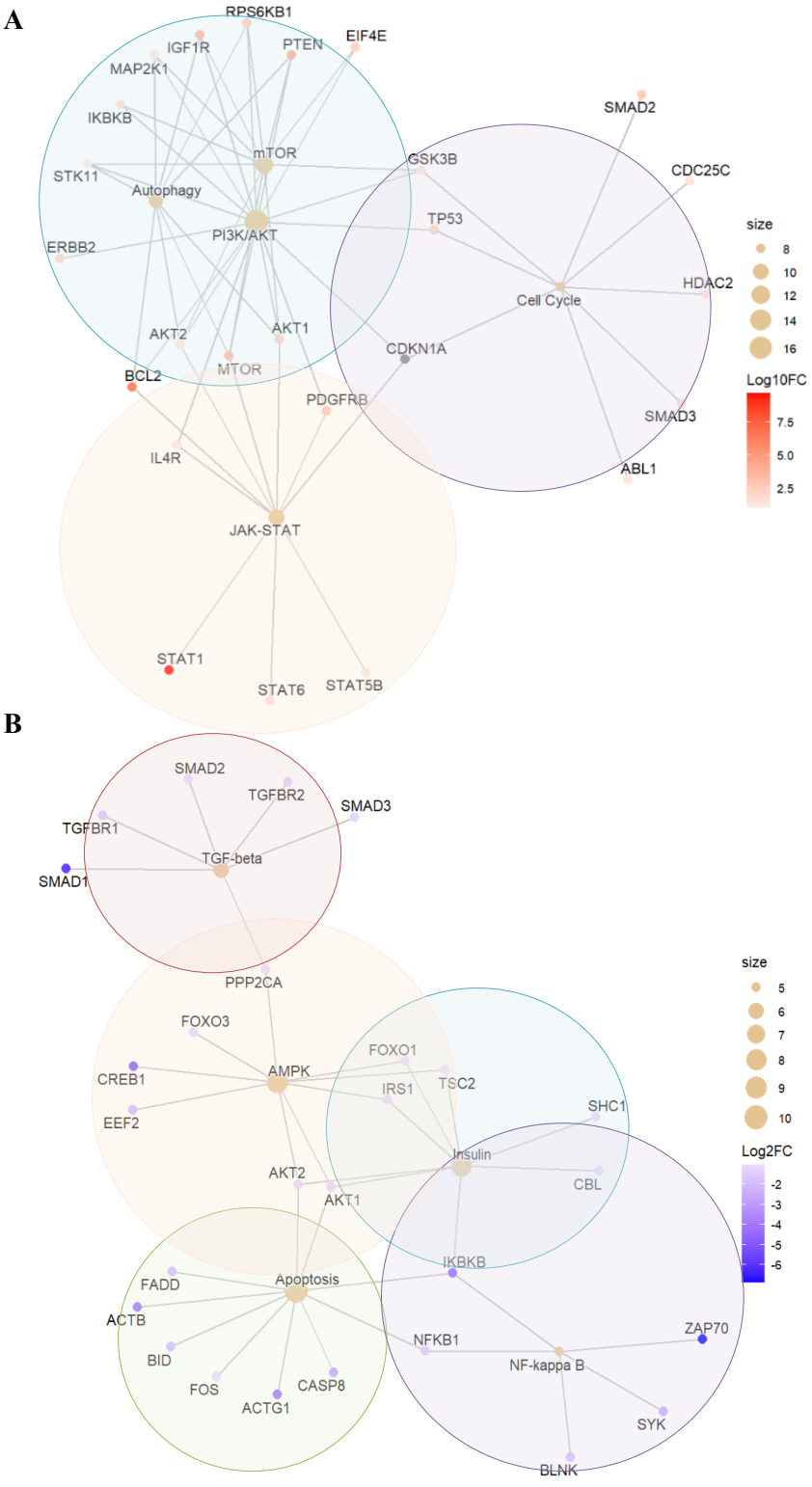

A
RPS6KB1
IGF1R
PTEN
EIF4E
MAP2K1
IKBKB
mTOR
STK11
Autophagy
PI3K/AKT
ERBB2
AKT2
AKT1
MTOR
BCL2
PDGFRB
IL4R
JAK-STAT
STAT1
STAT6
STAT5B
SMAD2
CDC25C
GSK3B
TP53
HDAC2
Cell Cycle
CDKN1A
SMAD3
ABL1
size
8
10
12
14
16
Log10FC
7.5
5.0
2.5
B
SMAD2
TGFBR2
SMAD3
TGFBR1
TGF-beta
SMAD1
PPP2CA
FOXO3
FOXO1
AMPK
CREB1
IRS1
TSC2
EEF2
SHC1
Insulin
AKT2
CBL
AKT1
IKBKB
FADD
Apoptosis
ACTB
NFKB1
ZAP70
BID
NF-kappa B
FOS
CASP8
ACTG1
SYK
BLNK
size
5
6
7
8
9
10
Log2FC
-2
-3
-4
-5
-6

Figure 3.15. Pathway Network Analysis of Proteins Regulated in BHB-Pretreated Primary Microglia Under Inflammatory Conditions. (**A**) Network analysis of proteins with increased phosphorylation in BHB+LPS-treated primary microglia. (**B**) Network analysis of proteins with reduced phosphorylation in BHB+LPS-treated primary microglia. Nodes in the (**A**) and (**B**) graphs represent signaling pathways or individual proteins associated with reduced phosphorylation-related signals following LPS stimulation compared with control cells. Edges indicate associations between proteins and their corresponding pathways. Pathway node size reflects the number of connected proteins, whereas node color intensity represents the magnitude of log2 fold change, with darker color indicating stronger downregulation. Red indicates proteins or pathways with increased phosphorylation signal, while blue indicates proteins or pathways with decreased phosphorylation signal; darker colors indicate a greater magnitude of change.

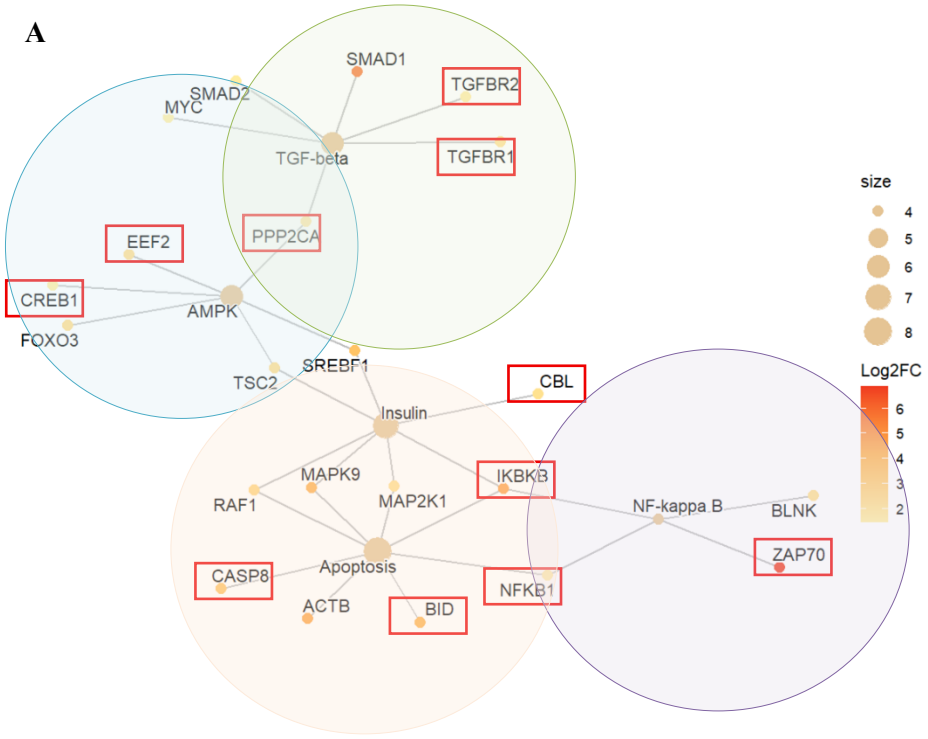
3.5.3 Differentially Phosphorylated Proteins Following CB2R Blockade in BHB-treated LPS-stimulated Primary Microglia

Pathway network analysis of the AM630+BHB+LPS group, compared with the BHB+LPS group, revealed that CB2R blockade modulated a wide range of proteins. In total, 41 proteins were upregulated across most of the analyzed pathways. Additionally, 16 proteins were downregulated and were mainly associated with the MAPK, PI3K/AKT, cell cycle, JAK/STAT, and autophagy pathways (Supplementary Figure 1 A, B).

However, the main purpose of this comparison was to identify proteins altered by BHB only when CB2R signaling remained functional. Blocking CB2R allowed us to isolate this receptor's contribution to BHB signaling. Therefore, we focused on proteins that showed opposite patterns of regulation between the BHB+LPS and AM630+BHB+LPS groups, specifically those upregulated in the BHB+LPS group but downregulated after CB2R blockade, and those downregulated in the BHB+LPS group but upregulated in the AM630+BHB+LPS group. This pattern suggested that their regulation by BHB may depend, at least in part, on intact CB2R signaling (Figure 3.16 A, B).

Pathways inhibited by BHB treatment but activated when CB2R was blocked included TGF-β, AMPK, insulin, apoptosis, and NF-κB. These findings suggest that CB2R blockade partially reversed the phosphorylation-associated signaling profile induced by BHB treatment. This effect was most evident in the IKKβ/NF-κB axis, indicating that CB2R signaling may contribute to the suppression of inflammatory signaling. In contrast, the changes observed in apoptosis-related and other signaling proteins likely reflect broader downstream remodeling of intracellular pathways.

Although several individual phosphorylation-related signals were decreased in the AM630+BHB+LPS group compared with the BHB+LPS group, no clearly defined signaling pathway was enriched among these proteins. Some of these proteins had previously shown increased phosphorylation after BHB treatment in LPS-activated microglia; however, after CB2R blockade, their reduction did not cluster into a consistent functional pathway or signaling hub. Therefore, these changes should be interpreted with caution as isolated phosphorylation events rather than as evidence of coordinated pathway regulation. Overall, the reduced phosphorylation signals observed after AM630 treatment do not provide strong mechanistic evidence for a specific CB2R-dependent pathway beyond the more clearly defined changes in previously mentioned pathways.



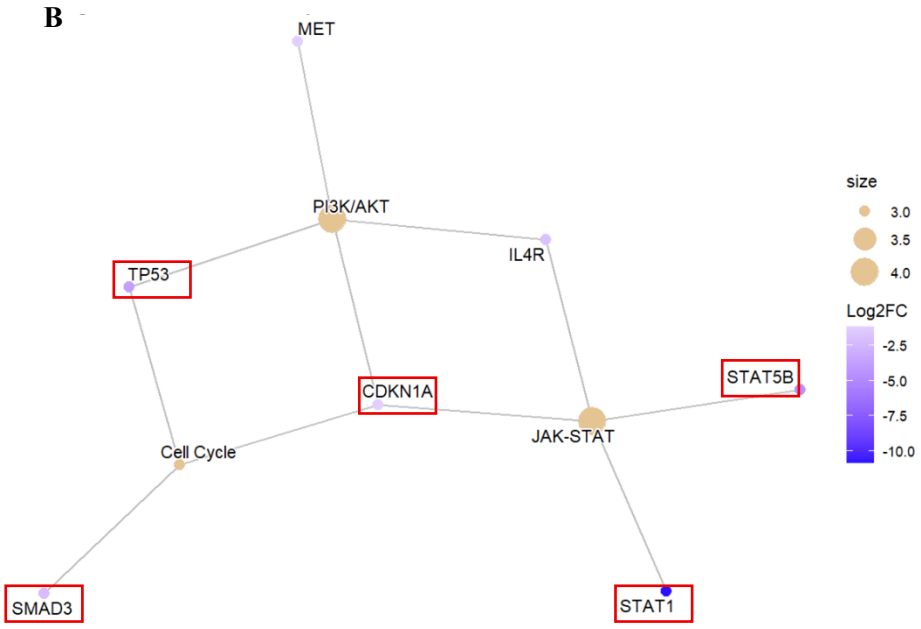


Figure 3.16. **Pathway Network Analysis of Proteins Regulated in AM-630+BHB+LPS-treated Primary Microglia.** (**A**) Network analysis of increased phosphorylation proteins in AM630+BHB+LPS-treated primary microglia. (**B**) Network analysis of downregulated phosphorylation-associated proteins in AM630+BHB+LPS-treated primary microglia. Nodes in the (**A**) and (**B**) graphs represent signaling pathways or individual proteins associated with reduced phosphorylation-related signals following LPS stimulation compared with control cells. Edges indicate associations between proteins and their corresponding pathways. Pathway node size reflects the number of connected proteins, whereas node color intensity represents the magnitude of log2 fold change, with darker color indicating stronger downregulation. Red indicates proteins or pathways with increased phosphorylation signal, while blue indicates proteins or pathways with decreased phosphorylation signal; darker colors indicate a greater magnitude of change.

4. DISCUSSION

BHB has been shown to improve memory, enhance cognitive function, and promote neuronal plasticity in various disease models, primarily through its anti-inflammatory effects (Yamanashi T et al., 2017). However, the precise mechanisms underlying these effects remain unclear. In this study, we demonstrate that the anti-inflammatory actions of BHB are mediated, at least in part, through CB2R signaling.

We initially hypothesized that BHB exerts anti-inflammatory effects in the DIO mouse model, which is well established to develop low-grade neuroinflammation (Henn RE et al., 2022; Cavaliere et al., 2019; Lainez et al., 2018), where BHB supplementation has been shown to reduce body weight and improve metabolic parameters (Heras V et al., 2025; Nasser S et al., 2022). In fact, BHB is widely recognized for its anti-inflammatory properties in aging mice and in neurodegenerative disease models. For instance, a ketogenic diet has been shown to attenuate age-related chronic neuroinflammation in mice (Nomura M et al., 2023). Additionally, BHB administration in the Alzheimer's (5xFAD) mouse model significantly reduced amyloid-β plaque burden, suppressed microgliosis, and inhibited NLRP3 inflammasome activation (Shippy DC et al., 2020). However, its specific effects on microglia have not been thoroughly investigated.

As a low-grade chronic inflammatory disease, obesity is linked to microglial dysfunction, including increased reactivity, which has been implicated in cognitive decline (Cope EC et al., 2018; Vijaya AK et al., 2024).To assess microglial function in the brain of DIO mice, we focused on three key aspects: morphology, phagocytic capacity, and the ability to engulf synaptic structures. Prior research has demonstrated that BHB treatment promotes a more branched microglial morphology, which is typically associated with a less pro-inflammatory, more surveillant state (Huang C et al., 2018). In line with this, we observed a transition from the rounded, amoeboid morphology typical of reactive microglia to a more complex, ramified structure in BHB-treated obese mice, indicating a shift toward a homeostatic, surveying phenotype. Moreover, BHB treatment appeared to direct microglia toward a more adaptive phagocytic profile by enhancing debris clearance while reducing synaptic engulfment. This selective modulation of phagocytic behavior supports a neuroprotective role for BHB in preserving synaptic integrity under conditions of metabolic stress.

Next, we aimed to investigate whether changes in ECS components accompany the immunomodulatory effects of BHB. CB2R is associated with immune modulation in the CNS, is highly expressed in microglia, and plays a

pivotal role in regulating inflammatory responses (Komorowska-Müller JA et al., 2020). Furthermore, MAGL is an enzyme that hydrolyzes the eCB 2-AG (Miller S et al., 2016), an agonist of both CB1R and CB2R, into arachidonic acid and glycerol (Jiang M et al., 2023). Inhibition of MAGL has been shown to elevate 2-AG levels, leading to neuroprotective effects in models of neurodegenerative diseases (Chen C, 2023). In a rat model of irritable bowel syndrome, a ketogenic diet restored epithelial integrity in the intestinal crypt base and was associated with upregulation of both CB1 and CB2 receptors (Gigante I et al., 2021), representing the first study to suggest that BHB-related mechanisms may be modulated via the ECS. However, to our knowledge, no prior studies have directly linked BHB effects in the brain to the ECS. In our study, we found that CB2R expression increased and MAGL expression decreased in the brains of obese mice treated with BHB, suggesting an enhanced 2-AG tone. These results support our hypothesis that BHB exerts its anti-inflammatory effects, at least in part, through CB2R signaling.

Before performing functional experiments, we compared different approaches for isolating primary microglia from adult mouse brain tissue. Although all tested protocols generated microglia-enriched cultures with acceptable purity, adult-derived cultures yielded relatively low cell numbers, limiting their suitability for repeated pharmacological treatments and downstream molecular analyses. This is consistent with the technical difficulty of isolating viable microglia from adult brain tissue, where higher myelin content, increased tissue complexity, and greater sensitivity to dissociation procedures can reduce both yield and experimental reproducibility (Lee JK & Tansey MG, 2013; Chen et al., 2017). Thus, while adult-derived microglia may provide higher biological relevance to the mature brain environment, their practical limitations made them less suitable for the experimental design of the present study.

In contrast, neonatal brain tissue is generally more permissive for microglial isolation, owing to lower myelin content, reduced tissue complexity, and a higher relative abundance and proliferative capacity of immature glial cells (Nikodemova & Watters, 2012; Bordt et al., 2020). These features were reflected in our experimental outcomes, as primary microglia isolated from mouse pups yielded substantially higher cell numbers and purity, making neonatal-derived cultures more appropriate for subsequent *in vitro* experiments requiring multiple downstream analyses. Therefore, neonatal microglia were selected as the primary *in vitro* model because they enabled reproducible, sufficiently powered functional and molecular analyses.

Nevertheless, this choice has important limitations. Neonatal microglia do not fully recapitulate the phenotype of adult microglia and may differ in their

transcriptional state, maturation profile, metabolic activity, and responsiveness to inflammatory stimuli (Gosselin et al., 2017; Hammond et al., 2019). Therefore, the findings obtained in neonatal-derived cultures should not be interpreted as a complete reflection of adult microglial behavior *in vivo*. Instead, this model should be viewed as a reductionist system that allows mechanistic investigation of microglial responses under controlled inflammatory conditions.

After establishing the primary microglial isolation protocol from the mouse brain, we proceeded to the *in vitro* phase of the study. This approach allowed us to investigate the effects of BHB directly in primary microglia under controlled inflammatory conditions. Specifically, this part aimed to determine whether BHB remains effective under strong pro-inflammatory stimulation and whether CB2R is required for BHB-mediated effects. Therefore, we focused on microglial function, pro- and anti-inflammatory signatures, and the main signaling pathways that may link BHB and CB2R signaling.

In our *in vitro* analysis, we found that BHB counteracted the detrimental effect of LPS by increasing phagocytosis and decreasing ROS synthesis, which is consistent with prior observations regarding BHB involvement in phagocytosis and ROS production (Julio-Amilpas A et al., 2015; Zhang Y et al., 2022; Rana AK et al., 2024; Jin LW et al., 2023). Additionally, we demonstrated that the phagocytic capacity of microglia was significantly reduced when cells were pretreated with the inverse CB2R agonist AM-630, accompanied by increased ROS production. These findings suggest that BHB's ability to enhance phagocytosis and reduce oxidative stress is dependent on functional CB2R signaling.

Moving forward, we aimed to identify key markers involved in the immunomodulatory effects of BHB and to determine whether CB2R blockade influences their expression.

We observed a pronounced effect on *Arg1* mRNA levels, which were significantly upregulated following BHB treatment in LPS-stimulated cells. This upregulation was abolished upon pharmacological blockade of CB2R, indicating that the expression of this marker is at least partially dependent on CB2R signaling. Arg1 is widely recognized as a marker of the anti-inflammatory microglial phenotype (Cherry JD et al., 2014), supporting the idea that BHB mediates its anti-inflammatory effects through Arg1 upregulation. Usually, when Arg1 is elevated, it competes with iNOS for its common substrate, L-arginine, thereby limiting substrate availability for iNOS-mediated NO production and contributing to the resolution of inflammation. However, since mRNA levels do not always directly correlate with protein quantity, we further assessed Arg1 protein expression. Our results

confirmed the mRNA findings, demonstrating that CB2R, consistent with previous studies, mediates part of the beneficial anti-inflammatory effects of BHB. CB2R blockade with AM-630 reduced Arg1 expression in various disease models (Jia J et al., 2016), whereas CB2R agonism typically produces the opposite effect (Ma L et al., 2015).

Interestingly, iNOS expression followed a similar pattern. As expected, LPS treatment increased iNOS expression (Watters JJ et al., 2002). However, cells pretreated with BHB before LPS exposure showed even higher iNOS expression than the LPS-treated group. These findings suggest that BHB may not exert uniform anti-inflammatory effects under all conditions, but rather that its effects may depend on the inflammatory and metabolic context of the cells (Shi X et al., 2014). Although this effect was not explored in greater detail in the present study, our findings indicate that most previous studies support an overall anti-inflammatory action of BHB. Therefore, the increased iNOS expression observed in the BHB+LPS group may reflect a context-dependent response resulting from the combined effects of robust inflammatory stimulation and BHB treatment. One possible explanation is that BHB did not merely suppress microglial activation but instead induced immunometabolic reprogramming in LPS-stimulated cells. In activated microglia, BHB has been shown to promote mitochondrial metabolism and the accumulation of tricarboxylic acid cycle-associated immunometabolites, while also increasing iNOS expression in the same experimental context (Benito A et al., 2020). Thus, the elevated iNOS levels observed here may reflect a metabolically remodeled microglial state rather than a purely stronger pro-inflammatory response. This interpretation is consistent with the current understanding that microglial activation does not follow a strict pro-inflammatory–anti-inflammatory dichotomy but rather involves diverse, overlapping functional states (Paolicelli RC et al., 2022).

Finally, we corroborated that BHB treatment following LPS stimulation significantly inhibited NF-κB signaling as previously reported by other research groups (Fu SP et al., 2015; Fu SP et al., 2014). Additionally, CB2R blockade reversed this effect, supporting the role of CB2R in modulating the NF-κB pathway (Morcuende A et al., 2022). Our results further reveal the convergence of CB2R and BHB actions in the NF-κB signaling pathway.

Taken together, our results demonstrate that BHB has an immunomodulatory effect in both an *in vivo* DIO model and an *in vitro* model of primary microglial cells. The data support that the beneficial effects of BHB are mediated, at least in part, by CB2R. The partial inhibition of BHB's effects by CB2R antagonism suggests convergence on a shared signaling pathway, positioning CB2R as a mediator of BHB-driven neuromodulatory effects. While individual

components of this mechanism have been described previously, our study is the first to trace the CB2R-dependent BHB process in microglia.

To gain broader insight into the intracellular mechanisms affected by BHB treatment and the involvement of CB2R in LPS-stimulated primary microglia, phosphorylation-associated signaling was assessed using a phospho-antibody microarray.

First, LPS stimulation induced broad remodeling of intracellular signaling pathways in primary microglia, consistent with previous studies showing that LPS-induced microglial activation involves simultaneous engagement of inflammatory and stress-responsive networks rather than a single linear pathway (Pulido-Salgado M et al., 2018; An J et al., 2020). In particular, in our study, LPS-driven microglial activation has been strongly linked to NF-κB, MAPK, PI3K/AKT, and JAK/STAT signaling, all of which contribute to cytokine production, inflammatory amplification, and changes in microglial functional state. Specifically, the phosphoproteomic microarray revealed that LPS activated the canonical PI3K/AKT/NF-κB signaling pathway. AKT activation in microglia is essential for LPS-induced inflammation and is mediated by its specific receptor. This PI3K/AKT pathway activation also regulates transcription factor activity, including NF-κB (Cianciulli A et al., 2016), consistent with our increased NF-κB nuclear translocation following LPS treatment. NF-κB itself is a key driver of inflammatory cytokine expression in response to pro-inflammatory stimuli such as LPS, β-amyloid, or tissue injury. Activation of this pathway commonly promotes the expression of inflammatory mediators, including TNF-α, IL-1β, IL-6, iNOS, and COX-2 (Subedi L et al., 2019), which explains our findings on TNF-α and iNOS upregulation and ROS production after LPS treatment, indicating that signaling pathway-related phosphorylation changes contribute to the inflammatory state observed in this model.

Additionally, the induction of phosphorylation of apoptosis-associated proteins (for example, p53, FADD, Caspase-8) suggests activation of stress-response and cell-death regulatory pathways, but this should not be interpreted as direct evidence of apoptosis without additional apoptotic markers. Proteins in the apoptosis hub are also stress-responsive transcription factors involved in DNA damage responses and senescence. Oxidative stress can induce their phosphorylation, consistent with cellular stress signaling in LPS-activated microglia (Chen K et al., 2003). This could be explained by the ROS production in microglial cells after LPS treatment in our *in vitro* study, suggesting that the observed phosphorylation changes in the apoptosis hub may reflect oxidative stress-associated signaling rather than apoptosis alone. In fact, proteins observed in the apoptosis hub also regulate inflammatory

signaling and have been described as upstream mediators of canonical and non-canonical NLRP3 inflammasome priming and activation (Gurung P et al., 2014). Therefore, this cluster most reliably indicates regulation of inflammatory stress and the balance between cell death and survival rather than apoptosis alone (Flicker M et al., 2013).

BHB can modulate microglial metabolism and promote beneficial effects in models of neuroinflammatory disease (Jin et al., 2023), which we corroborate in our DIO mouse model and microglial *in vitro* approach. To identify pathways potentially involved in the protective effects of BHB under inflammatory conditions, we compared the signaling profiles of the LPS+BHB group with the LPS-only group. BHB treatment in LPS-activated microglia was associated with increased phosphorylation of proteins mainly clustered within PI3K/AKT, JAK/STAT, mTOR/autophagy, and cell-cycle-related signaling networks. Disruption of microglial homeostasis is closely associated with altered autophagy and metabolic regulation, both of which are important for controlling inflammatory activation and cellular stress responses (Ou-Yang P et al., 2023; Lauro C and Limatola C, 2020). Therefore, the involvement of these pathways in the LPS+BHB group may suggest activation of a compensatory adaptive process. This may support microglial stress adaptation and functional recovery during inflammatory stimulation, consistent with our results showing reduced ROS production and increased microglial phagocytic activity after BHB treatment. Thus, while LPS alone induces a strongly inflammatory state, BHB appears to partially redirect this response toward mechanisms involved in cellular maintenance, stress adaptation, and inflammatory resolution.

Importantly, not all upregulated phosphorylation events were easily reconciled with the classical anti-inflammatory effects of BHB. For example, STAT1 phosphorylation is typically associated with pro-inflammatory signaling (He H et al., 2024). Nevertheless, this finding may help explain the previously observed induction of iNOS at both the protein and mRNA levels in this thesis, as STAT1 signaling can promote the expression of inflammatory mediators, including iNOS (Delgado, 2003). Together, these results suggest that BHB does not induce a uniformly anti-inflammatory signaling profile but rather reshapes microglial signaling in a more complex manner, combining anti-inflammatory/regulatory features with selected inflammatory or compensatory signaling events.

Importantly, NF-κB signaling appeared attenuated in the presence of BHB, suggesting a reduced inflammatory response in LPS-stimulated microglia. The reduction of NF-κB-associated phosphorylation after BHB treatment is consistent with the anti-inflammatory profile observed in our study. In

particular, this finding supports our previous results showing that BHB reduced LPS-induced NF-κB nuclear translocation, ROS production, and pro-inflammatory mediator expression. Similar inhibitory effects of BHB on LPS-induced NF-κB activation have also been reported in previous studies, further supporting the interpretation that suppression of NF-κB signaling represents one of the key mechanisms by which BHB limits inflammatory activation in microglia (Fu SP et al., 2014; Jayashankar SS et al., 2023).

Additionally, BHB reduced the activation of apoptosis-related proteins (for example, Caspase-8, FADD, BID), which also supports our findings in BHB attenuation of LPS-induced ROS production, NF-κB activation, and pro-inflammatory mediator expression. Since oxidative and inflammatory stress can engage cell-death-associated signaling pathways, the reduced activation of apoptosis-related proteins after BHB treatment may reflect a broader decrease in inflammatory stress rather than direct suppression of apoptosis (Kim et al., 2013; Orning et al., 2020; Jembrek et al., 2021). These proteins are classically involved in extrinsic apoptosis, but they also regulate inflammatory signaling and inflammasome priming/activation (Gurung P et al., 2014). Therefore, the reduction of these signals in the BHB+LPS group may support the idea that BHB dampens selected inflammatory stress pathways in activated microglia.

To determine whether CB2R is required for BHB's effects, we analyzed proteins differentially regulated in the AM630+BHB+LPS group. CB2R is primarily known to mediate anti-inflammatory and immunomodulatory effects in microglia, as discussed in the literature review. The changes observed and relevant in the microglial context were at the NF-κB node and in the apoptosis pathway, consistent with the previous comparisons.

CB2R blockade induced phosphorylation of IKKβ and p105, which are triggers of NF-κB activation (Zhang J et al., 2023). In contrast, pretreatment with BHB attenuated these effects, supporting our hypothesis that BHB's anti-inflammatory effects act through CB2R. Previous studies have shown CB2-mediated anti-inflammatory effects via NF-κB signaling, whereas AM-630 reverses these effects (Komorowska-Müller JA, Schmöle AC, 2020; Lisboa SF et al., 2016). This observation also supports our previous data and strengthens the interpretation that BHB suppresses NF-κB signaling under LPS-induced inflammatory conditions. Importantly, the re-induction of NF-κB-related phosphorylation after CB2R blockade with AM-630 suggests that CB2R signaling is required, at least in part, for the inhibitory effect of BHB on this pathway. However, whether BHB acts directly through CB2R-dependent mechanisms or converges on NF-κB through parallel CB2R-associated signaling remains to be clarified in further studies.

CB2R activation can suppress microglial inflammatory activation and shift microglia/macrophages toward a more anti-inflammatory phenotype (Lin L et al., 2017), consistent with a reduction in protein phosphorylation in the apoptosis-related node by BHB pretreatment, suggesting that the BHB-associated attenuation of this death-receptor/mitochondrial cell-death module may depend, at least in part, on CB2R signaling. In addition, CB2R agonist treatment reduced neuroinflammation and neuronal apoptosis in neuroinflammatory models, while AM630 produced opposite effects, supporting the idea that CB2R signaling can modulate inflammatory injury and cell-death-associated outcomes (Jiang F et al., 2022).

Overall, this study shows that BHB modulates microglial phenotype and function under metabolic and inflammatory stress conditions. In the DIO model, BHB shifted microglia toward a more ramified morphology, reduced inflammatory cytokine expression, and altered eCB-related markers, including increased CB2R and reduced MAGL expression. In primary microglia, BHB counteracted several LPS-induced effects by enhancing phagocytic activity, reducing ROS production, increasing Arg1 expression, and attenuating NF-κB signaling. Importantly, several of these effects were reversed by CB2R blockade, supporting the conclusion that CB2R signaling contributes, at least in part, to the immunomodulatory actions of BHB. The phosphorylation array further indicated that BHB not only suppresses microglial activation but also remodels multiple signaling networks involved in inflammation, metabolism, stress adaptation, and regulation of cell death. Among these, NF-κB signaling and apoptosis-related signaling emerged as the two main hubs affected by BHB treatment and sensitive to CB2R blockade. Specifically, the BHB-associated attenuation of these pathways was partially reversed by AM630, suggesting that CB2R contributes to the regulation of both inflammatory NF-κB signaling and inflammatory cell-death-associated signaling in LPS-stimulated microglia. Taken together, our data support a model in which BHB acts as a metabolic and immunomodulatory regulator of microglia, with CB2R potentially mediating its anti-inflammatory and protective effects.

Taken together, the two experimental parts of this dissertation complement each other and provide a broader understanding of the potential roles of β-BHB and CB2R signaling in regulating neuroinflammatory processes. The *in vivo* part of the study, performed in a diet-induced obesity model, enabled evaluation of BHB effects in a complex physiological context, in which metabolic alterations, systemic inflammation, brain immune responses, and microglial changes occur simultaneously. In this model, BHB treatment was associated with reduced inflammatory markers and changes in microglial morphology and function-related parameters, suggesting that BHB may

attenuate neuroinflammatory responses under metabolically challenged conditions.

In contrast, the *in vitro* experiments using primary microglial cultures provided a more controlled system for investigating microglia-specific responses and examining the possible involvement of CB2 receptors in BHB-mediated effects. The use of LPS stimulation enabled the assessment of microglial inflammatory activation, while pharmacological CB2R blockade helped to clarify whether CB2R signaling contributes to the observed effects of BHB. These experiments showed that BHB modulated several key features of microglial activation, including inflammatory mediator expression, ROS production, NF-κB signaling, and phagocytic activity, and that some of these effects were altered by CB2R inhibition. Therefore, the *in vitro* findings support the idea that CB2R may be one of the mechanisms through which BHB regulates microglial inflammatory responses.

Importantly, the two approaches address different but interconnected levels of the same biological question. The *in vivo* model demonstrates that BHB can influence neuroinflammatory changes in the brain under obesity-associated metabolic stress. In contrast, the *in vitro* model provides mechanistic insight into how BHB may act directly on microglial cells. Thus, the results from primary microglia help to interpret the changes observed in the animal model, while the *in vivo* findings support the physiological relevance of the cellular mechanisms identified *in vitro*. Together, these data suggest that BHB may contribute to the regulation of microglial inflammatory phenotype, at least in part through CB2R-associated signaling.

Further studies are needed to define the precise molecular mechanisms linking BHB, CB2R activation, and downstream inflammatory signaling pathways. In particular, future work should determine whether BHB directly interacts with CB2R or indirectly modulates this receptor via changes in endocannabinoid metabolism, receptor expression, or intracellular signaling networks. Additional studies using genetic CB2R knockdown or knockout models would help to confirm the receptor-specific contribution of CB2R to BHB-mediated effects. Moreover, it would be valuable to investigate the involvement of other BHB-sensitive pathways, including HCAR2 signaling, histone deacetylase inhibition, inflammasome regulation, and metabolic reprogramming, as these mechanisms may act in parallel with or upstream of CB2R-dependent responses.

Overall, this dissertation suggests that BHB is not only an alternative metabolic substrate but also a bioactive signaling molecule capable of modulating microglial inflammatory responses. By combining an obesity-associated *in vivo* model with mechanistic experiments in primary microglia,

this work provides evidence that CB2R signaling may contribute to the anti-inflammatory actions of BHB in neuroinflammatory conditions. These findings may serve as a basis for further studies investigating metabolic–endocannabinoid interactions as potential targets for modulating microglial function and neuroinflammation.

CONCLUSIONS

1. In the DIO mouse model, BHB markedly attenuated neuroinflammatory responses, as evidenced by reduced IL-1β and IL-6 expression, changes in microglial morphology consistent with a more ramified state, and modulation of phagocytic activity. These effects were accompanied by upregulation of CB2R and downregulation of MAGL, suggesting coordinated regulation of the endocannabinoid system.
2. The optimized protocol for primary microglia isolation from mouse pups achieved the most favorable balance between cell yield and culture purity among the methods evaluated, confirming its robustness and suitability for downstream *in vitro* functional and molecular analyses.
3. In LPS-stimulated primary microglia, BHB significantly enhanced phagocytic capacity and modulated inflammatory responses; these effects were partially diminished or reversed by CB2R antagonism with AM-630, indicating a contributory role of CB2R-mediated signaling.
4. In LPS-activated microglia, BHB was associated with changes in several intracellular signaling pathways related to inflammation, cell survival, metabolism, and adaptive responses. The partial reversal of these effects after CB2R blockade further supports the involvement of CB2R-dependent mechanisms in mediating BHB activity.

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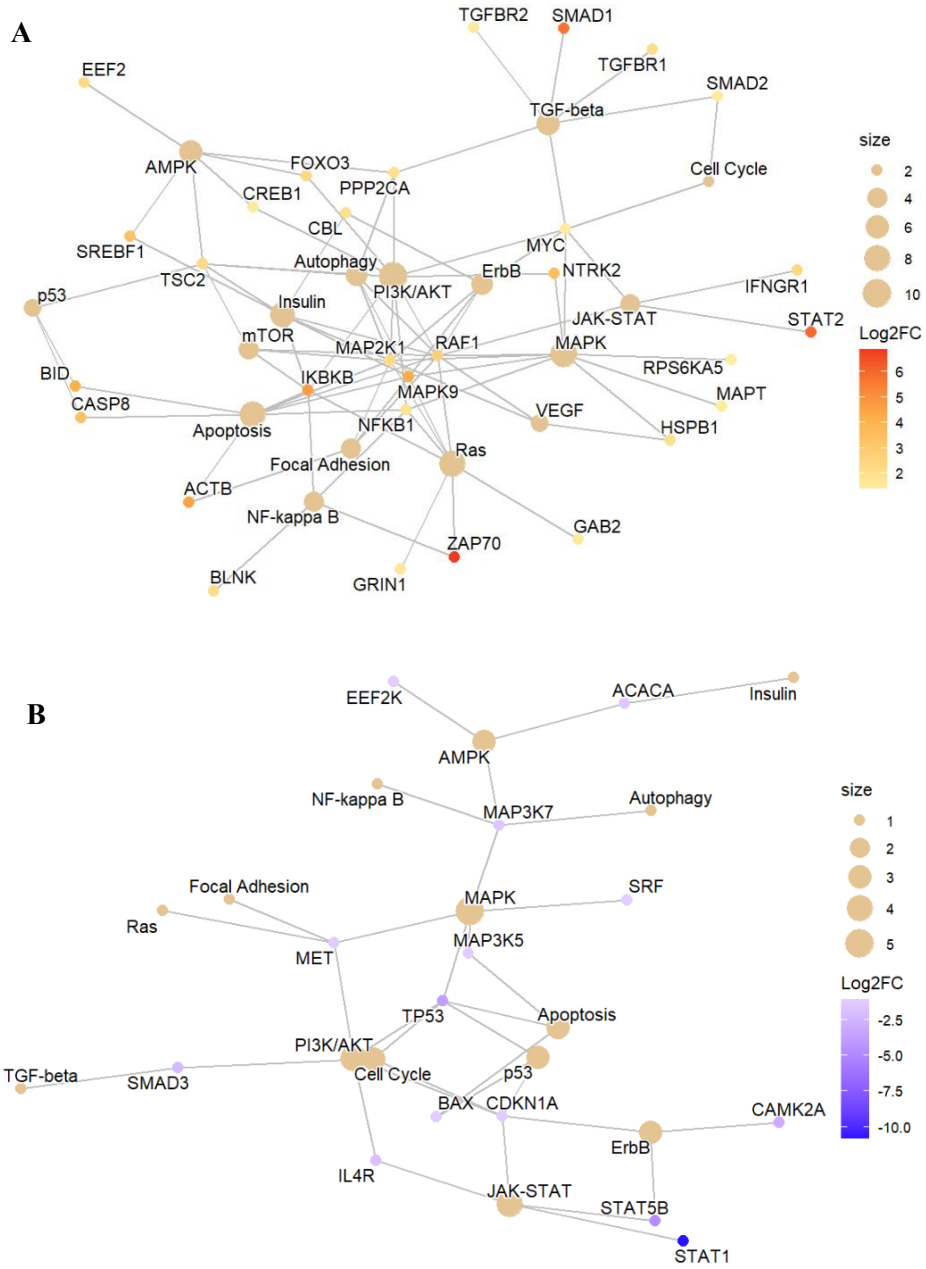
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Supplementary table S1. Antibodies used in this study

Antibody	Product code	Dilution and application
CD68	Abcam, #AB303565	1:350, IHC
PSD-95	SYSY antibodies, 124 011	1:250, IHC
Iba-1	WAKO, #019-19741	1:1000, IHC
Alexa Fluor 568-conjugated Goat anti-Mouse IgG	Invitrogen, #A-21124	1:1000, IHC
Alexa Fluor 488 conjugated Goat anti-Rabbit IgG	Invitrogen, #A-11008	1:1000, IHC
iNOS	Invitrogen, #PA1036	1:200, WB
Arg1	Invitrogen, PA5-85267	1:500, WB
CB2R	Invitrogen, #703485	1:500, WB
MAGL	Abcam, #ab24701	1:100, WB
IL-1ß	PeproTech, 500-P51-1MG	1:750, WB
α-Tubulin	Invitrogen, # PA5-85922	1:5000, WB
Horseradish peroxidase-conjugated Goat anti-Rabbit IgG	Jackson ImmunoResearch, 111-035-003	1:2000, WB
CD 11b-PE conjugated	Invitrogen, #12-0112-82	1:150, ICC
NF-κB	Invitrogen, #14-6731-63	1:100, ICC

Supplementary Table S2. Primers used in this study (Metabion)

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)
TNF-α	ATGGCCTCCCTCTCATCAGT	TTTGCTACGACGTGGGCTAC
Arg1	CTTGCGAGACGTAGACCCTG	TCCATCACCTTGCCAATCCC
IL-10	CGGGAAGACAATAACTGCACCC	CGGTTAGCAGTATGTTGTCCAGC
GAPDH	TGTCGTGGAGTCTACTGGTGTCTTC	CGTGGTTCACACCCATCACAA
NOX-2	CGAAGACAACTGGAAGGAA	GCTCVCACTAACATCACCAC
TGF-β	ACTGGAGTTGTACGGCAGTG	GGGGCTGATCCCGTTGATTT
pCW-5'PacI	CGCCTGGAGAATTGGTTAATTAAA TGAACGGACAGCTC	GAGCTGTCCGTTCATTTAATTAACCA ATTCTCCAGGCG
pCW3'PacI	CTTCCCATGGGAAGTGCTTAATTA ACTTAAATATTGAGGTCC	GGACCTCAATATTTAAGTTAATTAAG CACTTCCCATGGGAAG
SV40 LT	CCATTAATTAAAAGATGGATAAAG TTT	ACGTTAATTAAATGTTTCAGGTTC



Supplementary Figure 1. Differential phosphorylation and pathway network analysis of proteins regulated in AM-630+BHB+LPS-treated primary microglia. (**A**) Network analysis of upregulated phosphorylation-associated proteins in AM630+BHB+LPS-treated primary microglia. (**B**) Network analysis of downregulated phosphorylation-associated proteins in AM630+BHB+LPS-treated primary microglia.

SANTRAUKA

1. Santrumpos

BHB	–	β-hidroksibutiratas
CB2R	–	Antras kanabinoidinis receptorius
CD	–	Diferenciacijos antigenas
DIO	–	Dietos sukeltas nutukimas
EKS	–	Endokanabinoidinė sistema
IL	–	Interleukinas
LPS	–	Lipopolisacharidas
MAGL	–	Monoacilglicerolio lipazės
NF-κB	–	Branduolio faktorius kappa B
NLRP3	–	NOD tipo receptorių šeimos pirino domeną turintis 3 inflamasomos kompleksas
PSD95	–	Postsinapsinio tankio baltymas 95
ROS	–	Reaktyvios deguonies formos
TNF	–	Naviko nekrozės faktorius

2. Įvadas

Endokanabinoidinė sistema (EKS) yra sudėtinga reguliacinė sistema, moduliuojanti daugelį fiziologinių procesų nervinėje, imuninėje ir virškinamojo trakto sistemose (Șerban M et al., 2025). Ji apima kanabinoidinius receptorius, endogeninius kanabinoidus ir fermentus, atsakingus už jų sintezę bei metabolizmą (Rathod SS et al., 2023). Iš receptorių 2 tipo kanabinoidinis receptorius (CB2R) daugiausia yra išreiškiamas imuninėse ląstelėse, taip pat aptinkamas mikroglijoje, kur prisideda prie uždegiminio atsako ir mikroglijos aktyvacijos reguliavimo (Sobue A et al., 2024). Dėl šios priežasties CB2R sulaukia vis daugiau dėmesio kaip galimas neurouždegiminių ir neurodegeneracinių procesų reguliatorius, kadangi endogeniniai ir sintetiniai kanabinoidai gali mažinti mikroglijos prouždegiminį atsaką ir skatinti poslinkį link priešuždegiminio fenotipo (Saito VM et al., 2012; Young AP & Denovan-Wright EM, 2022). Mikroglija yra CNS reziduojančios imuninės ląstelės, svarbios smegenų homeostazės palaikymui ir neuropatologinių procesų reguliavimui (Le Thuc O et al., 2015). Tačiau ilgalaikės stimuliacijos sąlygomis mikroglija gali pereiti iš stebėsenos būsenos į reaktyvų fenotipą, kuriam būdinga ameboidinė morfologija ir padidėjusi prouždegiminių citokinų gamyba. Tokia užsitęsusi aktyvacija gali prisidėti prie lėtinio neurouždegimo, neuronų pažaidos ir neurodegeneracijos (Levtova N et al., 2017; Wang WY et al., 2015; Tong L et al., 2017). Pats neurouždegimas yra sudėtingas CNS atsakas į tokius dirgiklius kaip trauma, infekcija ar neurodegeneracinos ligos (Adamu A et al., 2024). Nors iš pradžių jis atlieka apsauginę funkciją, užsitęsęs arba sutrikęs neurouždegiminis atsakas gali pažeisti nervinį audinį (Le Thuc O et al., 2015). Lėtiniam neurouždegimui būdinga ilgalaikė mikroglijos aktyvacija, padidėjęs oksidacinis stresas ir uždegiminių mediatorių išsiskyrimas (Leszek J et al., 2016). Kadangi veiksmingos terapinės strategijos lėtinėms neurouždegiminėms būklėms gydyti tebėra ribotos, vis daugiau dėmesio skiriama gyvenimo būdu pagrįstoms strategijoms, įskaitant mitybos intervencijas (Thompson B et al., 2016; Kempermann G, 2019; Ornish D et al., 2024; Vijaya AK et al., 2024).

β-hidroksibutiratas (BHB), ketoninis kūnas, susidarantis ketozės ir protarpinio badavimo metu, sulaukia susidomėjimo dėl savo priešuždegiminio potencialo ir teigiamo poveikio neurouždegiminėmis sąlygomis (Newman JC and Verdin E, 2017; Yamanashi T et al., 2017; Polito R et al., 2023; Jiang Z et al., 2022; Jin LW et al., 2023). Nors nustatyta, kad BHB moduliuoja mikroglijos aktyvumą, o tokie keliai kaip NOD tipo receptorių šeimos pirino domeną turintis 3 inflamasomos kompleksas (NLRP3) ir branduolio faktoriaus kappa

B (NF-κB) signalo perdavimas yra siejami su šiuo poveikiu, nuo receptorių priklausomi mechanizmai, jungiantys BHB su mikroglijos uždegiminio atsako reguliavimu, vis dar yra nepakankamai ištirti (Fu SP et al., 2015; Youm YH et al., 2015). Nors sintetiniai kanabinoidinių receptorių agonistai yra prieinami (Schimrigk S et al., 2017; Abu-Sawwa R et al., 2020; Patti F et al., 2022), jų taikymą dažnai riboja nepageidaujamas poveikis (Prieto González JM and Vila Silván C, 2021). Todėl svarbu identifikuoti junginius, kurie galėtų netiesiogiai aktyvuoti su EKS susijusius kelius, tačiau tiesiogiai nesijungti prie kanabinoidinių receptorių ir vienas iš potencialių taikinių galėtų būti BHB.

Šiame tyrime iškėlėme hipotezę, kad BHB priešuždegiminį poveikį mikroglijoje sukelia per CB2R signalo perdavimą. Naudodami riebių pašarų sukelto nutukimo (DIO) pelių modelį, kuris laikomas žemo laipsnio lėtinio uždegimo modeliu (Avtanski D et al., 2019; Kiran S et al., 2021; Mandal M et al., 2025), vertinome mikroglijos funkcijas, CB2R ir monoacilglicerolio lipazės (MAGL) raišką bei prouždegiminių citokinų raišką pelių smegenyse. Siekdami išsamiau ištirti CB2R vaidmenį, optimizavome pirminės mikroglijos išskyrimo iš pelių smegenų protokolą ir atlikome *in vitro* eksperimentus, naudodami lipopolisacharidu (LPS) stimuliuotas mikroglijos ląsteles. Vertinome BHB ir CB2R antagonizmo poveikį fagocitozei, reaktyviųjų deguonies formų (ROS) gamybai, pro- ir priešuždegiminių genų raiškai bei NF-κB signalo perdavimui. Galiausiai, siekiant nustatyti signalinius kelius, dalyvaujančius BHB ir CB2R moduliacijos poveikiuose mikroglijoje, atlikome fosfo-antikūnų mikrogardelių analizę. Šie duomenys suteikia įžvalgų apie BHB ir CB2R sąveiką reguliuojant mikroglijos funkcijas ir pabrėžia šios sąveikos reikšmę neuruždegimo taikinių paieškai.

Šio tyrimo **tikslas** buvo įvertinti CB2R vaidmenį reguliuojant priešuždegiminį BHB poveikį mikroglijoje.

Šiam tikslui pasiekti buvo iškelti šie **uždaviniai:**

1. Išsamiai apibūdinti mikroglijos fenotipą DIO pelių modelyje ląsteliniu ir molekuliniu lygmenimis, įskaitant CB2R ir MAGL raiškos profilių įvertinimą.
2. Optimizuoti ir validuoti patikimą pirminės mikroglijos išskyrimo protokolą, pritaikytą tolimesniems *in vitro* tyrimams.
3. Ištirti BHB poveikį mikroglijos funkciniam aktyvumui ir genų raiškai *in vitro* LPS sukeltos uždegiminės aktyvacijos modelyje, ypatingą dėmesį skiriant CB2R tarpininkaujamiems mechanizmams.
4. Įvertinti BHB veikimo signalinius kelius ir nustatyti, kaip CB2R antagonizmas moduliuoja BHB sukeltus atsakus.

Mokslinis naujumas

Mikroglijos funckijų reguliavimo sutrikimai vis dažniau pripažįstami svarbiu veiksniu, prisidedančiu prie neuroužd egimo ir neurodegeneracinių ligų vystymosi. Nors nustatyta, kad BHB pasižymi priešuždegiminiu poveikiu mikroglijos ląstelėse, tikslūs molekuliniai mechanizmai, lemiantys šį poveikį, nėra išsamiai ištirti (Fu SP et al., 2014; Polito R et al., 2023). Be to, CB2R laikomas svarbiu mikroglijos aktyvacijos reguliatoriumi ir perspektyviu taikiniu neuroužd egiminėmis sąlygomis (Komorowska-Müller JA & Schmöle JC, 2020; Reusch N et al., 2022).

Šioje disertacijoje buvo tiriama BHB ir CB2R sąveika kaip galimas mechanizmas, reguliuojantis mikroglijos fenotipą uždegiminėmis sąlygomis. Literatūroje vis dar nepakankama informacijos apie bendrą BHB tarpininkaujamą metabolinį veikimą ir su CB2R susijusį mikroglijos atsako reguliavimą (Komorowska-Müller JA & Schmöle JC, 2020; Polito R et al., 2023). Šiame darbe, naudojant DIO pelių modelį ir pirmines mikroglijos kultūras, buvo įvertintas BHB poveikis mikroglijos būklei ir funkcijai tiek *in vivo*, tiek *in vitro* sąlygomis. Ši disertacija suteikia naujų žinių apie molekulinius mechanizmus, susijusius su BHB priešuždegiminiu poveikiu mikroglijai, ypatingą dėmesį skiriant su CB2R siejamo signalo perdavimo dalyvavimui.

Be to, šis darbas išplečia dabartinį supratimą apie BHB veikimą, identifikuojant viduląstelinius signalinius kelius, susijusius su jo poveikiu, taikant fosforilinimo pokyčius vertinančią fosfo-baltymų mikrogardelių analizę. Ši darbo dalis yra ypač nauja, nes signalo perdavimo keliai, lemiantys BHB poveikį uždegiminėmis sąlygomis aktyvuotai mikroglijai, ypač CB2R kontekste, iki šiol nebuvo išsamiai nagrinėti. Šie duomenys išplėtė mechanistinį BHB veikimo supratimą už funkcinių rodiklių ribų, atskleisdami signalinių kelių lygmens pokyčius, susijusius su siganlo perdavimu uždegimo sąlygomis ir metaboliniu reguliavimu – procesais, svarbiais mikroglijos aktyvacijai.

Apibendrinant, šios disertacijos rezultatai išplečia dabartines žinias apie metabolinio reguliavimo ir endokanabinoidinio signalo perdavimo sąsajas mikroglijos ląstelėse. Šis tyrimas gali prisidėti kuriant metabolines ir su EKS susijusias strategijas neuroždegimui moduliuoti.

Ginamieji teiginiai

1. BHB DIO modelyje sukelia teigiamus fenotipinius ir funkcinius mikroglijos ląstelių pokyčius, kartu moduliuodamas pagrindinius endokanabinoidinės sistemos komponentus.
2. Neonatalinės pirminės mikroglijos ląstelės yra patikimas ir tinkamas *in vitro* modelis, leidžiantis tirti molekulinius mechanizmus, susijusius su mikroglijos aktyvacija ir priešuždegimine moduliacija.
3. BHB poveikis mikroglijos ląstelių kultūrose pasižymi priešuždegiminiu poveikiu, rodančiu jo moduliacinį potencialą neurouždegiminio atsako atžvilgiu.
4. Farmakologinis CB2R antagonizmas slopina teigiamą BHB poveikį pirminėse mikroglijos kultūrose, išryškindamas nuo CB2R priklausomą veikimo mechanizmą.

3. Metodai

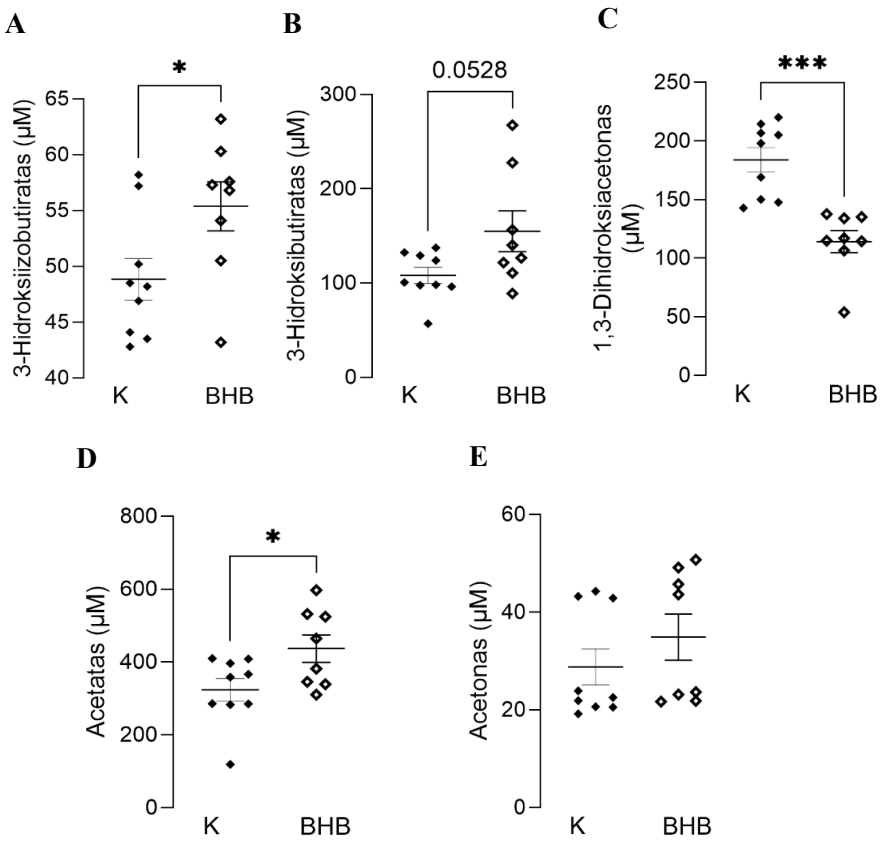
Šiame darbe taikytas kombinuotas *in vivo* ir *in vitro* tyrimo dizainas. *In vivo* dalyje naudotas riebių pašarų sukelto nutukimo pelių modelis, kuriame vienai gyvūnų grupei buvo papildmomai duodamas BHB. Po eksperimento iš gyvūnų surinktas kraujo serumas, kuriame tirti ketoniniai kūnai. Smegenų audinyje mikroglijos morfologija vertinta imunohistocheminiškai naudojant antikūnus, atpažįstančius Iba-1 žymenį, gauti vaizdai analizuoti kiekybiškai, analizuojant mikroglijos šakojimosi ir somos parametrus. To paties modelio mėginiuose taip pat tirta CB2R ir MAGL raiška bei prouždegiminių baltymų kiekis kiekybinio PGR, imunobloto bei ELISA metodais. *In vitro* dalyje pirmiausia buvo optimizuotas pirminės mikroglijos išskyrimo protokolas, įvertintas išskyrimo efektyvumas iš suaugusių ir naujagimių pelių smegenų, o gautos ląstelės augintos standartinėmis ląstelių auginimo sąlygomis. Ląstelių išskyrimo protokolo efektyvumas buvo vertinamas atsižvelgiant į ląstelių išeigą ir kultūrų grynumą žymint ląsteles imunocitochemiškai naudojant antikūnus, atpažįstančius mikroglijos biožymenį CD11b. Funkciniams eksperimentams mikroglija buvo veikta LPS uždegiminei būklei sukelti. BHB poveikiui įvertinti ląstelės prieš LPS poveikį buvo paveiktos BHB, o CB2R vaidmeniui nustatyti prieš BHB poveikį tiriamos ląstelės buvo veikiamos CB2R inversiniu agonistu AM630. Po poveikio vertinta mikroglijos fagocitinis aktyvumas, reaktyviųjų deguonies formų gamyba, atlikta genų raiškos analizė ir nustatyti NF-κB signalizacijos pokyčiai. Signalinių kelių pokyčiams įvertinti papildomai atlikta fosfo-antikūnų mikrogardelių analizė.

BHB priešuždegiminio poveikio analizė nutukusių pelių smegenyse

Šiame tyrime pirmiausia nagrinėjome BHB poveikį DIO pelių modelyje. Mūsų ankstesni duomenys parodė, kad BHB poveikis reikšmingai sumažino DIO pelių kūno svorį, tačiau reikšmingų kognityvinių funkcijų pokyčių nenustatyta (Mela V et al., 2025). Remdamiesi šiais duomenimis ir siekdami geriau suprasti BHB poveikį, toliau tyrėme, ar BHB šiame modelyje moduliuoja neuruždegiminius procesus ląsteliniu ir molekuliniu lygmenimis. Kad patvirtintume BHB papildų efektyvumą, matavome įvairių ketoninių kūnų koncentracijas pelių kraujo serume. Vėliau siekdami nustatyti, kaip BHB paveikė mikroglijos būseną, vertinome mikroglijos morfologiją ir fenotipą funkciniu ir molekuliniu lygmenimis.

Ketoninių kūnų pokyčiai kraujo serume po BHB poveikio

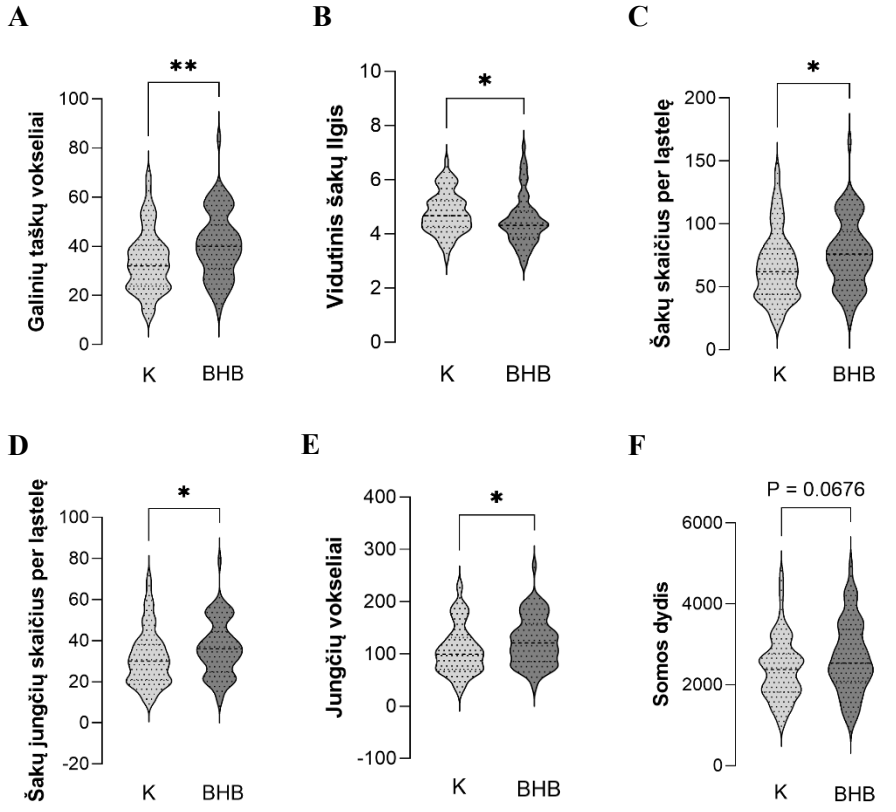
Pirmiausia nutukusių pelių, gavusių ir negavusių BHB, kraujo serume buvo ištirti įvairūs ketoniniai kūnai. BHB grupės pelių grupėje nustatytas reikšmingas 3-hidroksiizobutirato (1 A pav.; $p < 0.05$), ir acetato (1 D pav.; $p < 0.05$) padidėjimas. Taip pat buvo stebėta 3-hidroksibutirato didėjimo tendencija (1 B pav.; $p = 0.052$) ir nedidelis acetono padidėjimas (1 E pav.), o 1,3-dihidroksiacetono kiekis BHB veiktose pelėse sumažėjo (1 C pav.; $p < 0.001$) BHB grupėje. Šie rezultatai rodo, kad BHB pakeitė sisteminį su ketoniniais kūnais susijusį metabolinį profilį, taip patvirtinandami biologinį papildo aktyvumą šiame modelyje.



1 pav. Ketoninių kūnų pokyčiai kraujo serume kontrolinėje ir BHB grupėje. (**A**) 3-hidroksiizobutiratas; (**B**) 3-hidroksibutiratas; (**C**) 1,3-dihidroksiacetonas; (**D**) Acetatas; (**E**) Acetonas. K – kontrolinė grupė, BHB – BHB poveikio grupė. n = 8–9. Duomenys pateikti kaip vidurkis ± SEM; *$p < 0.05$; ***$p < 0.001$.

Ex vivo mikroglijos morfologiniai pokyčiai, indukuoti BHB poveikio

Toliau, kad nustatytume galimą BHB poveikį mikroglijos funkcinei būklei nutukusiose pelėse, siekėme įvertinti mikroglijos morfologinio kompleksiškumo ir šakojimosi pokyčius. Šiuo tikslu kiekybiškai įvertinome morfologinius parametrus hipokampo mikroglijoje, naudodami Iba-1 atpažįstantį antikūną.



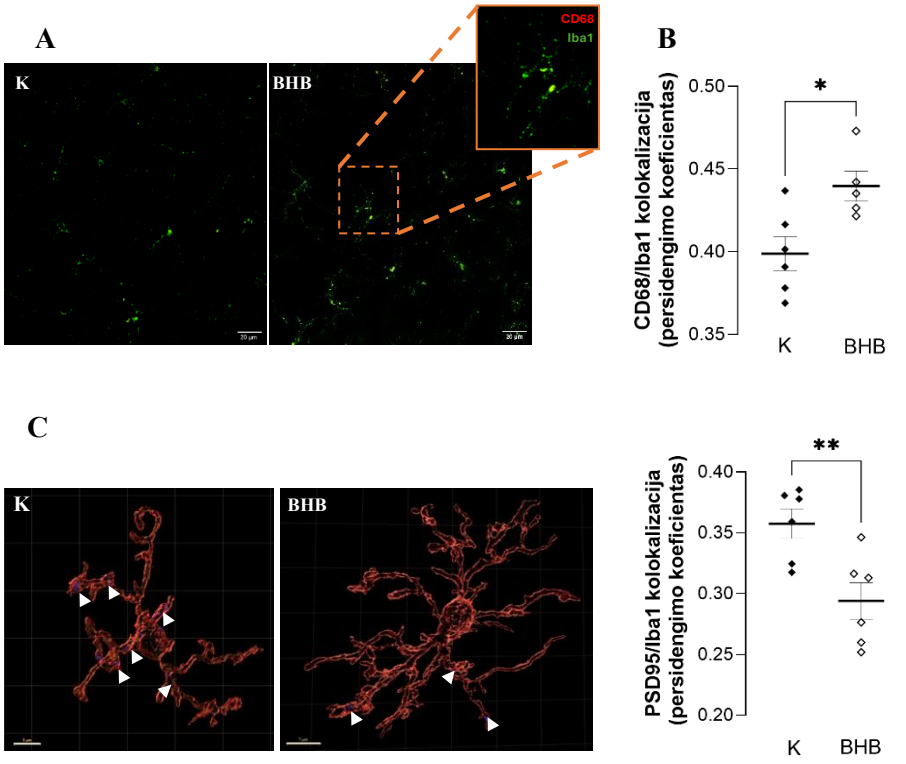
2 pav. BHB pakeitė nutukusių pelių hipokampo mikroglijos morfologiją. (**A**) Galinių taškų vokselių skaičius; (**B**) Vidutinis šakų ilgis; (**C**) Šakų skaičius ląstelėje; (**D**) Šakų jungčių skaičius ląstelėje; (**E**) Jungčių vokselių skaičius; (**F**) Somos dydis. K – kontrolinė grupė, BHB – BHB poveikio grupė. n = 6. Kiekvienam gyvūnui buvo analizuojama 10 – 15 ląstelių. Duomenys pateikiami kaip smuiko diagramos, rodančios individualių reikšmių pasiskirstymą; centrinė linija žymi vidurkį; *$p < 0.05$; **$p < 0.01$.

Analizė atskleidė reikšmingus pagrindinių mikroglijos struktūrinių parametrų skirtumus. BHB grupės pelių mikroglijoje lyginant su nutukusių pelių kontrolinių pelių grupe nustatytas reikšmingas galinių taškų vokselių skaičiaus padidėjimas (2 A pav.; $p < 0.01$), šakų skaičiaus padidėjimas (2 B pav.; $p < 0.05$), jungčių skaičiaus padidėjimas (2 C pav.; $p < 0.05$), jungčių vokselių skaičiaus padidėjimas (2 D pav.; $p < 0.05$) ir šakų ilgio sumažėjimas (2 E pav.; $p < 0.05$). Nors somos parametras nepasiekė statistinio reikšmingumo, buvo stebėta somos dydžio didėjimo tendencija (2 F pav.; $p = 0.068$) BHB pelių grupėje. Šie pokyčiai rodo padidėjusį mikroglijos morfologinį kompleksiškumą, kuris dažnai siejamas su mikroglijos funkcinės

būklės pokyčiais. Todėl galima manyti, kad BHB gali moduliuoti mikroglijos morfologiją nutukusių pelių hipokampe, tačiau vien morfologiniai požymiai nėra pakankami vienareikšmiškai apibrėžti neuroprotekcinį ar priešuždegiminį mikroglijos fenotipą.

BHB didina mikroglijos fagocitinį aktyvumą ir mažina sąveiką su sinapsėmis

Siekiant geriau apibūdinti BHB poveikį mikroglijos būklei, atlikome imunofluorescentinę kolokalizacijos analizę, naudodami antikūnus, atpažįstančius diferenciacijos antigeną (CD)68/Iba1 ir postsinapsinio tankio baltymą 95 (PSD95)/Iba1. Tai leido įvertinti mikroglijos fagocitinį aktyvumą ir sąveiką su sinapsėmis.



3 pav. BHB didina mikroglijos fagocitinį pajėgumą ir mažina sąveiką su sinapsėmis nutukusių pelių hipokampe. (**A**) Reprezentatyvus konfokalinės mikroskopijos vaizdas, rodantis CD68/Iba1 kolokalizaciją hipokampe; (**B**) CD68/Iba1 persidengimo koeficiento kiekybinis įvertinimas, parodantis padidėjusią kolokalizaciją BHB grupėje; (**C**) Reprezentatyvus IMARIS 3D rekonstrukcijos vaizdas, rodantis PSD95 lokalizaciją mikroglijoje; (**D**) PSD95/Iba1 persidengimo koeficiento kiekybinis įvertinimas, parodantis sumažėjusią kolokalizaciją BHB

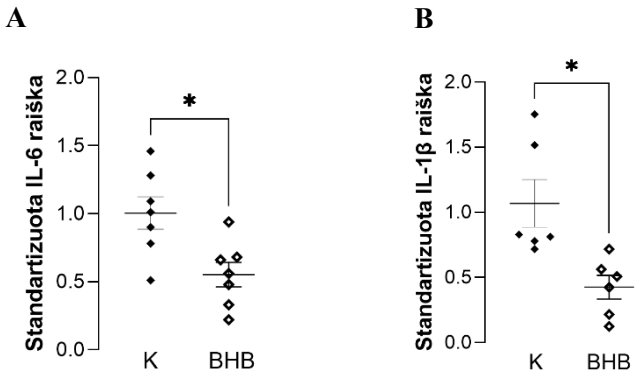
grupėje. K – kontrolinė grupė, BHB – BHB grupė. n = 6. Kiekvienam gyvūnui buvo analizuojama 10–15 ląstelių. Duomenys pateikti kaip vidurkis ± SEM; *$p < 0.05$; *$p < 0.01$.

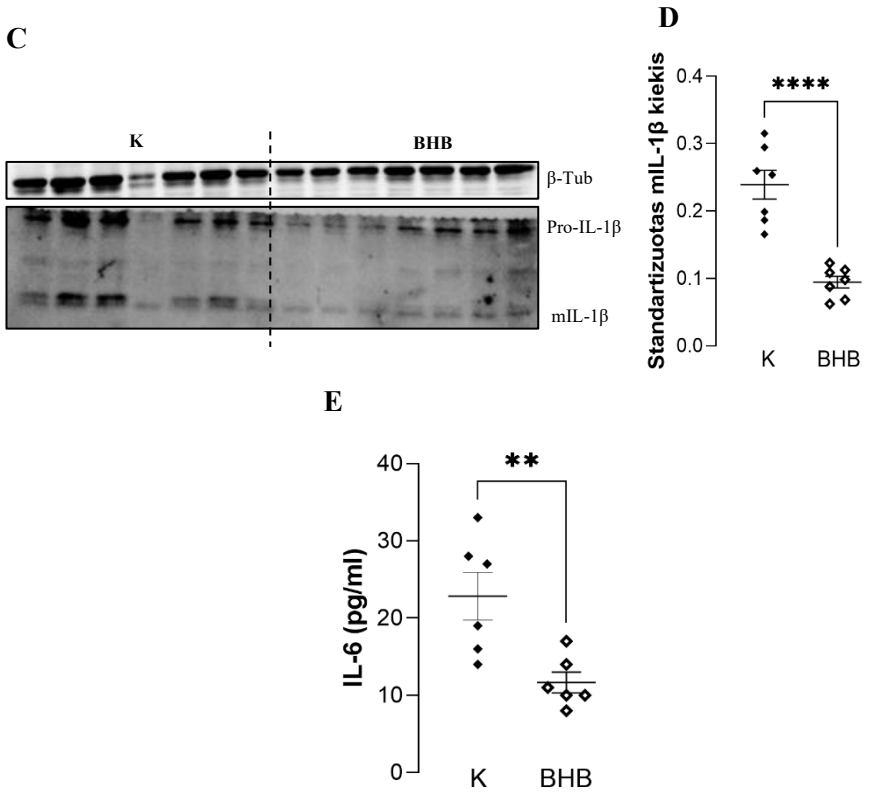
Kiekybinė persidengimo koeficiento analizė parodė reikšmingą CD68/Iba1 kolokalizacijos padidėjimą BHB paveiktų pelių grupėje, lyginant su kontrole (3 A, B pav.; $p < 0.05$). Tai rodo padidėjusį mikroglijos fagocitinį aktyvumą. Tuo tarpu PSD95/Iba1 kolokalizacija BHB grupėje buvo reikšmingai sumažėjusi (3 C, D pav.; $p < 0.01$), o tai reiškia sumažėjusią postsinapsinių elementų sąveiką su mikroglija.

BHB mažina IL-1β ir IL-6 raišką hipokampe ir žievėje

Siekiant įvertinti priešuždegiminį BHB poveikį nutukusiose pelėse, hipokampe buvo tirta *IL-1β* ir *IL-6* mRNR raiška. Nustatyta, kad BHB poveikis reikšmingai sumažino *IL-1β* ir *IL-6* raišką, lyginant su neveikta nutukusių pelių kontroline grupe (3.4 A, B pav.; $p < 0.05$). Šie rezultatai rodo, kad BHB silpnina su nutukimu susijusius neuroužd egiminius atsakus hipokampe.

Siekiant toliau įvertinti priešuždegiminį BHB poveikį nutukusiose pelėse, galvos smegenų žievės audinyje tyrėme IL-1β raišką imunobloto metodu, o IL-6 kiekį – ELISA metodu. BHB poveikis reikšmingai sumažino IL-1β raišką (5 A, B pav.; $p < 0.0001$) ir IL-6 kiekį, palyginti su nutukusių pelių kontroline grupe (5 C pav.; $p < 0.01$). Šie rezultatai rodo, kad BHB vartojimas silpnina su nutukimu susijusius neuroužd egiminius atsakus galvos smegenų žievėje.



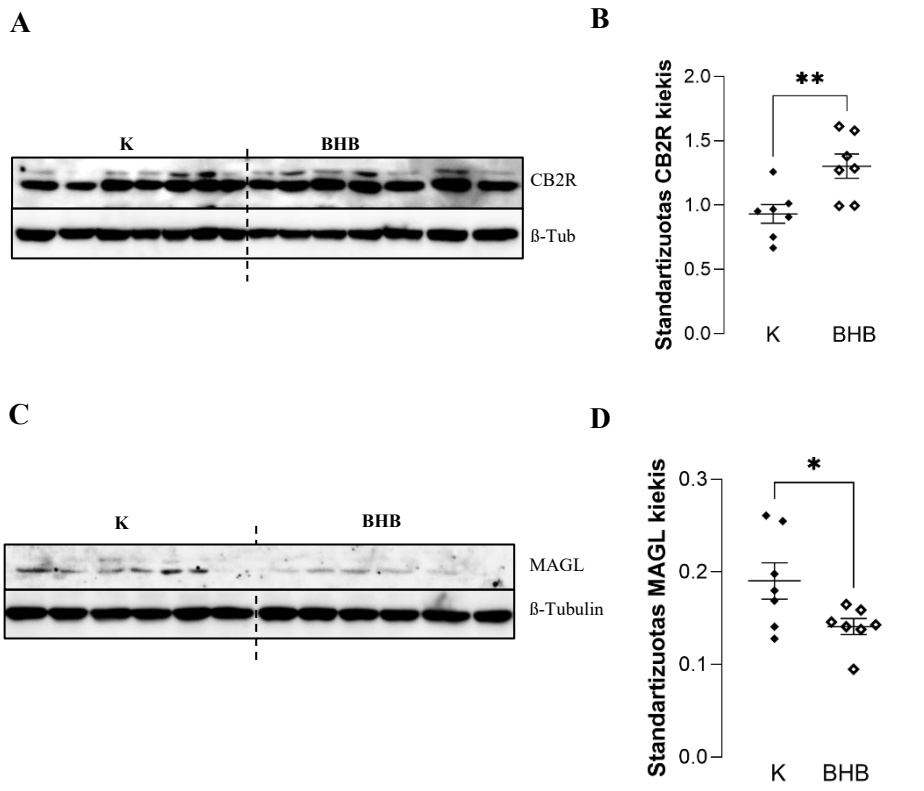


4 pav. BHB poveikis mažina prouždegiminių citokinų RNR kiekį nutukusių pelių hipokampe. (**A**) *IL-1β* RNR raiškos pokytis, lyginant su neveikta kontroline grupe. n = 7; (**B**) *IL-6* RNR raiškos pokytis, lyginant su neveikta kontroline grupe; (**C**) Reprezentatyvūs galvos smegenų žievės imunobloto duomenys iš kontrolinės ir BHB paveiktos grupės, rodantys pro-IL-1β, brandžios IL-1β formos (mIL-1β) ir β-tubulino raišką. (**D**) mIL-1β raiškos kiekybinis įvertinimas densitometrinės analizės būdu, normalizuotas pagal β-tubuliną; (**E**) IL-6 raiška, nustatyta ELISA metodu. n = 6. K – kontrolinė grupė, BHB – BHB grupė. Duomenys pateikti kaip vidurkis ± SEM; **p* < 0.05*;* ***p* < 0.01; *****p* < 0.0001.

BHB poveikis didina CB2R ir mažina MAGL raišką pelių hipokampe

Kadangi tikslūs BHB priešuždegiminio poveikio mechanizmai išlieka neaiškūs, iškėlėme hipotezę, kad BHB gali veikti moduliuodamas EKS, ypač per CB2R. Šiuo tikslu vertinome CB2R raišką, taip pat MAGL – pagrindinio fermento, atsakingo už endokanabinoidų degradaciją – raišką nutukusių kontrolinių pelių ir BHB paveiktų pelių hipokampe. BHB grupėje CB2R raiška buvo reikšmingai didesnė nei kontrolinėje grupėje (6 A, B pav.; $p < 0.01$). Šis rezultatas rodo galimą CB2R dalyvavimą hipokampe atsakant į

BHB poveikį. Kaip ir tikėjomės formuluodami hipotezę, MAGL raiška BHB grupėje buvo reikšmingai sumažėjusi (6 A, C pav.; $p < 0.05$), o tai leidžia manyti, kad BHB vartojimas gali būti susijęs su EKS signalo perdavimo pokyčiais.



5 pav. BHB moduliuoja CB2R ir MAGL raišką nutukusių pelių hipokampe. (**A**) Reprezentatyvūs hipokampo audinio imunobloto duomenys iš kontrolinės ir BHB grupės, rodantys CB2R ir β-tubulino raišką. (**B**) CB2R raiškos kiekybinis įvertinimas densitometrinės analizės būdu, normalizuota pagal β-tubuliną. (**C**) Reprezentatyvūs hipokampo audinio imunobloto duomenys iš kontrolinės ir BHB grupės, rodantys MAGL ir β-tubulino raišką. (**D**) MAGL raiškos kiekybinis įvertinimas densitometrinės analizės būdu, normalizuota pagal β-tubuliną. K – kontrolinė grupė, BHB – BHB grupė. n = 6–7. Duomenys pateikti kaip vidurkis ± SEM. *$p < 0.05$; **$p < 0.01$.

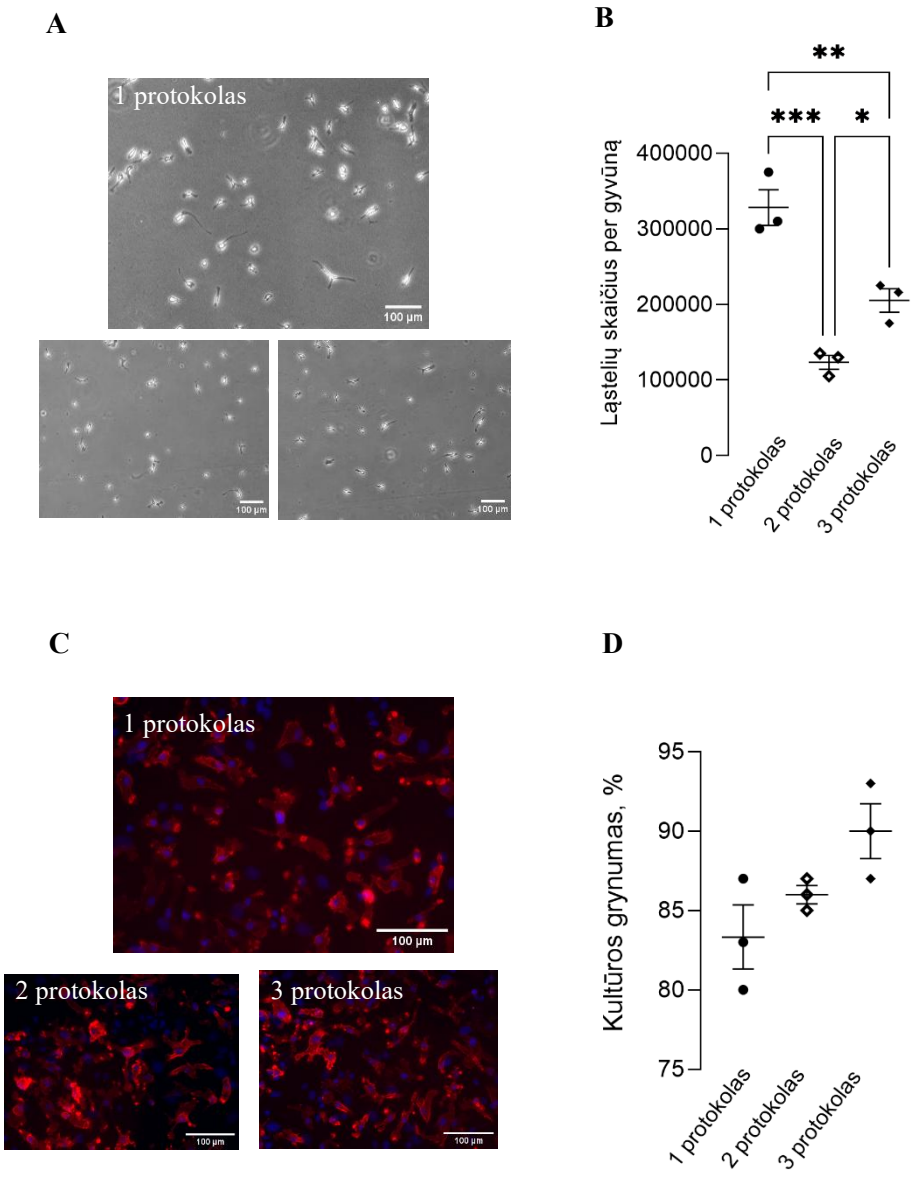
Pirminių pelių mikroglijos ląstelių išskyrimo protokolo optimizavimas

Siekiant išsamiau įvertinti BHB poveikį mikroglijai ir ištirti galimus šio poveikio mechanizmus, buvo pereita prie *in vitro* ląstelių kultūrų modelių. Tai sudaro galimybę tirti mikroglijos atsaką griežčiau kontroliuojamomis

sąlygomis ir tiesiogiai analizuoti galimus mechanistinius ryšius, kurie *in vivo* tyrimuose yra sunkiau atskiriami. Kaip pirmasis modelis buvo pasirinktos iš suaugusių pelių išskirtos pirminės mikroglijos ląstelės. Atsižvelgiant į tai, kad neuroužd egimas ir neurodegeneracija yra glaudžiai susiję su senėjimu, suaugusių pelių mikroglija laikyta tinkamesniu modeliu šių procesų tyrimui. Todėl šis modelis buvo pasirinktas siekiant geriau atspindėti uždegiminį kontekstą, kuriame BHB sukelta mikroglijos funkcijų moduliacija gali būti biologiškai reikšminga. Tačiau mikroglijos išskyrimas iš suaugusių pelių išlieka sudėtingas dėl mažos ląstelių išeigos ir nepakankamo kultūrų grynumo, o pakankamai efektyvus ir patikimas protokolas iki šiol nėra visuotinai prieinamas. Dėl šios priežasties buvo palyginti esami išskyrimo metodai ir optimizuotas vienas iš jų, siekiant padidinti ląstelių išeigą. Vėliau optimizuotas suaugusių pelių mikroglijos išskyrimo protokolas buvo palygintas su mikroglijos išskyrimo iš pelių jauniklių metodu, vertinant ląstelių išeigą ir gautų kultūrų grynumą.

Mikroglijos išskyrimas iš suaugusių pelių

Siekdami optimizuoti mikroglijos išskyrimo protokolą, pirmiausia dėmesį sutelkėme į ląstelių gavimą iš suaugusių pelių. Buvo įvertintos trys išskyrimo strategijos: 1 protokolas (iš pradžių aprašytas Bordt et al., 2020 ir vėliau modifikuotas mūsų laboratorijoje), 2 protokolas (Woolf et al., 2021) ir 3 protokolas (Lee JK and Tansey MG, 2013). Šie metodai buvo palyginti pagal ląstelių išeigą ir kultūrų grynumą. Palyginus tris išskyrimo strategijas nustatyta, kad mūsų optimizuotas protokolas užtikrino didžiausią mikroglijos ląstelių išeigą (3.5 A, B pav.; $p < 0.0001$). Kultūrų grynumo požiūriu statistiškai reikšmingų skirtumų tarp trijų išskyrimo metodų nenustatyta (3.5 C, D pav.). Didžiausias kultūros grynumas buvo pasiektas taikant 3 protokolą, po jo sekė 2 protokolas, o mūsų optimizuotas protokolas siekė mažiausią kultūros grynumą pagal CD11b žymenį.



6 pav. Mikroglijos išskyrimo iš suaugusių pelių smegenų įvertinimas. (**A**) Reprezentatyvus šviesinės mikroskopijos vaizdas, kuriame pavaizduota išskirta pirminė mikroglija, parodant ląstelių tankį ir morfologiją; (**B**) Ląstelių išeiga, gauta taikant tris skirtingus išskyrimo protokolus; (**C**) Reprezentatyvus išskirtos mikroglijos imunodažymo vaizdas: CD11b (raudona), branduoliai (mėlyna); (**D**) Iš suaugusių pelių išskirtos pirminės mikroglijos grynumas. n = 3. Duomenys pateikiami kaip vidurkis ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Šie rezultatai parodė, kad nors mikroglijos ląsteles buvo galima išskirti iš

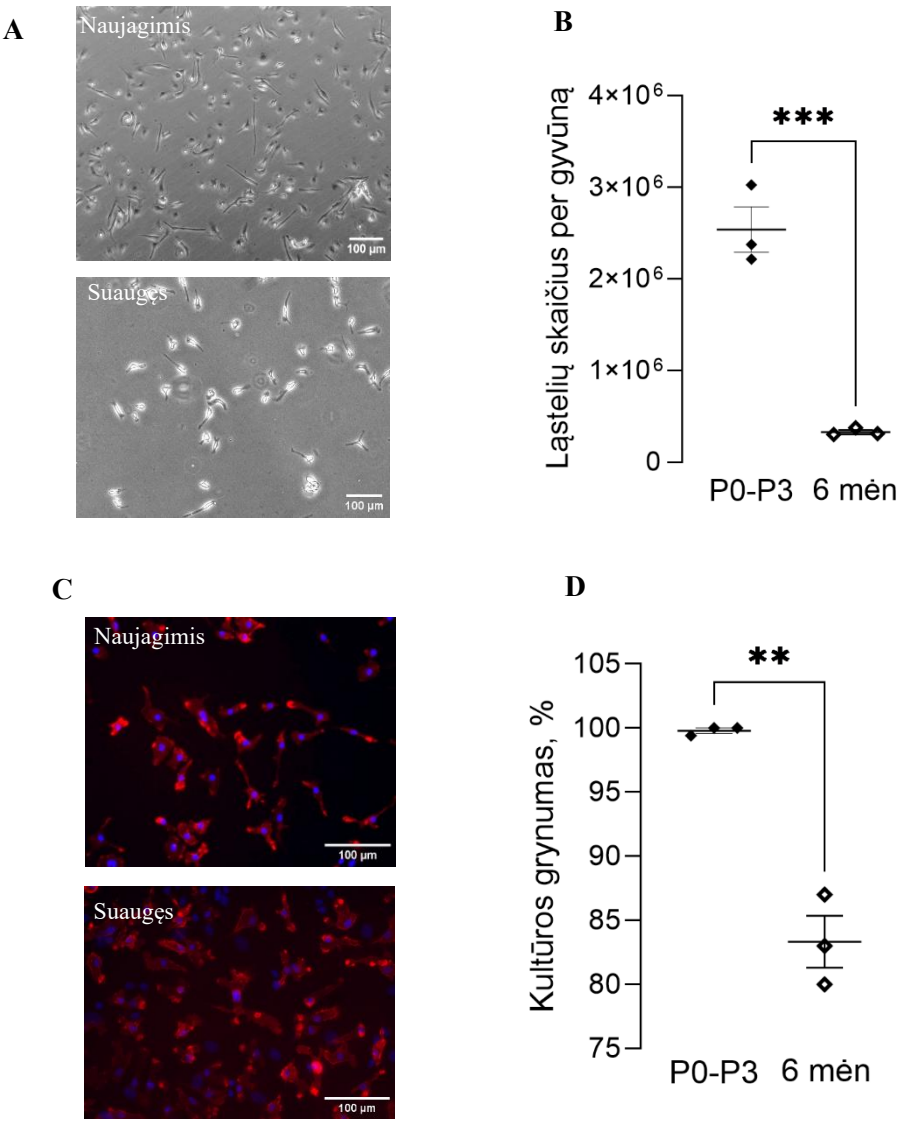
suaugusių pelių smegenų, tačiau tiek ląstelių išeiga, tiek kultūros grynumas išliko nepakankami mūsų tikslams. Todėl, siekiant sumažinti mechanistiniams tyrimams reikalingų gyvūnų skaičių, mikroglija buvo izoliuojama iš naujagimių pelių.

Mikroglijos išskyrimas iš naujagimių pelių

Siekiant palyginti mikroglijos išskyrimo efektyvumą iš jauniklių ir suaugusių pelių, įvertinome bendrą ląstelių išeigą ir kultūros grynumą. Išskirtų ląstelių išeiga buvo žymiai didesnė jauniklių grupėje nei suaugusių pelių grupėje. Iš jauniklių pelių smegenų mėginių buvo gaunama maždaug dešimt kartų daugiau ląstelių vienam gyvūnui – jų kiekis svyravo apie ~2,2 – 3 × 10^6 ląstelių/gyvūnui, tuo tarpu suaugusių pelių smegenų mėginiuose šis rodiklis buvo mažesnis – apie ~3,0 – 3,8 × 10^5 ląstelių/gyvūnui (7 pav. A, B; $p < 0.001$).

Mikroglijos žymens CD11b imunodažymas taip pat atskleidė kultūros grynumo skirtumus tarp amžiaus grupių. Kiekybinė analizė parodė, kad kultūros grynumas buvo žymiai didesnis iš jauniklių gautuose mėginiuose, siekė beveik 100 %, tuo tarpu iš suaugusių pelių gautų ląstelių grynumas buvo gerokai mažesnis – apie 80-87 % (7 pav. C, D; $p < 0.01$).

Atsižvelgiant į šiuos rezultatus, tolesniuose eksperimentuose buvo naudojamos iš jauniklių pelių smegenų išskirta pirminė mikroglija, nes tai leido užtikrinti patikimesnius rezultatus ir sumažinti naudojamų gyvūnų skaičių.



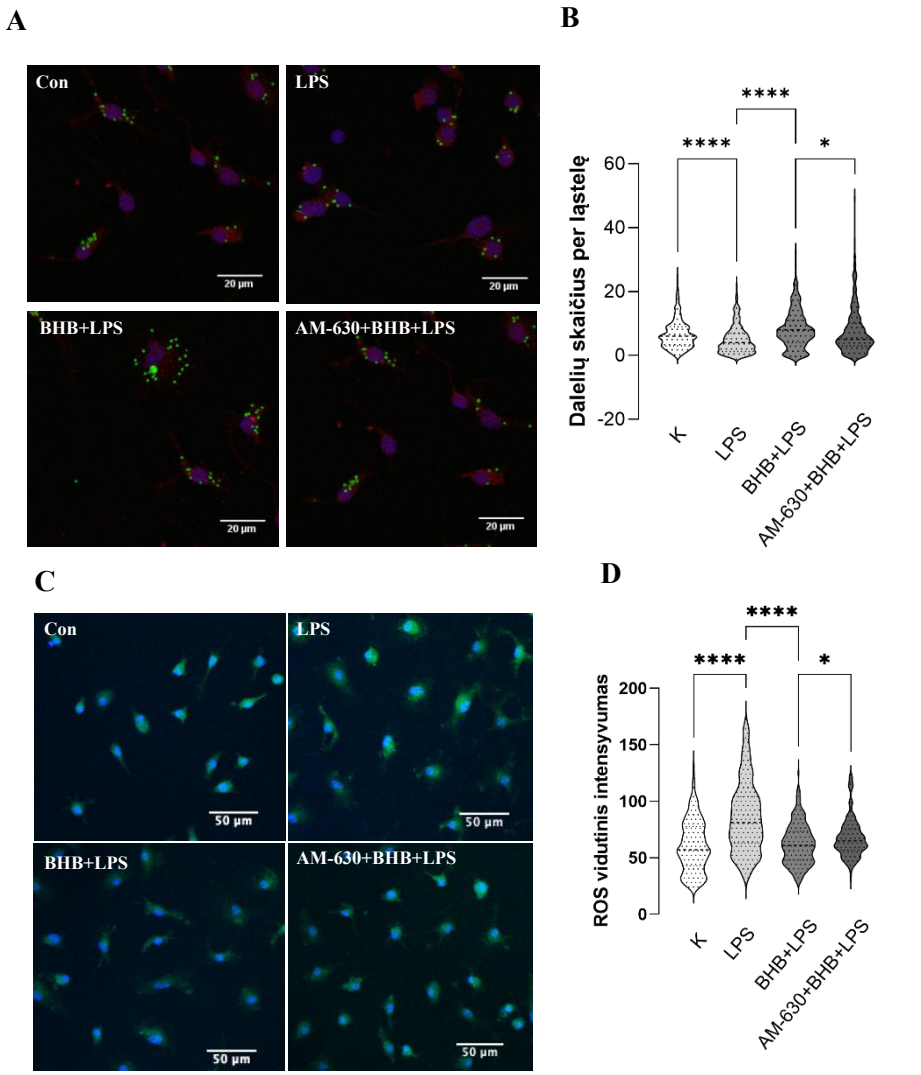
7 pav. Mikroglijos skyrimo iš jauniklių ir suaugusių pelių smegenų (1 protokoals) palyginimas. (**A**) Reprezentatyvus šviesinės mikroskopijos vaizdas, kuriame pavaizduota išskirta pirminė mikroglija, parodant ląstelių tankį ir morfologiją. (**B**) Ląstelių išeiga vienam gyvūnui iš jauniklių ir suaugusių pelių. (**C**) Reprezentatyvus išskirtos mikroglijos imunodažymo vaizdas: CD11b (raudona), branduoliai (mėlyna). (**D**) Iš jauniklių ir suaugusių pelių išskirtos pirminės mikroglijos grynumas. P0-P3 – iš naujagimių smegenų išskirtos ląstelės. 6 mėn – iš suaugusių pelių išskirtos ląstelės. n = 3. Duomenys pateikiami kaip vidurkis ± SEM. *$p < 0.05$; **$p < 0.01$.

BHB priešuždegiminis poveikis pirminėje pelių mikroglijoje priklauso nuo CB2R

Optimizavus pirminės mikroglijos išskyrimo metodiką, toliau buvo tiriamas CB2 receptoriaus vaidmuo BHB poveikyje. Tam tikslui ląstelės buvo stimuliuotos LPS, sukeliant stiprų uždegiminį atsaką. Siekiant įvertinti BHB poveikį, prieš LPS poveikį mikroglija buvo iš anksto paveikta šiuo ketoniniu kūnu. Norint nustatyti CB2R indėlį į priešuždegiminį BHB poveikį, prieš BHB poveikį ląstelės buvo paveiktos CB2R atvirkštiniu agonistu AM630. Pirmiausia buvo įvertintos mikroglijos funkcinės savybės, įskaitant fagocitozę ir ROS gamybą, o vėliau analizuota prouždegiminių ir priešuždegiminių genų raiška. Galiausiai buvo įvertinti NF-κB signalinio kelio pokyčiai, siekiant nustatyti, ar BHB poveikis ir CB2R dalyvavimas gali būti susiję su šiuo keliu.

CB2R blokada panaikina BHB sukeltus funkcinius pokyčius pirminėje mikroglijoje

Siekiant geriau suprasti tiesioginį BHB poveikį mikroglijos funkcijai, buvo atlikta *in vitro* funkcionalumo analizė, daugiausia dėmesio skiriant fagocitiniam mikroglijos aktyvumui ir ROS gamybai, taip pat vertinta, ar šiuos poveikius moduliuoja CB2R. Poveikis LPS reikšmingai slopino mikroglijos fagocitozę, sumažindamas vidutinį internalizuotų latekso dalelių skaičių, lyginant su kontroline grupe (8 pav. A, B; $p < 0.0001$). Kartu taikytas BHB ir LPS poveikis reikšmingai padidino fagocitinį aktyvumą, lyginant su vien LPS grupe (8 pav. A, B; $p < 0.0001$), o tai rodo, kad BHB skatina mikroglijos funkcinį atsistatymą uždegiminėmis sąlygomis. Kartu su BHB ir LPS taikytas CB2R antagonistas AM-630 sukėlė nedidelį, tačiau statistiškai reikšmingą fagocitozės sumažėjimą, lyginant su BHB+LPS grupe (8 pav. A, B; $p < 0.05$). Šie rezultatai rodo, kad CB2R bent iš dalies prisideda prie BHB sukelto fagocitozę skatinančio poveikio mikroglijoje uždegiminio streso sąlygomis.



8 pav. BHB ir CB2R blokados poveikis mikroglijos fagocitozei ir ROS gamybai uždegiminėmis sąlygomis. (**A**) Reprezentatyvūs fluorescencinės mikroskopijos vaizdai, rodantys pirminės pelių mikroglijos fagocitozę skirtingose eksperimentinėse grupėse. Žalia: internalizuotos fluorescuojančios latekso dalelės; raudona: mikroglijos žymuo CD11b; mėlyna: DAPI nudažyti branduoliai; (**B**) Smuiko diagrama, vaizduojanti mikroglijos fagocitozės pokyčius, n = 260-320 ląstelių. (**C**) Reprezentatyvūs fluorescencinės mikroskopijos vaizdai, rodantys pirminės pelių mikroglijos ROS gamybą skirtingose eksperimentinėse grupėse. Žalia: ROS signalas; mėlyna: DAPI nudažyti branduoliai; (**D**) Smuiko diagrama, vaizduojanti ROS kiekį, n = 200 ląstelių. K – kontrolinė grupė; LPS – LPS paveikta grupė (100 ng/mL); BHB+LPS – 1 val. išankstinis poveikis BHB prieš LPS; AM-630+BHB+LPS – 1 val. išankstinis poveikis AM630 (1 µM) prieš BHB ir LPS. Duomenys pateikiami kaip vidurkis ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.0001$.

Po poveikio LPS, ROS lygis reikšmingai padidėjo (8 pav. C, D; $p < 0.0001$) – tai atitinka aktyvuotą prouždegiminę mikroglijos būseną. Išankstinis poveikis BHB reikšmingai sumažino ROS gamybą, lyginant su LPS grupe (8 pav. C, D; $p < 0.0001$), patvirtindamas jo vaidmenį mažinant oksidacinį stresą. Svarbu tai, kad CB2R slopinimas iš dalies panaikino BHB ROS mažinantį poveikį ir padidino ROS gamybą, lyginant su BHB+LPS grupe (8 pav. C, D; $p < 0.05$). Šie rezultatai atitinka fagocitozės duomenis ir patvirtina, kad CB2R blokada gali skatinti prouždegiminį fenotipą. Apibendrinant, gauti duomenys pabrėžia CB2R svarbą reguliuojant priešuždegiminį ir homeostazinį poveikį BHB veikimą mikroglijoje.

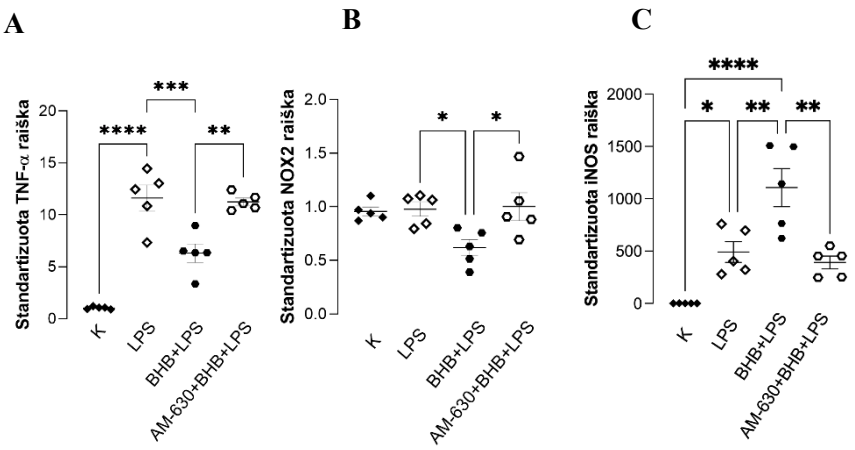
BHB ir CB2R poveikis uždegiminių genų raiškai pirminėse pelių mikroglijos ląstelėse

Toliau buvo tirta, ar BHB gali slopinti prouždegiminių genų, aktyvintų LPS stimuliacijos, raišką, ir ar šis poveikis yra tarpininkaujamas CB2R. Šiuo tikslu, naudojant kiekybinę PGR, buvo vertinami *TNF-α*, *NOX2* ir *iNOS* mRNR raiškos lygiai.

Kaip ir tikėtasi, LPS paveiktoje grupėje *TNF-α* mRNR raiška buvo reikšmingai padidėjusi (9 pav. A; $p < 0.001$). BHB sumažino *TNF-α* mRNR raišką (9 pav. A; $p < 0.001$). Ląstelėse, paveiktose AM-630 prieš BHB ir LPS poveikį, *TNF-α* mRNR raiška padidėjo, lyginant su BHB+LPS grupe (9 pav. A; $p < 0.01$). Šie duomenys rodo, kad BHB uždegiminėmis sąlygomis reikšmingai slopino *TNF-α* mRNR raišką, o tai atitinka mūsų rezultatus, gautus *in vivo*. Be to, padidėjimas, stebėtas blokuojant CB2R, leidžia manyti, kad CB2R dalyvauja reguliuojant TNF-α produkciją.

NOX2 mRNR raiška po LPS poveikio, lyginant su kontroline grupe, nepakito (9 pav. B). Tačiau grupėje, paveiktoje BHB prieš LPS stimuliaciją, NOX2 mRNR raiška buvo reikšmingai sumažėjusi, lyginant su vien LPS paveikta grupe (9 pav. B; $p < 0.05$). Be to, kai ląstelės prieš BHB ir LPS poveikį buvo paveiktos AM-630, *NOX2* mRNR raiška padidėjo, lyginant su BHB+LPS grupe (9 pav. B; $p < 0.05$). Šie duomenys atitinka anksčiau nustatytą ROS kiekio sumažėjimą ir leidžia manyti, kad BHB gali paveikti su oksidaciniu stresu susijusius mikroglijos signalinius kelius. Kadangi NOX2 yra vienas pagrindinių fermentinių ROS šaltinių, sumažėjusi *NOX2* mRNR raiška gali rodyti mažesnį su NOX2 susijusį oksidacinį aktyvumą. Vis dėlto, kadangi NOX2 aktyvumas nebuvo tiesiogiai matuotas, šie rezultatai turėtų būti vertinami kaip netiesioginiai įrodymai. Duomenys taip pat leidžia manyti, kad šis poveikis gali būti iš dalies susijęs su CB2R signalo perdavimu.

iNOS mRNR raiška po LPS poveikio reikšmingai padidėjo, lyginant su kontroline grupe (9 pav. C; $p < 0.05$). Įdomu tai, kad, priešingai nei tikėtasi remiantis priešuždegiminiu BHB poveikiu, poveikis BHB dar labiau padidino *iNOS* mRNR raišką, lyginant su vien LPS paveikta grupe (3.10 pav. C; $p < 0.01$). Be to, ląstelių veikimas AM-630 prieš BHB ir LPS poveikį reikšmingai sumažino *iNOS* mRNR raišką, lyginant su BHB + LPS grupe (3.10 pav. C; $p < 0.01$). Nors CB2R blokada dažniausiai siejama su prouždegiminių žymenų padidėjimu, šiuo atveju ji, priešingai, susilpnino iNOS raišką.



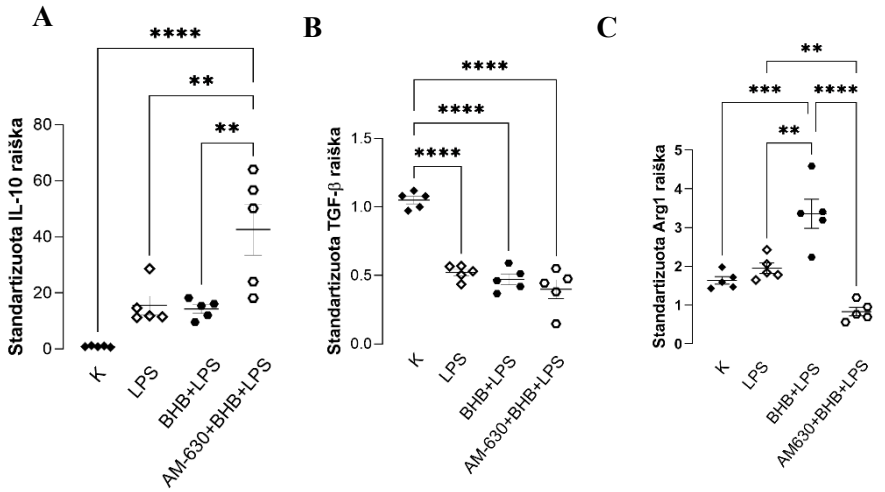
9 pav. Pro-uždegiminių citokinų raiškos moduliacija pirminėse pelių mikroglijos ląstelėse po LPS, LPS+BHB ir LPS+BHB+AM-630 poveikio. (**A**) *TNF-α;* (**B**) *NOX2*; (**C**) *iNOS*. Duomenys pateikiami kaip 2^-ΔΔCt su atskirais taškais, n = 5 biologiniai kartojimai kiekvienoje eksperimentinėje grupėje. Tiriamųjų genų raiška normalizuota pagal GAPDH geometrinį vidurkį. K – kontrolinė grupė; LPS – LPS paveikta grupė (100 ng/mL); BHB+LPS – 1 val. poveikis BHB prieš LPS; AM-630+BHB+LPS – 1 val. poveikis AM630 (1 μM) prieš BHB ir LPS. Duomenys pateikiami kaip vidurkis ± SEM; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

Priešuždegiminių genų raiškos reguliavimas pirminėje pelių mikroglijoje veikiant BHB ir CB2R vaidmuo

Toliau siekėme nustatyti, ar BHB didina priešuždegiminių genų raišką ir ar šis poveikis yra tarpininkaujamas per CB2R. Tam tikslui kiekybinės PGR metodu buvo įvertinta *IL-10*, *TGF-β* ir *Arg1* raiška.

Po poveikio LPS *IL-10* mRNR raiška padidėjo, tačiau šis skirtumas nebuvo statistiškai reikšmingas. BHB taip pat reikšmingai nepakeitė *IL-10* mRNR raiškos po LPS stimuliacijos. Kartu taikytas CB2R antagonistas padidino IL-10 *mRNR* raišką, lyginant su BHB+LPS grupe (10 pav. A; $p < 0.05$).

TGF-β mRNR raiškoje tarp tiriamųjų grupių reikšmingų pokyčių nenustatyta, išskyrus reikšmingą sumažėjimą po LPS poveikio, o tai rodo, kad nei BHB, nei CB2R neveikia per šį kelią (10 pav. B).
Kalbant apie *Arg1* mRNR raišką, kuri laikoma alternatyviai aktyvuotos mikroglijos žymeniu, po LPS poveikio buvo stebimas nedidelis jos padidėjimas, tačiau jis nebuvo statistiškai reikšmingas. Vis dėlto, poveikis BHB reikšmingai padidino *Arg1* mRNR raišką, lyginant su vien LPS paveikta grupe (10 pav. C; $p < 0.01$). Šis BHB sukeltas padidėjimas buvo visiškai panaikintas blokuojant CB2R, nes AM-630 sumažino *Arg1* mRNR raišką, lyginant su BHB+LPS grupe (10 pav. C; $p < 0.001$).

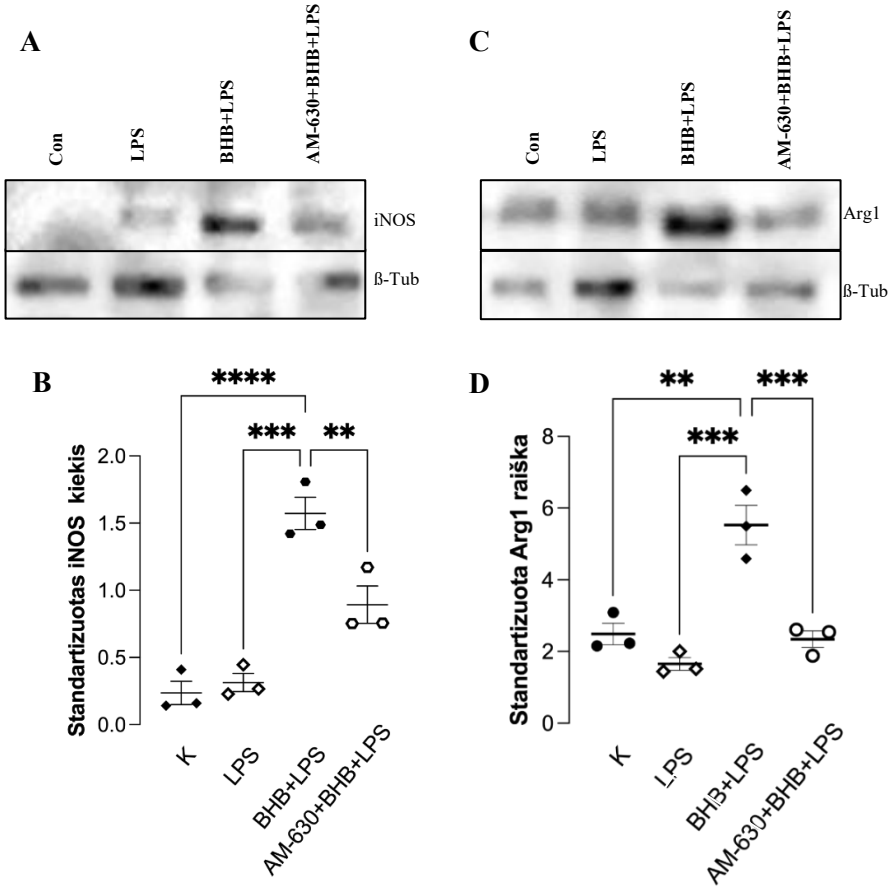


10 pav. Priešuždegiminių citokinų raiškos moduliacija pirminėse pelių mikroglijos ląstelėse po LPS, LPS+BHB ir LPS+BHB+AM-630 poveikio. (**A**) *IL-10*, (**B**) *TGF-β*, (**C**) *Arg1*. Duomenys pateikiami kaip 2^-ΔΔCt. Tiriamųjų genų raiška normalizuota pagal GAPDH geometrinį vidurkį. K – kontrolinė grupė; LPS – LPS paveikta grupė (100 ng/mL); BHB+LPS – 1 val. išankstinis poveikis BHB prieš LPS; AM-630+BHB+LPS – 1 val. išankstinis poveikis AM630 (1 μM) prieš BHB ir LPS. n = 5. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

BHB didina iNOS ir Arg1 baltymų raišką nuo CB2R priklausomu būdu

mRNR raiškos lygiai ne visada tiesiogiai koreliuoja su baltymų kiekiu ląstelėje. Todėl toliau siekta nustatyti, ar Arg1 ir iNOS baltymų lygiai atspindi transkripcijos lygmeniu stebėtus pokyčius.
Rezultatai parodė, kad poveikis vien tik LPS sukėlė nedidelį bei statistiškai nereikšmingą iNOS baltymo raiškos padidėjimą. Tačiau poveikis BHB prieš LPS stimuliaciją reikšmingai padidino iNOS raišką (11 pav. A, B; $p < 0.001$).

Šis BHB sukeltas padidėjimas susilpnėjo kartu taikant AM-630 (11 pav. A, B; $p < 0.05$), o tai rodo CB2 receptoriaus dalyvavimą.



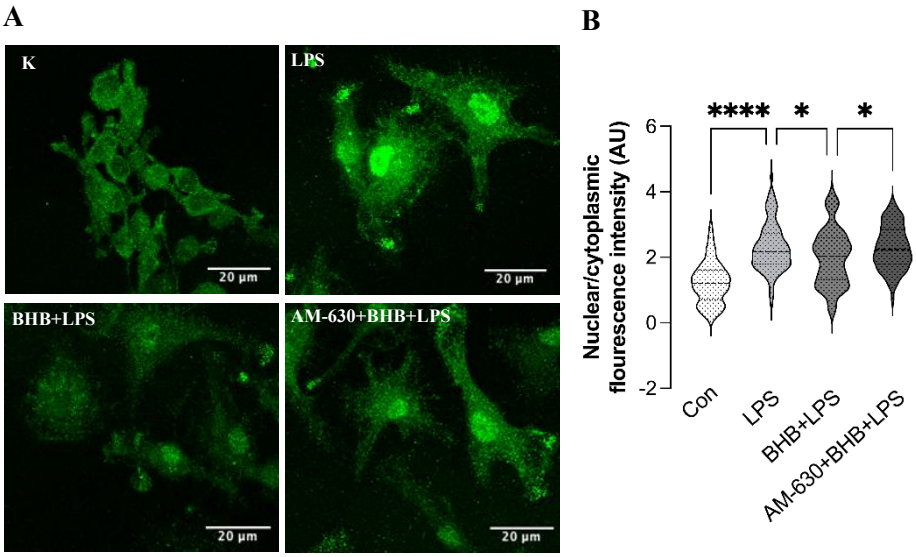
11 pav. BHB didina iNOS ir Arg1 baltymų raišką nuo CB2R priklausomu būdu. (**A**) Reprezentatyvus imunobloto vaizdas, rodantis iNOS ir β-tubulino raišką pirminėse mikroglijos ląstelėse; (**B**) iNOS raiškos kiekybinis įvertinimas, normalizuotas pagal β-tubuliną. (**C**) Reprezentatyvus imunobloto vaizdas, rodantis Arg1 ir β-tubulino raišką pirminėse mikroglijos ląstelėse. (**D**) Arg1 raiškos kiekybinis įvertinimas, normalizuotas pagal β-tubuliną. K – kontrolinė grupė; LPS – LPS paveikta grupė (100 ng/mL); BHB+LPS – 1 val. išankstinis poveikis BHB prieš LPS; AM-630+BHB+LPS – 1 val. išankstinis poveikis AM630 (1 μM) prieš BHB ir LPS. n = 3. Duomenys pateikiami kaip vidurkis ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

Panašiai Arg1 baltymo raiška atitiko jo, kaip priešuždegiminio žymens vaidmenį. Poveikis LPS sumažino Arg1 lygį, nors šis skirtumas nebuvo statistiškai reikšmingas (11 pav. C, D). Svarbu tai, kad kartu taikytas BHB ir

LPS padidino Arg1 baltymo raišką, lyginant su vien LPS grupe (11 pav. C, D; $p < 0.001$). Šis poveikis buvo panaikintas AM-630 ($p < 0.01$), patvirtinant nuo CB2R priklausomą veikimo mechanizmą

BHB priešuždegiminis poveikis yra susijęs su NF-κB signaliniu keliu ir susilpnėja blokuojant CB2R

Toliau buvo tirta, ar BHB slopina NF-κB signalinį kelią – pagrindinį mikroglijos imuninės funkcijos reguliatorių, skatinantį prouždegiminių citokinų ir chemokinų gamybą ir taip stiprinantį neurouždegimą. Be to, buvo siekiama nustatyti, ar CB2R blokada gali panaikinti slopinamąjį BHB poveikį šiam keliui.



12 pav. BHB sukeltas NF-κB branduolinės translokacijos slopinimas yra iš dalies panaikinamas blokuojant CB2R. (**A**) Reprezentatyvūs pirminės pelių mikroglijos fluorescencinės mikroskopijos vaizdai, nudažyti antikūnu prieš NF-κB p65 (žalia). (**B**) Smuiko diagrama, vaizduojanti NF-κB p65 branduolio ir citoplazmos intensyvumo santykį skirtingose tiriamose grupėse. K – kontrolinė grupė; LPS – LPS paveikta grupė (100 ng/mL); BHB+LPS – 1 val. išankstinis poveikis BHB prieš LPS; AM-630+BHB+LPS – 1 val. išankstinis poveikis AM630 (1 µM) prieš BHB ir LPS. N = 90. Duomenys pateikiami kaip vidurkis ± SEM. *$p < 0.01$; ****$p < 0.0001$.

Kaip ir tikėtasi, LPS stimuliacija reikšmingai padidino NF-κB lokalizaciją branduolyje, lyginant su kontroline grupe (12 pav. A, B; $p < 0.0001$), taip patvirtindama uždegiminio signalinio kelio aktyvaciją. Poveikis BHB prieš LPS poveikį reikšmingai susilpnino šį efektą (12 pav. A, B; $p < 0.05$), kas

atitinka literatūroje aprašomas BHB priešuždegimines savybes. Svarbu tai, kad kartu taikytas CB2R antagonistas AM-630 iš dalies panaikino BHB poveikį ir padidino NF-κB lokalizaciją branduolyje, lyginant su BHB+LPS grupe (12 pav. A, B; $p < 0.05$).
Šie rezultatai rodo, kad BHB savo priešuždegiminį poveikį bent iš dalies sukelia slopindamas NF-κB aktyvaciją – svarbų transkripcijos veiksnį mikroglijos tarpininkaujamame neuroužděgime ir šis procesas yra moduliuojamas CB2R.

BHB ir CB2R antagonizmo poveikis viduląsteliniams signaliniams keliams pirminėje mikroglijoje uždegiminėmis sąlygomis

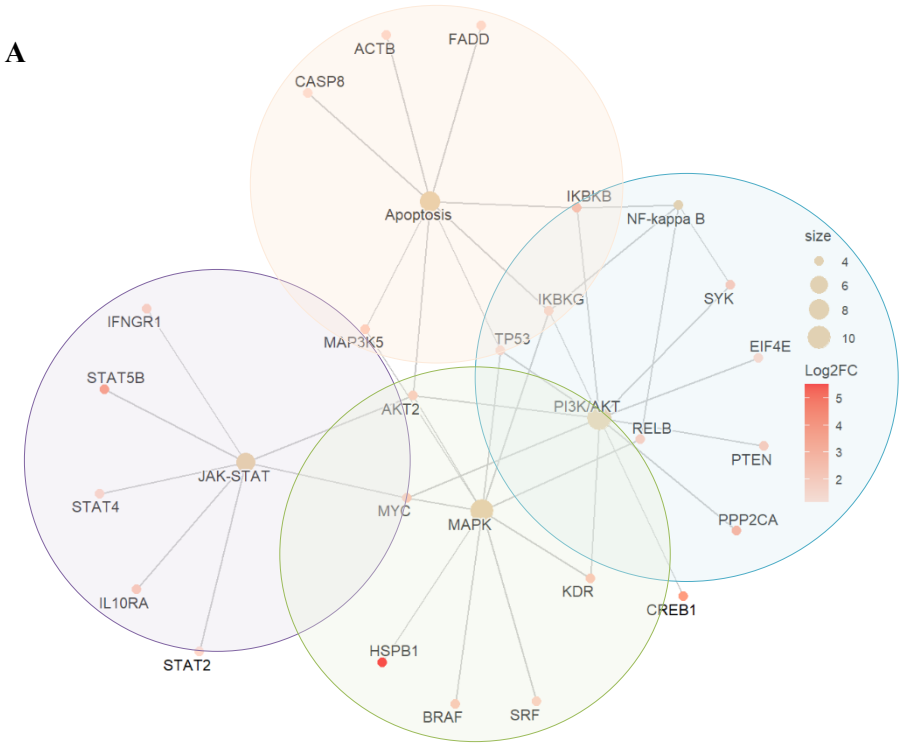
Galiausiai, siekiant ištirti molekulinius mechanizmus, lemiančius BHB poveikį, ir galimą CB2R vaidmenį, buvo įvertinti baltymų, susijusių su pagrindiniais viduląsteliniais signaliniais keliais, fosforilinimo būklės pokyčiai pirminėje mikroglijoje uždegiminėmis sąlygomis. Analizuoti baltymai buvo susiję su signaliniais keliais, dalyvaujančiais signalo perdavime uždegimo metu, metabolizmo reguliacijoje, ląstelių išgyvenamumo ir žūties, proliferacijos bei atsako į stresą procesuose (PI3K/AKT, AMPK, apoptozės, autofagijos, ląstelės ciklo, ErbB, sutelktinio sąlyčio, insulino, JAK/STAT, MAPK, mTOR, NF-κB, p53, Ras, TGF-β ir VEGF signalinius kelius).

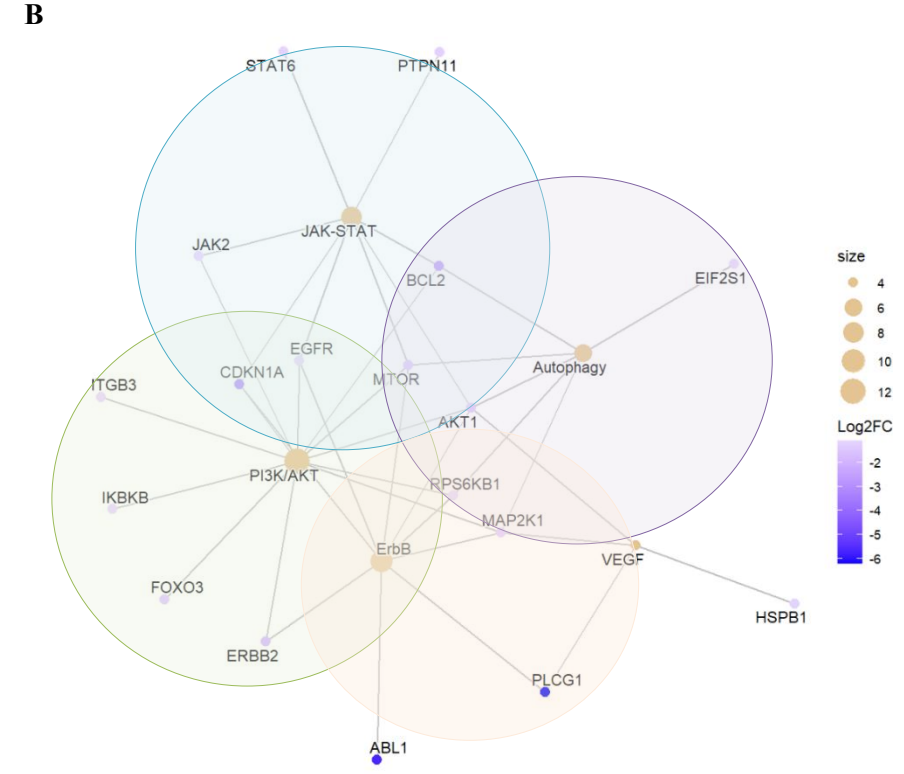
LPS stimulacijos poveikis signalinių molekulių fosforilinimo profiliui pirminėje pelių mikroglijoje

Siekiant apibūdinti uždegiminės stimuliacijos poveikį viduląsteliniams signaliniams procesams, buvo palyginti kontrolinių ir LPS paveiktų pirminių mikroglijos ląstelių baltymų fosforilinimo profiliai. LPS sukėlė plačius pokyčius tarpusavyje susijusiuose signaliniuose keliuose, siejamuose su uždegiminiu atsaku, ląsteliniu stresu, išgyvenamumu, apoptoze, augimu ir metabolizmu. Tarp baltymų, kurių fosforilinimas po LPS poveikio padidėjo, ryškiausi tinklo klasteriai buvo susiję su uždegiminiais signaliniais procesais, atsako į stresą keliais, apoptozės ir išgyvenamumo reguliacija bei su imuniniu atsaku susijusiu signalų perdavimu. Šie tarpusavyje susiję klasteriai leidžia manyti, kad LPS stipriai aktyvino signalinius modulius, dalyvaujančius mikroglijos atsake į uždegiminę stimuliaciją. Tokie keliai kaip JAK/STAT, MAPK, PI3K/AKT ir su apoptoze susiję signaliniai keliai sudarė pagrindinius tinklo mazgus, rodančius koordinuotą kelių, dalyvaujančių citokinų signalų perdavime, transkripcijos reguliacijoje, ląstelinio streso atsakuose ir uždegiminių mediatorių gamyboje, aktyvinimą (13 pav. A).

Priešingai, baltymai, kurių fosforilinimas po LPS poveikio sumažėjo, daugiausia telkėsi PI3K/AKT, JAK/STAT, ErbB ir autofagijos signaliniuose keliuose (14 pav. B). Tai rodo, kad LPS stimuliaciją lydėjo ne tik padidėjęs su uždegimu ir stresu susijusių signalinių baltymų fosforilinimas, bet ir sumažėjęs signalinių kelių, susijusių su augimo faktorių signalų perdavimu, ląstelių išgyvenamumu, metaboline adaptacija ir homeostazės reguliacija, komponentų fosforilinimas. Tiek padidėjęs, tiek sumažėjęs fosforilinimas tokiuose keliuose kaip PI3K/AKT ir JAK/STAT leidžia manyti, kad LPS šių signalinių kaskadų nevienodai aktyvino ar slopino, o veikiau sukėlė specifinį signalinių komponentų pertvarkymą.

Apibendrinant, šie rezultatai rodo, kad LPS sukėlė plataus masto nuo fosforilinimo priklausomų signalinių procesų pertvarkymą pirminėse mikroglijos ląstelėse. Užuot vienodai padidinęs uždegiminių signalinių kelių aktyvumą, LPS pakeitė atskirus signalinius mazgus uždegimo, išgyvenamumo, metabolizmo ir augimo faktorių signaliniuose tinkluose. Toks pokyčių pobūdis leidžia manyti, kad LPS sukelta mikroglijos aktyvacija apima koordinuotą viduląstelinių signalinių procesų reorganizaciją, vienu metu sustiprinant ir susilpninant pasirinktus fosforilinimo įvykius.


A
ACTB
FADD
CASP8
Apoptosis
IKBKB
NF-kappa B
size
4
6
8
10
IKBKG
SYK
IFNGR1
MAP3K5
TP53
EIF4E
Log2FC
5
4
3
2
STAT5B
AKT2
PI3K/AKT
RELB
JAK-STAT
PTEN
STAT4
MYC
MAPK
PPP2CA
KDR
IL10RA
CREB1
HSPB1
STAT2
BRAF
SRF


B
STAT6
PTPN11
JAK2
JAK-STAT
BCL2
EIF2S1
size
4
6
8
10
12
EGFR
CDKN1A
MTOR
Autophagy
ITGB3
AKT1
Log2FC
-2
-3
-4
-5
-6
PI3K/AKT
RPS6KB1
IKBKB
MAP2K1
ErbB
VEGF
FOXO3
HSPB1
ERBB2
PLCG1
ABL1

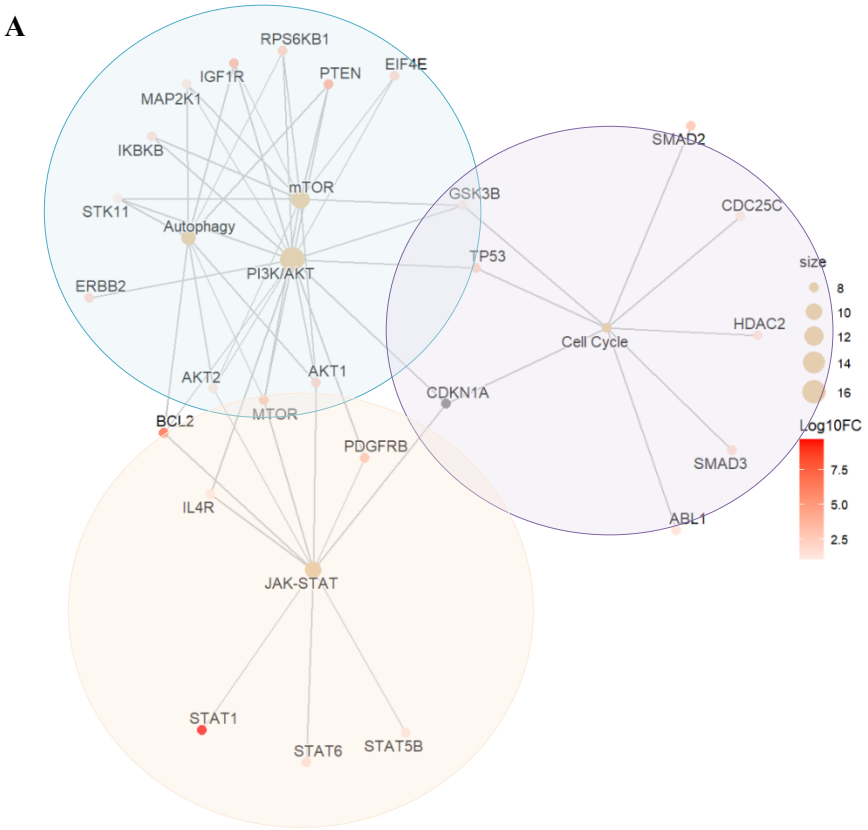
13 pav. Diferencinio baltymų fosforilinimo ir signalinių kelių tinklo analizė LPS paveiktoje pirminėje mikroglijoje. (**A**) Padidėjusio fosforilinimo baltymų signalinių kelių tinklo analizė LPS paveiktoje pirminėje mikroglijoje. (**B**) Sumažėjusio fosforilinimo baltymų signalinių kelių tinklo analizė LPS paveiktoje pirminėje mikroglijoje. Mazgai žymi signalinius kelius arba atskirus baltymus, susijusius su pakitusiais fosforilinimo signalais po LPS stimuliacijos, lyginant su kontrolinėmis ląstelėmis. Jungtys rodo sąsajas tarp baltymų ir jiems priskirtų signalinių kelių. Signalinio kelio mazgo dydis atspindi su juo susijusių baltymų skaičių, o mazgo spalvos intensyvumas parodo log2 pokyčio dydį – tamsesnė spalva reiškia stipresnį padidėjimą arba sumažėjimą.

BHB moduliuoja su fosforilinimu susijusius signalinius kelius LPS stimuliuotoje pirminėje mikroglijoje

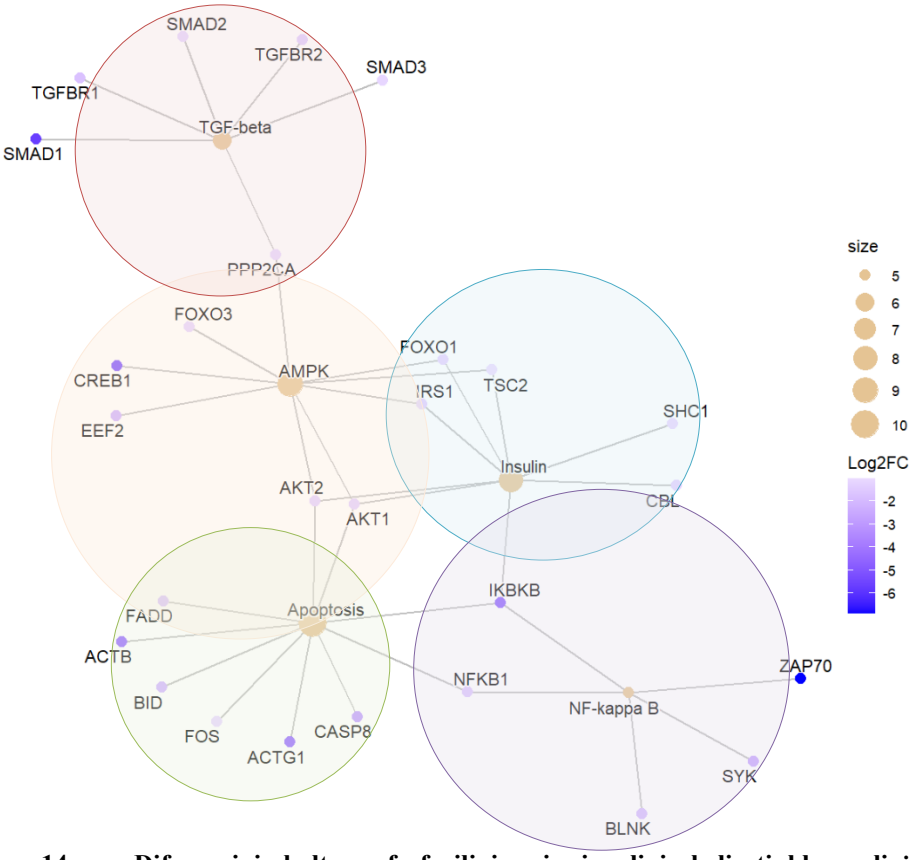
Siekiant išsamiau apibūdinti fosforilinimo pokyčius, susijusius su BHB poveikiu LPS aktyvintose pirminėse mikroglijos ląstelėse, buvo atlikta signalo perdavimo kelių tinklo analizė lyginant BHB+LPS ir LPS grupes. Baltymai, kurių fosforilinimas BHB+LPS grupėje padidėjo, daugiausia telkėsi PI3K/AKT/mTOR, JAK/STAT, autofagijos ir ląstelės ciklo susijusiuose tinkluose. PI3K/AKT/mTOR susijęs klasteris buvo viena iš ryškiausių signalo perdavimo grupių, rodanti, kad BHB poveikis buvo susijęs su fosforilinimo pokyčiais keliuose, dalyvaujančiuose ląstelių išgyvenamume, metabolinėje adaptacijoje ir su augimu susijusiame signalo perdavime. Šis klasteris buvo glaudžiai susijęs su autofagijos susijusiu signalo perdavimu, o tai leidžia manyti, kad BHB veikė adaptacinius ląstelių atsako kelius uždegiminėmis sąlygomis. Padidėjęs fosforilinimas JAK/STAT susijusiuose komponentuose taip pat rodo, kad BHB darė įtaką citokinams jautriems signalo perdavimo keliams LPS aktyvintoje mikroglijoje. Pažymėtina, kad STAT1 buvimas tarp baltymų, kurių fosforilinimas padidėjo, rodo, jog BHB poveikis buvo susijęs ne tik su priešuždegiminiu signalo perdavimu, bet ir su prouždegiminių arba interferonų susijusių signalo perdavimo komponentų aktyvinimu.
Priešingai, baltymai, kurių fosforilinimas po BHB poveikio sumažėjo, daugiausia buvo susiję su TGF-β, AMPK, insulino signalo perdavimo, apoptozės ir NF-κB susijusiais tinklais. Sumažėjęs fosforilinimas AMPK ir insulino susijusiuose keliuose rodo, kad BHB pakeitė metabolinio signalo perdavimo atsakus LPS aktyvintoje mikroglijoje. Sąsaja su TGF-β susijusiu signalo perdavimu rodo pokyčius keliuose, dalyvaujančiuose imuninėje reguliacijoje ir ląstelių homeostazėje, o sumažėjęs fosforilinimas šiame tinkle gali atspindėti reguliacinių arba priešuždegiminių signalo perdavimo komponentų slopinimą. Sumažėjęs fosforilinimas apoptozės susijusiuose tinkluose rodo, kad BHB poveikis taip pat apėmė signalo perdavimo

komponentus, susijusius su ląstelės likimu ir streso atsako reguliacija. Tuo pačiu metu NF-κB susijusių komponentų buvimas tarp baltymų, kurių fosforilinimas sumažėjo, rodo uždegiminio signalo perdavimo keli7 slopinimą LPS stimuliacijos sąlygomis.

Apibendrinant, šie duomenys rodo, kad BHB sukėlė selektyvų nuo fosforilinimo priklausomo signalo perdavimo pertvarkymą LPS aktyvintose pirminėse mikroglijos ląstelėse. Sumažėjusio fosforilinimo NF-κB susijusiuose keliuose ir padidėjusio citokinams jautrių komponentų, tokių kaip STAT1, fosforilinimo derinys rodo, kad šiame modelyje BHB neveikia kaip paprastas, globalus priešuždegiminis reguliatorius. Vietoje to, BHB, tikėtina, keičia aktyvintos mikroglijos uždegiminio signalo perdavimo būseną, vienu metu slopindamas kai kuriuos uždegiminius kelius, tačiau išlaikydamas arba stiprindamas pasirinktus prouždegiminius, metabolinius ir adaptacinius signalo perdavimo komponentus.



B



14 pav. Diferencinio baltymų fosforilinimo ir signalinių kelių tinklo analizė BHB paveiktoje pirminėje mikroglijoje uždegiminėmis sąlygomis. (**A**) Padidėjusio fosforilinimo baltymų signalinių kelių tinklo analizė BHB+LPS paveiktoje pirminėje mikroglijoje. (**B**) Sumažėjusio fosforilinimo baltymų signalinių kelių tinklo analizė BHB+LPS paveiktoje pirminėje mikroglijoje. Mazgai žymi signalinius kelius arba atskirus baltymus, susijusius su pakitusiais fosforilinimo signalais BHB+LPS grupėje, lyginant su LPS grupe. Jungtys rodo sąsajas tarp baltymų ir jiems priskirtų signalinių kelių. Signalinio kelio mazgo dydis atspindi su juo susijusių baltymų skaičių, o mazgo spalvos intensyvumas parodo log2 pokyčio dydį – tamsesnė spalva reiškia stipresnį sumažėjimą.

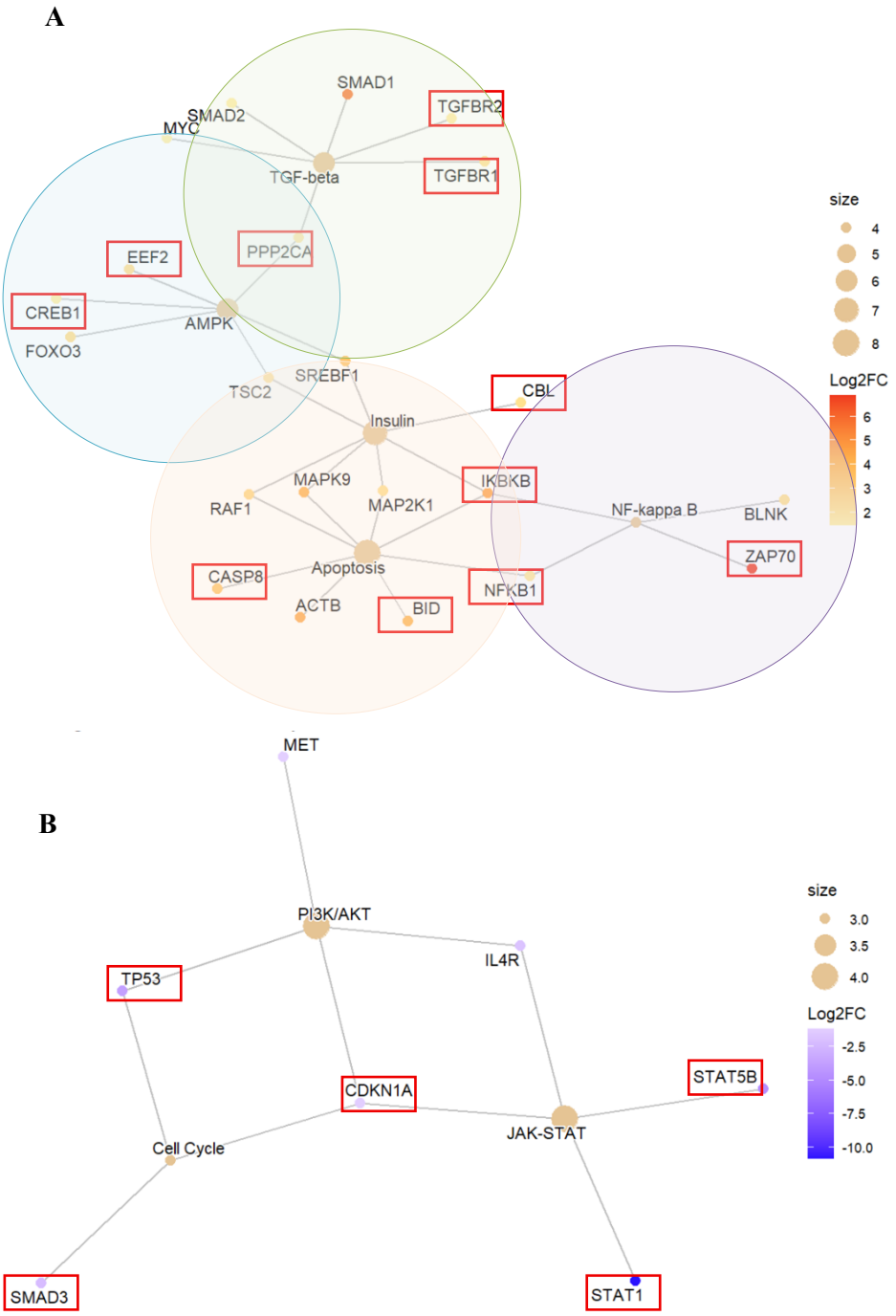
Fosforilinų baltymų pokyčiai po CB2R blokados BHB paveiktoje, LPS stimuliuotoje pirminėje mikroglijoje

AM630+BHB+LPS grupės, lyginant su BHB+LPS grupe, signalo perdavimo kelių tinklo analizė parodė, kad CB2R blokavimas paveikė platų baltymų spektrą. Iš viso padidėjęs fosforilinimas buvo nustatytas 41 baltymuose. Be to, 16 baltymų fosforilinimas sumažėjo, o šie baltymai daugiausia buvo susiję su MAPK, PI3K/AKT, ląstelės ciklo, JAK/STAT ir autofagijos keliais.

Tačiau pagrindinis šio palyginimo tikslas buvo nustatyti baltymus, kurių fosforilinimo pokyčiai po BHB poveikio pasireiškė tik esant funkcionuojančiam CB2R signalo perdavimui. CB2R blokavimas leido įvertinti šio receptoriaus indėlį į BHB sukeltus fosforilinimo pokyčius. Todėl tolesnėje analizėje buvo sutelktas dėmesys į baltymus, kurių reguliacijos kryptis buvo priešinga BHB+LPS ir AM630+BHB+LPS grupėse: t. y. į baltymus, kurių fosforilinimas padidėjo BHB+LPS grupėje, bet sumažėjo po CB2R blokavimo, ir į baltymus, kurių fosforilinimas sumažėjo BHB+LPS grupėje, bet padidėjo AM630+BHB+LPS grupėje. Toks priešingas reguliacijos modelis rodo, kad šių baltymų fosforilinimo pokyčiai po BHB poveikio gali bent iš dalies priklausyti nuo nepažeisto CB2R signalo perdavimo (15 pav.).

Keliai, kuriuose fosforilinimas po BHB poveikio buvo sumažėjęs, tačiau vėl padidėjo užblokavus CB2R, apėmė TGF-β, AMPK, insulino, apoptozės ir NF-κB signalo perdavimo kelius. Šie duomenys rodo, kad CB2R blokavimas iš dalies pakeitė BHB sukeltą fosforilinimo profilį. Ryškiausi pokyčiai buvo stebimi NF-κB susijusiame kelyje, o apoptozės, TGF-β, AMPK ir insulino signalo perdavimo keliai taip pat sudarė aiškiai išskiriamus tinklo mazgus. Taigi, CB2R blokavimas padidino fosforilinimą keliuose signalo perdavimo tinkluose, kuriuose po BHB poveikio fosforilinimas buvo sumažėjęs (15 pav. A).

Nors kelių atskirų baltymų fosforilinimas AM630+BHB+LPS grupėje sumažėjo, palyginti su BHB+LPS grupe, tarp šių baltymų nebuvo nustatytas aiškiai praturtintas signalo perdavimo kelias. Dalis šių baltymų anksčiau pasižymėjo padidėjusiu fosforilinimu po BHB poveikio LPS aktyvintose mikroglijos ląstelėse, tačiau po CB2R blokavimo jų sumažėjęs fosforilinimas nesudarė nuoseklaus funkcinio kelio ar aiškaus signalo perdavimo tinklo mazgo. Todėl šie pokyčiai buvo vertinami atsargiai kaip pavieniai fosforilinimo įvykiai, o ne kaip koordinuoto konkretaus kelio reguliavimo įrodymas. Bendrai sumažėję fosforilinimo signalai po AM630 poveikio nesuteikė tvirto pagrindo išskirti papildomą specifinį CB2R priklausomą kelią, išskyrus aiškiau apibrėžtus pokyčius anksčiau minėtuose signalo perdavimo tinkluose (15 pav B).



15 pav. Diferencinio baltymų fosforilinimo ir signalinių kelių tinklo analizė AM-630+BHB+LPS paveiktoje pirminėje mikroglijoje. (**A**) Padidėjusio fosforilinimo baltymų signalinių kelių tinklo analizė AM630+BHB+LPS paveiktoje pirminėje

mikroglijoje. (**B**) Sumažėjusio fosforilinimo baltymų signalinių kelių tinklo analizė AM630+BHB+LPS paveiktoje pirminėje mikroglijoje. Mazgai žymi signalinius kelius arba atskirus baltymus, susijusius su pakitusiais fosforilinimo signalais po gydymo AM630+BHB+LPS, palyginti su BHB+LPS grupe. Jungtys rodo sąsajas tarp baltymų ir jiems priskirtų signalinių kelių. Signalinio kelio mazgo dydis atspindi su juo susijusių baltymų skaičių, o mazgo spalvos intensyvumas parodo log2 pokyčio dydį – tamsesnė spalva reiškia stipresnį sumažėjimą.

5. Diskusija

Yra žinoma, kad BHB gerina atmintį, kognityvines funkcijas ir skatina neuronų plastiškumą, daugiausia per priešuždegiminius mechanizmus, nors tikslūs šio poveikio keliai išlieka neaiškūs (Yamanashi T et al., 2017). Šiame tyrime parodėme, kad BHB pasižymi imunomoduliaciniu poveikiu tiek DIO pelių modelyje, tiek pirminėje mikroglijos kultūroje, o šis poveikis bent iš dalies yra tarpininkaujamas CB2R. Kadangi DIO yra siejamas su žemo laipsnio neuroužd egimu (Henn RE et al., 2022; Cavaliere et al., 2019; Lainez et al., 2018), o BHB pasižymi priešuždegiminiu poveikiu senėjimo ir neurodegeneracinių ligų modeliuose (Nomura M et al., 2023; Shippy DC et al., 2020), šiame tyrime pirmiausia vertinome, ar BHB gali keisti mikroglijos fenotipą nutukusių pelių smegenyse.

In vivo sąlygomis BHB pakeitė mikroglijos morfologiją iš labiau apvalios, reaktyvios formos į labiau šakotą ir kompleksišką struktūrą, rodančią labiau stebėsenai/homeostazei būdingą fenotipą. Tai atitinka ankstesnius duomenis, teigiančius, kad BHB skatina labiau šakotą mikroglijos morfologiją ir mažina uždegiminę aktyvaciją (Huang C et al., 2018). BHB taip pat pagerino mikroglijos funkcinę pusiausvyrą, didindamas su ląstelių liekanų šalinimu susijusią fagocitozę ir mažindamas sąveiką su sinapsėmis, taip palaikydamas galimą neuroapsauginį BHB vaidmenį metabolinio streso sąlygomis. Kartu BHB padidino CB2R ir sumažino MAGL raišką. Kadangi CB2R yra gausiai išreiškiamas mikroglijoje ir reguliuoja CNS imuninius atsakus (Komorowska-Müller JA et al., 2020), o MAGL skaido 2-AG, CB1R/CB2R agonistą (Miller S et al., 2016; Jiang M et al., 2023), šie rezultatai leidžia spręsti apie sustiprėjusį endokanabinoidinį tonusą ir palaiko hipotezę, kad BHB gali veikti iš dalies per su CB2R susijusius mechanizmus.

Norėdami tai ištirti detaliau, optimizavome pirminės mikroglijos išskyrimo protokolą. Pirma, palyginome suaugusių gyvūnų mikroglijos išskyrimo protokolus ir nustatėme, kad mūsų modifikuotas fermentinės disociacijos protokolas, adaptuotas pagal Bordt et al., pasižymėjo didžiausia išeiga ir leido išvengti magnetinių dalelių naudojimo (Bordt EA et al., 2020). Suaugusių gyvūnų mikroglija yra fiziologiškai aktualesnė lėtinio arba su amžiumi susijusio neuroužd egimo tyrimams, tačiau jų išskyrimas yra techniškai sudėtingesnis ir gaunama mažiau ląstelių. Priešingai, naujagimių mikroglijos pasižymėjo didesne išeiga ir kultūros grynumu, tikėtina, dėl mažesnio mielino kiekio ir didesnio proliferacinio pajėgumo, nors jos gali skirtis nuo suaugusių mikroglijos transkripciniu profiliu ir atsaku į dirgiklius (Nikodemova & Watters, 2012; Gosselin et al., 2017; Hammond et al., 2019). Todėl naujagimių mikroglijos kultūros buvo pasirinktos kaip praktiška platforma

mechanistiniams tyrimams, tačiau pagrindinių rezultatų validavimas suaugusių gyvūnų mikroglijose išlieka svarbus.

Naudojant pirminę naujagimių mikrogliją nustatėme, kad joje BHB susilpnino kelis LPS sukeltus poveikius. BHB padidino fagocitinį aktyvumą ir sumažino ROS gamybą, o tai atitinka ankstesnius tyrimus, siejančius BHB su pagerėjusia fagocitoze ir mažesniu oksidaciniu stresu (Julio-Amilpas A et al., 2015; Zhang Y et al., 2022; Rana AK et al., 2024; Jin LW et al., 2023). Svarbu tai, kad šie poveikiai buvo sumažinti arba panaikinti AM-630. Tai leidžia manyti, jog funkcionalus CB2R prisideda prie BHB moduliuojamo fagocitozės sustiprinimo ir oksidacinio streso slopinimo.

BHB taip pat padidino Arg1 raišką po LPS stimuliacijos, o CB2R blokada šį poveikį panaikino. Kadangi Arg1 dažnai siejamas su priešuždegiminiu mikroglijos fenotipu (Cherry JD et al., 2014), o CB2R blokada, kaip parodyta ankstesniuose tyrimuose, gali sumažinti Arg1 raišką, (Jia J et al., 2016; Ma L et al., 2015), mūsų duomenys patvirtina CB2R vaidmenį BHB imunomoduliacinio veikimo metu. Įdomu tai, kad iNOS ekspresija BHB+LPS grupėje taip pat buvo padidėjusi. Tai rodo, kad BHB ne visomis sąlygomis sukelia vienodai priešuždegiminę būseną, bet gali skatinti nuo konteksto priklausomą imunometabolinį persitvarkymą, ypač esant stipriai LPS stimuliacijai (Shi X et al., 2014; Benito A et al., 2020).

Atsižvelgiant į priešuždegiminį BHB profilį, BHB slopino LPS sukeltą NF-κB aktyvaciją, kaip anksčiau buvo parodyta kituose tyrimuose (Fu SP et al., 2014; Fu SP et al., 2015). AM-630 šį poveikį panaikino, rodydamas, kad CB2R bent iš dalies yra reikalinga BHB tarpininkaujamam NF-κB slopinimui. Ši BHB ir CB2R konvergencija NF-κB signalo perdavimo kelyje buvo papildomai patvirtinta fosfo-antikūnų mikrogardelių analize, kuri buvo atlikta siekiant įvertinti BHB poveikį viduląsteliniams mechanizmams ir CB2R dalyvavimui LPS stimuliuotose pirminėse mikroglijos ląstelėse, gautose iš naujagimių pelių smegenų. Mūsų rezultatai parodė, kad LPS stimuliacija sukėlė platų signalo perdavimo kelių pertvarkymą, apimantį NF-κB, MAPK, PI3K/AKT ir JAK/STAT kelius. Tai atitinka ankstesnius tyrimus, rodančius, kad LPS sukelta mikroglijos aktyvacija nėra vieno linijinio kelio rezultatas, bet apima kelių uždegiminių ir streso atsako tinklų aktyvinimą (Pulido-Salgado M et al., 2018; An J et al., 2020). LPS aktyvintas PI3K/AKT/NF-κB kelias gali skatinti uždegiminių mediatorių, įskaitant TNF-α, IL-1β, IL-6, iNOS ir COX-2, raišką (Cianciulli A et al., 2016; Subedi L et al., 2019). Tai dera su mūsų ankstesniais duomenimis, rodančiais padidėjusią NF-κB translokaciją į branduolį, TNF-α ir iNOS raišką bei ROS gamybą po LPS poveikio.

LPS taip pat paveikė apoptozės susijusių baltymų fosforilinimą, tačiau šie pokyčiai neturėtų būti laikomi tiesioginiu apoptozės įrodymu be papildomų apoptozės žymenų. Tokie signalai gali atspindėti oksidacinį stresą, DNR pažaidos atsaką, uždegiminio signalo perdavimą ir ląstelės žūties / išgyvenamumo pusiausvyros reguliavimą (Chen K et al., 2003; Gurung P et al., 2014; Flicker M et al., 2013).

BHB poveikis LPS aktyvintai mikroglijai buvo susijęs su padidėjusiu PI3K/AKT, JAK/STAT, mTOR/autofagijos ir ląstelės ciklo susijusių tinklų baltymų fosforilinimu. Kadangi BHB gali moduliuoti mikroglijos metabolizmą ir pasižymi teigiamu poveikiu neuroužd egimo modeliuose (Jin et al., 2023), šie pokyčiai gali rodyti adaptacinių procesų aktyvinimą uždegiminėmis sąlygomis. Tai dera su mūsų duomenimis, kad BHB sumažino ROS gamybą ir padidino mikroglijos fagocitinį aktyvumą.

Vis dėlto BHB poveikis nebuvo vienareikšmiškai priešuždegiminis. Pavyzdžiui, STAT1 fosforilinimas dažniausiai siejamas su prouždegiminiu signalo perdavimu (He H et al., 2024) ir gali padėti paaiškinti šiame darbe stebėtą iNOS padidėjimą, nes STAT1 gali skatinti iNOS raišką (Delgado, 2003). Todėl BHB šiame modelyje greičiau pertvarko mikroglijos signalo perdavimą, o ne vienodai slopina visus uždegiminius kelius.

Svarbiausias priešuždegiminis BHB poveikis buvo susijęs su NF-κB kelio slopinimu. BHB sumažino NF-κB susijusį fosforilinimą, o tai atitinka mūsų ankstesnius rezultatus, rodančius sumažėjusią NF-κB translokaciją į branduolį, ROS gamybą ir prouždegiminių mediatorių raišką. Panašus BHB poveikis LPS sukeltam NF-κB aktyvinimui aprašytas ir ankstesniuose tyrimuose (Fu SP et al., 2014; Jayashankar SS et al., 2023). Be to, BHB sumažino apoptozės susijusių baltymų aktyvinimą, kas greičiausiai atspindi mažesnį uždegiminį ir oksidacinį stresą, o ne tiesioginį apoptozės slopinimą (Kim et al., 2013; Orning et al., 2020; Jembrek et al., 2021).

CB2R blokavimo analizė parodė, kad ryškiausi BHB poveikiui jautrūs pokyčiai buvo NF-κB mazge ir apoptozės susijusiame kelyje. AM630 padidino NF-κB susijusį fosforilinimą, kurį BHB buvo sumažinęs, o tai rodo, kad CB2R gali dalyvauti BHB sukeltame NF-κB slopinime. Tai dera su ankstesniais tyrimais, rodančiais, kad CB2R tarpininkaujamas priešuždegiminis poveikis gali būti susijęs su NF-κB kelio slopinimu, o AM630 šį poveikį panaikina (Komorowska-Müller JA, Schmöle AC, 2020; Lisboa SF et al., 2016). Panašiai, CB2R gali būti susijęs ir su BHB poveikiu apoptozės signalams, nes CB2R aktyvinimas mažina neuroužd egimą ir apoptozę, o AM630 gali sukelti priešingą poveikį (Jiang F et al., 2022).

Apibendrinant, mūsų duomenys rodo, kad BHB moduliuoja mikroglijos morfologiją, funkcijas, uždegiminį profilį ir viduląstelinį signalo perdavimą

metabolinio ir uždegiminio streso sąlygomis. DIO pelių modelyje BHB skatino labiau šakotą mikroglijos fenotipą, mažino uždegiminius žymenis ir keitė su EKS susijusius baltymus. Pirminėse mikroglijos ląstelėse BHB didino fagocitozę, mažino ROS, didino Arg1 raišką ir slopino NF-κB. Kelių šių poveikių panaikinimas AM-630 leidžia daryti išvadą, kad CB2R prisideda prie BHB imunomoduliacinių veiksmų. Vis dėlto, baltymų fosforilinimo duomenys taip pat rodo, kad BHB ne tiesiog slopina mikroglijos aktyvaciją, bet pertvarko kelis signalinius kelius, susijusius su uždegimu, metabolizmu, streso adaptacija ir ląstelių žūties reguliacija. Taigi, BHB veikia kaip metabolinis ir imunomoduliacinis junginys, o CB2R yra svarbus jo priešuždegiminio ir apsauginio poveikio mediatorius.

6. Išvados

1. DIO pelių modelyje BHB reikšmingai susilpnino neuroužd egiminį atsaką, kuris pasireiškė sumažėjusia IL-1β ir IL-6 raiška, mikroglijos morfologijos poslinkiu link labiau šakotos, homeostatinės būsenos ir fagocitinio aktyvumo moduliavimu. Šiuos pokyčius lydėjo padidėjusi CB2R raiška ir sumažėjusi MAGL raiška, rodanti koordinuotą endokanabinoidinės sistemos reguliaciją.
2. Optimizuotas pirminės mikroglijos iš pelių jauniklių išskyrimo protokolas užtikrino didžiausią ląstelių išeigą ir kultūros grynumą tarp vertintų metodų, taip patvirtindamas jo patikimumą ir tinkamumą tolimesniems *in vitro* funkciniams ir molekuliniams tyrimams.
3. LPS stimuliuotose pirminėse mikroglijos ląstelėse BHB reikšmingai padidino fagocitinį efektyvumą ir moduliavo uždegiminius atsakus. Šie poveikiai buvo iš dalies susilpninti arba panaikinti AM-630, o tai rodo, kad CB2R prisideda prie BHB poveikio.
4. BHB moduliavo kelis viduląstelinius signalinius kelius, susijusius su uždegimu, ląstelių išgyvenimu, metabolizmu ir adaptaciniu atsaku LPS aktyvuotoje mikroglijoje. Dalinis šių poveikių panaikinimas po CB2R blokados papildomai patvirtina CB2R priklausomų mechanizmų dalyvavimą tarpininkaujant BHB poveikiui.

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Vijaya AK†, **Iešmantaitė M**†, Mela V, Baltriukienė D, Burokas A. Microglia isolation from aging mice for cell culture: A beginner's guide. Front Cell Neurosci. 2023 Jan 19;17:1082180. doi: 10.3389/fncel.2023.1082180
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LIST OF CONFERENCE PRESENTATIONS

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CURRICULUM VITAE

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