Brain Derived Neurotrophic Factor (BDNF) as a Mediator of Microglia-induced Neuroinflammation

by

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Abstract

Microglia are the primary immunocompetent cells that protect the brain from environmental stressors; however, their activation can also have deleterious effects on brain functioning. Indeed, environmental toxins and microbial agents can induce microglial driven inflammatory processes that increase levels of pro-inflammatory cytokines and induce a cytotoxic environment. Recent therapeutic strategies have sought to determine how to modulate microglia, so as to favour their neuroprotective effects, while minimizing toxic outcomes. BDNF is one of the most commonly expressed neurotrophins in the brain and is important for the regulation of plasticity, synapse formation, and general neuron health. Yet, little is known about how exogenous BDNF directly effects microglial activity. We hypothesized that BDNF would have a modulatory effect on inflammation in isolated microglia cultures in the context of a bacterial endotoxin. To this end, we indeed found that a BDNF treatment following LPS-induced inflammation attenuated the release of both IL-6 and TNF-α in primary microglia. In neurons, LPS-activated microglial media was able produce a minor inflammatory response, while secondary treatment of BDNF reduced this effect. Interestingly, LPS activated microglial media alone was found to increase production of anti-inflammatory IL-4 in neurons. We speculate that BDNF plays a role in regulating microglia activation and localized microglia-neuron crosstalk may be crucial in preventing damaging effects of inflammatory mechanisms.

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List of Abbreviations

$A_{2A}R$	Adenosine 2A receptors
Aβ	Amyloid beta
AD	Alzheimer's disease
AKT	Protein kinase B
ALS	Amyotrophic lateral sclerosis
α-syn	A-synuclein
ATP	Adenosine triphosphate
BDNF	Brain derived neurotrophic factor
cAMP	Cyclic adenosine monophosphate
CBP	CREB-binding protein
CD200	Cluster of differentiation 200
CD200R	Cluster of differentiation 200 receptor
CNS	Central nervous system
CREB	cAMP response element binding protein
CX3CL1	C-X3-C-motifchemokine ligand 1
CX3CR1	C-X3-C-motifchemokine receptor 1
DAMP	Damage-associated molecular patterns
DIV	Days *in vitro*
ERK	Extracellular signal-regulated kinase
HD	Huntington's disease
IGF-1	Insulin-like growth factor-1
1GF-1R	Insulin-like growth factor-1 receptor
IL-1β	Interleukin1β
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-10	Interleukin-10
JNK	c-Jun N-terminal
LBP	LPS-binding protein
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
LTP	Long-term potentiation
MAC1	Macrophage antigen complex
MAPK	Mitogen-activated protein kinase
mBDNF	Mature BDNF
MD-2	Myeloid differentiation factor 2
mTOR	Mammalian target of rapamycin
MyD88	Myeloid differentiation primary response 88 protein
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
P2X4R	P2X4 purinoreceptors
P75NTR	P75 neurotrophin receptor
PAMP	Pathogen-associated molecular patterns
PD	Parkinson's disease
PDL	Poly-D-lysine

PI3K	Phosphatidylinositol 3-kinase
PRR	Pattern-recognition receptors
RhoA	Ras homolog gene family member A
ROS	Reactive oxygen species
SNARE	SNAP receptor
SNpc	Substantia nigra pars compacta
TGF-β	Transforming growth factor-β
TLR	Toll-like receptor
TNFα	Tumore necrosis factor-α
TrkB	Tyrosine receptor kinase B

1. Introduction

1.1. The Innate Immune Response and Neuroinflammation

Immunity is a fundamental defensive process that all organisms possess and at the cellular level, is mediated by a diverse set of cell types including macrophages, monocytes, B-cell, T-cell, and other leukocytes (white blood cells) (Charles A Janeway et al., 2001). The immune response can be separated into the innate and adaptive immune branches. The innate immune response is rapid and relatively non-specific and mediated mainly by neutrophils and macrophages which release cytokines, reactive oxygen species (ROS) and proteases (Colton, 2009). They also phagocytize foreign particles and pathogens, and induce recruitment of other immune cells (van Rees et al., 2016). Adaptive immunity is comprised of T-cells and B-cells that are much more specifically directed and maintain some degree of immunological memory (Kolaczkowska and Kubes, 2013). Immunity within the central nervous system (CNS) is almost solely dependent upon the brain's specialized microglial cells, which rapidly respond to any perturbations in their microenvironment that might stem from viral and bacterial infection, stroke and traumatic brain injury (Zarruk et al., 2018; Dwyer et al., 2020a).

All immune cells, microglia included, express pattern-recognition receptors (PRRs) that are important in the detection of pathogen-associated molecules and initiation of the innate immune system (Medzhitov, 2001). The most crucial PRRs in the immune response are the Toll-like Receptors (TLRs) which have specified functionality based on different pathogen-associated molecular patterns (PAMPs) (Kaisho and Akira, 2006). In response to infection or cell injury, microglia and other immune cells can recognize PAMPs and induce a specified immune response (Colton, 2009). As in the periphery, upon activation,

microglia promote the production of inflammatory factors particularly beneficial for the removal of pathogens and any cellular debris (Streit, 2002). In the short-term, this is beneficial, and the immune response responds by clearing any damaged tissue and any unnecessary pro-inflammatory markers via anti-inflammatory factors and mechanisms (Nayak et al., 2014). However, in periods of prolonged stress, chronic neuroinflammation can create a cytotoxic environment due to high levels of pro-inflammatory factors and other neurotoxic factors (Block et al., 2007; Hickman et al., 2018).

In addition to their role as immune defenders of the CNS, microglia also have critical roles in brain development and health (Streit, 2002; Vilhardt, 2005; Hughes and Appel, 2020). During neural development, microglia play an important role in phagocytosis of neural stem cells, myelin modification, and synaptic pruning (Nayak et al., 2014; Hughes and Appel, 2020). Various neurotrophic factors such as brain derived neurotrophic factor (BDNF) and insulin-like growth factor-1 (IGF-1) are released by microglia and have been shown to play an important role in neural development and the formation of neural circuits (Ness and Wood, 2002; Parkhurst et al., 2013). Into adulthood, microglia continue to assist with synaptic plasticity, neurogenesis (especially in the hippocampus), as well as neural circuit maintenance and function (Schafer et al., 2012; Diaz-Aparicio et al., 2020).

Microglia have a diverse range of activation states from pro-inflammatory phenotypes, to anti-inflammatory phenotypes (Saitgareeva et al., 2020). Sometimes broadly categorized as ‘M1’ and ‘M2’, respectively, these are often seen as extremes on each end of the microglia polarization spectrum (Subhramanyam et al., 2019). Indeed, the response of microglia to neurotoxic insults is quite diverse, often with multiple activation

states being active in a localized populations of cells (Mills et al., 2000). These heterogenous group of cells work in combination to induce the most optimal response to any given neurological insult (Lobo-Silva et al., 2016). Microglial activation can be triggered by a multitude of environmental factors (e.g. pathogens and toxicants), neurogenic factors (e.g. trauma, injury) and systemic factors (e.g. viral or bacterial threats) (Griffiths et al., 2009; Cherry et al., 2014; Nayak et al., 2014). It has also been shown that age, prolonged periods of stress, and genetics can all play a role in activation of microglia (Villegas-Llerena et al., 2016; Wang et al., 2018). Consequently, microglia dysfunction has been implicated in a wide range of developmental disorders, neuropsychiatric disorders, and neurodegenerative disorders (Lull and Block, 2010; Subhramanyam et al., 2019; Kwon and Koh, 2020).

In recent years, researchers have looked towards the possible modulation of microglial activity as a therapeutic pathway for Parkinson's disease (PD), Alzheimer's disease (AD), and other neurological disorders (Wilms et al., 2007; Subramaniam and Federoff, 2017; Ahmad et al., 2019). Through suppression of inflammatory -inducing states, and enhancing of anti-inflammatory states, it is believed that there may be valid therapeutic approaches that could protect neuron functionality.

In addition to neuroinflammatory microglial responses, PD, AD and in fact virtually all neurodegenerative disease also have fundamental deficits in various aspects of neuroplasticity (Blasko et al., 2004; Wilms et al., 2007; Tang and Le, 2016). Among these, reductions in BDNF might be directly contributing neuronal vulnerability and synaptic loss that gives rise to motor and cognitive deficits (Garzon et al., 2002; Sakata et al., 2009; Petzold et al., 2015). Although clinical pathology is closely related to BDNF deficits at the

level of the neuron, accumulating evidence suggests that microglia might also be important both as a source of BDNF and their ability to respond to the tropic factor (Coull et al., 2005; Gomes et al., 2013). Whatever the case, BDNF is a known to be a widely expressed neurotrophic factor that is beneficial for neuron health, regulating differentiation, survival, and synaptic plasticity (Ghosh et al., 1994; Kowiański et al., 2017).

BDNF normally binds to the receptor tropomyosin receptor kinase B (TrkB) which is commonly found on neurons (Brigadski and Leßmann, 2020), however there is also evidence that TrkB expression occurs on microglia (Gomes et al., 2013; Prowse and Hayley, 2021). In fact, recent research has found that disruption of the TrkB pathway in microglia increases their activation and production of pro-inflammatory cytokines (Wu et al., 2020). However earlier works oppose these findings (Frisén et al., 1993), and with little overall supporting data, there is still a great deal of research needed to determine the role of BDNF in microglia. Hence, as will be discussed later, a primary focus of the present thesis is the direct impact of BDNF on isolated microglia (both unstimulated and in the context of an inflammatory challenge).

1.2. LPS and BDNF

Microglia do appear to be able to pre- and post-synaptically release BDNF, albeit at relatively low levels, in association with events such as learning and memory tasks (Coull et al., 2005; Lu et al., 2014; Kowiański et al., 2017; Brigadski and Leßmann, 2020). Following its release, BDNF binds the TrkB receptor, triggering a downstream effect that induces synaptic plasticity and synaptic formation. However, there are several isoforms

including, pro-BDNF and pre-pro-BDNF which are two precursor forms of the protein (Matsumoto et al., 2008; Foltran and Diaz, 2016). As newly formed pre-pro-BDNF travels from the endoplasmic reticulum to the Golgi apparatus, the pre-region is cleaved, leaving pro-BDNF to be packaged into intracellular vesicles and transported to the axon terminals, which can result in both pro-BDNF and mature BDNF (mBDNF) being released at the synapse (Kowiański et al., 2017). The ratio of pro-BDNF to mBDNF varies depending on the stage of development, with pro-BDNF being more abundant in early postnatal periods and mBDNF being more abundant in adulthood (Yang et al., 2014). While mBDNF preferentially binds to TrkB, the pro-BDNF form typically binds to a complex made of the p75 neurotrophin receptor (p75NTR) and sortilin (Nykjaer et al., 2004). With a greater affinity towards p75NTR, pro-BDNF induction leads to the activation of c-Jun N-terminal (JNK), Ras homolog gene family member A (RhoA), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathways (Palasz et al., 2020). These pathways generally trigger apoptosis, neuronal growth cone development and neuronal survival, respectively (Kowiański et al., 2017).

mBDNF-TrkB pathways induce many intracellular signalling cascades that are important in synaptic plasticity and long-term potentiation (LTP) (Opazo et al., 2003; Itoh et al., 2016), dendritic spine growth (Gorski et al., 2003; Orefice et al., 2013), and anti-apoptotic processes (Petersén et al., 2001). Emerging evidence has posited that modulation of BDNF and the BDNF-TrkB signalling pathway may play a role in the prevention and modulation of age-associated neurodegenerative disorders, epilepsy, cancers and even perception of pain (Cappoli et al., 2020; Colucci-D'amato et al., 2020; Lin et al., 2020). With BDNF being hypothesized and a therapeutic agent, recent research has been focused

on increasing endogenous release of BDNF (Palasz et al., 2020; Xia et al., 2021; Jin et al., 2022) to ameliorate both neurological and peripheral problems alike.

In microglia, there is competing evidence as to whether BDNF is primarily upregulated or downregulated during inflammatory activation states (Prowse and Hayley, 2021). A majority of studies done using classical activators of microglia, such as LPS, have shown an overall decrease in expression of BDNF, but a few have indicated an upregulation (Gomes et al., 2013; Parkhurst et al., 2013; Hu et al., 2020). This is believed to be due to the relative complexity of microglia and the downstream transcription factors that are involved (Colton, 2009). Lipopolysaccharide (LPS), a major outer surface membrane component of gram-negative bacteria, is the main PAMP that binds to TLR4 found on microglia (Ciesielska et al., 2021). TLR4 is a PRR also expressed on macrophages, monocytes and dendritic cells, where it induces adaptive immune responses, including the secretion of pro-inflammatory cytokines (Lim and Staudt, 2013).

LPS induces activation of TLR4 through the LPS-binding site that consists of LPS-binding protein (LBP) and glycerophosphatidylinostil (GPI)-anchored protein CD14 (Ciesielska et al., 2021). Forming a complex with myeloid differentiation factor 2 (MD-2), LBP and CD14 (Kim and Kim, 2017), LPS activates many downstream targets including the myeloid differentiation primary response 88 protein (MyD88)-dependent pathway (Akira et al., 2006; Rodríguez-Gómez et al., 2020). Recruitment of MyD88 creates a signalling cascade facilitating NF-κB meditated inflammatory signalling (Sughra et al., 2010; Zhang et al., 2022). Through this pathway, it is common to see a downregulation of BDNF (Koss et al., 2019; Chang et al., 2020). The MyD88-dependent pathway is also

important in the recruitment of anti-inflammatory factors, such as IL-10, to aid in the termination of inflammation (Chanteux et al., 2007).

Another ‘classic’ activator of microglia, adenosine triphosphate (ATP), can provoke an upregulation of BDNF, and this is believed to occur through P2X4 purinoceptors (P2X4R) and adenosine 2A receptors ($A_{2A}R$) (Trang et al., 2009; Gomes et al., 2013). Indeed, cyclic AMP-response element-binding protein (CREB) is a downstream target for both P2X4Rs and $A_{2A}Rs$ and has also been shown to be the main transcription factor for the production of BDNF (Wen et al., 2010; Esvald et al., 2020). Through activation of the $A_{2A}R$ via adenosine (Gomes et al., 2013), LPS priming of microglia can stimulate BDNF expression (Elkabes et al., 1998; Chang et al., 2020). Similarly, P2X4R activates the phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR) cascade, which ultimately phosphorylates CREB to induce BDNF expression (Kowiański et al., 2017). P2X4R is also important in the soluble N-ethylmaleimide-sensitive factor attachment protein (SNAP) receptor (SNARE)-dependent release of BDNF at the synapse (Trang et al., 2009). Taken together, it seems that a balance between activation of multiple receptor types and pathways can modulate the homeostatic balance in BDNF expression in microglia.

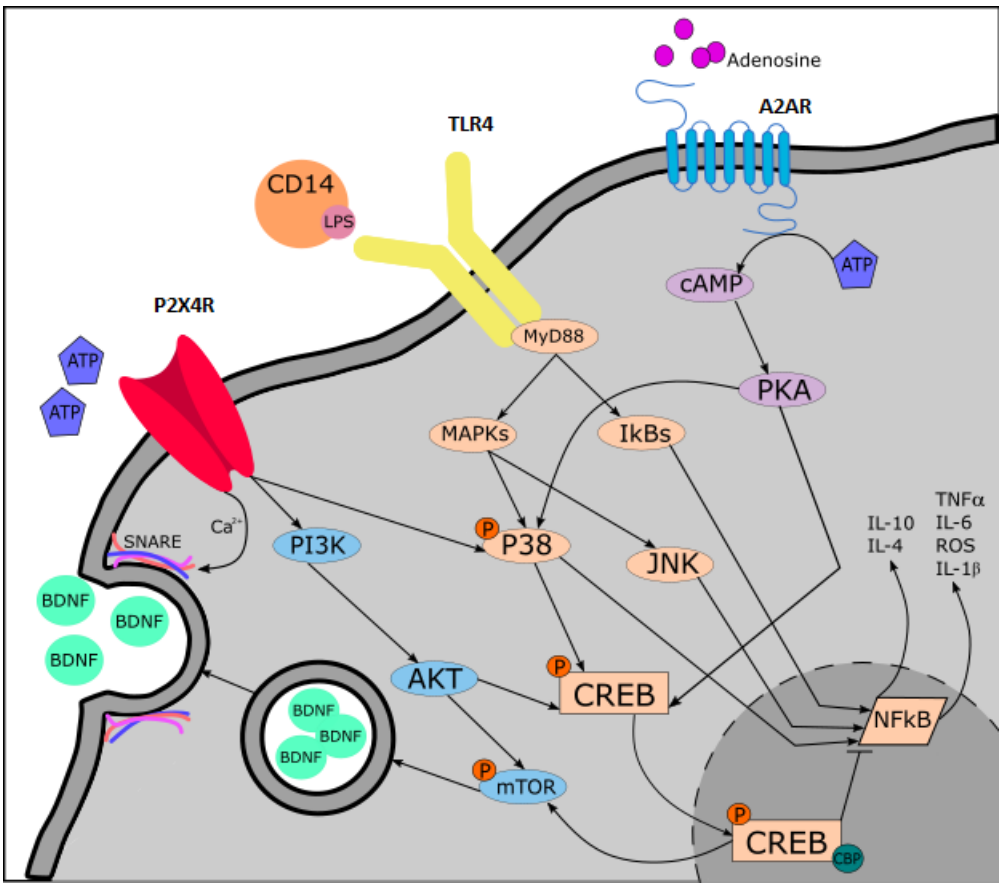

Figure 1. Activation Pathways that may regulate BDNF and Inflammatory Cytokines

Microglial signalling pathways for purinergic signalling as well as LPS mediated inflammation. LPS is able to bind to TLR4 with assistance from CD14 and through the MyD88 pathway, is able to induce activation of both CREB and NK-κB through MAPK and IκBs depending on intended action on the microglia. P2X4R, activated by ATP is able to assist in the recruitment of BDNF packaged vesicles to the cell membrane through PI3K/AKT/mTOR pathway as well as P38 to interact with phosphorylated CREB translocation and inhibition of NF-κB. Through adenosine $A_{2A}R$ is capable of inducing translocation of CREB as well through cAMP/PKA/CREB pathway. Figure modified from Prowse and Hayley, 2021.

1.3. Microglia Polarization and Cytokines

Microglial are phenotypically diverse, capable of producing both pro-inflammatory and anti-inflammatory factors (Soehnlein and Lindbom, 2010). Polarization of microglia is characterized by the presentation of certain cytokines, receptors, and other cell-surface markers. Classically activated microglia, which can be induced with LPS and other cytotoxic events is associated with the production of pro-inflammatory factors, such as TNF-α, IL-1β, IL-6, tumour necrosis factor-α (TNF-α), and ROS (Lund et al., 2006; Subhramanyam et al., 2019). In contrast, the alternatively active phenotype that is primarily associated tissue repair and resolution of the inflammatory response is associated with anti-inflammatory cytokines, such as IL-4, IL-13, IL-10, and transforming growth factor-β (TGF-β) and can also induce a downregulation of ROS (Wang et al., 2015). Upon encountering an activating stimulus, microglia can undergo a rapid and dramatic change from a resting ramified state to various intermediates morphologies, with the most extreme being the adoption of an amoeboid morphology (Orihuela et al., 2016). Usually, for acute or modest stressors, there is a balance of active microglia to ensure homeostasis (Cherry et al., 2014). This balance between pro-inflammatory and anti-inflammatory states is key for limiting cell death and preventing irreparable damage to important brain regions (Aloisi, 2001; Block et al., 2007). This is usually accomplished by a two-step process, wherein the pro-inflammatory microglia initially remove any microbial or other threats, followed by a shift to an anti-inflammatory phenotype, resulting in further debris clearance, tissue repair, and extracellular matrix reconstruction (Cherry et al., 2014). The dynamic action of microglia is crucial for a healthy immune response. Commonly seen in neurodegenerative disorders and autoimmune disorders, an unbalanced microglia response results in

uncontrolled production of pro-inflammatory cytokines and ROS (Wang et al., 2015; Chu et al., 2018). During severe environmental stress or long-term stressors, microglia can adopt a hyperactive state and induce chronic inflammation that causes damage to surrounding healthy tissue (Minghetti et al., 2005; Hayley et al., 2021). This is thought to occur in many cases of PD and AD, where high levels of inflammatory markers induce a cycle of chronic inflammation with slow but constant degradation of the brain (Zhang et al., 2005; Meyer-Luehmann et al., 2008).

Activation of NF-κB in times of stress has been found to target genes in the nucleus responsible for the transcriptional action of pro-inflammatory cytokines such as IL-1β, IL-6, TNFα, as well as the secretion of ROS (Chang et al., 2020). As an important transcription regulator, it is categorized as a rapid-acting transcription factor due to the fact that no protein synthesis is required for activation of NF-κB, only needing initiation by specific membrane bound proteins and receptors (Lien et al., 2000; Ainbinder et al., 2002). The NF-κB family of proteins are important modulatory proteins that integrate signalling from TNF receptors, TLRs, and IL receptors (Escoubet-Lozach et al., 2011; Rodríguez-Gómez et al., 2020). NF-κB essential modulator (NEMO)-dependent pathways, such as that seen in LPS-induced inflammation, are important for the induction of TNF-α gene transcription and the release of TNF-α (Chung and Benveniste, 1990). In the presence of LPS, NF-κB is mediated via the MyD88-dependent pathway, wherein the phosphorylation of MAPKs IκB and IKKγ induces NF-κB mediated expression of pro-inflammatory molecule associated genes (Kaisho and Akira, 2006; Ciesielska et al., 2021).

Other receptors and membrane bound proteins that may also be involved in the promotion of the pro-inflammatory phenotype include macrophage antigen complex

(MAC1) and NAPDH oxidase. The MAC1 (also known as integrin CD11b/CD18, CR3 heterodimer) is a key adhesion molecule and important in the recognition of several different PAMPs and damage associated molecular patterns (DAMPs) that can induce phagocytosis (Ross and Vetvicka, 1993; Le Cabec et al., 2002) and mediate migration and adhesion of leukocytes in the inflammatory response (Akiyama and McGeer, 1990; Griffiths et al., 2009). While it is not fully understood whether CD11b is expressed more readily on activated microglia, its expression is increased on most microglia in individuals with inflammatory disorders (Walker and Lue, 2015). The activation of NADPH oxidase through MAC1 may play a crucial role in the production of ROS in the microglia (Qin et al., 2004; Block et al., 2007). Finally, p38 is important in microglial proliferation, apoptosis, and differentiation, as well as in the activation of ROS (Trang et al., 2009). Interestingly, it has also been shown p38 can induce production of BDNF under certain situations (Trang et al., 2012).

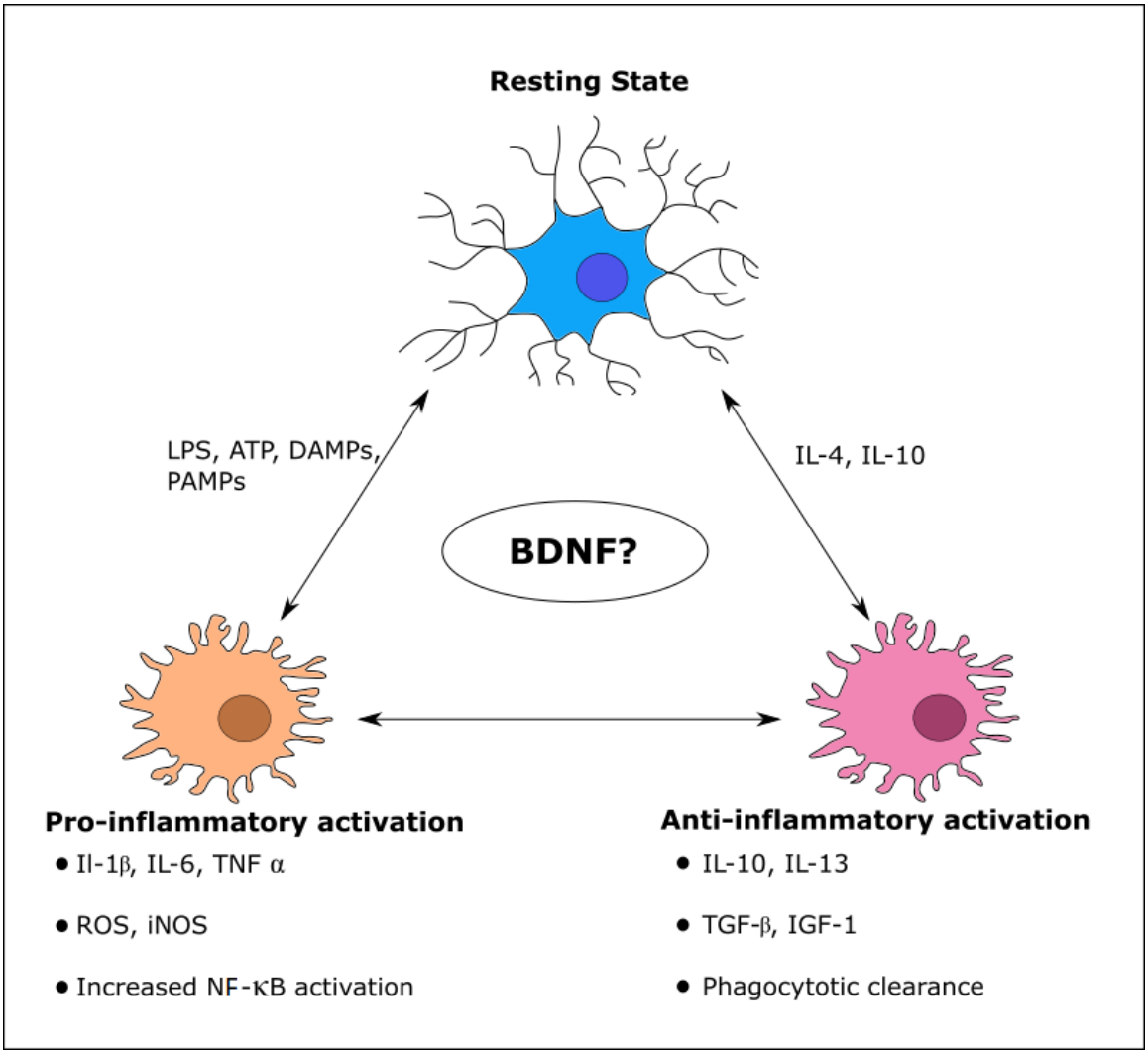

Figure 2. Microglia Polarization

In normal physiological states microglia are in a ramified resting state, surveying the surrounding area for threats. When pro-inflammatory threats are present such as LPS, ATP, PAMPs, or DAMPs microglia take on an ameboid phenotype and begin to produce pro-inflammatory cytokines, oxidate stressors, as well as increased activity on NF-κB pathways associates with these releasable pro-inflammatory factors. Anti-inflammatory microglia are capable of interacting with other communication molecules such as IL-4 or IL-10 to induce production of trophic factors, and other cytokines important for the proliferation of inducing a neuroprotective state. It is still not well understood how BDNF interacts directly with microglia or how it may affect the polarization states of microglia.

1.4. Neuroinflammatory Processes in Neurodegenerative Disorders

Overactivation of microglial driven neuroinflammatory has been well documented in neurodegenerative disorders like AD, PD, Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS) (Zhang et al., 2005; Block et al., 2007; Subhramanyam et al., 2019). Correspondingly, it has been shown that β-amyloid (Aβ) and α-synuclein (α-syn) can induce classical microglial activation, much like that of LPS (Ii et al., 1996; Austin et al., 2006), which if prolonged, can result in the destruction of neurons (Cherry et al., 2014; Tang and Le, 2016). In such neurodegenerative diseases, the overactivation of pro-inflammatory phenotypes creates a cascading effect of chronic inflammation, that may essentially overwhelm the ability of microglial to mount an anti-inflammatory response (with reduced levels of neurotrophins and anti-inflammatory cytokines) that facilitates recovery and tissue repair (Cherry et al., 2014).

In the case of PD, the hallmark pathology is a loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) (Hirsch et al., 2016) coupled with the aggregation of α-syn and formation of Lewy bodies (Braak et al., 2003; Glass et al., 2010). Additionally, there is increasing evidence that shows microglia may contribute to the pathogenesis of PD (Wilms et al., 2007; Subhramanyam et al., 2019). In fact, there are several PD related genes that also regulate microglia function, including LRRK2, Parkin, and Phosphatase and tensin homolog (PTEN)-induced kinase 1 (PINK1) (Gao and Hong, 2011). Certain mutations in these genes is directly associated with early onset familial forms of PD (Tomiyama et al., 2015). Even under normal resting conditions these genes are crucial for phagocytosis, production of ROS, and surveillance (Dwyer et al., 2020b). However, even more importantly, polymorphisms in some of these genes, notably LRRK2 and PINK1,

may convey vulnerability to environmental toxins and microbes and hence, might be aligned with the more commonly occurred idiopathic form of PD that develops later in life (Wallings and Tansey, 2019; Quinn et al., 2020).

The SNpc is particularly vulnerable to environmental toxins and some have speculated that this could be at least in part, related to the particularly robust microglial response in this brain region (Fiorenzano et al., 2021). Also, the resident SNpc dopamine neurons are constantly under very high basal metabolism and generate substantial oxidative stress by products (Brichta et al., 2014). During times of stress, these sensitive neurons containing high levels of dopamine, breakdown and introduce high levels of ROS and other redox active molecules into the system which can interact with microglia (Zigmond et al., 2002), which can adopt inflammatory states that may in turn, damage neurons (Zhang et al., 2005).

AD is the leading cause of dementia in the elderly population and has been long understood to have a neuroimmune component (Rogers et al., 1988). With neural damage beginning in the cortex and slowly moving inwards to regions such as the hippocampus and amygdala, overtime this results in impaired memory, loss of emotional control, and overall cognitive decline. One of the main proteins implicated in this pathology is Aβ which is a pro-inflammatory factor that activates microglia to produce factors such as NO, TNFα, and ROS (Ii et al., 1996; Veerhuis et al., 1999). Interestingly, Aβ is also directly toxic to neurons, which further induces recruitment of active microglia and proinflammatory factors (Ii et al., 1996; Amidfar et al., 2020). These changes promote the induction of more microglia to the site in order to phagocytize accumulating Aβ and amyloid plaques (Meyer-Luehmann et al., 2008). However, it is thought that over time (due to aging and ongoing

cytotoxicity) the ability of microglia to phagocytose and clear plaques diminishes, which leads to aggregation and neurotoxicity (Sheng et al., 1998; Blasko et al., 2004).

HD is a rare autosomal-dominant neurodegenerative disorder that has wide effects on an individual's movement, cognitive ability, and behaviour (Walker, 2007). Characterized by progressive degeneration of the striatum, and cortex in advanced stages, the disease is caused by the mutations in the protein huntingtin (HTT) (Ghosh and Tabrizi, 2018). Mutant HTT (mHTT) is translated to have a polyglutamine repeat on the N-terminus and high levels of mHTT mRNA are found to be present in microglia, promoting expression of many pro-inflammatory associated genes even in the absence of a pro-inflammatory stimuli (Crotti et al., 2014).

ALS is associated with general loss of motor neurons throughout the cortex, brainstem, and spinal cord (Hardiman et al., 2017). There are several ALS-associated genes (SOD1, C9orf72, TDP43) with varying effects in the progression of ALS, however a common factor with these genes is the broad effects on the ability of microglia to regulate the inflammatory response and prevent microglia-associated neurotoxicity (Turner et al., 2004; Lall and Baloh, 2017). It is now commonly accepted that ALS is strongly associated with high levels of pro-inflammatory markers and overactive-microglia present at sites of atrophy that are tied with accelerated onset of the disease (Yamanaka et al., 2008).

Taken together, it is clear that microglia and the inflammatory response play a key role in the progression of many neurodegenerative diseases. Interestingly however, dysregulation of trophic pathways also occurs in neurodegenerative diseases. With regards to PD, BDNF has been shown to be decreased in the nigrostriatal pathway and consequently, treatments that promote BDNF and TrkB expression may induce

neuroprotective effects (Palasz et al., 2020). In AD, Aβ has been shown to interfere with the CREB-BDNF pathway that induces BDNF gene translation in the nucleus of the neuron (Fahnestock, 2011). In HD, lower levels of BDNF were associated with mHTT expression in humans and in mouse models over-expression of BDNF has been shown to attenuate neuronal dysfunction (Ciammola et al., 2007; Giralt et al., 2011). Interestingly, hyper-activity of the BDNF/TrkB pathway has been seen in ALS and has been shown to enhance motor neuron susceptibility to excitotoxic insults (Kafitz et al., 1999; Tremblay et al., 2019). However, treatment avenues for ALS are still looking at modulation of the BDNF/TrkB pathway via A_{2a}Rs (Komaki et al., 2012).

1.5. Research Objectives

Much evidence indicates that BDNF can act directly on neurons to convey neuroprotective consequences. Yet, very little is known about how BDNF can impact microglial cells and how this in turn, might influence neurons. Hence, the present thesis sought to assess whether some of the beneficial effects of BDNF might be related to microglial activation state. Specifically, (a) can BDNF prime microglia to adopt a protective phenotype and (b) can BDNF offset the inflammatory consequences of an inflammatory challenge (LPS). Essentially, this thesis seeks to better understand the role of BDNF during the neuroimmune response as a possible therapeutic approach to reduced inflammation caused by overactive microglia. As well as further define the interactions between microglia and neurons in an attempt to better understand the neuroinflammatory role in neurodegeneration.

We hypothesize that: 1. LPS would induce microglia to produce a cytotoxic environment. 2. BDNF would induce microglia to promote anti-inflammatory factors. 3. Treating LPS-primed microglia with BDNF would diminish the traditional pro-inflammatory response.

2. Materials and methods

Unless otherwise specific, materials were sourced from Sigma-Aldrich.

2.1. Animals and Primary Culture

C57BL/6 mice were purchased from Charles River Laboratories and were used for *in vitro* culture. Animals were left on a normal 12-hour light cycle, given ab libitum access to water and lab chow. Animals were harem mated (2 females to 1 male) for 2-14 days for neuronal and microglial cultures. Cortical neuron tissue was collected from dams that were euthanized by rapid decapitation and 5 to 10 embryonic Day 16 (E16) pups retrieved (per litter). Microglial cultures were collected from 5 to 10 postnatal Day 1-3 (P1-3) pups (per litter).

Primary cultures were produced in 24 well plates on autoclaved, dH_2O rinsed, coverslips. Both E16 primary neurons and P1-3 microglia were seeded at a concentration of 1 $x10^5$ cells/ml. Each 6 well row on a 24 well plate was assigned either vehicle, BDNF, LPS or LPS and BDNF treatments.

Collection of cortical mouse neurons was adapted from Hilgenberg and Smith (2007) as well as Gaven and colleagues (2014), with primary neurons being isolated from the cortex, mildly trypsinized with trypLE and seeded on Poly-D-lysine (PDL) coated wells

at 10ug/ml in complete neurobasal media (CNB: 2% B-27 [Gibco], 0.5% penicillin/streptomycin, 0.25% GlutaMAX, 97.25% Neurobasal media). E16 cortical neurons were incubated for 7-10 days (DIV7-10) in a humidified environment (37ºC, 5% CO_2). New CNB, without GlutaMAX, was placed on the cells the day after surgery to remove debris and then every 2-3 days and on DIV7-10 cells were utilized for experiments (Hilgenberg and Smith, 2007; Gaven et al., 2014).

Collection of primary P1-3 microglia was adapted from Saura and colleagues (2003) as well as Schildge and colleagues (2013). In this case, microglia were collected from a mixed glial culture extracted from the cortex following, mildly trypsinized (trypsinization solution: 1:1:1 dilution ratio of DMEM, versine, and 0.25% trypsin), and seeded on PDL coated (10ug/ml) T75 flasks in complete media at 1.0 x 10^5 cells/ml with 2-3 cortices per flask. New complete media (CM: 10% fetal bovine serum [FBS], 1% penicillin/streptomycin, 89% high glucose DMEM) was placed on the mixed glia culture the day after surgery to remove debris. Half media changes were then conducted every 4-6 days. Microglia were isolated from the mixed glial culture between DIV25 and DIV30 to yield highest confluency of microglia. The microglia were then plated immediately into culture at 1.0 x10^5 cells/ml for experimental use (Saura et al., 2003; Schildge et al., 2013).

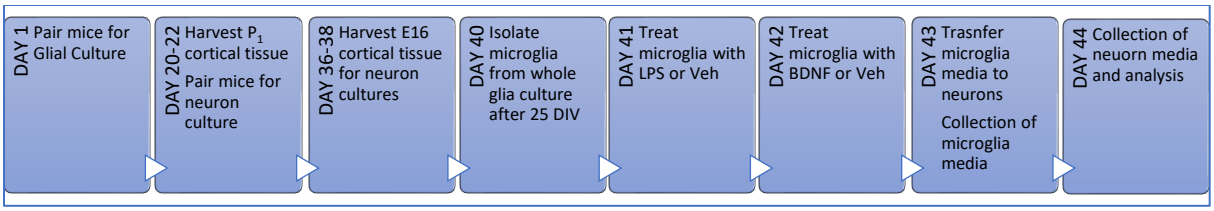

Figure 3. Primary Culture Timeline

WT littermates were harmed mated for up to one week to ensure pregnancy and then cortical tissue was extracted from the pups on day of birth (P1) for whole glial cultures; WT littermates for neuron cultures were harem mated on the same day for 2 days to ensure the day of pregnancy is known; on the 16th day of pregnancy (E16) embryos are collected and neuronal cultures are collected; after 25 days of proliferation (25 DIV) microglia are isolated from whole glial culture; the following day microglia are treated with LPS or Veh for 24 hours; followed by either BDNF or Veh treatment for 24 hours; media is transferred to neurons cultures for 24 hours and then all media is collected.

2.2. Treatments

Neurons and microglia were left to culture separately in 24 well plates for the duration of the experiment. Microglia cultures were treated for 24 hours with either (1) vehicle (PBS), (2) LPS at 100ng/ml, (3) BDNF at 50ng/ml, or (4) a pre-treatment of LPS (100 ng/ml) for 24 hours with a secondary treatment of BDNF (50ng/ml) for an additional 24 hours. Separately, neurons were treated with transplanted media from primed microglia cultures resulting in 4 distinct treatment groups that correspond to the treatments for microglia cultures. Neurons were treated for 24 hours with media from (1) vehicle treated microglia, (2) microglia media primed with LPS at 100ng/ml, (3) microglia media primed with BDNF at 50ng/ml, (4) microglia pre-treated with LPS (100 ng/ml) for 24 hours, with a secondary treatment of BDNF (50ng/ml) for a subsequent 24 hours.

2.3. Lactate dehydrogenase (LDH) Assay

Cell death in microglia was quantified using the LDH assay (Thermo-Fisher). To measure LDH levels, Microglia were seeded in triplicate on a 96 well plate at $5x10^4$ cells/ml. After treatments, 50µl of media was collected from each group and added to a 96-well plate, followed by 50µl of substrate media and incubated for 30min at room temperature in the dark. At the end of incubation, stop-buffer was added, and the plate was read at 490nm and 680nm. Percentage of cytotoxicity was calculated by subtracting background absorbance (680nm) from the absorbance of the medium from cell treatment (490nm) and dividing by the maximum LDH release which accounts for spontaneous release of LDH from untreated microglia. Maximum LDH release was obtained by adding lysis buffer (1:10) to treatment groups 45 min prior to end of experiment.

2.4. Measurement of Cytokines by ProQuantum Immunoassay kits

Levels of TNFα, IL-6 and IL-4 in the media after treatment were measured using ProQuantum immunoassay kits (Thermo-Fischer). Media was collected from each experimental group and control. Levels of TNFα, IL-6 and IL-4 were measured according to manufacturer's instruction.

2.5. Statistical Analysis

All data was analyzed by cell treatment ((Vehicle vs LPS) vs (Vehicle vs BDNF)) in a two-way ANOVA with significant interactions further analyzed by means of Fisher's

LSD planned follow up comparisons ($p < 0.05$) where appropriate. All data was analyzed using the statistical software Prism (version 9), all data is presented in the form of marginal mean ± standard error of the mean (mean ± SEM), and differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. BDNF Reverses LPS Induced Microglial and Neuronal IL-6 Response

IL-6 levels were analyzed using a two-way ANOVA to explore the differences across treatment groups in cultured primary microglia and neurons. The analysis revealed a significant LPS x BDNF interaction for IL-6 levels in primary microglia ($F(1,8) = 112.3$, $p < 0.01$). Indeed, microglial levels of IL-6 were remarkably increased in the LPS treated cells, relative to controls or those that received BDNF ($p < 0.05$). A significant LPS x BDNF interaction was also evident for IL-6 levels within primary neurons ($F(1,8) = 26.48$, $p < 0.01$). Paralleling the microglia, the neuronal levels of IL-6 were significantly elevated in the cells that were cultured with LPS in the absence of BDNF, relative to the remaining groups ($p < 0.05$; Fig. 4).

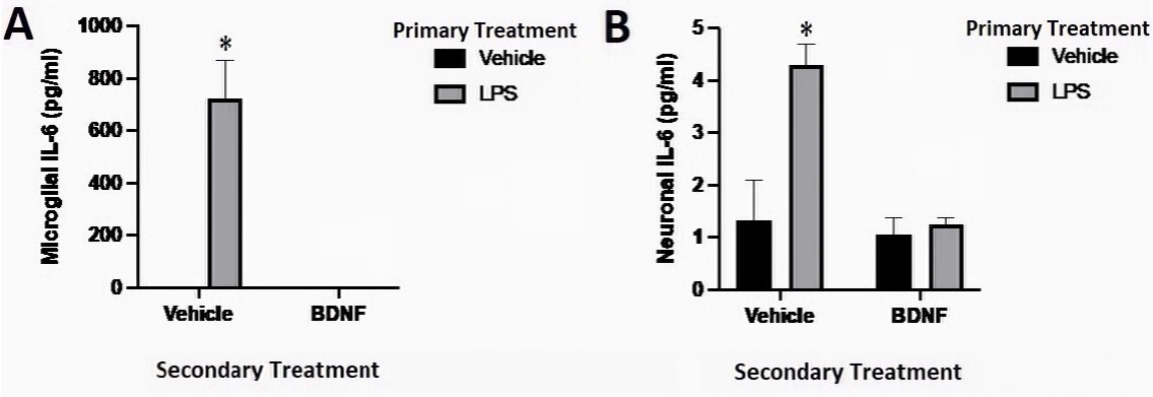

Figure 4. IL-6 Concentration in Microglia and Neurons

A. Levels of IL-6 in microglia media treated with LPS,Veh was found the be significantly increased when compared to Veh,Veh treatment group. IL-6 levels in microglia media treated with LPS,BDNF was found to be significantly decreased when compared the LPS,Veh treatment group. **B.** Levels of IL-6 in neuron media treated with LPS,Veh-activate microglia media, was found to be significantly increased when compared to neurons treated with media from Veh,Veh microglia. IL-6 levels in neurons treated with microglia media from LPS,BDNF treatment group were shown to be significantly lower compared to neurons treated with media from the LPS,Veh group.

3.2. BDNF Reverses LPS induced Microglial response in TNFα

The interaction between the LPS and BDNF treatments just missed statistical significance for TNF-α levels within primary microglia ($F(1,8) = 4.13$, $p = 0.07$). Given our *a priori* hypothesis and the fact that LPS treatment alone did provoke an obvious TNF-αelevation in microglia (albeit variable), we conducted post hoc comparisons. This revealed that LPS treatment increased microglia culture levels of TNF-α compared to vehicle treatment ($p < 0.05$; Fig. 5) and once again, this was reversed by BDNF treatment. In the case of the cultured neurons, there was no significant differences in TNF-α levels between the groups.

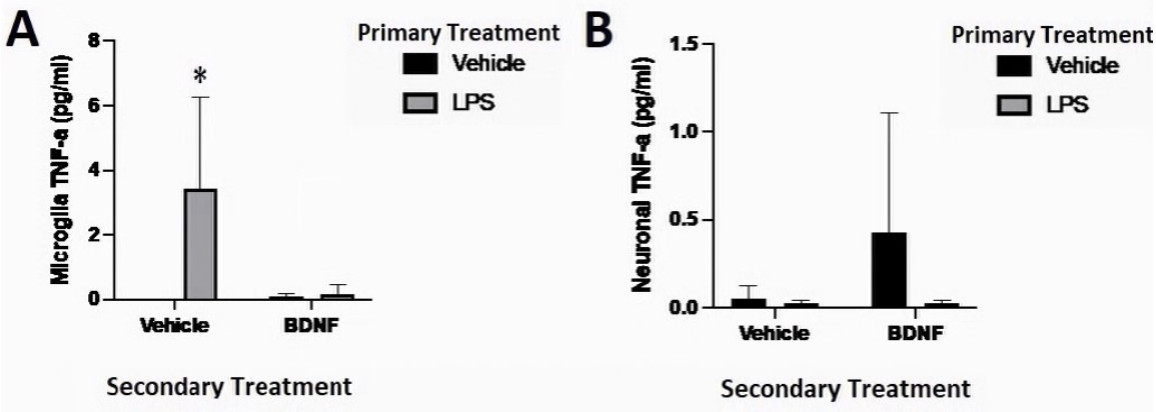

Figure 5. TNFα Concentration in Microglia and Neurons

A. Levels of TNFα in microglia media treated with LPS,Veh was found the be significantly increased when compared to Veh,Veh treatment group. TNFα levels in microglia media treated with BDNF and LPS was found to be significantly decreased when compared the LPS,Veh treatment group. **B.** There were no significant differences in TNFα levels across treatment groups in neurons.

3.3. LPS Alone Influences Neuronal Levels of IL-4

The ANOVAs failed to reveal any significant differences between the groups for IL-4 levels within the microglial cultures. Similarly, there was no significant interaction effect or main effect for BDNF treatment with regards to neuronal IL-4 levels. However, there was a significant main effect for LPS treatment in the neuronal cultures ($F(1,8) = 6.26$, $p < 0.05$). Indeed, treatment of the neuronal cultures with LPS elevated neuronal IL-4 levels above that of vehicle exposure ($p < 0.05$) and this occurred irrespective of BDNF treatment (Fig. 6).

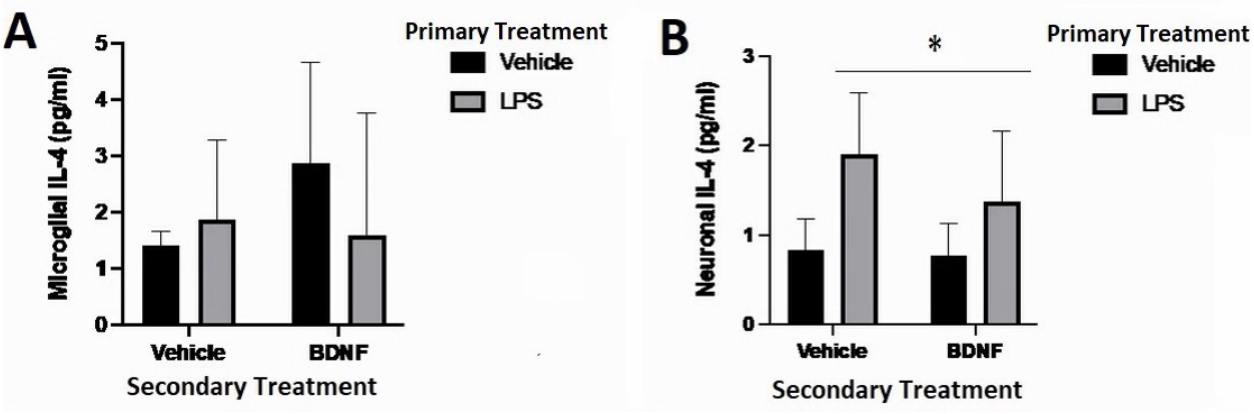

Figure 6. IL-4 Concentration in Microglia and Neurons

A. There were no significant differences in IL-4 levels across treatment groups in microglia. **B.** However, IL-4 concentration was significantly increased in neurons treated with LPS-activated microglia media, regardless of a secondary treatment of BDNF or Vehicle on the microglia. Levels of IL-4 in neurons treated with LPS,Veh microglia media were increased when compared to neurons treated with Veh,Veh microglia media. While levels of IL-4 in neurons treated with LPS,BDNF microglia media were increased when compared to neurons treated with Veh,BDNF.

3.4. Multi-hit Model Assists in Cell Viability of Activated Microglia

Analysis of LDH levels in microglia was used to measure cell viability and cell cytotoxicity. Analysis revealed that there was a significant LPS x BDNF interaction for LDH levels in the microglial culture ($F_{1,8}$=91.35, $p<0.0001$). Further analysis shows that LPS significantly increased LDH release from microglia compared to control microglia media ($p < 0.05$). Curiously, LPS and BDNF treatment together greatly reduced LDH release, to an extent below controls and the remaining groups ($p < 0.05$; Fig. 7).

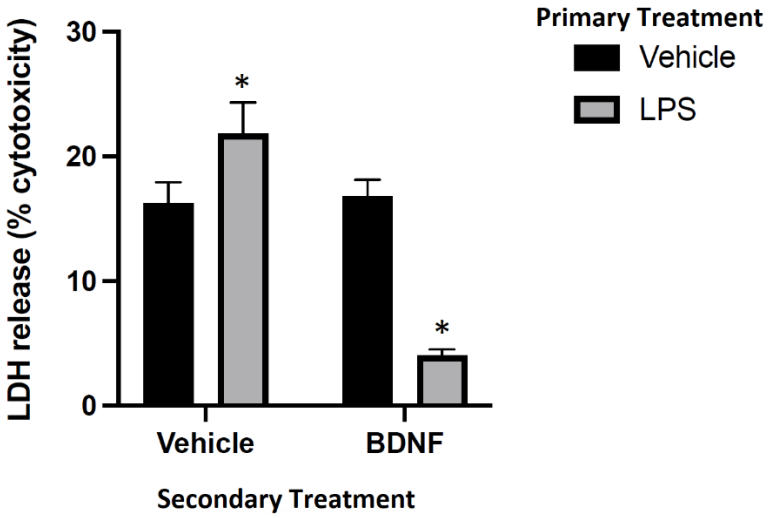

Figure 7. LDH Release in Microglia Media as an index of Cytotoxicity

The release of LDH in microglia that was treated with LPS,Veh was significantly increased when compared to the Veh,Veh treatment group. LDH release in microglia treated with BDNF and LPS was found to be significantly decreased when compared both the LPS,Veh and the Veh,BDNF treatment group.

4. Discussion

The microglial driven inflammatory immune response has been implicated in various neurological disorders and conversely, the trophic factor, BDNF, is known to have neuroprotective action in these conditions (Lu et al., 2013; Palasz et al., 2020). Numerous reports demonstrate that BDNF provides trophic support by binding to its TrkB receptor on neurons (Lu et al., 2015; Xu et al., 2017; Jin, 2020). This facilitates neurons survival, as well as promote a host of neuroplastic actions, including synaptogenesis and dendritic remodelling (Itoh et al., 2016; Kowiański et al., 2017). Yet, very little is known regarding the impact of BDNF upon the primary immunocompetent cells of the brain; namely, microglia. To this end, we posited that BDNF can induce a shift in the microglial functioning, towards a protective and to anti-inflammatory phenotype. Accordingly, this

study evaluated the impact of exogenously applied BDNF on the LPS induced inflammatory response in isolated microglia and neurons.

We found that LPS induced the expected robust inflammatory response in microglia and most importantly, BDNF treatment ameliorated this effect. Indeed, exposure of microglia to BDNF reversed the LPS induced elevations of extracellular released TNF-α and IL-6. A similar effect was observed in neurons, wherein an inflammatory response was triggered by exposure to media from LPS activated microglia. This it was thought, might provide some insight into microglia-neuron soluble factor interactions. In this case, the LPS-activated microglial media increased extracellular IL-6 levels in the cultured neurons and once again, BDNF completely reversed this inflammatory response. Interestingly, IL-4 levels (while not affected by BDNF) were increased in the neuronal media after exposure to media from LPS-activated microglia, suggesting the possibility that a protective anti-inflammatory neuronal response was elicited. IL-4 does in fact play a crucial role, when released by neurons, ensuring repair and debris clearance via glial cells (Zhao et al., 2015).

The ability of LPS to reliably induce comparable *in vivo* and *in vitro* inflammatory responses in humans and rodents makes it particularly useful for testing novel anti-inflammatory treatment approaches (Nava Catorce and Gevorkian, 2016; Brooks et al., 2020; Seemann et al., 2017)). Although LPS is an inflammatory insult in its own right, it also works well in 'double-hit' models, wherein low doses can prime immune cells for more vigorous responding in the context of another insult (La Vitola et al., 2021). Indeed, such double hit LPS-priming models have found that the secondary insult, such as a second LPS dose (Chae, 2018), or stressor (Espinosa-Oliva et al., 2011), or α-synuclein (La Vitola

et al., 2021), can trigger a hyper-immune state characterized by greater expression of inflammatory markers. Our present work suggests that using the trophic "protective" factor, BDNF, as a second hit following LPS produced a potentially protective reduction of inflammatory cytokine secretion.

Consistent with the present findings, Wu and colleagues (2020), showed that *in vitro* treatment of BV2 cells with BDNF attenuated the impact of LPS. Interestingly, they showed that BDNF as a pre-treatment to LPS, rather than post-treatment (as in the current study), produced the greatest reduction in pro-inflammatory cytokine production, while also increasing activation of the TrkB-CREB pathway. Phosphorylation of CREB was found to increase after BDNF treatment of microglia, whereas LPS-induced phosphorylation of NF-κB p65 was decreased.

It has been shown BDNF may also have anti-inflammatory effects following insults other than LPS. For instance, previous work by Jiang and colleagues (2011) found that that exogenous BDNF decreases the pro-inflammatory factor expression that was provoked by cerebral ischemia. Indeed, intranasal administration of BDNF in rats was shown to reduce mRNA expression pro-inflammatory factors, such as TNF-α but increased expression of anti-inflammatory IL-10 (Jiang et al., 2011). As well, BDNF treatment decreased pro-inflammatory factors and increased anti-inflammatory factors in the context of a bacterial infection (Xu et al., 2017). This suggests that the communication between microglia and neurons *in vivo* may be important for modulation of the innate inflammatory response. It is important to note however that *in vivo* administration allows for microglia to interact with neurons and other immune cells that can alter the microglial activation compared to an *in vitro* model.

While microglia and to a certain extent, astrocytes, are generally considered the main regulator of cytokines and other chemokines (Hanisch, 2002; Kwon and Koh, 2020), neurons also respond to and can release small concentrations of cytokines (Ringheim et al., 1995; Stow et al., 2009). Accordingly, we presently found that concentration of IL-6 in microglia media was 100 times higher than that of IL-6 in the neuronal media.

4.1. Neuron-Microglia Crosstalk

Communication between neurons and glial cells is essential for the maintenance of homeostasis. This involves both secretory communicating molecules, as well cell-to-cell contact involving membrane bound molecules (Pósfai et al., 2019; Fenner et al., 2021). Crosstalk between neurons and microglia is constant, even in healthy brains, microglia are always surveying for any potential threats (Kierdorf and Prinz, 2017). This is observed throughout development into adulthood, where the removal of apoptotic neurons and their programmed cell death is required for maintaining strong neural networks (Dekkers and Barde, 2013; Dekkers et al., 2013). In adulthood, microglia have an important role in the modulation of synaptic plasticity and can assist in cognitive and other functions (Tremblay et al., 2011; Ikegami et al., 2019). For instance, microglia were shown to modulate proliferation of neural progenitor cells and impact adult neurogenesis in the dentate gyrus (Xu et al., 2017; Wu et al., 2020), which is important for spatial memory and emotional responses to stressors (Vukovic et al., 2012; Gebara et al., 2013; Diaz-Aparicio et al., 2020).

BDNF might be an important messenger between neurons and glia; particularly, as a restorative factor during times of infection, injury or cellular stress. For instance, inflammatory-associated damage of neurons causes the release of ATP that acts as a DAMP signaling molecule, which in turn can induce the release of BDNF from microglia via P2XRs and P2YRs (Calovi et al., 2019); Fields and Burnstock, 2006). The release of trophic factors such as BDNF from microglia can then enhance neuronal plasticity to either aid in neuronal survival or compensate for any neuronal loss. In fact, microglial BDNF may be crucial for LTP and the regulation of inhibitory transmission in neuronal networks (Ferrini and De Koninck, 2013; Parkhurst et al., 2013; Kato et al., 2016).

Cell membrane integrity of microglia was measured by assessing extracellular levels of LDH present in the media. Any spillover from intracellular LDH indicates membrane rupture/leakage and likely consequent cellular death. LPS alone was found to reduce cell integrity by producing greater levels of LDH in media, whereas this effect was reversed by a secondary treatment with BDNF. In fact, LDH media levels were reduced below that of control levels in response to BDNF following previous LPS exposure. This suggests that BDNF may be modulating cell death mechanisms activated by LPS to such a degree that even the basal level of cell pathology that is associated with the culturing process itself was even inhibited. Yet, it should be noted that BDNF alone had no impact of LDH levels, indicating that the impact of the trophic factor was only evident in the context of an LPS provoked inflammatory environment.

We observed that IL-4 production was induced in neurons through some endogenous factor when treated with LPS-primed microglial media. Increased production of IL-4 has been seen in other models of neuronal stress, such as cerebral ischemia (Zhao

et al., 2015), with levels of IL-4 released from neurons after focal ischemia, with elevated concentration in neuronal culture media as long as 24-48 hours. Furthermore, IL-4 was able to increase phagocytosis in microglia, as well as increase expression of BDNF (Zhao et al., 2015). This suggests a protective model, where active neurons releasing IL-4 are able to induce recovery and/or debris clearance by microglia. The LPS-activated microglia media may be creating a low-level cytotoxic environment for the neurons in which IL-4 release is upregulated to counter cytotoxicity. Thus IL-4 might counter toxicity by its anti-inflammatory effects or by inducing protective growth factors, such as BDNF, GDNF or IGF-1 (Liu et al., 2016). In fact, microglia and neurons can produce such trophic factors in response to injury or LPS challenge (Suh et al., 2013). These growth factors not only aid in cellular survival (Ueno et al., 2013), but are also known to provoke the further release of IL-4 from adjacent cells (Granja et al., 2019), essentially creating a feedback loop such that IL-4 could amplify protective responses.

Pro-inflammatory cytokines serve not only as modulators of immune defences, but can also impact multiple aspects of synaptic transmission and plasticity (Costello et al., 2011; Santello et al., 2011). In fact, IL-6 plays an important role in memory and LTP in the hippocampus, where in this case, increased levels of the cytokine reduced learning (Ma and Zhu, 2000) and correspondingly, IL-6 knockout improved radial maze task performance (Braida et al., 2004). IL-6 is also upregulated in many neurodegenerative disorders, including PD, AD and MS (Maimone et al., 1997; Guzman-Martinez et al., 2019) and similarly, we presently found that IL-6 was greatly increased in LPS-stimulated microglia and neurons. It is understood however that IL-6 production is stimulated in neurons by pro-inflammatory cytokines IL-1β and TNFα as shown by Ringheim and

colleagues (1995), and later shown to be activated through multiple signalling pathways, including CREB (Tsakiri et al., 2008a, 2008b).

Finally, it is of interest to determine the mechanisms through BDNF might reduce the microglial release of cytokines observed in the present work. It seems likely that inhibition of microglia through BDNF is dependent in some way upon the TrkB-CREB pathway. Indeed, it was found that BDNF interfered with the inflammatory transcription factor, NFkB, while simultaneously increasing activity of the trophic transcription factor, CREB (Wu et al., 2020). Yet, exploring this is beyond the scope of the present thesis.

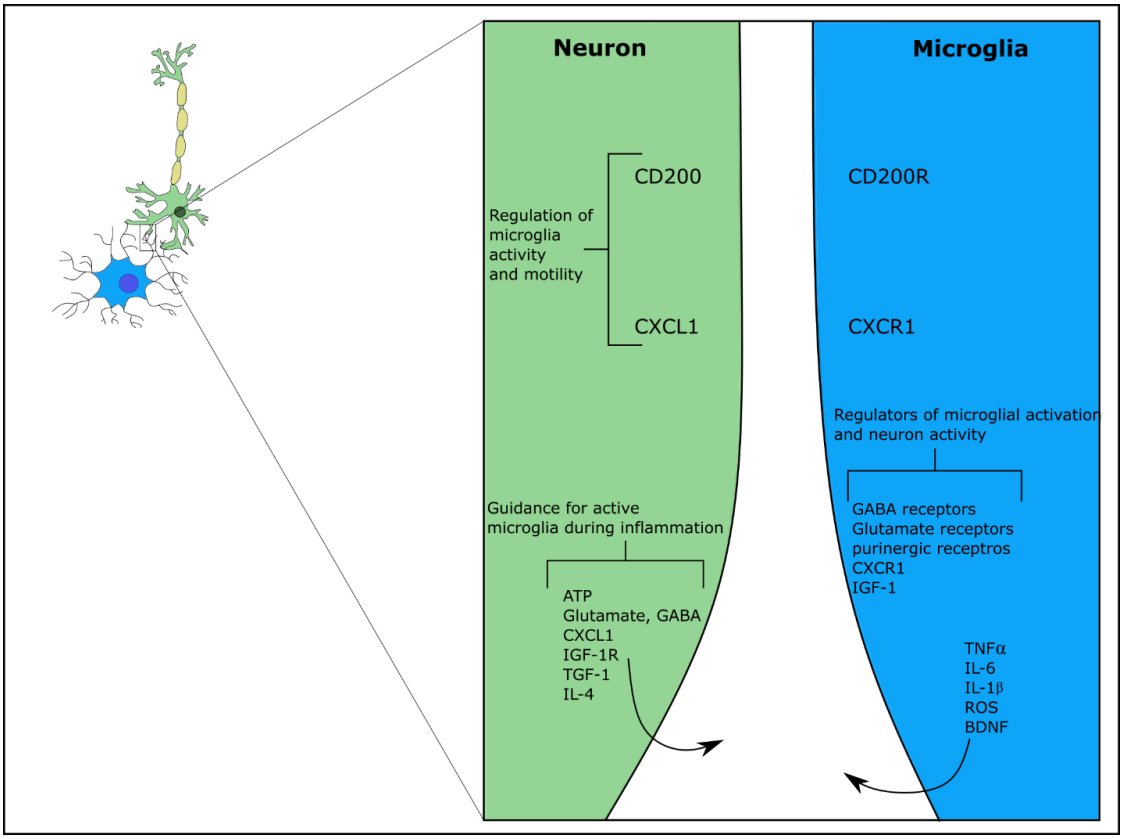

Figure 8. Bidirectional signaling between Microglia and Neurons

Neuron-microglia communication is regulated by a multitude of factors including releasable factors as well as contact-dependent factors. CX3CL1 (fractalkine) and CD200 both play crucial roles in neurons regulation over ramified microglia and activation of their receptors on microglia trigger signal specific pathways that can activate microglia to release many soluble factors important in neuroprotection. Neurons are also capable of communicating with neurons via glutamatergic and purinergic signalling which play a role in guidance of microglia during inflammation.

5. Future Work

The connection between microglia and neurons is essential for a robust inflammatory responses (Kierdorf and Prinz, 2017; Szepesi et al., 2018) with many *in vivo* studies, assessing these interactions (Jiang et al., 2011; Xu et al., 2017). Yet, very few *in vitro* studies have isolated these cell types in order to more directly assess their interactions. A follow-up study where co-culture between neurons and microglia to monitor how BDNF modulates inflammation would be novel in its own right. It is also important to determine the action of other chemokines such as fractalkine and CD200, while widening the scope of this study to look at other factors that control cytokine production and cell membrane integrity. To better understand TrkB-CREB activity, inhibition of CREB with 666-15 (Li et al., 2016) or TrkB with ANA-12 (Cazorla et al., 2011) could be used to determine the requirement of TrkB-CREB pathways.

To the best of our knowledge, few studies have assessed endogenous activation of microglial BDNF (Trang et al., 2012; Long et al., 2020). As far as we are aware, recent work by our study presents the particularly novel finding that media from LPS activated microglia can directly impact neuronal cytokine production. Further research into neuron-microglia communication and the role of BDNF in microglia may be important for the development of treatments targeting inflammatory aspects of neurodegenerative disorders.

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