

AMERICAN UNIVERSITY OF BEIRUT

CROSSTALK BETWEEN BRADYKININ AND
SPHINGOSINE-1-PHOSPHATE EXACERBATES
INFLAMMATION AND OXIDATIVE STRESS IN
MICROGLIA

by
NADINE SALAH HASSAN

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ABSTRACT OF THE THESIS OF

Nadine Salah Hassan

for

Master of Science

Major: Biochemistry

Title: Crosstalk between Bradykinin and Sphingosine-1-Phosphate Exacerbate Inflammation and Oxidative Stress in Microglia

Background: Neurodegenerative diseases (NDs) are a set of disorders that involve the death of neurons in the brain. These disorders are described as progressive and irreversible due to the brain's limited regenerative capacity and thus causing debilitating consequences to the lives of ND patients. The underlying pathogenesis of NDs is still elusive to this date. Therefore, there is no cure for NDs, and therapies are restricted to symptom relief treatments that can only slow down the progression of these disorders. Bradykinin (BK) is an active peptide in the kallikrein-kinin system found to be involved in mechanisms that are prevalent in both diabetes, inflammation, and stroke in diabetic patients that involves the microglia. Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid that is involved in inflammation through the modulation of microglia activation. However, there is limited knowledge on the involvement of BK and S1P in inflammatory pathways when studied in conjunction, given that both molecules activate G-protein coupled receptors.

Aims: The aim of this study was to explore the action of BK and S1P in brain resident macrophages known as microglia. We hypothesize that BK and S1P exacerbate inflammation and oxidative stress through crosstalk signaling cascades.

Methods: For the purpose of this study, immortalized murine microglial cells (N9) were stimulated with 0.1 μM BK and/or 1 μM S1P to obtain baseline data regarding inflammatory and oxidative stress markers. Inhibitors of Bradykinin-2-Receptor (B2R) and of Sphingosine Kinase (SK) were utilized to explore the possibility of crosstalk among BK and S1P. The gene expression of inflammatory mediators, oxidative stress markers, BK receptors, and S1P related markers were measured through qPCR to assess for changes in their mRNA levels.

Results: Our results show that inflammatory and oxidative stress markers were significantly elevated when N9 cells were stimulated with BK. Moreover, BK was able to increase mRNA levels of Sphingosine Kinases (SK1 and SK2) and S1P receptors, implicating a possibility of crosstalk between BK and S1P.

Conclusion: BK stimulated the expression of various inflammatory and oxidative markers in microglial cells. Although BK was also capable of inducing the expression of SK1 and SK2 and S1P receptors, further studies are needed to establish a mechanistic pathway through which BK is inducing this expression.

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CHAPTER I

INTRODUCTION

A. Neurodegenerative Diseases

1. Overview

When it comes to regeneration, most of the human body tissues are capable of this process which provides them with an enhanced healing ability. However, in the case of the nervous system, the regenerative capacity of neurons is scarce, leading to poor disease prognosis especially when dealing with neurodegenerative diseases. This limited ability of neuro-regeneration is attributed to the high complexity of neuronal structural and functional networks [1]. The term “neurodegenerative diseases” is a collective term that refers to a set of conditions that involve the death of neurons in the brain. These diseases are described as progressive and irreversible, leading to a loss of function either on the cognitive or motor levels. The typical characterization of neurodegenerative diseases (NDs) includes demyelination, loss of dendrites and neuronal death. Although the incidence of neurodegenerative disorders increases with age, usually mid-to-late adult life, the causes of neuronal degeneration are still elusive. However, common underlying pathophysiology of neurodegenerative diseases involve inflammation and oxidative stress [2, 3].

Brain disorders, including NDs, are responsible for approximately 25% of global death and disability [4]. Furthermore, because of the constant increase in life expectancy, NDs currently represent a global public health burden [5]. NDs are described as a broad spectrum of diseases that involve the loss of protein homeostasis and accumulation of misfolded and aggregated proteins [6]. Moreover, NDs are said to be “conformational diseases” due to the phenomenon where proteins lose their

physiochemical properties, leading to their deposition in the brain as aggregates [7]. NDs include but are not limited to: Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and Amyotrophic Lateral Sclerosis (ALS). Each disease has its own etiology and set of symptoms. However, as mentioned above, the loss of a set of certain neuronal populations is common to all these diseases [5]. The classification of NDs is based on the anatomical region of the brain that is affected and hence the set of symptoms that are expressed, the proteins that undergo conformational changes, altered biochemical properties, and the neurons or glial cells that are involved in the pathological process [7].

According to Tesco and Lomoio, who extensively discuss the pathophysiology of NDs, mention that axonal damage and hence the disruption of axonal transportation are responsible for NDs. They also elucidate that axonal transport can decline with age thus reinforcing that age is a significant risk factor. However, according to their review, there are other factors that are at interplay in the development of NDs including neuroinflammation, oxidative stress and mitochondrial dysfunction [5]. Aggregation of proteins with altered physiochemical properties might be the hallmark of NDs, but a distinction should be made between pathology and pathogenesis. As protein aggregation is at the core of pathology of NDs, the pathogenesis of these diseases is still elusive [8]. Despite the establishment of an intimate relation between microglia activation, oxidative stress and neuroinflammation in the brain, molecular classification and understanding of pathogenic pathways in NDs is still needed [7].

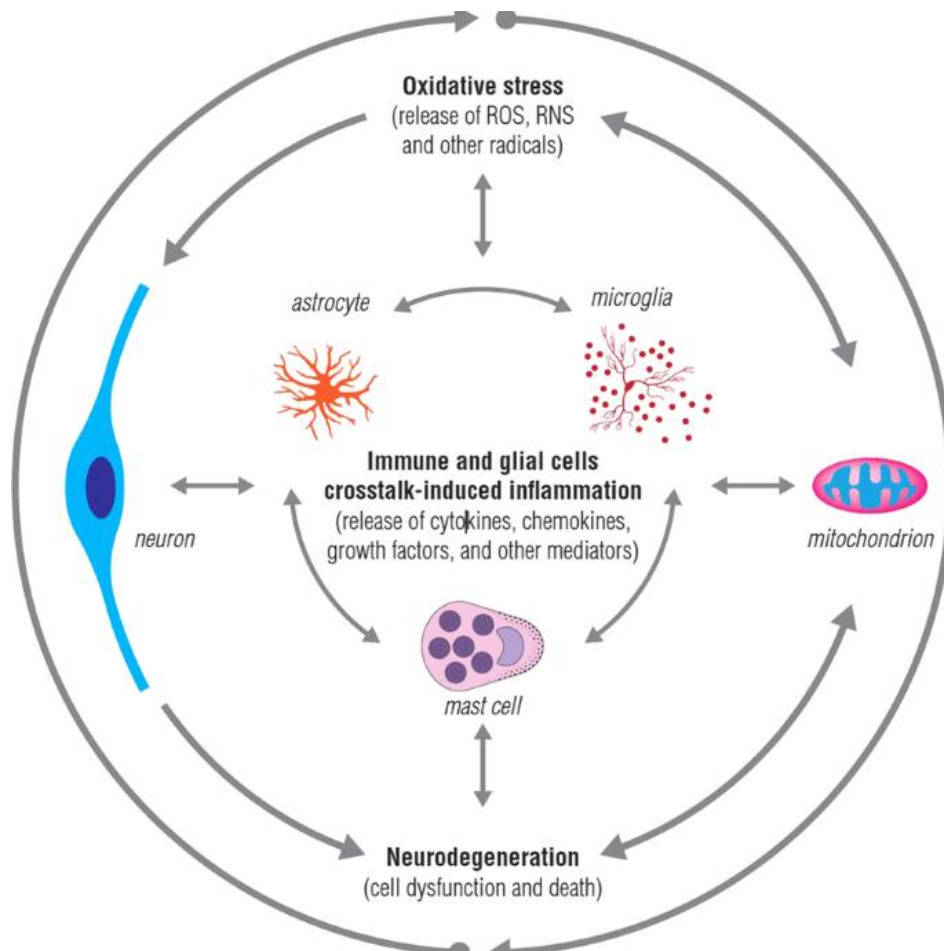


Figure 1. Scheme showing interaction among different factors implicated in the pathogenesis of neurodegeneration including inflammation, oxidative stress, mitochondrial dysfunction and others, adapted from Rekatsina et al. [2].

2. *Alzheimer's Disease*

It is agreed that the most common neurodegenerative disease is Alzheimer's disease [2, 6, 8-10]. AD is the most frequently occurring type of dementia that affects more than 46.8 million people worldwide, with the highest prevalence in the elderly [11]. In a previous study by Gotovac et al., it is predicted that the estimated number of patients with AD is set to increase to 115 million by the year 2050 [9]. The hallmark pathological process of AD involves the deposition of extracellular neuritic plaques containing Amyloid Beta aggregates (A β 42) and intraneuronal neurofibrillary tangles composed of phosphorylated protein tau (3R/4R tau) [6]. There is evidence for

involvement of inflammation and production of free radicals in this deposition and toxicity, however, the underlying mechanisms are still not fully understood [10]. Described as chronic, progressive, and irreversible disease, the most prominent symptom of AD is a sporadic decline in episodic memory followed by a global decline in other cognitive functions such as language and attention. These symptoms are also accompanied by a change in mood and behavior and eventually, AD patients face debilitating consequences where they are unable to perform daily life activities independently [2, 4, 11]. Unfortunately, there are no developed treatments to cure AD. However, current treatment regimens aim to alleviate symptoms temporarily and slow down the progression of the disease [4].

3. Parkinson's Disease

Parkinson's disease is the second most common neurodegenerative disease following AD and it is estimated that it affects around 5 million people each year with a higher incidence in males than females [11]. In the early stages of the disease, movement related symptoms are prominent including tremors, stiffness, and bradykinesia as a cardinal sign of PD. As PD progresses, patients lose 50 to 70% of all dopaminergic neurons in the substantia nigra. Hence worsening of motor symptoms is seen in addition to a decline in cognitive function progressing all the way to dementia in advanced stages [2, 11]. The pathology of this disease, which involves a marked decrease in dopamine levels in the brain and an increase in acetylcholine levels, is known to be caused by alteration of the acetylcholine-GABA-dopamine closed-circuit [11]. However, the underlying molecular mechanisms to the pathogenesis of the disease remain poorly understood. Nevertheless, there are evidence of interplay between

neuroinflammation and oxidative stress to be explored [2, 11]. The current treatment for PD is by L-dihydroxyphenylalanine, a precursor of dopamine. However, this medication has debilitating side effects on patients which calls for a more effective treatment for PD [9].

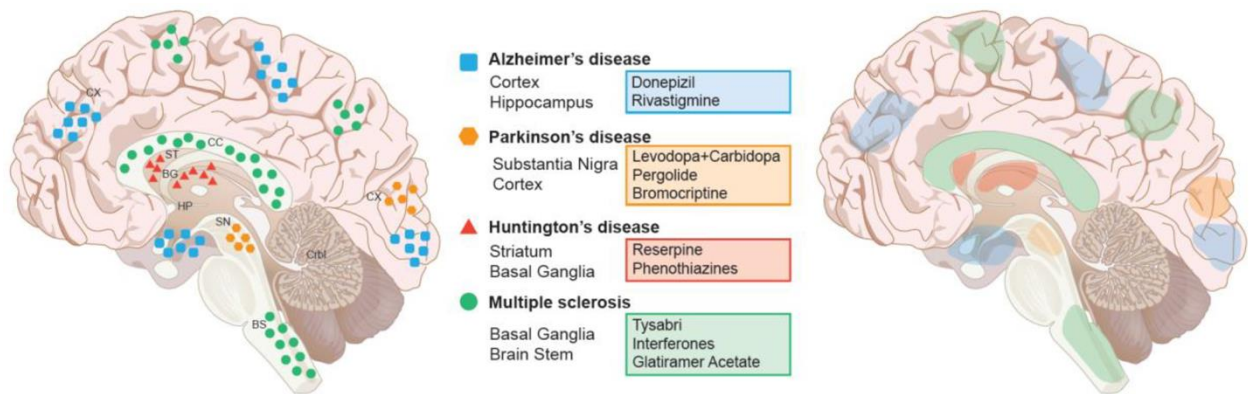


Figure 2. Scheme showing different neurodegenerative diseases and their associated anatomical regions and suggested treatments, adapted from Hussain et al. [12].

B. Microglia

Microglia are the smallest cells in the Central Nervous System (CNS) and are considered the main effector cells in the CNS immunity [12]. Although microglia constitute 5 to 12% of all the cells in the brain, they have 3 essential functions that tremendously impact the brain's homeostasis. These functions are summarized into sensing, housekeeping, and protection against injury. Despite their small number, microglial cells form a network that spans the CNS and are proven to have a dynamic function even when they are in a "resting state". Microglial cells have a ramified shape with multiple branches and projections that are necessary for the constant surveillance of the CNS which allows them to perform other functions including housekeeping and neuroprotection. Housekeeping functions of microglial cells are not limited to surveillance and migration towards site of injury but are also found to be involved in synaptic pruning, an essential event for the development and homeostasis of the CNS.

Considering that these cells are the main immune cells in the CNS, their most remarked function remains their contribution to protection of the surrounding neurons from any injury or infection through participation in neuroinflammation [13].

Like every other cell, the structure of a microglial cell determines its function. The ramified shape of microglia is essential for its surveillance capacity since these cells protrude and retract their projections to cover long distances and multiple areas of the brain. However, when microglial cells are activated and become engaged in phagocytosis or inflammation, they lose their ramified shape and transform into an ameboid shape where cell bodies become enlarged, and projections are shortened to be more localized towards the site of insult or injury [14]. For the purpose of performing their functions, microglial cells express a wide range of receptors, making them highly potent players in the immunity and protection of the CNS. Microglial cells express receptors that include but are not limited to Toll-like receptors and coreceptors that play a role in detection of non-self and self-antigens, scavenger and tyrosine kinase receptors that play a role in phagocytosis and endocytosis, as well as chemokine, cytokine, and immune receptors that regulate and mediate inflammation [14].

Microglial cells can be activated by many factors including pathogens, apoptotic cells as well as abnormal protein aggregates. The activation of microglia is not limited to the release of cytokines and chemokines that mediate inflammation, but also includes the production of nitric oxide and reactive oxygen species that corroborate oxidative stress. It has been widely studied that upon activation, microglia can assume two phenotypes referred to as M1 and M2. Although these are recognized as two separate phenotypes, the M1 and M2 are at interplay to maintain homeostasis and regulate the function of microglial cells. The M1 phenotype is also known as classical activation

where a cascade of pro-inflammatory events occurs and cytokines such as interleukins IL-6, IL-1 β , and tumor necrosis factor alpha (TNF- α) are released in addition to reactive oxygen species due to the expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidases known as NOXs. The M2 phenotype on the other hand is known as the alternative or anti-inflammatory phenotype where anti-inflammatory cytokines and growth factors are released to mediate wound healing, debris clearance and tissue repair. Hence, following the activation of the M1 phenotype it is necessary to have an efficient activation of the M2 phenotype to attenuate the effects of M1 and keep a tight control on pro-inflammatory events (Fig. 1) [14, 15].

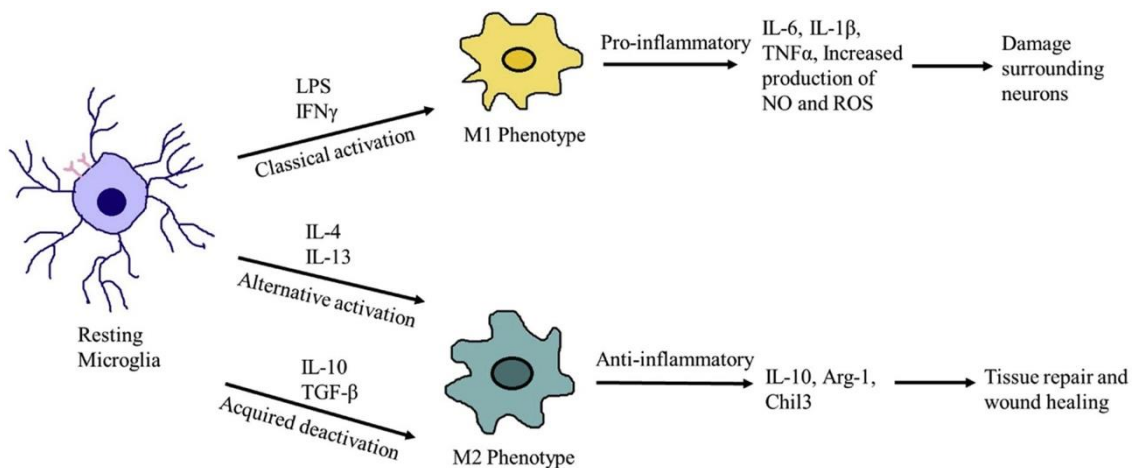


Figure 3. Scheme representing the M1 and M2 phenotype of microglia adapted from Subramanyam et al. [16]

Microglia have a well-established and important role in the protection of the CNS. However, according to many studies, the regulation of microglia or other glial cells, such as astrocytes, is considered to be a complicated process that is still under study to be fully understood [16]. With regards to microglia, it has been demonstrated that CNS trauma, ischemia, infection, and toxic insult can initiate chronic activation of microglial cells which has detrimental effects on surrounding neurons, leading to toxicity and thus neuronal death, a major contributor to neurodegenerative diseases [15,

17]. In AD, for instance, studies have shown that microglial cells have a protective role initially by the endocytosis and phagocytosis of amyloid beta plaque aggregates [13]. However, aberrant and excessive activation of microglia leads to chronic inflammation and hence worsening of the pathologic condition. The collective of this evidence suggest that microglial cells not only can lead to disease progression, but also to disease initiation [15].

C. Factors Affecting NDs

1. Neuroinflammation

Neuroinflammation is characterized a complex cascade of events in response to injury or trauma that involves activation of glial cells and induction of inflammatory cytokines as well as reactive oxygen species [18]. Factors that can contribute or initiate to neuroinflammatory diseases are multiple and include stroke, trauma, infections, and vascular disease. Moreover, pathologies such as hypertension, diabetes, and depression are considered as “silent contributors” to neuroinflammation. It has been hypothesized that the aforementioned diseases can contribute to small vessel disease (SVD), leading to chronic low-grade inflammation systematically and in the CNS. This uncontrolled and sustained inflammatory response is found to contribute to dementia or neurodegenerative diseases [3]. Another contributing factor to aberrant and chronic state of neuroinflammation is aging, and it’s not a coincidence that aging is also a major risk factor for neurodegenerative diseases. Due to aging, the inflammatory status in the CNS is altered owing to the change in the number and morphology of microglial cells. Studies have shown that microglial cells lose their ramified shape and self-renewal ability, and their projections become shortened, causing them to be more vulnerable to

damage. This change in morphology is also associated with alterations in the homeostatic functions of microglia and hence, elderly people are more prone to infections and neuronal damage [17, 19]. Moreover, the cellular senescence of microglia is described as pro-inflammatory in nature and contributes to increased expression of pro-inflammatory cytokines and interleukins including IL-6, IL-1 β and TNF- α (Fig. 2) [20].

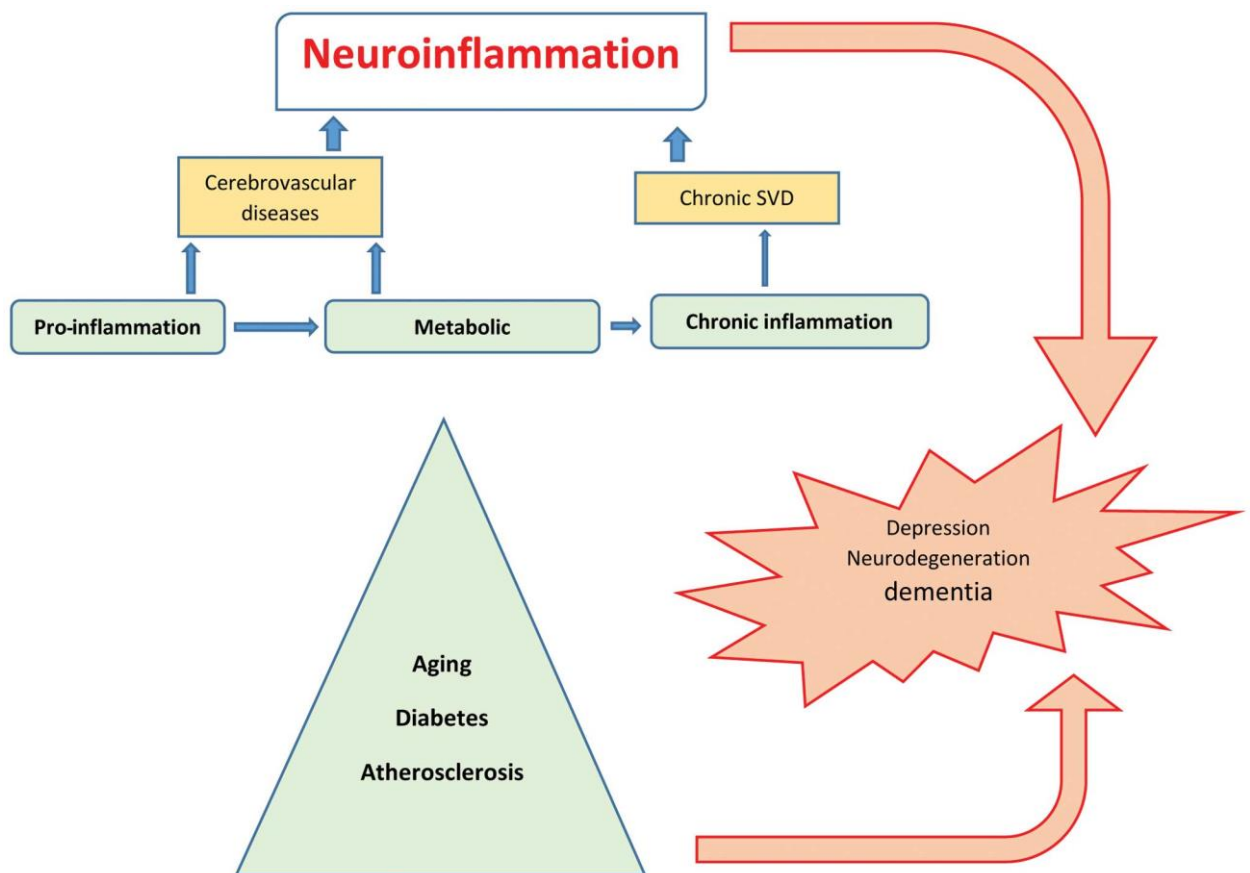
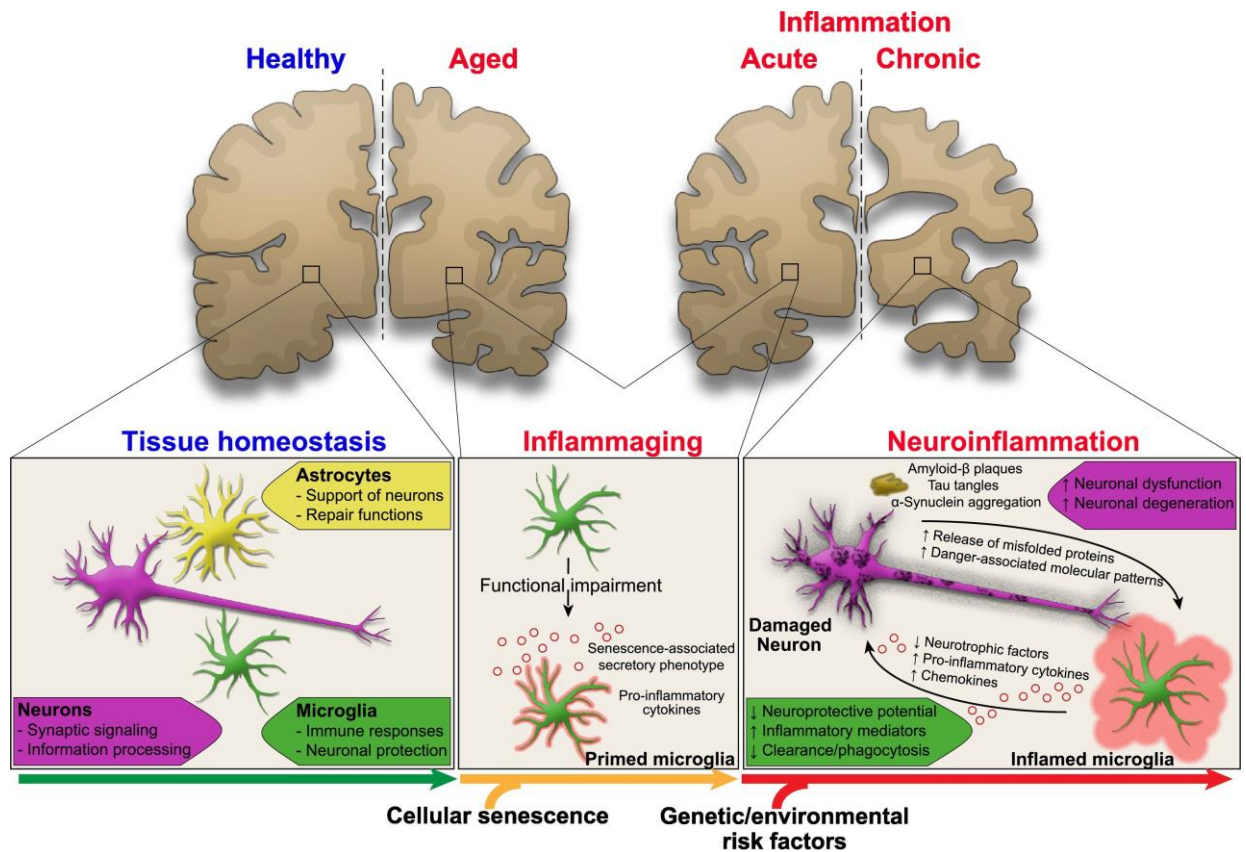


Figure 4. Scheme showing the role of neuroinflammation in neurodegenerative diseases, adapted from Chen et al. [3]



Trends in Immunology

Figure 5. Scheme showing contribution of aging to microglia impairment and chronic neuroinflammation adapted from Scheiblich et al. [22].

2. Oxidative Stress

When an imbalance between oxidants and antioxidants occurs in the biological system, the resulting event is known as oxidative stress. This happens either when there is an excess of reactive oxygen species (ROS) production or malfunctioning of the antioxidant system [21]. ROS are defined as a group of oxygen-containing free radicals and are characterized as highly reactive molecules due to their unpaired valence electrons. There are multiple mechanisms for the generation of ROS that include the electron transport chain in the mitochondria, the nicotinamide adenine dinucleotide oxidase (NOX), and uncoupled endothelial nitric oxide synthase, among others [22]. The disrupted equilibrium between pro-oxidants and antioxidants leads to the excess production and accumulation of ROS which in turn can cause damage to cellular lipids,

proteins, and DNA. Therefore, irregular production of ROS can lead to cellular damage which implicates oxidative stress in the pathogenesis of various diseases including autoimmune disorders, cancer, and neurodegenerative diseases [23]. In microglial cells, it has been established that they secrete a number of inflammatory mediators as well as ROS which are involved in the mechanistic pathways of various CNS diseases. There is also evidence that cytotoxic activation of microglial cells results in damaged neurons and eventually cell death which is a marker in NDs. Furthermore, it has been recognized that there is an interaction between neuroinflammatory mediators such as cytokines and oxidative stress agents which exacerbate neurodegeneration through a vicious loop where neuroinflammation aggravates oxidative stress and in turn, oxidative stress enhances inflammation [24].

In a review by Cahill-Smith et al., it was shown that NADPH oxidase 2 (NOX2) is the primary NOX is involved in oxidative stress in neuronal and glial cells. Moreover, evidence show that NOX2 is a major source of ROS and a key player in cerebrovascular disease. In microglial cells, NOX2 is highly activated by neurotoxic factors induced by neuroinflammation. This enhanced activation of NOX2 stimulates the excess production of ROS which leads to neuronal damage as well as activation of pathways implicated in inflammation and apoptosis including mitogen-activated protein kinases (MAPK), extracellular signal-regulated kinases (ERK) and nuclear factor kappa B (NFκB) [25]. In a recent article by Jurcau, it has been discussed that increased ROS levels play a role in inducing inflammation. High levels of ROS activated the NFκB pathway, leading to elevated production of TNF-α which induces activation of microglial cells. In turn, excessive activation of microglial cells leads to degradation of synapses causing functional impairments that are prominent in NDs. Furthermore, in the mentioned

review, it is discussed that the brain is particularly vulnerable to oxidative stress due to various reasons. Among these reasons is that microglia produce high level of superoxide via NOXs and that oxidized messenger RNA causes mutations and truncation of proteins, leading to misfolding and aggregation often seen in NDs [26].

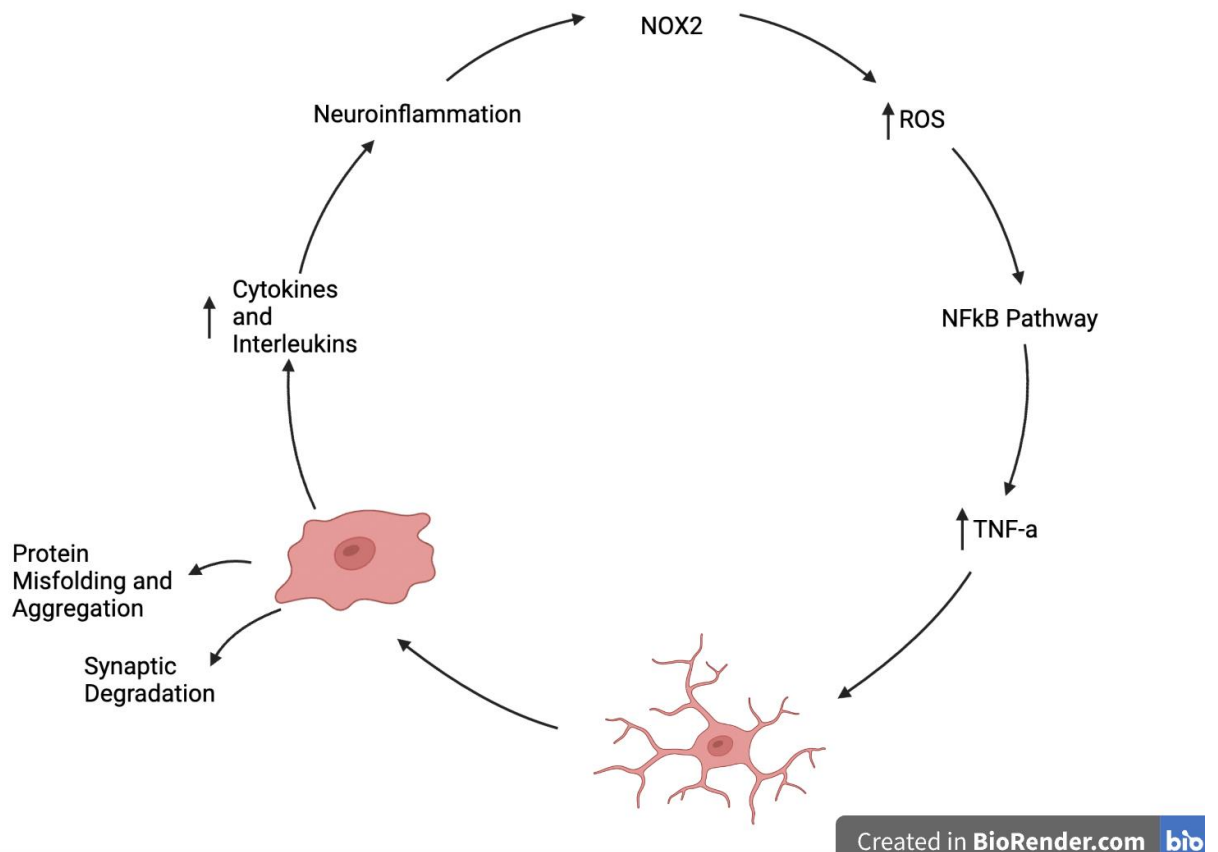


Figure 6. Scheme representing interplay between oxidative stress and neuroinflammation in contribution to neurodegeneration, created with BioRender.com.

D. Plasma Kallikrein-Kinin System: Bradykinin

The plasma kallikrein-kinin system (KKS) consists of plasma proteins that are known to respond to pathological conditions and tissue injury [27]. Key players of the KKS include plasma pre-kallikrein, Factor XII, and high molecular weight kininogen [28]. Plasma pre-kallikrein is cleaved by the action of activated Factor XII to produce plasma kallikrein (PK), a serine protease, that in turn, cleaves high molecular weight

kininogen to generate bradykinin (BK) [29]. BK is considered a potent inflammatory mediator that goes further to stimulate its two receptors: B1R and B2R. B2R is found to be constitutively expressed in vascular and neuronal cells, whereas B1R is usually upregulated during pathological conditions which include inflammation, among others [30]. The activation of both B1R and B2R is implicated in acute and chronic inflammation through the activation of the NF κ B pathway which leads to further production of inflammatory cytokines and chemokines including TNF- α , IL-1 β and IL-6 [31].

Inflammation is known to be a first-line defense mechanism against injury, trauma, and infection. During the process of inflammation, a series of events takes place to facilitate the transport of inflammatory mediators to the site of insult. These events include increased vasodilation to enhance vascular permeability. Besides being known as an important pro-inflammatory mediator, BK also has a recognized role in vasodilation. BK has been found to mediate neurogenic inflammation through vasodilation and plasma extravasation as well as induction of oxidative stress which has been implicated in neuronal damage [32]. In a recent study by Singh et al., it was shown that BK has a key role in the pathogenesis of AD. BK levels have been found to be critically elevated in AD patients with severe cognitive impairment, implicating that BK is not only involved in the initiation of AD but also its progression through sustained inflammatory pathways [33].

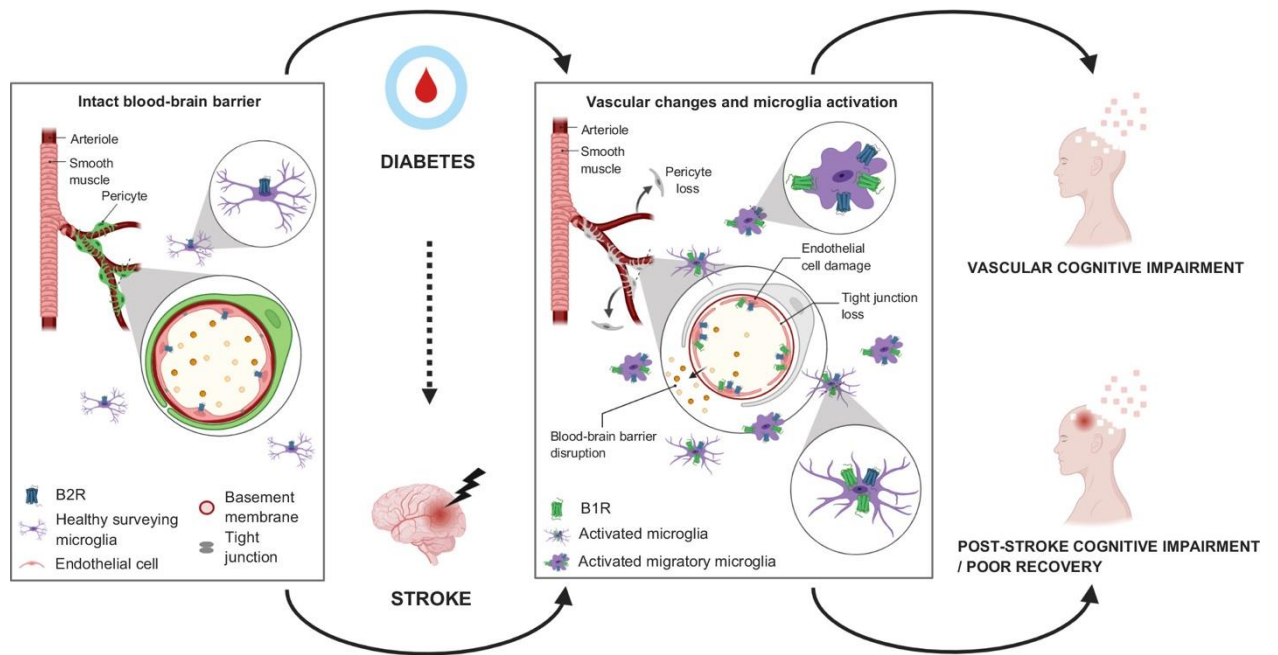


Figure 7. Contribution of BK to vascular cognitive impairment post-stroke, adapted from Barić and Radmilović [36].

E. Sphingosine-1-Phosphate

Sphingosine-1-Phosphate (S1P) is a potent lysophospholipid signaling molecule that is involved in cellular homeostasis on an extra and intracellular level [34]. The production of S1P is tightly regulated by two enzymes: Sphingosine Kinases (SK) 1 and 2 that catalyze the phosphorylation of sphingosine into S1P, and S1P lyase which promotes the degradation of S1P [35]. SK1 is bound to the plasma membrane and the effects of S1P generated by SK1 seem to be opposite to that generated by SK2 which localizes to the nucleus. SK2 is less studied than SK1, and while SK1 produces S1P that mediate proliferation and cell growth, S1P produced by SK2 leads to apoptosis and inhibition of proliferation [36]. To mediate its effects, S1P binds to 5 different G-protein coupled receptors, namely Sphingosine-1-Phosphate receptor 1 through 5 (S1PR1-5) [37]. The description of the S1P and S1PR receptor function is complex due to the ubiquitous distribution of these receptors in all bodily organs and their function

not only depends on their location but also on the state of activation of the cells where they are expressed [38]. Moreover, different S1P receptors bind to different alpha subunits of GPCRs, mediating different effects. The alpha subunits of GPCRs are: G_q , G_i , $G_{12/13}$ and G_s [39]. S1PR1 is known to bind to G_i , promoting cell survival, migration, and proliferation. S1PR2 binds to G_i , $G_{12/13}$, and G_q , most likely leading to suppression of cell proliferation. S1PR3 binds to G_i , $G_{12/13}$, and G_q as well, however, this promotes cell proliferation, migration, and inhibition of apoptosis similar to S1PR3. S1PR4 and 5 are less studied than the other 3 receptors but have been implicated in cell proliferation and inflammation [36, 40].

In the CNS, all S1P receptors are found to be expressed. For example, in astrocytes, S1PR1 and S1PR3 are implicated in proliferation, migration and gap junction communication. On the other hand, in microglial cells, S1PR1, 2 and 3 are expressed and are involved in the induction of proinflammatory cytokines [41]. Moreover, studies have shown that in microglia, the accumulation of S1P leads to the activation of S1P-S1PR2 axis which not only induces proinflammatory effects but also impairs autophagy [42]. Not only are S1PRs involved in the mediatory effects of S1P, but also at the level SK1 is vital for the regulation of inflammation, especially in microglia. A study by Nayak et al. showed that the inhibition of SK1 attenuated the induction of proinflammatory cytokines as well as nitric oxide production and is being considered for therapeutic implications of neurodegenerative diseases [43].

In terms of NDs, studies have shown that dysregulation of the SK1-S1P-S1PR1 axis plays a role in the pathogenesis of these diseases [44, 45]. S1P was found to enhance the pathogenesis of neurodegenerative diseases through multiple mechanisms including neuroinflammation and oxidative stress. However, S1P activates pathways

that are also involved in the dysregulation of protein and lipid transport in addition to the deposition of neurotoxic proteins which are at the basis of many neurodegenerative diseases [36]. There is evidence that the addition of S1P to microglial cells has deprives microglia of oxygen and glucose which drives these cells towards apoptosis. Moreover, chronic neuroinflammation mediated by S1P in the CNS through persistent activation of microglia induced impaired cognition and synaptic loss and hence, sustained neurodegeneration [46]. In a study by Gaire et al., it has been shown that S1PR3 plays a major role in the activation of microglia and its polarization towards the M1 phenotype which induces proinflammation. Furthermore, this study has implicated S1PR3 as a therapeutic target in brain ischemia because this receptor was found to activate the ERK1/2 pathway which led to the sustained activation of NFκB pathway and thus, the upregulation of pro-inflammatory cytokines and interleukins [47].

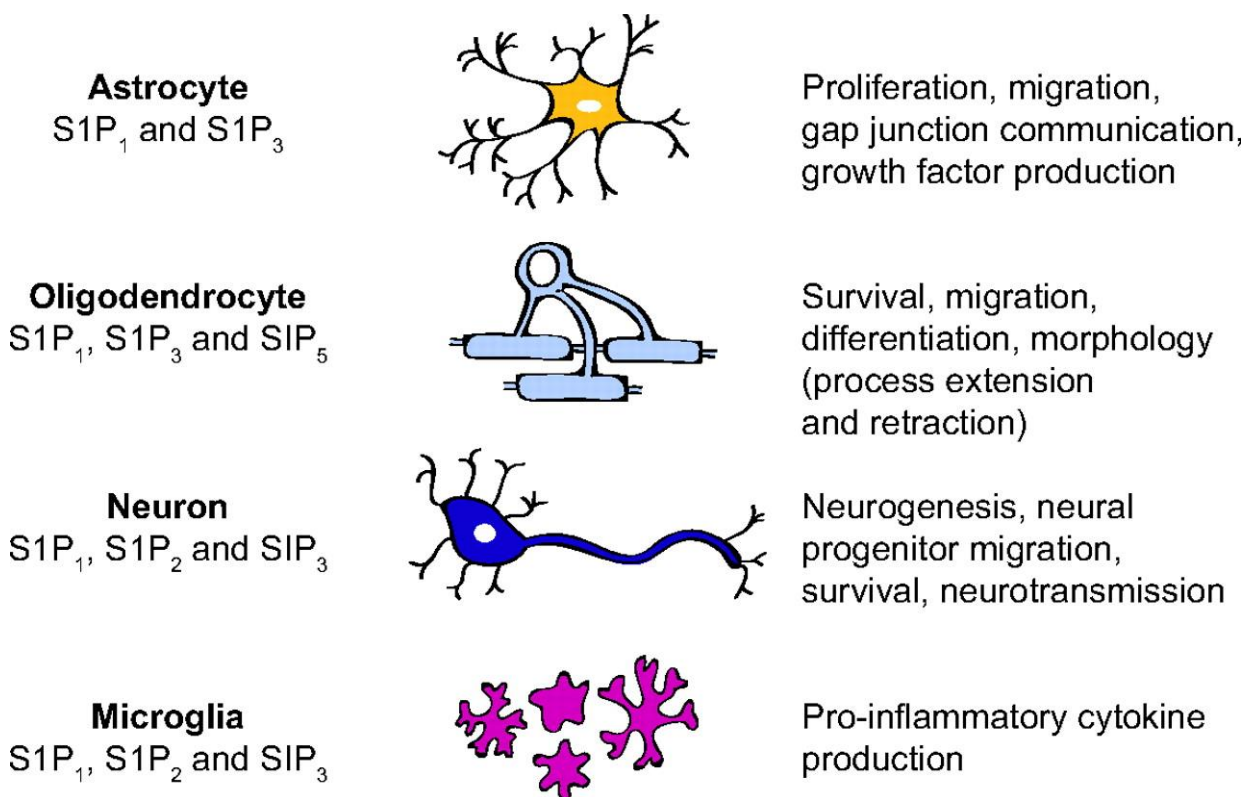


Figure 8. Distribution of S1P receptors in different brain cells, adapted from Soliven et al. [44].

CHAPTER II

AIM OF THE STUDY

Based on what was found in the literature, it is clear that there is an intricate role for both neuroinflammation and oxidative stress in the pathogenesis of neurodegenerative diseases. Moreover, BK and S1P have been proven to have a role in mediating both processes: inflammation and oxidative stress. Therefore, the aims of this study are:

1. Investigate the effect of BK and S1P on microglial cells.
2. Explore role of BK and S1P in inflammation and oxidative stress.
3. Study the possibility of crosstalk between BK and S1P in the induction of inflammation and oxidative stress in microglia.

CHAPTER III MATERIALS AND METHODS

A. Cell culture

Murine microglial cell line, N9, a generous gift from EnCor biotechnology (Gainesville, FL, United States), was utilized in this study. N9 microglial cells are derived from the brain of mice, and they are developed by immortalizing primary microglial cells using the v-myc oncogene of an avian retrovirus. Hence, this cell line has been genetically modified to accommodate increased proliferation and adherence, however still maintaining common phenotypical characteristics with primary microglial cells [48]. N9 cells were cultured in Dulbecco's Modified Eagles Medium/Nutrient Mixture (DMEM) F-12 Ham medium (Sigma-Aldrich, United Kingdom) supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin (PS) and incubated in a humidified incubator at 37°C and 5% CO₂ for two days. The cells were then split into two flasks at a ratio of 1/6 after they had reached 80-90% confluence.

B. Cell count optimization

To achieve an optimal seeding density for N9 cells, N9 cells were seeded at different densities in a 6-well plate. The seeded densities were: 150,000, 200,000, 350,000, 400,000 and 600,000 cells/well.

C. Treatment

N9 cells were seeded into 12-well or 96-well plates, depending on the experiment being performed. After 24 hours of seeding, the cells were starved for another 24 hours in DMEM F-12 Ham medium, supplemented with 1% FBS and 1% PS

(starvation media) to achieve quiescence (80% confluence). The experiments were divided as follows:

1. Assessment of Inflammation and Oxidative Stress

For this experiment, N9 cells were seeded in 12-well plates at a density of 150,000 cells/well for RNA and protein extraction. After 24 hours, the media was discarded and replaced with starvation media. The conditions for this experiment: control (media only), LPS at 100 ng/ml (diluted in water), BK at 0.1 μ M (diluted in 0.1M acetic acid), and S1P at 0.1 μ M and 1 μ M (diluted in DMSO and NaOH). S1P at a concentration of 1 μ M was then used for the remainder of the experiments.

2. Receptor inhibitors

Receptor inhibitors of B2R (HOE140, Axon Medchem BV, Cedarlane) and Sphingosine Kinase (SphKI1, CAS 1177741-83-1 Calbiochem) were used at a concentration of 1 μ M each [49]. The inhibitors were diluted in DMEM F-12 starvation media (1% FBS) from the initial stock of 1 mM for HOE140 and 10⁻²M for SphkI1. These inhibitors were then added to the cells for 30 minutes prior to the stimulation with BK and S1P. The cells were then harvested for RNA extraction after 24 hours.

D. MTT Cell Activity Assay

To assess for cell activity under the effect of S1P, different concentrations were prepared from the stock solution of 1 mM (Cayman, Germany). The concentrations were: 0.1 μ M, 0.5 μ M, 1 μ M, and 5 μ M. N9 cells were seeded in 96-well plates at a density of 20,000 cells/well. After 24 hours, the cells were starved using starvation

media to achieve 80% quiescence. Following another 24 hours, the mentioned treatments were prepared in DMEM F-12 Media with 1% FBS and the cells were treated and left in the incubator at 37°C and 5% CO₂ for 24 hours. The media was then aspirated and cells were treated with 10 µL MTT (Thiazolyl Blue Tetrazolium Bromide) reduction assay dye (Sigma-Aldrich, United States, M5655) and 90 µL of DMEM F-12 supplemented with 1% PS. The plates were left in the incubator for 3 hours before 150 µL of MTT stop solution (Isopropanol, triton X-100, and 37% HCL) was added to the cells. The optical density (OD) or absorbance was then measured at a wavelength of 595 nm. The percentage of cell activity was then measured using the following formula, where cell culture media was used as blank:

$$\% \text{ Activity} = \frac{(\text{SampleOD} - \text{blankOD})}{(\text{controlOD} - \text{blankOD})} \times 100$$

E. NBT Assay

To quantify the production reactive oxygen species (ROS) by microglial cells, Nitroblue Tetrazolium Assay (NBT) was performed. N9 cells were seeded in 96-well plates at a density of 20,000 cells/well in complete media (DMEM F-12 supplemented with 10% FBS and 1% PS). The media was then discarded after 24 hours and starvation media was added to the cells. After another 24 hours, the cells were treated using the following conditions: control (media only), BK (0.1 µM), S1P (1 µM), and the antagonists HOE140 (1 µM) and SphkI1 (10⁻⁵ M) for 30 minutes each prior to treatment with BK and S1P prepared in DMEM F-12 starvation media. Following 24 hours, the media was aspirated and 100 µL of NBT solution was added. The cells were then incubated at 37°C and 5% CO₂. After 1 hour, the NBT solution was removed, and cells

were washed using 100 μL of Methanol and left to air dry. Following this step, the formazan crystals are solubilized using 120 μL of potassium hydroxide (KOH) at a concentration of 2M and 140 μL of DMSO. The OD was then measured using an 96-well microplate reader at a wavelength of 630 nm.

F. Total RNA extraction and quantification

Following treatment, the cells were washed with Phosphate Buffered Saline for 1 minute. After this step, total RNA was extracted using Qiazol Lysis reagent (Qiagen, Hilden, Germany, 79306). Qiazol was added to the cells in each well and scraped thoroughly. The cell lysates were then transferred into Eppendorf tubes and vortexed for 30 seconds. 100 μL chloroform were added to each tube and vortexed well to attain homogeneity. The tubes were incubated for 5 minutes on ice before being centrifuged for 15 minutes at 12,000 g and 4°C. After centrifugation, the aqueous phase containing RNA was collected and transferred into new Eppendorf tubes. 200 μL of isopropanol was added to the RNA to promote its precipitation and vortexed again for 15 seconds and then incubated on ice for 5 minutes. The samples were centrifuged again for 15 minutes at 12,000 g and 4°C. Thereafter, a pellet of RNA could be seen. The supernatant is then discarded, and the RNA pellet is washed in 400 μL of 75% Ethanol before the tubes are centrifuged for 5 minutes at 7,500 g and 4°C. The previous step is repeated again before the supernatant was discarded and the tubes were left to air-dry under a fume hood for 10 minutes. Finally, the RNA pellets were resuspended in 22 μL of RNase/DNase free water and placed in the oven at 60°C for 5 minutes to further inactivate any RNase activity. RNA samples were then quantified, and their purity was evaluated using the DeNovix DS-11FX Spectrophotometer.

G. Reverse Transcription

To convert RNA into cDNA, 1 µg of the extracted RNA was taken and transcribed into cDNA at a final volume of 20 µL. The High Capacity Reverse Transcriptase kit (Thermo Fisher Scientifics, 004007363) was used for the process of transcription and the reaction mix was done in a BioRad T100 Thermo Cycler machine (Bio-Rad laboratories, California, USA). The conditions for the experiment were set at: 25°C for 10 minutes, 37°C for 2 hours, 85°C for 5 minutes and finally at 4°C.

H. Real Time Polymerase Chain Reaction (RT-qPCR)

The oligonucleotides for PCR were synthesized and purchased from Macrogen Inc (Seoul, South Korea). Primers were centrifuged at 1,000 RPM for 1 minute before being reconstituted with 300 µL RNase/DNase free water to obtain a stock concentration of 100 µM, and working solutions of 50 µM were prepared and stored at -20°C. For every reaction mix, 2.5 µL of SYBR Green Supermix 4X (iTaq™ Universal SYBR Green Supermix, Bio-Rad Laboratories, 1725121), 4.8 µL of RNase/DNase free water, and 0.1 µL each of forward and reverse primers were added into each tube of cDNA sample. Results were calculated using the $\Delta\Delta\text{CT}$ method and normalized to the housekeeping gene GAPDH.

Table 1. List of primers used for RT-qPCR and their forward and reverse sequences.

Primer	Forward	Reverse
GAPDH	CGTCCCGTAGACAAAATGGTGAA	GCCGTGAGTGGAGTCATACTGGAACA
TNF- α	CGTCAGCCGATTTGCTATCT	CGGACTCCGCAAAGTCTAAG
IL-6	AGTTGCCTTCTTGGGACTGA	TCCACGATTTCCAGAGAAC

IL-1 β	CCGTGGCTTCTAGTGCTGAC	TGTCCTCATCCTGGAAGGTC
TGF- β	CCGTGGCTTCTAGTGCTGAC	CTCCGTTTCACCAGCTCCA
CTGF	AATGTCAGTGCGCAGCCGAAGCA	AGGGGTCACGCTCCGTACACAG
B1R	CCATCAGTCAGGACCGCTAC	AGGGACGACTTTGACGGAAC
B2R	CTGGGTGTTTGGAGAGGTGT	ACGAGCATCAGGAAGCAGAT
NOX 1	GGATCCATGGCCTGGGTGGGAT	GGATGCCTGCAACTCCCCTTATGG
NOX 2	CACACTGACCTCTGCTCCTG	AGCATTGAATAGCCCCTCCG
NOX 4	TGTTGGGCCTAGGATTGTGT	CAGGACTGTCCGGCACATAG
Sphk1	GAAGGGCAAGCATATGGAAC	ACCATCAGCTCTCCATCCAC
Sphk2	GACCTGTCCCTCAATGGTGG	GCTGTTTTGAGAGCGTTGGG
S1PR1	CCGTCTTCACTCTGCTCCTG	CGACTGGCCTTGAGATGTT
S1PR2	GTGGCTCTGTACGTCCGAAT	ATGGTGACCGTCTTGAGCAG
S1PR3	TCCTCAACTCGGCCATGAAC	TCCCCTTGCCCTTGACTAGA
ITGAM	GACGTGAATGGGGACAAACT	GAGGCTGGCTATTGATGCTC

I. Statistical Analysis

Statistical analysis was done using GraphPad Prism 9, (version 9.4.1 for Mac, GraphPad software, La Jolla, CA 92037, USA). One-Way Analysis of Variances (ANOVA) was used for the comparison of more than 2 groups, and it was followed by post-hoc Turkey's multiple comparison test. $p < 0.05$ was considered statistically significant.

CHAPTER IV RESULTS

A. Optimization of N9 cells count for seeding

To achieve the optimal seeding density for N9 cells, we aimed at 70 to 80% confluence after 24 hours. The cell densities that were seeded are the following: 100,000 – 200,000 – 300,000 – 450,000 and 600,000 cells each in a different well. After 24 hours, the cells were observed under the microscope and the optimal density with 70 to 80% confluence was found to be between 300,000 to 450,000 cells/well (Fig. 9).

N9 Seeding Optimization

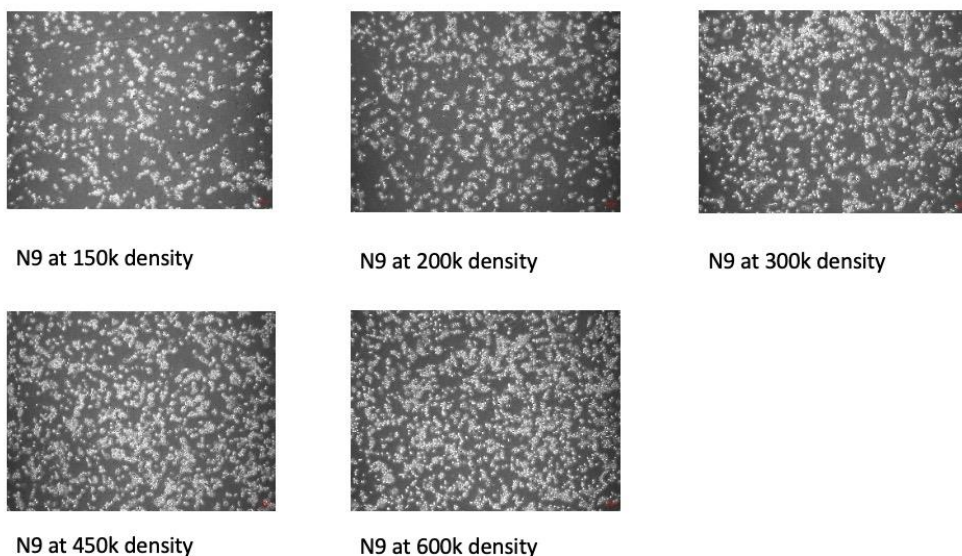


Figure 9. N9 cells images taken at 5X amplification for every cell density seeded in 6 well plate.

B. MTT Assay for cell viability of N9 cells under effect of S1P

The cell activity assay (MTT) was previously performed in our lab for BK [49]. Similarly, we were interested in testing activity of N9 cells under varying concentrations of S1P. According to the results obtained in Fig. 10, it has been shown

that S1P has no significant effect on the cell activity of N9 cells and thus was not considered toxic for these cells.

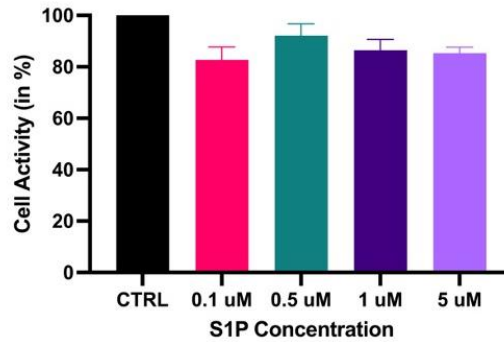


Figure 10. Cell activity assay for N9 cells. MTT Assay was performed on N9 cells. Results are expressed as percent activity (n=4).

C. Role of BK and S1P in the induction of inflammation in Microglia

To evaluate the role of BK and S1P in the induction of inflammation in microglial cells, N9 cells were stimulated for 24 hours with LPS as a positive control, BK or S1P at the following concentrations 100 ng/ml, 10^{-7} M and 10^{-6} M, respectively. The results obtained from qPCR are shown in Fig. 11. The mRNA expression of the pro-inflammatory markers interleukin-6 (IL-6), interleukin-1 β (IL-1 β), and tumor necrosis factor alpha (TNF- α) showed a significant increase upon stimulation with BK compared to control. Unlike IL-6 (Fig. 11A), there was a slight increase in the expression of IL-1 β and TNF- α upon stimulation with S1P although no statistical significance is shown (Fig. 11B and 11C).

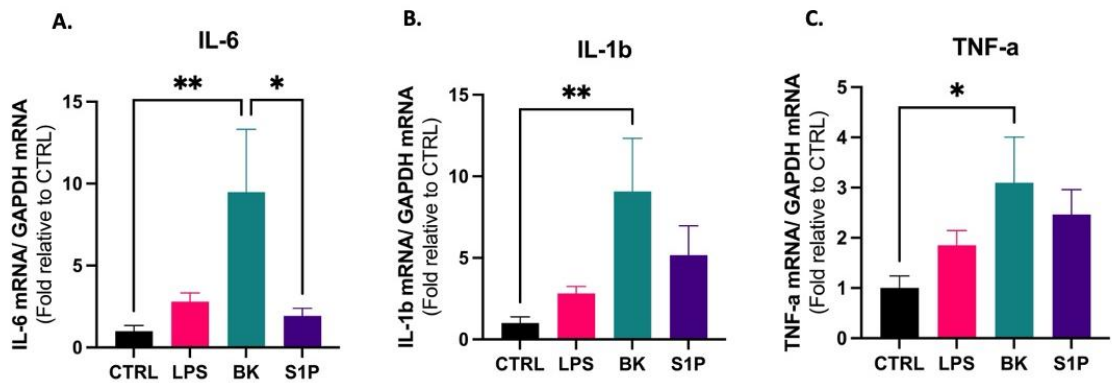


Figure 11. Expression of inflammatory markers in N9 cells. Bar graph plots showing mean and SEM for the fold changes in mRNA levels of (A.) IL-6, (B.) IL-1 β and (C.) TNF- α expressed relative to CTRL and to GAPDH mRNA levels. (* $p \leq 0.05$, ** $p \leq 0.01$, $n = 6$).

D. BK and S1P induce growth factors in Microglia

To evaluate the full effect of BK and S1P on microglia, growth factors such as connective tissue growth factor (CTGF) and transforming growth factor beta (TGF- β) were evaluated. As shown in Fig.12, LPS induced no significant increase in the expression of either CTGF or TGF- β . However, looking at CTGF mRNA levels, a significant increase in CTGF expression is observed upon stimulation with both BK and S1P (Fig. 12A). Although a similar trend can be seen for TGF- β upon stimulation with BK and S1P, the increase is not significant compared to the control (Fig.12B).

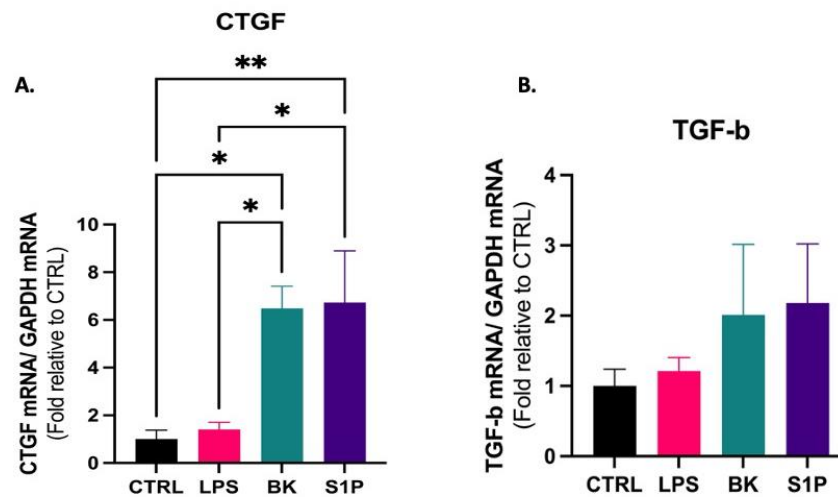


Figure 12. Expression of growth factors in N9 cells. Bar graph plots showing mean and SEM for the fold changes in mRNA levels of (A.) CTGF and (B.) TGF- β expressed relative to CTRL and to GAPDH mRNA levels. (* $p \leq 0.05$, ** $p \leq 0.01$, $n = 6$).

E. Activation markers of Microglia upon stimulation with BK and S1P

Microglial cell activation was also assessed in response to stimulation with LPS, BK and S1P. CD11b was used as a marker of activation for microglial cells. There was an observed increase in the expression of CD11b upon stimulation with LPS, BK and S1P, however, only a significant increase in CD11b expression levels can be seen with BK compared to the control (Fig. 13).

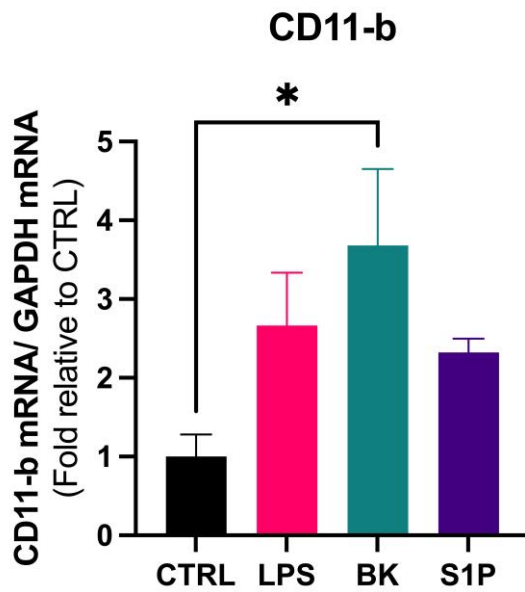


Figure 13. Expression of microglia activation marker in N9 cells. Bar graph plots showing mean and SEM for the fold changes in mRNA levels of CD11-b expressed relative to CTRL and to GAPDH mRNA levels. (* $p \leq 0.05$, ** $p \leq 0.01$, $n = 6$).

F. Bradykinin Receptors Expression

The gene expression of BK receptors, B1R and B2R, were also assessed when N9 cells were exposed to LPS, BK and S1P. After treatment of N9 cells with LPS, BK and S1P for 24 hours, data from qPCR showed that BK and S1P both induced an increase in the expression of B1R and B2R. However, the one-way ANOVA test showed that, although a similar trend can be seen for both genes, the increase in B1R expression was more significant with both BK and S1P compared to the control (Fig. 14A). Furthermore, looking at data from B1R and B2R mRNA levels, it was seen that the increase in receptors expression upon stimulation with BK is insignificant compared to S1P.

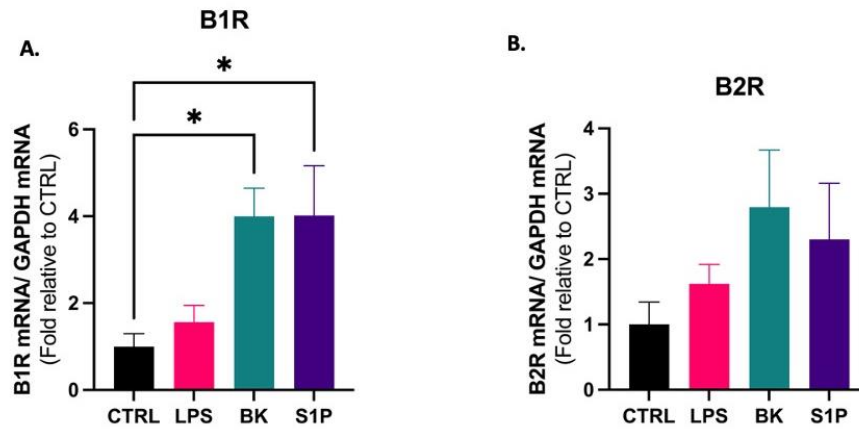


Figure 14. Expression of bradykinin receptors in N9 cells. Bar graph plots showing mean and SEM for the fold changes in mRNA levels of (A.) B1R and (B.) B2R expressed relative to CTRL and to GAPDH mRNA levels. (* $p \leq 0.05$, ** $p \leq 0.01$, $n = 6$).

G. Sphingosine-1-Phosphate related markers in microglia

We also sought out to evaluate the expression of genes related to S1P in microglial cells, therefore the mRNA levels of Sphingosine Kinases 1 and 2 (SK1 and SK2), enzyme catalyst for the formation of S1P from ceramides, and Sphingosine-1-Phosphate Receptors 1, 2 and 3 (S1PR1, S1PR2, and S1PR3) were measured:

1. *Sphingosine Kinase 1 and 2*

For both the expression of SK1 and SK2, a significant increase in mRNA levels was observed upon stimulation with S1P compared to the CTRL. No other significant increase can be seen with either LPS or BK, although a slight increase upon stimulation

with BK can be observed (Fig. 15A and B).

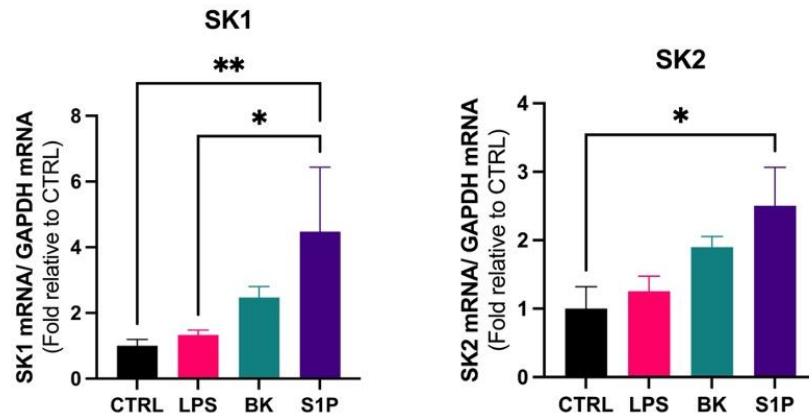


Figure 15. Expression of sphingosine kinases in N9 cells. Bar graph plots showing mean and SEM for the fold changes in mRNA levels of (A.) SK1 and (B.) SK2 expressed relative to CTRL and to GAPDH mRNA levels. (* $p \leq 0.05$, ** $p \leq 0.01$, $n = 6$).

2. *Sphingosine-1-Phosphate Receptors in microglia*

To obtain a more inclusive look at markers related to S1P role in microglial cells, the mRNA levels of S1P receptors were also assessed. S1PR1, S1PR2 and S1PR3 have been found to be expressed in the central nervous system with distinct roles [34]. According to data obtained from qPCR, the expression of all S1P receptors increased significantly upon stimulation with BK compared to the control (Fig. 16A, B, and C). It was also observed that the highest increase was seen in S1PR3 (Fig. 16C).

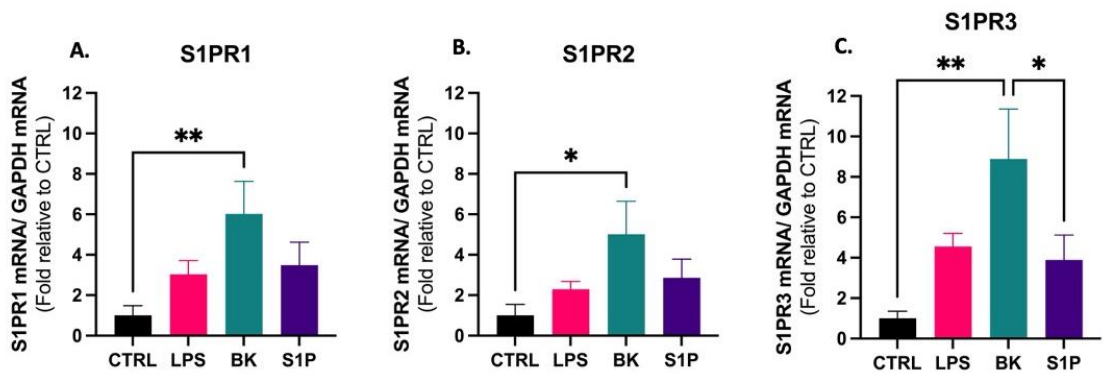


Figure 16. Expression of inflammatory markers in N9 cells. Bar graph plots showing mean and SEM for the fold changes in mRNA levels of (A.) S1PR1, (B.) S1PR2 and (C.) S1PR3 expressed relative to CTRL and to GAPDH mRNA levels. (* $p \leq 0.05$, ** $p \leq 0.01$, $n = 6$).

H. Crosstalk between BK and S1P in Microglia

To investigate the probability of crosstalk between BK and S1P, N9 cells were stimulated with BK and S1P at 10^{-7} M and 10^{-6} M, respectively. Inhibitor of B2R, HOE140, was added to N9 cells at a concentration of 10^{-6} M for 30 minutes before stimulation with either BK or S1P for 24 hours. Similarly, inhibitor for Sphingosine Kinase (SKi) was added for 30 minutes also at a concentration of 10^{-6} M before stimulation with either BK or S1P. The same genes that were measured previously were repeated and the results are as follows:

1. *Inflammatory Markers:*

Consistent with previous results, a significant increase in all inflammatory markers expression is seen upon stimulation with BK. Looking at the mRNA expression of IL-6, the increase seen with BK stimulation compared to BK+HOE140 was not significant (Fig.17A). This could mean that BK is increasing the expression of IL-6 via B2R. Moreover, BK significantly increased the expression of IL-1 β compared to CTRL

and to BK+SKi (Fig. 17B). This might implicate that BK can also induce the expression of IL-1 β via an S1P-dependent pathway. Lastly, looking at gene expression of TNF- α in Fig. 17C, stimulation with BK induced a significant increase in mRNA levels compared to both CTRL and BK+HOE140, but no significant change was seen when Sphingosine Kinase was inhibited prior to stimulation with BK (BK+SKi). This finding suggests that BK was upregulating the expression of TNF- α via its B2R receptor.

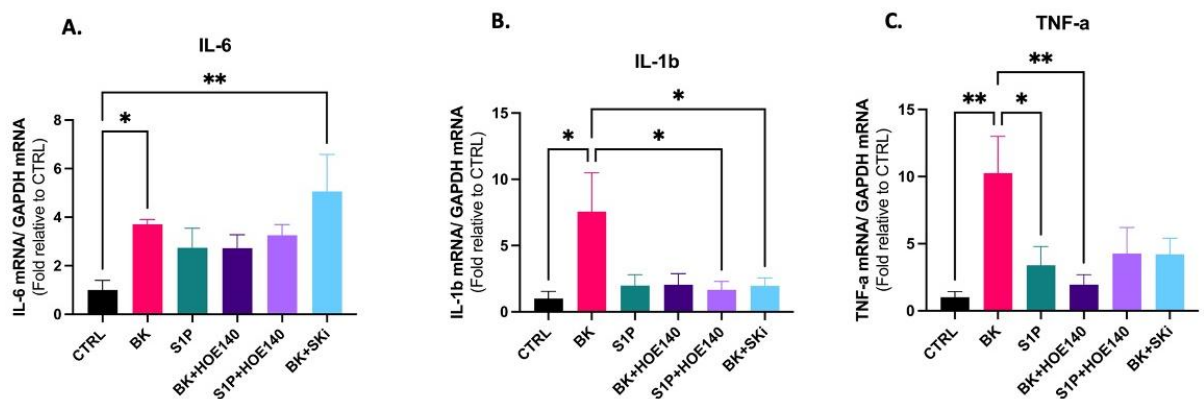


Figure 17. Expression of inflammatory markers in N9 cells. Bar graph plots showing mean and SEM for the fold changes in mRNA levels of (A.) IL-6, (B.) IL-1 β and (C.) TNF- α expressed relative to CTRL and to GAPDH mRNA levels. (* $p \leq 0.05$, ** $p \leq 0.01$, $n = 6$).

2. Growth Factors in Microglia:

Growth factors, namely CTGF and TGF- β , were evaluated again when inhibitors of B2R and Sphingosine Kinase were introduced. As shown in Fig 18. below, BK stimulated a significant increase in the mRNA expression levels of both CTGF and TGF- β . Interestingly, when looking at CTGF gene expression levels, we noticed that there was a significant increase in mRNA levels upon stimulation with BK compared to BK+SKi (Fig. 18A). This could suggest that BK was increasing the expression of CTGF through a S1P-dependent pathway. On the other hand, when looking at TGF- β

expression in Fig. 18B, there was a significant increase in mRNA levels when cells were stimulated with BK compared to when B2R inhibitor was introduced (BK+HOE140). Therefore, this means that BK was working to induce TGF- β through its receptor B2R. Moreover, S1P did stimulate an increase in TGF- β although it is statistically insignificant. However, this increase was significant when compared to cells that have been inhibited by HOE140 followed by S1P stimulation (S1P+HOE140) (Fig. 18B). This finding suggests that S1P might be increasing TGF- β through a B2R dependent pathway.

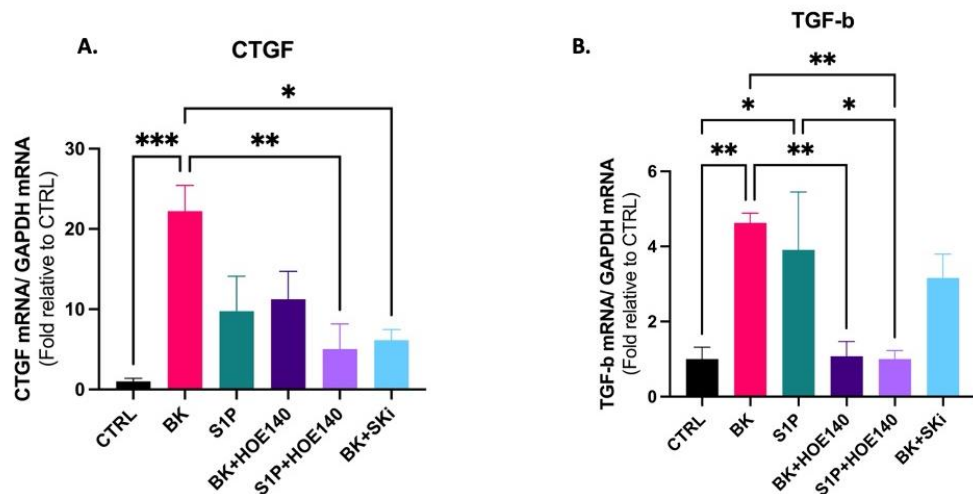


Figure 18. Expression of fibrotic markers in N9 cells. Bar graph plots showing mean and SEM for the fold changes in mRNA levels of (A.) CTGF and (B.) IL-1 β expressed relative to CTRL and to GAPDH mRNA levels. (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, $n = 6$).

3. *Bradykinin Receptors:*

There is no previous evidence of interaction between BK and S1P. For this purpose, we were interested in studying the expression of Bradykinin receptors after introducing inhibitors. Although no significant results could be seen for B1R, the data was still consistent with what has been represented above, both BK and S1P increased the expression of B1R (Fig. 19A). For mRNA levels of B2R, there was a significant

increase upon stimulation with BK compared to control and compared to cells inhibited with Sphingosine Kinase inhibitor followed by stimulation with BK (Fig. 19B). This could suggest the presence of crosstalk among BK and S1P dependent pathways.

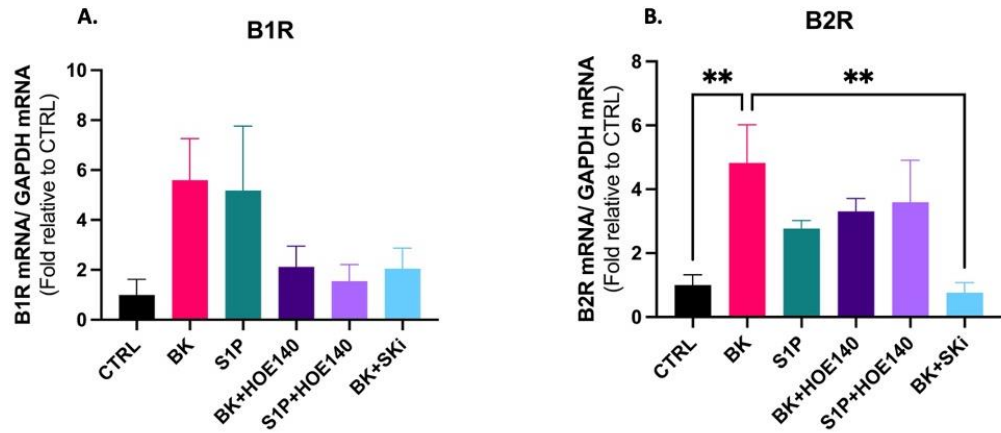


Figure 19. Expression of bradykinin receptors in N9 cells. Bar graph plots showing mean and SEM for the fold changes in mRNA levels of (A.) B1R and (B.) B2R expressed relative to CTRL and to GAPDH mRNA levels. (* $p \leq 0.05$, ** $p \leq 0.01$, $n = 6$).

4. *Sphingosine-1-Phosphate related markers:*

a. Sphingosine Kinase:

Since our aim is to evaluate crosstalk among both molecules of BK and S1P, it was also important to inspect the gene expression levels of Sphingosine Kinase 1 and 2, in addition to S1P receptor 1, 2 and 3. Our data in Fig. 20 showed that stimulation with BK significantly increased mRNA levels of SK1 and SK2 compared to CTRL and to S1P. Moreover, this increase was also significant when compared to mRNA levels of BK+HOE140.

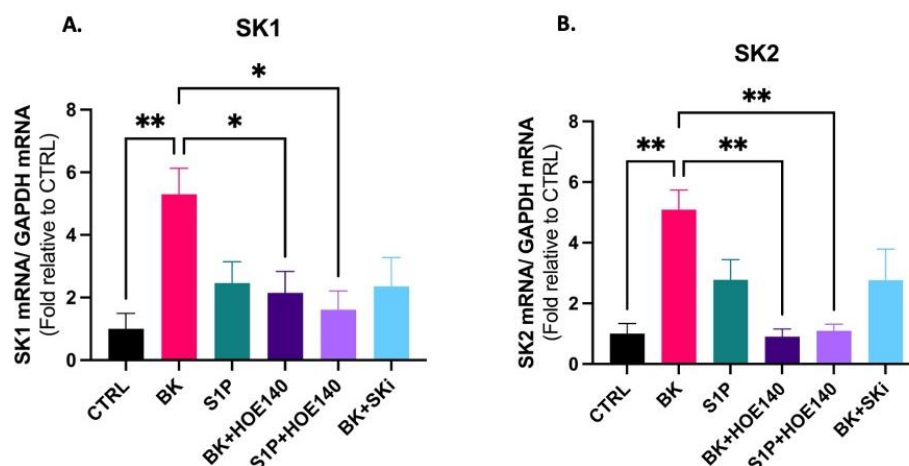


Figure 20. Expression of sphingosine kinases in N9 cells. Bar graph plots showing mean and SEM for the fold changes in mRNA levels of (A.) SK1 and (B.) SK2 expressed relative to CTRL and to GAPDH mRNA levels. (* $p \leq 0.05$, ** $p \leq 0.01$, $n = 6$).

b. Sphingosine-1-Phosphate Receptors

The gene expression levels of S1P receptors were measured again after introducing the inhibitors. Bar graphs in Fig. 21 show that stimulation with BK significantly increased mRNA levels of all 3 receptors. Despite an increase that can also be seen upon stimulation with S1P, this increase in receptors mRNA levels was not statistically significant. Although the degrees of significance vary from receptor expression to the other, it can be observed that there was a significant decrease in mRNA levels with BK+HOE140 and BK+SKi. These findings might implicate that BK can induce the upregulation of S1P receptors in microglia via both B2R and S1P dependent pathways.

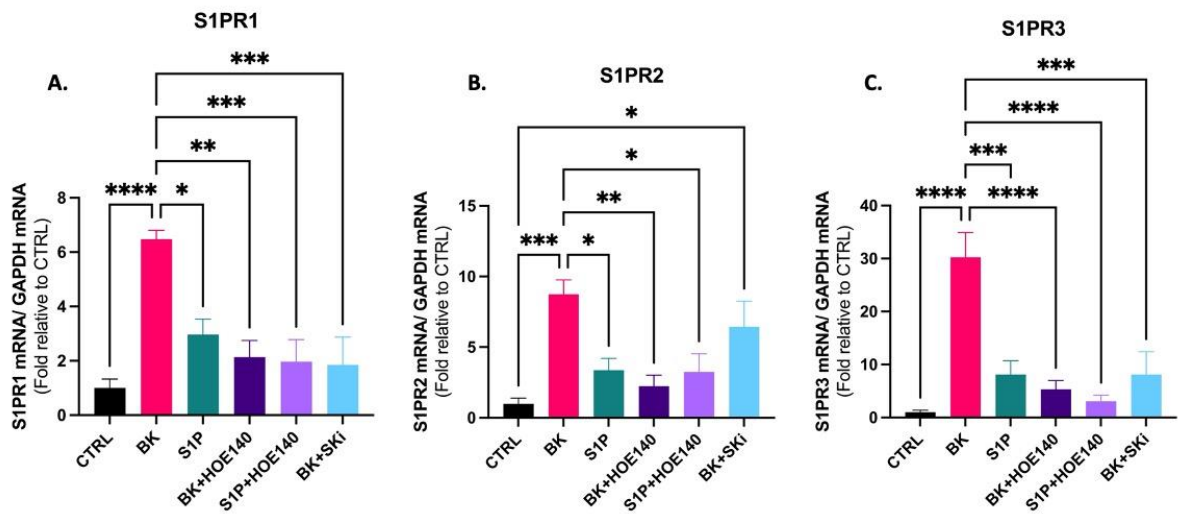


Figure 21. Expression of inflammatory markers in N9 cells. Bar graph plots showing mean and SEM for the fold changes in mRNA levels of (A.) S1PR1, (B.) S1PR2 and (C.) S1PR3 expressed relative to CTRL and to GAPDH mRNA levels. (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, $n = 6$).

I. Exploring the possibility of crosstalk among BK and S1P to induce oxidative stress in microglia

NADPH Oxidases (NOXs) have been proven to be upregulated in traumatic brain injury and neurodegenerative diseases [50]. For this reason, we were interested in measuring the gene expression of NOXs in microglia as an indicator of oxidative stress since NOXs are primary generators of reactive species [51]. In Fig. 22, the mRNA levels of NOX 1, 2 and 4 are shown relative to control and mRNA levels of GAPDH. Upon stimulation with BK, it is observed that there was a significant increase in gene expression levels for all NOXs in N9 cells. For both NOX 1 and 2, there was a significant decrease in mRNA levels with BK+HOE140 and BK+SKi compared to stimulation with BK (Fig. 22A and B). This could indicate that BK is inducing the expression of NOX 1 and 2 via B2R and a S1P dependent pathway, similar to what has been seen with S1P receptors.

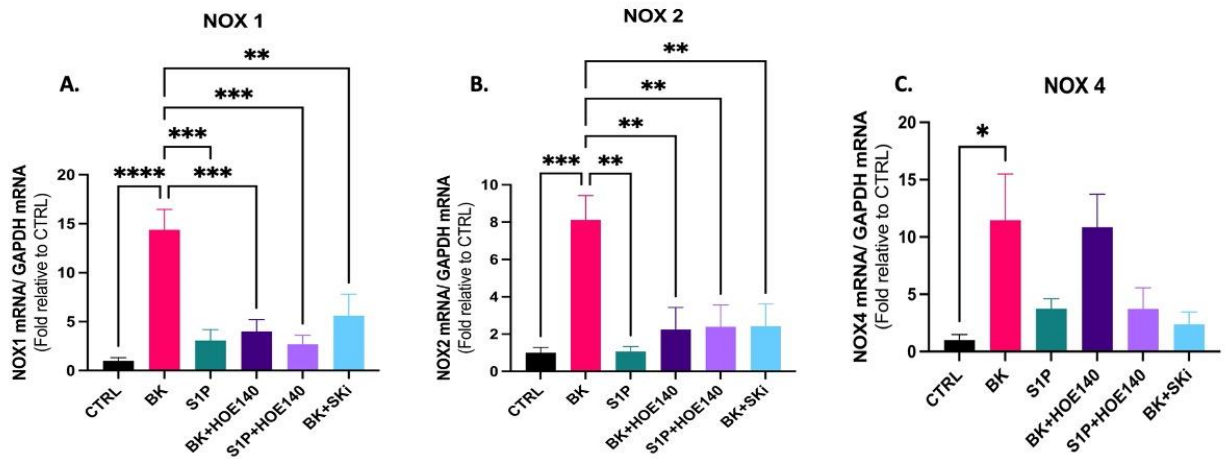


Figure 22. Expression of oxidative stress markers in N9 cells. Bar graph plots showing mean and SEM for the fold changes in mRNA levels of (A.) NOX 1, (B.) NOX 2 and (C.) NOX 4 expressed relative to CTRL and to GAPDH mRNA levels. (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, $n = 6$).

CHAPTER V DISCUSSION

Multiple studies have shown that inflammation and oxidative stress are involved in the pathogenesis of neurodegenerative diseases. However, the mechanistic pathways that could be involved in the process are still not fully mapped or understood. The plasma kallikrein kinin system has been implicated in inflammatory processes in various organ systems including respiratory, gastrointestinal, ophthalmic, and neuronal. B2R receptor is known to be constitutively expressed in cells under normal conditions and to have implications in acute inflammation. On the other hand, B1R is expressed in pathological conditions and has a role in chronic inflammation as well as induction of oxidative stress [31, 52]. A study done by Noda et al. has provided evidence that BK receptors, mainly B2R, are expressed in microglial cells using both RT-PCR and immunofluorescence, and that they have a key role in CNS inflammatory processes [53]. Another study have provided evidence that kinins, including BK through the action of its receptors, are involved in inflammatory processes that are linked to multiple neurodegenerative diseases including AD, PD and MS [54]. In a recent review by Rex et al., it has been reported that BK can mediate its effects through several pathways; of which BK can lead to brain injury and inflammation through a Protein Kinase C gamma (PKC- δ) dependent activation of mitogen activated protein kinase 1/3 (MAPK1/3) and nuclear factor-kappa B (NF- κ B) pathway [52]. Another study by Khan and Elkon focused on the role of BK through an extracellular signal-regulated kinase (ERK 1/2) pathway that is specifically involved in Alzheimer's disease. Their findings have shown that BK activates several pathways including the PKC and MAPK

pathways leading to phosphorylation of ERK 1 and ERK 2 which could be utilized as a clinical indicator of Alzheimer's disease [55].

Sphingosine-1-Phosphate has become the interest of many studies due to its diverse roles in multiple organs. The effects of S1P have been mostly explored in relation to Multiple Sclerosis, post-stroke, and ischemia events. Many of these studies provide evidence that S1P is involved in inflammatory processes that are mediated through sphingosine kinase 1 and S1P's receptors including S1PR1, S1PR2 and S1PR3 [56-59]. Moreover, a study by Karunakuran et al. showed that the S1P-S1PR2 axis is involved in proinflammatory effects in microglia in addition to impaired autophagy [42]. In another study, it was proven that the inhibition of SK1 induced a decrease in the expression of pro-inflammatory cytokines and nitric oxide in LPS-stimulated microglia, implicating that SK1 can be a therapeutic target of neuroinflammation and neurodegeneration [43]. With regards to S1P receptors, both S1PR1 and S1PR2 have been implicated in the pathogenesis of neurodegenerative diseases through potentiation of cerebrovascular inflammation [60, 61]. It is also important to note that studies have shown that the ERK 1/2 pathway implicated in the signaling cascade of BK is also necessary to phosphorylate SK1 to induce its translocation to the plasma membrane to catalyze the production of S1P [62, 63]. However, to date there are no studies that provide evidence of crosstalk between BK and S1P.

In this study, N9 cells were stimulated with BK and S1P to study their effects on microglial cells. Our results show that both BK and S1P induced an increase in the gene expression of proinflammatory and fibrotic markers as well as mediators of oxidative stress. Furthermore, both molecules also increased the mRNA levels of the microglial activation marker CD11b with a significant increase upon stimulation with BK.

Moreover, we have established that BK can induce the expression of SK1 and SK2 as well as S1PR1, 2 and 3 and that S1P has the potential to induce B1R and B2R. Following this step, we utilized the inhibitor of B2R, HOE140, and an inhibitor of Sphingosine Kinase to study the probability of crosstalk between BK and S1P. We found that BK induces the expression of pro-inflammatory cytokines most likely through its receptor B2R. This is observed as there was a slight but insignificant decrease in the expression of the pro-inflammatory markers IL-6 and IL-1b upon stimulation with BK after inhibition of B2R. Most likely, conducting further experiments would help elucidate the data and allow us to obtain significant results. However, it is worth noting that TNF- α mRNA expression decreased significantly upon stimulation with BK and HOE140 as compared to BK alone, clearly indicating that BK stimulated TNF- α via B2R. On the other hand, looking at the expression of fibrotic markers, data showed that BK might induce an increase in CTGF through an S1P dependent pathway. Moreover, TGF- β gene expression was decreased upon stimulation with S1P and HOE140 as compared to S1P alone, suggesting that S1P could potentially be working through a B2R dependent mechanism.

Investigation of the expression of BK receptors revealed that B2R mRNA levels were reduced significantly when Sphingosine Kinase was inhibited despite stimulation with BK, suggesting that Sphingosine Kinase might have a role in the upregulation of B2R receptors. Our data also revealed that inhibition of B2R caused a significant decrease in the expression of SK1 and SK2, suggesting the involvement of B2R in the upregulation of Sphingosine Kinases. Moreover, it was shown that while BK could significantly increase the expression of S1P receptors, this increase was markedly reduced when both B2R and sphingosine kinase were inhibited which suggests a

probable crosstalk between B2R and Sphingosine Kinases or S1P to induce the upregulation of S1PR1, 2 and 3.

In this study, we were also interested in investigating the effect of BK and S1P on inducing oxidative stress in microglial cells since oxidative stress is also implicated in the pathogenesis of neurodegenerative diseases [7]. Our data has shown that BK can induce the expression of NOX 1, 2 and 4, however, S1P showed no significant increase in any of the NOXs. Moreover, inhibiting both B2R and Sphingosine Kinase has markedly decreased the expression of NOX 1 and 2 upon stimulation with BK, implicating that BK can also induce oxidative stress through both B2R and S1P dependent pathways.

In conclusion, our results suggest the involvement of both BK and S1P in mediating inflammation in microglia, but only BK seemed to have a notable effect in inducing oxidative stress. Our data also maps a preliminary pathway that implies crosstalk between BK and S1P. Figure 15 depicts a proposed pathway for crosstalk among BK and S1P based on this study's findings and what we hope to be achieved in the future. We have established that BK can induce a significant increase in the expression of SK1 and 2 which catalyzes the production of S1P that in turn acts through its S1PR receptors. But is BK stimulating a direct expression of SK1 and 2 or is it acting through its B2R receptor? Furthermore, we have found that S1P might induce the expression of B2R, but this increase was not significant. Therefore, more studies can be conducted to determine whether S1P can stimulate B2R. As mentioned before, ERK1/2 seems to be a pathway that both BK and S1P mediate their effects through. But could ERK1/2 be the pathway involved in the crosstalk between the two molecules to induce inflammation and oxidative stress? In light of these findings, more thorough

investigation is needed to establish a mechanistic pathway which certainly defines this crosstalk among the two molecules. Both BK and S1P are known to mediate their effects through G-protein coupled receptors (GPCRs) and in turn, GPCRs mediate their effects as monomers, dimers or through crosstalk pathways. Both bradykinin receptors and sphingosine-1-phosphate receptors have been shown to be able to heterodimerize with other GPCRs. For instance, B2R has been proven to heterodimerize with thromboxane TP receptors, dopaminergic receptors, beta adrenergic receptor and angiotensin II type 1 receptor, among others [64-67]. Furthermore, a recent study has shown that S1PR5 forms a heterodimer with a cannabinoid receptor, G-protein coupled receptor 55 (GPCR55) [68]. Heterodimerization, however, is not the only way that two GPCRs can interact and crosstalk. Other crosstalk mechanisms include modulation of scaffold or kinase proteins and regulation of gene receptor expression [64].

CHAPTER VI FUTURE DIRECTIVES

Considering evidence of crosstalk among BK and S1P in this study, it is important to design future studies that can have more in depth investigation of mechanisms in which this crosstalk occurs. The possibility of heterodimerization among B2R and S1PR can be explored through fluorescence resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET) techniques. N9 cells can also be stimulated with BK and S1P at the same time to study whether they have an additive or synergistic effect. Moreover, signaling cascades downstream of B2R and S1PR can be studied since both molecules share common downstream signaling pathways such as ERK1/2 pathway. As this study has been conducted *in vitro*, it would strengthen our findings if a similar experimental design can be conducted *in vivo* to further study effects of BK and S1P on inflammation and its impact on neurodegenerative diseases.

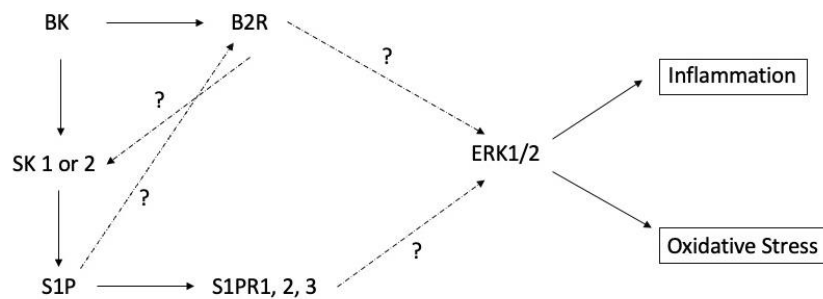


Figure 23. Schematic representation of probable crosstalk pathway between BK and S1P. Straight arrows indicate what is known or have been shown in this study. The dashed arrows indicate proposed pathways that could be studied in the future.

CHAPTER VII LIMITATIONS

This study had several limitations related to a lack of time and certain materials. We were interested in the study of inflammatory and oxidative stress markers at the protein level using western blot but due to lack of certain material this was not possible at this time. Also, due to the lack of certain inhibitors such as inhibitors for S1PR1 or S1PR3, we were not able to study the full effect of these receptors in mediating inflammation and oxidative stress and whether they are directly involved in crosstalk.

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