Kinin B1 Receptor Mediates Bidirectional Interaction between Neuroinflammation and Oxidative Stress

By

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May 2023

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Abstract

Hypertension is the leading risk factor for cardiovascular disease and affects nearly half the adults in the United States. It is well established that hypertension is a low-grade inflammatory condition and is associated with increased release of proinflammatory cytokines, elevated oxidative stress levels, and increased activity of the kallikrein-kinin system (KKS). The KKS is a family of vasoactive proinflammatory peptides that play a vital role in regulating blood pressure. Activation of kinin B1 receptor (B1R) results in increased inflammation and vasoconstrictive effects which can ultimately lead to hypertension. Previously, we showed that angiotensin II (Ang II) can upregulate B1R expression and can induce oxidative stress and neuroinflammation in primary neurons. However, the order at which this occurs has not yet been investigated. In this study, we aim to determine the relationships between neuroinflammation, oxidative stress, and activation of B1R in both primary hypothalamic neurons and primary hypothalamic microglia. Following stimulation with tumor necrosis factor (TNF), lipopolysaccharide

(LPS), or hydrogen peroxide (H_2O_2), we were able to identify a significant increase in reactive oxygen species production, inflammation, and B1R expression. Furthermore, we showed that B1R blockade using a B1R specific antagonist can attenuate these effects in both neurons and microglia. Together, these data provide novel evidence that the interaction between neuroinflammation, oxidative stress, and B1R activation in the brain is bidirectional and that blocking B1R may serve as a potential therapeutic target for neuroinflammation and oxidative stress in various disease pathologies.

KININ B1 RECEPTOR MEDIATES BIDIRECTIONAL INTERACTION BETWEEN NEUROINFLAMMATION AND OXIDATIVE STRESS

A Thesis

Presented to the Faculty of the Department of Pharmacology and Toxicology

East Carolina University

In Partial Fulfillment of the Requirements for the Degree

Masters of Science in Biomedical Sciences

By

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May 2023

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CHAPTER 1: Introduction

1.1 Hypertension overview

Hypertension remains the dominant risk factor in the development of cardiovascular disease, which is the leading cause of death worldwide (39). Nearly half the adults in America have hypertension, and of these, only one out of every three have their condition under control (10). Of these with hypertension, half of the individuals are unaware that they have this condition and of the half that is aware, only one-half of those have their condition under control (10). Hypertension on its own is asymptomatic, leaving undiagnosed patients with the detrimental risk of end organ damage. Therefore, uncontrolled- and undiagnosed- hypertension could result in end organ damage and severe cardiovascular and renal complications.

Resistant hypertension is becoming increasingly common and can be defined as hypertension that is poorly responsive to three concurrent antihypertensive agents following six months (42). A nonpharmacological, early intervention is lifestyle modification. Evidence shows that lifestyle modifications can decrease the need for drug therapy as well as reduce the risk of heart disease, stroke, end-stage renal disease, and peripheral vascular disease (23). However, the ability to maintain a healthy lifestyle is not sufficient or easy to comply with for all, therefore, majority of hypertensive patients will require pharmacological intervention to control and maintain their blood pressure. First line therapeutics for the treatment of hypertension

include diuretics, beta blockers, angiotensin-converting enzyme (ACE) inhibitors, calcium channel blockers (CCBs), and angiotensin receptor blockers (ARBs) (Figure 1) (19, 23). The etiology behind hypertension is still rather complex and unknown, leaving many patients with uncontrolled hypertension. Therefore, there is a detrimental need for novel antihypertensive modalities.

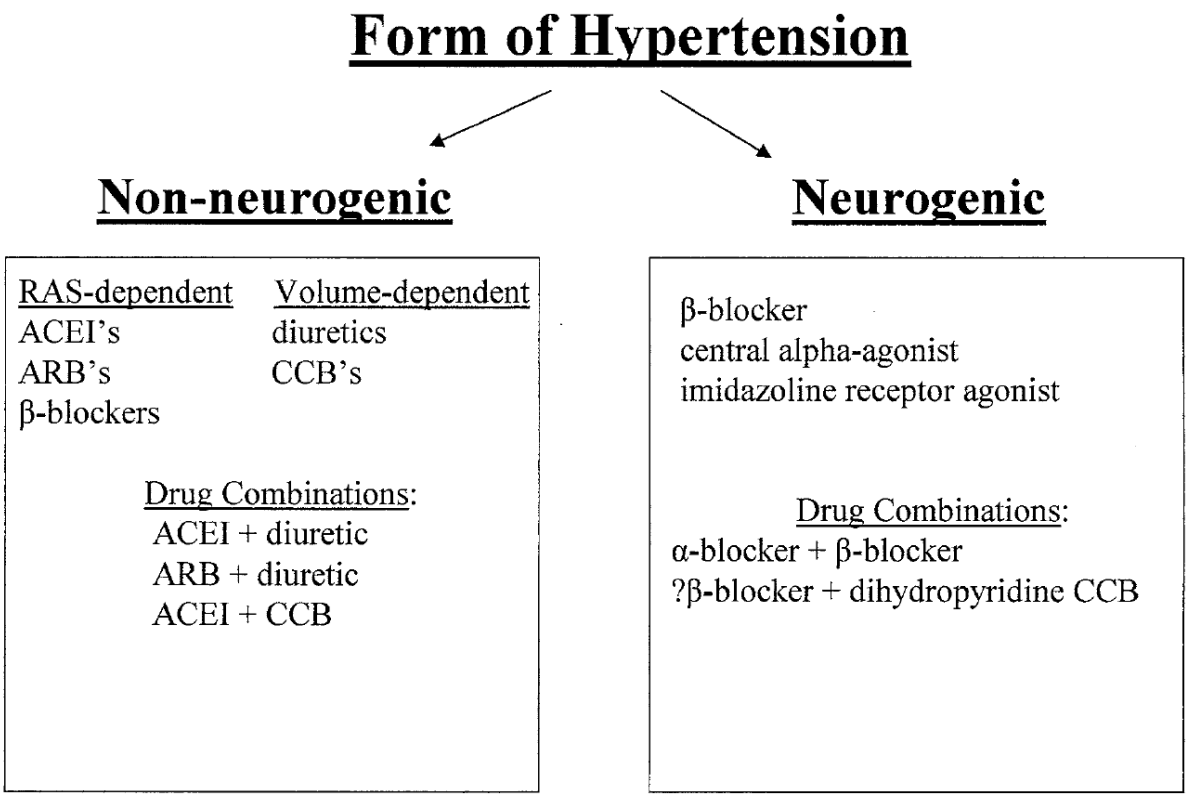

Figure 1. Proposed algorithm for selection of antihypertensive agents in individuals with neurogenic and non-neurogenic hypertension. RAS (renin-angiotensin system); ACEI (angiotensin-converting enzyme inhibitor); ARB (angiotensin receptor antagonist); CCB (calcium channel blocker). Resistant hypertension occurs as a result of a patient taking 3 or more of these agents concurrently at their optimal daily dose and seeing no significant reduction in blood pressure. Adapted from (19).

1.2 Neurogenic Hypertension

Neurogenic hypertension can be defined as hypertension where the sympathetic nervous system is a dominant factor as the driving force of hypertension (20). This is determined by elevated levels of norepinephrine and plasma catecholamines in hypertension that is unresponsive to first line treatments (16). An increased sympathetic tone has been previously investigated to involve a variety of hormones and cytokines such as oxidative stress, inflammatory cytokines, vasopressin, and tumor necrosis factor alpha (TNF) (20). Within the brain, there are major regions that play critical roles in initiating and maintaining increased blood pressure. Circumventricular organs include the subfornical organ, area postrema, median eminence, organum vasculosum of the lamina terminals, posterior pituitary, and subcommissural organ. These structures surround the brains ventricle and lack a distinct blood brain barrier (BBB), making them an easy target for blood pressure control (20). Other regions include medullary structures (regulate cardiovascular functions), nucleus tractus solitarius (cardiorespiratory reflex integration), caudal ventrolateral medulla (transmits baroreceptor inputs to RVLM), and the rostral ventrolateral medulla (resting cardiovascular function) (Figure 2) (19, 20). Most important to our lab and this study is the paraventricular nucleus (PVN) of the hypothalamus. This is a cluster of neurons within the hypothalamus that aids in development, neuroendocrine roles, growth, regulation of body fluid balance, and cardiovascular outputs (20). Stimulating glutamatergic neurons in the PVN causes autonomic dysfunction, leading to increased blood pressure, which allow the PVN to be a potential target in managing neurogenic hypertension.

As previously described, first line therapeutics involve targeting the RAS as well as diuretics, alpha and beta blockers, and so forth. In majority of patients with hypertension, the underlying mechanisms are the RAS and sodium/volume, therefore, successful treatment typically involves ACEIs, ARBs, calcium channel blockers, and/or diuretics. Whereas, in neurogenic hypertension, the main treatment options consist of alpha- and beta-adrenergic receptor blockers (20). However, neurogenic mechanisms often coexist with volume/sodium and RAS mediated mechanisms as well, therefore combining agents may be the most beneficial in some cases. One of the fundamental issues when managing neurogenic hypertension is due the BBB. The BBB plays a vital role in protecting the CNS from foreign material, but the downfall to this is they also prevent the permeation of therapeutics into the CNS.

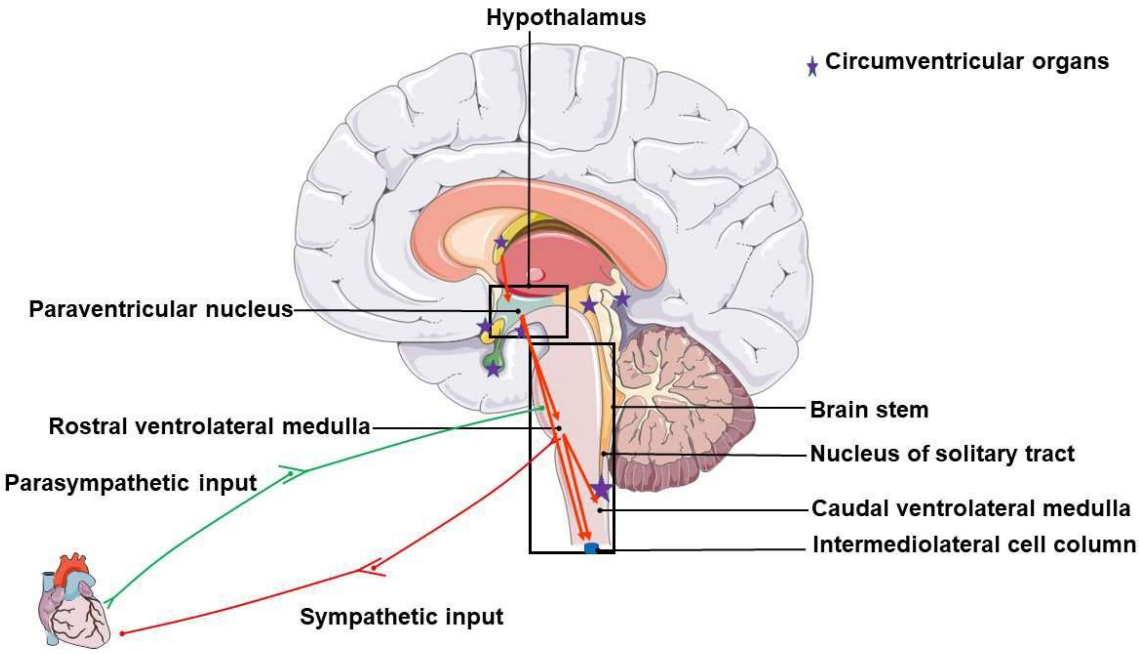

Figure 2. Brain regions that contribute to neurogenic hypertension. Purple stars indicate regions of the circumventricular organs. Adapted from (16).

1.3 Kallikrein-kinin system

The kallikrein-kinin system (KKS) is a family of vasoactive proinflammatory peptides that play an integral part in regulating blood pressure and inflammation and can often result in vasoconstriction and inflammation (36). Kinins are the key peptides of the KKS and have the ability to act as vasoactive hormones and inflammatory mediators (24). The KKS can be further broken down into inactive kinin precursor proteins, High Molecular Weight, and Low Molecular Weight Kininogens (HMWK/LMWK) (21). Following this, the precursor proteins can be further cleaved by tissue kallikrein (LMWK) or plasma kallikrein (HMWK) into bradykinin or kallidin. Bradykinin and its active metabolite des-arg9-bradykinin mediate their physiological effects through two G-protein coupled receptor subtypes, kinin B1 (B1R) and B2 (B2R) receptors (13, 17, 43). B2R is constitutively expressed within most tissues, whereas B1R expression is low under physiological conditions and is upregulated under stress conditions such as in the presence of inflammation and oxidative stress (15, 35). Furthermore, the acute phase of inflammation and pain response is associated with B2R, and the chronic phase of inflammatory response is associated with B1R (35).

Within the nervous system, the KKS is involved in the cerebral cortex, thalamus, hypothalamus, choroid plexus, and spinal cord (17). More specifically, expression of B1R is found on neurons, microglia, and astrocytes within the brain (35). When B1R is activated, it can influence immune responses through microglial activation and cytokine production. B1R expression can be activated through its own agonists (DAKD and DABK), bacterial endotoxins (LPS), and inflammatory cytokines (TNF or IL-6) (28, 35).

Therefore, the activation of B1R may play a vital role in neuroinflammation and the pathogenesis of cardiovascular disease.

It is well recognized that B1R plays a significant role in mediating inflammatory response in several cardiovascular diseases such as hypertension, diabetes, stroke, heart failure, and atherosclerosis (Figure 3) (35). Previous studies have shown that expression of B1R is upregulated in the hypothalamus of deoxycorticosterone acetate-salt-treated (DOCA) hypertensive mice and that specifically, B1R expression is increased in the neurons of the PVN within hypertensive mice (34). Along with this, an increase of proinflammatory cytokines was also seen which supports the idea of B1R upregulations and neuroinflammation in the pathogenesis of hypertension.

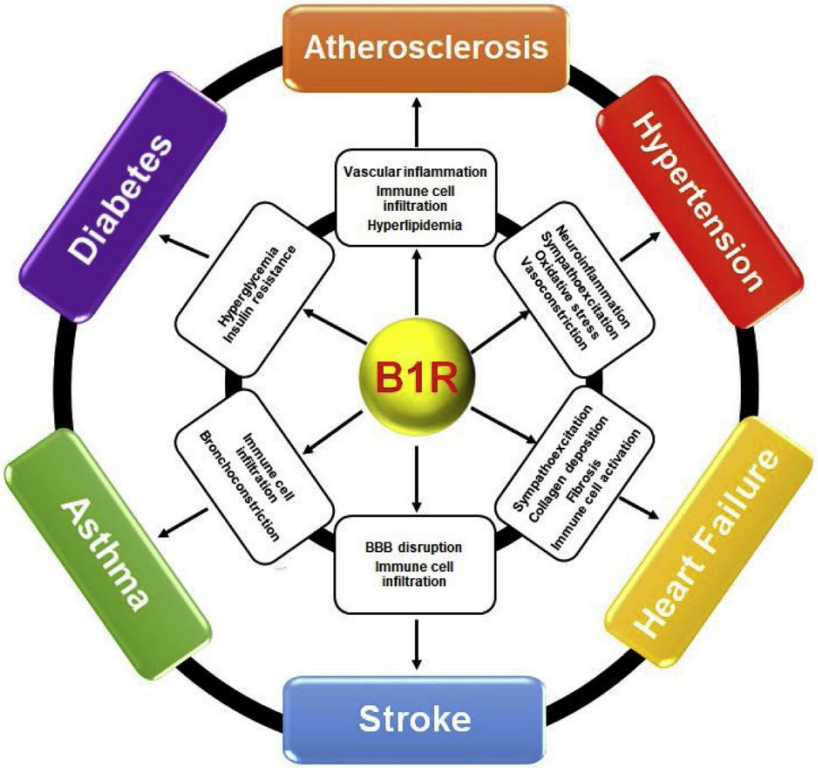

Figure 3. Schematic representation of kinin B1R mediated processes involved in cardiovascular diseases. Activation of kinin B1 receptor (B1R) can play a pivotal role in cardiovascular disease pathogenesis by modulating various physiological and pathophysiological processes such as immune cell infiltration, sympathoexcitation, oxidative stress, and neuroinflammation. Adapted from (35).

1.4 Neuroinflammation and oxidative stress in hypertension

Over the years, there has been accumulating evidence that implicates proinflammatory cytokines and reactive oxygen species (ROS) are related to the pathophysiological features found in hypertension (44). Furthermore, cells that participate in innate and adaptive immune responses lead to blood pressure elevation by driving the generation of ROS in cardiovascular control organs including the nervous system, vasculature, and kidney (6). Within the brain, the PVN has been established to be a vital central cardiovascular regulation that models sympathetic nerve activity as well as blood pressure in hypertension. The brain is specifically sensitive to oxidative damage due to the high and specific metabolic activity, therefore neuronal cells are highly susceptive to damage from oxidative stress (12). In the PVN specifically, ROS promotes the development of hypertension through an impaired balance between sympathetic and parasympathetic tones of the autonomic nervous system (44).

Oxidative stress is characterized by an excessive production of ROS which overwhelms the antioxidant systems, resulting in an altered oxidation reduction state (38). Under physiological conditions in the body, ROS are required for normal functions, however, under pathological conditions, excessive ROS production leads to oxidative stress, DNA damage, protein oxidation, and lipid peroxidation (12, 38). The major sources of ROS production are mitochondria and inflammation (6, 12). Mitochondrial derived ROS have been shown to play a role in central regulation of systemic cardiovascular functions and is often associated with hypertension in various experimental models (7). Within the mitochondria, an imbalance between ROS and mitochondrial antioxidants has been found to contribute to the pathogenesis of hypertension.

Microglia activation has been shown to be a hallmark characteristic in the development of hypertension. In cardiovascular disease, once microglia are activated, they release proinflammatory cytokines in response to pathological alterations (44). Therefore, once hypertension is established, these activated microglia promote inflammation which can exacerbate hypertension. In the brain, microglia express both B2R and B1R receptors, and evidence has shown that neuropeptides, such as kinins, can regulate brain inflammation and can affect microglial function (1).

Generation of ROS have the ability to stimulate transcription factors, chemokine and cytokine production, proinflammatory genes, and recruit and activate inflammatory and immune cells that play a significant role in cardiovascular inflammation (Figure 4) (38). Conversely, evidence suggests that activation of inflammatory responses in the PVN can contribute to ROS production, ultimately driving central mediated blood pressure elevation (6). Therefore, inflammatory responses can induce oxidative stress, as well as ROS generation can induce immune activation, allowing us to believe that they operate cooperatively in the pathogenesis of hypertension. The interaction shown here between oxidative stress and inflammation suggests that targeting one in treatment may also limit the other.

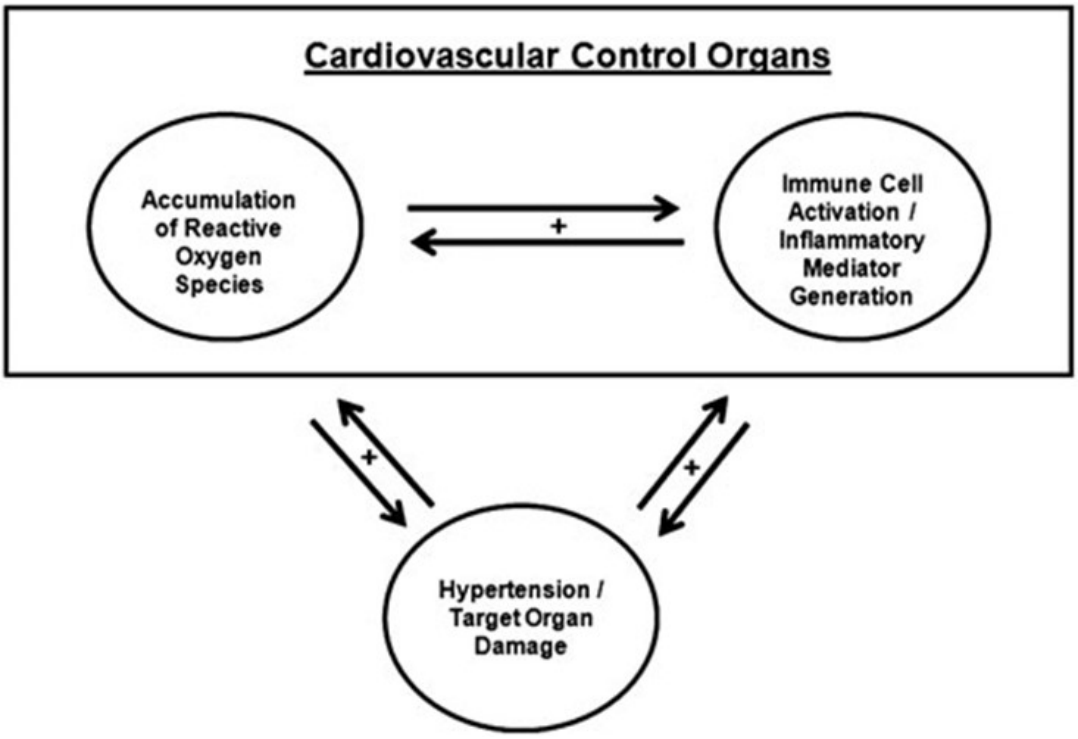

Figure 4. Oxidative stress and inflammatory responses act synergistically in the pathogenesis of hypertension. Enhanced immune responses result in blood pressure elevation and the ensuing end-organ injury by driving reactive oxygen species (ROS) generation in cardiovascular control organs, including the vasculature, the kidney, and the nervous system. Conversely, ROS generated within these cardiovascular control organs can promote activation of circulating immune cells that exaggerates hypertensive responses following an initial injury signal. Adapted from (6).

1.5 Main hypothesis and specific aims

Based on the literature and previous studies carried out in our lab relating to kinin B1 receptor activation and its role in hypertension, I formulated the hypothesis: neuroinflammation and oxidative stress can be mediated in the brain by blocking kinin B1 receptor.

Prior studies utilizing DOCA mice models of hypertension revealed that B1R expression is upregulated in the brain in hypertensive mice (34). Furthermore, this study also showed that protein levels of neuroinflammatory markers were significantly increased in DOCA-salt hypertensive mice, indicating elevated neuroinflammation. In turn, this effect was reduced in B1R knockdown mice (34). Blockade of B1R by use of knockdown mouse also blunted DOCA-salt-induced oxidative stress in the brain (34). In primary hypothalamic neurons treated with angiotensin II (Ang II), B1R expression was significantly upregulated. Along with B1R expression, proinflammatory cytokine production and the oxidative potential was significantly increased in neurons stimulated by Ang II (25). Interestingly, pretreatment with R715, a B1R specific antagonist, was able to ameliorate Ang II induced B1R expression, inflammatory markers, and oxidative potential (25).

Therefore, the aims of this project are:

Aim 1: Determine the interaction between B1R activation, neuroinflammation, and oxidative stress in primary hypothalamic neurons and primary hypothalamic microglia.

Aim 2: Determine if B1R blockade using B1R specific antagonists can prevent neuroinflammation and oxidative stress in primary hypothalamic neurons and primary hypothalamic microglia.

CHAPTER 2: Materials and Methods

2.1 Primary Hypothalamic Neuronal Isolation

Primary hypothalamic neurons were cultured and maintained from neonatal mice pups as described in our previous studies (25, 26, 40). The experimental protocols used for breeding mice were approved by East Carolina University Animal Care and Use Committee (AUP #W261) and were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Mouse pups were deeply anesthetized prior to decapitation using isoflurane (4%) in an oxygen flow (1 L/min). The brain was collected using sterile dissection scissors into a 10 cm petri dish containing Hank's Balanced Salt Solution (HBSS) (Gibco 14,175-079). Hypothalamus was removed and transferred to a 35 mm plate then tissue was minced into small pieces using a sterile blade. The minced tissue was transferred to a conical tube and was rinsed twice using HBSS and then digested in a 15 mL conical tube with HBSS containing 1% trypsin (T1426 Sigma-Aldrich, St. Louis, MO, USA) and 1.5 kU/mL DNaseI (D5025 Sigma Aldrich) for 10 min at 37℃. Following enzymatic dissociation. The tissue was rinsed twice with HBSS containing 20% FBS followed by two rinses with HBSS. Using HBSS containing DNaseI, the tissue was further homogenized using a pipette with a 1 mL pipette tip (six times) followed by using a 200 uL pipette tip (six times) attached to a 10 mL serological pipette. Following dissociation, cells were spun down by centrifugation and the cell pellet was collected and resuspended in complete Neurobasal culture medium supplemented with 2% B27, 0.5 mM GlutaMax, and penicillin/streptomycin (100 U/mL and 100 ug/mL) (Gibco). Neurons were then plated into poly-L-lysine-coated cell culture

plates and grown in a humidified atmosphere of 5% CO2-95% air at 37℃ at a density of 50,000 cells per mL. Cells are cultured for four days then treated with cytosine arabinofuranoside (Ara-C, 2 uM, Sigma Aldrich C1768) to suppress non-neuronal cell proliferation. Neurons were cultured for at least 10 days prior to experimentation. Neurons were treated with either LDABK (300nM), H2O2 (10 uM), TNF (10ng), or LPS (100ng) for 24 hours with or without pretreatment of B1R selective antagonists, R715 (10uM) or SR (10uM), for two hours prior to stimulation. All drug treatments were dissolved in sterile PBS as a vehicle control. Concentrations used were derived from preliminary data generated or previous studies within the lab.

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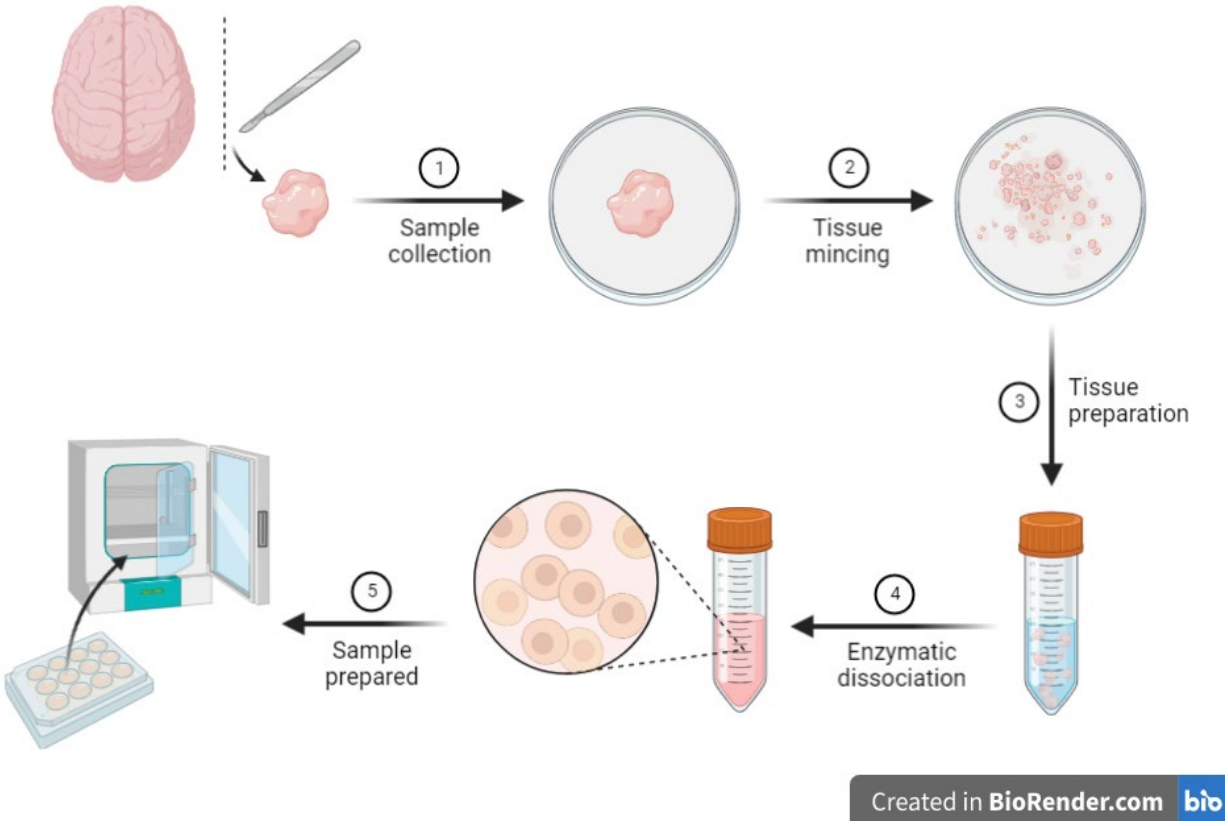

Figure 5. Schematic of primary hypothalamic neuron isolation from neonatal mice pups. Created using BioRender.com.

DRUGS	Concentration
Vehicle (DMSO or 1xPBS)	
LDABK	300 nM
R715	10 uM
TNF	10 ng
LPS	100 ng
H_2O_2	10 uM
SR	10 uM

Table 1. List of pharmacological agents used and their concentrations.

2.2 Primary Microglia Isolation

Neonatal mice pups were deeply anesthetized prior to decapitation using isoflurane (4%) in an oxygen flow (1 L/min). The brain was collected using sterile dissection scissors into a 10 cm petri dish containing Hank's Balanced Salt Solution (HBSS) (Gibco 14,175-079). Hypothalamus was removed and transferred to a 35 mm plate then tissue was minced into small pieces using a sterile scalpel blade. The minced tissue was rinsed with HBSS then transferred to a 50 mL conical tube containing 2.5% trypsin (Gibco #15090-046) in HBSS and incubated in 5% CO2-95% air at 37℃ for 15 min. After incubation, 1.5 mL of trypsin inhibitor (R-007-100) and 100uL of DNase was added for digestion then spun down by centrifugation. The supernatant was aspirated, and the pellet was titrated using DMEM+4.5 g/L D-Glucose + 110 mg/L sodium + 5 mL pen strep + 50 ml FBS. The tissue was then further homogenized using a pipette with a 1 mL pipette tip (six times) followed by using a 200 uL pipette tip (six times) attached to a 10 mL serological pipette. The homogenous cell suspension was then transferred to a 15 ml conical tube and was spun down by centrifugation. The supernatant was aspirated, and the pellet was resuspended by culture media. Cells were plated into poly-L-lysine-coated T-75 flasks at a density of 50,000 cells/cm2 and grown in a humidified atmosphere of 5% CO2-95% air at 37℃. The following day, media was removed and replaced with fresh warmed media. Cells were cultured for 5-10 days, or until 70% confluency was reached. Once cells reached confluency, we tapped the flask to loosen the microglial cells and removed the media containing these cells. Microglia were then plated into poly-L-lysine-coated cell culture plates and grown in a humidified atmosphere of 5% CO2-95% air at 37℃. After two hours of incubation in the cell culture plate, media was removed,

and we added back fresh warmed media. Microglia must adhere prior to experimentation. Cultured microglia were validated using immunofluorescence labeling with a microglia and macrophage-specific marker, IBA1 (Ionized calcium binding adaptor molecule 1), a glial cell-specific marker, GFAP (glial fibrillary acidic protein), and a neuron-specific cytoskeletal marker, MAP2 (microtubule-associated protein 2). Once validated that we have primary hypothalamic microglia cultures, microglia were treated with either LDABK (300 nM), H_2O_2 (10 uM), TNF (10 ng), or LPS (100 ng) for 24 hours with or without pretreatment of R715 (10 uM) or SR (10 uM) for two hours prior to stimulation. All drug treatments were dissolved in sterile PBS as a vehicle control. Drug concentrations were determined by preliminary data and previous studies from the lab.

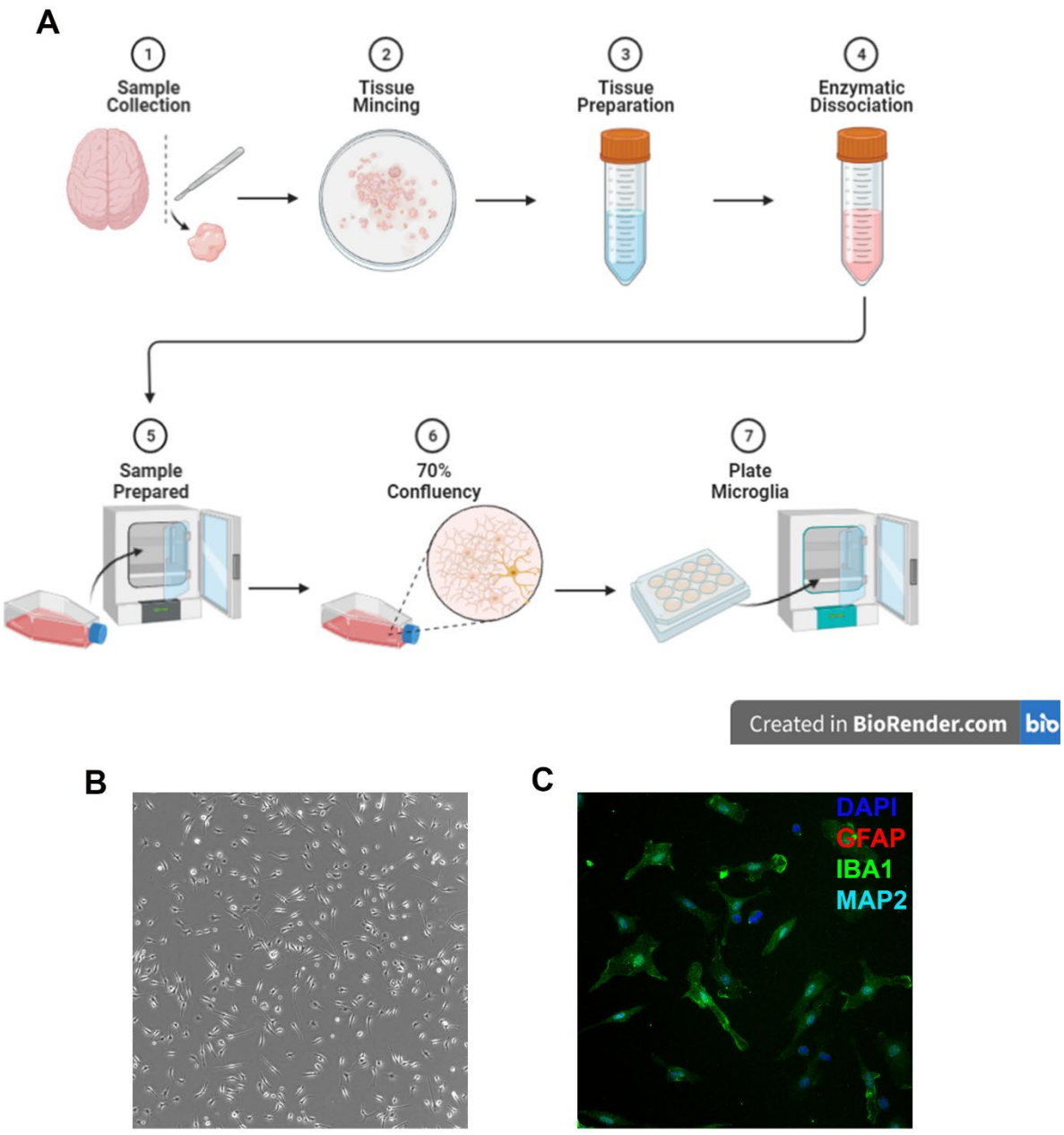

Figure 6. Schematic overview of (A) primary microglia isolation from neonatal mice pups. (B) Live cell imaging of microglia following full culture protocol. (C) Triple immunocytochemistry staining to reveal cultured microglia (green), with no indication of neurons (teal) or astrocytes (red). Created using Biorender.com.

2.3 Immunocytochemistry (neurons)

Once neurons were cultured for at least 10 days on poly-L-lysine-coated glass cover slips in 12-well plates and treated with various agonists for time points, they were fixed with 4% paraformaldehyde for 15 min. Neurons were rinsed three times for 5 min using 1xPBS followed by permeabilization with 1xPBS containing 0.1% Triton X-100 for 10 min. Neurons were blocked with 2% normal donkey serum in 1XPBS containing 0.2% Tween20 for 1 h at room temperature. Following this, cells were incubated overnight at 4℃ with MAP2 antibody (NBP3-05552, lot 16278-011222, Novus Biologicals, 1:500) diluted in 1xPBS containing 1% normal donkey serum and 0.2% Tween20. The next morning, cells were rinsed with 1XPBS containing 0.2% Tween20 three times then incubated with appropriate Alexa Fluor conjugated secondary antibodies for 2 h at room temperature. Neurons were washed three times with 1xPBS + 0.2% Tween20 then double immunostaining was performed with appropriate antibodies for detection of B1R (#ABR-011, Alomone labs, 1:500 dilution) or TNF (Cell Signaling 11948S, 1:500 dilution). Coverslips were mounted onto slides using ProLong Gold antifade reagent with DAPI (Invitrogen). Images were captured using an ECHO Revolve Microscope.

Antibody	#	Dilution	Buffer
B1R	Alomone AAR-011	1:500 (o/n)	1% NDS, 0.2% Tween20, 1xPBS
MAP2	Novus - NBP3-05552	1:500 (o/n)	1% NDS, 0.2% Tween20, 1xPBS
TNF	cell signaling 11948S	1:250 (o/n)	1% NDS, 0.2% Tween20, 1xPBS
IBA1	WAKO 011-27991	1:1000 (o/n)	1% BSA, 0.3% Tritonx100, 1xPBS
GFAP	Invitrogen 53-9892-82	1:1000 (2hr)	1% NDS, 0.2% Tween20, 1xPBS

Table 2. Primary antibodies used with reference number, dilution, and buffer.

2.4 Immunocytochemistry (microglia)

Once cells are grown to confluency and microglia are cultured onto poly-L-lysine-coated glass cover slips in 12-well plates and treated with various agonists, they were fixed with 4% paraformaldehyde for 15 min. The microglia were washed three times with 0.3% Triton X-100 before blocking with 1% BSA and 0.3% Triton X-100 in 1xPBS for two hours. Microglia were incubated overnight at 4℃ with primary antibody IBA1 (WAKO 011-27991, 1:1000 dilution) in 1% BSA and 0.3% Triton X-100 in 1xPBS. The following morning, cells were washed three times with 0.3% Triton X-100 in 1xPBS prior to incubation with appropriate Alexa Fluor conjugated secondary antibody for 2 h at room temperature. Cells were washed three times with 0.3% Triton X-100 in PBS then double immunostaining was performed with appropriate antibodies for detection of B1R (#ABR-011, Alomone labs, 1:500 dilution) or TNF (Cell Signaling 11948S, 1:500 dilution). Coverslips were mounted onto slides using ProLong Gold antifade reagent with DAPI (Invitrogen). Images were captured using an ECHO Revolve Microscope.

2.5 DHE Measurement of ROS

Dihydroethidium (DHE) is relatively specific for the measurement of superoxide and was used to measure the levels of reactive oxygen species produced. DHE exhibits a blue fluorescence until it is oxidized and interacts with the cell's DNA, resulting in a bright fluorescent red staining of the nucleus. The media was removed from primary neurons and microglia that have been cultured in 12-well poly-L-lysine glass coverslips and were washed twice with room temperature 1xPBS. Cells were given 10uM DHE

(Invitrogen D11347) and incubated in a light protected humidified chamber at 37℃ for 15 minutes. Following the 15 minutes, DHE solution was removed, and coverslips were mounted onto slides using Prolong Gold antifade reagent with DAPI. Images were obtained with a fluorescent microscope (TEXRED, Echo Revolve). Mean fluorescence intensity of the image was quantified using ImageJ software (NIH). To further confirm these results, we also measured ROS production by spectofluorometry using DHE probes in neurons. Neurons were cultured in 48-well plates and were briefly treated for their respective groups. The media was removed from the 48-well plate and neurons were rinsed twice with room temperature PBS prior to incubation with 10 uM DHE solution. Following a 15-minute incubation at 37℃, the plate was read via spectrofluorometer (Tecan Infinite m200), and fluorescence of the neurons was detected at an excitation wavelength of 488 nm and emission wavelength of 610 nm. Data are expressed as total fluorescence (relative fluorescent units, RFU).

2.6 MitoSOX/MitoTracker (neurons)

To measure relative mitochondrial ROS production, we used MitoSOX red (Life Technologies M36008) fluorescent signal and normalized it to MitoTracker (Invitrogen M7514) fluorescent signal as described previously (31). MitoSOX red is generated as a byproduct of oxidative phosphorylation and is highly selective for measuring superoxide production in the mitochondria of live cells. MitoTracker is used to label active mitochondria in the cell. To do this, we cultured neurons in a poly-L-lysine coated 48-well plate and treated them for their respective groups. Following this, DMSO was added to create stock solutions of 5 mM MitoSOX and 1 mM MitoTracker. In a 15 ml conical tube containing HBSS, MitoSOX red was added to a final concentration of 2.95 uM and

MitoTracker was added to a final concentration of 50 nM. Cells were incubated with this solution for 30 min at 37℃, then this was removed and replaced with warmed HBSS. The plate was read via spectrofluorometer (Tecan Infinite m200) at 510-nm excitation and 580-nm emission for MitoSOX red signal and 490-nm excitation and 516-nm emission for the MitoTracker green signal. The MitoSOX fluorescent signal (amount of mitochondrial ROS production) was normalized to MitoTracker fluorescent signal (total mitochondrial content).

2.7 H2DCFDA (microglia)

To measure active ROS levels in microglia, we performed an CM-H2DCFDA assay (Invitrogen C6827). CM-H2DCFDA is a chloromethyl derivative of H2DCFDA and is an indicator of ROS in cells, specifically live cells as it exhibits better retention for long term studies. To do this, we cultured microglia in a poly-L-lysine coated 48-well plate and treated them for their respective groups. Shortly before beginning experimentation, H2DCFDA was reconstituted in DMSO to make the stock solution. The media was removed from the microglia culture plate and cells were incubated with 10 uM H2DCFDA probe in warmed HBSS. Cells were incubated at 37℃ for 30 minutes. The probe solution was then removed and warmed HBSS was returned to the cells and fluorescence was analyzed via spectrofluorometer (Tecan Infinite m200) with excitation 488nm and emissions 530nm.

2.9 Statistical Analysis

Statistical analyses were performed using GraphPad Prism 9 (GraphPad Software). Data are presented as mean ± standard error of the mean (SEM). Using one-way analysis of variance followed by Tukey's multiple comparisons test, multiple comparisons were made, and differences were considered statistically significant at $p<0.05$.

CHAPTER 3: Results (Neurons)

3.1 B1R activation induces neuroinflammation and ROS production in primary hypothalamic neurons.

B1R expression can be induced by its own agonists, such as LDABK, as previously shown (4, 25, 41). To confirm the role of a B1R selective agonist, LDABK, in the activation of neuroinflammation and reactive oxygen species generation we first used primary neurons that were cultured from the hypothalamus of neonatal mice. The neurons were cultured for 10 days prior to treatment with LDABK (300 nM, 24 h) with or without pretreatment using R715 (10 uM, 2h), a B1R specific antagonist. Using immunofluorescence, we were able to confirm that LDABK treatment increases B1R expression as shown in Figure 7A (25, 40). These increases in expression were prevented following a 2 h pretreatment with R715 which further indicates that LDABK induces B1R expression and can be blocked using B1R antagonism. Reactive oxygen species have been shown in previous studies to play an intricate role in the development of neurogenic hypertension (2, 29). To investigate the role of B1R activation on ROS production, we treated the neurons with dihydroethidium (DHE) and measured the oxidative potential within the cells. Treatment with LDABK enhanced DHE red fluorescence, whereas this increase was diminished using a B1R antagonist (Figure 7B). These results indicated that LDABK-induced B1R activation increases the generation of ROS in primary hypothalamic neurons. To confirm these findings, we used a microplate DHE assay and once again showed that LDABK stimulation results in a significant rise in

ROS production, and that this can be mitigated by use of a specific B1R antagonist (Figure 7D). Mitochondria are known to be a significant source of ROS production, but the role of mitochondrial oxidative stress is rather unknown in the pathophysiology of hypertension (7). To determine this, we performed an assay combining MitoSOX and MitoTracker to determine the generation of mitochondrial ROS normalized to the total mitochondrial content. Our results showed that LDABK stimulation significantly increases mitochondrial ROS production, but the prior use of B1R antagonist SR can prevent this increase (Figure 7E). Lastly, to determine if B1R activation can induce neuroinflammation, we used double immunostaining to determine proinflammatory cytokine TNF expression. We found that LDABK increases TNF expression as indicated by an increase in red fluorescence, and that pretreatment with SR can ameliorate this increase (Figure 7C). These results provide evidence that B1R activation in primary hypothalamic neurons induces neuroinflammation and increases ROS generation, and that B1R antagonism may attenuate these effects.

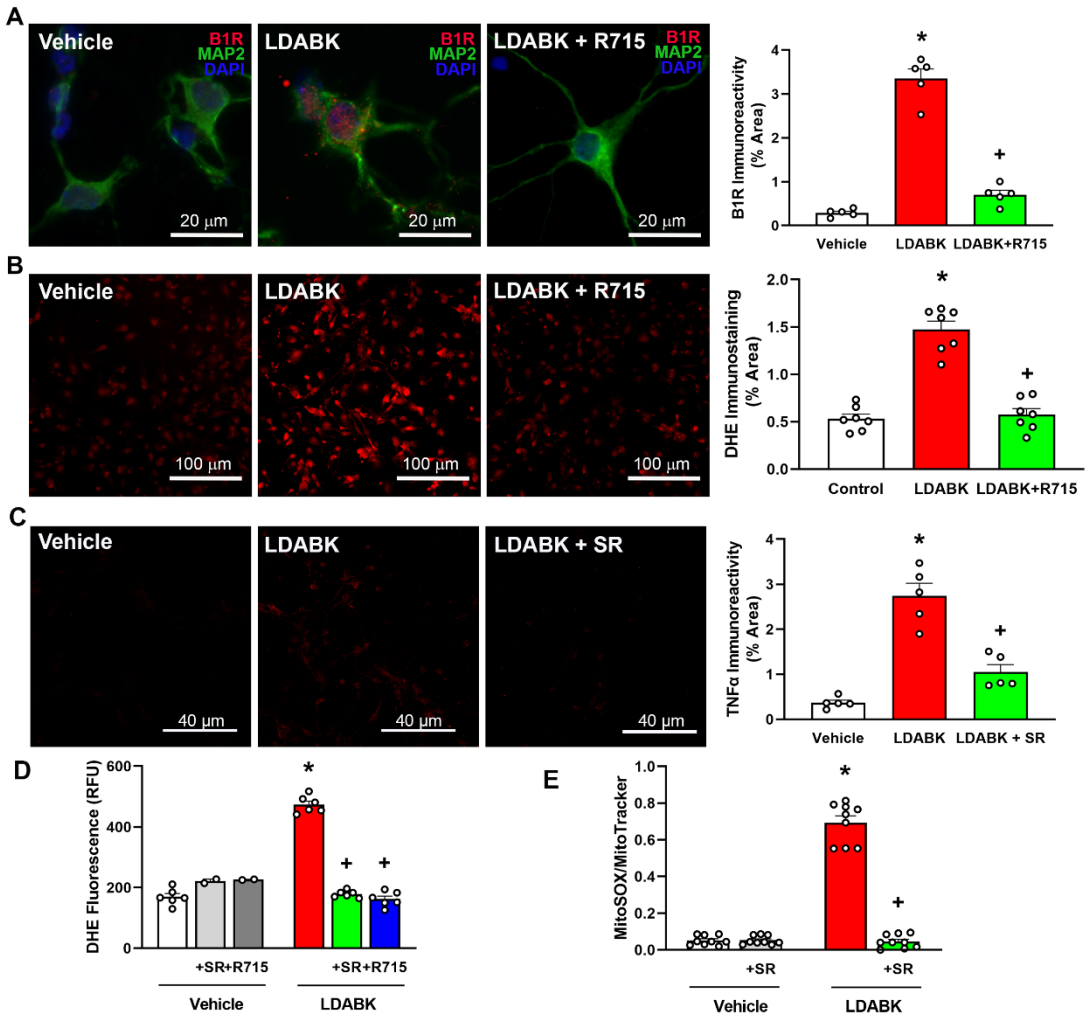

Figure 7. LDABK treatment on primary neurons enhances B1R expression. (A) Immunocytochemistry fluorescence and quantification for B1R expression (n=5/group). (B) Superoxide production measured by DHE fluorescence (n=7/group). (C) Immunofluorescence staining and quantification for TNF expression (n=5/group). (D) ROS generation using DHE microplate assay (n=6/group). (E) MitoSOX mitochondrial oxidative stress production normalized to MitoTracker total mitochondrial content (n=9/group). Statistical significance: One-way ANOVA followed by Tukey's multiple comparison test. *p<0.05 compared to vehicle. +p<0.05 compared to LDABK.

3.2 TNF stimulation increases expression of B1R and ROS in hypothalamic neurons.

It is known that B1R activation participates in neuroinflammation and is a crucial factor in the pathogenesis of neurogenic hypertension (38). Therefore, we stimulated our primary neurons with TNF, a proinflammatory cytokine, to determine if blocking B1R could prevent inflammation induced oxidative stress and B1R expression. We first determined if TNF (10 ng, 24 h) could induce B1R activation and found that TNF significantly amplifies B1R expression (Figure 8C). In addition, B1R blockade was able to attenuate this affect, allowing us to believe that B1R blockade can prevent inflammation induced B1R expression. Since TNF initiates a proinflammatory cascade which stimulates other cytokines, chemokines, and produces ROS, we sought to investigate the role of B1R blockade on inflammation-induced oxidative stress (33). To identify this, we treated primary neurons with 10ng TNF with or without 2 h preincubation of 10uM R715 and measured DHE immunofluorescence. The results indicated that pretreatment with R715 reduces TNF induced ROS generation as shown by a decrease of red fluorescence compared to the TNF treated alone group (Figure 8D). Using a DHE microplate assay, we were able to further confirm these results (Figure 8B). A MitoSOX/MitoTracker assay was used once again to determine the levels of mitochondria oxidative stress relative to the total mitochondrial content. Our data showed that TNF stimulation significantly increased oxidative stress and that B1R blockade can partially minimize this effect (Figure 8A). Furthermore, we wanted to determine if B1R blockade could reduce neuroinflammation stimulated by TNF. We found that pretreatment with SR can lessen the effects of TNF induced inflammation as shown by immunofluorescence (Figure 8E).

This data concludes that B1R blockade can mitigate the rise in inflammation induced B1R expression and ROS generation.

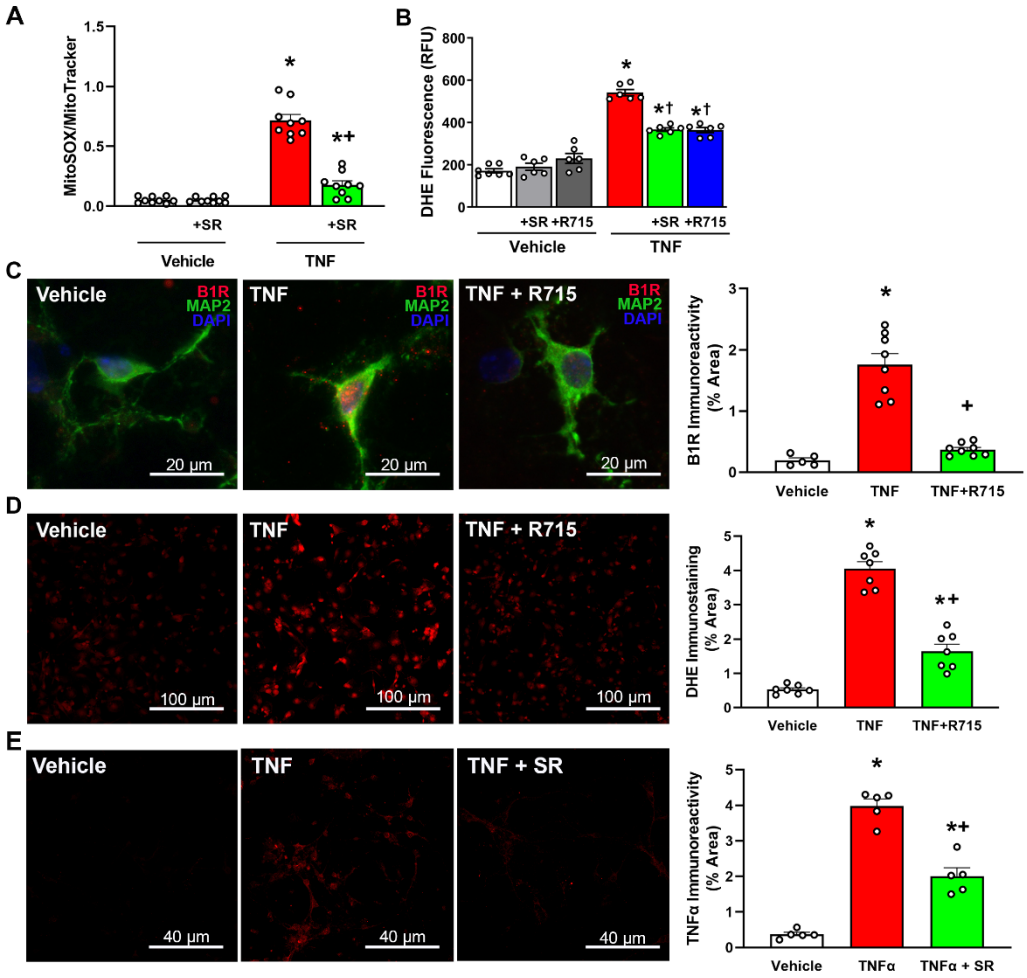

Figure 8. TNF stimulation increases neuroinflammation in primary hypothalamic neurons. (A) MitoSOX mitochondrial oxidative stress production normalized to MitoTracker total mitochondrial content (n=9/group). (B) ROS generation using DHE microplate assay (n=6/group). (C) Immunofluorescence staining and quantification for B1R expression (n=5/group). (D) Superoxide production measured by DHE fluorescence (n=7/group). (E) Immunofluorescence staining and quantification for TNF expression (n=5/group). Statistical significance: One-way ANOVA followed by Tukey's multiple comparison test. *p<0.05 compared to vehicle. +p<0.05 compared to TNF.

3.3 LPS stimulation increases expression of B1R, ROS, and neuroinflammation in primary hypothalamic neurons.

LPS has been shown to induce neuroinflammation through the stimulation and release of proinflammatory cytokines, generation of oxidative stress and inducing inflammatory responses (14). Immunocytochemistry revealed that stimulation with 100 ng LPS can induce B1R activation whereas 2-h pretreatment with R715 prior to stimulation was able to significantly attenuate these effects (Figure 9C). Next, we investigated the role of LPS in reactive oxygen species production. DHE immunofluorescence showed that once again LPS can induce ROS production, and that this increase can be suppressed following pretreatment with a B1R antagonist (Figure 9D). To confirm this, we carried out a DHE microplate assay where the results revealed a similar trend, allowing us to conclude that B1R blockade can mitigate LPS induced ROS production (Figure 9B). Levels of mitochondria oxidative stress were determined using a MitoSOX/MitoTracker assay and once again confirmed that B1R blockade can lessen LPS-induced oxidative stress (Figure 9A). Since LPS has been shown to cause the release of inflammatory cytokines, we used immunofluorescence to determine the role of LPS on TNF expression in primary neurons (14, 27). Our data shows that LPS stimulation induces TNF expression in primary neurons, and that B1R antagonist SR can mitigate this increase (Figure 9E). This data allows us to believe that LPS acts, in part, through a B1R mediated pathway.

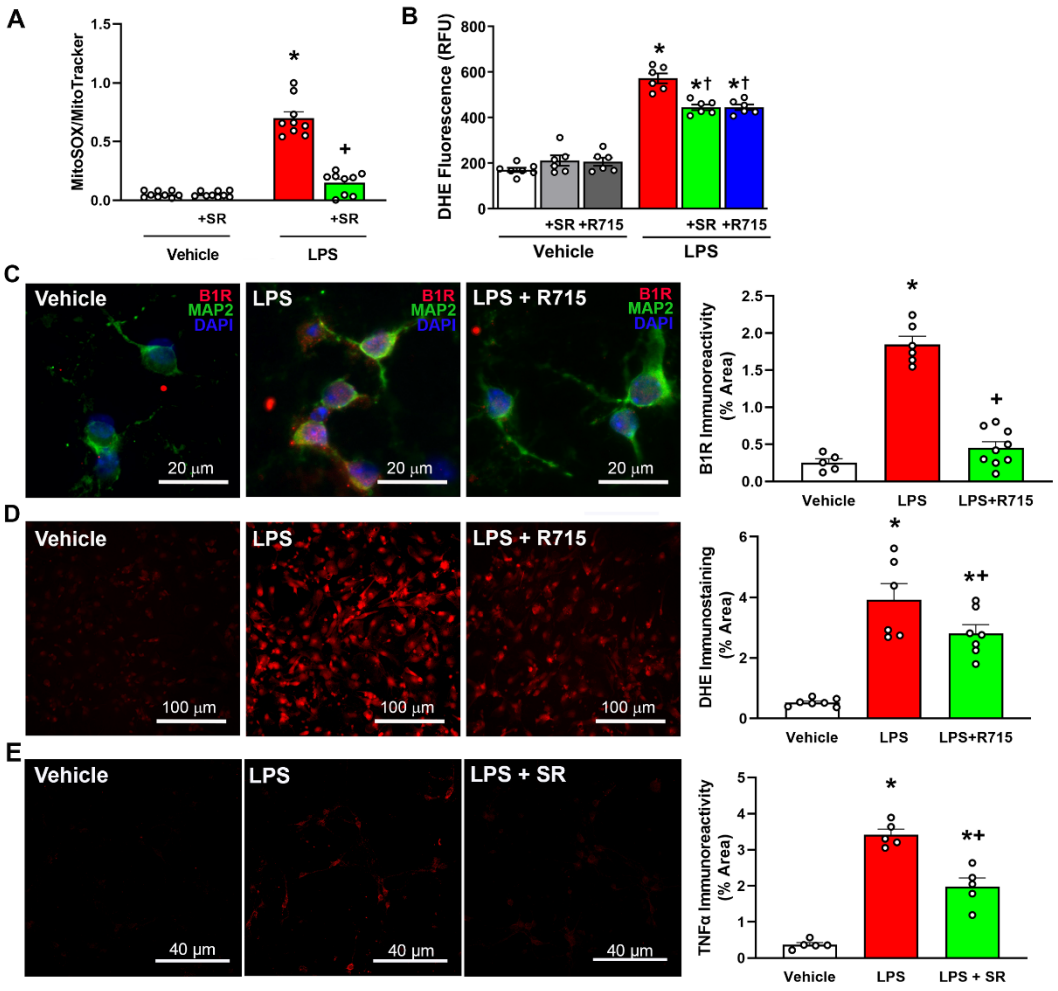

Figure 9. B1R blockade reduces LPS effects in primary hypothalamic neurons. (A) MitoSOX mitochondrial oxidative stress production normalized to MitoTracker total mitochondrial content (n=9/group). (B) ROS generation using DHE microplate assay (n=6/group). (C) Immunofluorescence staining and quantification for B1R expression (n=5-9/group). (D) Superoxide production measured by DHE fluorescence (n=7/group). (E) Immunofluorescence staining and quantification for TNF expression (n=5/group). Statistical significance: One-way ANOVA followed by Tukey's multiple comparison test. *p<0.05 compared to vehicle. +p<0.05 compared to LPS.

3.4 B1R blockade prevents hydrogen peroxide induced oxidative stress and neuroinflammation in hypothalamic neurons.

Previous literature has indicated that oxidative stress is a key component in the pathogenesis of hypertension and other cardiovascular diseases (2, 29). Oxidative stress is associated with an excessive production of ROS such as superoxide anion, hydrogen peroxide, and hydroxyl radical. To model oxidative stress in our neuron cultures, we treated primary hypothalamic neurons with 10 uM hydrogen peroxide for 24 h, with or without pretreatment of a B1R selective antagonist. Using immunocytochemistry, we were able to identify that following stimulation with hydrogen peroxide, B1R is upregulated in neurons. Furthermore, B1R blockade resulted in an attenuation of this effect (Figure 10A). Now knowing that hydrogen peroxide-induced oxidative stress can be minimized by blocking B1R, we sought out to determine its role in ROS generation. DHE staining showed that, as predicted, hydrogen peroxide elevates ROS production, and that B1R blockade mitigates this increase (Figure 10B). To further confirm these results, we used a DHE microplate assay and confirmed that B1R blockade by SR and R715 can partially decrease levels of hydrogen peroxide induced ROS (Figure 10D). Furthermore, we wanted to examine the role of B1R blockade in the mitochondria. Using a MitoSOX/MitoTracker assay, we found that hydrogen peroxide increases mitochondrial oxidative stress levels relative to total mitochondria content. Inversely, B1R antagonism can partially prevent this increase in mitochondrial oxidative stress (Figure 10E). Increase in ROS production, such as an increase in hydrogen peroxide, stimulates the activation of signaling proteins, chemokines, and cytokine production (38). Therefore, we wanted to investigate the role of hydrogen peroxide in neuroinflammation, and if B1R blockade could

prevent hydrogen peroxide-induced neuroinflammation. TNF expression was measured using immunofluorescence and our data reveals that hydrogen peroxide increases TNF expression, and that pretreatment with SR provides an attenuation of this effect (Figure 10C). These results indicate that there is a relationship between B1R upregulation and oxidative stress in neurons, and that B1R antagonism may help to limit these effects.

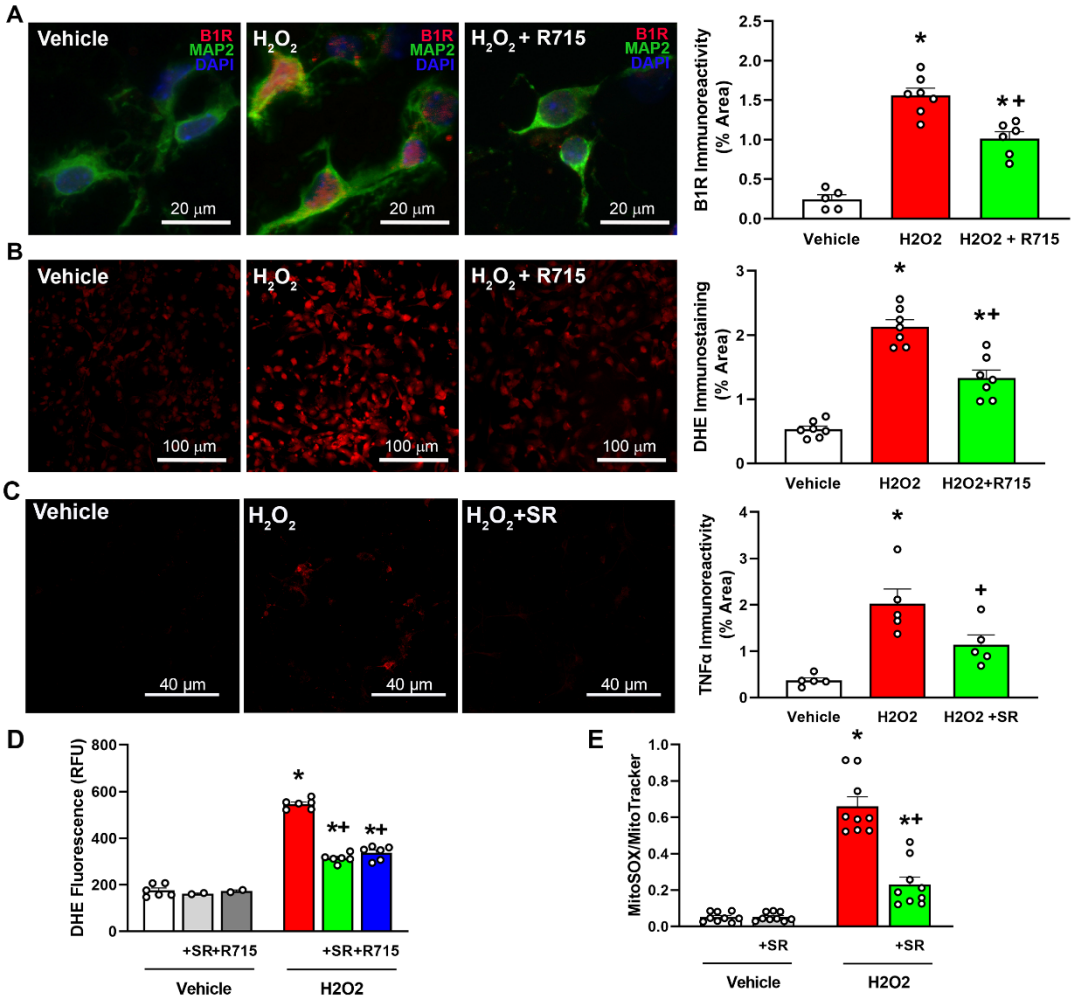

Figure 10. Hydrogen peroxide increases ROS production in primary hypothalamic neurons. (A) Immunocytochemistry fluorescence and quantification for B1R expression (n=5/group). (B) Superoxide production measured by DHE fluorescence (n=7/group). (C) Immunofluorescence staining and quantification for TNF expression (n=5/group). (D) ROS generation using DHE microplate assay (n=6/group). (E) MitoSOX mitochondrial oxidative stress production normalized to MitoTracker total mitochondrial content (n=9/group). Statistical significance: One-way ANOVA followed by Tukey's multiple comparison test. *p<0.05 compared to vehicle. +p<0.05 compared to H2O2.

CHAPTER 4: Results (Microglia)

4.1 B1R agonism induces ROS production and neuroinflammation in primary microglia.

In the brain, neuropeptides, such as kinin B1R, can regulate brain inflammation and affect microglial functions (1). It has been shown that B1R is expressed in microglia within the brain, however it is unknown if use of B1R selective antagonists can meditate neuroinflammation and oxidative stress in microglia. Microglia were cultured from the hypothalamus of neonatal mice pups and grown until 70% confluent and could be plated into cell-culture plates. Microglia were treated with 300 nM LDABK (24 h) with or without 2 h pretreatment of B1R antagonist. Using double immunostaining, we confirmed previous findings that LDABK induces B1R expression and found that it is attenuated using R715 (Figure 11A). Microglia are the resident immune cells of the brain, and they are known to induce neuroinflammation through the production of proinflammatory cytokines and reactive oxygen species (5). To measure ROS production, we used DHE immunofluorescence and showed that LDABK increases the oxidative potential, and this can be prevented with pretreatment of B1R antagonist SR (Figure 11B). To confirm the production of ROS due LDABK stimulation, we used a CM-H2DFDA assay to measure general oxidative stress levels. LDABK stimulation increased oxidative stress in primary microglia, but this effect was mitigated by use of SR (Figure 11D). We then measured proinflammatory cytokine TNF and showed that LDABK significantly enhances expression of TNF. Interestingly, use of a B1R antagonist was able to diminish this effect

(Figure 11C). These results indicate that blocking B1R may be able to mitigate the effects of neuroinflammation and ROS levels in primary hypothalamic microglia.

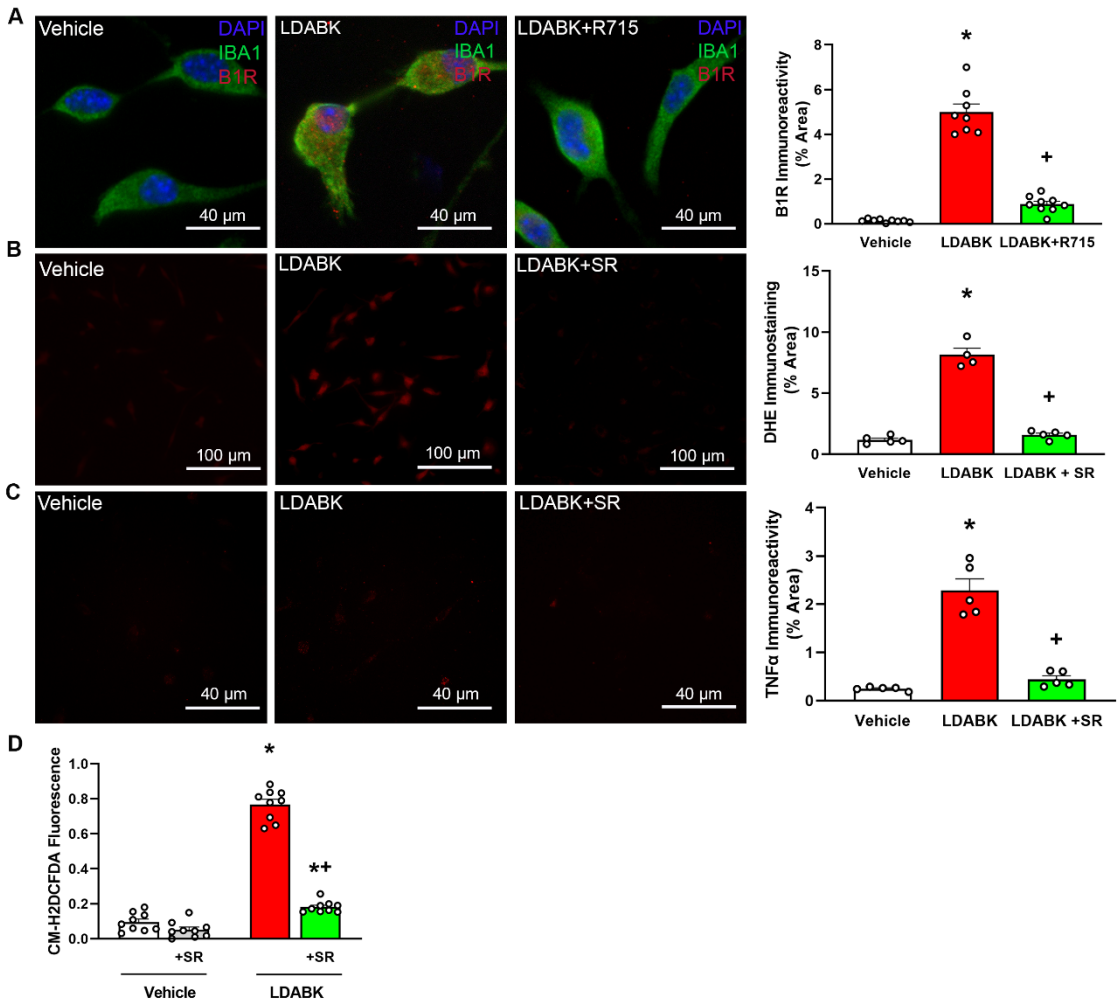

Figure 11. B1R blockade prevents LDABK induced B1R expression in primary hypothalamic microglia. (A) Immunostaining and quantification of B1R expression (n=8-10/group). (B) ROS production indicated by DHE red fluorescence (n=5/group). (C) Inflammation measured by TNF expression (n=5/group). (D) General levels of oxidative stress measured by CM-H2DFDA assay (n=9/group). Statistical significance: One-way ANOVA followed by Tukey's multiple comparisons test. * $p < 0.05$ compared to vehicle. + $p < 0.05$ compared to LDABK.

4.2 B1R antagonism attenuates TNF induced neuroinflammation and oxidative stress in primary microglia.

Microglia are known as the resident immune cells of the brain as they act as the first line of defense in the central nervous system (5). Due to this, they are known to cause neuroinflammation and oxidative stress. To determine the role of inflammation in microglia and if this can be blocked using B1R antagonism, we stimulated primary hypothalamic microglia with 10ng TNF. Using immunofluorescence, we found that TNF induces B1R activation and that blocking B1R can mitigate this increase in expression (Figure 12A). Inflammatory responses can heighten ROS generation, which leads to the pathogenesis of hypertension (6). To identify if B1R blockade in microglia could prevent inflammation-induced oxidative stress, we performed DHE immunofluorescence and revealed that TNF increases immunofluorescence, and pretreatment with SR was able to minimize this increase as shown by a decrease in red fluorescence (Figure 12B). To further confirm these results, we used a DHE microplate assay and once again showed that TNF induces ROS production, and that pretreatment with SR can mitigate this increase. To measure general oxidative stress levels, we used a CM-H2DFDA assay. Results showed that TNF stimulation significantly increase oxidative stress levels, but this increase can be partially reduced using a B1R antagonist (Figure 12D). Furthermore, we wanted to determine if B1R blockade could prevent TNF induced neuroinflammation in primary microglia. Using immunofluorescence, we found that B1R blockade partially lessens the expression of TNF due to TNF stimulation (Figure 12C). Altogether, this data provides evidence that B1R blockade may help to lessen the levels of TNF induced neuroinflammation, oxidative stress, and B1R activation.

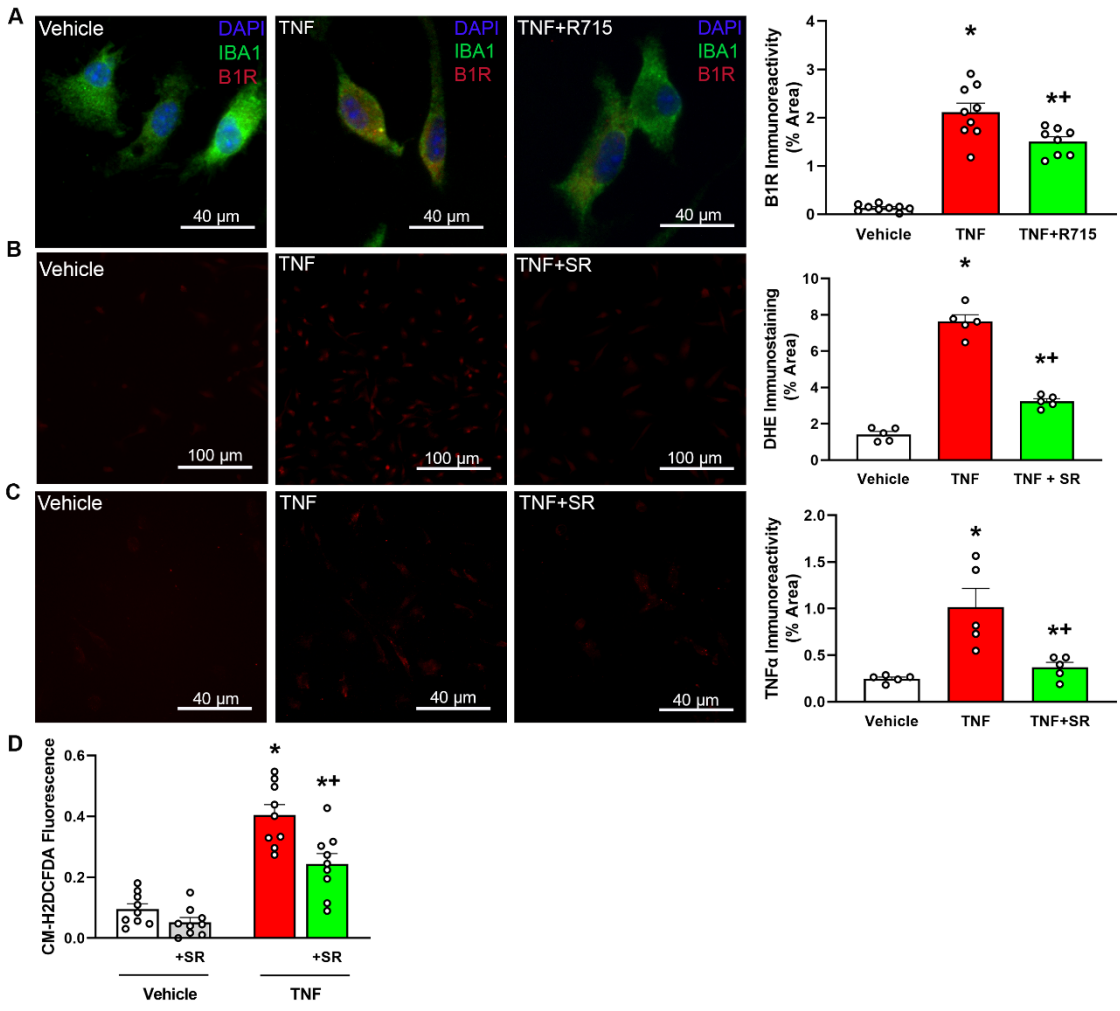

Figure 12. TNF stimulation amplifies B1R expression in primary microglia. (A) B1R expression as indicated by immunofluorescence and quantification (n=9/group). (B) Red DHE fluorescence indicates ROS generation (n=5/group). (C) Neuroinflammation as indicated by TNF immunoreactivity (n=5/group). (D) General oxidative stress levels in microglia as determined by CM-H2DFDA (n=9/group). Statistical significance: One-way ANOVA followed by Tukey's multiple comparisons test. * $p < 0.05$ compared to vehicle. + $p < 0.05$ compared to TNF.

4.3 LPS stimulation in primary microglia induces B1R expression, ROS production, and neuroinflammation

Microglia activation is associated with neuroinflammation and serves as a hallmark characteristic of hypertension (30). Studies have previously shown that LPS can induce morphological changes of microglia which results in proliferation and activation (11). To investigate the role of LPS on B1R activation in microglia, we used immunofluorescence and found that LPS induces B1R expression as indicated by increased red fluorescence. In turn, blocking B1R using a specific antagonist was able to lessen these effects (Figure 13A). Since previous studies have shown that LPS induces neurotoxicity through increasing oxidative stress and activating proinflammatory cytokines, we wanted to investigate the role of B1R blockade on LPS induced oxidative stress and inflammation (14). Initially, we performed DHE immunofluorescence to show that LPS induces ROS production in microglia. Consequently, pretreatment with SR can mitigate this increase (Figure 13B). To measure the general oxidative stress levels, a CM-H2DFDA assay was used and showed that B1R blockade was able to once again minimize LPS induced oxidative stress (Figure 13D). To determine the role in inflammation, we measured TNF immunoreactivity and was able to show that LPS induces TNF expression, and that pretreatment with SR partially diminishes this as indicated by the difference in red fluorescence (Figure 13C). These results provide evidence that B1R blockade may aid in prevention of LPS induced neuroinflammation, oxidative stress, and B1R upregulation.

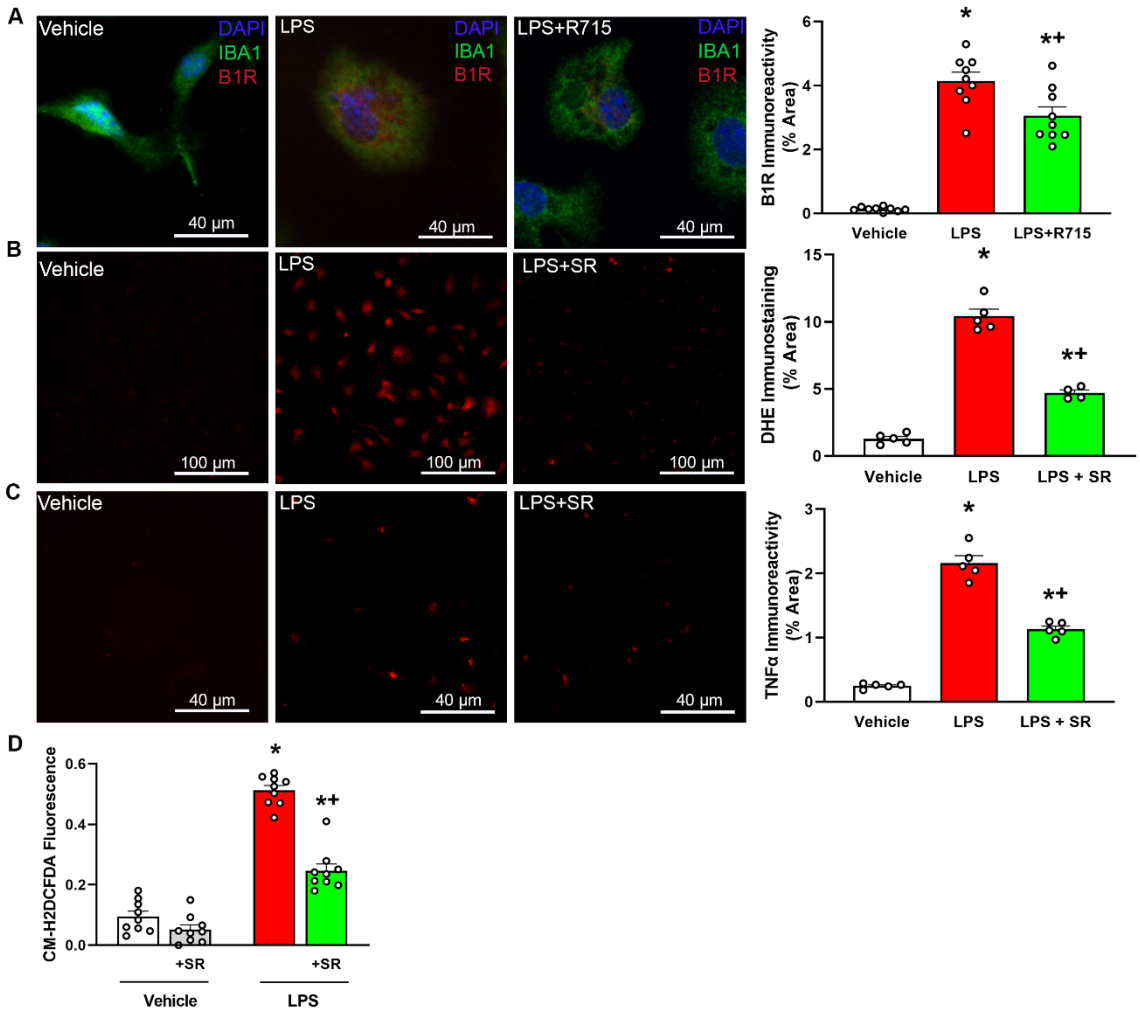

Figure 13. Role of bacterial endotoxin LPS in primary hypothalamic microglia. (A) B1R upregulation measured by immunocytochemistry (n=9-10/group). (B) Measurement of superoxide production by DHE staining (n=5/group). (C) Expression of TNF measured using immunofluorescence (n=5/group). (D) CM-H2DFDA was used to show levels of oxidative stress (n=9/group). Statistical significance: One-way ANOVA followed by Tukey's multiple comparisons test. * $p < 0.05$ compared to vehicle. + $p < 0.05$ compared to LPS.

4.4 Hydrogen peroxide stimulation induces B1R expression and neuroinflammation in primary hypothalamic microglia.

Prior studies have identified that ROS, specifically hydrogen peroxide, are involved in cell signaling and often regulate cell proliferation in microglia (18). In this present study, we sought to determine the role of hydrogen peroxide induced ROS production to determine its role in neuroinflammation and B1R upregulation. Initially, we cultured primary hypothalamic microglia until 70% confluency was reached, then plated them in 12 well-cell culture plates before treating them with 10uM hydrogen peroxide for 24 h with or without 2 h pretreatment of selective B1R antagonists. We then used immunofluorescence to determine the role of hydrogen peroxide on B1R activation. We found that 24 h stimulation with hydrogen peroxide upregulates B1R expression, and that use of a B1R antagonist, R715, can mitigate this effect (Figure 14A). Furthermore, DHE immunofluorescence was used to determine levels of ROS production. Hydrogen peroxide significantly increased ROS production in primary microglia as indicated by increased red fluorescence, however, this increase was mitigated by 2 h pretreatment with SR (Figure 14B). To measure general oxidative stress levels, we then performed a CM-H2DFDA assay and confirmed that hydrogen peroxide can significantly upregulate oxidative stress, as predicted since it is a ROS on its own. Interestingly, blocking B1R was able to partially reduce this effect in primary hypothalamic microglia (Figure 14D). Furthermore, we wanted to determine the role of hydrogen peroxide-induced oxidative stress on neuroinflammation. Using immunocytochemistry, we measured TNF expression and found that B1R blockade can significantly reduce hydrogen peroxide induced TNF expression (Figure 14C). In summary, this data provides evidence that hydrogen

peroxide- induced oxidative stress plays a role in neuroinflammation and B1R activation, and that the kinin B1 receptor may mediate these interactions.

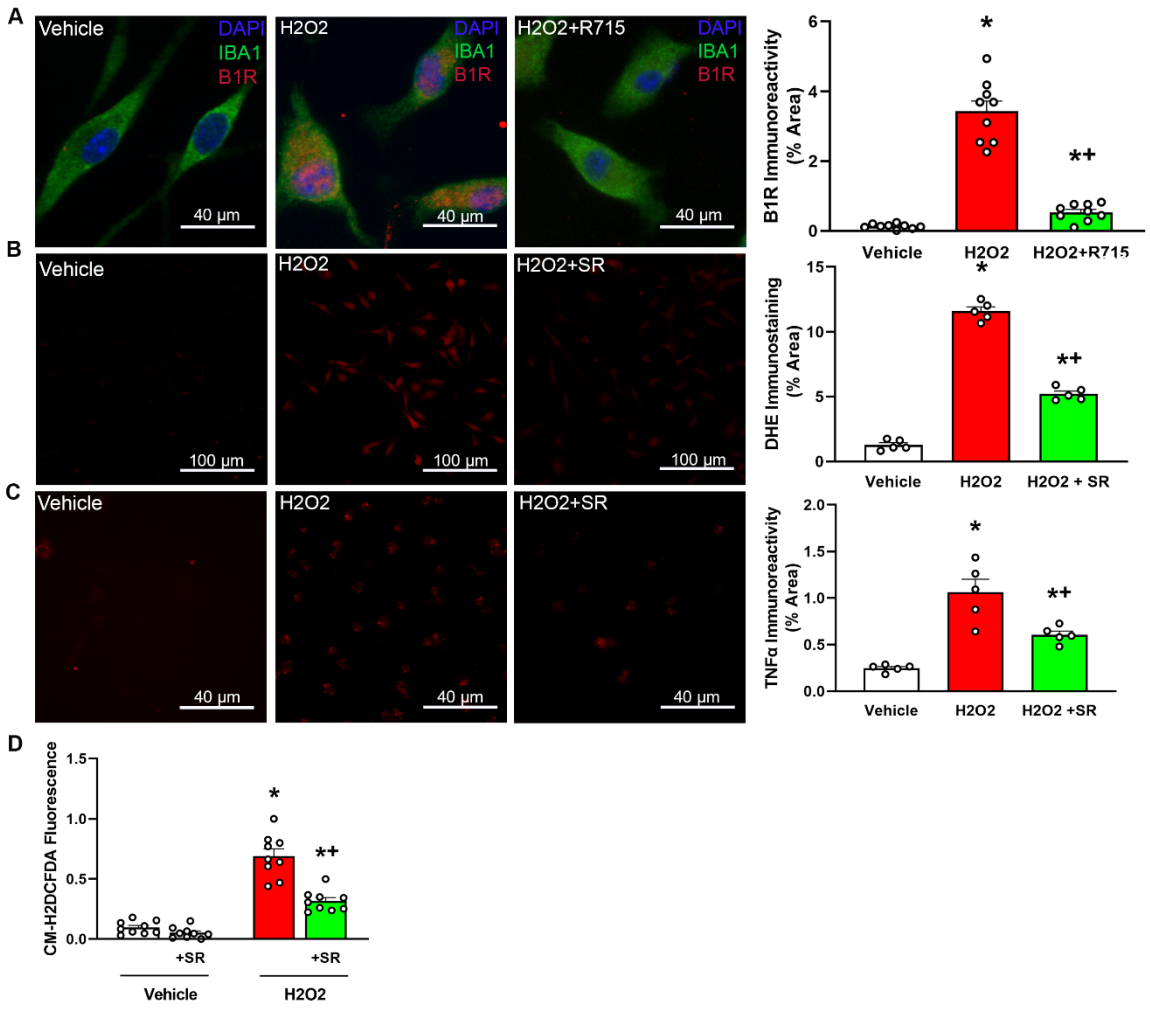

Figure 14. Effect of hydrogen peroxide on microglia. (A) Immunofluorescence used to reveal B1R expression (n=9-10/group). (B) DHE staining and quantification for ROS generation (n=5/group). (C) Neuroinflammation measured through TNF immunostaining and quantification (n=5/group). (D) Levels of general oxidative stress indicated by CM-H2DFDA assay (n=9/group). Statistical significance: One-way ANOVA followed by Tukey's multiple comparisons test. * $p < 0.05$ compared to vehicle. + $p < 0.05$ compared to H2O2.

CHAPTER 5: Discussion

It is well established that hypertension is associated with increased activity of the KKS (34). Specifically, activation of kinin B1R can lead to inflammation and vasoconstriction, supporting the idea that B1R plays a role in the pathogenesis of hypertension. To activate B1R in our cellular model, we stimulated primary hypothalamic neurons with a B1R agonist, LDABK. LDABK is an active metabolite of bradykinin and can endogenously active B1R (17). In this present study, we confirm that LDABK upregulates B1R expression using immunofluorescence. In turn, use of B1R antagonist prior to LDABK stimulation can attenuate this effect in primary hypothalamic neurons. Furthermore, we were able to confirm that B1R activation increases reactive oxygen species production resulting in increased levels of oxidative stress, as indicated through DHE immunostaining and microplate assay, and MitoSOX/MitoTracker. Inversely, B1R blockade was able to blunt B1R induced-oxidative stress in hypothalamic primary neurons, signifying that kinin B1 receptor may mediate oxidative stress. Additionally, we wanted to determine the role of B1R blockade in neuroinflammation, and we once again found that blocking B1R results in an attenuated effect of B1R induced-TNF expression. Taken together, this data provides evidence to a possibility that B1R can mediate neuroinflammation and oxidative stress in primary hypothalamic neurons.

It is well documented that B1R is expressed on neurons, microglia, and astrocytes within the brain (35). However, to our knowledge, the role of B1R blockade on neuroinflammation and oxidative stress in microglia is relatively unknown. Recently it has been reported that neuropeptides, such as kinin B1R, regulate brain inflammation and

can in turn affect microglial functions (1). To confirm this, we performed immunocytochemistry and confirmed that B1R is upregulated in microglia following stimulation with LDABK, a B1R agonist. Furthermore, pretreatment with B1R selective antagonists was able to attenuate B1R upregulation in primary hypothalamic microglia. After confirming B1R expression in microglia, we sought to determine the role of B1R and B1R blockade in oxidative stress. Oxidative stress is the excessive production of ROS which results in altered oxidation-reduction states (38). To measure this, we used DHE immunofluorescence, DHE microplate assay, and CM-H2DFDA. Results indicated that LDABK-induced B1R activation increased ROS generation and general oxidative stress levels, and that B1R blockade was able to blunt this in primary hypothalamic microglia. It is well established that in times of inflammation, B1R is upregulated (3, 22). Using immunofluorescence to determine TNF expression, we found that LDABK-induced B1R activation significantly upregulates TNF expression, and in turn, B1R blockade can prevent this increase. This data in both primary microglia and neurons display that B1R blockade can mediate LDABK induced neuroinflammation and oxidative stress.

In patients with known hypertension or prehypertension, patients often demonstrate an elevated circulating level of TNF (37). TNF is a proinflammatory cytokine and is vital in initiating and sustaining the inflammatory cascade, which also results in a production of more cytokines and reactive oxygen species (33). Furthermore, recent evidence has suggested that blocking TNF decreases oxidative stress while delaying the progression of hypertension in various experimental models (9, 33). Knowing that B1R blockade can prevent LDABK-induced B1R expression, oxidative stress, and neuroinflammation, we sought to investigate the role of TNF in these pathways.

Immunofluorescence revealed a significant upregulation of B1R expression, ROS production, mitochondrial oxidative stress, and TNF expression in primary neurons and microglia from the hypothalamus of neonatal mice. Interestingly, blockade of B1R using a B1R specific antagonist was able to partially ameliorate the effects of TNF in these cells. These results indicate that B1R blockade may also partially block TNF, which results in reduced ROS generation, less neuroinflammation, and no rise in B1R expression.

Similar to TNF, hypertensive patients also display increased levels of LPS compared with normotensive patients (8). LPS is a bacterial endotoxin purified from Escherichia coli O55:B5 and is known to increase production of cytokines and induce vascular dysfunction (8, 30). To determine the role of B1R blockade on LPS, we used double immunocytochemistry and showed that preincubation of B1R antagonist can eradicate LPS induced B1R expression in primary neurons and primary microglia. In both neurons and microglia, LPS has been shown to be an inducer of oxidative stress. Conversely, in microglia, LPS have been shown to cause microglial activation, which is a hallmark of hypertension in cells. Our data in this present study indicates that LPS induces ROS production in neurons as indicated by DHE and MitoSOX/MitoTracker. In microglia, LPS stimulation shows an activation of microglia as indicated by an ameboid shape. ROS and general oxidative stress levels were remarkedly increased as shown by DHE fluorescence and CM-H2DFDA. Furthermore, B1R blockade was able to partially mitigate LPS induced- ROS generation and oxidative stress in both primary hypothalamic neurons and microglia. However, B1R blockade was unable to prevent LPS induced microglia activation. Since inflammation plays a substantial role in hypertension, we examined the role of LPS on neuroinflammation. Previous studies have shown that LPS induces

amplification of inflammatory markers such as TNF and IL-6 (1). Data confirmed that LPS stimulation significantly increases TNF expression in both neurons and microglia, but once again, B1R blockade can lead to a partial reduction in this. These data provide evidence for B1R blockade serving as a mediator for LPS induced B1R expression, neuroinflammation, and oxidative stress.

Oxidative stress has been thought to play a role in the pathophysiology of hypertension, although there is no confirmation that oxidative stress is a primary cause of hypertension (38). However, there is unambiguous evidence that increased levels of ROS are associated with hypertension (38). A specific ROS that has been implicated in hypertension, vascular remodeling, and cytokine production is hydrogen peroxide. Hydrogen peroxide is a second messenger that plays an intracellular role in intercellular signaling and is a paracrine mediator of cardiovascular dysfunction (32). We determine the role of hydrogen peroxide on B1R expression in neurons and microglia using immunofluorescence. We demonstrated that H_2O_2 increases B1R expression, and that blocking B1R can reduce this effect in microglia and neurons. Furthermore, since hydrogen peroxide is a reactive oxygen species on its own, we sought to investigate how B1R blockade could play a role in H_2O_2-induced oxidative stress. Between the neurons and microglia, we determined ROS production, mitochondrial oxidative stress production, and general oxidative stress levels. In all models, we discovered that, as predicted, hydrogen peroxide significantly increases ROS generation and oxidative stress levels. However, B1R antagonism was only able to partially ameliorate this increase. Our data also shows that hydrogen peroxide-induced oxidative stress leads to an increase in neuroinflammation as indicated by an increased expression of TNF. Once more, B1R

blockade was able to mitigate this effect. In all, this data provides the first evidence to the role of B1R blockade on hydrogen peroxide induced oxidative stress.

Taken together, this study provides evidence to a bidirectional interaction between B1R activation, neuroinflammation, and hypothalamic stress in primary neurons and microglia. We provide straightforward evidence as to how oxidative stress and neuroinflammation are, in part, mediated through the B1R pathway and that blocking B1R may serve as a therapeutic model for a variety of diseases and conditions. Although we were not able to differentiate the order at which these events occur, this study allowed us to identify a casual role of B1R and oxidative stress, further confirming its part in the development of various pathologies such as neurodegenerative diseases, cardiovascular disease, and hypertension.

To our knowledge, this study is the first to examine the bidirectional role between B1R activation, oxidative stress, and neuroinflammation in primary hypothalamic microglia and neurons. Our lab has previously used primary neuronal cultures as a model as they provide us with a high resolution compared to immortalized cells (2, 25, 40). However, this was the first study we performed using primary hypothalamic microglia. Future studies will be needed to fully identify which events occur in what order, while also looking at these interactions in other models which as in vivo models and neuron-glia cocultures. Nevertheless, the data in this study provides unambiguous evidence to support the role of B1R activation in microglia and neurons and show the beneficial effects B1R blockade has on mitigating the effects of oxidative stress and neuroinflammation.

CHAPTER 6: References

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APPENDIX: IACUC APPROVAL LETTER

1/10/22

ECU

Animal Care and Use Committee
003 Ed Warren Life Sciences Building | East Carolina University | Greenville NC 27834 - 4354
252-744-2436 office | **252-744-2355** fax

January 10, 2022

Srinivas Sriramula, Ph.D.
Department of Pharmacology and Toxicology, ECU

Subject: Protocol W261, original approval date 07/16/2010

Dear Dr. Sriramula:

The amendment#3 to your Animal Use Protocol entitled, "Neuroimmune Mechanisms of Kinin B1 Receptor in Hypertension" (AUP#W261) was reviewed by this institution's Animal Care and Use Committee on 01/07/2022. The following action was taken by the Committee:

"Approved as submitted"

Please contact Aaron Hinkle prior to any hazard use

A copy of the protocols is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP/Amendment and are familiar with its contents.

Sincerely yours,

SB McRae

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/GD

enclosure