THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF BIOLOGY

REGUALTION BY THE RON RECEPTOR TYROSINE KINASE TO ATTENUATE CENTRAL NERVOUS SYSTEM INFLAMMATION IN A MODEL OF ATHEROSCLEROSIS

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ABSTRACT

Neurodegeneration is the leading cause of cognitive impairment leading to functional disability in aging populations. The debilitating symptoms and progression of neurodegenerative diseases, such as Alzheimer's Disease, aging associated dementia, and Parkinson's disease, are mainly characterized by chronic central nervous system inflammation. The Ron receptor tyrosine kinase (Ron) is expressed in tissue resident cells in the brain, including microglia and neurons. Most importantly, expression of Ron on macrophages has been identified to attenuate inflammation. The objective of this study was to further understand the neuroprotective role of the Ron receptor and its ligand, macrophage-stimulation protein (MSP), in regulating inflammation in the brain in both vascular structures and cells including microglia and astrocytes. Specifically, the significance of the inducible enzyme iNOS in triggering inflammation, as well as the dynamics of NLRP3 inflammasome activation were assessed in the CNS. Apolipoprotein E-knockout (ApoE-/-) and Ron receptor/ApoE-/- double knockout (DKO) transgenic mice were developed and maintained on a high-fat-high-cholesterol diet (HFHCD) for 18 weeks. Immunohistochemical analysis indicated that a loss of Ron lead to the accumulation of smooth-muscle arterial iNOS in small to large CNS vessels ($p<0.05$), as well as surrounding cellular accumulations of iNOS. Additionally, increases in GFAP concentrations signified microgliosis that was triggered by the loss of Ron. Finally, Ron was observed to attenuate lipopolysaccharide (LPS)-induced activation of the NLRP3 inflammasome upon activation by its ligand, MSP. This study was beneficial in determining the key players involved in neuroprotection by Ron and highlighting the importance of activation by its ligand. By understanding and targeting inflammatory regulators such as the Ron receptor, prevention treatments for chronic inflammation caused by neurodegenerative diseases can be advanced.

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Chapter 1: Introduction

1.1: Neurodegeneration

Chronic central nervous system (CNS) inflammation is an underlying characteristic of neurodegenerative disorders, including Alzheimer's Disease (AD), aging associated dementia, and Parkinson's disease (PD).[8] Sadly, in 2016 over 5 million Americans suffered from AD, and rates of AD diagnoses are predicted to increase drastically over time. These chronic conditions contribute to significant cognitive impairment leading to functional disability. The dynamics of these diseases are very complex due to multiple pathological factors contributing to symptoms. AD specifically, is characterized by oxidative stress, mitochondrial damage, glutamate excitotoxicity, neuroinflammation, neurofibrillary tangle (NFT) formation, and β-amyloid (Aβ) plaque formation.[2] During disease states, inflammation works to combat the cause of cellular stress and eliminate damaged cells in tissues all over the body. Lately, many literature studies have highlighted contrasting, detrimental effects of neuroinflammation, as it can worsen the progression of dementia specifically. In some studies, the induction of neuroinflammation actually triggered intracellular Aβ deposits and tau phosphorylation (proteins that accumulate in neurofibrillary tangles with phosphorylation disruption).[2] This inflammation then tends to progress uncontrollably, causing irreversible neuropathology.

AD, aging associated dementia, and PD are currently incurable. Due to the multifactorial nature of neurodegenerative diseases, extensive research on neuronal physiologies and key players of cellular repair during disease states is vital in understanding and working towards

treatments for AD, aging associated dementia, and Parkinson's Disease. Studying the early development of inflammation can be beneficial in determining how to combat these diseases before they cause severe, irreversible damage. Therefore, this research aims to further study the earlier stages of neuroinflammation by studying the role of a key signaling protein, called the Ron receptor tyrosine kinase, and its vital actions in controlling CNS inflammation.

1.2: The Ron Receptor Tyrosine Kinase

Receptor d'origine Nantais (Ron) is a growth factor receptor present on the surfaces of epithelial cells and tissue resident macrophages. Specifically, Ron can be found on microglia in the central nervous system (CNS), but also cells in the liver, kidney, testes, bone, lung, breast, and epithelia of the digestive tract. The receptor belongs to the Met protooncogene family of transmembrane receptor tyrosine kinases and contains 1400 amino acids.[17] It is synthesized as a single 185kDa chain and cleaved before it is expressed on cell surfaces. The ligand for the Ron receptor is hepatocyte growth factor-like protein (HGFL), also referred to as macrophage stimulating 1 (MST1) or macrophage-stimulation protein (MSP), that is produced in the liver in its inactive form.[8] MSP is activated by proteolytic cleavage by membrane-associated proteases. When MSP binds to the Ron receptor as seen in Figure 1, Ron activation leads to the induction of multiple signaling cascades, which can then trigger a range of pleiotropic responses.[17] Specifically, in the CNS, Ron can be found in neuronal and microglial populations and serves as a negative regulator of inflammation to repress genes that cause cell death by regulating macrophage activity.

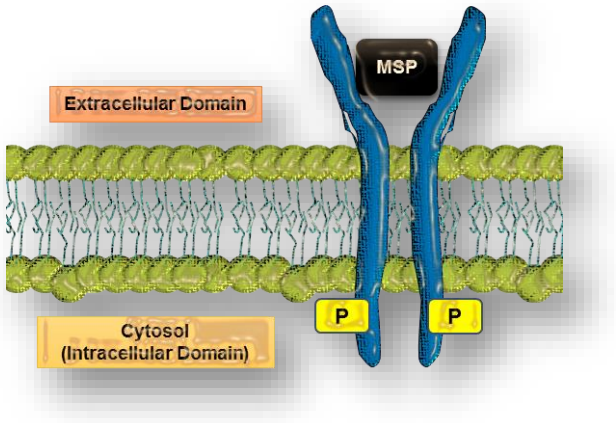

Figure 1. MSP binding to Ron receptor on surface of cell, *(illustrated by Adwitia Dey, PhD student)*

1.3: Ron Regulates Macrophage Heterogeneity

The innate immune system regulates disease onset and progression in the CNS through macrophage activity. Tissue macrophages play an important role in the induction, maintenance, and resolution of inflammation. During non-inflammatory conditions, macrophages display an alternatively activated M2 phenotype that supports tissue remodeling, repair, and homeostasis. Alternatively, the classically activated M1 macrophage phenotype is induced by environmental stimuli and promotes the release of nitrogen radicals and pro-inflammatory cytokines. These phenotypes are categorized by arginine metabolism within the macrophage- where higher expressions of inducible NO synthase (iNOS) converts L-arginine to L-citrulline in M1, and Arginase-1 (Arg-1) catabolizes L-arginine to L-ornithine in M2.[9]

The Ron receptor tyrosine kinase is a macrophage-stimulating protein receptor located on the extracellular matrix of the cell that, upon MSP-dependent activation, has been shown to support anti-inflammatory processes and subsequent tissue repair. Ron is involved in regulating macrophage heterogeneity primarily through activation of the M2 state.[8] Ron orders macrophage

activity in response to inflammatory conditions by regulating the production of cytokines and chemokines. Ron activation inhibits pathogen- and cytokine-induced inflammatory gene expression to repress inflammation. The significant role of Ron in macrophage heterogeneity was shown through previous laboratory studies revealing that an in vivo deletion of the extracellular ligand binding domain of Ron (Ron -/-) promotes inflammatory (MI) macrophages and limits reparative (M2) macrophage activation.[9]

1.4: Ron's Protective Role

Additionally, as a regulator of inflammation, Ron is characterized by its protective role in various inflammatory diseases. Previous studies have revealed a defensive role of the Ron receptor in the progression of diet-induced obesity. Obesity is an inflammatory disease that contributes to the development of heart disease, type 2 diabetes, atherosclerosis, and hepatic steatosis. Nearly one-third of the world's population is obese or overweight, with rates tripling world-wide since 1975. Obesity is classified as a chronic inflammatory disease and is mediated by the activation of inflammatory macrophages. During disease states, bone marrow monocyte-derived macrophages (BMDMs) are recruited to inflamed tissues and produce pro-inflammatory cytokines which exacerbate inflammation.[22] A longitudinal study of adults found that a higher BMI throughout an individual's 30s and 40s lead to an earlier onset of Alzheimer's dementia.[5] Ron is expressed in a subpopulation of macrophages during chronic inflammation that is induced by obesity. When activated by its ligand MSP, Ron has been found to reduce obesity mediated inflammation in CNS tissue. Furthermore, a loss of Ron has been shown to accelerate the degree of neuroinflammation in chronic inflammatory models of diet induced obesity.[8]

1.5: Significance of Research

This research aims to study the progression of neurodegeneration by further analyzing the role of the Ron receptor tyrosine kinase in controlling CNS inflammation. Specifically, this project focuses on studying the actions of Ron and its ligand MSP in regulating inflammation of vascular structures in the brain, as well as promoting astrocytic and microglial health. The regulation of the inflammatory enzymes such as inducible nitric oxide synthase (iNOS) and inflammasomes involved in innate immunity are studied to further quantify the impact of regulation by the Ron receptor in the CNS.

Vascular structures and iNOS regulation

Cerebral blood vessels are vital for delivering oxygen and nutrients throughout the brain, as well as for trophic signaling from cerebrovascular cells to neurons and glia. Neural activity is most dependent on blood flow since it requires the most energy in the brain. Increases in blood flow is dependent on many factors, one being nitric oxide (NO) regulation.[14] NO is released continuously through blood flow from endothelial cells in the vascular system. Neuronal NOS (nNOS, NOS-1), iNOS (NOS-2), and endothelial NOS (eNOS, NOS-3) are three isoforms of NOS that synthesize NO. iNOS is primarily found in macrophages and can be stimulated by the release of cytokines.[11] Greater levels of iNOS has been linked to increases in Aβ-plaques and accelerated AD progression.[20] Overproduction of NO stimulates inflammatory cascades and causes NO to form into superoxide anion ($O2^{-}$), that then causes accumulation of the reactive oxygen species (ROS) and peroxynitrite ($ONOO^{-}$).[11] Peroxynitrite disrupts cerebrovascular homeostasis by reducing the activity of eNOS.[4] Therefore, for optimal vascular function, NO levels must be maintained and can be achieved by targeting regulators of iNOS activity, such as the macrophage-stimulating Ron receptor.

NLRP3 in innate immunity

Excessive oxidative stress can lead to the assembly of the multiprotein inflammatory complexes called the inflammasomes.[5] The inflammasome contains a Nucleotide-binding domain and Leucine-rich Repeat (NLR) containing proteins. NLRs sense stress or damage to cells and stimulate the assembly of the inflammasome.[16] Inflammasome complexes then regulate the activation of caspase-1 which cleaves inflammatory cytokines from its pro-to active forms of Interleukin 1β (IL-1β) and Interleukin 18 (IL-18).[12] Nod-like receptor protein 3 (NLRP3) is expressed in the myeloid lineage monocytes and macrophages where it can sense cellular stress signals, particularly ROS. The inflammatory M1 state of microglia during neurodegenerative diseases has been linked to increased NLRP3 activation and interleukin (IL)-Iβ accumulation. As a major player in the inflammatory progression of neurodegenerative diseases, regulation of NLRP3 activation can help to attenuate the progression of AD.[16] Furthermore, understanding the role of the Ron receptor in inflammasome activation could provide more insights on its neuroprotective role and suggest advancements to therapies that target NLRP3 activation.

Neuronal and Glial Health

Cognitive skills and brain functioning is largely dependent on neuronal and glial health. Neuronal inflammation is a key characteristic of many neurodegenerative disorders. Dysregulation of iNOS leads to uncontrolled neuroinflammation, which is seen in AD and aging associated dementia. Glial cells, such as microglia and astrocytes, serve as macrophages and support cells for neurons, respectively, and respond to CNS injury as seen in Figure 2.[19] When there is damage to neurons, astrogliosis, an increase in the activation of astrocytes, and glial scarring can occur. This is partially caused by an upregulation of glial fibrillary acidic protein (GFAP), that is involved in cell communication and maintenance.[10] Additionally, microglial cells

respond directly to inflammation in efforts to rid CNS tissue of damaged cells. Microgliosis can occur which is characterized by the upregulation of ionized calcium-binding adapter molecule (IBA1).[15] Studying the responses of glial cells to neuronal health in regards to the Ron receptor can lead to a better understanding of the dynamics CNS inflammation.

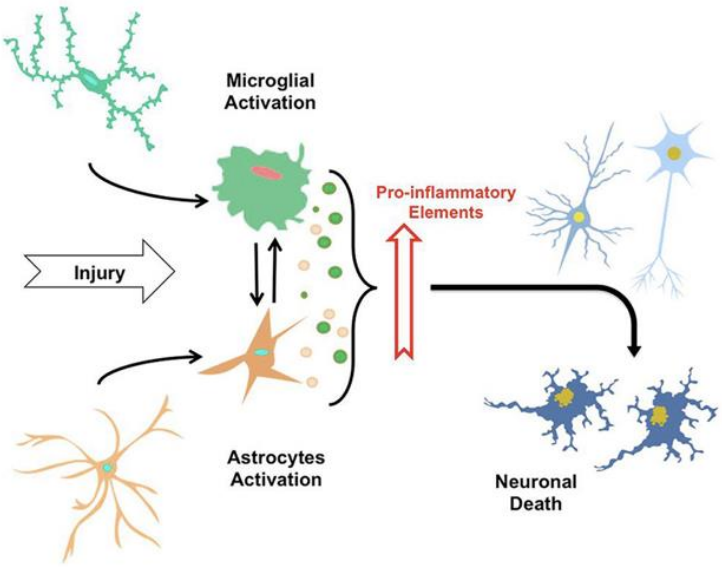

Figure 2: Glial response to CNS stress[19]

(Morales, I., Guzman-Martinez, L., Cerda-Troncoso, C.,Farias, G.A., Maccioni, R.B. (2014) Neuroinflammation in the pathogenesis of Alzheimer's disease. A rational framework for the search of novel therapeutic approaches. Front. Cell. Neurosci. ***8****, doi: 10.3389/fncel.2014.00112)*

Significance of MSP activation

Ron receptor activation is dependent on the binding of its ligand, MSP. In vitro classical M1 or alternative M2 macrophage activation can be induced in microglia by lipopolysaccharide (LPS) or IL-4 respectively.[8] The decrease in macrophage M2 phenotypes has been shown to further exacerbate neuroinflammation in various neurodegenerative diseases, and a balance of both M1 and M2 phenotypes is necessary for proper CNS health. During inflammatory states, MSP is activated and binds to the Ron receptor. Upon activation by MSP, Ron promotes tissue

repair and maintains homeotic states within cells. Previous research noted that the loss of Ron responsiveness to MSP induces neuroinflammation. In a recent study by Dey, et. al., MSP activation of the Ron receptor decreased the LPS induced expression of TNFα in human microglial cells (Figure 3). Additionally, by administering exogenous MSP, inflammatory cascades triggered during stress can be attenuated.[8]

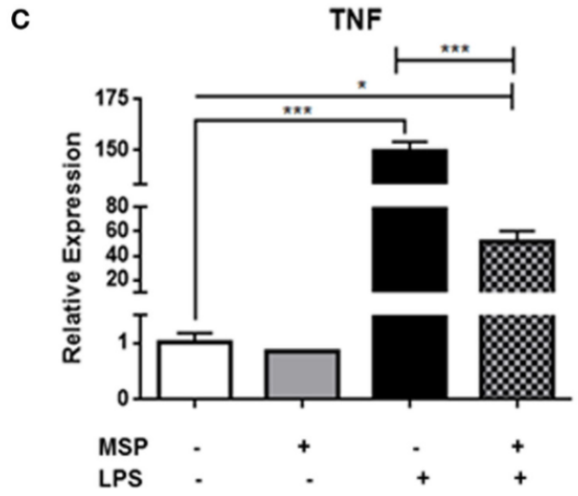

Figure 3: Ron activation by MSP suppressed LPS-induced upregulation of TNFα in human macrophage cells[8]

(Reproduced with permission from Dey et.al)

Experiments for this study were conducted using an atherosclerosis model, in which the ApoE protein is knocked out, to study CNS inflammation in mice on high-fat-high-cholesterol diets. The role of Ron in regulating CNS inflammation is analyzed, stressing that a loss of Ron leads to greater neuroinflammation and exacerbated disease states.

Chapter 2: Materials and Methods

2.1: Mouse Models and Diets

Wild-type and apolipoprotein E knockout (ApoE-/-) mice on the C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Ron knockout (Ron -/-) mice were generated with C57BL/6 mice by targeted disruption of the Ron gene, so that the ligand binding domain of Ron was deleted. Ron -/- and ApoE -/- mice were also crossed used to generate ApoE -/- and Ron -/- (double knockout, DKO) mice. Both male and female mice were used for the study. Mouse genotypes were assessed using PCR. ApoE-/- (n=18) and DKO (n=18) models were fed a high-fat-high-cholesterol diet (HFHCD; diet number F6334; Bioserv) for 18 weeks. On a caloric basis, the HCD contained 1.25% cholesterol. Animals were maintained in a humidity and temperature-controlled room on a 12-hour light/dark cycle. These experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at The Pennsylvania State University. Mice were euthanized by CO_2 narcosis. Whole brain tissues were isolated and submerged in 10% buffered formalin, before temporary storage in 70% ethanol.

2.2: Solutions

Sodium citrate buffer: A solution of sodium citrate buffer was made for antigen retrieval by dissolving 2.94g of Tri-sodium citrate in 1000mL of distilled water. The pH was adjusted to 6.0 using 1N HCl, and then 0.5mL of Tween 20 was mixed into the solution.

T-PBS: 10x PBS was prepared by dissolving 800g NaCl, 20g KCl, 144g $Na_2HPO_4 \bullet 2H_2O$, and 24g KH_2PO_4 in 8L of double distilled water. The solution was mixed and then topped off to 10L with double distilled water. The pH was adjusted to 6.8 using HCL and/or NaOH. 1X PBS was prepared by mixing 100mL of 10x PBS in 900mL of double distilled water, and the pH was adjusted to 7.4 using HCl and/or NaOH.

20% Goat Serum Blocking Buffer: 100% goat serum was purchased from Sigma-Aldrich (St. Louis, MO) and diluted to 20% goat serum with 1x T-PBS.

2.3: Immunofluorescent (IHC) Labeling

Isolated brains were fixed in formalin, tissue processed and embedded in paraffin for sectioning. Fixation and sectioning of brain tissue were conducted at the Huck Institutes Histology Core, Penn State UP. A microtome was used to obtain transverse and coronal sections of the brains. Before staining, the sections were deparaffinized (xylene and ethanol) and antigen retrieved for 20 minutes by boiling slides in a sodium citrate buffer (10mM sodium citrate, 0.05% tween-20, pH 6.0). Tissues were blocked with 20% goat serum, and concurrently stained with a primary antibody overnight at 4° Celsius. Antibodies used for detection included iNOS (1:100) from Cell Signaling Technology (Danvers, MA), Beta tubulin (1:100) from Proteintech (#10094-1-AP), NLRP3 (1:100) a gift from Dr. Teresa Alnemri (Thomas Jefferson University, Philadelphia, PA), Ron β (C-20) from Santa Cruz Biotechnology (sc-322), and GFAP (1:100) from Cell Signaling Technology (Danvers, MA) (#12389). Tissues were then incubated with a secondary antibody (Alexa Fluor®594 Goat Anti-Rabbit IgG, Invitrogen, 1:450) for 1 hour in 20% goat serum in T-PBS (1x). For subsequent storage and visualization, tissues were mounted

with medium (Prolong® Diamond Antifade Reagent containing DAPI, Invitrogen) containing 1% 4', 6-diamidino-2-phenylindole (DAPI), a fluorescent nuclear stain, and covered with glass cover slips (VWR® SuperSlips™ Micro Cover Glasses, Rectangular, No. 1, 24 X 55mm, CA48382-128). Slides were stored at 4° Celsius and then imaged using an Olympus BX-51 fluorescence photomicroscope (Olympus, Inc. BS-2 series) and DP71 image capture software (Olympus Inc.).

2.4: Cell Culturing

Human microglia cell line CHME-3 was obtained as a gift from Dr. A. Henderson (Boston University). CHME-3 cells were maintained in Complete DMEM media (GIBCO, Gaithersburg, MO), supplemented with 100% fetal bovine serum (10mL) (GIBCO, Gaithersburg, MO), 1% ciprofloxacin (Santa Cruz, San Juan; CAS93107-08-5), sodium pyruvate (1.1mL, Sigma-Aldrich, St. Louis, MO; P2256), and non-essential amino acids. Cell culture plates were stored at 37° Celsius with an atmosphere setting of 5% CO_2.

2.5: In Vitro Stimulation

Recombinant human MSP-(Cys672Ala) protein was obtained from R&D Systems (Minneapolis, MN) and culture grade lipopolysaccharide (LPS) was obtained from Sigma-Aldrich (St. Louis, MO). CHME-3 cells were treated with MSP (100ng/mL) overnight and then stimulated with LPS (100ng/mL) for 4-hours. Cells were counted using a hemocytometer with 0.2% Trypan blue dye (1:5) from Thermo Fisher (Bellefonte, PA), and re-suspended in T-PBS (1x) to a final concentration of 5x10^5 cells/mL. Cells were then immobilized onto slides using a

Cytospin 3 centrifuge and fixated by plunging in ice cold methanol for 5 minutes. Slides were stored at 4° Celsius until staining. To prepare for staining, cells were incubated for 1 hour with 20% goat serum in T-PBS (1X). Primary antibodies (iNOS, NLRP3, and Caspase-1 a gift from Dr. Teresa Alnemri [Thomas Jefferson University, Philadelphia, PA]) were diluted (1:100) in T-PBS and incubated with tissues overnight at 4° Celsius. Secondary staining, mounting, and imaging were conducted as previously described in 2.3.

2.6: Statistics

Values are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using unpaired paired Student T-TestDifferences were considered significant at $p<0.05$. Graphing was done using GraphPad Prism 5.0 (San Diego, CA).

Chapter 3: Results

3.1 Loss of Ron leads to increased vascular iNOS

The Ron receptor is important for maintaining CNS homeostasis and decreasing inflammation. Vascular function can be obstructed during inflammatory conditions and could thus be reliant on Ron receptor functioning. Inflammation was analyzed in smaller arterioles to larger arteries in the entorhinal cortex to observe the effects of a loss of Ron functioning in the CNS of mice placed on a HFHCD. Excess iNOS levels have been shown to lead to reduced activity of eNOS in endothelial cells and thus disrupt cerebrovascular homeostasis. Upon staining for iNOS, a noticeable increase in the expression of iNOS in DKO mice compared to ApoE-/- mice was observed. Figure 4 displays representative images of CNS vessels that were imaged to measure iNOS associated inflammation. Vessels are divided into three categories to effectively quantify iNOS accumulation: small, medium, and large vessels. The quantification of the vascular accumulation of iNOS can be seen in Figure 5, which represents the fluorescent densities of iNOS in respect to small, medium, and large vessels ($p<0.05$). The loss of Ron by obstruction of its ligand binding site appears to cause an accumulation of iNOS in all sizes of CNS vessels, as seen by the black bars (Figure 5).

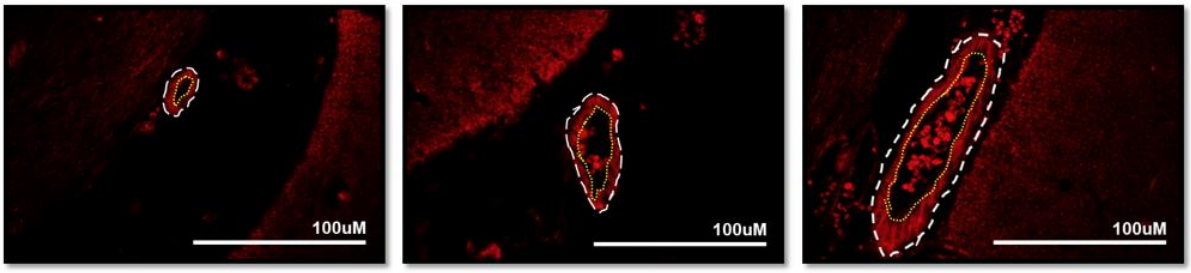

Figure 4: Small, medium, and large vessels in the entorhinal cortex.

Age-matched ApoE-/- and DKO mice were placed on a HFD for 18-weeks. Transverse sections of brain tissue from ApoE-/- and DKO mice were stained with an iNOS antibody overnight and a red fluorescent secondary antibody for 1 hour. Slides were mounted and imaged using a fluorescence photomicroscope.

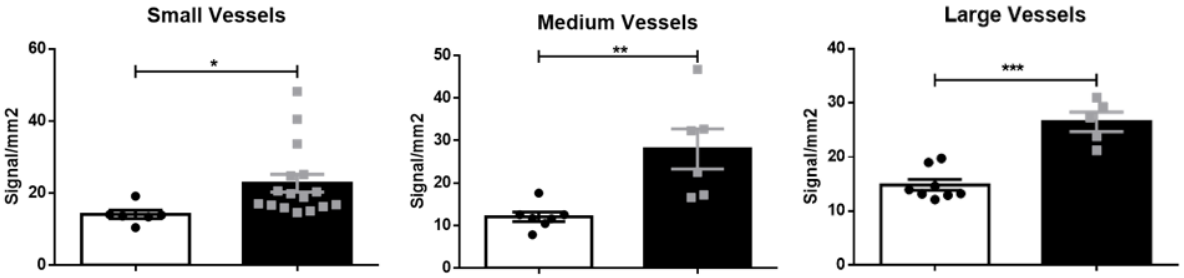

Figure 5: Loss of Ron leads to iNOS accumulation in small, medium, and large vessels in the entorhinal cortex.

Vessels were measured and the densities of fluorescent markers were assessed using ImageJ software. The mean results for vessels were graphed to represent the signal density per mm^2 for ApoE-/- versus DKO mice ($n \geq 6$ for each vessel size).

3.2 Loss of Ron leads to increased neuronal iNOS

Ron is expressed on cells outside of macrophages and assists in repair during inflammatory conditions. Dysregulation of iNOS leads to uncontrolled neuroinflammation, a key characteristic of AD and aging associated dementia.[20] In order to confirm the protective role of Ron, mice with an obstruction of Ron's ligand binding domain (DKO mice) were compared to ApoE -/- mice to assess cellular health. Non-microglia CNS cells in DKO mice had notable increases in iNOS concentration when compared to brain tissue from ApoE-/- mice (Figure 6), confirming that the loss of Ron negatively affects neuronal health through iNOS accumulation. Additionally, iNOS staining did not coincide with nuclear staining, suggesting that iNOS deposits reside outside the nucleus of CNS cells.

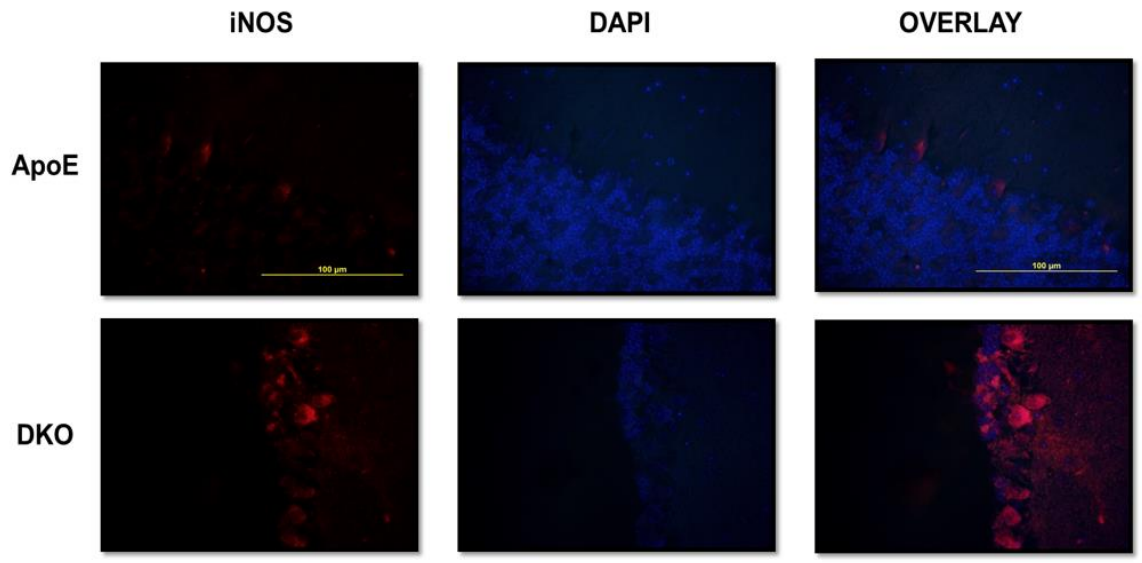

Figure 6: Loss of Ron causes iNOS accumulation in CNS cells of DKO mice.

Transverse sections of brain tissue from ApoE-/- and DKO mice were stained with an iNOS antibody overnight and a red fluorescent secondary antibody for 1 hour to assess CNS inflammation. DAPI served as a nuclear and chromosome counterstain to localize iNOS concentrations. Stained tissues were mounted on slides and imaged using a fluorescence photomicroscope.

3.3 Loss of Ron leads to increased astrocytic inflammation

Astrocytes and microglia respond to CNS stress and injury through astrogliosis and microgliosis, respectively.[10,15] As previously studied, the loss of Ron detrimentally affects CNS health through M1 mediated inflammation and increases in iNOS expression in cells.[9] To further assess CNS macrophage health with respect to the loss of Ron, GFAP and IBA-1 levels were assessed through fluorescent staining of DKO mice (created by deletion of the ligand binding domain of the Ron receptor) and ApoE-/- mice. GFAP concentration had a visibly significant increase in the astrocytes of DKO mice compared to ApoE mice (Figure 7). In contrast, IBA1 concentrations did not appear to have a significant increase in DKO mice (Figure 8). Additionally, both GFAP and IBA1 accumulated outside the nucleus. The data suggests that the loss of Ron triggered astrogliosis, signified by the upregulation of GFAP.

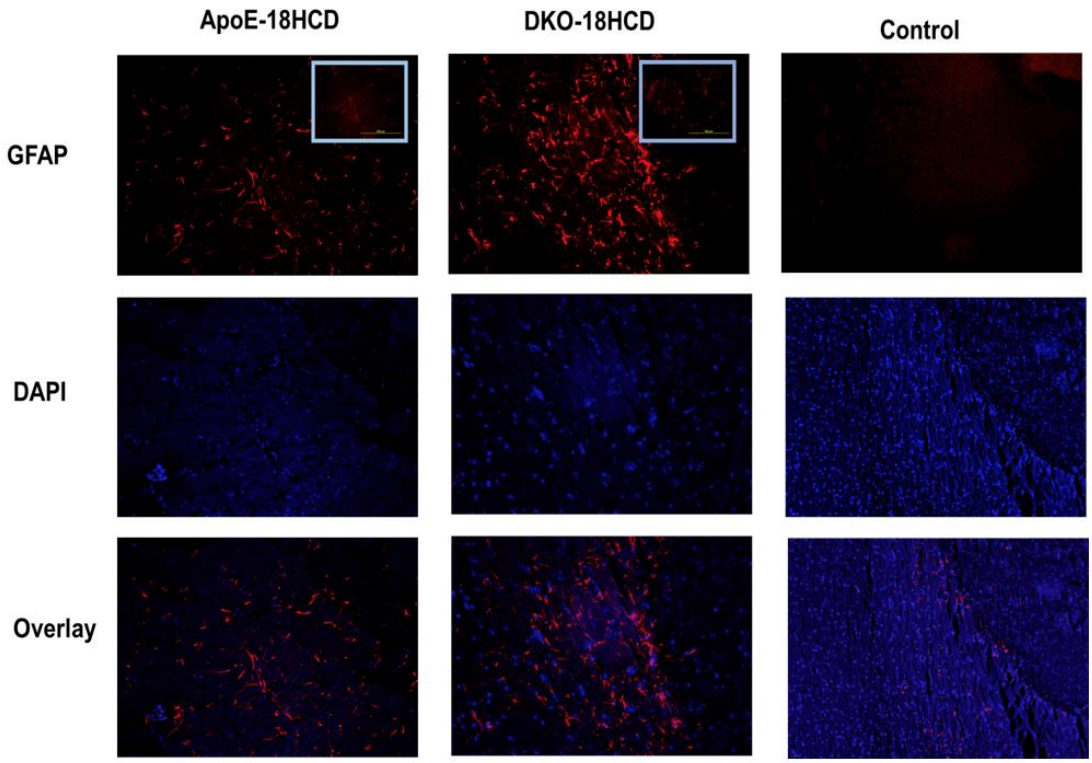

Figure 7: Loss of Ron causes an upregulation of GFAP in astrocytes of DKO mice.

Coronal sections of brain tissue from ApoE-/- and DKO mice were stained with a GFAP antibody overnight and a red fluorescent secondary for 1 hour to assess astrogliosis in response to the loss of Ron receptor functioning. DAPI served as a nuclear and chromosome counterstain to localize GFAP deposits. Control sections had no GFAP staining. Stained sections of brain tissue were mounted on slides and imaged using a fluorescence photomicroscope.

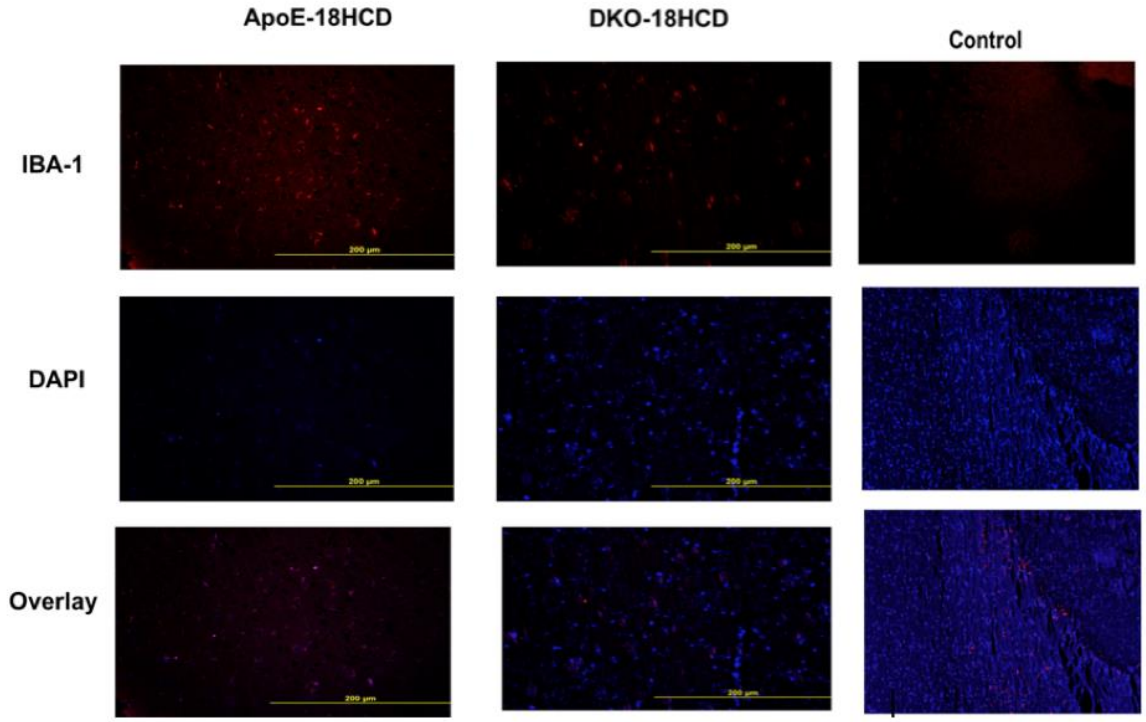

Figure 8: IBA1 concentrations in microglia of DKO and ApoE-/- mice.

Coronal sections of brain tissue from ApoE-/- and DKO mice were stained with an IBA1 antibody overnight and a red fluorescent secondary for 1 hour to assess microgliosis in response to the loss of Ron receptor functioning. DAPI served as a nuclear and chromosome counterstain to localize IBA1 deposits. Control sections had no IBA1 staining. Stained sections of brain tissue were mounted on slides and imaged using a fluorescence photomicroscope.

3.4 MSP-stimulated Ron activation attenuates inflammasome activation

MSP binding to the Ron receptor induces the expression of Ron in a positive feedback manner. Lipopolysaccharide (LPS) can induce inflammation in CNS tissue through M1-mediated activation of macrophages.[8] NLRP3 responds to cellular stress signals and responds by triggering inflammatory cascades.[16] Upon activation by MSP, Ron attenuates inflammation in macrophages through activation of the M2 state.[8] In order to further study the role of the MSP binding site in inflammasome activation, CHME-3 cells were pretreated with LPS in the presence or absence of MSP, and NLRP3 concentration was observed. The data in Figure 9 confirms that the role of Ron in decreasing inflammation depends on activation by MSP. Upon activation of Ron, inflammasome activation is notably decreased in MSP-stimulated cells (Figure 9).

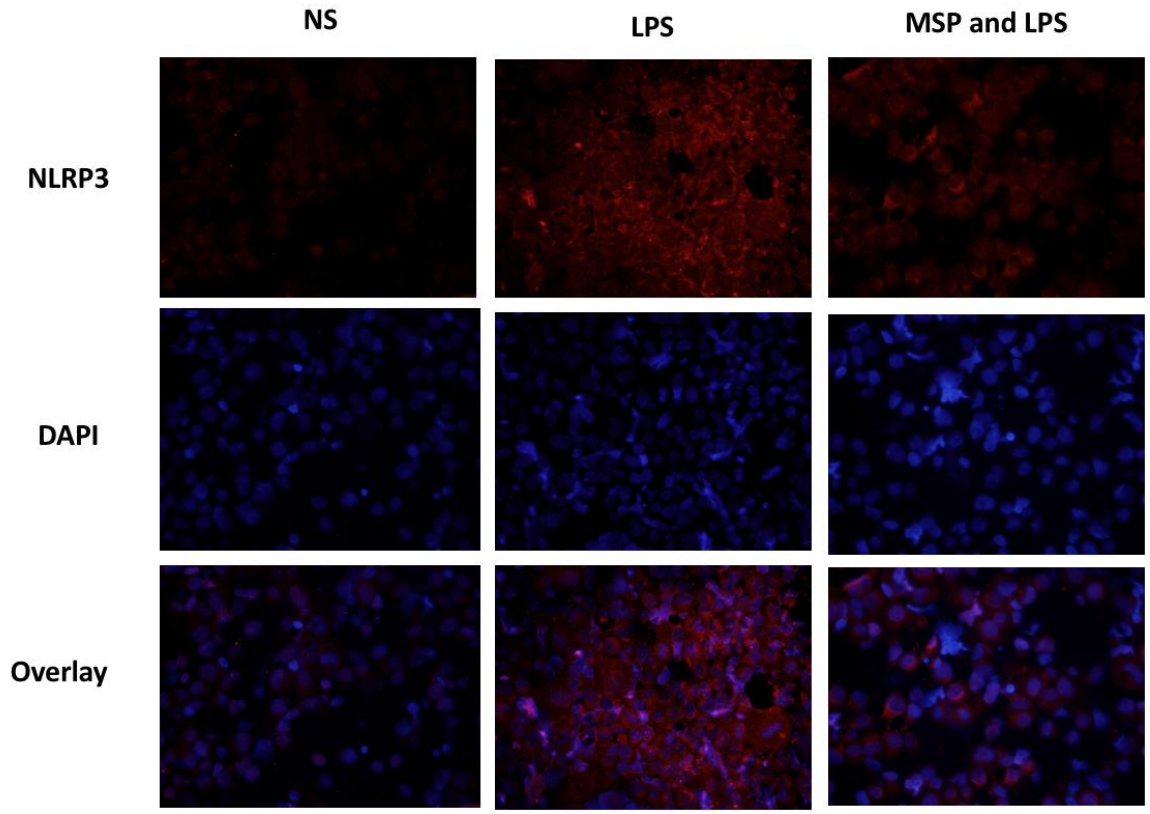

Figure 9: Ron suppresses lipopolysaccharide (LPS)-induced upregulation of NLRP3 in CHME-3 cells.

CHME-3 cells were stimulated for 4 hours with LPS in the presence or absence of Ron agonist MSP. After fixation on slides, cells were stained with an NLRP3 antibody overnight and red fluorescent secondary for 1 hour to assess inflammasome activation in cells. NS cells were used as controls to validate the inflammatory effects of LPS treatment with or without MSP stimulation. DAPI served as a nuclear and chromosome counterstain to localize NLRP3 accumulation. Immuno-stained CHME-3 cells were mounted on slides and imaged using a fluorescence microscope.

Chapter 4: Discussion

This project aimed to further understand the progression of neurodegeneration by studying the role of the Ron receptor tyrosine kinase in controlling CNS inflammation. Specifically, this research analyzed the role of Ron and its dependency on the ligand MSP in regulating inflammation in vascular and cellular structures in the brain. The expression of iNOS, GFAP, IBA1, and NLRP3 was studied to identify the key players involved regulation of CNS inflammation by Ron. The data from this study further confirmed that upon activation by its ligand MSP, the Ron receptor tyrosine kinase is largely responsible for maintaining CNS homeostasis and decreasing inflammation through regulation of iNOS and NLRP3 activation. Additionally, Ron signaling affects the states of glial cells that respond to CNS stress.

iNOS and NLRP3 suppression through MSP-dependent Activation of Ron

This study confirmed that Ron receptor signaling is dependent on the binding of activated MSP to Ron's ligand binding domain. Upon activation, Ron works to reduce inflammation through the induction of an anti-inflammatory M2 state in macrophages. MSP is produced by the liver and circulates through blood in its inactive form. MSP is activated when it is cleaved by proteases such as matriptase, hepsin, and hepatocyte growth factor-A (HGF-A), which are activated at sites of inflammation.[3] Figure 9 shows that when cells were not pretreated with MSP, LPS treatment induced M1 macrophage activation by NLRP3 inflammasome activation. Upon treatment with MSP, NLRP3 expression was significantly lower, suggesting the role of Ron activation in the suppression of inflammasome complex assembly.

Transgenic mice were used to further understand the regulation of CNS inflammation by the Ron receptor. As seen in Figure 6, DKO mice displayed higher levels of iNOS expression in CNS tissue when compared to ApoE-/- mice. iNOS resides mainly in macrophages and its expression is increased during inflammatory (M1) macrophage states. A previous study showed that the Ron receptor works to decrease inflammation by inhibiting the NF-$_K$B pathway, specifically by suppression of IKKβ. The NF-$_K$B pathway is responsible for activating transcription factors for the expression of both iNOS and the prostaglandin-synthesizing enzyme, cyclooxygenase-2 (COX-2).[23] Therefore, when Ron receptor activity is diminished through obstruction of its ligand binding domain, macrophages reside in inflammatory M1 states where they express greater amounts of iNOS, as confirmed in Figure 6.

Both iNOS and COX-2 generate reactive oxygen species (ROS), which has been linked to the induction and assembly of inflammasomes.[5] A previous study by Hua et al. revealed that COX-2 also increases the amount of damaged mitochondria and the release of mitochondrial DNA into the cytosol, which causes an increase in NLRP3 activation.[13] So upon binding of the ligand for Ron, MSP, an anti-inflammatory M2 state is maintained in macrophages by the inhibition of iNOS and COX-2 expression by the NF-$_K$B pathway. In contrast, when Ron activation is diminished, the expression of iNOS and COX-2 significantly increase, leading to cellular stress, and contributing to the activation of the NLRP3 inflammasome pathway.

Glial Cell Activation during Inflammatory States

Glial cells play a critical role in maintaining neuronal health and responding to CNS damage. The brain has almost 10 times more astrocytes than neurons that function to support neuronal activities. Mature astrocytes produce GFAP, an intermediate filament that influences the shape and movement of astrocytes.[1] During neurodegenerative stress, increases in the

expression of GFAP signify astroglial activation and gliosis, which is confirmed by the results in Figure 7. The mass activation of astrocytes through increased expression of GFAP contributes to the loss of neurons and increases cognitive impairment. Therefore, determining a relationship between Ron receptor regulation and GFAP production could provide insight for the treatment of neurodegenerative disorders. A study by Brahmachari et al. found that NO is a potent regulator of GFAP expression and regulates its expression independent of microglial activation. The study concluded that increases in GFAP were caused by NO regulation of the GC-cGMP-PKG pathway.[1] This data supports the findings in Figures 6 and 7 that the loss of Ron receptor functioning results in increased iNOS production, which produces excess NO that increases GFAP production.

Furthermore, microglia serve as macrophages in the brain to rid CNS tissue of damaged or nonfunctional cells. Although there are significantly less microglia than astrocytes-constituting about 5% of glial cells-, they are still primary responders during inflammatory events. IBA1 is expressed in microglia as a calcium binding protein and is increased during inflammation, reflecting microglial activation.[15] Although the loss of Ron receptor functioning has been linked to increased CNS inflammation, the results in Figure 8 do not depict a visible increase in microglial IBA1 expression in DKO mice. A study by Mathys et al., found that two microglia cell states reside that are distinct from the microglia state in healthy brains based on relative gene expressions.[18] These two unhealthy microglia states are different from each other in that one occurs early in neurodegeneration and the other is later in time. Another study by Ito et al., found that IBA1 expression in ischemic brains is time dependent and peaked soon after neuronal injury, as a marker of microglia activation in the brain.[15] Therefore, although the loss of Ron does not seem to affect IBA1 expression, the increase in IBA1 to signal activation of

microglia could have occurred early on in mouse development, prior to when brain tissue was collected at 18 weeks, and decreased expression over time. A future direction to study this hypothesis could be to measure IBA1 concentrations earlier and later in the development of ApoE-/- and DKO mice.

Ron regulates Vascular Inflammation

The brain relies on a constant supply of blood to meet its high oxygen and energy demands. Preserving the health of vascular structures in the brain is thus essential for delivering blood and maintaining homeostasis in CNS tissue. This study explored the relationship between the Ron receptor and vascular inflammation. The results in Figure 5 suggest that a loss of Ron receptor signaling leads to an increase in vascular iNOS concentrations. The buildup in iNOS concentration could correlate with an increase in COX-2 production due to their increased transcription in the absence of Ron regulation. The accumulation of ROS could then trigger inflammasome activation. The inflammation and potential damage to endothelial cell function that results from the loss of Ron could obstruct blood flow, leading to neuronal damage and further cognitive impairment. Furthermore, in a study on a post-ischemic brain, Nogawa et al. stated that the excess NO produced by iNOS has also been shown to activate COX-2 and increase its toxicity by accelerating production of proinflammatory prostaglandins.[21] This finding reveals the importance of the Ron receptor in regulating iNOS production of NO and suggests that regulating NO production through iNOS inhibition may be a strategy for reducing inflammasome activation.

Chapter 5: Conclusion

Overall, this study further confirms that Ron receptor tyrosine kinase is a major regulator of CNS inflammation. By studying the roles of other key players involved in CNS inflammation, such as iNOS, GFAP, IBA1, and NLRP3, a better understanding of Ron's regulatory pathway can be understood and techniques for the treatment of various neurodegenerative disorders can be advanced. This study has shown that, upon activation by its ligand MSP, Ron works to decrease inflammation by inhibiting the production of inducible enzymes iNOS and COX-2 which subsequently reduces inflammasome activation in CNS tissue. Studies on microglia and astrocytes also confirmed that a loss of Ron causes enough CNS inflammation to activate glial cells in response and worsen neurodegeneration. From the studies on vascular inflammation, Ron has shown to maintain CNS homeostasis by attenuating inflammation in blood vessels that could lead to the obstruction of endothelial cells and decreased blood flow in the brain. Studies should be extended to determine more key players in the signaling pathway of the Ron receptor during inflammatory conditions, as well as possible methods of activating the receptor during chronic disease states in efforts increase M2 anti-inflammatory macrophages.

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