TRAFFIC-GENERATED AIR POLLUTION-EXPOSURE MEDIATED EXPRESSION OF FACTORS ASSOCIATED WITH PROGRESSION OF MULTIPLE SCLEROSIS IN A FEMALE POLIPOPROTEIN E KNOCKOUT MOUSE MODEL

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Dissertation Prepared for the Degree of

DOCTOR OF PHILOSOPHY

UNIVERSITY OF NORTH TEXAS

December 2020

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Adivi, Anna. *Traffic-Generated Air Pollution-Exposure Mediated Expression of Factors Associated with Progression of Multiple Sclerosis in a Female Apolipoprotein E Knockout Mouse Model*. Doctor of Philosophy (Biology), December 2020, 98 pp., 3 tables, 18 figures, 1 appendix, references, 213 titles.

Environmental air pollution is one risk factor associated with the onset and progression of multiple sclerosis (MS). In this project, we investigated the effects of ubiquitous traffic-generated pollutants, namely a mixture of gasoline and diesel vehicle exhaust (MVE), on signaling pathways associated with the pathophysiology of MS in the central nervous system (CNS) of either ovary intact (ov+) or ovariectomized (ov-) female Apolipoprotein (Apo) $E^{-/-}$. Specifically, we investigated whether a subchronic inhalation exposure to MVE (200 PM $\mu g/m^3$; 6 hr/d, 7d/wk, 30d) vs. filtered air (FA) controls altered myelination, T cell infiltration, blood-brain barrier (BBB) integrity, or production of reactive oxygen species (ROS) and expression of neuroinflammation markers in the CNS ov+ and ov- Apo $E^{-/-}$ mice. Our results revealed that inhalation exposure to MVE resulted in increased demyelination and CD4+ and CD8+ T cell infiltration, associated with alterations in BBB integrity. Disruption of the BBB was evidenced by decreased tight junction (TJ) protein expression, increased matrix metalloproteinase (MMPs) activity, and increased permeability of immunoglobin (Ig) G, which were more pronounced in the MVE ov- group. Moreover, MVE-exposure also promoted ROS and neuroinflammatory signaling in the CNS of ov+ and ov- mice, compared to FA groups. To analyze mechanisms that may contribute to MVE-exposure mediated inflammatory signaling in the CNS, we examined the NF-κB signaling pathway components, namely IKK subunits, IKKα, and IKKβ, as well as RelA. MVE -exposure did not alter the expression of either IKKα and IKKβ or RelA. However, increased expression of IKKα and IKKβ mRNA was observed in both FA ov- and MVE ov- groups, indicating female sex steroid hormone signaling involvement. Investigation of hormone

receptors expression revealed a reduction in cerebral ERα mRNA expression, compared to ov+ mice; however, MVE-exposure resulted in an even further decrease in expression of ERα mRNA, while ERβ and PRO A/B transcript expressions were unchanged across groups.

Collectively, these study findings revealed that subchronic inhalation exposure to MVE mediates alterations in ER expression in the CNS of ApoE$^{-/-}$ female mice, associated with altered cerebrovascular integrity and increased ROS production and inflammatory signaling. These detrimental outcomes in the CNS, resulting from MVE-exposure, are further associated with increased CD4+/CD8+ infiltration and local demyelination in the CNS of female ApoE$^{-/-}$mice, which are hallmarks of MS. Such findings suggest that exposure to ubiquitous traffic-generated air pollutants may contribute to pathologies that exacerbate demyelinating diseases in the CNS of females.

ACKNOWLEDGMENTS

This dissertation has been completed with the support of all my dissertation committee members. I want to express my sincere appreciation to my major professor Dr. Amie Lund for all her support, training, and guidance during my 6 years of education and research under her guidance.

I would like to thank all my committee members, Dr. Warren Burggren, Dr. Rebecca Cunningham, Dr. Aaron Roberts, and Dr. Wang Xiaoqiang, for serving on my research committee for my Ph.D. degree. I'm thankful for their insightful comments and valuable critiques that were provided in support of my research project and writing. When I started my PhD, I wasn't speaking English fluently and I was difficult to understand at times, so I'm grateful for the patience shown with me throughout the years.

I appreciate my laboratory colleagues, especially JoAnn Lucero, who collaborated with me and trained me on the methodologies and techniques necessary for working in the lab. She has trained me on most of the lab skills and methodologies I needed to complete my research project.

Finally, I would like to express my immense appreciation to my family for all of the support, and motivation that they have given me during these years. This project has been finalized with the assistance of all these people.

So, thank you, every single person that played a part in my educational journey over these past few years, I thank you for being with me.

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CHAPTER 1

INTRODUCTION

1.1 Aims of Study

Hypothesis: Inhalation exposure to traffic-generated air pollutants induces pathways associated with the onset and/or progression of multiple sclerosis.

> Aim 1: Determine if inhalation exposure to vehicle emissions (diesel + gasoline engine) at environmental levels results in altered brain pathology, associated with the pathology of multiple sclerosis (MS).

Multiple sclerosis (MS) is characterized by demyelination and neuronal damage. Demyelination decreases the conduction velocity in neurons or can result in an axonal loss. The focus of this aim is mainly to evaluate the adverse effects of mixed vehicle exhaust (MVE)-exposure on cerebral myelination and the infiltration of T cells (CD4+/CD8+) associated with brain lesions in MS. Even though a link between exposure and MS has been confirmed, the pathways associated with air pollution alteration in MS pathophysiology, particularly myelination, are still unclear.

Sub Aim 1: Since the MS disease is more prominent in females, this aim investigated the effects of sex hormones on signaling pathways associated with demyelination and alteration of brain lesions markers alongside demyelination. We measured gross brain weight, T cell staining by immunohistochemistry as a marker of the brain lesions. CD4+ and CD8+ T cells were stained and analyzed. Finally, we have checked whether the presence of ovary altered the expression of CD4+/CD8+ or not.

> Aim 2: Clarify whether signaling molecules and pathways associated with the onset/progression of MS are deregulated through inhalation exposure to vehicle emissions in ApoE$^{-/-}$ mice on a high-fat diet.

Myelin oligodendrocyte glycoprotein (MOG) autoantibodies are found exclusively in the central nervous system and involved in demyelination. This study investigated how the MOG

altered in response to MVE inhalation and whether sex hormones. For Aim 2, the hormone receptors' role, including estrogen and progesterone receptors in MS pathology, was studied. We measured the alteration in estrogen receptors' signaling and progesterone receptors via the histology and quantitative reverse transcription PCR (RT-qPCR).

Sub Aim 2: We quantified the expression of inflammatory markers such as IL-1, IL-1β, INFγ, TNF-α, IL-6, and oxidative stress mediators (ROS), using DHE staining, immunohistochemistry, and quantitative reverse transcription PCR (RT-qPCR). Also, we have investigated whether the presence of ovary affected each endpoint or not.

Aim 3: Determine pathways involved in blood-brain barrier (BBB) disruption in response to inhalation exposure to mixed exhaust vehicle emissions.

Blood-brain barrier (BBB) disruption occurs via the alteration in tight junction proteins (TJs) or an increase in cell adhesion molecules such as ICAM, and VCAM, and upsurge of metalloproteinases (MMPs) which implicated in the degradation of extracellular matrix (ECM), and TJs. This Aim investigated the signaling pathways associated with MVE – mediated BBB disruption via the alteration of matrix metalloproteinases (MMPs) -2/9 expression and activity, TJ protein expression, and the extravasation of Immunoglobulin G (IgG) using immunofluorescence staining, in situ zymography and quantitative reverse transcription PCR (RT-qPCR). Also, we determined whether hormone/ovary status altered expression of this endpoint, related to exposure.

1.2 Significance

Approximately 2.5 million people worldwide have been diagnosed with multiple sclerosis (MS), characterized by neuronal demyelination and axonal damage (National MS Society, 2018). MS is 2-3 times more predominant in women than men (Trapp and Nave, 2008). Besides gender, genetics, geographical location, viral infection, vitamin D deficiency, and

environmental factors are all associated with the initiation and progression of MS pathologies (Alonso, and Hernán, 2008). It has been confirmed that air pollution exposure is linked to MS's incidence and progression (Tateo et al., 2019). For example, a higher prevalence of MS is reported in locations where air pollutants such as particulate matter (PM), sulfur dioxide (SO_2), and nitrogen oxides (NOx) levels are high (Heydarpour et al., 2014). Moreover, ozone (O_3) and PM_{10} have been linked to the occurrence of MS and the frequency of relapses (Jeanjean et al., 2018). Diesel exhaust PM exposure lower the differentiation of effector T cells in a dose-response manner and suppressed T regulatory cells (Treg) differentiation in vitro and exacerbated experimental autoimmune encephalomyelitis (EAE) in vivo. (O'Driscoll et al., 2018). While there are a handful of epidemiologic studies showing associations between air pollution exposure and progression or exacerbation MS, there is a lack of comprehensive research studies investigating and identifying the mechanism of actions of pollutants in contribution to the central nervous system (CNS)-related disorders, such as MS. Additionally, it has been well documented that autoimmune diseases, such as MS, occur with higher frequency in women, and MS symptoms decline in some women during the pregnancy. Despite the fact much of the available literature is from studies in males, this project focused on identifying the effects of mixed vehicle engine (gasoline + diesel) exhaust on the pathophysiologic pathways associated with MS in a female apolipoprotein (Apo) $E^{-/-}$ mouse model. $ApoE^{-/-}$ mice model is an animal model to study atherosclerosis, cardiovascular, respiratory disease, and inhalation toxicology. $ApoE^{-/-}$ knockout mice have also been used to study Alzheimer's disease, and the ApoE alleles do not affect MS. Also, we wanted to compare our results from female $ApoE^{-/-}$ mice to the effects of similar studies on male $ApoE^{-/-}$ mice in our lab. Mice were placed on a high-fat diet to try to be consistent with the western diet. However, the amount of fat percentage

may be high compared to some country's diet, but it makes sense at a global level. We wanted to investigate whether exposure to traffic-generated air pollutants resulted in different outcomes related to the female central nervous system (CNS). Therefore, our experimental design included analyses of exposures in both ovariectomized and ovary-intact female mic*e*. Our overarching hypothesis for the study is that inhalation exposure to traffic-generated (vehicle engine) air pollution results in the induction of pathways associated with the pathophysiology of MS, associated with blood-brain barrier disruption the CNS of female ApoE$^{-/-}$ mice.

1.3 Background

MS is a demyelinating and inflammatory disease of presumed autoimmune origin, which affects the CNS and peripheral nervous system (PNS) (Trapp and Nave, 2008). Axons are wrapped by a fatty layer called the myelin sheath, which is supplied by either oligodendrocyte in CNS or Schwann cells in the PNS, and functions as both an insulator and to speed up the transmission of the action potential down the length of the axon (Knowles, 2017). Myelin sheath increases the velocity and frequency of impulse transmission within the CNS and between the CNS and PNS (Fields, 2008). Demyelination, often accompanied by axonal damage, is the hallmark feature of MS (Popescu, 2013). T-cell mediated inflammation is also known to play a vital role in MS pathology, which is associated with the degree of demyelination present in the CNS. In MS, reactive T-cells subtypes, such as CD4+ and CD8+, travel across a disrupted blood-brain barrier (BBB) and trigger an inflammatory response and activation of microglia that damage the myelin sheath (Minagar and Alexander, 2003). CD4+ are macrophage cells bind to major histocompatibility complex (MHC) II and phagocytize the myelin sheath, while CD8+ are cytotoxic cells that bind to MHC class I and induce cytotoxic effects in the CNS, including inflammation and ROS production. Increased infiltration of CD8+ cells into the CNS has also

been reported to induce neuronal apoptosis (Medana et al., 2000).

1.3.1 Etiology of Multiple Sclerosis

Many factors, including race, sex, genetics, geographical location, viral infection, and air pollutants, alter the frequency of MS in humans (Milo and Kahana, 2010). MS most frequently occurs in Caucasians, with the highest incidence occurring between age 25 and 35 years (Ascherio, and Kassandra, 2007). Previous studies also report a genetic association of the human leukocyte antigen HLA-DR2 haplotype, which poses antigens to the immune system to initiate a T-cell response, with increased incidence of MS (Sawcer et al., 2014). A large percentage of MS patients (between 28-33%) express the HLA-DRB1*15:01 haplotype (Mangalam et al.,2013). The HLA is a complex of genes classified into three groups based on their tissue origin, structure, and functions (Gfeller and Michal, 2018). Class I and II of MHC genes encode codominant expressed HLA cell antigens, and class III genes encode different modules of the supplement system; all play critical roles in the function of the immune system (Hoppenbrouwers and Rogier, 2010).

Interestingly, the risk of MS can change across generations, often dependent on geographical location. For example, it has been reported that when migrants from low-risk geographical locations relocated to high-risk geographical locations, they maintain their low risk; however, their children tend to have a risk level associated with that of the (higher risk) new region (Gale and Martyn, 1995). The risk of MS varies from 30.5% in offspring where both parents have been diagnosed with MS, compared to 2.49% in offspring who have only one parent with MS (Tietje et al., 2013). Interestingly, some studies suggest that the genetic risk for developing MS is more strongly associated with their maternal vs. paternal lineage (Hoppenbrouwers et al., 2008). While the initiating events that trigger the pathogenesis of MS

remain uncertain, outcomes from at least a few studies suggest that a combination of infectious and non-infectious factors likely initiate MS in patients with a genetic predisposition (Kountouras et al., 2007). In agreement with this premise, 99.5% of all MS patients are seropositive for Epstein-Barr virus, and expression of latent viral proteins is frequently detected in the brains of MS (Serafini et al., 2007). Vitamin D deficiency is another factor associated with frequency of MS relapses, and associated disability (Smolders et al., 2008). Regardless of the factors associated with the initiation of MS, the progression of MS is associated with an increase in inflammation and BBB disruption.

MS is more prevalent in females vs. males, with a respective average ratio of 2.9:1 (Harbo et al., 2013). The occurrence of MS is currently increasing in females, while the rate remains constant in males. The cause is not clear, but it is believed that sex hormones are involved in the onset and progression of MS, as well as may contribute to different outcomes of therapy, including response to interferon-β treatment (Magyari et al., 2014). The effects of steroids hormones on MS will be explained in more detail in section 1.6, below. Another strong risk factor for MS is exposure to air pollution. Recent epidemiological studies have reported that exposure to environmental air pollutants is associated with increased incidence and/or relapse of MS (Jeanjean et al., 2018). The effects of air pollution on MS, as well as some of the proposed mechanisms of toxicity are described in more detail in section 2.7.

1.3.2 Multiple Sclerosis and Blood Brain Barrier Disruption

The blood brain barrier (BBB) is a barrier between the systemic circulation and the brain parenchyma, which consists of cerebrovascular endothelial cells, pericytes, and their basal lamina, which are surrounded and reinforced by the foot processes of astrocytes and perivascular macrophages (Minagar and Alexander, 2003). The BBB serves as both a physical and chemical

barrier that controls the influx of most complexes from the blood into the brain tissue (Ronaldson, and Davis, 2011). Endothelial cells are connected by TJ proteins, including claudins and occludin, which are transmembrane proteins that form a “zipper-like” barrier and regulate paracellular diffusion (Balda and Matter, 2008). Cell Adhesion molecules (CAMs) including intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1, belong to the immunoglobin superfamily. Increased expression of both ICAM-1 and VCAM-1 in the cerebral microvasculature have been associated with BBB disruption, increased leukocyte trafficking, and increased inflammatory responses in MS (Elovaara et al, 2000). Of importance for the current study, inhalation exposure to traffic-generated air pollution results in altered BBB integrity and transport, which is association with decreased TJ protein expression, in both animal and human studies (Oppenheim et al., 2013). Furthermore, air pollution-mediated alterations in BBB permeability have been positively correlated with increased occurrence of CNS disorders, including stroke, dementia-related diseases, and MS (Genc et al., 2012). Our knowledge about the mechanism of air pollution-exposure and alterations in the integrity of the cerebral microvasculature is not complete, but inflammation, ROS production, and upregulation of MMP-2/9 activity are associated with traffic-generated air pollution exposure-mediated BBB disruption (Oppenheim et al., 2013; Suwannasual et al., 2018, 2019).

1.3.3 Multiple Sclerosis and Inflammatory Pathways

Inflammatory signaling is a hallmark of MS disease and is associated with the increased translocation of immune cells into the brain due to BBB disruption (Miljković and Spasojević, 2013). After T cells are activated in the periphery by stimuli (i.e., genetic, environmental, pathogenic, etc.), they migrate to the CNS and initiate an inflammatory response (Dejaco et al., 2006). Key inflammatory markers associated with MS are interleukins (ILs), tumor necrosis

factor (TNF)-α, interferons (INFs), reactive oxygen species (ROS), and their respective signaling pathways (Kallaur et al., 2017). In MS patients, some of these inflammatory factors are also found upregulated in the systemic circulation, in addition to the CNS. For example, serum levels of IL-1β, IL-17, TNF-α, and INF-γ were found to increase in patients with more progressed cases of MS, indicating these factors may serve as a biomarker for the severity of the disease state (Magnano et al., 2004). TNF-α is a cytokine secreted by macrophages and microglia in the brain, which has been reported to mediate oligodendrocyte necrosis, resulting in demyelination (Kemanetzoglou et al., 2017). IL-1 expressed in various cells, including neurons, astrocytes, is also believed to play a role in MS pathologies via damage to the blood-brain barrier (Warabi, 2007). Increased expression of another proinflammatory cytokine, IL-1β, has been reported to induce MS-associated neurodegenerative damage via a p53-mediated apoptosis pathway (Rossi et al., 2014).

IL-17 and INF-γ are also known to promote leukocyte recruitment, drive inflammation at the BBB, and translocate into the CNS via ICAM-1-mediated transport pathways during demyelinating pathologies, including those associated with the progression of MS (Levesque et al., 2013). Interestingly, researchers have shown that dendritic cells (DCs) are observed close to invading T cells (i.e., CD4+ cells) and macrophages in the brain lesions of MS patients (Legroux, and Arbour, 2015). Also, DCs are able to invade the blood vessels of non-inflamed regions of the brain, suggesting they may serve as the initial antigen-presenting cell recognized by invading encephalitogenic effector/memory T cells (Greter et al., 2005). Expression of both CD4+ and CD8+ cells are found associated with MS lesions in the CNS, with CD4+ cells prevailing in acute injuries, and CD8+ cells being observed more commonly in chronic lesions (Chitnis., 2007). It has been reported that CD4+ lymphocytes are MHC class II-restricted, mostly

polarized as Th1 cells initiate CNS inflammation by producing inflammatory cytokines such as IFN-γ, IL-2, TNF-α, and lymphotoxin (Akdis et al., 2011). Stimulated myelin-reactive CD4+ cells are present in the blood and cerebrospinal fluid of MS patients and may function as a possible biomarker for the pathology's progression. CD8+ bound to MHC class I are frequently observed in MS lesion, as well, and exert their cytotoxic effects through induction of Fas ligand (FasL) and apoptosis of the cells in the CNS (Deb et al., 2010).

1.3.4 Multiple Sclerosis and Female Steroid Hormones

One of the causative factors linked to the prevalence of MS appears to be steroid (sex) hormones, as MS is reported to be 2- to 3- times more common in females compared to males (across age groups) (Voskuhl and Gold, 2012); however, the reason for this inequality has not been well characterized. In the female, the physical adjustment starts with pubertal development that results in the activation of the hypothalamic-pituitary-gonadal (HPG) axis established before birth but remains inactive until the onset of puberty. The HPG signaling in the female consist of a feedback cycle that regulates the hypothalamic secretion of gonadotropin-releasing hormone (GnRH), which acts on the anterior pituitary and stimulates the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Both FSH and LH are involved in gametogenesis in the female, whereby FSH stimulates maturation of the ovarian follicle, and LH signals ovulation of the mature follicle or oocyte (Fisher 1998). Menopause occurs when menstrual periods stop forever, which is characterized by a sustained increase of FSH. Menopause typically starts between 45-55 years of age. While both males and females have similar signaling via the HPG axis, the gonad hormones differ. Estrogen and progesterone secreted from the ovary in response to pituitary hormones, while males release androgens from the testes. Thus, one possible difference between MS rates in females vs. males could be in response to sex hormone-

related pathways, specifically estrogen and progesterone (Marques, 2018). The biological functions of estrogen are mediated via two nuclear estrogen receptors (ER): ERα and ERβ, which are universally expressed in the body, whereas progesterone exerts its effects via the progesterone receptor (PR, isoforms A or B). Estrogen has been described in the literature to exert an anti-inflammatory or an "immune protective" response in patients (Jiang et al., 2010; Laffont et al., 2015). Interestingly, MS relapses appear to decline in pregnancy, specifically in the third trimester, suggesting that sex steroid hormones may impact MS susceptibility (Voskuhl and Gold, 2012). Since the main female sex hormones, estrogen and progesterone, are elevated during pregnancy, particularly in the third trimester, and both hormones are involved in immune response, that may mediate the documents decline in the relapses. Progesterone has an anti-inflammatory role and decreased IFN-γ production and IL's production, including IL-2, IL-17, IL-23 and CD4+ and CD8 + in EAE mice (Ysrraelit et al., 2019). The therapeutic effects of sex hormones in MS were experimentally investigated, in one study, by using an ERβ ligand agonist treatment. There was no significant difference observed in the onset of MS; however, activation of the ERβ receptor appeared to stimulate recovery during the chronic phase of MS (Wisdom et al., 2013). Additionally, studies with ERα agonist-treatment have reported an anti-inflammatory effect on the systemic immune system and were associated with reduced CNS inflammation (Tiwari-Woodruff et al., 2007). Progesterone treatment in EAE mice reduced the severity of courses and reduced inflammatory response and demyelination in the spinal cord (Garay et al., 2007). Progesterone has also been shown to promote myelin regeneration and have a neuroprotective influence in demyelinating lesions in the CNS (Almad et al., 2011). Further roles for sex hormones in the relapse and/or progression of MS have been discovered via studies in pregnant patients with MS. For instance, a study in pregnant MS patients reported that these

patients have a significantly reduced inflammatory profile and relapse rate during the third trimester of gestation when estriol (one of the three main endogenously produced estrogen types) levels are the highest (Confavreux et al., 1998). Moreover, relapse rates rebound in the postpartum time, which is correlated to a decline in serum estriol levels (Gilli et al., 2010). This premise was further established through a study where MS patients treated with estriol presented with considerably less lesion number and volume, as measured by MRI (Sicotte et al., 2002). One possible mechanism for these findings may be due to progesterone-mediated production of the progesterone-induced binding element (PIBF) by lymphocytes. In humans, PIBF increases throughout pregnancy and falls considerably after birth (Polgár and Todd, 2008). High concentrations of PIBF have been reported to promote differentiation of CD4+ T cells into helper T cell type 2 (Th2) cells that secrete high concentrations of anti-inflammatory cytokines, such as IL-4, IL-5, and IL-10 (Szekeres and Polgar, 2010). Other reasons can be the result of alteration in human chorionic gonadotropin (hCG) produced by syncytiotrophoblast cells after embryonic implantation modifies dendritic cell activity via a decrease in T-cell activation and cytokine production (Bansal et al.,2012).

1.3.5 Environmental Air Pollutants and Multiple Sclerosis

While the majority of studies in the literature show that environmental-exposure is a risk factor in MS disease, extensive research into which types of environmental factors involved in the etiology of MS are lacking. The majority of previously published studies have focused on the link between smoking and MS susceptibility. Not surprisingly, nearly all have detected a significant detrimental effect. Cigarette smoking has been reported to result in increased BBB permeability, associated with a decreased expression of TJ proteins (Mazzone et al., 2010). Air pollutants, including particulate matter (PM), sulfur dioxide (SO_2), nitrogen oxides (NO_x), and

ozone (O_3) are connected to the incidence of MS and the development of relapses (Jeanjean et al., 2018). In addition to the mentioned air pollutants, diesel exhaust alters Th1 and Treg differentiation in both experimental autoimmune encephalomyelitis (EAE) and in vitro (O'Driscoll et al., 2018). Results of a population-based cohort study revealed living close to highways increased the incidence of a neurological disorder such as Alzheimer's, Parkinson's, and MS in Canada (Chen et al., 2017). Additionally, environmental air pollutants have also been implicated as a chronic source of neuroinflammation and ROS that can produce neuropathology and CNS diseases (Oppenheim, H. et al., 2013). Exposure to PM_{10} initiates immune responses by increasing the expression of proinflammatory cytokines and increasing ROS, increasing BBB breakdown, and activation of autoreactive T cells (Bergamaschi et al., 2018).

Considering the multiplicity of variables involved in the pathophysiology of demyelinating diseases, including MS, such as sex and environmental factors, and lack of clear understanding of their effects, we investigated the hypothesis that inhalation exposure to traffic-generated air pollution results in the induction of pathways associated with the pathophysiology of MS, associated with blood-brain barrier disruption, in the CNS of female Apo $E^{-/-}$ mice. Furthermore, we utilized both ovary-intact and ovariectomized female mice to assess hormone vs. exposure-mediated outcomes, or interactions thereof, in the CNS.

CHAPTER 2

TRAFFIC-GENERATED AIR POLLUTION– EXPOSURE MEDIATED EXPRESSION OF FACTORS ASSOCIATED WITH DEMYELINATION IN A FEMALE ApoE$^{-/-}$ MOUSE MODEL*

2.1 Abstract

Epidemiology studies suggest that exposure to ambient air pollution is associated with multiple sclerosis (MS) progression in humans. The pathophysiology of MS results from an autoimmune response involving increased inflammation and demyelination in the central nervous system (CNS), which is higher in young (adult) females. Exposure to traffic generated air pollution is associated with neuroinflammation and other detrimental outcomes in the CNS; however, its role in the progression of pathologies associated with MS has not yet been fully characterized in a female model. Thus, we investigated the effects of inhalation exposure to mixed vehicle emissions (MVE) in the brains of both ovary-intact (ov+) and ovariectomized (ov-) female Apolipoprotein (ApoE$^{-/-}$) mice. Ov+ and ov- ApoE$^{-/-}$ mice were exposed to either filtered air (FA, controls) or mixed gasoline and diesel vehicle emissions (MVE: 200 PM μg/m$_3$) for 6 hr/d, 7 d/wk, for 30 d. We then analyzed MVE-exposure mediated alterations in myelination, the presence of CD4+ and CD8+ T cells, reactive oxygen species (ROS), Myelin oligodendrocyte protein (MOG), and expression of estrogen (ER) and progesterone (PROA/B) receptors in the CNS. MVE-exposure mediated significant alterations in myelination across multiple regions in the cerebrum and increased CD4+ and CD8+ staining. There was also an increase in ROS production in the CNS of MVE-exposed ov- and ov+ ApoE$^{-/-}$ mice. Ov- mice

* This chapter has been submitted for peer-review at Environmental Pollution as: Adivi A, Lucero J, Simpson N, McDonald JD and Lund AK. Traffic-Generated Air Pollution– Exposure Mediated Expression of Factors Associated with Demyelination in a Female Apolipoprotein E-/- Mouse Model.

displayed a reduction in cerebral ERα mRNA expression, compared to ov+ mice; however, MVE exposure resulted in an even further reduction in ERα expression, while ERβ and PRO A/B were unchanged across groups. These findings collectively suggest that inhaled MVE-exposure may mediate estrogen receptor expression alterations associated with increased CD4+/CD8+ infiltration into the CNS, regional demyelination, and production ROS.

Key words: Air Pollution, Female, Multiple sclerosis; Inflammation, ROS

2.2 Introduction

Recent statistics estimate that more than 2.5 million people worldwide have been diagnosed with multiple sclerosis (MS), an autoimmune disease of the central nervous system (CNS) (National MS Society, 2018). MS is considered a disease of the CNS associated with demyelination and neuronal damage (Peterson and Fujinami, 2007). Myelin sheath is a fat layer (70% lipid and 30% protein) that wraps around nerves and is produced by oligodendrocytes in the CNS (Jackman et al., 2009). Myelin oligodendrocyte glycoprotein (MOG) is localized to the outer layer of a myelin sheath of the CNS and expressed by oligodendrocytes (Narayan et al.,2018). While the function of MOG is not clear, it is believed to perform as a cellular adhesive molecule, involved in the regulation of the stability of oligodendrocyte microtubule to facilitate complement cascade (Wynford-Thomas et al., 2019). The progression of MS and relapse rates increased in patients with antibodies to MOG (Spadaro et al., 2016).

In addition to MOG, reactive oxygen species (ROS) such as superoxide ($O2^{-\bullet}$) and hydroperoxyl radical ($HO2^{-\bullet}$) are bioproduct of electron transport chain in mitochondria and overproduction of ROS in response to inflammation participate in different mechanisms triggering the pathogenesis of MS (Ortiz et al., 2013). Overproduction of ROS accelerates the initiation of lipid peroxidation cascade, which results in demyelination and the death of

oligodendrocytes (Smith et al., 1999). ROS play a vital function in the signaling of the molecules that target T cell activation and differentiation, whereas overproduction of ROS causes damage to these biomolecules and cellular organelles and their functioning (Gnanaprakasam et al., 2018). CD4+ and CD8+ are part of the adaptive immune system, CD4+ bind to major histocompatibility complex (MHC) class II, which are antigen-presenting cells act as macrophage cells present in human MS lesion, while CD8+ bind to MHC class I and promotes cytotoxic immune response (Denic et al., 2013). MS is associated with T cell infiltration and inflammation in the CNS, leading to lesions characterized by demyelination and axonal loss (Brück, 2005). During MS, the integrity of the blood-brain barrier (BBB) impaired, leading to an increase of translocation of activated T cells, such as CD8+ and CD4+, from the circulation into the parenchyma (Ortiz et al., 2014). The increase in the infiltration of activated T cells is associated with an inflammatory response and activation of microglia, which can damage the myelin sheath (Minagar and Alexander, 2003). CD8+ cells in the CNS are also associated with the induction of neuronal apoptosis via the ligation of cell death receptors such as Fas ligand (FasL) and/or Fas receptor (Volpe et al., 2016).

Ambient air pollution is made of different components including carbon dioxide (Co_2), nitrogen oxides (NO_x), particulate matter ($PM_{2.5}$ and PM_{10}), volatile organic compounds (VOCs), ozone (O_3), and heavy metals (Kampa and Castanas, 2008). Previous studies demonstrated that ambient air pollution and PM_{10} exposure increased MS relapses by four-fold (Oikonen et al., 2003). Outcomes from both human studies and animal models show a strong correlation between exposure to environmental air pollution and detrimental CNS consequences including neuroinflammation, neurodegeneration, and BBB disruption (Block and Calderón-Garcidueñas et al., 2009; Suwannasual et al., 2018). Air pollution is associated with neurodegenerative diseases

such as Alzheimer's disease (AD), and MS via the release of cytokines, ROS overproduction in the CNS (Block and Calderón-Garcidueñas, 2009).

Sex hormones are also believed to contribute to the onset or progression of MS. The highest prevalence of MS is reported in young females (ages 20-40 yrs.), with rates of diagnosis approximately 2-3 times higher than that reported in men (Noonan, 2002). The manifestation of MS appears to be relatively low in early life but occurs with more frequency during young adulthood, and then drops back off around age 50 (Ascherio et al., 2012). Also, MS relapses decline by 80% in the third trimester of pregnant MS patients, when both estrogen and progesterone levels are elevated (Harbo et al., 2013). Moreover, estrogen has also been reported to have neuroprotective effects, treatment with an estrogen receptor-beta (ERβ) agonist effectively improved clinical courses of MS and provide neuroprotection in EAE (Spence et al.,2013). The upsurge of both ERα and ERβ have been associated with the reduction of demyelination and axonal loss in the EAE animal model of MS (Maglione et al., 2019). Together, the outcomes suggest an alteration in sex-steroid hormone production and signaling play an essential role in the etiology and/or pathology of the disease. The observation further confirms this premise that pregnancy appears to provide "protective effects" of relapse in MS patients, via an estrogen-mediated reduction in proinflammatory cytokine expression (Soldan et al., 2003). During the development of MS, it is hypothesized that CD4+/CD8+ cells are activated in the periphery in response to an "insult" or stimuli of genetic, environmental, and/or pathogenic origin (Dejaco et al., 2006). These T cells then travel to the CNS, where they are believed to initiate an inflammatory cascade causing demyelination and axonal loss (Lubezki and Stankoff, 2014). The mechanisms involved are still under investigation since exposure to ubiquitous environmental air pollutants, such as vehicle engine emissions, has been established to induce

detrimental CNS pathologies related to the occurrence of MS. Furthermore, very little information exists on the effects of traffic-generated pollutant-exposures in the CNS of females. Therefore, this study investigated the hypothesis that inhalation exposure to a mixture of gasoline and diesel vehicle emissions (MVE) mediates the induction of pathways associated with the demyelination in female ApoE$^{-/-}$ mice. To further delineate the role of hormone signaling contributions in the observed MVE mediated CNS effects, we utilized female ApoE$^{-/-}$ mice with ovaries, and those that had been ovariectomized, in our exposure studies. Although, ApoE$^{-/-}$ mice were considered not to be a suitable model for MS study; it has been confirmed as the right strain for study inhalation toxicology (Sasso et al.,2016). Despite everything, this study will provide a new perspective of air pollution effect on the female mice model and new information regarding understanding the sex bias in autoimmune diseases specifically demyelinating diseases via in alteration myelination, ROS production, and initiation of lesion markers CD4+ and CD8+ in the brain of female ApoE$^{-/-}$ mice.

2.3 Materials and Methods

2.3.1 Animals and Inhalation Exposure Protocol

Six-to-eight-week-old female Apo E$^{-/-}$ mice, 16 ovariectomized and 16 ovary-intact, were obtained from Taconic (Albany, NY). All mice were placed on a high fat diet (TD88137 Custom Research Diet, 21.2% fat, 1.5g/kg cholesterol diet; Harlan Teklad, Madison, WI) for two weeks prior to the onset of exposures and were maintained on the same diet throughout the 30-day exposure. Cytology (vaginal swabs) was utilized to determine the stage of estrous at the beginning of the exposure. Mice were then grouped to receive either mixed vehicle emissions (MVE: 200 PM μg/m^3), which was created from a mixture of exhausts generated by GM gasoline engine (50 PM μg/m^3) and a Yanmar diesel generator system (~150 PM μg/m^3) or

filtered air (FA, controls) for 6 hr/d, 7 d/wk, for 30 d, as previously described (Lund et al., 2009). Characterization and measurement of PM and gases were conducted daily, as previously described (Lund et al., 2011). Mice were singly housed in standard shoebox cages within an Association for Assessment and Accreditation of Laboratory Animal Care International approved rodent housing facility ($2m^3$ exposure chambers) for the duration of the study, which maintained a constant temperature (20–24°C) and humidity (30–50% relative humidity). Mice had access to chow and water ad libitum throughout the study period, except during daily exposures when chow was removed. All animal protocols were approved by the Lovelace Respiratory Research Institute's Animal Care and Use Committee (AAALAC-accredited Assurance #A3083-01; USDA-registered facility #85-R-003) and followed the Guide for the Care and Use of Laboratory Animals issued by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.3.2 Tissue Collection

At the end of the assigned exposure protocol, the animals were anesthetized with Euthasol®, euthanized by exsanguination; and brain tissues were collected. The meninges were gently removed from the brains, the brains weighed, dissected (coronal plane/cut at roughly Bregma 0 – Bregma -2.92 mm) and a portion fixed in HISTOCHOICE (VWR, Irving, TX) at 4°C overnight. Fixed tissue was then rehydrated in 30% sucrose/PBS (weight/vol) at 4°C overnight, and embedded in Tissue Freezing Medium (TBS, IMEB Inc., San Marcos, CA) for sectioning. The remaining regions of the brain not fixed for histology were frozen in liquid nitrogen and stored at -80°C for future molecular assays.

2.3.3 Cerebral Myelin Staining

Embedded frozen brain tissues were cut on a cryostat in serial 10µm sections, placed on

Superfrost™ Plus slides (ThermoFisher, Richardson, TX), and stored at -80°C. For myelination staining, a Brain – StainTM Imaging Kit (ThermoFisher #B34650) was utilized, following the manufacturer recommended the protocol. Green (GFP) was used to stain myelin, and red (RFP) was used for Nissl staining. Stained slides were imaged at 10x, 20x, and 40x using the inverted fluorescent microscope with epifluorescence optics (EVOS Fl, ThermoFisher) and analyzed with ImageJ software (NIH, Bethesda, MD). Slides with no primary antibody treatment were used as negative controls. N = 5 animals for each group, 2 slides each animal, with 2 sections on each slide were used for quantification and analysis. For microscopy and analysis, brains were divided into six regions (Fig. 2.1). Region 6 was excluded from myelin staining quantification. The same area and size were measured in each region for quantification in Image J. All myelin staining is reported as total fluorescence staining per until area for the defined region (Fig. 2.1).

2.3.4 CD4+/CD8+ Staining and Quantification

Sections of 10 µm frozen cerebral tissue between were processed through immunohistochemistry for CD4+ or CD8+ cell expression. Briefly, sections were treated in cold acetone for 30 min at RT and rinsed with 1X PBS 3 times. Then slides were blocked with a solution of 1X PBS-T, and Bovine Serum Albumin (60 mg/2 ml vol/ vol) for 1 hr at RT. Tissue sections were then incubated with biotinylated primary antibodies CD4+, and CD8+ (Affymetrix eBioscience/ThermoFisher #36-0041-85 and 36-0081-85, respectively) in a dark chamber overnight at 4°C. Samples were rinsed 3 times with 1X PBS, and the substrate was added using an ABC detection kit (#PK-6100; Vector Laboratories, Burlingame, CA), following the manufacturer protocol and incubated for 1 hr in the dark chamber at RT. Slides were developed by using a Vector Red substrate kit (#SP-5100 0; Vector Laboratories), following the manufacturer protocol. Slides were then counterstained with hematoxylin and cover-slipped.

Sections were imaged by microscopy at 10X and 40X, N = 5 from each group, 2-3 slides per animal, with 2 sections each. Histological analysis was conducted in regions of the brain, as described in the previous section and shown in Fig. 2.1. Image J cell counter software was used to quantify the expression of CD8+ and CD4+ (red staining) in each defined region. Samples without primary antibody were used as a negative staining control to confirm staining specificity.

2.3.5 Real Time RT-qPCR

RNA was isolated from cerebral tissue using an RNeasy Mini kit (Qiagen, Valencia, CA), per kit instructions. cDNA was synthesized using an iScript cDNA Synthesis kit (BIORAD, Hercules, CA; Cat. #170-8891). Real-time PCR analyses of ERα, ERβ, progesterone receptors A/B (PRO A/B), or GAPDH (house-keeping gene) were conducted using specific primers (Table 2.1) and SYBR Green detection (SSo Advanced Universal SYBR Green Supermix, Biorad, Hercules, CA; Cat #172-5271), following manufacturer's protocol. Real-time PCR analyses were run on a Biorad CFX96 Touch™ Real-Time PCR Detection System (BioRad). ΔΔCT values measured using CFX Manager™ Software and normalized to GAPDH on triplicate samples, as previously described by our laboratory (Lund et al., 2009). Results are expressed as mean normalized gene expression as a percentage of GAPDH controls.

2.3.6 Dihydroethidium (ROS) Staining

Dihydroethidium (DHE) was conducted on 20μm thick cerebral sections and the production of ROS in $ApoE^{-/-}$ mice was imaged and quantified, as previously described by our laboratory (Oppenheim, H- A. et al., 2013). Sections were imaged by fluorescent microscopy at 10X from an N = 5 from each group, 2-3 slides per animal, with 2 sections each through all 6 regions. We quantified average DHE expression in the cortex of female $ApoE^{-/-}$ (per unit area), using Image J software, in all 6 regions.

2.3.7 Double Immunofluorescence

10 μm frozen sections of the cerebrum from Bregma 0 through -2.5mm for immunofluorescent labeling, as previously described by our laboratory (Suwannasual et al. 2018), utilizing primary antibody for estrogen receptor α (1:500; Abcam, Cambridge, MA, # ab32063), and von Willebrand factor (vWF: 1:1000; Abcam #11713). Alexa-Fluor 488 (1:250, Thermo Fisher Scientific #A32731) and Alexa Fluor 555 (1:250) were used for the secondary antibody. Slides were imaged by EVOS fluorescent microscopy (EVOS Fl, Thermo Fisher Scientific) at 40x with the proper excitation/emission filter, and digitally recorded. Images were analyzed by Image J software (NIH, Bethesda, MD) by a blinded technician. Colocalization was measured by analyzing total fluorescence from at least 4-5 vessels (less than 50 μm in size) from the overlaid images (2 sections per slide, 2 slides per animal, and n = 5 per group).

2.3.8 Statistical Analysis

Data are shown as mean ± SEM. Sigma Plot 10.0 was used to analyze all statistical endpoints. A two-way ANOVA with Tukey post-hoc was used for statistical comparisons between the exposure and/or ovary +/- groups, as well as the exposure x ovary status interaction, as indicated in the results and figure legends. All data is represented as mean ± standard error (S.E.). A $p \leq 0.050$ was considered a statistically significant difference for all measured endpoints.

2.4 Results

2.4.1 Gross Tissue Weight

Our data shown there is no significant difference between the brain weight in the MVE-exposed groups vs. Filtered Air (FA) controls. However, the body weights were much higher in the ovariectomized (both MVE and FA) groups compared to the study groups with ovaries; thus,

the brain/body weight ratios were significantly lower in those groups. (Table 2.1). The significant change in weight was likely not due to the exposures, since it was observed in the both the exposed and control ovariectomized mice, thus it is likely due to alterations in food intake and/or alterations in metabolism and/or downstream activity of the gonadal hormones' estrogen or progesterone; however, these endpoints were not assessed in this study.

Table 2.1: Brain (wet) and body weights at necropsy.

Female Mouse Ovary / Exposure Groups	Brain Weight (g)	Body Weight (g)	Brain W/Body W Ratio
Ovary intact + FA	0.400 ± 0.012	23.10 ± 0.249	0.0174 ± 0.0007
Ovary intact + MVE	0.391 ± 0.007	23.78 ± 0.621	0.0172 ± 0.0007
Ovariectomized + FA	0.398 ± 0.010	28.19 ± 1.209*	0.0139 ± 0.0009*
Ovariectomized + MVE	0.388 ± 0.005	28.21 ± 1.394*	0.0135 ± 0.0006*

Abbreviations: FA = filtered air (controls); MVE = mixed gasoline and diesel engine exhaust (200 PM µg/m^3); g = grams = W = weight; *$p \leq 0.050$ compared to ovary intact + FA.

2.4.2 Quantification of Myelination in the Cerebrum of Female Ovary-Intact and Ovariectomized ApoE$^{-/-}$ Mice Exposed to MVE

As demyelination is a hallmark of MS, we assessed the effects of MVE-exposure on myelination in the cerebrum (regions 1-5, as indicated in Fig. 2.1) of our female study animals. Compared to ovary intact (ov+) FA controls (Fig. 2.2 A-C), we observed a decrease in myelin staining in the cortex of MVE-exposed female ov+ ApoE$^{-/-}$ mice (Fig. 2.2 D-F). Results of demyelination in cortex for exposure, $F = 19.095$; $p < 0.001$, for ovary status $F = 38.950$; $p < 0.001$; and for exposure x ovary status interaction, for exposure x ovary interaction $F = 0.112$; $p = 0.742$. Furthermore, we also observed a decrease in myelination in the cerebrums of both FA ov- (Fig. 2.2 G-I; $p = 0.030$) and MVE ov+ (Fig. 2.2 J – L; $p < 0.001$) exposed animals, compared to FA ov+. For exposure. Graphic representation of myelination in the cortex of ApoE$^{-/-}$ mice is shown in Fig. 2.2 M. Compared to FA ov+ (Fig. 2.3 A-C), quantification of

myelination in the corpus callosum (region 5, as indicated in Fig. 2.1 of female ApoE$^{-/-}$ mice showed no significant alterations in myelination in the MVE-exposed ov + (Fig. 2.3 D-F), FA ov- (Fig. 2.3 G-I), nor MVE ov- (Fig. 2.3 J-L) animals, as indicated in Fig. 2.3 M.

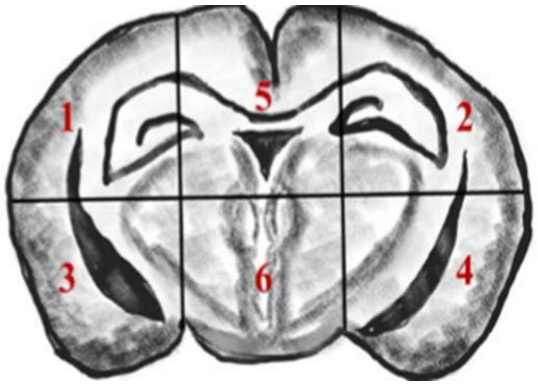

Figure 2.1: Schematic of regions used for imaging/analysis of the cerebrum of ApoE$^{-/-}$ mice for histological endpoints.

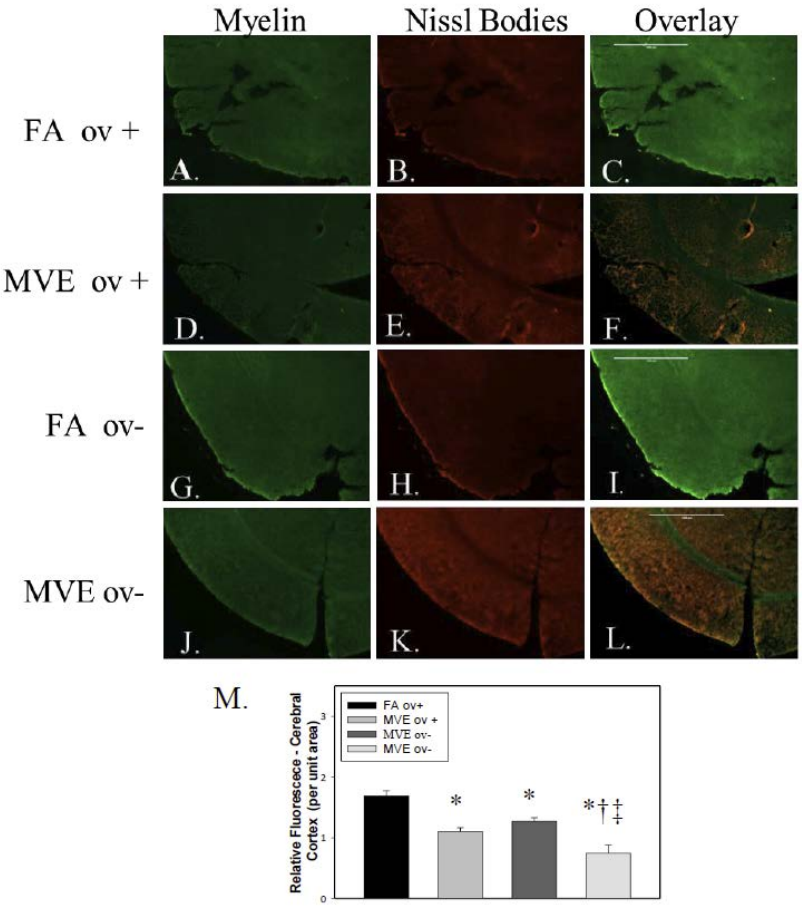

Figure 2.2: Representative staining of myelination in the cerebral cortex (regions 1-4) of female ApoE-/- mice, on a high fat diet, with either ovaries (ov+) or ovariectomized (ov-) and exposed to either filtered air (FA, control A –C ovary intact; and G-I ovariectomized) or mixed gasoline and

diesel vehicle exhaust (MVE: exposed; 200 PM µg/m3 for 6 hrs/d , 7d/wk for 30 d); (D-F ovary intact; J-L ovariectomized). Green stain represents myelin, red stain represents Nissl staining, and the overlay represents neurons with the myelin sheath. Quantification per unit area, and the unit of the area measured was kept consistent within each region quantified, across all animals in the study. Scale bar = 100 um. N = 5 animals for each group, 2 slides each animal, with 2 sections on each slide were used for quantification and analysis; $p < 0.001$. Graph of normalized myelination in cortex represented in the panel Results represent mean ± SEM. M. *$p \leq 0.050$ compared to FA ov+ group; †$p \leq 0.050$ compared to MVE ov+; ‡ $p \leq 0.050$ compared to FA ov-.

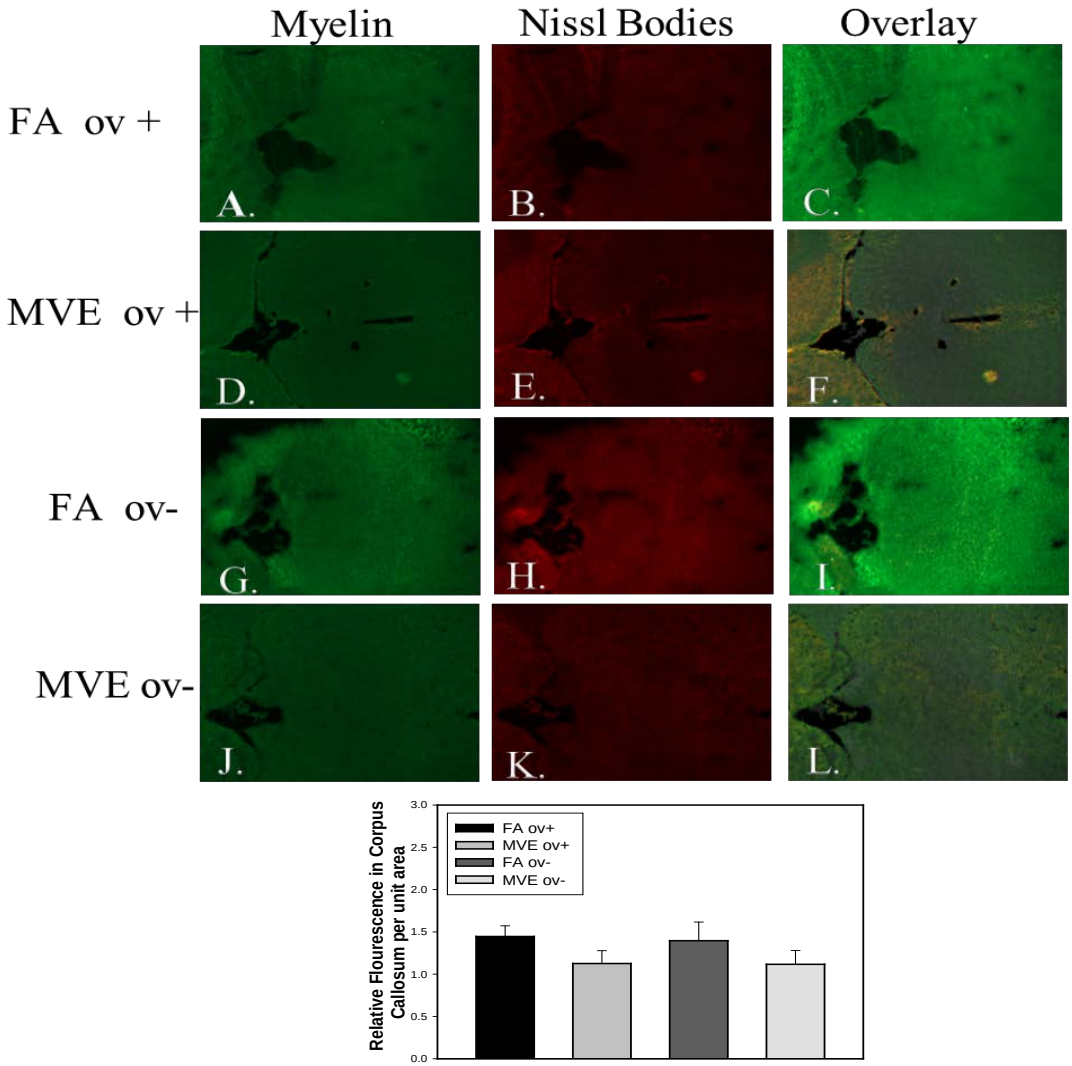

Figure 2.3: Representative myelination in the corpus callosum of female ApoE-/- mice (region 5) on a high-fat diet, with either ovaries (ov+) or ovariectomized (ov-) and exposed to either filtered air (FA, control A-C ovary intact; and G-I ovariectomized) or mixed gasoline and diesel vehicle exhaust (MVE: exposed; 200 PM µg/m3 for 6 hrs/d,7d/wk for 30 d); (D-F ovary intact; J-L ovariectomized). Green stain represents myelin, red stain represents Nissl staining, and the overlay represents neurons with the myelin sheath. Quantification per unit area. Scale bar = 100 um. N = 5 animals for each group, 2 slides each animal, with 2 sections on each slide were used for quantification and analysis; p = 0. 725.Graph of normalized myelination in cortex represented in the panel M.

Analysis of myelin staining across different regions of the cerebrum revealed that there is a significant decrease in myelination in the MVE-exposed groups both MVE ov- and MVE ov+, compared to FA groups, across regions 3 and 4 (Figs. 2.4 A and B, respectively).

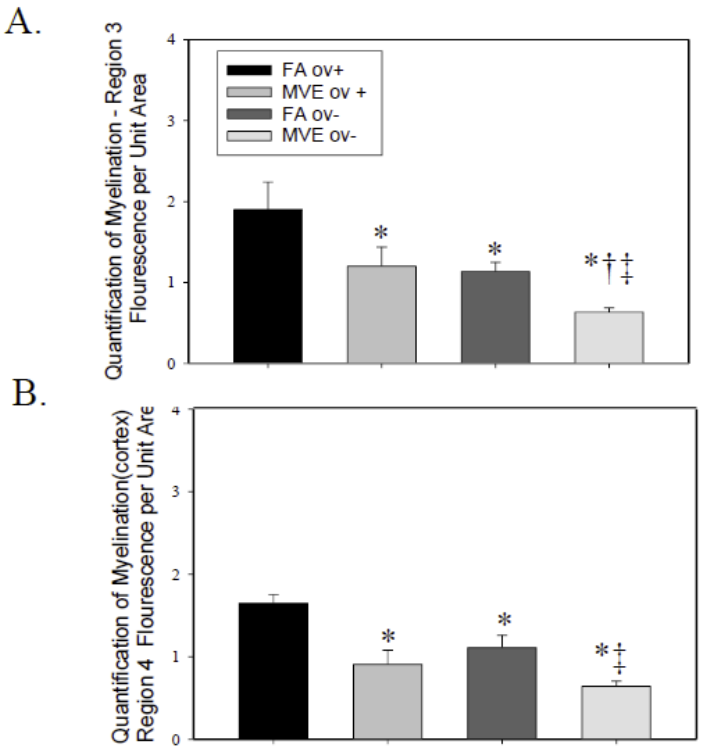

Figure 2.4: Quantification of normalized myelination in (A) region 3, and (B) region 4 in the cerebrum of female ApoE-/- mice, on a high-fat diet, with ovaries (ov+) or ovariectomized (ov) and exposed to either filtered air (FA control) or mixed exhaust (MVE: mixed gasoline and diesel emissions, 200 PM µg/m3 for 6 hrs /day, 7 d/wk, for 30 d). Scale bar = 100 um. N = 5 animals for each group, 2 slides each animal, with 2 sections on each slide were used for quantification and analysis. * p ≤ 0.050 compared to FA ov+; †p ≤ 0.050 compared to MVE; ‡ p ≤ 0.050 compared to FA ov-.

2.4.3 Expression of CD4+ and CD8+ Cells in the Cerebrum of Female ApoE$^{-/-}$ Mice Exposed to MVE

MS lesions in the brain are typically associated with increased infiltration of CD4+ and CD8+ cells. Therefore, we analyzed the expression of the T lymphocyte subtypes in the cerebrum of female ov+ and ov- ApoE$^{-/-}$ mice exposed to MVE vs. FA, using immunohistochemical staining. Compared to FA ov+ (Fig .2.5 A), we observed a significant increase of CD4+ in the cerebrum in both the MVE ov+ (Fig. 2.2 B; p = 0.005) and MVE ov-

(Fig. 2.5 D) female ApoE$^{-/-}$ animals, as presented in Fig. 2.5 E. The F value for exposure was 138.631; the F value for ovary status was 16.675; the F value for exposure x ovary status interaction = 20.171. No differences were noted in cerebral CD4+ levels between FA ov+ (Fig. 2.5 A) and FA ov- (Fig. 2.5 C) female ApoE-/- mice.

2.4.4 CD8+ Cells in the Cerebrum of Female ApoE$^{-/-}$ Mice Exposed to MVE

MS lesions in the brain are typically associated with increased infiltration of CD4+ and CD8+ cells. Therefore, we analyzed the expression of the T lymphocyte subtypes in the cerebrum of female ov+ and ov- ApoE$^{-/-}$ mice exposed to MVE vs. FA, using immunohistochemical staining. Compared to FA ov+ (Fig. 2.5 A), we observed a significant increase of CD4+ in the cerebrum in both the MVE ov+ (Fig. 2.5 B) and MVE ov- (Fig. 2.5 D) female ApoE-/- animals, as presented in Fig. 2.5 E. For exposure was $F = 32.989$; $p < 0.001$; for ovary status the $F = 2.498$; $p = 0.130$; for exposure x ovary status interaction $F = 0.0917$; $p = 0.765$. No differences were noted in cerebral CD4+ levels between FA ov+ (Fig. 2.5 A) and FA ov- (Fig. 2.5 C) female ApoE$^{-/-}$ mice. Immunohistochemical staining of cerebral tissue was performed to analyze the expression of CD8+, resulting from ovary status and/or exposures. Compared to FA ov+ (Fig. 2.6 A) we observe a significant increase of CD8+ in the cerebrum with MVE-exposure in both the MVE ov+ (Fig. 2.6 B, $p = 0.010$) and MVE ov- (Fig. 2.6 D; $p < 0.001$) female Apo E$^{-/-}$ animals, as presented in Fig. 2.6 E. For the exposure $F = 9.564$, $p \leq 0.001$; for ovary status $F = 0.773$, $p = 0.525$; for the exposure x ovary interaction $F = 0.0746$, $p = 0.788$. No differences were noted in cerebral CD8+ levels between FA ov+ (Fig. 2.6 A) and FA ov- (Fig. 2.6 C) female Apo E$^{-/-}$ mice.

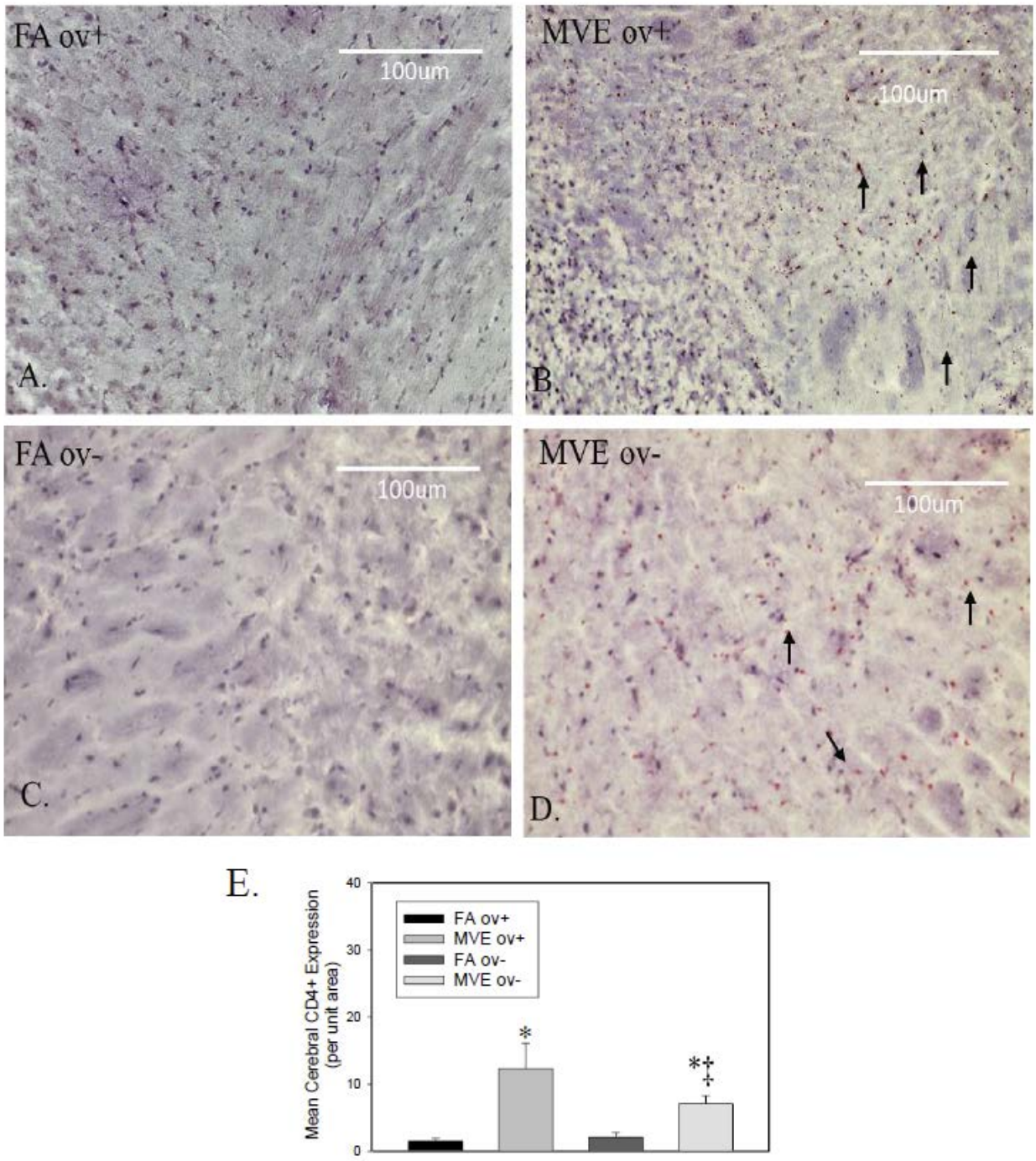

Figure 2.5: Representative CD4+ staining in the cerebrum of female ApoE$^{-/-}$ mice with ovaries (A, B) or ovariectomized (C, D), and exposed to either filtered air (FA: A, C) or mixed vehicle exhaust (MVE: B, D; mixture of gasoline and diesel emissions at 200 PM µg/m^3 for 6 hr /d, 7 d/wk for 30 d). (E) Displays quantification of CD4+ expression per unit area in the cerebrum± S.E. N = 5 animals for each group, 2 slides each animal, with 2 sections on each slide were used for quantification and analysis. Scale bar = 100 µm. *p ≤ 0.050 compared to FA ov+; ‡ p ≤ 0.050 compared to FA ov-.

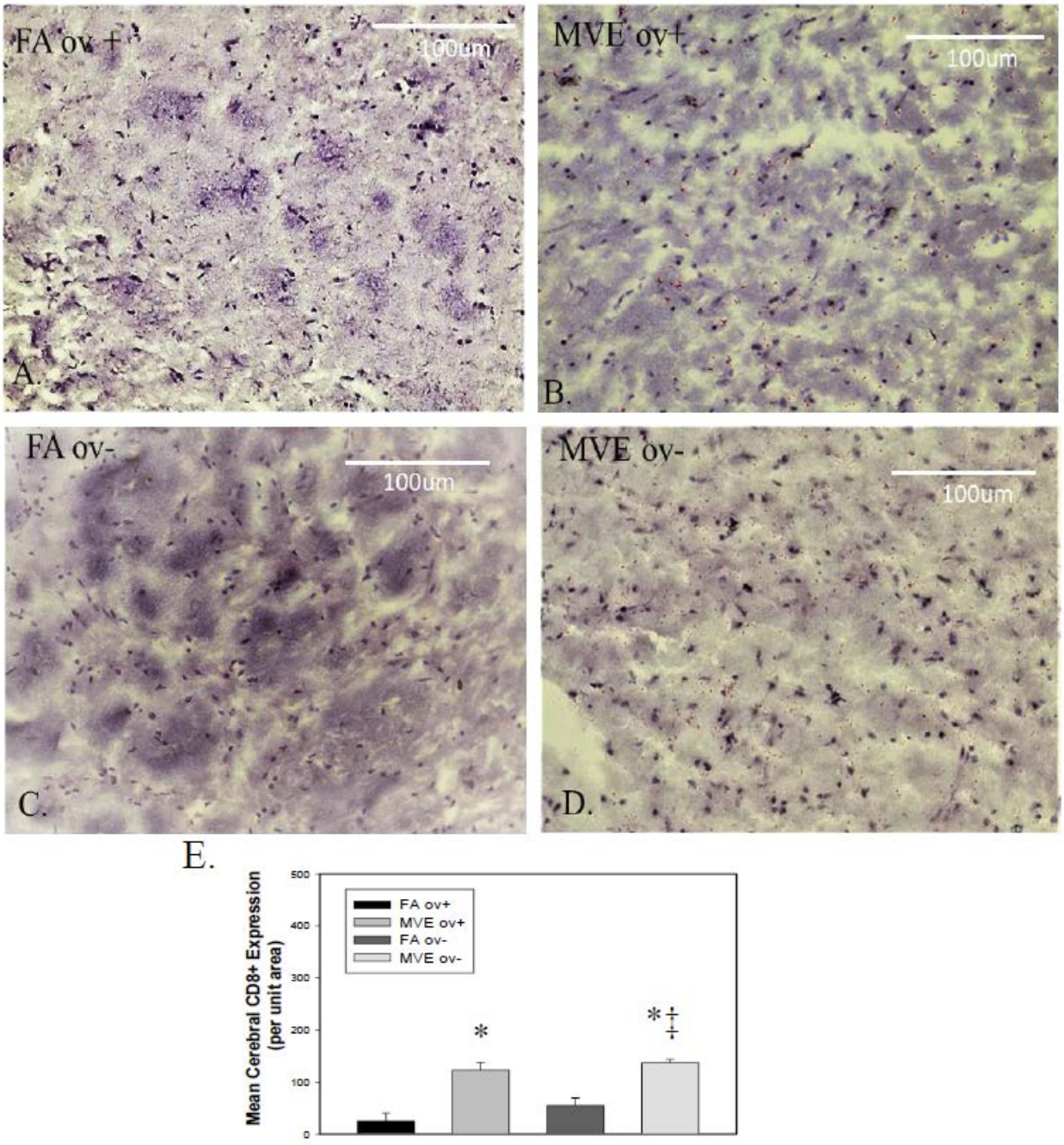

Figure 2.6: Representative CD8+ staining in the cerebrum of female ApoE$^{-/-}$ mice with ovaries (A, B) or ovariectomized (C, D) , and exposed to either filtered air (FA: A, C) or mixed vehicle exhaust (MVE: B, D; mixture of gasoline and diesel emissions at 200 PM µg/m^3 for 6 hr /d, 7 d/wk for 30 d). (E) Shows quantification of CD8+ expression per unit area in the cerebrum ±S.E. N = 5 animals for each group, 2 slides each animal, with 2 sections on each slide were used for quantification and analysis. Scale bar = 100 µm. *p ≤ 0.050 compared to FA ov+ group; ‡ p ≤ 0.050 compared to FA ov-

2.4.5 Expression of Estrogen and Progesterone Receptors in Ovary-Intact and Ovariectomized Female ApoE$^{-/-}$ Exposed to MVE

Female sex hormone-signaling may be a key factor contributing to the increase in the occurrence of MS in females. As such, we analyzed the expression of estrogen and progesterone receptors in the cerebrum of our study animals. ERα receptor expression in the cerebral microvasculature of female ApoE $^{-/-}$ mice was not significantly altered in response to MVE-exposure in this study Fig. 2.7 A-L., as quantified in Fig. 2.7 M. However, real-time RT-qPCR analysis of ERα mRNA expression from the cerebral tissue of MVE-exposed female ApoE$^{-/-}$ mice revealed a decrease in expression of ERα receptor subtype in MVE ov+ and MVE ov- animals, which was also observed in the FA ov- animals, compared to FA ov+ controls (Fig. 2.7 N). for exposure F = exposure = 4.671, p = 0.042; for ovary status F = 17.680, and for the exposure x ovary status interaction F = 3.641, p = 0.070. Neither ERβ, nor progesterone A/B, receptors showed any statistical change in cerebral mRNA expression across exposure and/or control groups (Figs. 2.8 A and 2.8 B, respectively). One possible reason for the difference in results of microvascular expression of ERα receptor and mRNA level measured from total brain tissues and ERα receptor is that the ERα receptor is ubiquitously expressed in multiples cells in the CNS, such as astrocytes and oligodendrocytes (Platania et al., 2003).

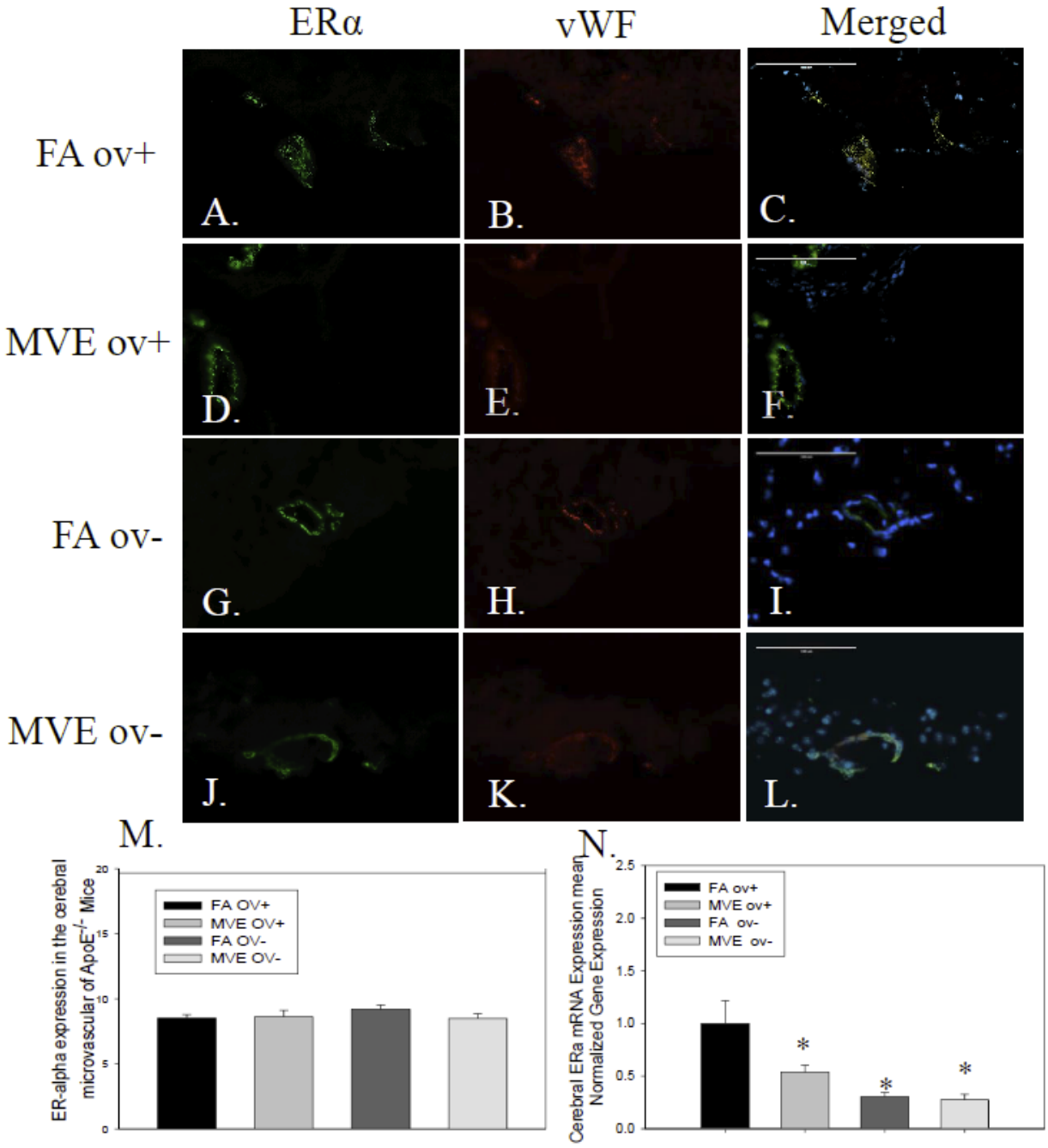

Figure 2.7: Microvascular expression of Erα quantification of Erα (M), and RT-qPCR quantification of Erα (N) in female ApoE$^{-/-}$ mice with ovaries (ov+) or ovariectomized (ov-), on the high-fat diet, exposed to either filtered air (FA) or mixed vehicle emissions (MVE: 200 PM ug/m^3 for 6 hrs /day, 7 d/wk, 30 d; n = 5 per group; 2 sections each, 5 vessels per section). Scale bar = 100 µm. *p ≤ 0.050 compared to FA ov+ group, †p ≤ 0.050 compared to MVE ov+.

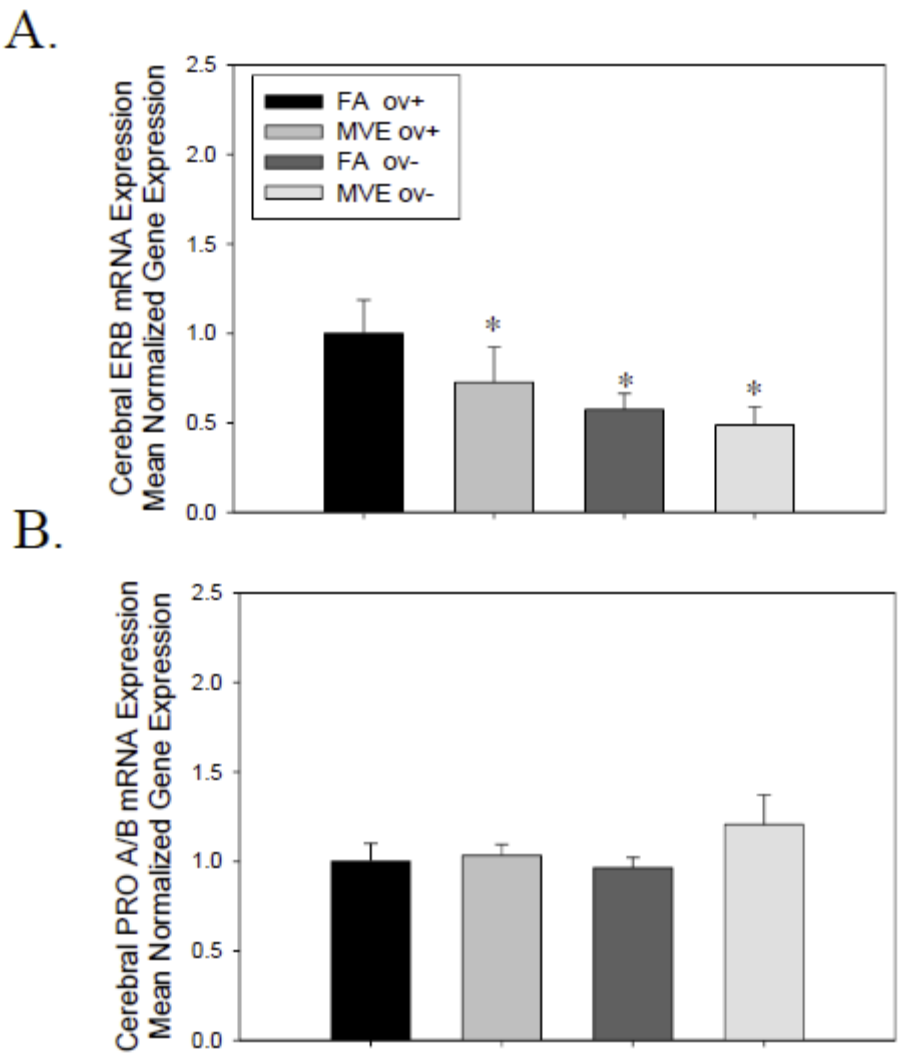

Figure 2.8: Mean normalized gene expression of (A) estrogen receptor (ER) β, and (B) progesterone receptors A/B in female ApoE$^{-/-}$ mice with ovaries (ov+) or ovariectomized (ov-), on the high-fat diet, exposed to either mixed vehicle emissions (MVE: 200 PM ug/m^3 for 6 hrs /day, 7 d/wk, 30 d) or filtered air (FA), as quantified by Real time RT-qPCR. N = 6 per study group.+.

2.4.6 Expression of ROS in Ovary-Intact and Ovariectomized Female ApoE$^{-/-}$ Exposed to MVE

Dihydroethidium (DHE) staining of cerebral tissue was performed to analyze the production of ROS resulting from MVE-exposure. Compared to FA ov+ (Fig. 2.9 A), and FA ov-(Fig. 2.9 C) we observe a significant increase of ROS production in the cerebrum in both the MVE ov+ (Fig. 2.9 B) and MVE ov- (Fig. 2.9 D) groups, as presented in Fig. 2.9 E. The F value

for exposure = 6.506, p = 0.014; for ovary status F = 1.333, p = 0.254 and the for-exposure x ovary status interaction F = 1.242, p = 0.271 No differences were noted in cerebral ROS production between FA ov+ (Fig. 2.9 A) and ov- (Fig. 2.9 C) female Apo $E^{-/-}$ mice.

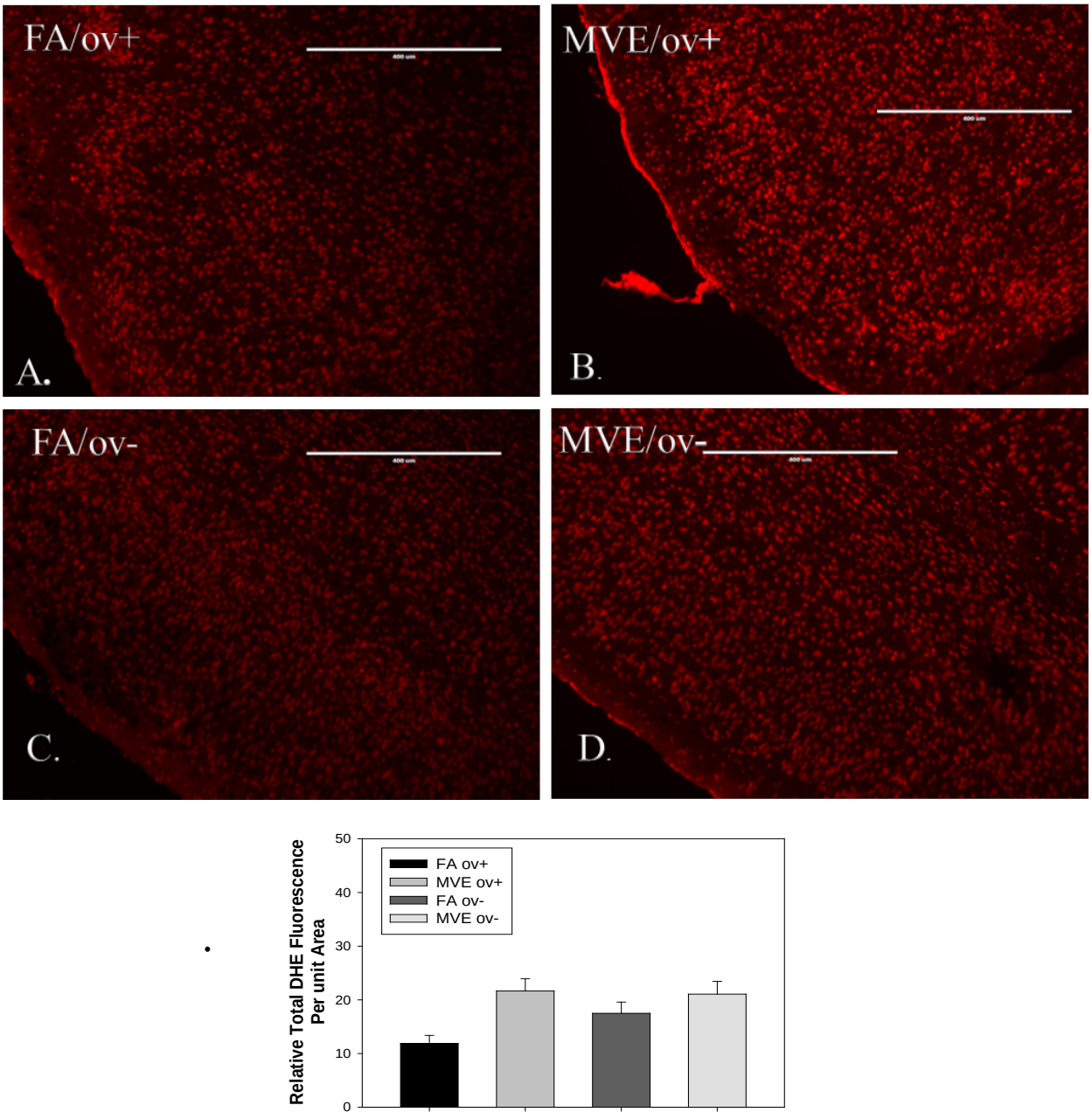

Figure 2.9: Representative DHE staining in the cerebrum of female $ApoE^{-/-}$ mice with ovaries (ov+; A, B) or ovariectomized (ov-; C, D), and exposed to either filtered air (FA: A, C) or mixed vehicle exhaust (MVE: B, D; 200 PM µg/m^3 for 6 hr /d, 7 d/wk for 30 d). (E) Quantification of DHE fluorescence per unit area. N = 6 per study group, Scale bar = 400 µm *p ≤ 0.050 compared to FA ov+ group.

2.4.7 Cerebral mRNA Expression of Mylein Oligodendrocyte in Female ApoE$^{-/-}$ Mice

MOG belongs to immunoglobulin superfamily and is expressed on the outer layer of myelin sheath and an increase in expression is thought to be involved in the process of demyelination. Our results showed the MOG was increased at the transcript level in the cerebrum of the MVE -exposed groups compared to FA groups, regardless of the ovary status (Fig. 2.10). For exposure F = 4.794, p = 0.045; for ovary status = 0.199, p = 0.662, and for the exposure x ovary status interaction F = 0.0820, p = 0.779.

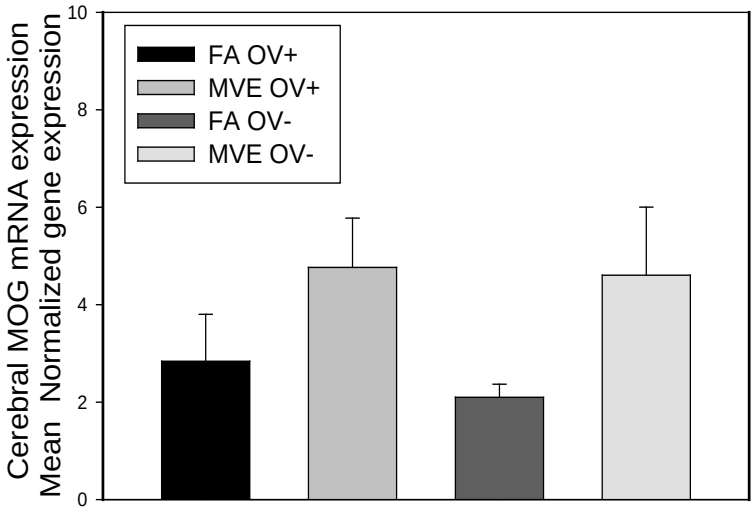

Figure 2.10: Mean normalized cerebral expression of MOG in female ApoE$^{-/-}$ mice with ovaries (ov+) or ovariectomized (ov-), on the high-fat diet, exposed to either mixed vehicle emissions (MVE: 200 PM ug/m^3 for 6 hrs /day, 7 d/wk, 30 d) or filtered air (FA), as determined by Real time RT-qPCR. N = 6 per study group. *p ≤ 0.050 compared to FA ov+ group.

2.5 Discussion

The World Health Organization (WHO) has reported that air pollution exposure is responsible for around 7 million deaths per year (WHO, 2018). Air pollution exposure contributes to detrimental CNS occurrences such as stroke, neuroinflammation, and neurodegeneration (Block and Calderón-Garcidueñas, 2009). However, there are currently conflicting reports in the literature about whether exposure to air pollution contributes to

pathologies in the CNS associated with MS's etiology, including demyelination (Angelici et al., 2016; Bai et al., 2018). Moreover, there is even less understood regarding the role of steroid hormone-receptor contributions in these air pollution exposure-mediated outcomes. Therefore, we investigated whether inhalation exposure to traffic-generated pollutants was associated with the induction of factors in the CNS, associated with the demyelination in a female mouse model. The rationale for utilizing female $ApoE^{-/-}$ mice (+/- ovaries) was threefold: (1) since MS has a higher prevalence in females (Gold, S- M., and Voskuhl, R-R., 2009), we have tried to investigate whether the presence of sex hormones such as estrogen and/or progesterone may mediate and/or exacerbate the effects of MVE- exposure in the CNS; (2) to be able to translate findings to similar exposure scenarios and endpoints from our previously published studies in $ApoE^{-/-}$ male mice (Lucero, J. et al., 2017); and (3) since $ApoE^{-/-}$ mice model is considered a good model for an inhaled toxicology study, we have tried to investigate how inhaled MVE - exposure may alters pathways associated with demyelination, T cell activation, and ROS production.

Findings from our study suggest that inhalation exposure to traffic-generated air pollutants may promote demyelination in certain regions of the brain. Furthermore, the presence or absence of ovaries, and presumably sex hormone signaling appeared to play a role in the degree of MVE mediated demyelination observed. This was evidenced by decreased levels of myelination observed in the brains of MVE- exposed ov-, compared to MVE-exposed ov+ female mice. Additionally, within the same regions that we observed a decrease in myelination, we also observed a significant increase in the presence of CD4+ and CD8+ cells. Importantly, CD4+ and CD8+ T cells are known to be upregulated in MS lesions, thus can serve as a in the brain susceptible to lesion formation (Chitnis, T., 2007). CD8+ are cytotoxic cells that stimulate

apoptotic signaling of the myelin sheath, while CD4+ cells are reported to be involved in the direct destruction of the myelin sheath (Patel, J. and Balabanov, R., 2012). Interestingly, the reduction in myelination was more evident in the brains of the MVE-exposed ov- mice, while the presence of CD4+ and CD8+ positive cells appeared to be more strongly correlated to the exposure itself, as there were no statistical differences noted between these endpoints in the cerebrums of MVE ov- vs. MVE ov+ mice. Furthermore, the expression of CD8+ and CD4+ across different regions of the brain were not statistically different within each exposure/ovary group. MVE-mediated demyelination varied across different regions of the brain in our study. We measured higher degrees of demyelination across regions 3 and 4 of the brain (as indicated in Fig. 2.1), in comparison to the other regions of the brain. Importantly, regions 3 and 4 are associated with structures including the basolateral and amygdaloid nucleus, pyriform (olfactory), lateral olfactory tract (LOT), lateral cortex ventricle, and subthalamic nucleus. Likewise, outcomes of neuroinflammation studies in the CNS, resulting from prolonged exposure to diesel exhaust, report that regions of the brain including the frontal cortex, hippocampus, cerebellum, striatum, and olfactory bulb are more susceptible to induced proinflammatory signaling with exposure to air pollution (Gerlofs-Nijland et al., 2010). Thus, it is plausible that different regions of the brain are more "susceptible" to detrimental outcomes in the CNS, associated with the progression of MS, resulting from inhalation exposure to environmental air pollutants.

In addition to demyelination and increased infiltration of CD4+ and CD8 + cells in the CNS, MVE- exposure also increased MOG mRNA expression, compared to FA control groups, regardless of ovary status. As previously mentioned, MOG is exclusively present in the CNS and has been reported to play a role in demyelination. The presence of MOG antibody (MOG-

Abs) is a biomarker used for both the diagnosis of neuromyelitis optica spectrum disorder (NMOSD) and MS (Hacohen, Y., and Banwell, B., 2019). Thus, MVE-mediated increases in cerebral expression of MOG transcript may be correlated with the alterations in myelination observed with these exposures.

We also observed a significant increase in ROS production in the cerebrum of MVE-exposed ov- and ov+ female ApoE$^{-/-}$ mice. In MS, increased ROS production is associated with demyelination and axonal damage. This premise is further confirmed by reports showing elevated ROS in the CNS of both EAE mice and MS patients (Witherick et al., 2011). Increased ROS production has been correlated with the incidence and severity of MS, likely due to the activation of phagocytosis of the myelin sheath (Van der Goes et al., 1998). Microglial activation in the CNS has been shown to promote ROS production and the expression of inflammatory factors (Lassmann, H., and Hossen, J., 2011). Further studies are required to determine whether inflammation is driving the increased ROS by treating animals with an anti-inflammatory agent such as COX-2 inhibitors, NF-KB inhibitors, or TNF-alpha inhibitors, and see if ROS levels decrease in the brain. If we want to test if ROS is driving the brain's inflammation, we can treat the animals with anti-oxidants such as superoxide dismutase (SOD), catalase, NADPH oxidase inhibitors, such as apocynin, and see if the inflammation is decreased. Exposure to multiple ubiquitous environmental air pollutants have been shown to directly result in the activation of microglia in both animal and human studies (Campbell et al., 2009; Santiago-López et al., 2010). Nevertheless, further studies are required to determine if microglial activation is involved in ROS production in the CNS of MVE-exposed female mice.

Analysis of female steroid hormone receptors in the cerebrum of our study animals showed a reduction in the expression of ERα mRNA in our ovariectomized groups (both FA and

MVE). Interestingly, we also observed a significant decrease in mRNA ERα in the MVE-exposed ov+ mice. Nevertheless, Erβ expression in cerebral microvascular of female ApoE $^{-/-}$ mice did not show a significant change in response to MVE inhalation or even though the presence of ovaries. Estrogen receptors are nuclear transcriptional proteins that regulate the immune system and are implicated in the alteration of innate and adaptive immune responses via the activation of dendritic cells and toll-like receptor (TLR) signaling (Kovats, S., 2015). Downregulation of ERα is associated with increased TNF-α expression and macrophage infiltration, which may account for a mechanism that contributes to the increased occurrence of autoimmune diseases in females compared to males (Panchanathan et al., 2010). Signaling via the ERα has been reported to provide protective effects in the CNS via inhibiting inflammatory cell recruitment in EAE mice (Subramanian et al., 2003). Thus, the observed MVE-exposure mediated decrease in ERα in the CNS of ov+ females in the current student may account for a link between air pollution. The decrease in ERα receptor mRNA expression we observed in the brain of ov+ mice exposed to MVE may result from negative feedback due to the presence of estrogen-mimetics in environmental air pollutants. These include compounds such as polycyclic aromatic hydrocarbons (PAHs), metalloestrogens, phthalates, and alkylphenols (Fucic et al., 2012), many of which have been shown to act as ligands at the estrogen receptors, thereby altering signaling activities (Carpenter et al., 2002). Although we did not observe any MVE exposure- mediated change in expression of PRO A/B receptor mRNA, progesterone can contribute to the pathophysiology of MS by inhibition of T helper cells (Hughes, G-C., 2012). Further mechanistic/inhibitor studies are necessary to characterize further the role of the female sex hormones and/or receptor-mediated signaling in mediating effects of inhaled environmental air pollutants in autoimmune disorders and/or the CNS.

As previously mentioned, inhaled traffic generated air pollutants have been reported to increase ROS production, inflammation, and BBB permeability in the CNS of both Apo $E^{-/-}$ and C57Bl/6 wild type mice, in addition to promoting microglial activation, all of which have been reported to contribute to the progression of MS (Mumaw et al., 2016; Suwannasual et al., 2018). However, the components of air pollution mediating these detrimental outcomes in the CNS have not yet been fully characterized. Additional studies are necessary to determine the component(s) of MVE that mediates the observed induction of inflammation, production of ROS, CD4+/CD8+ infiltration, decreased myelination, and alterations in ER expression within the cerebrum of female mice. While utilizing female $ApoE^{-/-}$ mice for the current study allows us to compare MVE-exposure mediated outcomes in the CNS to our previous studies in male mice (Oppenheim, H. et al., 2013; Lucero et al., 2017; Suwannasual et al., 2018), which is of great importance for understanding exposure and toxicity outcomes across sexes, it can also be viewed as a limitation of the current studies.

The $ApoE^{-/-}$ mice in this study were placed on a high-fat diet to initiate atherosclerosis also to characterize the effects of air pollution exposure in the progression of cardiovascular disease in a female model. However, previous studies suggest that the consumption of a high-fat diet can also exacerbate inflammation in the CNS (Timmermans et al., 2014). Additionally, the concentration of MVE used for the exposures was chosen for comparison to previous studies and/or endpoint in male mouse models (Lucero et al., 2017; Lund et al., 2011; Oppenheim et al., 2013; Suwannasual et al., 2018; Suwannasual et al., 2019). While these concentrations are high in comparison to most daily human environmental exposure scenarios, they may occur near roadways during peak traffic hours, occupational settings, and/or regions with high annual levels of air pollution (Lin et al., 2018; WHO Database., 2018). Nevertheless, this study provides a

foundation for future studies in additional animal models, and exposure scenarios/time points, to further investigate mechanisms involved in air pollution-mediated pathogenesis of MS.

2.6 Conclusion

In conclusion, inhalation MVE-exposure resulted in the initiation of pathways associated with the demyelination in the CNS of female Apo $E^{-/-}$ mice. Demyelination was significant in MVE-exposed groups, which appeared to be more pronounced in regions that include the basolateral and amygdaloid nucleus, pyriform (olfactory), LOT, lateral cortex ventricle, and the subthalamic nucleus. Additionally, the degree of demyelination appears to be mediated by the presence of female sex hormones (or hormone signaling), as demyelination was observed to be exacerbated in the cerebrum of MVE-exposed ovariectomized female Apo $E^{-/-}$ mice. Infiltration of CD4+ and CD8+ immune cells increased ROS production in the CNS of female $ApoE^{-/-}$ mice appeared to be primarily mediated by MVE exposure. However, the interaction between the MVE exposure and the presence (or lack) of ovaries also appeared to mediate the increased presence of CD4+/CD8+ cells. Measurement of estrogen receptors expression in the cerebrum of female $ApoE^{-/-}$ revealed that MVE-exposure promoted a significant reduction in ERα receptor expression; however, neither ERβ receptor nor PRO A/B receptor mRNA expression was altered across exposure/control groups. Collectively, our findings suggest that MVE-exposure induces immune cell infiltration, ROS production, associated with increased demyelination.

2.7 Acknowledgements

We would like to thank the Inhalation Exposure group, in the Environmental Respiratory Health Program, at Lovelace Biomedical and Environmental Research Institute for the characterization and monitoring of the animal exposures.

2.8 Funding

This work was supported by National Institute of Environmental Health Sciences at National Institute of Health grants [R00ES016586 and R15ES026795 to A.K.L.], as well as internal funding from the University of North Texas to A.K.L. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health or the Environmental Protection Agency.

2.9 Conflict of Interests

Funding from grants received from the National Institute of Environmental Health were used to conduct some the exposures and studies described in this manuscript; however, the authors declare no conflict of interest or financial gains to these entities associated with this publication.

2.10 Author Contributions

A.A.: investigation, formal analysis, validation, writing – original draft; J.L.: project administration, formal analysis, investigation, writing – review & editing; N.S.: investigation, formal analysis, writing – review & editing; J.D.M.: methodology, resources, writing – review & editing; A.K.L.: conceptualization, funding acquisition, formal analysis, resources, supervision, writing – review & editing.

CHAPTER 3

EXPOSURE TO MIXED EXHAUST AIR POLLUTION TRIGGERS ALTERATIONS IN THE INTEGRITY OF THE BRAIN MICROVASCULAR IN ApoE$^{-/-}$ MICE ON A HIGH-FAT DIET*

3.1 Abstract

Traffic-generated air pollutants have been correlated with alterations in blood-brain barrier (BBB) integrity, which is associated with pathologies in the central nervous system (CNS). Much of the existing literature investigating the effects of air pollution in the CNS has predominately been reported in males, with little known regarding the effects in females. As such, this study characterized the effects of inhalation exposure to mixed vehicle emissions (MVE), as well as the presence of female sex hormones, in the CNS of female ApoE$^{-/-}$ mice, which included cohorts of both ovariectomized (ov-) and ovary-intact (ov+) mice. Ov+ and ov- were placed on a high-fat diet and randomly grouped to be exposed to either filtered-air (FA) or MVE (200 PM/m^3; 50µg PM/m^3 gasoline engine + 100µg PM/m^3 from diesel engine emissions) for 6 hr/d, 7d/wk, for 30d. MVE-exposure resulted in altered cerebral microvascular integrity and permeability, as determined by the decreased immunofluorescent expression of tight junction (TJ) proteins, occludin, and claudin-5, and increased IgG extravasation into the cerebral parenchyma, compared to FA controls, regardless of ovary status. Associated with the altered cerebral microvascular integrity, we also observed an increase in matrix metalloproteinases (MMPs) -2/9 activity in the MVE ov+, MVE ov-, and FA ov- groups, compared to FA ov+. There was also elevated expression of intracellular adhesion molecule (ICAM)-1, inflammatory

* This chapter has been submitted for peer-review in Toxicology Letters as: Adivi A, Lucero J, Simpson N, McDonald JD, Lund AK. "Exposure to Mixed Exhaust Air Pollutants Triggers Alterations in the Integrity of the Brain Microvascular in ApoE$^{-/-}$ mice on a High-fat Diet."

interleukins (IL-1, IL-1β), and tumor necrosis factor (TNF-α) mRNA in the cerebrum of MVE ov+ and MVE ov- animals. IκB kinase (IKK) subunits IKKα and IKKβ mRNA expressions were upregulated in the cerebrum of MVE ov-and FA ov- mice. Our findings indicate that MVE exposure mediates altered integrity and transport in the cerebral microvasculature correlated with increased MMP-2/9 activity and inflammatory signaling, regardless of female hormones present.

Key words: Air pollution, Female, Brain, Inflammatory markers, BBB disruption

3.2 Introduction

Exposure to traffic-generated air pollutants is positively correlated to neuroinflammation and neurodegeneration, as well as neurovascular disruption of the blood-brain barrier (BBB), which contribute to multiple central nervous system (CNS) disease-states (Block and Calderón-Garcidueñas, 2009; Calderón-Garcidueñas, 2008; Oppenheim et al., 2013). The BBB consists of endothelial cells, pericytes, astrocytes, and microglia, which acts as a physical and chemical barrier and contributes to brain homeostasis (Villabona-Rueda et al., 2019). Disruption of the BBB integrity can alter the efflux and influx of neurotoxins, macromolecules, and nutrients in the brain (Sanchez-Covarrubias et al., 2014). The endothelial cells that contribute to the BBB are "sealed" by proteins, including tight junction (TJs) proteins, such as occludin and claudins (Luissint et al., 2012). BBB disruption is a hallmark of multiple CNS disorders, including multiple sclerosis (MS) and Alzheimer's disease (Rempe et al., 2016; Minagar and Alexander, 2003; Zenaro et al., 2017). Exposure to traffic-generated particulate matter (PM) has been shown to alter BBB integrity, which is also associated with neuroinflammation in both children and adults (Calderón-Garcidueñas et al., 2008). Furthermore, our laboratory has previously reported that exposure to traffic-generated air pollution has been shown to promote BBB disruption in male Apolipoprotein (Apo) $E^{-/-}$ and C57BL/6 mice via decreased expression of TJ proteins,

associated with induction of matrix metalloproteinase (MMP)-2/9 activity and inflammatory signaling (Oppenheim et al., 2013; Suwannasual et al., 2018; Lucero et al., 2017). MMPs are enzymes expressed by various CNS cells, such as endothelial cells, meninges, astrocytes, and microglial cells in an inactive form, which are proteolytically activated under neuroinflammatory conditions (Fujioka et al., 2012; Mirshafiey et al., 2014). Increased activity of gelatinases, MMP-2 and MMP-9, has been reported to degrade TJ proteins, including claudins and occludin, which leads to altered permeability of the BBB (Manicone, and McGuire, 2008; Chen et al., 2009).

Elevated expression of inflammatory markers including interleukins (ILs), such as IL-1β, and tumor necrosis factor (TNF)-α, are associated with recruitment of immune cells to the brain and progression of CNS pathologies, such as multiple sclerosis (MS) (Özenci et al., 2000; Wilson et al., 2010). Exposure to traffic-generated air pollutants, such as diesel exhaust, has also been reported to increase neuroinflammatory signaling markers, including IL-1β, IL-6, and TNFα in the midbrain (Levesque et al., 2011). TNF-α is known to alter BBB permeability by modifying the cellular distribution of junctional adhesion molecules (JAMs), as well as through mediating the induction of expression of adhesion molecules, (VCAM)-1, and intercellular adhesion molecule (ICAM)-1, which promote the transmigration of leukocytes to the CNS (Daneman, and Prat, 2015). In addition to mediating inflammatory signaling, IL-1β has been shown to induce MMP-9 expression via the activation and translocation of NF-κB (p65) (Cheng et al., 2010), and increase the expression of genes encoding chemokines and adhesion molecules ICAM-1 and VCAM-1 (Cheng et al., 2010; Lin et al., 2007). NF-κB composed of p65 or (RelA), RelB, c-Rel, NF-κB1, and NF-κB2 (Israël, 2010). IκB kinase (IKK) subunits, IKKα and IKKβ, are involved in NF-κB signaling in the canonical and non-canonical pathways, in

response to different stimuli, including proinflammatory cytokines (Oeckinghaus and Ghosh, 2009). IKKα and IKKβ, along with the IKKγ, comprise the IKK complex, which, when activated, phosphorylates IκB. NF-κB dimers are then activated by phosphorylation of IκB, resulting in degradation by the proteasome. NF-κB is then transferred to the nucleus, where it serves as a transcription factor to activate target genes (Collins et al., 2016). In the non-canonical pathway, IKKα activation phosphorylates p100 leading to the release and translocation of the p52/RelB active heterodimer and subsequent NF-κB activation (Oeckinghaus and Ghosh, 2009).

There is much debate in the literature regarding the role of sex hormones in CNS disorders; some report that sex hormones are protective, while others report that the benefit of hormones is age-dependent, with estrogen replacement can be harmful after age 60 (Alzheimer's Association International Conference, 2018). The presence of 17β-estradiol (E2) has been shown to suppress the expression of MMPs (Na et al., 2015), which may account for a protective mechanism of female sex hormones in BBB integrity. Much of the available literature on the effects of air pollution exposure in females focus on pregnancy-related outcomes, effects in the cardiovascular system, or lung function, with very little information available on the effects of air pollution exposure on the CNS of women either pre- or post-menopausal. In the current study, we investigated differential effects of female sex hormones in mediating (or mitigating) the outcomes in the CNS from inhalation exposure to traffic-generated air pollution by using both ovary-intact and ovariectomized female ApoE$^{-/-}$ mice. When the ApoE$^{-/-}$ mouse is fed a high-fat diet, it develops atherosclerosis with etiology similar to that observed in humans. As the majority of the human population has some degree of atherosclerosis, and many individuals in the Western world consume a diet rich in fats, utilization of the ApoE$^{-/-}$ mouse model allows us

to analyze the outcomes of air pollution exposure in a baseline model with increased susceptibility (Sasso et al., 2016; Godfrey and Reardon, 2012).

3.3 Materials and Methods

3.3.1 Animals and Exposures

Six-to-eight-week-old ovary-intact (ov+; n = 16) and ovariectomized (ov-; n = 16) female Apo $E^{-/-}$ mice were purchased from Taconic (Albany, NY). All mice were fed a high-fat diet (TD88137 Custom Research Diet, 21.2% fat, 1.5g/kg cholesterol diet; Harlan Teklad, Madison, WI) for two weeks before beginning exposures and were maintained on the same diet during the 30-days of exposure. Mice were then randomly grouped to be exposed to either mixed gasoline and diesel vehicle exhaust (MVE: 200 PM $\mu g/m^3$: 50 PM $\mu g/m^3$ gasoline engine + 150 PM $\mu g/m^3$ from diesel engine emissions; n = 8 ov+ and n = 8 ov-), or filtered air (FA; n = 8 ov+, n = 8 ov-) for 6 hr/d, 7 d/wk, for 30 d. The emissions were generated and characterized daily, as previously published by our laboratory (Lund et al., 2011; Suwannasual et al., 2019). Mice were singly housed in standard shoebox cages to prevent the stress and physical injury within an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) approved rodent housing facility ($2m^3$ exposure chambers) for the duration of the study, which was maintained at a steady temperature (20-24°C) and humidity (30-50% relative humidity). Mice had free access to chow and water *ad libitum* throughout the study period, except during daily exposures when chow was removed. All animal protocols approved by the Lovelace Respiratory Research Institute's Animal Care and Use Committee (AAALAC-accredited Assurance #A3083-01; USDA-registered facility #85-R-003) and followed the guidelines form the Care and Use of Laboratory Animals released by US National Institutes of Health (NIH) Publication No. 85-23, revised 1996).

3.3.2 Tissue Collection

At the end of the 30d exposure protocol, the animals were anesthetized with Euthasol®, euthanized by exsanguination, and the brains were carefully removed from the skull, meninges gently removed, and weighed. The brains were then cut (coronal plane/cut at roughly Bregma 0 – Bregma -2.92 mm) and fixed in HISTOCHOICE (VWR, Irving, TX) at 4°C overnight. Brain tissues were then rehydrated in 30% sucrose/PBS (weight/vol) at 4°C overnight, embedded in Tissue Freezing Medium (TBS, IMEB Inc., San Marcos, CA) and frozen in at -80°C freezer prior to sectioning. The remaining regions of the brain not fixed for histology were snap-frozen and stored at -80°C for future molecular assays.

3.3.3 Double Immunofluorescent Staining

10 μm frozen sections of the cerebrum from Bregma 0 through -2.5mm were used for immunofluorescent labeling, as previously described by our laboratory (Suwannasual et al. 2018), using the following primary antibodies: occludin (1:500; Abcam, Cambridge, MA, #168986), claudin-5 (1:500; Abcam #15106), IgG (1:500, Abcam #6708), and von Willebrand factor (vWF: 1:1000; Abcam #11713). Donkey-anti mouse IgG (H+L) Alexa-Fluor 594 (1:250, Thermo Fisher Scientific, Waltham, MA #A32744) and Goat-anti rabbit IgG (H+L) Alexa-Fluor 488 (1:250, Thermo Fisher Scientific #A32731) were used for the secondary antibodies. Slides were imaged by EVOS fluorescent microscopy (EVOS Fl, Thermo Fisher Scientific) at 40x with the proper excitation/emission filter, and digitally recorded. Images were analyzed by Image J software (NIH, Bethesda, MD) by a blinded technician. Only cerebral vessels ≤ 50 μm in size were used for analysis. Colocalization was quantified by calculating total fluorescence from the overlaid images from at least 4-5 vessels for each section (2 sections per slide), 2 slides per animal, and n = 6 per group were utilized for analysis.

3.3.4 In situ Zymography

MMP-2/9 activity was measured by the in situ zymography technique on 10 μm thick cerebral sections, as previously described by our laboratory (Oppenheim et al., 2013). Slides were imaged by fluorescent microscopy, and 40x images were used for analysis and densitometry quantification via Image J software (NIH). Analysis was performed on at least 4 vessels per section, 2 sections per slide, 3 slides per mouse, n = 6 samples per group. Background fluorescence was subtracted from each image, prior to statistical analysis. Only vessels less than 50μm in size were used for analysis.

3.3.5 Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Gene expression of IL-6, MMP-9, VCAM-1, ICAM-1, claudin-5, and occludin were analyzed utilizing SYBR green (SsoAdvanced SYBR Green Supermix, BIORAD, Hercules, CA) assays with the appropriate forward and reverse primers (Table 3.1) by real-time RT-qPCR. Cerebral tissue was homogenized utilizing a Tissue Lyser system and RNA isolated using and All Prep DNA/RNA/miRNA kit (Qiagen, Germantown, MD), following the manufacturer protocol. Real-time qRT-PCR was completed and analyzed in the BIORAD CX. GAPDH was used for internal control, and results were analyzed and normalized, from n = 6 animals for each group, as previously described (Suwannasual et al., 2019).

3.3.6 Statistical Analysis

A two-way ANOVA with posthoc Tukey's test was used to analyze the statistical significance between ovary status and exposure, as well as the interaction between both factors, for each endpoint. Statistical analyses were performed using Sigma Plot 10.0 (Systat, San Jose, CA). A $p < 0.050$ was considered statistically significant.

Table 3.1: Primer sets utilized for real-time RT-qPCR.

Gene/Primer	Sequence (5' – 3")
Mouse occludin FP	CTCCCATCCGAGTTTCAGGT
Mouse occludin RP	GCTGTCGCCTAAGGAAAGAG
Mouse claudin-5 FP	TTCGCCAACATTGTCGTCC
Mouse claudin-5 RP	TCTTCTTGTCGTAGTCGCCG
Mouse IL-1β FP	CCTCCTTGCCTCTGATGG
Mouse IL-1β RP	AGTGCTGCCTAATGTCCC
Mouse TNF-α FP	CCCCAGTCTGTATCCTTCT
Mouse TNF-α RP	ACTGTCCCAGCATCTTGT
Mouse VCAM-1 FP	ACTTTCTATTTCACTCACACCAGCC
Mouse VCAM-1 RP	ATCTTCACAGGCATTTCAAGTCTCT
Mouse ICAM-1 FP	CCATAAAACTCAAGGGACAAGCC
Mouse ICAM-1 RP	TACCATTCTGTTCAAAAGCAGCA
Mouse IL-1 FP	GAAGAGATGTTACAGAAGCC
Mouse IL-1 RP	CATGCCTGAATAATGATCAC
Mouse IKKα FP	CCAGAACAGTACTCCATTGCCAGA
Mouse IKKα RP	TGGCATGGAAACGGATAACTGA
Mouse IKKβ FP	TGGCATGGAAACGGATAACTGA
Mouse IKKβ RP	CTGGAACTCTGTGCCTGTGGAA
Mouse MMP-9 FP	GACAGGCACTTCACCGGCTA
Mouse MMP-9 RP	CCCGACACACAGTAAGCATTC
Mouse RelA FP	TGTTGCCCACTTCAGGTTGT
Mouse RelA RP	AGTGGAAGCCCTGTCCTAGT
Mouse IL-6 FP	GGCCTTCCCTACTTCACAAG
Mouse IL-6 RP	CACTAGGTTTGCCGAGTAGATCTC
Mouse GAPDH FP	CATGGCCTTCCGTGTTCCTA
Mouse GAPDH RP	GCGGCAGTCAGATCCA

FP, forward primer; RP, reverse primer; IL-1β, interleukin-1 beta; TNF-α, tumor necrosis factor alpha; VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intracellular adhesion molecule-1, IKKα, inhibitor of nuclear factor kappa-B kinase subunit alpha; IKKβ, inhibitor of nuclear factor kappa-B kinase subunit beta; MMP-9, matrix metalloproteinase-9; RelA (p65), v-rel avaian reticuloendotheliosis viral oncogene homolog A; IL-6, interleukin-6; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

3.4 Results

3.4.1 Mice Exposed to MVE Display Decreased TJ Protein Expression in the Cerebral Microvasculature

To elucidate the effects of MVE exposure on modifications in BBB integrity, we analyzed the expression of cerebral microvascular TJ proteins, claudin-5, and occludin. Compared to FA ov+ (Figs. 3.1 A-C) and FA ov- (Figs. 3.1 G-I), we observed a significant decrease in claudin-5 expression in the cerebral microvasculature of MVE ov+ (Figs. 3.1 D-F) and MVE ov- (Figs. 3.1 J-L) female ApoE $^{-/-}$ mice, as quantified in Fig. 3.1M. For exposure, $F = 34.271$, $p < 0.001$; for ovary status $F = 34.380$, $p < 0.001$; and for exposure x ovary status $F = 1.178$, $p = 0.309$. MVE-exposed groups either MVE ov and MVE ov- compared to controls FA ov+ and FA ov-; $p < 0.001$, as shown in (Fig.3.1 A-I). In agreement with these findings, cerebral claudin-5 mRNA expression was also downregulated in the MVE-exposed vs. FA-exposed female mice (Fig. 3.1N). For exposure, $F = 4.617$, $p = 0.045$, for ovary status, $F = 4.617$, $p = 0.275$; and for exposure x ovary interaction, $F = 0.00296$, $p = 0.957$. Interestingly, cerebral microvascular claudin-5 was increased in the FA ov- mice compared to the FA ov+ female mice (Fig. 3.1 M), although this relationship was not observed at the transcript level (Fig. 3.1 N).

Similar to the results of claudin-5 expression in the cerebral microvasculature, compared to FA ov+ (Figs. 3.2 A-C) and FA ov- (Figs. 3.2 G-I), occludin expression was decreased in the cerebral microvasculature of both MVE ov+ (Figs. 3.2 D-F) and MVE ov- (Figs. 3.2 J-L) female ApoE$^{-/-}$ mice, as quantified in Fig. 3.2M. For exposure, $F = 8.135$, $p = 0.021$; for ovary status, $F = 0.0999$, $p = 0.760$; and for exposure x ovary interaction, $F = 0.0507$, $p = 0.827$. However, there were no statistical alterations in cerebral occludin mRNA expression quantified across any study groups (Fig. 3.2N). Although mRNA expression was assessed on whole-brain (cerebrum) homogenates, which may not be indicative of vascular-specific expression (Fig. 3.2M.)

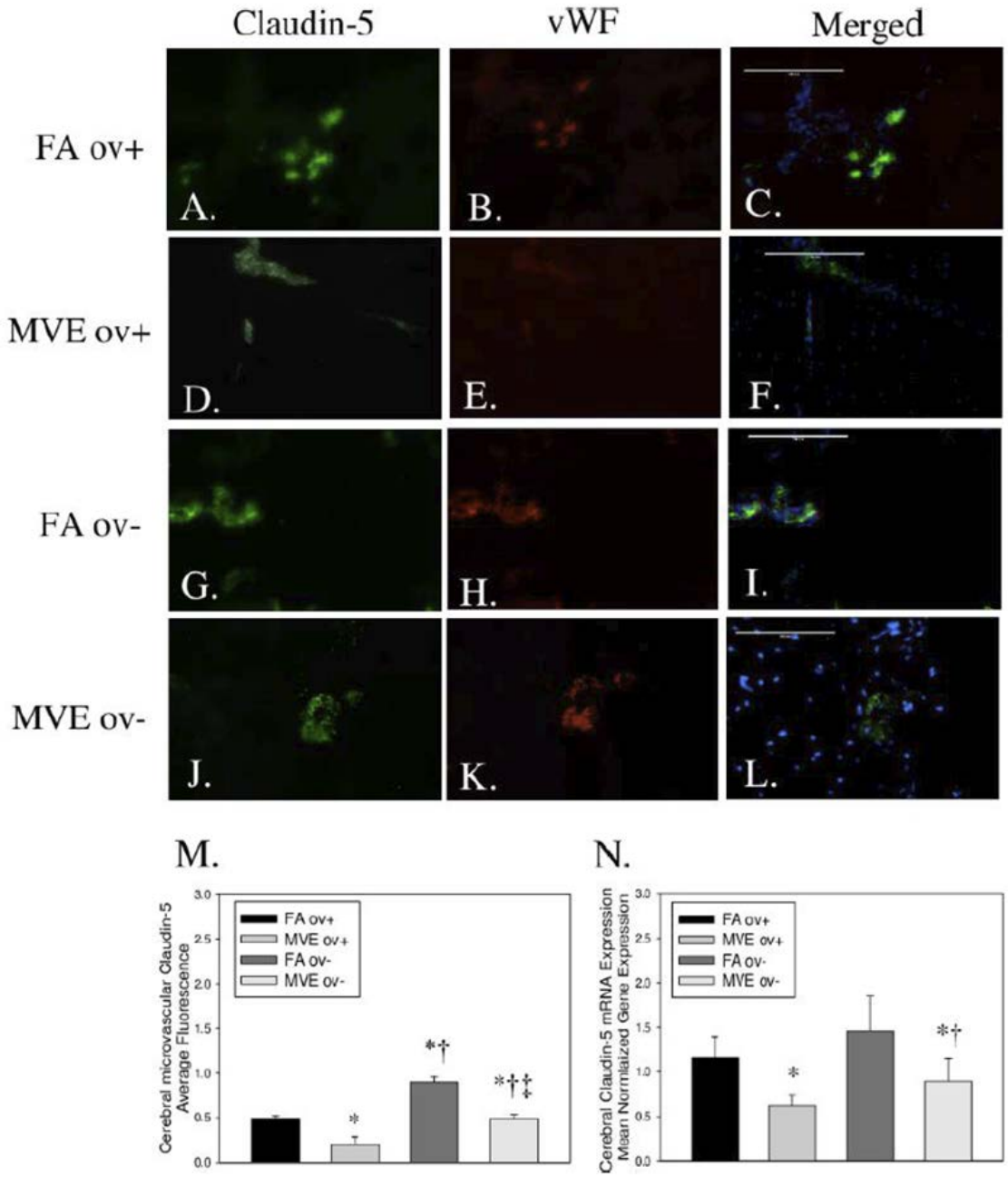

Figure 3.1: Representative immunofluorescence expression of claudin-5 (red) and vWF (green) in cerebral microvessels from female ApoE-/- mice with ovaries (ov+) or ovariectomized (ov-) exposed to either filtered air (FA) or mixed vehicle emissions (MVE: 200 µg/m^3 PM of mixed gasoline and diesel engine emissions) for 6 hrs/d, 7 d/wk, for 30 d. (A-C) FA ov+; (D-F) MVE ov+; (G-I) FA ov-; and (J-L) MVE ov-. Overlay panels (right-side panels: C, F, I, L) are merged images of the red and green fluorescence channels. Blue fluorescence is Hoechst stained nuclei. (M) Quantification graph of microvascular claudin-5 expression; (N) mean normalized cerebral claudin-5 expression, as quantified by RT-qPCR. Scale bar = 100 um. A minimum of 4-5 vessels (<50 µm) per section (2 sections per slide), 2 slides per animal, and n = 6 per group were utilized for analysis. Results represent mean ± SEM. *p ≤ 0.050 compared to FA ov+; †p ≤ 0.050 compared to MVE ov+; ‡ p ≤ 0.050 compared to FA ov-.

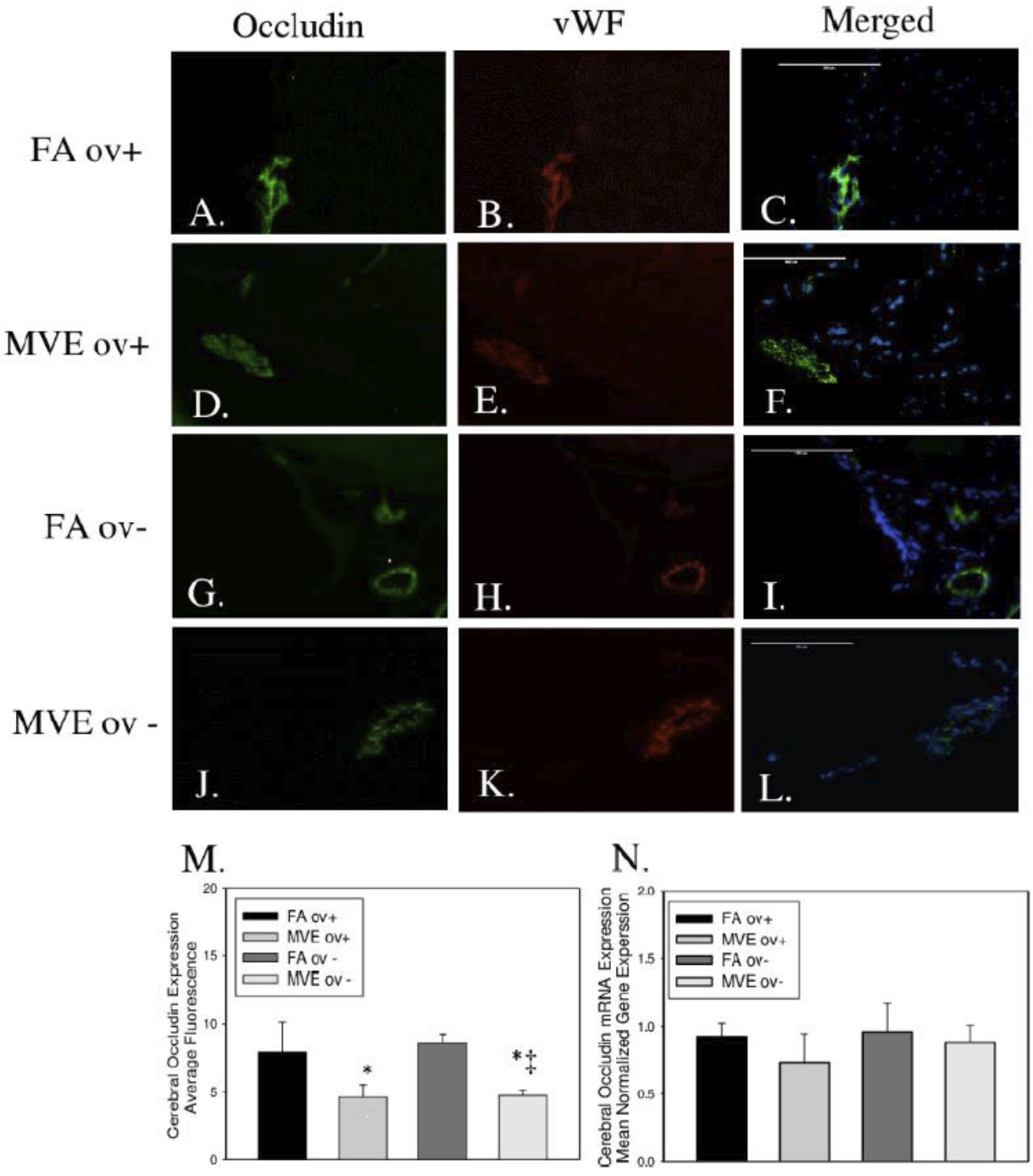

Figure 3.2: Representative immunofluorescence expression of occludin (red) and vWF (green) in cerebral microvessels from female ApoE$^{-/-}$ mice with ovaries (ov+) or ovariectomized (ov-) exposed to either filtered air (FA) or mixed vehicle emissions (MVE: 200 µg/m^3 PM of mixed gasoline and diesel engine emissions) for 6hrs/d, 7 d/wk, for 30 d. (A-C) FA ov+; (D-F) MVE ov+; (G-I) FA ov-; and (J-L) MVE ov-. Overlay panels (right-side panels: C, F, I, L) are merged images of the red and green fluorescence channels. Blue fluorescence is Hoechst stained nuclei. (M) Quantification graph of microvascular occludin expression; (N) mean normalized cerebral occludin expression, as quantified by RT-qPCR. Scale bar = 100 um. A minimum of 4-5 vessels (<50 µm) per section (2 sections per slide), 2 slides per animal, and n = 6 per group were utilized for analysis. Results represent mean ± SEM. *p ≤ 0.050 compared to FA ov+; ‡ p ≤ 0.050 compared to FA ov-.

3.4.2 Mice Exposed to MVE Display Upregulation in the Activity of MMPs Expression in the Cerebral Microvasculature

MMP-2/9 can alter BBB integrity by degrading TJ and basal lamina proteins. We have previously reported that MVE-exposure upregulates MMP-2/9 activity in the cerebral microvasculature of male ApoE$^{-/-}$ and C57BL/6 wild-type mice (Oppenheim et al., 2013, Lucero et al., 2017, Suwannasual et al., 2019). We, therefore, analyzed MMP-2/9 activity in the female cerebral microvasculature, using in situ zymography. Compared to FA ov+ (Figs. 3.3 A-C), we observed a significant increase in MMP-2/9 activity in the cerebral microvasculature of MVE ov+ (Figs. 3.3 D-F), FA ov- (Figs. 3.3 J-L), as quantified in Fig. 3.3 N. For exposure, $F = 29.439$ $p < 0.001$; for ovary status, $F = 12.955$, $p = 0.007$; and for exposure x ovary status interaction, $F = 0.234$, $p = 0.642$. However, at the transcript level, there was no statistical difference in MMP-9 mRNA expression noted across any groups (Fig. 3.3 N). Such results indicate that either total cerebral MMP-9 expression is not altered with exposures or ovary status, MMP-2 is the primary mediator of the increase in gelatinase activity (in situ zymography) observed, and/or MMP-9 activity is altered by changes in tissue inhibitor of MMPs (TIMP) interactions.

3.4.3 Mice Exposed to MVE Display an Increase IgG Extravasation into the Cerebral Parenchyma

IgG antibodies are large MW proteins that generally do not pass across an intact BBB. Thus, IgG translocation from the blood into the CNS parenchyma can be used as an indicator of BBB disruption and increased permeability. Therefore, we assessed vascular permeability by quantifying IgG translocation into the cerebral parenchyma. The distance from the edges of vessels to the furthest point of IgG diffusion into the parenchyma was quantified. Compared to FA ov+ (Figs. 3.4 A-C), we quantified a significant increase in IgG extravasation into the brain parenchyma in the MVE ov + (Figs. 3.4 D-F), FA ov- (Figs. 3.4 G-I), and the MVE ov- (Figs.

3.4 J-L) female ApoE$^{-/-}$mice, as quantified in Fig. 3.4 M. For exposure, $F = 43.858$, $p \leq 0.001$; for ovary status, $F = 45.653$, $p \leq 0.001$; and for exposure x ovary status interaction, $F = 5.107$, $p = 0.054$.

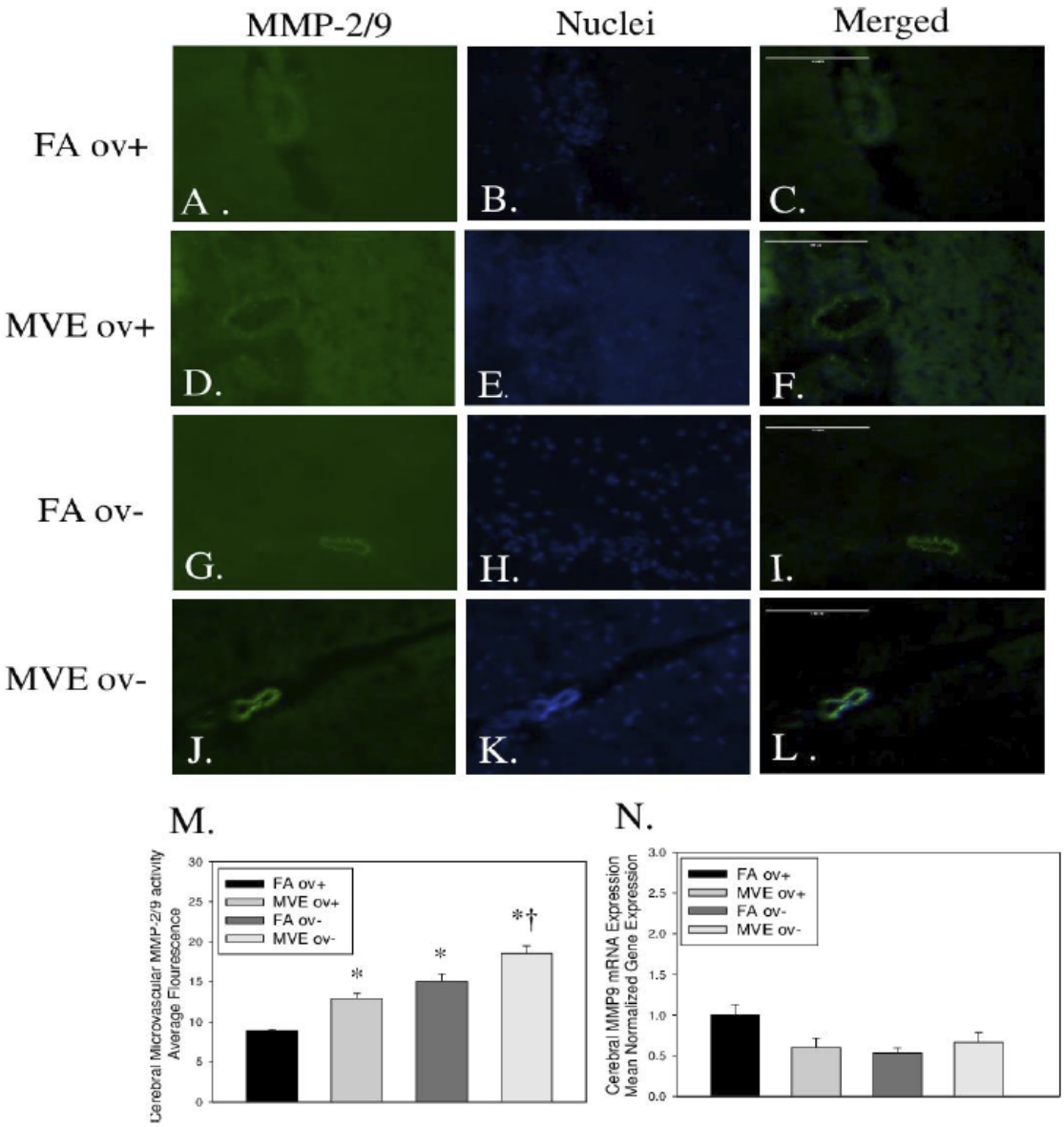

Figure 3.3: Representative MMP-2/9 (gelatinase, green) activity in the cerebral microvasculature of female ApoE$^{-/-}$ mice with ovaries (ov+) or ovariectomized (ov-) and exposed to either filtered air (FA) or mixed vehicle emissions (MVE: 200 µg/m^3 PM of mixed gasoline and diesel engine emissions) for 6hrs/d, 7 d/wk, for 30 d. (A-C) FA ov+; (D-F) MVE ov+; (G-I) FA ov-; and (J-L) MVE ov-. Blue fluorescence is Hoechst-stained nuclei. Scale bar = 100 µm. A minimum of 4-5 vessels (<50µm) per section (2 sections per slide), 2 slides per animal, and n = 6 per group were utilized for analysis. Results represent mean ± SEM. *$p \leq 0.050$ compared to FA ov+; †$p \leq 0.050$ compared to MVE ov+.

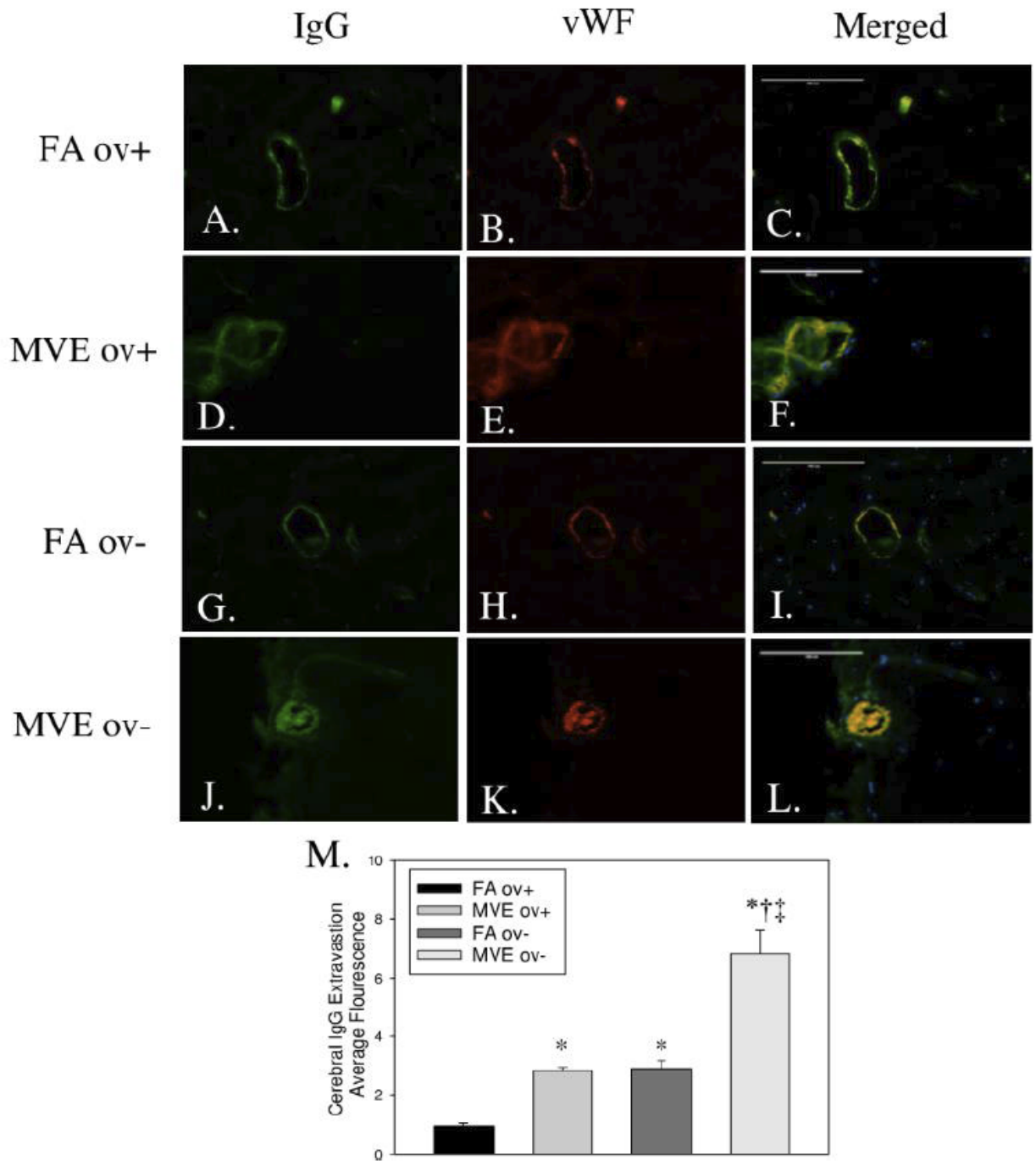

Figure 3.4: Immunofluorescence analysis of IgG (green) extravasation from the microvasculature into the cerebral parenchyma in female ApoE$^{-/-}$ mice with ovaries (ov+) or ovariectomized (ov-), exposed to either mixed vehicle emissions (MVE: 200 PM µg/m^3 for 6 hrs /day, 7 d/wk, 30 d) or filtered air (FA). Red fluorescence = endothelial cell marker, von Willebrand (vWF). Overlay = merged IgG and vWF fluorescence (co-localization). Scale bar is 100µm. A minimum of 4-5 vessels (<50 µm) per section (2 sections per slide), 2 slides per animal, and n = 6 per group were utilized for analysis. Results represent mean ± SEM. *p ≤ 0.050 compared to FA ov+; †p ≤ 0.050 compared to MVE ov+; ‡ p ≤ 0.050 compared to FA ov-.

3.4.4 MVE-Exposure Alters Cerebral Transcript Expression of Cell Adhesion Molecules

Increased expression of endothelial cell adhesion molecules, ICAM-1, and VCAM-1 indicates endothelial cell activation and associated with BBB disruption (Sumbria et al., 2016; Shimizu et al., 2018). Thus, we analyzed the cerebral ICAM-1 and VCAM-1 mRNA expression in our study animals. MVE-exposure results in a significant increase in cerebral ICAM-1 mRNA transcript expression regardless of ovary status, compared to FA exposed female ApoE$^{-/-}$ mice (Fig. 3.5 A). For exposure, $F = 5.927$, $p = 0.025$, for ovary status, $F = 0.00350$, $p = 0.953$; and for interaction between exposure and status of ovaries, $F = 0.0620$, $p = 0.806$. Conversely, we observed no difference in cerebral VCAM-1 mRNA expression across any of our study groups (Fig. 3.5 B).

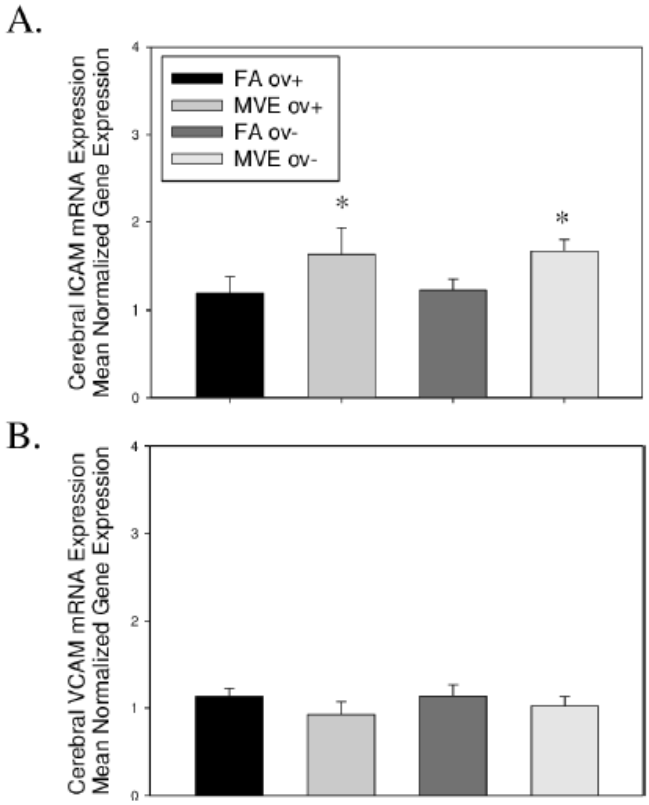

Figure 3.5: Mean normalized gene expression of cerebral (A) intracellular adhesion molecule (ICAM)-1 and (B) vascular cell adhesion molecule (VCAM)-1 in female ApoE$^{-/-}$ mice with ovaries (ov+) or ovariectomized (ov-), exposed to either mixed vehicle emissions (MVE: 200 PM µg/m^3 for 6 hrs /day, 7 d/wk, 30 d) or filtered air (FA), as determined by real-time RT-qPCR. Results represent mean ± SEM. *$p \leq 0.050$ compared to FA ov+ group.

3.4.5 Expression of Inflammatory Markers in the Cerebrum of Female Ovary-Intact and Ovariectomized ApoE$^{-/-}$ Mice Exposed to MVE

To determine if the observed MVE-mediated alteration in BBB integrity is associated with inflammatory signaling, we analyzed the cerebral expression of IL-1β, IL-6, and TNF-α mRNA in our study animals. MVE-exposure significantly increased the expression of cerebral IL-1β mRNA (Fig. 3.6 A) and TNF-α (Fig. 3.6 B) in both MVE ov+ and MVE ov- female ApoE$^{-/-}$ mice, compared to both the FA ov+ and FA ov- groups. Results of mRNA IL-1β for exposure, $F = 7.420$, $p = 0.004$; for ovary status, $F = 0.0597$, $p = 0.810$; and interaction between exposure and ovary status, $F = 0.855$, $p = 0.442$ (Fig. 3.6 A). Likewise, we observed an increase of TNF-α mRNA in the cerebrum of both MVE ov+ and MVE ov- mice, compared to the FA exposure groups (Fig. 3.6 B). For exposure, $F = 29.581$, $p < 0.001$; for ovary status, $F = 0.013$ $p = 0.911$; for the interaction between exposure and ovary status, $F = 3.314$, $p = 0.084$. IL-1 increased in response to exposure, $F = 6.273$, $p = 0.024$; for ovary status, $F = 0.231$, $p = 0.638$; and for interaction between exposure and ovary status, $F = 0.00399$, $p = 0.950$; (Fig. 3.6 C). We did not observe any alterations in cerebral IL-6 mRNA expression across any of the groups within our study (Fig. 3.6 D)

3.4.6 MVE Exposure and mRNA Expression of RELA, IKKa, and IKKb in Female ApoE$^{-/-}$ Mice

Increased expression of inflammatory signaling in the brain can lead to increased NF-κB activation in the CNS, which in turn can lead to further induction of neuroinflammation-mediated pathways resulting in altered neurovascular permeability and neuronal cell death (Shih et al., 2015). Increased activation of kinases IKKα and IKKβ, which are catalytic subunits of IKK, also results in activation of NF-κB through promoting degradation of the inhibitory subunit IκBα Moreover, activated Rel A, also known as p65, is also involved in the activation and

modification of NF-κB, which in turn acts as a transcriptional factor for induction of multiple signaling pathways. As such, we analyzed the expression of cerebral RelA, IKKα, and IKKβ in the brains of our study animals. We did not observe any statistical difference in cerebral RelA mRNA expression across any of our study groups (Fig. 3.7 A).

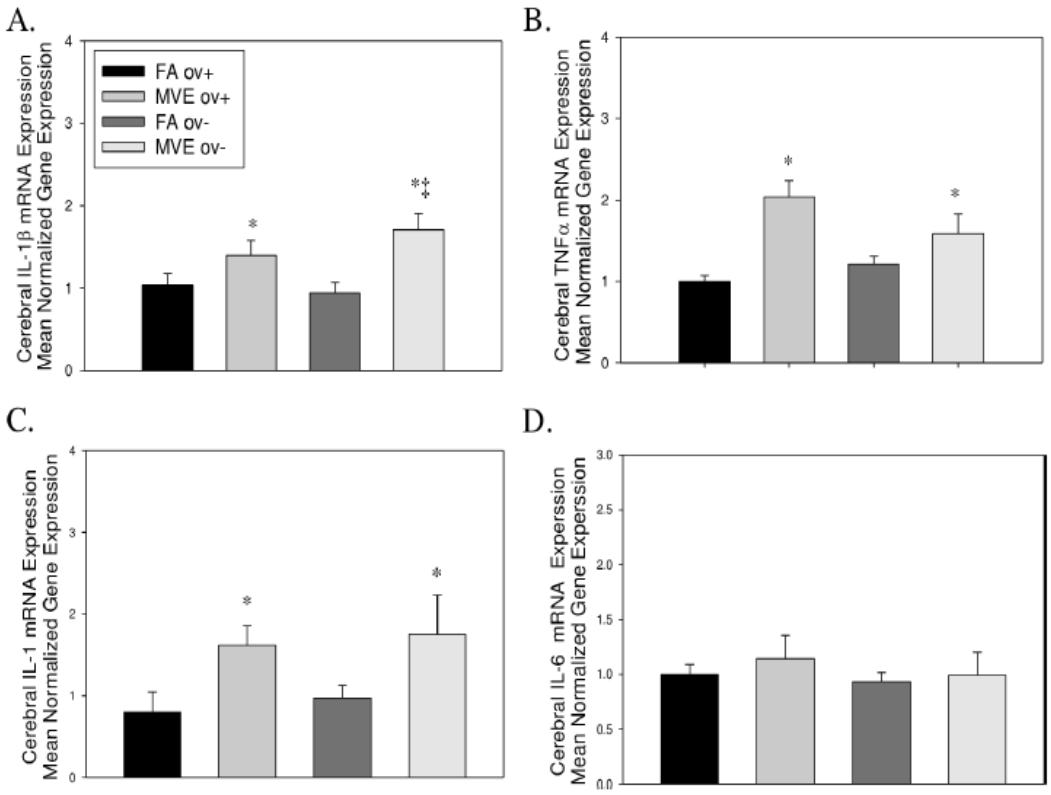

Figure 3.6: Mean normalized gene expression of cerebral (A) interleukin (IL)-1β mRNA, (B) tumor necrosis factor (TNF)-α mRNA, (C) IL-6, and (D) IL-1 mRNA in female ApoE$^{-/-}$ mice with ovaries (ov+) or ovariectomized (ov-), exposed to either filtered air (FA) or mixed exhaust from gasoline and diesel engines (MVE: 200 PM μg/m^3) for 6 hrs /day, 7 d/wk for 30 d, as determined by real-time RT-qPCR. Results represent mean ± SEM. *p ≤ 0.050 compared to FA ov+ group; ‡ p ≤ 0.050 compared to FA ov-.

Interestingly, we did observe a significant increase in both IKKα (Fig. 3.7 B) and IKKβ (Fig. 3.7 C) mRNA transcript expression in the cerebrum of both FA ov- and MVE ov- female Apo E$^{-/-}$ mice. The presence of ovaries (and presumably female hormones) mediated the alteration in mRNA expression of these kinases, as determined via a 2-way ANOVA. Results of IKKα for exposure, F = 0.269, p = 0.610; for ovary status, F = 4.754, p = 0.042; and for interaction

between exposure and ovary status, F = 0.330, p = 0.573. Similarly, for IKKβ, the F value for exposure = 0.0001, p = 0.991; for ovary status, F = 9.707, p = 0.006; and for interaction between exposure and ovary status, F = 0.703, p = 0.413. We did not observe a significant interaction between exposure x presence of ovary for RELA, IKKα, or IKKβ mRNA transcript expressions.

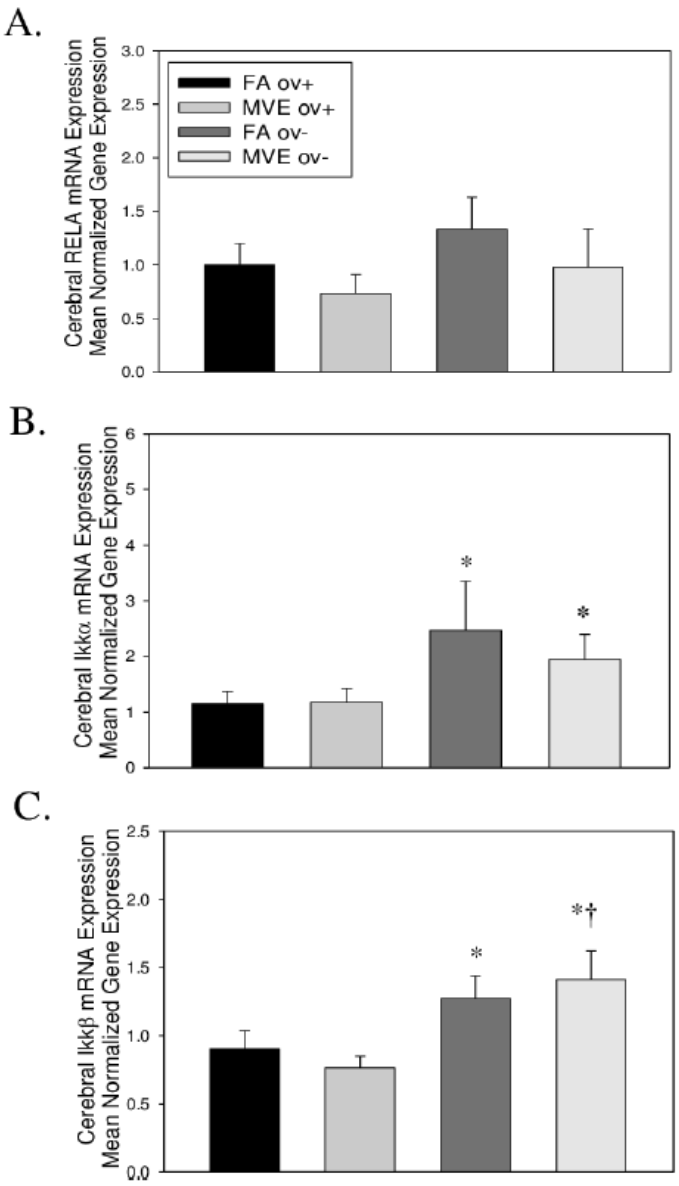

Figure 3.7: Mean normalized gene expression of cerebral (A) RELA, (B) inhibitor of nuclear factor kappa-B kinase subunit (IKK) α, and (C) IKKβ mRNA in female ApoE$^{-/-}$ mice with ovaries (ov+) or ovariectomized (ov-), exposed to either filtered air (FA) or mixed exhaust from gasoline and diesel engines (MVE: 200 PM μg/m^3) for 6 hrs /day, 7 d/wk for 30 d, as determined by real-time RT-qPCR. Results represent mean ± SEM. *p ≤ 0.050 compared to FA ov+; †p ≤ 0.050 compared to MVE ov+.

3.5 Discussion

Traffic-generated pollution is a complex mixture of particulate matter (PM), gases, organic compounds, and different metals, each of which is capable of producing detrimental effects in the CNS (Babdjouni et al., 2017; Block and Calderón-Garcidueñas, 2009; Genc et al., 2012). Collectively, inhaled air pollutants have been reported to damage the brain via alteration of structure and function of the cerebral vascular, neuroinflammation, and neurodegeneration (Block and Calderón-Garcidueñas, 2009). For example, $PM_{2.5}$ exposure has been linked to increased neurodegeneration and neuroinflammation, associated with the pathogenesis of Alzheimer's disease, as early as childhood, in highly polluted regions (Calderón-Garcidueñas et al., 2020; Calderón-Garcidueñas et al., 2020; González-Maciel et al., 2017). Exposure to other traffic-generated air pollutants, including NO_2/NO_x, and carbon monoxide (CO), is also associated with an increased risk of dementia (Peters et al., 2019). Ehsanifar et al., 2019). Reports from multiple rodent studies confirm that exposure to diesel exhaust and diesel PM results in increased pro-inflammatory cytokines in the CNS, including TNF-α and IL-1β (Gerlofs-Nijland et al., 2010; Levesque et al., 2011a; Levesque et al., 2011b). Our laboratory has reported that exposure to MVE results in increased production of ROS and neuroinflammation, and altered cerebral microvascular integrity and permeability in the brain (Oppenheim et al., 2013; Suwannasual et al., 2018; Suwannasual et al., 2019; Block et al, 2009). In our previous studies, we observed that the altered BBB integrity was associated with increased MMP-2/9 activity and decreased TJ protein expression in the cerebral microvasculature of both C57BL/6 and $ApoE^{-/-}$ mice; however, these studies were only conducted in male mice. As such, we conducted the current inhalation exposure studies in ov+ and ov- female $ApoE^{-/-}$ in order to determine (1) whether the brains of female mice have similar detrimental CNS outcomes, and (2)

whether the presence of female hormones alter any of the previously reported outcomes observed in the CNS of males.

In agreement with our previous studies in male mice, in the current study, we observed that MVE-exposure mediated altered cerebrovascular integrity through the degradation of TJ proteins claudin-5 and occludin. Moreover, the decrease in microvascular integrity was associated with increased BBB permeability, as evidenced by increased IgG extravasation from the blood into the cerebral parenchyma in the MVE-exposed female ApoE$^{-/-}$ mice, regardless of ovary status. BBB disruption is often associated with increased IgG expression in the brain due to decreased TJ protein expression leading to increased permeability. This premise has been confirmed through multiple studies in brains from aged patients and those with CNS disorders, such as Alzheimer's disease and MS, which are associated with decreased BBB integrity (Bake et al., 2009; Ryu and McLarnon, 2009; Syndulko et al., 1993). Based on our results, the alteration in cerebral microvascular integrity was mediated by the MVE-exposure, independent of ovary/hormone status, as there was a significant decrease in TJ proteins, claudin-5, and occludin, in both the MVE ov+ and MVE ov- groups. There was a statistical increase in claudin-5 expression in the cerebral microvasculature of the FA ov- group, compared to the FA ov+, suggesting the loss of female hormones does not alter the BBB integrity via expression of these TJ proteins. Additionally, while the cerebral claudin-5 mRNA transcript findings were similar to those observed in the cerebral microvasculature, the cerebral analysis of occludin mRNA expression showed no statistical changes across groups. This may be because occludin is expressed in other cell types in the brain, including astrocytes and neurons (Bauer et al., 1999), and thus analyzing transcript expression in a cerebral homogenate is not indicative of what is occurring within the microvasculature. Interestingly, while MVE-exposure resulted in a

significant increase in IgG extravasation into the cerebral parenchyma, we also observed an increase in IgG expression in the FA ov- mice, compared to the FA ov+, suggesting that altered BBB permeability was associated with the loss of female hormones and MVE-exposure. It has been shown that long term exposure to $PM_{2.5}$ on results in ovarian dysfunction, abnormal hormone synthesis and oocyte maturation, and also accelerated the depletion of primordial follicles by increasing apoptosis and altered the ovarian endocrine functioning, and resulted in irregular estrous cycles (Zhou et al., 2020). These findings are in agreement with previous studies that have described age-related changes leading to increased BBB permeability, as assessed by IgG expression, but no change in claudin-5 protein levels in the microvasculature of reproductive senescent female mice (Bake et al., 2009). However, these authors observed dysregulation in microvascular claudin-5 localization within the cerebral microvasculature, suggesting that even though it is present, it is not properly located in the TJs between endothelial cells allowing unregulated transport (Bake et al., 2009). While we did not directly assess claudin-5 localization within the cerebral microvasculature, it is plausible that similar outcomes occur in our ov- mice due to lack of female hormone signaling, leading to increased BBB permeability, which is even further exacerbated with MVE exposure. This premise is further supported through study findings that show estradiol protects BBB integrity, even in the presence of inflammatory conditions (Maggioli et al., 2016).

Correlated with increased IgG extravasation, we observed a significant induction of MMP-2/9 (gelatinase) activity in the cerebral microvasculature in FA ov- female $ApoE^{-/-}$ mice, which was even further induced in the microvasculature of the MVE ov+ and MVE ov- groups. MMP-2/9 activity has been well characterized to contribute to the impairment of neurovascular function during aging, as well as after ischemic stroke (Chen et al., 2017; Lee et al., 2012).

Moreover, increased MMP-9 activity has been reported to mediate BBB disruption and leukocyte infiltration during CNS disorders such as seizures and MS (Li et al., 2013; Choi et al., 2017). Increased MMP-2/9 activity alters BBB integrity through the degradation of TJ proteins between the brain microvascular endothelial cells that comprise the BBB, thereby allowing for increased permeability (Rosenburg and Yang, 2007). MMP-9 decreased in EAE and MS patients in response to estriol treatment via mediating of ERα (Gold et al.,2009). While ApoE deficiency has previously been reported to promote BBB disruption via increased MMP-9 activity (Zheng et al., 2014), calling into question the use of $ApoE^{-/-}$ in the current study, we have previously reported that MVE-exposures also alters BBB integrity, associated with increased MMP-2/9 activity, in male $ApoE^{-/-}$ and also C57BL/6 wild-type mice on a high-fat diet (Suwannasual et al., 2019). Furthermore, the observed induction of MMP-2/9 activity, coupled with increased IgG extravasation (permeability) in the FA ov- group, is in agreement with previous reports that estrogen attenuates TJ disruption via repression of MMP transcription in the BBB (Na et al., 2015). Collectively, our results suggest that MVE-exposure exacerbates BBB disruption in females lacking ovary-produced hormones.

In addition to altered BBB integrity and permeability, we observed that MVE-exposure also mediated the induction of ICAM-1 mRNA in the cerebrum of female $ApoE^{-/-}$, regardless of the presence of ovaries/female hormones. ICAM-1 is involved in leukocyte adhesion to the cerebrovascular endothelium, which can allow for increased leukocyte migration into the CNS. Furthermore, the upregulation of endothelial ICAM-1 is associated with cytoskeletal rearrangement and cellular signaling associated with increased BBB permeability in multiple CNS disorders (Huber et al., 2006). While typically minimally expressed under physiologic conditions, the expression of ICAM-1 significantly increases with inflammation in the brain

(Jander et al., 1996; Rossi et al., 2011). A previous study of air pollution effects in an aged population reported increased circulating ICAM-1 and VCAM-1 associated with exposure multiple components of air pollution, including particle number, $PM_{2.5}$, and NO_2, suggesting air pollution-exposure mediates alterations in endothelial cell dysfunction. (Bind et al., 2012). Additionally, we have previously reported that MVE-exposure mediates increased ICAM-1 and VCAM-1 mRNA in the cerebral microvasculature of Apo $E^{-/-}$ male mice (Lucero et al., 2017). Interestingly, MVE-exposure did not increase VCAM-1 mRNA expression in the cerebrum of the females, in the current study, even at double the exposure concentration (200 $\mu g/m^3$ PM). This may be due to the utilization of cerebral tissue homogenate, in the current study, compared to using only cerebral microvessels for transcript analysis in the male $ApoE^{-/-}$ mouse study (Lucero et al., 2017).

Correlated to our altered cerebral microvascular integrity and permeability findings, we also observed increased expression of inflammatory markers, TNFα, IL-1β, and IL-1 mRNA in the cerebrum of female $ApoE^{-/-}$ exposed to MVE, compared to FA controls. In agreement with the previous endpoints, the ovary/hormone status did not appear to alter the expression of these factors in the brain, as there was no significant difference between ov- vs. ov+ animals. Exposure to traffic-generated air pollutants has been well characterized to contribute to increased inflammatory signaling in the brain and cerebrovasculature (Block and Calderón-Garcidueñas, 2009; Hahad et al., 2020); however, relatively few studies have investigated mechanistic outcomes specifically in the female brain.

In addition to increased inflammatory signaling, MVE-exposure also mediated elevations in IKKα and IKKβ mRNA expression, but only in ov- mice. However, this same outcome was also observed in the brains of FA ov- mice, suggesting ovary status, not the exposure, mediated

the alteration in cerebral IKKα and IKKβ mRNA expression. IKKα and IKKβ, involved in canonical and classical NF-κB activation, are the catalytic subunits of IκB-kinase (IKK) that mediates NF-κB activation. Upon stimulation, IKK phosphorylates the inhibitor of NF-κB, IκB-α, leading to ubiquitination and proteasomal degradation of IκB-α, allowing for the activation NF-κB signaling pathways. TNF-α and IL-1 are both known to mediate the activation of canonical NF-κB, resulting in the initiation of transcription of several signaling pathways that play a critical role in cell survival (Galeone et al., 2013; Liu et al., 2017). We observe an upregulation of TNF-α and IL-1 in the cerebrum of both MVE ov- and MVE ov+ female mice, but not FA ov- mice, while we observe increased cerebral IKKα and IKKβ mRNA expression only in the brains of ov- female mice (both MVE and FA). Estrogen signaling is known to be neuroprotective; therefore, the absence of the hormone-mediated signaling pathways in ov- mice may lead to activation of NF-κB signaling. In agreement with this premise, 17β-estradiol administration has been shown to decrease inflammatory signaling via modulating the NF-κB signaling pathway through an estrogen receptor alpha (ERα)-mediated mechanism. (Ghisletti et al., 2005). Thus, loss of estrogen signaling in the ov- mice may lead to decreased inhibition or regulation of the NF-κB signaling in the brain. Regardless, this alteration in cerebral IKKα and IKKβ mRNA expression in the current study does not appear to be mediated by MVE-exposure.

While the current study adds to the foundation of knowledge on the effects of traffic-generated air pollution exposure-mediated outcomes in the CNS, there are some limitations that should be noted. The concentration of MVE chosen for the current study (200 $\mu g/m^3$ PM) would be considered high for most environmental exposures; however, it is within the range of occupational exposure scenarios, as well as daily $PM_{2.5}$ levels observed in heavily populated regions worldwide (Pronk et al., 2009; Costa et al., 2017; IQAir). Additionally, we only

investigated the reported endpoints at one exposure time point (30 d subchronic exposure), and thus cannot confirm these detrimental CNS outcomes also occur in acute or chronic exposure scenarios. However, acute exposure to diesel exhaust (6 hr, 250-300 $\mu g/m^3$) has been reported to result in significant elevations in oxidative stress and inflammation (TNF-α, IL-1β) in the brains of both male and female C57BL/6 mice (Cole et al., 2016). Lastly, while utilizing the $ApoE^{-/-}$ mouse model allowed us to compare outcomes in the brain and neurovasculature to previous studies in male mice from our laboratory (Oppenheim et al., 2013; Lucero et al., 2017), the use of this model for the current study can also be viewed as a limitation. ApoE is known to contribute to TJ protein expression and BBB stability (Nishitsuji et al., 2011); thus, it is possible the lack of ApoE expression in the brain exacerbated the reported outcomes in the current study. However, we have also observed similar outcomes in the brains of wild-type mouse models exposed to MVE (Suwannasual et al., 2018; Suwannasual et al., 2019), which are in agreement with reported outcomes of traffic-generated air pollution exposure in the brains of humans (Calderón-Garcidueñas et al., 2008).

3.6 Conclusion

The results of this study show that inhalation exposure to MVE results in altered cerebral microvascular integrity, as evidenced by decreased expression of TJ proteins, claudin-5, and occludin, in the cerebral microvasculature of female $ApoE^{-/-}$ mice. Moreover, the altered neurovascular integrity was associated with increased MMP-2/9 (gelatinase) activity and correlated with increased permeability, as determined by increased measured IgG extravasation. MVE-exposure was also associated with elevated cerebral expression of adhesion molecule, ICAM-1, and inflammatory factors TNF-α, IL-1β, and IL1, independent of the presence of ovaries/female hormone signaling. Ovariectomized (ov-) control mice also displayed significant

induction of gelatinase activity in the cerebral microvasculature, associated with increased IgG extravasation; however, there was no concurrent alteration of TJ protein expression or increase in inflammatory signaling compared to the ovary-intact (ov+) FA ApoE$^{-/-}$ mice. We also observed an increase in cerebral IKKα and IKKβ mRNA transcript expression was also increased in both the FA ov- and MVE ov- animals, suggesting altered hormonal signaling may contribute to this observed outcome. Collectively, our results suggest that traffic-generated air pollution exposure alters neurovascular integrity and promotes inflammation in the CNS of females.

3.7 Acknowledgements

We would like to thank the Chemistry and Inhalation Exposure group, in the Environmental Respiratory Health Program, at Lovelace Biomedical and Environmental Research Institute for the characterization and monitoring of the animal exposures.

3.8 Funding

This work was supported by National Institute of Environmental Health Sciences at National Institute of Health [R00ES016586 to A.K.L.], the University of North Texas Research Initiation Grant (RIG) [GA93601 to A.K.L], and Environmental Protection Agency Center Grant RD-83479601 [Project 3 to A.K.L., coPI and Project 2 to J.D.M., coPI] for the animal exposures. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health or the Environmental Protection Agency.

3.9 Conflict of Interests

Funding from grants received from the National Institute of Environmental Health and U.S. Environmental Protection Agency were used to conduct some the exposures and studies

described, herein; however, the authors declare no conflict of interest or financial gains to these entities associated with this publication.

3.10 Author Contributions

A.A.: investigation, formal analysis, validation, writing – original draft; J.L.: project administration, formal analysis, investigation, writing – review & editing; N.S.: investigation, formal analysis, writing – review & editing; J.D.M.: methodology, resources, writing – review & editing; A.K.L.: conceptualization, funding acquisition, formal analysis, resources, supervision, writing – review & editing.

CHAPTER 4

DISCUSSION AND CONCLUSION

Air pollution accounts for an estimated 4.2 million deaths and ranked fifth in all health risk factors globally (Hadad et al., 2020). The effects of air pollution exposure on detrimental outcomes in the CNS is of growing interest in the scientific and medical fields, as there are multiple recent epidemiologic and research studies that provide strong associations between environmental air pollution exposure and altered cognitive and motor function, mental health status, and CNS disorders such as Alzheimer's disease and MS in both young and aged populations (Calderón-Garcidueñas et al., 2020; Costa et al., 2020; Lopuszanska and Samardakiewicz, 2020; Shou et al., 2020; Thiakaratne et al., 2020; Ventriglio et al., 2020;). However, there are still many unknowns related to the mechanistic signaling pathways that contribute to these pathologies in the brain.

While ambient air pollution exposure is a complex mixture of gases and PM, which can vary in composition across different locations, the primary components in most urban settings is from traffic-derived sources (resulting from combustion in gasoline or diesel engines). Studies from different regions consistently report air pollution-exposure contributes to injury and disorders in the CNS (Babadjouni et al., 2017; Calderón-Garcidueñas et al., 2019). For example, studies from the highly polluted region of Mexico City report that exposure to ambient pollution, predominately consisting of traffic-generated pollutants, is linked to the onset of pathologies associated with Alzheimer's disease in the brains of children under 18 years old (Calderón-Garcidueñas et al., 2019; Calderón-Garcidueñas et al., 2019; Mansour et al., 2019). Additionally, exposure to PM from biomass-burning and diesel exhaust PM has been reported to increase inflammation, ROS production, and signaling pathways associated with neurodegeneration in

male wild-type mice (Milani et al., 2020). Also, exposure to a mixture of common traffic-generated pollutants, SO_2 and $PM_{2.5}$, was reported to result in neuronal apoptosis and neurodegeneration in both in vitro and in vivo exposure models (Ku et al., 2017). Exposure to air pollutants, as well as smoking, are also well-known risk factors for the onset of MS (Waubant et al., 2019). Ambient air pollution exposure, including PM_{10}, NO_2, SO_2, and O_3, has been linked to MS incidence and increased relapses via the promotion of neuroinflammation and oxidative stress in the brain (Corona-Vázquez et al., 2019; Roux et al., 2017; Jeanjean et al., 2018; Heydarpour et al., 2014; Ashtari et al., 2018; Börü et al., 2018; Bergamaschi et al., 2018). An epidemiologic study in Finland reported over a 4-fold increase in MS relapse rates when PM_{10} levels were in the highest quartile (Oikonen et al., 2003). Moreover, exposure to air pollutants has been reported to result in the activation of T lymphocytes and contribute to autoimmune diseases including Lupus, and rheumatoid arthritis, and MS (Zhao et al., 2019). However, there are also conflicting reports in the literature. For example, a cohort study conducted in the US showed no link between air pollution and MS (Palacios et al., 2017). The different outcomes observed cross epidemiologic studies likely pertain to differences across the studies, including sample size, age of participants, as well as geographical location. While there is still some debate in the literature as to whether exposure to air pollution is associated with the progression of MS, there are many studies that have reported that these exposures upregulated signaling pathways in the CNS associated with MS pathology. For example, diesel engine exhaust exposure has been shown to mediate increased expression of inflammatory factors, IL-β and TNF-, in the brains of rats through both direct olfactory exposure routes and also through increased systemic inflammation (Gerlofs-Nijland et al., 2010). Furthermore, in vivo exposure to SO_2 resulted in an induction of ROS and NF-κB signaling in the hippocampus in rodent studies (Sang et al., 2011).

Similarly, inhaled exposure to air pollution and resulting systemic signaling was reported to promote oxidative stress and increase the expression of the inflammatory marker, nitric oxide synthases iNOS, as well as IL-1α, IL-1β, IL-3, IL-6, TNF-α, microglia activation across various regions in the brain in exposed mice (Milani et al., 2020; Cole et al., 2018).

Inhaled air pollutions, including PM and gaseous components, can influence the CNS and MS pathophysiology via various mechanisms. First, inhaled air pollutants enter the CNS directly by the passage of air pollutants via the nasal cavity to the olfactory bulb (Genc et al., 2012). Second, inhaled air pollutants can modify the MS pathology via crossing the crossing alveolar-capillary barrier in the lung and enter the systemic circulation and alter BBB integrity and T cell translocation and demyelination. BBB acts as an impenetrable barrier and controls the influx and efflux of external components to the brain. We have previously reported that exposure to traffic generated air pollutants, namely a mixture of gasoline and diesel engine emissions (MVE), alters BBB integrity and permeability in male $ApoE^{-/-}$ and C57BL/6 wildtype mice (Oppenheim et al., 2013; Lucero et al., 2017; Suwannasual et al., 2019; Peeples,2020; Woodward et al.,2018). In addition to air pollution, female sex hormones alter BBB integrity and permeability via a different mechanism. Findings from experimental studies show that female sex hormones, particularly estrogen, have vasoprotective effects via signaling of Erα binding to estrogen response elements (ERE) and altering-endothelialization and endothelial nitric oxide production (Brouchet et al., 2001; Darblade et al.,2002). Likewise, E2 treatment increased the expression of Claudin-5 on the promoter, mRNA, and protein levels in mice (Burek et al., 2010). However, there is a knowledge gap regarding mechanistic associated with the effects of air pollutants and female sex hormones or possible interaction between them on pathways associated with MS pathophysiology, particularly at the molecule level. Our previous findings in male mice

revealed that MVE exposure mediated a reduction in TJ proteins' expression, occludin, and claudin-5, associated with increased MMP-2/9 activity in the cerebral microvasculature. We also analyzed cerebral microvascular claudin -5 and occludin expression in the female ApoE$^{-/-}$ mice in the current study. In agreement with our previous studies in male mice, we observed a decrease in the expression of both claudin -5 and occludin TJ proteins in the cerebral microvasculature in the MVE-exposed females, regardless of the presence of ovaries. Although our results did not show sex hormones effected on BBB integrity, previous studies showed estrogen increase in occludin expression in female mice brain and altered BBB's integrity (Kang et al., 2006). It has also been confirmed that estrogen has anti-inflammatory effects, decreases iNOS activity, and decreases lymphocyte trafficking (Cignarella et al., 2009; Maggioli et al., 2016). It has also been verified that estrogen treatment decreased MMP-9 in EAE via ERα receptor and decreased T cell infiltration associated with MS (Gold et al., 2009). Associated with the decrease in TJ protein expression in the cerebral microvasculature of MVE-exposed mice, we observed increased MMP-2/9 gelatinase activity. Interestingly, we also observed a significant increase in MMP-2/9 activity in the cerebral microvasculature of FA ov- ApoE$^{-/-}$ mice, indicating that loss of female steroid hormone signaling can promote the activity of gelatinases, which are associated with the degradation of TJ proteins and diminished BBB integrity. These findings indicate that MVE exposure mediates alterations in TJ protein expression in female ApoE$^{-/-}$ mice's cerebral microvasculature, associated with increased MMP-2/9 activity.

Associated with altered cerebral microvascular integrity, we also observed that MVE-exposure mediated increased IgG extravasation from the circulation into the cerebral parenchyma, which was further exacerbated in the MVE ov- mice compared to the MVE ov+ mice. As protein transport from the blood across the BBB is limited, increased IgG expression in

the parenchyma is an indicator of altered BBB permeability and transport. We also noted a significant increase in IgG extravasation in the cerebrum of FA ov- mice. It appears that lack of ovaries (and corresponding sex hormones) are just as detrimental as MVE on BBB permeability possibly via the alteration in ovarian-endocrine change and alteration in hormone change in response to MVE inhalation, as described by a previous study in mice (Zhou et al., 2020). Also, 17β-estradiol has been revealed to modify the expression of occludin, and loss of ovaries increased expression and arrangement of relocalization of endothelial connexin Cx43 (Okamoto and Suzuki, 2012). When coupled with the previously mentioned results, we observed increased BBB permeability and transport associated with increased MMP-2/9 activity in the cerebrum of MVE-exposed and FA ov- female $ApoE^{-/-}$ mice. Such results indicate that MVE-exposure and female steroid hormone signaling can independently mediate alterations in cerebral microvascular integrity and permeability; however, the combined exposure and loss of female hormones results in a further increase in BBB permeability.

In MS, alterations in cerebral microvascular integrity and permeability are also associated with increased T cell extravasation into the parenchyma resulting in increased inflammation and demyelination (Sallusto et al., 2012). Expression of adhesion molecules, ICAM-1 and VCAM-1, on the BBB's endothelial cells, mediate "crawling" of T cells prior to diapedesis across the BBB (Lyck and Engelhardt, 2012). A previous study reported that the extravasation of CD4+ across the BBB increased when cerebral microvascular endothelial expression of ICAM-1 expression was elevated, regardless of the inflammatory status of the BBB (Abadier et al., 2014). We observed that exposure to MVE mediated a significant increase in cerebral ICAM-1 mRNA transcript expression, regardless of the presence of ovaries. Correlated with the increase in ICAM-1 mRNA, we also measured a significant increase in CD4+ and CD8+ in the brains of

both MVE ov+ and MVE ov- mice. CD4+ are macrophages cell and modify the myelin sheath, while CD8+ are cytotoxic cells and involved in the apoptosis of neurons via activation of Fas-mediated p53 (Medana et al.,2007). CD4+ and CD8+ are increased in actively demyelinating lesions in MS patients and EAE; however, CD8+ are typically more numerous than CD4+ (Battistini et al., 2003). Collectively, such findings suggest that MVE-exposure mediates increased alterations in BBB integrity, permeability, and T cell translocation in the brains of female ApoE$^{-/-}$ mice, regardless of the presence of female steroid hormone signaling. As such, it is plausible that exposure to traffic-generated pollutants can exacerbate pathologies in the CNS associated with the pathogenesis of MS.

In addition to increased CD4+ and CD8+ expression in the brains of MVE-exposed female ApoE$^{-/-}$ mice, we also observed elevated MOG mRNA transcript expression, regardless of the presence of ovaries and female hormones. An increase in myelin oligodendrocyte glycoprotein antibody (MOG-Ab) is associated with demyelination (Hacohen and Banwell, 2019). In the current study, MVE-exposure was associated with increased demyelination in the brains of female ApoE$^{-/-}$ mice; however, the degree of demyelination was varied across different regions of the brain. For example, we observed more demyelination throughout regions 3 and 4 of the brain in comparison to the other regions of the brain (see Fig. 2.1). Notably, regions 3 and 4 are related to structures involving the basolateral and amygdaloid nucleus, pyriform (olfactory), lateral olfactory tract (LOT), lateral cortex ventricle, and subthalamic nucleus. The current study findings are in agreement with a previous report, which revealed that exposure to diesel exhaust resulted in proinflammatory signaling in the frontal cortex, hippocampus, cerebellum, striatum, and olfactory bulb compared to other regions of the brain (Gerlofs-Nijland et al., 2010). Interestingly, the decrease in myelination was higher in the brains of the MVE-

exposed ov- mice compared to MVE ov+, and we also observed significantly more demyelination in the FA ov- compared to FA ov+, indicating that the presence of female steroid hormones is protective against demyelination in the current model. However, the presence of CD4+ and CD8+ positive cells appeared to be more connected to the exposure itself, as there were no statistical differences noted between these endpoints in the cerebrums of MVE ov- vs. MVE ov+ mice. Furthermore, the expression of CD8+ and CD4+ across different regions of the brain were consistent. Such findings indicate that there are other factors involved in the loss of myelination in the brains of the FA ov- female ApoE$^{-/-}$ mice.

Increase inflammatory signaling is a hallmark of the pathophysiology of MS. An elevation of proinflammatory cytokines such as TNF-α, IL-1, and IL1-β has previously been reported in MS (Florindo, 2014). IL-1 is involved in the processing of proinflammatory cytokines and induce apoptosis (Lin et al., 2017). Our current findings revealed we had an increase of cerebral TNF-α and IL1-β mRNA, while IL-6 mRNA did not change significantly. In addition to inflammation and demyelination, it increased ROS production is also associated with the progression of MS (Florindo, 2014). ROS production has been reported to be associated with demyelination and axonal damage in MS and Experimental Autoimmune Encephalomyelitis (EAE) mice, a rodent model of MS (Witherick et al., 2011). In addition to others, we have previously reported that exposure to MVE induces ROS in the brains of male C57BL/6 and ApoE$^{-/-}$ mice (Oppenheim et al., 2013; Lucero et al., 2017). We also observed a significant increase in ROS production in the cerebrum of MVE-exposed ov- and ov+ female ApoE$^{-/-}$ mice. Increased ROS production has been correlated with the incidence and severity of MS, likely due to the activation of phagocytosis of the myelin sheath (Van der Goes et al., 1998). ROS production occurs through multiple mechanisms in the brain, including through increased

NADPH oxidase and/or xanthine oxidase activities, as well as through mitochondrial respiration (Abramov et al., 2007). Air pollutants such as O_3 and NO_x have been reported to stimulate NADPH isoform 4 (NOX4) activity, leading to activation of NADPH oxidase located in the cell membrane and increased superoxide production (Lodovici and Bigagli, 2011; Zorov et al., 2014). While consistently see increased ROS production in the vasculature and CNS with MVE-exposure, in C57BL/6 wild type and male and female $ApoE^{-/-}$ mice, further mechanistic studies are necessary to determine the source(s) of exposure-mediated ROS production.

We also analyzed the expression of female steroid hormone receptors in the cerebrum and cerebral microvasculature of our study animals. While there was a decrease in the cerebral expression of ERα mRNA in our ovariectomized groups (both FA and MVE), as well as the MVE exposed ov+ females, there was no difference in ERα expression in the cerebral microvasculature. Notably, estrogen receptor signaling is known to regulate the immune system via the activation of dendritic cells and toll-like receptor (TLR) signaling (Kovats, 2015). Moreover, down-regulation of ERα is associated with increased TNF-α inflammatory signaling and macrophage infiltration (Panchanathan et al., 2010). ERα signaling has been shown to exert protective effects in the CNS via inhibiting inflammatory cell recruitment in a MS rodent model (EAE mice) (Polanczyk et al., 2003). While MVE-exposure, and ovary removal, mediated a significant decrease in cerebral ERα mRNA levels, there was no statistical change in cerebral expression of either ERβ or PRO A/B receptors noted. As we only analyzed hormone receptor expression in the current study, further mechanistic/inhibitor studies are required to investigate the role of the female sex hormones and/or receptor-mediated signaling in the initiation of effects of inhaled environmental air pollutants on autoimmune disorders in the CNS.

Finally, in an effort to begin to understand mechanistic pathways that may be contributing to inflammatory signaling, related to either female hormone status or MVE-exposure, we quantified the cerebral expression of regulatory subunits of NF-κB signaling, including RelA, and IKKα and IKKβ, which are subunits of IκB kinase. Increased IκB kinase will activate NF-κB signaling through phosphorylation of its inhibitor, IκBα, leading to its dissociation and degradation. NF-κB subunits RelA/p65, c-Rel, RelB are activated in response to inflammation and ROS and reported to regulate expression of ICAM-1 and VCAM-1 mRNA, via alterations in regulatory IκB subunits in both MS and EAE rodent models of MS (Yue et al., 2018; Brand et al., 1996). Also, NF-κB inhibition in astrocytes inhibits inflammatory cytokine expression and reduces the severity of EAE via a degradation-resistant form of an inhibitor of nuclear factor-kappa B or IκBα (Liu et al., 2017). A previous study reported that inhaled exposure to $PM_{2.5}$ resulted in increased hypothalamic IL-6, TNF-α, and IKKβ mRNA expression, as well as increased microglial cell activation (Liu et al., 2014). Furthermore, when administered a central (intracerebral) IKKβ inhibitor during $PM_{2.5}$ exposures, inflammatory markers were diminished both locally and in the systemic circulation, indicating that alterations in IKK subunits mediate induction of NF-κB inflammatory signaling pathways (Liu et al., 2014). We also observed an increase in IKKα and IKKβ mRNA levels in the cerebrum of both the FA and MVE ov-; however, cerebral IKKβ mRNA was significantly induced in the MVE ov- compared to the FA ov- mice. Such findings indicate that loss of female steroid hormone signaling may mediate increased NF-κB-mediated inflammatory signaling in the brain, which is further exacerbated with MVE exposure. Interestingly, unlike in the MVE ov- female mice, we did not observe any statistical changes in the expression of inflammatory mediators (IL-1β, TNF-α, IL-1) in the brains of FA ov- mice, correlated with the induced IKK subunit expression. This

may be due to increased anti-inflammatory signaling pathway responses in the brains of FA ov- animals that are blunted with MVE-exposures. Further mechanistic studies are necessary to confirm whether alterations in IKKα and IKKβ mRNA expression mediate induced NF-κB endpoints in the brains of MVE-exposed animals. Summary of air pollution and sex hormones effects on pathophysiology associated with MS represented in Fig. 4.1.

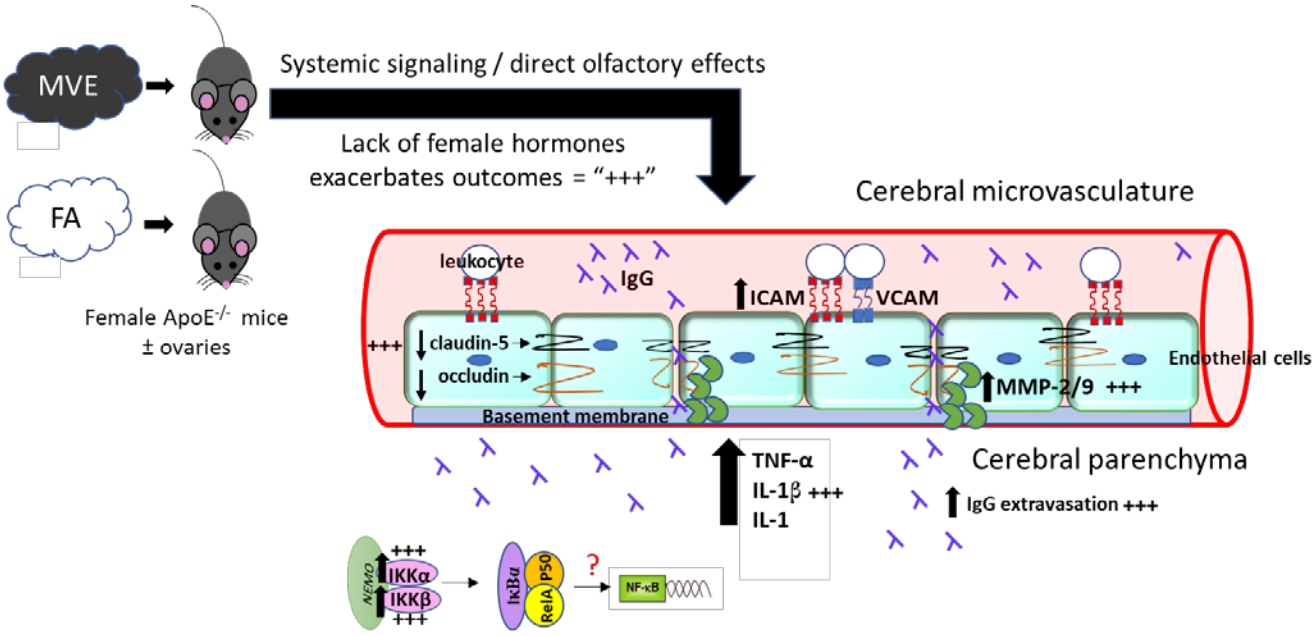

Figure 4.1: **Schematic results of air MVE-exposure and sex hormones on pathways associated with onset and development of MS in the brain of female ApoE$^{-/-}$ mice and the underlying mechanism.**

In confirmation of this project's hypothesis and its aims, air pollution altered molecular and signaling pathways associated with the onset and progression of MS, including demyelination, neuroinflammation, T cell activation, and BBB disruption. Also, MVE increased BBB permeability, and this study proved the IgG extravasation to brain parenchyma in response to inhaled MVE. Similarly, in support of our hypothesis that sex hormones alter some of the mechanisms associated with the pathophysiology of MS, the current research also revealed that the lack of sex hormones was associated with demyelination and altered MMP-2/9 activity, which is known to degrade TJ proteins such as occludin and claudin-5. Also, sex hormones

affect the NF-κB inflammatory signaling pathways in the CNS. The results of this project and evidence that support our hypothesis and aims are summarized and presented in Table 4.1.

Table 4.1: **Summary of the result of MVE air pollution exposure and sex hormones on pathophysiologic outcomes in the CNS associated with MS.**

Endpoints	Sex Hormones	Exposure	Interaction	Hypothesis was Supported
Demyelination	Yes ↑	Yes↑	Yes ↑	Yes
CD4/CD8 (IH)	No	Yes ↑	No	Yes, for MVE No for sex hormones
ROS (DHE)	No	Yes ↑	No	Yes, for MVE No for sex hormones
MOG (IF)	No	Yes ↑	No	Yes, for MVE No for sex hormones
Inflammatory markers (RT-qPCR)	No (only IL-1β)	Yes↑	Yes, for IL-1β ↑	Yes, for MVE No for sex hormones except IL-1β
Tight junctions (IF)	Yes (claudin-5) in ovariectomized ↓	Yes ↓	Yes ↓	Yes, for MVE No for sex hormones
Cell adhesion molecules	No	Yes (ICAM) ↑	Yes↑	Yes, for MVE No for sex hormones
IgG extravasation (IF)	Yes↑	Yes ↑	Yes↑	Yes
Ikkα/ Ikkβ (RT-qPCR)	Yes ↑	No	No	No for MVE Yes, for sex hormones
RelA (RT-qPCR)	No	No	No	No
MMP	Yes ↑	Yes ↑	Yes ↑	Yes

4.1 Conclusion

The results of this study collectively reveal that exposure to traffic-generated air pollutants (MVE) promotes neuroinflammation, T cell activation, and BBB disruption. The MVE-mediated alterations in BBB integrity and permeability were associated with increased MMP-2/9 activity and decreased TJ protein expression of claudin-5 and occludin. Additionally, we observed that the diminished presence of sex hormones in the ovariectomized mice, combined with MVE exposures and a high-fat diet, mediate pronounced inflammatory responses, BBB integrity, and demyelination in the CNS. The analysis of contributing signaling pathways

show that ROS production, along with increased expression of cerebral TNFα, IL-1β, and IL-1 mRNA in female ApoE$^{-/-}$ was directly linked to MVE-exposure. Based on the current results, the presence of ovaries/female sex hormones did not affect the expression of inflammatory markers in the CNS. Conversely, expression of the subunits of IκB, IKKα and IKKβ were elevated in ovariectomized animals FA ov- and MVE ov-, indicating that the loss of female steroid hormone signaling may regulate NF-κB-mediated signaling in the brain, thereby increasing inflammation. Additionally, NF-κB signaling is known to increase ICAM and VCAM expression; however, we observed an increase in cerebral ICAM-1 in both MVE ov+ and MVE ov-, suggesting additional factors in the cerebral microvasculature driving ICAM mRNA expression. Interestingly, in addition to decreased ERα mRNA expression in the cerebrum in ov- mice, we also observed that MVE-exposure downregulated ERα mRNA expression. Based on the current results, it is plausible that MVE-exposure is modulating estrogen signaling in the CNS of females through alterations in receptor expression. Such findings may provide insights into understanding pathways by which exposure to environmental air pollution may contribute to the etiology of CNS demyelinating disorders, such as MS. Moreover, identifying contributing signaling pathways that promote these detrimental outcomes in the CNS may provide targets for the treatment of MS, as well as prevention of MS relapses, especially in individuals who live in regions of consistently high levels of air pollution.

4.2 Future Experiments

TLR receptors are an important part of the innate immune system, which recognizes pathogens via pathogen-associated molecule patterns (PAMPs) or pattern recognition receptors (PRRs) and defend the body (Lai and Gallo, 2008). Also, TLR receptors are modulators of cytokines and chemokines and trigger inflammation associated with the pathophysiology of MS

(Gooshe et al., 2014). In addition to inflammation, TLR receptors also alter the myeloid differentiation factor 88 (MyD88) and alter the NF-κB pathway. Future studies will provide more information regarding the mechanistic pathways by which air pollution and/or sex hormones alter the expression of and signaling through the TLR receptors, as well as whether signaling through these receptor subtypes is associated with MS pathophysiology. For this aim, we can use immunofluorescence staining, RT-q PCR, and both in vivo and in vitro inhibitor studies.

In addition to TLR receptors, heat shock proteins such as Hsp70/90, which are ATP-dependent molecular chaperones involved in various cellular processes, alter protein folding and remodeling (Genest et al., 2019). Since the misfolding of heat shock proteins in the brain is associated with neurodegenerative disease (Lackie et al.,2017), a mechanistic study investigating the effects of air pollution on Hsp70/90 signaling pathways associated with MS pathology. To study the role of heat shock proteins in air pollution mediated pathology in the brain related to MS, we can use mass spectrometry or immunohistochemistry and immunofluorescence assays in EAE mice model.

Finally, in view of the fact that MS disease is sex bias disease, using sex hormone treatment or sex hormone receptor antagonist, in the EAE mice model, in combination with air pollution exposures can provide significant insight into the synergistic or antagonists actions of the exposure on sex hormone signaling in the CNS. Also, this can provide information about the fluctuation in sex hormone levels, in combination with environmental exposures, can lead to an alteration in signaling pathways associated with MS onset, and also relapses.

APPENDIX

PRIMER SEQUENCES USED FOR EVALUATION OF BRAIN REFERENCE GENES FOR RT-QPCR ENDPOINTS

Gene/Primer	Sequence (5’ – 3”)
Mouse ERα FP	TGGGCTTATTGACCAACCTAGCA
Mouse ERα RP	AGAATCTCCAGCCAGGCACAC
Mouse ERβ FP	GACTGTAGAACGGTGTGGTCATCAA
Mouse ERβ RP	CCTGTGGAGGTAGGAATGCGAAAC
Mouse PRO A/B FP	GACCACATCAGGCTCAACGAG
Mouse PRO A/B RP	AGTGCTGCCTAATGTCCC
Mouse TNF-α FP	CCCCAGTCTGTATCCTTCT
Mouse TNF-α RP	ACTGTCCCAGCATCTTGT
Mouse VCAM-1 FP	ACTTTCTATTTCACTCACACCAGCC
Mouse VCAM-1 RP	ATCTTCACAGGCATTTCAAGTCTCT
Mouse ICAM-1 FP	CCATAAAACTCAAGGGACAAGCC
Mouse ICAM-1 RP	TACCATTCTGTTCAAAAGCAGCA
Mouse IL-1 FP	GAAGAGATGTTACAGAAGCC
Mouse IL-1 RP	CATGCCTGAATAATGATCAC
Mouse IKKα FP	CCAGAACAGTACTCCATTGCCAGA
Mouse IKKα RP	TGGCATGGAAACGGATAACTGA
Mouse IKKβ FP	TGGCATGGAAACGGATAACTGA
Mouse IKKβ RP	CTGGAACTCTGTGCCTGTGGAA
Mouse MMP-9 FP	GACAGGCACTTCACCGGCTA
Mouse MMP-9 RP	CCCGACACACAGTAAGCATTC
Mouse RelA FP	TGTTGCCCACTTCAGGTTGT
Mouse RelA RP	AGTGGAAGCCCTGTCCTAGT
Mouse IL-6 FP	GGCCTTCCCTACTTCACAAG
Mouse IL-6 RP	CACTAGGTTTGCCGAGTAGATCTC
Mouse MOG FP	GCTAATTGAGACCTATTTCTC
Mouse MOG FP	AGCAATAAACAGGTGGAAGGTC
Mouse occludin FP	CTCCCATCCGAGTTTCAGGT
Mouse occludin RP	GCTGTCGCCTAAGGAAAGAG
Mouse claudin-5 FP	TTCGCCAACATTGTCGTCC
Mouse claudin-5 RP	TCTTCTTGTCGTAGTCGCCG
Mouse IL-1β FP	CCTCCTTGCCTCTGATGG
Mouse IL-1β RP	AGTGCTGCCTAATGTCCC
Mouse GAPDH FP	CATGGCCTTCCGTGTTCCTA
Mouse GAPDH RP	GCGGCAGTCAGATCCA

FP, forward primer; RP, reverse primer; IL-1β, interleukin-1 beta; TNF-α, tumor necrosis factor alpha; VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intracellular adhesion molecule-1, IKKα, inhibitor of nuclear factor kappa-B kinase subunit alpha; IKKβ, inhibitor of nuclear factor kappa-B kinase subunit beta; MMP-9, matrix metalloproteinase-9; RelA (p65), v-rel avaian reticuloendotheliosis viral oncogene homolog A; IL-6, interleukin-6. Myelin oligodendrocyte glycoprotein (MOG), GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

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