ENDOTHELIAL MINERALOCORTICOID RECEPTORS IN CEREBROVASCULAR AND COGNITIVE FUNCTION IN ANGIOTENSIN II-HYPERTENSION

By

Janice Marie Díaz-Otero

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Pharmacology and Toxicology- Doctor of Philosophy

2019

ABSTRACT

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Vascular cognitive impairment and dementia (VCID) is a growing public health issue that lacks effective treatments. VCID is described as a spectrum of cognitive deficits that have a cerebrovascular origin, meaning there is an impairment in the structure and function of small arteries and arterioles, including parenchymal arterioles (PAs) and capillaries. My studies focused on the PAs that are critical regulators of blood flow to the cerebral microcirculation. These are considered the weak link or a bottle-neck in the perfusion of the cerebral microcirculation. Importantly, occlusion of a single PA produces a microinfarct and cognitive dysfunction. Hypertension, a primary risk factor for VCID, causes artery remodeling and impaired endothelial function, which leads to impaired autoregulation of cerebral blood flow. The mechanisms by which artery remodeling and impaired autoregulation occur with hypertension remain unknown. Elevated aldosterone levels and mineralocorticoid receptor (MR) activation have been linked to vascular damage in hypertension. In rat models of hypertension, MR antagonism prevents hypertensive artery remodeling, but the signaling mechanisms and the specific cell types involved in the process have not been identified because MR antagonists inhibit the actions of aldosterone in all cell types in the arteries. Endothelial MR signaling in peripheral arteries plays a critical role in endothelial dysfunction. The endothelial MRs regulate vasodilation in peripheral arteries through calcium-activated potassium (K_{Ca}) channels, in particular, small conductance (SK_{Ca}) and intermediate conductance (IK_{Ca})

channels. These channels can be activated by calcium influx through transient receptor potential (TRP) channels, such as the V4, and we recently showed that TRPV4 channels mediated PA endothelium-dependent dilation. The studies in the peripheral vasculature suggest that the effects of MR activation are vascular bed-specific and that we need to study different vascular beds separately. My studies tested the hypothesis that in hypertension endothelial MR activation will result in PA inward remodeling, impaired TRPV4-mediated dilation, and reduced cerebral blood flow. Furthermore, I proposed that the cerebrovascular dysfunction caused by endothelial MR activation would result in cognitive impairment. I addressed these hypotheses using a pharmacological and genetic approach. My studies show that MR signaling at the level of the endothelium mediates PA inward hypotrophic remodeling and impaired endothelium-dependent dilation. I also show that MR signaling mediates cognitive dysfunction in hypertension, but this was not specific to endothelial MR signaling. My studies also show that TRPV4 channels are important regulators of cognitive function. My studies could have wide reaching implications because identification of the cell specific actions of MR signaling could allow us to better define the downstream mechanisms of MR-mediated changes in hypertension. This could contribute to the development of better therapeutic approaches to improve cerebrovascular health in hypertensive patients, a rapidly growing concern in our aging population.

This thesis is dedicated to my Mom,
you are my inspiration in life.
Thank you for your unconditional love and support.

ACKNOWLEDGEMENTS

My Ph.D. would not have been possible without the support and guidance of many people. I will always be in debt to you all.

First and foremost, my mentor Dr. Anne M. Dorrance. Thanks for taking a chance with me and for guiding me into becoming my own scientist. Your passion for science and your patience, empathy, and knowledge have inspired me every single day for the last five years. The way you care about every student and our research made me enjoy coming into lab every day. I can only hope that one day I can be as amazing a scientist and person as you are.

Thanks to my co-mentor, Dr. William F. Jackson, for always challenging me to be critical in the way I think about my science. Thanks for your endless patience, for always cheering me up and for getting excited about the research we do. You are a great role model and I could not have achieved any of this without your support and guidance.

To my committee members Drs. Gregory Fink and Richard Mortensen for your time, guidance and invaluable inputs.

Thanks to every member of the lab because they have made all these years a lot of fun. A special mention goes to my wonderful undergraduate, Jessica Yen, because of her responsibility and enthusiasm toward our projects. I also want to thank the faculty and administrative personnel of the Department of Pharmacology and Toxicology. Your help, dedication, and hard work are appreciated.

I could not have achieved this without the support of my family and friends. Thanks to my parents because you taught me to always follow my dreams. To my grandparents

for the unconditional love and always guiding me to be the best version of myself. To the loves of my life, my siblings Rafael and Jesús Andrés, and cousins Diemi, Hewill, and WilGabriel, because you are the reason I never give up. To my cousins, aunts and uncles because you taught me that no distance is too far, and no time is too long. Even in the distance you always made sure I felt loved and supported.

I also want to thank my best friends and soul sisters Drs. Viviana Vidal Anaya and María Alejandra Bernard Flores for their unconditional love and support. A special mention goes to the most amazing friend I could have ever asked for, Dr. Fernando Javier Del Valle, because you always believed in me even when I stopped believing in myself. Your patience, love and support were truly the glue that kept the pieces together all these years. To my friends, Naiomy Ríos, Neldyn Rosado, and Patricia Perez for all the fun times and your support.

Lastly, but not least, to my guardian angels my daddy Rafael Díaz and cousin Wilfredo David Otero because I know that even if we are no longer physically together you are always by my side.

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KEY TO ABBREVIATIONS

11β-HSD2	11 Beta Hydroxysteroid Dehydrogenase Type 2
4-HNE	4-hydroxynonenal
4α-PDD	4-alpha-phorbol 12,13-didecanoate
AA	Arachidonic Acid
ACE	Angiotensin Converting Enzyme
aCSF	artificial Cerebrospinal Fluid
ACTH	Adrenocorticotropic Hormone
AT_1R	Angiotensin Type 1 Receptor
AngII	Angiotensin II
BBB	Blood Brain Barrier
BCAO	Bilateral Common Carotid Artery Occlusion
BCAS	Bilateral Common Carotid Artery Stenosis
BDNF	Brain Derived Neurotrophic Factor
BK_{Ca}	Big Conductance Calcium-activated Potassium Channels
Ca^{2+}	Calcium
CADASIL	Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy
cAMP	Cyclic Adenosine Monophosphate
CASL	Continuous Arterial Spin Labeling
CCh	Carbachol
cGMP	Cyclic Guanosine Monophosphate

CICR	Calcium Induced Calcium Release
Col	Collagen
COOH	Carboxyl
COX	Cyclooxygenase
cSVD	Cerebral Small Vessel Disease
CYP2C9	Cytochrome P450 2C9
DAG	Diglycerol
DOCA	Deoxycorticosterone
EC-MR	Endothelial Cell Mineralocorticoid Receptor
ECMRKO	Endothelial Cell Mineralocorticoid Receptor Knockout
EDH	Endothelial-derived Hyperpolarization
EDHF	Endothelial-derived Hyperpolarization Factor
EETs	Epoxyeicosatrienoic Acids
EGTA	Ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic Adic
eNaC	Epithelial Sodium Channel
eNOS	Endothelial Nitric Oxide Synthase
EPHESUS	Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study
EPL	Eplerenone
GFAP	Glial Fibrillary Acidic Protein
GPER	G protein-coupled Estrogen Receptor
GR	Glucocorticoid Receptor
HRE	Hormone Response Element

HSP	Heat Shock Protein
HZ	Hertz
ICAM-1	Intracellular Adhesion Molecule-1
IK_{Ca}	Intermediate Conductance Calcium-activated Potassium Channel
IL	Interleukin
IM	Intramuscular
iNOS	Inducible Nitric Oxide Synthase
IP_3	Inositol trisphosphate
K^+	Potassium
K_{Ca}	Calcium-activated Potassium Channel
KCl	Potassium Chloride
KH_2PO_4	Monopotassium Phosphate
K_{ir}	Inward Rectifier Potassium Channel
L-NAME	Nitro-L-arginine Methyl Ester
MAP	Mean Arterial Pressure
MAPK	Mitogen Activated Protein Kinase
MCA	Middle Cerebral Artery
MCP	Monocyte Chemoattractant Protein
MEJ	Myendothelial Gap Junction
MEP	Myoenothelial Gap Projection
$MgSO_4 \cdot 7H_2O$	Magnesium Sulfate
Mg^{2+}	Magnesium

mmHg	Millimeters of Mercury
MMP	Matrix Metalloproteinases
MR	Mineralocorticoid Receptor
mV	Millivolt
Na^+	Sodium
NaCl	Sodium Chloride
NH_2	Amine
nNOS	Neuronal Nitric Oxide Synthase
NO	Nitric Oxide
NORT	Novel Object Recognition Test
NOS	Nitric Oxide Synthase
NOX	NADPH Oxidase
NR3C2	Nuclear Receptor Subfamily 3 Group C Member 2
NTS	Nucleus Tractus Solitarius
NVC	Neurovascular Coupling
NVU	Neurovascular Unit
PAs	Parenchymal Arterioles
PCA	Posterior Cerebral Artery
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
PGI_2	Prostaglandin I_2
PKA	Protein Kinase A
PKC	Protein Kinase C

PKG	Protein Kinase G
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLCγ1	Phospholipase C isoform γ1
PSS	Physiological Salt Solution
PVM	Perivascular Macrophage
RAAS	Renin Angiotensin Aldosterone System
RALES	Randomized Aldactone Evaluation Study
RANTES	Regulated on Activation, Normal T cell Expressed and Secreted
ROS	Reactive Oxygen Species
SHR	Spontaneously Hypertensive Rat
SHRSP	Stroke-prone Spontaneously Hypertensive Rat
SK_{Ca}	Small Conductance Calcium-activated Potassium Channel
SMC	Smooth Muscle Cell
SNP	Sodium Nitroprusside
SOCE	Store-operated Calcium Channel
SPIR	Spironolactone
SQ	Subcutaneous
SR	Sarcoplasmic Reticulum
SYP	Synaptophysin
TGFβ	Transforming Growth Factor Beta
Th	T helper cell

TNFα	Tumor Necrosis Factor Alpha
Tp	Thromboxane Receptor
TRP	Transient Receptor Potential
TRPA	Transient Receptor Potential Ankyrin
TRPA1	Transient Receptor Potential Ankyrin 1
TRPC	Transient Receptor Potential Canonical
TRPM	Transient Receptor Potential Melastatin
TRPML	Transient Receptor Potential Mucolipin
TRPP	Transient Receptor Potential Polycystic
TRPV	Transient Receptor Potential Vanilloid
TRPV4	Transient Receptor Potential Vanilloid 4
UCAO	Unilateral Common Carotid Artery Occlusion
UTP	Uridine Triphosphate
VCAM-1	Vascular Cellular Adhesion Molecule 1
VCID	Vascular Cognitive Impairment and Dementia
WKY	Wystar Kyoto
WKY-*Trpv4*^{em4Mcwi}	Transient Receptor Potential Vanilloid 4 Knockout

CHAPTER 1

The Cerebral Vasculature in Health and Chronic Hypertension: Focus on Endothelial Mineralocorticoid Receptors and Parenchymal Arterioles

1. Overview

The brain is a highly perfused organ that accounts for only 2% of the body weight but receives 15-20% of the cardiac output. The brain consumes a large amount of energy, but it lacks energy stores. Thus, cerebral blood flow is tightly regulated to provide the oxygen, glucose and all other energy requirements needed to maintain brain activity. Interruptions in cerebral perfusion, even if acute, result in neuronal death and brain damage (111). Cerebral blood flow is tightly regulated by myogenic tone, endothelium-dependent dilation and neurovascular coupling (111).

The arterioles in the peripheral and cerebral vasculature have similar structures in that they all contain smooth muscle and endothelial cells. However, the cerebral vasculature should be studied as an independent entity because the endothelial cells in the cerebral arteries are highly specialized; they lack fenestrations and have tight junctions (28). The cerebral endothelium provides the structural basis of the blood brain barrier (BBB). The cerebral arteries are different in that they do not have an external elastic lamina but instead have a well-developed internal elastic lamina (142). Also, a paucity of elastic fibers in the medial layer and a thin adventitia (28). In this chapter I will discuss the cerebral vascular anatomy and its physiology. I will describe how in hypertension endothelial mineralocorticoid receptor (MR) signaling mediates hypertensive artery remodeling and impaired endothelium-dependent dilation. I will also discuss the role for transient receptor potential (TRP) channels in the hypertension-associated changes in vascular dysfunction. In addition, I will discuss how the

cerebrovascular dysfunction associated with hypertension increases the risk for cognitive decline and the possible mechanisms by which this occurs.

1.1 – Anatomy of the cerebral circulation

1.1.1 – Extracranial arteries

Blood flow to the brain is supplied by two pairs of large extracranial arteries: the right and left internal carotid arteries and the right and left vertebral arteries (142). The internal carotid arteries originate at the bifurcation of the left and right common carotid arteries; they provide 80% of the total perfusion to the cerebrum (208). The vertebral arteries arise from subclavian arteries, run along the spinal cord and converge at the base of the brainstem to form the basilar artery; they provide 20% of the total cerebral perfusion and are an important source of brain stem perfusion (208). At the base of the cerebrum the basilar artery, the internal carotid arteries and other communicating arteries join the arteries to form an anastomotic ring known as the circle of Willis (142). This structure provides collateral blood flow when one of the major arteries becomes occluded, this minimizes ischemic damage (28).

1.1.2 – Circle of Willis and intracranial arteries

The Circle of Willis is composed of the posterior communicating arteries, posterior cerebral arteries (PCA), internal carotid arteries, anterior cerebral arteries and the anterior communicating artery (Figure 1.1). The middle cerebral arteries (MCA) are not considered part of the circle of Willis, but they instead branch off of it. These main arteries divide into smaller pial arterioles that run along the surface of the brain and provide blood flow to the cerebral cortex. The anterior cerebral arteries have cortical and central branches to supply the anteromedial regions of the brain (142). The central branches provide blood

support to the corpus callosum, caudate nucleus, anterior putamen and septum pellucidum (208). The cortical branches supply regions that include the frontal lobe, olfactory lobe, medial orbital gyrus, straight rectus, medial frontal gyrus, cingulate gyrus, and paracentral lobule (208). These regions are involved in motor control of the legs, memory, insight, mood, judgment and emotions (15). The MCAs and their branches supply the largest region including 5 regions of the temporal lobe, angular, anterior, and posterior, parietal, central, precentral, prefrontal, and orbitofrontal cortex (208). These regions are involved in motor control, hearing, speech, writing, understanding, mood and judgment (16). Lastly, the PCAs and their branches supply regions of the hippocampus, the occipital and temporal lobes (106, 208). These regions are associated with sensory information processing and memory (106).

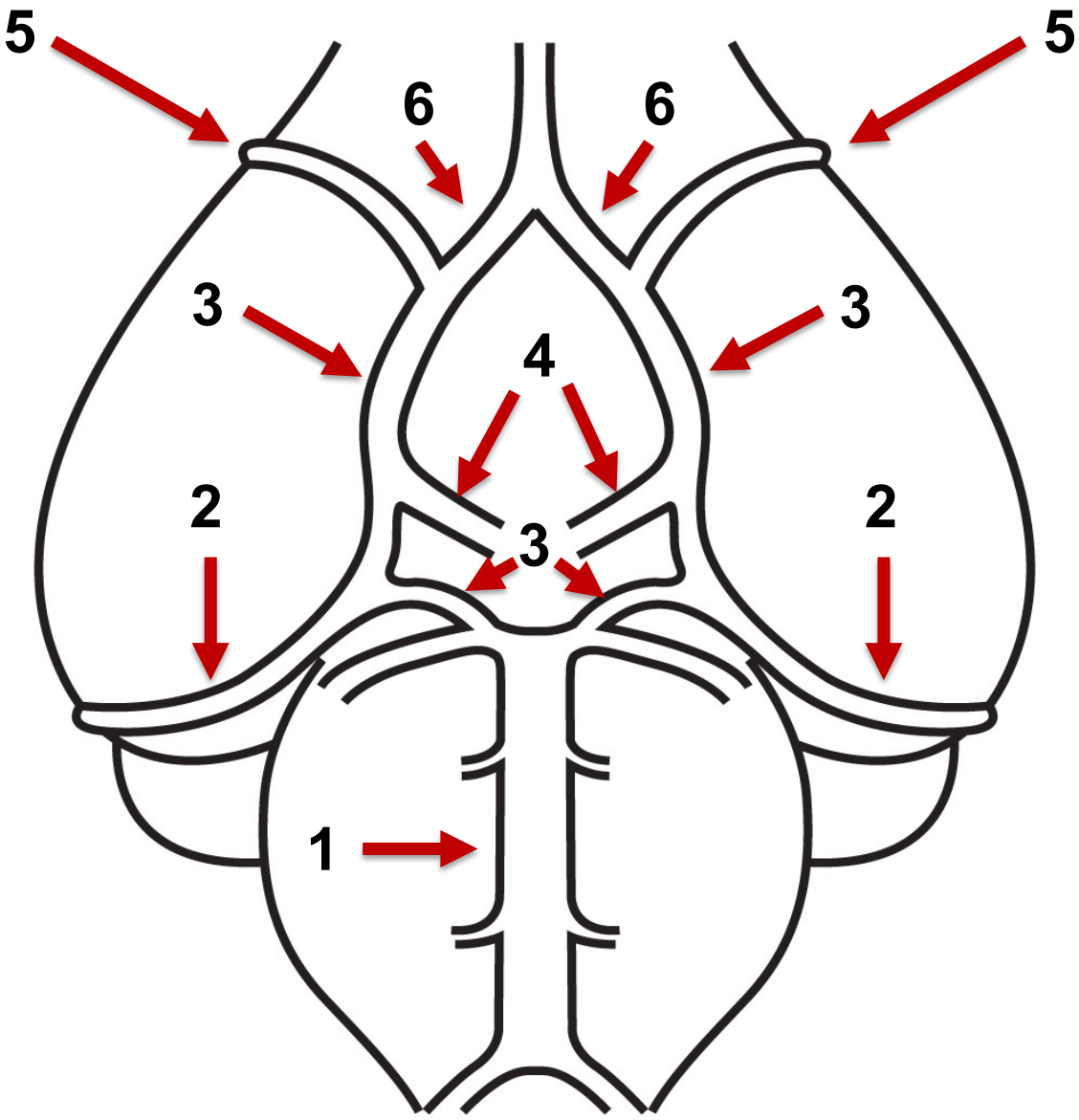

Figure 1.1 Major arteries and the circle of Willis in rats and mice. 1. Basilar artery; 2. Posterior cerebral arteries; 3. Posterior communicating arteries; 4. Internal carotid arteries; 5. Middle cerebral arteries; 6. Anterior cerebral arteries. Figure courtesy of Daniel Bollman.

1.1.3 – Pial arteries

Pial arteries are on the surface of the brain within the pia-arachnoid and they contribute to cerebrovascular resistance. These arteries continue to branch into smaller arteries and arterioles giving rise to a high number of anastomoses, or connections, between vessels. These anastomoses connect the anterior to the middle circulation or the middle to the posterior circulation and provide collateral blood flow in situations when perfusion is reduced because an artery is occluded. The pial arteries and arterioles receive innervation from the peripheral nervous system also known as extrinsic innervation.

1.1.4 – Parenchymal arterioles

The pial arteries continue to branch into penetrating arterioles that are located in the Virchow-Robin space almost perpendicular to the pial arteries. As the penetrating arterioles continue to dive further into the brain parenchyma they turn into parenchymal arterioles (PAs) (Figure 1.2). These arterioles provide blood flow to discrete regions of the cortex and the microcirculation. Both pial arterioles and PAs have endothelial cells but PAs are different in that they only have one layer of smooth muscle cells (SMC). There are other differences between the pial arteries and PAs. The PAs lack extrinsic innervation from the peripheral nervous system, instead receive intrinsic innervation from the neuropil (29) and are mostly surrounded by astrocytic endfeet (111). PAs are not connected to each other by collateral vessels thus, they are considered the weak link in perfusion, in fact, occlusion of one PA results in a microinfarct and cognitive decline (180). There are also some functional differences between the two vessel types: PAs have greater basal myogenic tone and this tone is regulated by endothelium-derived

hyperpolarization factors (EDHF) in addition to nitric oxide (NO). These arterioles do not contract in response to neurotransmitters like norepinephrine (29). They are also important regulators of neurovascular coupling (NVC). This is the process by which cerebral blood flow is regulated and matched to the metabolic demands of the brain (74). This thesis will be focused on understanding the mechanisms by which hypertension alters the function and structure of PAs.

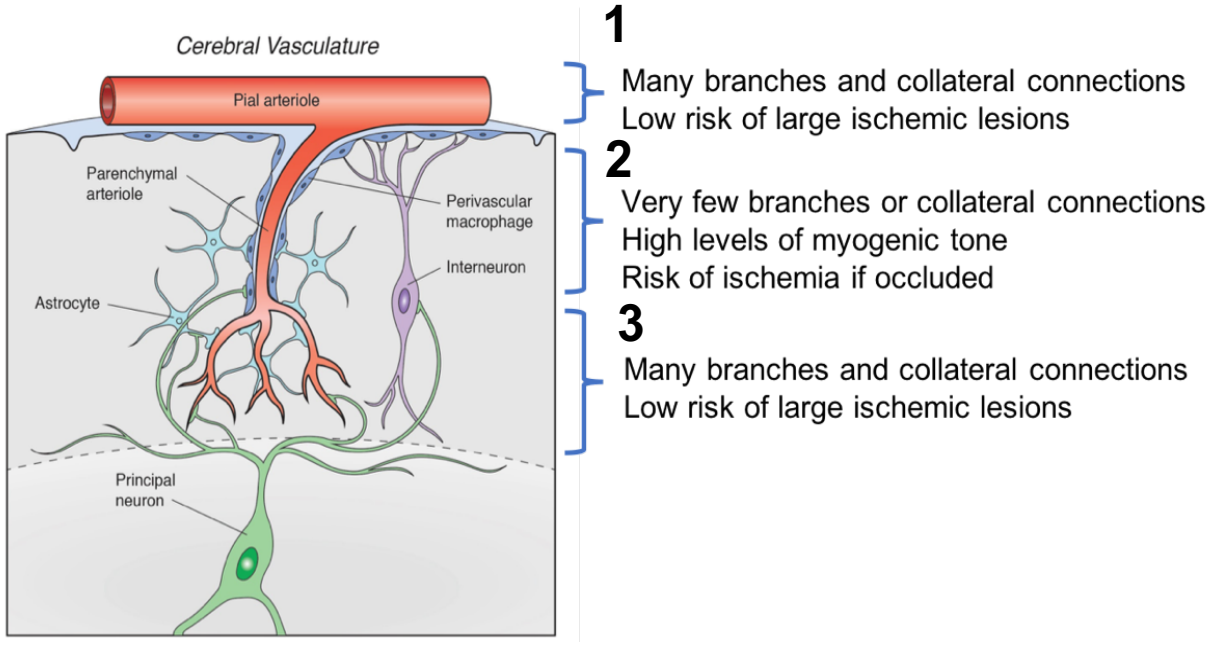

Figure 1.2 Schematic of the cerebrovascular tree. The cerebrovascular tree can be divided in three levels: level 1. The pial arteries and arterioles cover the surface of the brain. These are connected to each other by many collateral arteries/arterioles, this lowers the risk for ischemia; level 2. The parenchymal arterioles dive into the brain parenchyma to perfuse the microcirculation. These arterioles are not connected to each other and thus are considered the weak link in or a bottleneck in perfusion. The risk for ischemic damage is higher at this level; level 3. The capillaries branch off the parenchymal arterioles and have many branches and collateral connections. The risk for ischemic damage is lower at this level.

1.1.5 – Capillaries

The brain has an extensive network of capillaries that are less than 10μm in diameter and perfuse the microcirculation. The capillaries are structurally different from upstream arteries and arterioles because they are composed of endothelial cells but lack SMCs. Capillaries are mostly surrounded by pericytes. The density of capillaries varies depending on the metabolic demands of the brain with higher density in the grey matter compared to the white matter (128). Approximately 90% of the capillary network is continuously perfused to supply the metabolic demands of the brain (84). Capillaries are connected to parenchymal venules that then return blood to the central sinus in the cortex.

1.1.6 – Veins

There is a significant gap in our understanding of the physiology and pathophysiology of the cerebral venous system when compared the cerebral arterial system. The focus of this thesis is on the arterial side of the cerebral circulation therefore the discussion of veins will be limited. The venous system is composed of dural sinuses and cerebral veins that are divided into superficial cortical veins and central veins (221). The superficial cortical veins drain the blood from the cortex and subcortical white matter and the central veins from the deep white and gray matter (162).

1.2 – Physiology of the cerebral arteries and arterioles

Maintenance of constant blood flow to the brain and the ability to match metabolic demands to perfusion are controlled by cerebrovascular autoregulation and vascular tone. In this section I will discuss the mechanisms responsible for cerebral artery autoregulation, myogenic tone, endothelium-dependent cerebral artery dilation and the regulation of cerebral blood flow.

1.2.1 – Cerebrovascular autoregulation and myogenic tone

Myogenic tone is an intrinsic property of the arteries to maintain an active contractile force in the SMCs of the wall, whereas myogenic reactivity is the ability of the artery to change tone in response to fluctuations in intralumenal pressure. Cerebrovascular autoregulation is a process by which the brain maintains constant cerebral blood flow in the face of fluctuations in pressure (12, 182). This process is also called the Bayliss effect and is a consequence of the ability of the arteries to reduce their lumen diameter in response to increases in pressure; this keeps blood flow constant (12, 194). In healthy adults, blood flow remains constant over a range of blood pressures; this is known as the autoregulatory range and it is typically from 50mmHg to 150mmHg (192); at either end of the range cerebral blood flow is directly proportional to blood pressure. Pressures below the autoregulatory range will result in artery collapse and ischemia. However, pressures above the autoregulatory range lead to force-mediated dilation and edema formation (Figure 1.3). Recent studies in humans suggest a role for sympathetic vasoconstriction (101) and cholinergic vasodilation (102) in the control of cerebral autoregulation. Blood flow itself also plays a role in the regulation; it can induce contraction or dilation in the cerebral arteries dependent on the segment of the cerebrovascular tree studied (135). At the basic level, autoregulation is controlled by myogenic reactivity and at higher pressures this is particularly important because the arteries have to constrict to prevent increased flow and damage to downstream capillaries (12, 182).

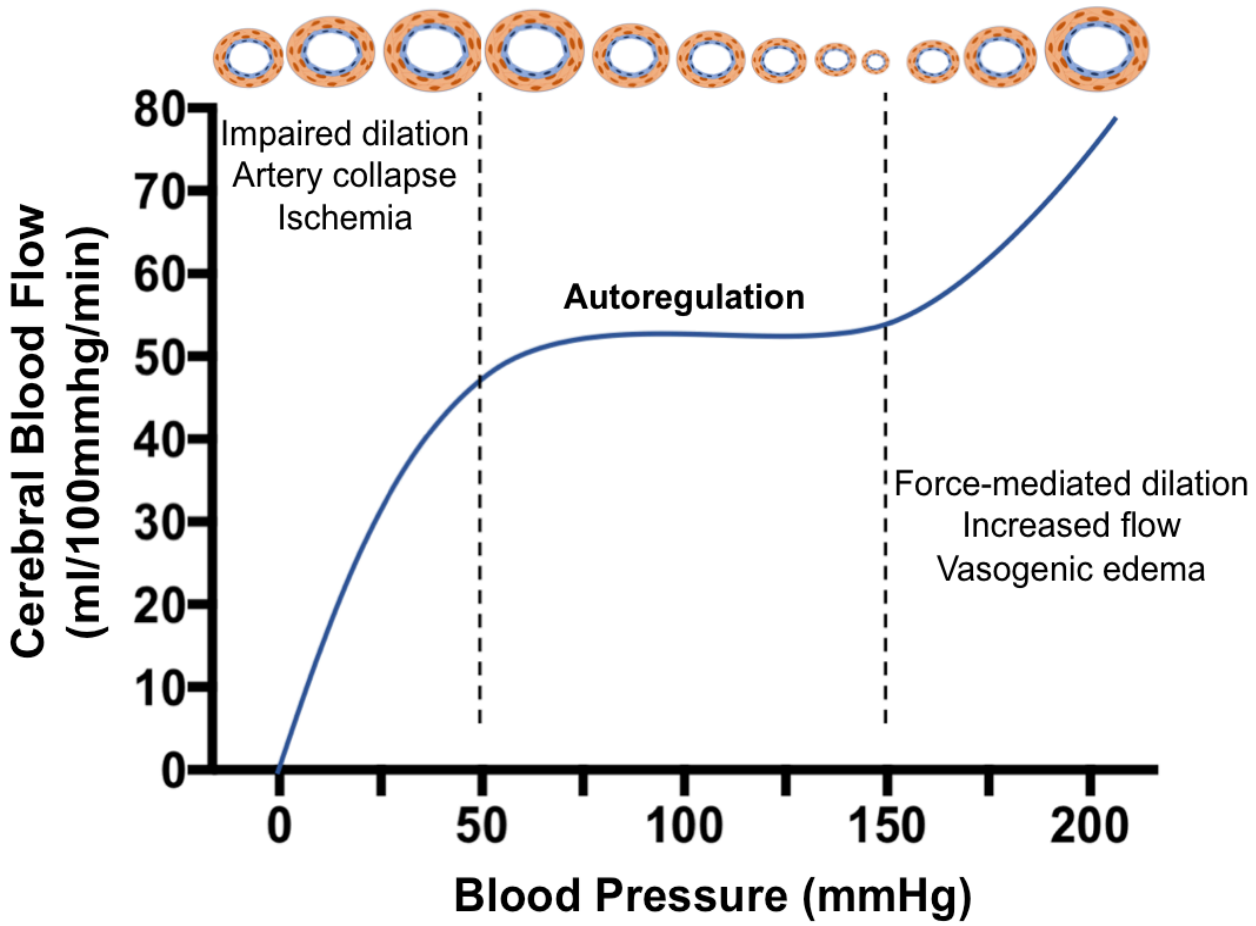

Figure 1.3 Autoregulation in cerebral arteries. Cerebral perfusion is maintained constant between 50mmHg and 150mmHg due to myogenic reactivity in the cerebral arteries. Intralumenal pressures below the autoregulatory range result in reduced blood flow because of impaired dilation, artery collapse and ischemia, whereas pressures above the range lead to force-mediated dilation, increased flow and vasogenic edema.

The generation of myogenic tone is regulated by several factors including intraluminal pressure, calcium (Ca^{2+}) channel activity, resting potassium (K^+) conductance, and the sensitivity of the contractile signaling pathways to Ca^{2+}. Increased pressure leads to SMC depolarization due to activation of TRP channels and this activates L-type voltage-gated Ca^{2+} channels such as the $Ca_v1.2$ (28). The increased intracellular Ca^{2+} increases myosin light-chain (MLC) phosphorylation and promotes constriction of the arteries (28). Numerous studies have shown a role of phospholipase C (PLC) in the generation of myogenic tone (119, 184, 190, 245). Activation of the $\gamma1$ isoform of PLC (PLC$\gamma1$) is the pressure sensor of myogenic constriction in the cerebral arteries. Inositol triphosphate (IP_3) generated by PLC$\gamma1$ in response to pressure, sensitizes IP_3 receptors to Ca^{2+} influx mediated by the TRPC6 channel, synergistically increasing IP_3 receptor-mediated Ca^{2+} release to activate TRPM4 channels, leading to SMC depolarization and constriction of the cerebral arteries (92). IP_3 receptor and protein kinase C (PKC) activation by diglycerol (DAG) have been proposed to contribute to myogenic tone in cerebral arteries (62, 91, 183). A recent study also shows that angiotensin type 1 receptor (AT_1R) is the primary sensor of intraluminal pressure in PCAs (198). Other studies also show that chloride channels contribute to myogenic tone in rat cerebral arteries (176). During tone initiation there is little change in SMC Ca^{2+}, but Ca^{2+} sensitivity is increased during myogenic reactivity (28). Some of the mechanisms implicated in this increased Ca^{2+} sensitivity include activation of PKC and RhoA-Rho kinase pathways (28).

A negative feedback mechanism to control myogenic vasoconstriction involves the activation of large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) and voltage-dependent K^+ channels (129). Voltage-dependent K^+ channels have been found in the SMC of cerebral arteries (129). BK_{Ca} are expressed in cerebral artery SMC and when activated by intracellular Ca^{2+} they cause SMC hyperpolarization and attenuate myogenic tone (116). In recent years, several studies have also suggested that TRP channels are also critical regulators of tone in cerebral arteries (58). The role of TRP channels in SMC and tone generation will be discussed in section 1.8. The myogenic process is initiated by the vascular SMCs, but the endothelium can regulate tone by releasing factors such as NO (68), and prostacyclin (154) and by causing endothelium-derived hyperpolarization (EDH) (93). The exact mechanisms that are responsible for basal tone and cerebrovascular resistance are dependent on the artery studied. The regulation of myogenic tone in the PAs is in part regulated by voltage-gated Ca^{2+} channels. Studies in isolated PAs show that increasing the intraluminal pressure to 40mmHg results in SMC depolarization from -60mV to -35mV leading to Ca^{2+} influx via L-type voltage gated- Ca^{2+} channels (177) and eventually the generation of about 40% myogenic tone. PAs generate more tone than pial arteries and this might be due to the lack of negative feedback from BK_{Ca}-mediated hyperpolarization under resting conditions (37). Vascular tone can also be enhanced by the constrictor agents Angiotensin II (AngII) or endothelin-1 (194).

1.2.2 – Endothelial regulation of cerebral artery dilation

The endothelium is the common component in all vascular structures from the large conductance arteries to the smallest capillaries. Endothelial cells in the brain form a continuous layer linked together by connexins and tight junction proteins including

claudins. Healthy endothelial cells are required for the proper functioning of brain activity. The endothelium can be considered as the master regulator of vasodilation because it controls many of the signaling pathways that regulate SMC contractility and thus cerebral blood flow. The endothelial cells also play an increasingly well recognized role in NVC through the production of vasoactive factors such as NO, EDHF and prostaglandins (Figure 1.4).

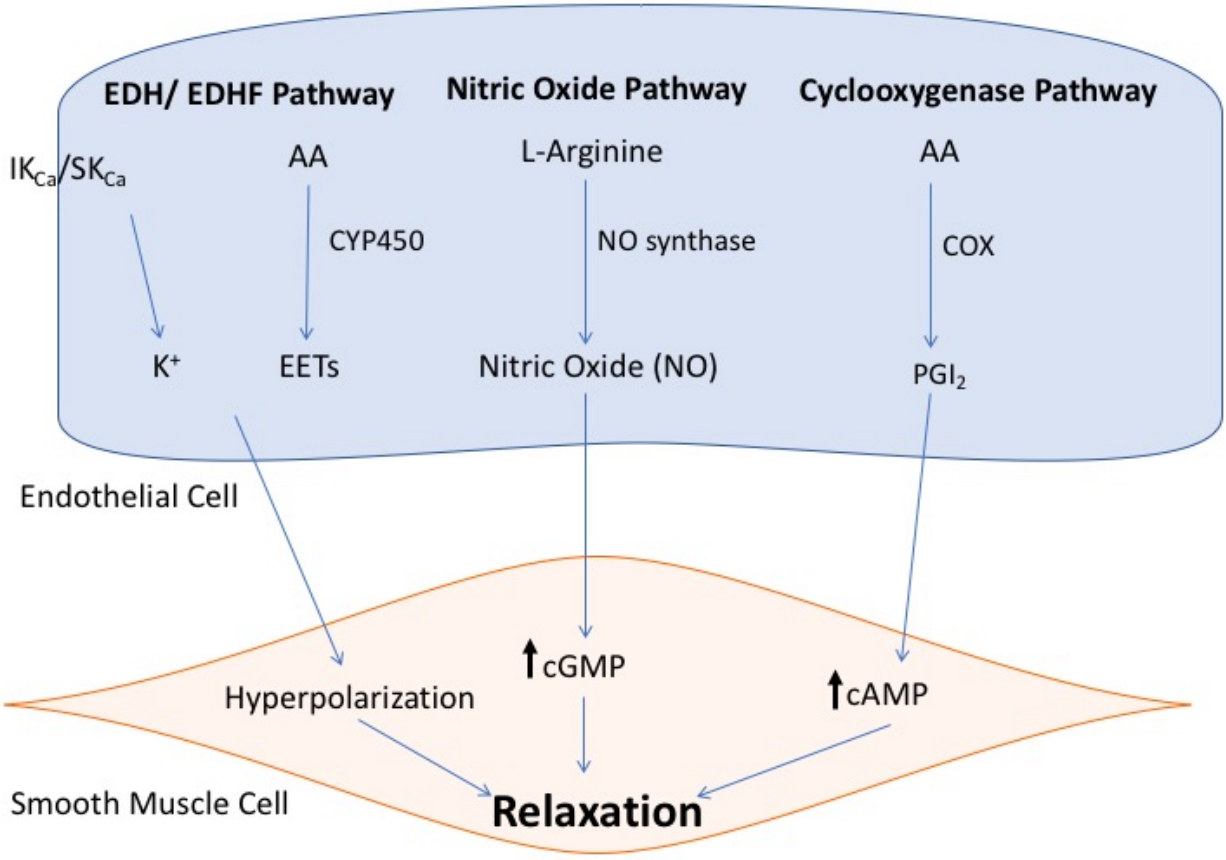

Figure 1.4 Vasodilation pathways. There are several mechanisms that mediate cerebral artery-endothelium-dependent dilation including EDH/EDHF, NO and COX pathways.

1.2.2.1 – Nitric Oxide

NO is the most widely studied vasodilator in the cerebral vasculature and it is also an important regulator of basal myogenic tone in pial arteries (68). NO is produced by the enzyme NO synthase (NOS) that converts L-arginine to L-citrulline in the presence of the co-factor tetrahydrobiopterin (167). There are three major isoforms of NOS: endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS); eNOS is the most important in the cerebral vasculature (131). eNOS regulates vascular tone but some studies suggest that nNOS may also be expressed in SMCs and play a role in the regulation of tone (247). NO, produced in the endothelium by eNOS, diffuses to the SMCs where it binds to soluble guanylate cyclase stimulating the production of cyclic guanine monophosphate (cGMP) (114). This then activates protein kinase G (PKG), leading to the opening of K^+ cannels, hyperpolarization and dilation (216). The production of NO can also stimulate voltage-gated K^+ channels and Ca^{2+}-activated K^+ (K_{Ca}) channels through soluble guanylate cyclase independent-mechanisms (149). NO can also activate TRP channels (272).

The physiological importance of NO has been highlighted by studies showing that it regulates blood pressure and cerebrovascular function (210). It is important to note that the effect of NO in cerebrovascular function is dependent on the location in the cerebrovascular tree of the arteries being studied (66, 82), and sometimes it is difficult to define the effects of blood pressure from the direct effects of NO in the vasculature. NO released from the endothelium can mediate endothelium-dependent dilation in pial arteries and arterioles (139). NO has beneficial effects beyond dilation, because it modulates platelet aggregation, inflammation and cell proliferation. Pathological states

such as hypertension limit eNOS activity and reduce NO bioavailability (2, 171, 266). The reduction in NO leads to increased reactive oxygen species (ROS) generation and oxidative stress (2). It also results in endothelial dysfunction and reduced perfusion after ischemia (109). Recent studies suggest that inhaled NO is a promising therapeutic agent for cerebral ischemia by targeting the collateral circulation to produce dilation (227). The role of NO in PA endothelium-dependent dilation is more controversial with some studies showing that EDH plays a major role in the dilation of these arterioles (45, 48).

1.2.2.2 – Prostaglandins

Cyclooxygenase (COX) breaks down arachidonic acid (AA) to produce vasodilator substances such as prostaglandin I_2 (PGI_2) or prostacyclin, prostaglandin E_2 (PGE_2), and prostaglandin D_2 (PGD_2). There are three COX isoforms, COX1-3, that are all expressed in cerebral arteries (127). Prostaglandins increase cyclic adenosine monophosphate (cAMP) levels and protein kinase A (PKA) activity in SMCs. The activation of PKA leads to opening of K^+ channels to induce hyperpolarization, activates sarco/endoplasmic reticulum Ca^{2+}-ATPase to reduce intracellular Ca^{2+} concentration, leads to closure of voltage-gated Ca^{2+} channels and artery relaxation (191). Very interestingly, PGE_2 constricts PAs from rats and mice through activation E-prostanoid receptors on SMCs (36), whereas PGI_2 release from the endothelium in response to muscarinic receptor activation elicits PA dilation through a G_s-coupled prostanoid receptor on SMCs (230).

1.2.2.3 – Endothelium-derived hyperpolarization/endothelium-derived hyperpolarization factors

Numerous studies have shown that there is clearly a dilation that is not NO or COX dependent; this is EDHF/EDH-mediated dilation (19, 96, 99, 118, 156). In EDHF dilation there is a clear signaling molecule that is released from the endothelium that causes SMC relaxation in arteries, in the presence of inhibitors of COX and NOS. Some EDHFs include hydrogen peroxide and epoxyeicosatrienoic acids (EETs) and these can cause maximal artery dilation (273) but it is possible that more EDHFs remain to be identified (118). One or more EDHFs may exist in the same artery and the exact contribution of an EDHF to vasodilation may vary. Shear stress and activation of various endothelial receptors such as the muscarinic receptors cause the formation of EDHFs (24, 76). EDH is the vasodilation that occurs as a result of the activation of K^+ channels in the endothelium causing it to hyperpolarize; this hyperpolarization is conducted to the SMC via the myoendothelial gap junction and causes hyperpolarization of the SMCs cells and dilation (85). It is becoming clearer, especially in the smaller vessels, that EDH-mediated dilation can occur even in the absence of an EDHF signaling molecule, by the activation of the K_{Ca} channels in the endothelium (79).

As mentioned, EDH-mediated dilation of cerebral arteries and arterioles is regulated by the activation of K_{Ca} channels, particularly intermediate (IK_{Ca}) and small (SK_{Ca}) conductance channels, expressed in endothelial cells. Opening of IK_{Ca}/SK_{Ca} channels hyperpolarizes the endothelium and this hyperpolarization then travels to SMCs through myoendothelial gap junctions to hyperpolarize the smooth muscle and cause relaxation by deactivating voltage-gated Ca^{2+} channels (249). IK_{Ca}/SK_{Ca} can be activated by Ca^{2+}

influx through TRP channels such as TRP vanilloid 4 (TRPV4) and increases in intracellular Ca^{2+} through IP_3 receptors. The role of TRP channels in the vasculature will be discussed in more detail in section 1.5.

The physiological role of EDHFs in the maintenance of cerebral blood flow varies along the cerebrovascular tree. EDH is an important mediator of endothelium-dependent dilation in large cerebral arteries (273) but it does not appear to regulate basal tone in the MCA because inhibition of IK_{Ca}/SK_{Ca} does not change myogenic tone (30). However, dilation in the PAs appears to be mediated mostly by EDH (274) and inhibition of IK_{Ca}/SK_{Ca} increases basal myogenic tone (30). The neurovascular unit (NVU) regulates cerebral perfusion and astrocytes are key cells in this process. It is possible that astrocytes control perfusion through activation of EDHF pathways. Astrocytes produce epoxygenase metabolites of AA that can cause dilation in the arteries (103). Other mechanism may also include K^+ channels. Studies by Longden *et al.* show that in capillary endothelial cells extracellular K^+ , a byproduct of neural activity, activates the inward rectifier K^+ (K_{ir}) 2.1 channel to produce hyperpolarization that results in upstream PA dilation and increased blood flow in the capillary bed (150). The studies previously mentioned suggest that the exact contribution of EDHF to endothelium-dependent dilation varies between the vascular bed, experimental models and pathological conditions.

1.2.3 The neurovascular unit and neurovascular coupling

In the 1960-70s it was recognized that brain activity increases blood flow specifically to the activated area, and it was not until recent years that the concept of the NVU was developed (111). The NVU is composed of astrocytes, glia, neurons and vascular cells (Figure 1.5); these cells work together to control NVC (17, 55). NVC, or functional

hyperemia, links the metabolic demands of the neurons to cerebral blood flow by integrating signaling between all the cells in the unit. The composition of the NVU varies depending on the location of the artery in the cerebrovascular tree (111). At the level of the PAs, the NVU is composed of the vascular cells, astrocytes, glia, neurons and pericytes. At the level of the capillaries, the NVU does not contain SMC, but the capillaries are mostly surrounded by pericytes. Active neurons and astrocytes release substances that regulate dilation of the arterioles and capillaries (112). This process allows for delivery of sufficient of nutrients and the removal of waste from the active brain regions. Some of the signaling molecules involved in the process include K^+ ions, neurotransmitters such as GABA and acetylcholine, NO, adenosine, prostanoids and AA metabolites (44). If blood flow is to be increased by NVC the vessels upstream of the microcirculation must also dilate to ensure sufficient perfusion. This is driven by what is referred to as conducted or propagated dilation.

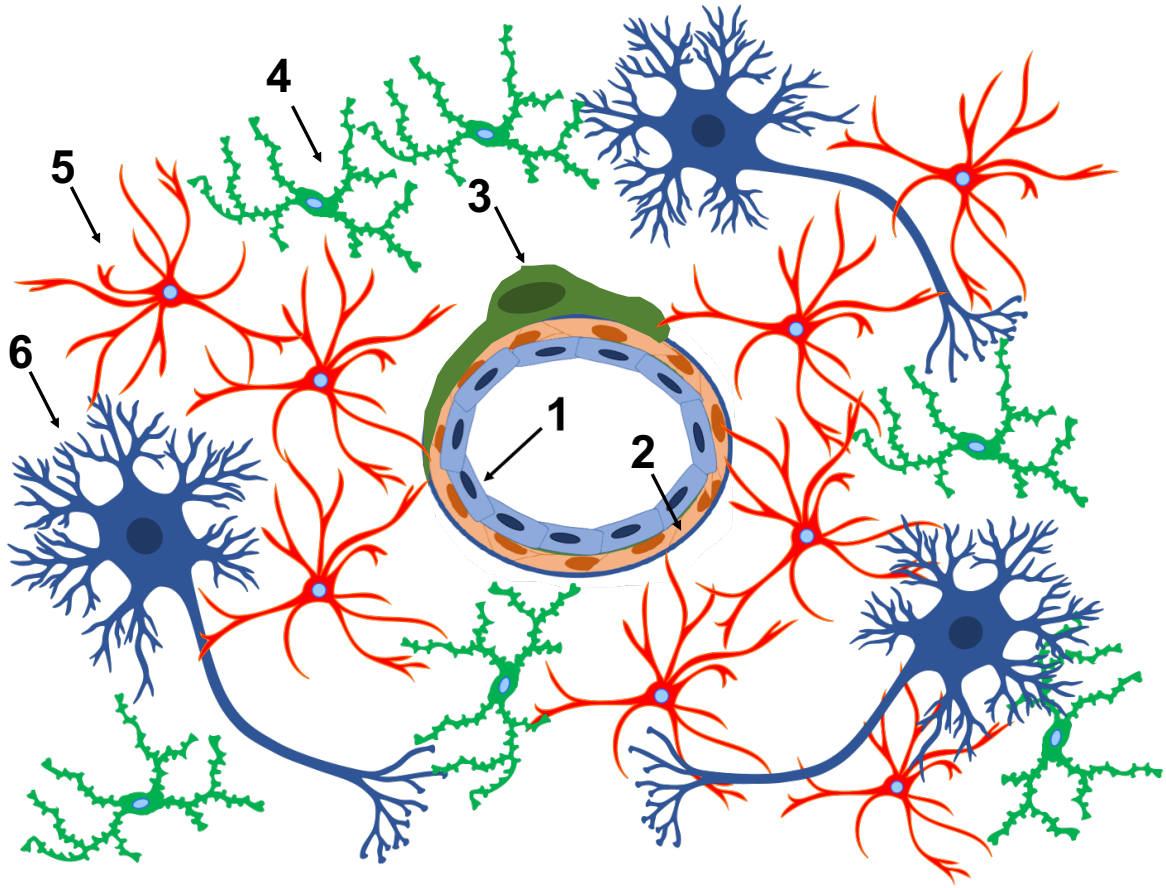

Figure 1.5 Neurovascular unit. The neurovascular unit is composed of 1. endothelial cells; 2. smooth muscle cells; 3. pericytes; 4. microglia; 5. astrocytes; and 6. neurons that act in together to control neurovascular coupling and regulate cerebral perfusion. The coverage of pericytes changes along the cerebrovascular tree. PAs are covered by few pericytes while the capillaries are covered mostly by pericytes.

1.3 – Hypertension and the cerebral vasculature

Chronic increases in blood pressure lead to alterations in the structure and function of the cerebral arteries and arterioles that have the potential to reduce cerebral perfusion (194). Increased vascular resistance is a hallmark of hypertension; this is caused by an increase in the vessel contractility, reduced dilation and structural changes resulting in arteries with reduced lumen diameters (194). Vascular resistance is the force that opposes blood flow; flow is inversely proportional to resistance. In the cerebral circulation approximately 50% of the resistance is carried by pial arteries and arterioles; PAs carry 30-40% of the vascular resistance (28). This section will summarize some of the relevant alterations to the cerebral arteries caused by hypertension.

1.3.1 – Cerebral artery remodeling

The term remodeling refers to changes in the passive structure of arteries in Ca^{2+} free and zero flow conditions; inward remodeling is a reduction in the lumen diameter, whereas outward remodeling refers to arteries with larger lumens (194). These changes in the lumen diameter of the arteries sometimes are accompanied by changes in the wall thickness (170). Hypotrophic remodeling occurs when there is a reduction in arterial wall thickness; during eutrophic remodeling the wall thickness is unchanged; and there is an increase in the wall thickness or area with hypertrophic remodeling (194). Artery remodeling in hypertension is the consequence of an initial response of the arterial wall to normalize the increased wall stress that occurs in response to the increased pressure. However, the remodeling becomes maladaptive when hypertension is sustained and this can impair the regulation of blood flow, leading to vascular and end-organ damage (194). Older hypertensive patients have reduced cerebral perfusion as a result of artery

remodeling, independent of atherosclerosis (13). In several rodent models of hypertension, inward remodeling of large pial arteries has been observed (197, 214). Less is known about remodeling of the PAs. The reductions in the lumen diameter are important particularly in situations when the cerebral arteries become maximally dilated such as during cerebral ischemia.

The mechanisms responsible for the hypertension-associated cerebral artery remodeling are still being investigated. Activation of the renin-angiotensin-aldosterone-system (RAAS) is the most well studied mechanism of remodeling. Studies show that lowering blood pressure with RAAS inhibitors such as angiotensin converting enzyme (ACE) inhibitors improves cerebral artery remodeling but lowering blood pressure alone with beta-blockers does not (194). Also, studies from our lab show that MR antagonism improves MCA remodeling in stroke-prone spontaneously hypertensive rat (SHRSP), independent of changes in blood pressure (214, 215). Inflammation is another potential mechanism for remodeling that is being investigated. The inflammatory response could be mediated by reactive astrocytes and microglia. Both the reactive astrocytes and activated microglia release a number of inflammatory cytokines such as interleukin (IL) -1, IL-6, tumor necrosis factor alpha (TNFα), and free radicals. Reactive astrocytes and microglia also express matrix metalloproteinase enzymes (MMP) such as MMP-9 that can mediate the migration of T lymphocytes to white matter lesions in hypertensive rats (123). MMP activation can mediate the artery remodeling. Studies in our lab show that MMP inhibition with doxycycline prevents the MCA remodeling in SHRSP (200). Also, in SHRSP inhibition of TNFα improves MCA structure (200). Macrophages also play a role in artery remodeling. The brain has two different macrophage populations, the

perivascular macrophages (PVM) and the choroid plexus macrophages. The PMVs are in close association with the vessel wall of the pial arteries and PAs, thus they can release pro-inflammatory cytokines to mediate the remodeling (71). Depletion of peripheral macrophages with clodronate prevents MCA remodeling in SHRSP (196). Other potential mechanisms that have been proposed to be involved in artery remodeling include oxidative stress, peroxisome proliferator-activated receptor γ activation, and chloride channel activation among many others (194).

1.3.2 – Impaired endothelial function

Impaired endothelium-dependent dilation is a hallmark of hypertension and has been reported in the brachial artery of patients with essential hypertension (187, 188). Reduced brachial artery dilation to acetylcholine has also been reported in normotensive subjects with a family history of essential hypertension implying that impaired endothelial function is not just a consequence of the high blood pressure but it may also play a role in the progression of the disease (222). Similar impairments in endothelium-dependent dilation have been observed in pial arteries and PAs in animal models of hypertension (48, 156, 157).

In the pial arteries, the impaired vasodilation is the result of a reduction in NO production by the endothelium is not the result of a change in vascular sensitivity to NO because NO donors elicited a similar dilation in SHRSP and Wistar Kyoto (WKY) rats (158). The reduced NO bioavailability in the pial arteries could be a consequence of reduced NO production by NOS. Incubation of the pial arterioles with the Ca^{2+} ionophore A23187, a receptor-independent activator of NOS, elicits pial arteriole dilation in WKY rats and this was attenuated in SHRSP (268). The reduced NOS activity could be the

result of reduced protein expression or it could be a consequence of NOS uncoupling; when NOS is uncoupled the electrons are diverted to oxygen instead of L-arginine resulting in the formation of superoxide instead of NO. The pial arterioles from hypertensive rats have reduced eNOS expression and less eNOS phosphorylation, a marker of eNOS activation. This causes a reduction in eNOS activity and further reduces NO bioavailability. The reduced NO bioavailability could also be a consequence of NO degradation by superoxide which is increased in hypertensive subjects (248). The superoxide produced reacts with NO to produce peroxynitrite to reduce NO availability and this is detrimental to the vasculature (189).

In the PAs, where EDH plays a major role, hypertension also impairs endothelium-dependent dilation (156). However, the mechanisms by which hypertension impairs the EDH-mediated dilation in the cerebral arterioles are not very well understood. We can use the studies in the peripheral arteries to identify possible mechanisms for the hypertension-associated PA dysfunction. For example, in mesenteric arteries from SHRSP, the impaired EDH-mediated dilation was a consequence of downregulation of TRPV4 and SK_{Ca} channels (223). It is possible that similar mechanisms impair EDH dilation in the cerebral vasculature. In fact, Matin *et al.*, showed that TRPV4-mediated dilation was impaired in the PAs from SHRSP (156).

1.3.3 – Impaired cerebral artery autoregulation

Impaired cerebral autoregulation has been reported in chronic hypertension, stroke and Alzheimer's disease (50, 67, 181). Hypertensive patients have decreased blood flow suggesting that cerebrovascular resistance is increased to counteract the high perfusion pressure (112). Chronic hypertension causes a shift of the autoregulatory curve to the

right which can then result in cerebral perfusion dysregulation (112). For example, in spontaneously hypertensive rat (SHR) the autoregulatory range for the PCA is from 65 to 190mmHg whereas in the normotensive WKY rats the range is from 45 to 150mmHg (194). The shift in the autoregulatory curve is a consequence of increased myogenic tone and impaired dilation of the cerebral arteries (194). Remodeling of the arteries and arterioles with hypertension also contributes to the shift in the autoregulatory range because of the reduced lumen diameter and increased cerebrovascular resistance (70). The shift in the lower end of the autoregulatory curve increases the susceptibility for ischemic damage, especially in situation where blood flow becomes dependent on the lumen diameter of the arteries like in hypertension (70). This is because hypertension impairs the ability of the arteries to dilate and it also reduces the lumen diameter of the arteries and this combination greatly increases the risk of ischemia (70). This has been shown in the MCAs from SHRSPs fed with high salt-diet; the MCAs of these rats lose their ability to autoregulate. The loss in autoregulation is important in the development of cerebral small vessel disease (cSVD) because the increased blood flow that occurs when autoregulation is impaired will be transferred to the downstream arterioles increasing the risk for microhemorrhages (194).

1.3.4 – Impaired neurovascular coupling

NVC and cerebral artery autoregulation work together to regulate brain perfusion. In animal models, NVC is assessed by measuring changes in cerebral perfusion in response to a sensory stimulus such as whisker stimulation. NVC is impaired in hypertensive rats and mice (113, 125, 239). In a study using middle-aged SHR, treatment for 10 weeks with losartan and verapamil did not prevent the impaired NVC (22). The impaired NVC in

hypertension causes hypoperfusion of the active regions in the brain which increases the risk for cognitive impairment. The mechanisms for the impaired NVC in hypertension have not been fully elucidated but artery remodeling and impaired dilation are involved.

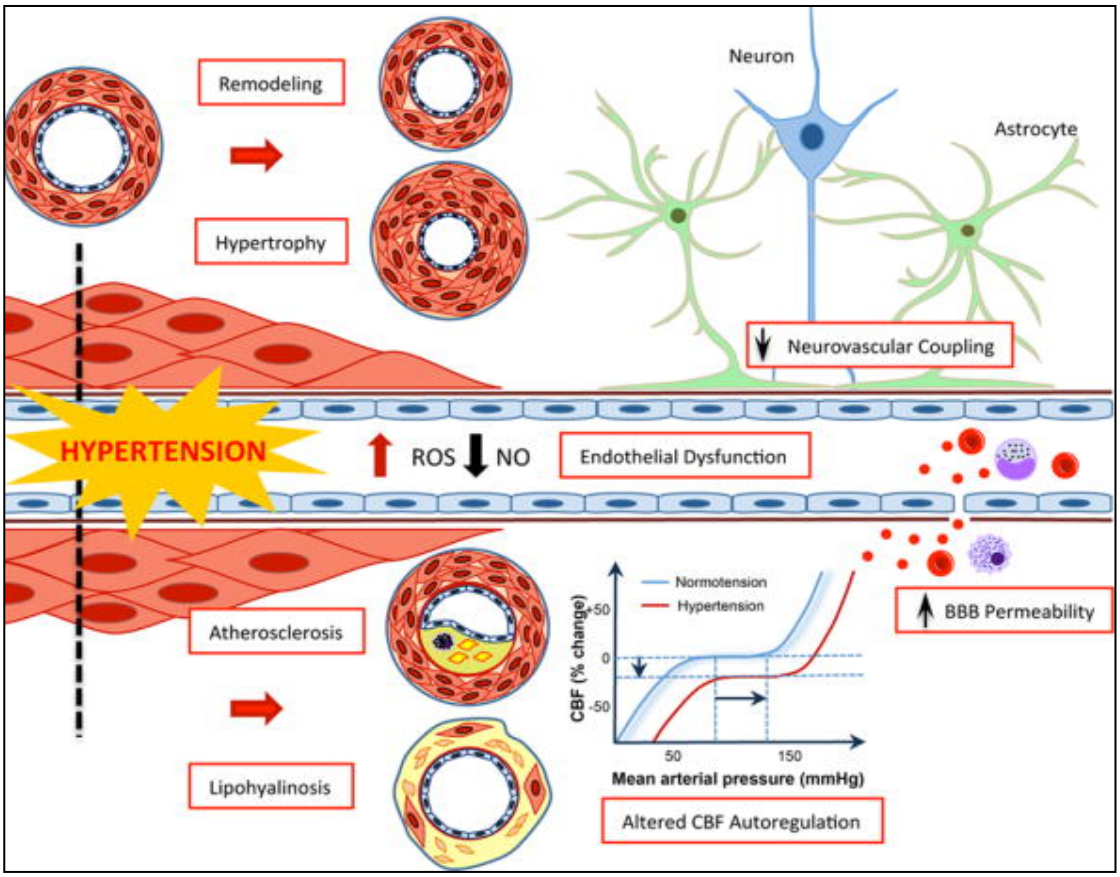

Figure 1.6 Effects of hypertension on the cerebral arteries. Hypertension causes cerebral artery remodeling, endothelial dysfunction, impaired neurovascular coupling, altered autoregulation of cerebral blood flow and blood brain barrier breakdown. With permission from (70).

1.3.5 – Angiotensin II- dependent hypertension

AngII-dependent hypertension is one of the most widely studied models of hypertension. AngII is a component of the RAAS and it has been shown to have wide ranging detrimental effects on the cerebral vasculature (194). The most effective drugs to treat hypertension, ACE inhibitors directly inhibit AngII production, thus the AngII treated mouse is a clinically relevant model.

Studies using ex vivo techniques in isolated arteries have shown that AngII-induced hypertension impairs endothelium-dependent dilation of cerebral pial arteries and arterioles (42, 43, 71). AngII treatment increases the production of ROS through NAPDH oxidases (NOX) such as NOX2 or NOX4 in the cerebral arteries from mice and rats (25, 42, 125). In the pial arterioles, the impaired endothelium-dependent dilation is in part mediated by NOX2 because AngII administration in NOX2 deficient mice did not cause impaired pial arteriole dilation (26). AngII also causes inward remodeling in pial arterioles (11). Recent studies show that slow pressor infusion of AngII causes BBB breakdown allowing AngII to enter the perivascular space (71). Feeding a salt diet to the AngII hypertensive mice does not exacerbates the BBB breakdown but it causes further increases in blood pressure (253). In the perivascular space, AngII can act through the PVMs to impair endothelium-dependent dilation and NVC (71).

There are also studies showing that infusion of AngII into the cranial window has the same effects as infusion into the mouse suggesting that AngII may have direct effects on the cerebral vasculature that are unrelated to the increase in blood pressure (71). Studies in cerebral arteries, including in human, have shown that AngII causes vasoconstriction (43). The effects of AngII occur, via activation of AT_1R and require ROS generation (71).

Recent studies have also reported that AngII-hypertensive mice have impaired cognitive function (71, 163). All these studies show that AngII-induced hypertension is a suitable model to assess the effects of hypertension on the structure and function of PAs. In the current study, I use an AngII-hypertensive mouse model to explore the effects of hypertension on the structure and function of PAs.

1.4 – Mineralocorticoid Receptors

The MR is a nuclear receptor that acts as a ligand-activated transcription factor. This receptor is encoded by the nuclear receptor subfamily 3 group C member 2 (NR3C2) gene localized on chromosome 4 q3.1 region in humans. It has three functional domains: N-terminal domain, ligand-binding domains and DNA-binding domain (Figure 1.7). It was first described in the renal epithelium as a regulator of sodium balance, but further studies demonstrated expression of the MR in the brain, arteries (both SMC and endothelium), heart, macrophages and other cells suggesting that MR has other roles in the vasculature beyond blood pressure regulation. More importantly, MR activation has been linked to vascular damage in hypertension and stroke (51–53, 159, 214, 215). This section will summarize the role for MRs in the cerebral vasculature and cognition.

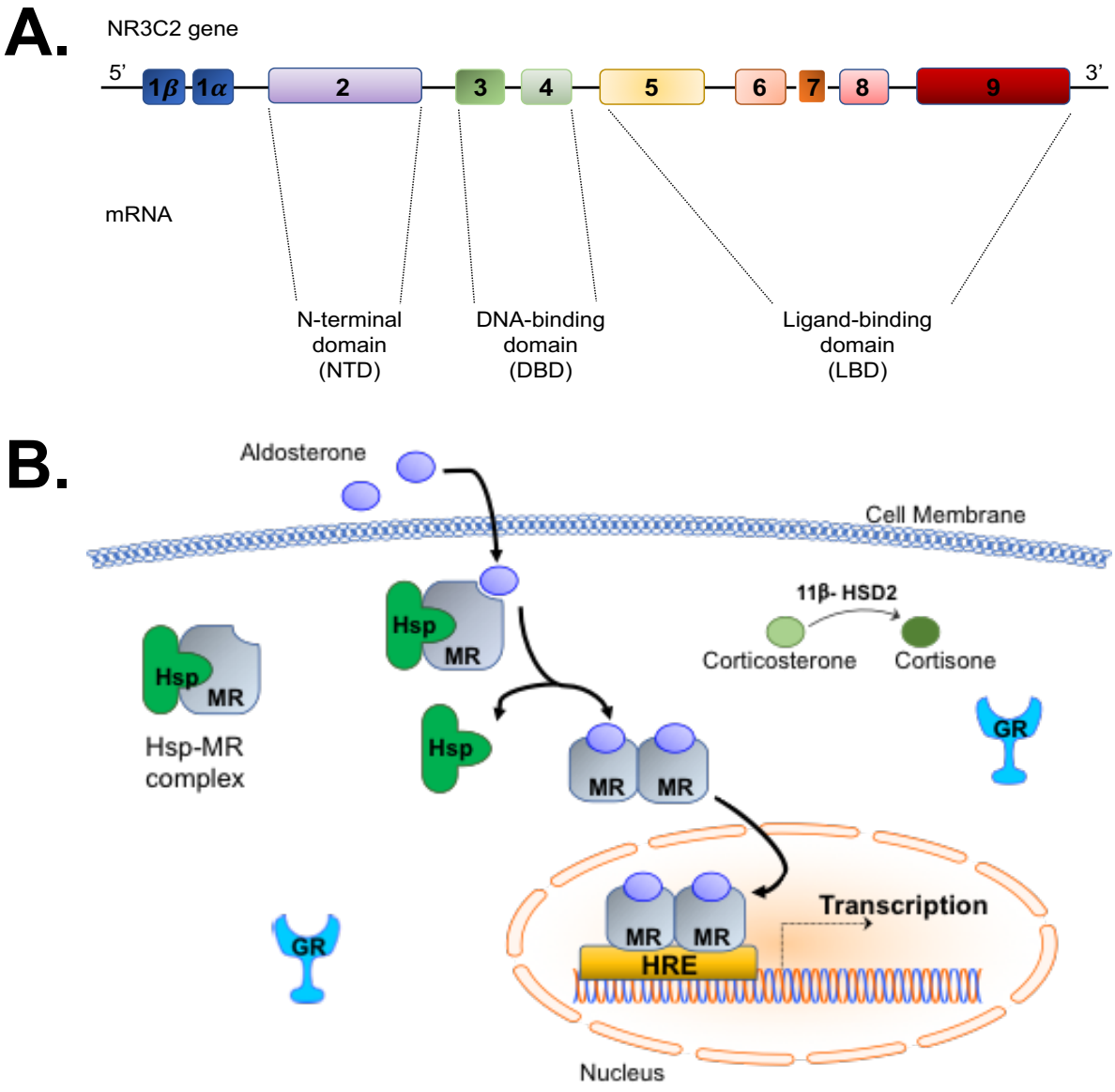

Figure 1.7 Mineralocorticoid Receptors. A. The MR (NR3C2) gene contains 10 exons and the N-terminal, ligand-binding and DNA-binding domains. B. Under normal conditions, the MR is bound to heat shock proteins (HSP) in the cytoplasm. When aldosterone activates the MR, the HSP-MR complex dissociates. Then the MR forms a dimer and translocate to the nucleus where it binds the hormone response element (HRE) and causes transcription of several genes. Corticosterone can also bind to the MR; however, endothelial cells express 11β-HSD2 that convert corticosterone into cortisone. Cortisone cannot bind to the MR. Glucocorticoid receptors (GR) are also expressed in the endothelial cells.

1.4.1 – Mineralocorticoid receptors in the vasculature

MR is activated by glucocorticoids, cortisol, corticosterone (in rodents) and the mineralocorticoid aldosterone (41). Glucocorticoids and cortisol circulate at concentrations 1,000 (total plasma) and 100 (free steroid) higher than aldosterone (77). Under the correct conditions the MR can be activated by steroid hormones other than aldosterone and corticosterone resulting in vascular damage (78, 203, 204). The selectivity of the MR for aldosterone is dependent on the enzyme 11β-hydroxysteroid dehydrogenase type II (11β-HSD2), which metabolizes cortisol and corticosterone to their inactive metabolites, cortisone and 11-dehydrocorticosterone, that cannot bind the MR. Endothelial cells and SMC express 11β-HSD2, therefore the MR in both cell types will have higher selectivity for aldosterone. In tissues such as the macrophages, cardiomyocytes and neurons that do not express 11β-HSD2, the MR will be activated mostly by other glucocorticoids. The MR can also mediate non-genomic effects that do not involve gene transcription, but it is not clear which receptor mediate the non-genomic effects. A study suggested the non-genomic effects could be mediated through the G protein-coupled estrogen receptor (GPER) in endothelial and SMCs (98). However, this has not been replicated in other studies (98).

Macrophages do not express 11β-HSD2 therefore the MR is predominantly activated by glucocorticoids. MR promotes activation of the pro-inflammatory M1 macrophage phenotype to increase the production of TNFα, RANTES (regulated on activation, normal T cell expressed and secreted), IL-6 and other cytokines in hypertension and stroke. Aldosterone activation of dendritic cells also increases IL-6 and transforming growth factor β (TGFβ). MR activation also promotes T lymphocyte

differentiation to the pro-inflammatory T helper cell (Th) 1 and Th17 cells (14). Using macrophage-specific MR knockout mice, Rickard *et al.*, showed that macrophages are involved in cardiac fibrosis and hypertension (117). These studies suggest that aldosterone and MR activation modulate innate and adaptive immunity.

In the smooth muscle, MR regulates blood pressure and vasoconstriction in hypertension. SMC MR-knockout mice are protected from age-associated increases in blood pressure (160). Inducible knockout of the MR in 4-month-old mice prevented the increase in blood pressure that normally occurs with age (117). These mice are less responsive to AngII and the mesenteric arteries generated less myogenic tone (117). SMC-MR deletion prevented the increase in blood pressure induced by AngII but not by aldosterone-salt treatment (117). SMC-MR also play a role in carotid artery remodeling and vascular calcification in hypertension (133). The role of MR expression in endothelial cells will be described in section 1.4.3.

The best understood effects of MR activation by aldosterone are outside the vasculature. MR activation in the kidney regulates sodium (Na^+) and water balance to modulate blood pressure. AngII and aldosterone are both elevated in patients with congestive heart failure and two clinical trials, Randomized Aldactone Evaluation Study (RALES) and Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study (EPHESUS), reported that blocking the actions of the MR significantly reduced the risk of death from cardiac causes (78). MR are also expressed in pre-adipocytes and are important in adipocyte differentiation and mitochondrial function (193, 251). In the brain, MRs are expressed in neurons in the hippocampus, discrete nuclei of the hypothalamus, amygdala, nucleus tractus solitarius (NTS), cerebral cortex, and

Purkinje cells of the cerebellum (87, 88, 90). The role for the MR in most of these neurons is unknown, particularly those in the cortex and cerebellum. MR in the paraventricular nucleus of the hypothalamus modulate blood pressure and renal function by increasing sympathetic activity in the arteries, heart and kidney (90). Aldosterone infusion in the fourth ventricle of rats results in activation of neuronal activation in the NTS to alter Na^+ appetite without any changes in blood pressure (279) while infusion in the third ventricle produces hypertension without altering Na^+ appetite (89).

MR is highly expressed in hippocampal neurons where it plays a role in cognition, learning and coping with stress. In the hippocampus, the MR is mostly bound by corticosterone and other steroids. This is because aldosterone does not cross the BBB very well despite its lipophilicity, which is in part because the protein transporter P-glycoproteins pumps aldosterone back across the endothelium (83); therefore most of the cells in the brain are exposed to low levels of aldosterone. In healthy men MR blockade impairs selective attention and working memory performance but enhances long-term memory (107, 185, 261). These divergent effects could be because the MR/glucocorticoid receptor (GR) ratio was disturbed. Both MR and GR are important for cognitive function; GRs are mainly involved in memory consolidation and MR in memory acquisition, therefore in healthy conditions imbalances in GR and MR activation could impair cognition. However, this seems to be different in cardiovascular risk settings. MR blockade with spironolactone (SPIR) in a mouse model of diabetes protected cognitive function (124). Recent studies in patients with essential hypertension show that increases in plasma aldosterone levels are correlated with poor cognitive function and that MR antagonism with eplerenone (EPL) improves cognitive function in these patients (65, 233,

265). These studies suggest that MR signaling is important for cognitive function and that during hypertension MR activation impairs cognition. This impaired cognition could be the result of the damage to the arteries caused by MR activation and not a direct effect of MR activation on neurons. In the current study I will explore the role of MR activation in cognitive function in AngII-hypertensive mice.

1.4.2 – Mineralocorticoid receptor antagonists

10-15% of hypertensive patients have hyperaldosteronism and could benefit from MR antagonist therapy (23). The therapeutic potential of MR antagonists is broadened by studies showing that blocking the MR can be beneficial regardless of which hormone activates the receptor (202). SPIR and EPL are two clinically available MR antagonists. SPIR is a competitive MR antagonist that was first developed as an anti-hypertensive drug. SPIR is potent but it can also bind other steroid receptors, therefore it has anti-androgenic and progestogenic effects (34, 97, 143, 240). The significant side effects of SPIR lead to the development of a second MR antagonist. EPL is more selective than SPIR but less potent (80, 108). These are also different in their metabolism. SPIR has two active metabolites that have anti-MR activity (108), whereas EPL has no active metabolites (33). Canrenone is one of the active metabolites of SPIR and is clinically used to reduce blood pressure (47). The active metabolite is also easier to administer in laboratory studies because it has less anti-androgenic effects and can be administered in the drinking water (7, 8).

MR antagonism with SPIR and EPL prevents MCA (214) and PA (197) remodeling in SHRSP in males without lowering blood pressure. EPL also improves endothelial function in the MCA from SHRSP (159). The beneficial effects of the MR antagonists on

the cerebral vasculature were independent of changes in blood pressure suggesting direct effects on the vasculature. MR blockade in females did not prevent MCA remodeling in SHRSP and they had increased MR expression compared to the males (213). In the same study, the potential role of estrogen was also explored. The authors found that in ovariectomized-SPIR treated SHRSP, MCA remodeling was prevented. Canrenoic acid prevents MCA remodeling and white matter injury in a rat model of obesity (199). Canrenoic acid administration also improves cerebral artery structure and perfusion two weeks post-stroke in SHRSP. MR activation also causes cerebral artery remodeling and vascular injury caused by cerebral ischemia (53). In models of cardiac fibrosis, it has been proposed that one of the mechanisms of action of MR antagonists is a reduction in vascular inflammation by reducing macrophage infiltration (212, 259, 275) and Th17 cell activation (3). Most of the studies that have been conducted have assessed the effects of MR blockade on preventing cerebral artery remodeling and endothelial dysfunction. A study in our lab showed that MR blockade with SPIR for 6 weeks prevents the MCA remodeling in SHRSP with established hypertension (214). In the future, we need more studies that assess reversing the established remodeling and endothelial dysfunction in hypertension because this would be a more clinically relevant strategy.

The anti-androgenic and other side effects such as hypokalemia caused by SPIR and EPL led to the development of non-steroidal MR antagonists. Finerenone (BAY 94-8862) is a potent MR antagonist that has no activity for the other steroid hormone receptors. It is in phase III clinical trials for chronic heart failure but it can also have beneficial effects in chronic kidney disease and other cardiovascular diseases (18, 175). Aldosterone synthase blockers could also be beneficial. In preclinical trials, FAD286

showed beneficial effects in models of hypertension and heart failure (72, 141, 169) and reduced aldosterone production (164). However, FAD286 also significantly reduced cortisol production limiting its clinical usefulness. A second aldosterone synthase inhibitor developed was LCI699 and it is in phase II and III clinical trials for Cushing's syndrome and pituitary adrenocorticotrophic hormone (ACTH) hyposecretion, respectively (4, 104).

Given the beneficial effects of MR antagonists beyond blood pressure, there has been an increased interest in repurposing MR antagonists as vasculoprotective agents. In the current study, I will explore the benefit of MR antagonism on PA structure and function, and cognitive function in AngII-hypertension.

1.4.3 – Endothelial mineralocorticoid receptors

As previously mentioned, MRs are expressed in endothelial cells, SMCs and macrophages around the arteries. Therefore, it has been difficult to define the downstream mechanisms and vascular cell types involved in MR signaling because the antagonists inhibit the actions of aldosterone in all cell types in the arteries. Endothelial MR (EC-MR) signaling in peripheral arteries plays a critical role in cardiovascular injury and impaired endothelium-dependent dilation (120, 211). Cell-specific MR knockout models have been developed to understand the role of EC-MR signaling in endothelial dysfunction during cardiovascular disease. In the current project I will use an endothelial specific MR knockout (ECMRKO) mouse where the knockout is driven by VE-Cadherin Cre. To generate the knockout, a Cad-Cre mouse was crossed with the mrl2 mouse, which has exons 5 and 6 of the MR floxed (Figure 1.8). This is the only available true ECMRKO strain; other knockouts available utilize the Tie-2 promoter that also deletes the MR from myeloid cells making it more difficult to tease out the role of the EC-MR versus

the myeloid MR (168, 211). The ECMRKO mouse I will use in these studies has normal blood pressure and hormone regulation under control conditions. These knockouts along with mice that overexpress human MR in endothelial cells have allowed a better understanding about the role for EC-MR in blood pressure regulation, vascular injury, aging and inflammation in the peripheral circulation. However, the role for the EC-MR in any cerebral artery remains to be defined. As previously mentioned, endothelial cells in the cerebral endothelium are anatomically unique therefore we cannot extrapolate findings in the periphery to the brain.

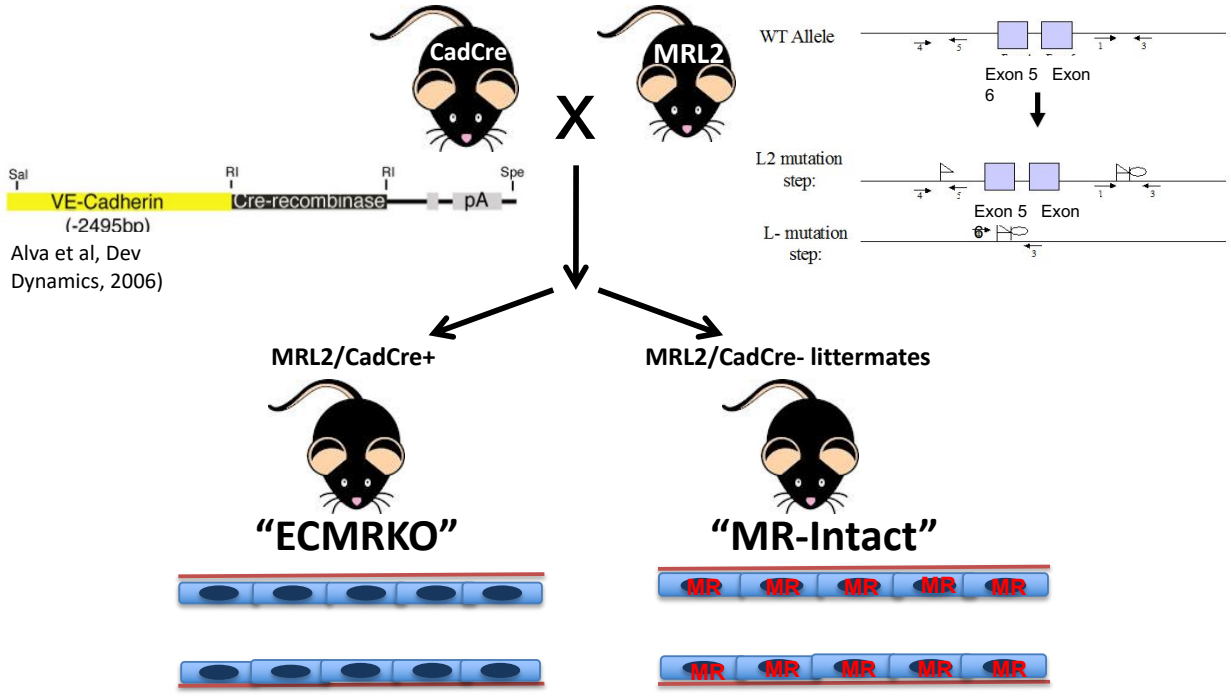

Figure 1.8 Schematic of the endothelial MR knockout mouse. The EC-MR deletion was obtained by crossing a Cad-Cre mouse, which expresses Cre-recombinase under control of the VE-cadherin promoter, with the mrl2 mouse, which has exons 5 and 6 of the MR floxed. Schematic was provided by Katelee Barett Mueler, Tufts University, Boston.

EC-MR expression is increased in the microvasculature of SHR rats (117). EC-MR overexpressing mice have increased blood pressure along with increased vasoconstriction in mesenteric arteries (178), interestingly, endothelium-dependent dilation was not altered in these EC-MR overexpressing mice (178). These EC-MR overexpressing mice had a trend in lower plasma aldosterone levels and no change in corticosterone (178). MR overexpression in endothelial cells impairs thrombus formation suggesting EC-MR activation may have beneficial anti-thrombotic actions in healthy vessels. In contrast, EC-MR deletion does not alter blood pressure suggesting that under basal conditions the EC-MR does not regulate blood pressure but overexpression or activation can alter vascular function (168). The role of EC-MR in vasodilation depends on the disease state and vascular bed studied. In control animals, ECMRKO mice have normal blood pressure and endothelial function (168).

In settings of cardiovascular risk factors including hypertension, EC-MR contributes to the development of endothelial dysfunction. Mueller *et al.*, utilized EC-MR knockout driven by the VE-cadherin promoter to show that the EC-MR regulates mesenteric artery vasodilation but not coronary artery function in response to AngII-hypertension in males (168). Furthermore, EC-MR deletion does not change blood pressure in a model of deoxycorticosterone (DOCA)/salt hypertension (152). EC-MR deletion in DOCA/salt-hypertension prevents vascular inflammation and fibrosis (211). EC-MR also promotes vascular inflammation by increasing the expression of the cell adhesion molecule, intracellular adhesion molecule-1 (ICAM-1), promoting leukocyte adhesion to human coronary artery endothelial cells (25). Leukocyte adhesion to endothelial cells promotes leukocyte recruitment from the systemic circulation into the artery wall to produce

inflammation. Rickard *et al.* showed that EC-MR deletion reduced cardiac macrophage infiltration and fibrosis in DOCA/salt-induced hypertension and cardiac inflammation (211). In later studies, Lother *et al.* showed that cardiac inflammation requires vascular cell adhesion molecule 1 (VCAM-1) in endothelial cells (152). The EC-MR may contribute to vascular stiffness via the epithelial Na^+ channel (eNaC), the target of the MR in the kidney. eNaC, expressed in the endothelium, contributes to the classical MR regulation of blood pressure control. ENaC is upregulated by aldosterone treatment in human endothelial cells resulting in decreased eNOS activity and NO bioavailability (120). eNaC also mediates Na^+ transport into the endothelium and contributes to myogenic tone in rat PCAs (56). MR activation increases eNaC in the endothelium where it can contribute to endothelial dysfunction and arterial stiffness (138).

MR activation mediates different endothelial functions such as cell adhesion of inflammatory cells and but also production of ROS. EC-MR deletion prevents increased superoxide production in cerebral arteries (49) and impaired aortic endothelium-dependent dilation in mice fed a Western diet (120). Recent studies have reported sex differences in the role of EC-MR signaling in vascular dysfunction in obesity. These studies suggest that females have higher sensitivities to EC-MR than males (40). In females, EC-MR deletion prevents aortic impaired dilation, vascular stiffness and cardiac dysfunction in a model of Western-diet-induced obesity (120, 121).

The EC-MR also regulates vasodilation in peripheral arteries through IK_{Ca}/SK_{Ca} channels (161). These channels can be activated by Ca^{2+} influx through TRP channels such as the TRPV4 and TRP ankyrin A1 (TRPA1); the role of these channels will be explained in later sections. Dysfunction in the IK_{Ca}/SK_{Ca} is linked to cardiovascular

disease (161), and these channels are emerging as therapeutic targets (231). IK_{Ca}/SK_{Ca} are expressed in cerebral arteries and arterioles with the exception of capillaries where K_{ir} channels regulate most of the dilation (236). In rat choroidal arteries, aldosterone and MR activation increases the expression of the SK_{Ca} channels (278). The activity of SK_{Ca} has also been implicated in the development of hypertension (56). Cipolla *et al*. determined that IK_{Ca}/SK_{Ca} channels are involved in endothelium-dependent dilation of PAs (30). However, the effects of MR activation on Ca^{2+}-mediated dilation in PAs have not been investigated. The unique anatomy of the cerebral endothelium prevents the extrapolation of the findings in the periphery to the brain. My studies will assess, for the first time, how EC-MR activation regulates TRPV4- and IK_{Ca}/SK_{Ca}-mediated dilations in the cerebral microcirculation. I will focus on the PAs because impaired function of these arterioles may result in cerebral hypoperfusion causing impaired cognition and worsen the outcome of cerebral ischemia.

1.5 – Transient Receptor Potential Channels

TRP channels are a superfamily of cation channels that have different degrees of selectivity for various ions including Ca^{2+} and Na^{+}. The mammalian genome encodes 28 members of the TRP superfamily that are involved in the regulation of vascular function and dysfunction in disease. TRP channels are polypeptides of 553-2,022 amino acids that contain six transmembrane domains and intracellular amine (NH_2) and carboxyl (COOH) termini of variable length. TRP channels are divided in six families: vanilloid (TRPV), canonical (TRPC), ankyrin (TRPA), melastatin (TRPM), polycystic (TRPP), and mucolipin (TRPML) (Figure 1.9). This section will summarize the role of TRP channels in

the vasculature paying particular attention to the role of TRPV4 channels in the regulation of myogenic tone and endothelial function.

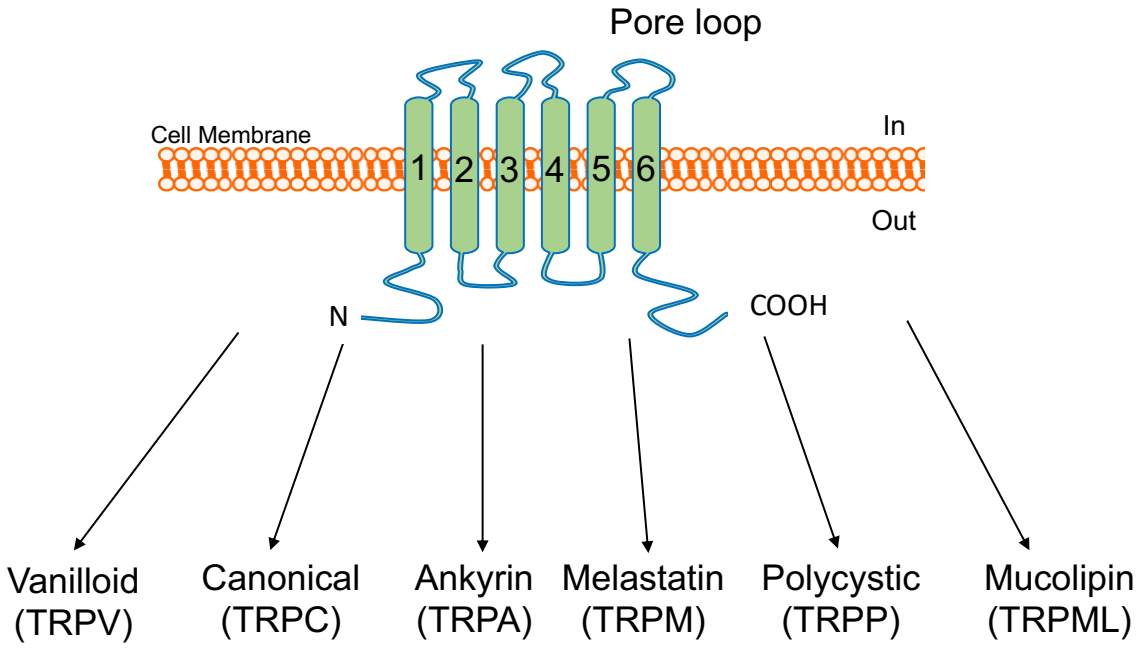

Figure 1.9 Transient Receptor Potential Channels. TRP channels are composed of six membrane-spanning helices with intracellular N- and COOH- terminals. There are six subfamilies for TRP channels found in mammals: vanilloid, canonical, ankyrin, melastatin, polycystic, and mucolipin.

1.5.1 – Transient Receptor Potential channels in the vasculature

TRP channels in the vasculature contribute to the regulation of vasomotor tone, endothelium-dependent dilation and control of blood flow. The next two sections will summarize the important finding on SMC and endothelial cell TRP channels in vascular regulation.

1.5.1.1 – Smooth muscle cell transient receptor potential channels

In the vascular SMC TRP channels are involved in the regulation of vascular tone through different mechanisms that include store-operated Ca^{2+} entry (SOCE), receptor-mediated tone, and mechanical stimulation of the channels. TRP channels from the canonical family such as TRPC1, TRPC4 and TRPC5 in the vessels in the cerebral cortex of rabbits, pial arteries and rat mesenteric arteries contribute to SOCE into the cytoplasm and replenish Ca^{2+} stores in the cells (100, 147, 264). Other members of the canonical family such as TRPC3 and TRPC6 are involved in receptor-mediated vasoconstriction and myogenic tone. TRPC3 and TRPC6 are expressed in the SMC of several vascular beds including mesenteric arteries (219), aorta (219) and cerebral arteries (209, 256, 262) where they control GPCR-mediated vasoconstriction. TRPC3 channels in SMC are activated by G-protein coupled receptor agonists such as endothelin-1 (220) and AngII (148). In the cerebral pial arteries, TRPC3 channel activation regulates receptor-mediated vasoconstriction but not myogenic tone (209). In cerebral artery SMC, TRPC3 channel activation requires coupling to IP_3 receptor which then leads to Ca^{2+} entry through voltage-gated Ca^{2+} channels and contraction (262). In rabbit mesenteric artery myocytes, application of low levels of AngII (1nM) activates TRPC6 channels through the AT_1R

(238). TRPC6 activation also regulate myogenic tone in several vascular beds including the cerebral arteries (256).

In cerebral arteries, Ca^{2+} entry through TRPC6 channels, activated by PLCγ1 dependent generation of DAG or by mechanical stimulation, leads IP_3 receptor-mediated Ca^{2+} release from the sarcoplasmic reticulum (SR) which then activates nearby TRPM4 channels (92). TRPM4 is impermeant to Ca^{2+} but its activation depolarizes the membrane, activating voltage-gated Ca^{2+} channels, increasing intracellular Ca^{2+} and leading to vasoconstriction. The expression of this channel has also been observed in the SMC from rat aorta, mesenteric, pulmonary and cerebral arteries (63, 115, 270). TRPM4 channels are important in autoregulation of cerebral blood flow through regulation of myogenic tone (63). TRPM4 channels coupled to purinergic receptors, modulate the development of myogenic tone in PAs (145). Another member of the melastatin family, TRPM5 is also impermeant to Ca^{2+} (20). TRPM8 mediates vasoconstriction in the aorta (122).

Members of the polycystic family such as TRPP1 and TRPP2 channels are also involved in the regulation of myogenic tone in mesenteric arteries. In human cerebral artery SMC, TRPP2 channel is expressed at higher levels than TRPP1. TRPP2 channel deletion in mice reduces cerebral artery myogenic tone by ~39% over the intralumenal pressures of 40mmHg to 100mmHg. These studies suggest that TRPP2 differentially regulates the myogenic responses in cerebral arteries and mesenteric arteries (174).

The vanilloid (TRPV) family also plays an important role in vasoconstriction and myogenic tone. TRPV1 channel activation mediates vasoconstriction in mesenteric arteries and aorta (172). TRPV2 channels are expressed in SMC of aorta, mesenteric arteries and the basilar artery where they regulate tone (206). Also, SMC TRPV4

channels have been proposed to regulate myogenic tone in different vascular beds. The role of SMC-TRPV4 channels in the regulation of myogenic tone will be discussed in section 1.5.2.

1.5.1.2 – Endothelial cell transient receptor potential channels

In vascular endothelial cells, TRP channels contribute to endothelium-dependent dilation. Endothelial cells and SMC are separated by the internal elastic lamina. Projections of the endothelial cells protrude through fenestrations of the internal elastic lamina so that the endothelial cells are physically connected to the SMC. These projections are known as the myoendothelial projections (MEPs) which express IP_3 receptors (183). Some MEPs contain gap junctions, these are known as myoendothelial junctions (MEJs) and the following channels are also expressed in these regions: IK_{Ca} (59), TRPA1 (59) and TRPV4 (235) channels. TRPV1, TRPV3, TRPV4, TRPA1, TRPC3, and TRPC4 channels have all been suggested to be involved in endothelium-dependent dilation (57, 59–61, 201, 225).

TRPV1 channel is activated by capsaicin and stimulates Ca^{2+} influx, eNOS phosphorylation and increased NO production to cause endothelium-dependent dilation of mesenteric arteries (267). In rat cerebral arteries, TRPV3 channel activation with carvacrol increases intracellular Ca^{2+} and activates EDH-dependent mechanisms of dilation (60). Recent studies have shown that TRPV3 activation causes PA dilation (201). The role of TRPV4 channels in endothelium-dependent dilation will be discussed in section 1.5.3.

TRPA1 channels are activated by electrophilic compounds, allicin, allyl isothiocyanate, ROS and ROS metabolites such as 4-hydroxy-2-nonenal (4-HNE) (130). These channels are present in the cerebral artery endothelium but not in the mesenteric, coronary, dermal or renal arteries (242) suggesting they play an important role in dilation. TRPA1 activation causes pial artery (242) and PA endothelium dependent-dilation. TRPA1 channels are abundant in the cerebral endothelium where they colocalize with IK_{Ca} channels. They also colocalize with NOX2 and studies have shown that stimulation of NOX2 increases TRPA1 sparklets and cause cerebral artery dilation (242). The TRPA1-induced cerebral artery dilation was blunted by SK_{Ca}/IK_{Ca} or K_{ir} channel inhibition but was insensitive to NOS and COX blockade (59) suggesting that EDH plays a major role in the cerebral artery dilation.

TRPC3 channels can also contribute to EDH-mediated dilation of cerebral arteries (132). These channels also promote cerebral artery remodeling during hypertension (195). TRPC1, TRPC2, TRPC6 and TRPV4 channels have been suggested to regulate endothelial permeability (58). The role of TPRV4 channels in endothelium-dependent dilation will be discussed in section 1.5.3. Taken together all these studies suggest that the regulation of tone and dilation in the vasculature is complex and may require the activity of more than one channel.

1.5.2 – Transient Receptor Potential Vanilloid 4 channels in myogenic tone and vasoconstriction

TRPV4 channels are expressed in the brain, liver, fat, spleen, heart and lungs (75). In the brain they are expressed in endothelial cells, SMC, astrocytic end feet that surround the vessels, microglia and hippocampal neurons. TRPV4 mutations are associated with

inflammatory, metabolic and musculoskeletal disorders (207). These channels are more permeable to Ca^{2+} than magnesium (Mg^{2+}), can be activated by cell swelling, warm temperatures and chemicals agonists such as GSK1016790A and 4-α-phorbol 12,13-didecanoate (4α- PDD) (146, 255). The unitary conductance of the TRPV4 channel is 30-60 pS at -60mV and 88-100 pS at +60mV (58).

The role of TRPV4 channels in vasoconstriction has been explored in different vascular beds. In the mouse aorta, TRPV4 causes a constriction that involves a COX-generated Tx receptor (TP) agonist that acts through mitogen-activated protein kinase (MAPK) and Src kinase signaling. This vasoconstriction was potentiated by AT_1R activation through PKC (218). TRPV4 channels also contribute to serotonin-induced pulmonary vasoconstriction (263). Other studies in the same vascular bed showed that chronic hypoxia increases the expression of TRPV4 channels and facilitates hypoxia-induced myogenic tone in pulmonary arteries (269). In the renal arteries of neonatal pigs, TRPV4 channels act as mechanosensors and regulate myogenic tone (232). In the uterus of pregnant rats, pharmacological activation of TRPV4 resulted in contraction (271).

Inhibition of TRPV4 channels in mesenteric arteries increases myogenic tone at low intralumenal pressures (20mmHg and 40mmHg) (10), those studies suggest that TRPV4 channels mediate a Ca^{2+}-pulsar-dependent vasodilation influence at these pressures by generating a large-amplitude Ca^{2+} pulsar that promote EDH and suppresses myogenic constriction (10). In MCAs, traumatic brain injury induced BK_{Ca} channel activation via TRPV4-dependent pathways in SMCs and this impairs pressure-induced constriction (243). In PAs, TRPV4 channel deletion does not alter basal myogenic tone but reduces myogenic tone after cerebral hypoperfusion (27). TRPV4 channels in

astrocytes are also key regulators of pressure-induced PA tone, when PA flow/pressure increases, astrocytes have an increase in intracellular Ca^{2+} and contributed to the increased tone (126).

Together these studies suggest that TRPV4 channels play a role in the regulation of vascular tone in different vascular beds. In the current study I will explore if EC-MR signaling and TPRV4 channels modulate the generation of myogenic tone of PAs during AngII-hypertension.

1.5.3 – Transient Receptor Potential Vanilloid 4 channels in dilation

TRPV4 channels can mediate dilation through different pathways such as EETs, flow, agonists and Ca^{2+} signaling in the endothelium. EETs produced by endothelial cells act as EDHFs in some vascular beds (24, 64, 153). EETs mediate mesenteric artery dilation through TRPV4 channels and an EDH-dependent mechanism (61). Half of the dilation was lost when the endothelium was disrupted suggesting that EETs can act on TRPV4 channels in endothelial cells and SMCs (61). Further studies showed that EETs produced endogenously activate Ca^{2+} influx through TRPV4 channels in an autocrine manner to elicit artery dilation (254).

Flow-induced dilation is initiated when the endothelium senses the shear stress caused at the vascular wall when blood flow velocity increases (66). Several studies have reported that TRPV4 channels are involved in flow-induced dilation. TRPV4 channels are activated indirectly by cell swelling through a force-sensitive signaling pathway (58). In rat gracilis arteries, TRPV4 channel activation with the agonist 4α-PDD or shear stress resulted in dilation and this response was blunted by the TRPV4 antagonist ruthenium red (134). In the same study, the authors show that NOS inhibition attenuated the flow-

induced dilation but not the agonist-induced dilation. The 4α-PDD induced dilation was blunted by IK_{Ca}/SK_{Ca} channel inhibition suggesting that shear stress increases NO production and Ca^{2+} through TRPV4 channels leading to artery dilation, while pharmacological activation of TRPV4 mediates dilation through EDH-dependent mechanisms (134). TRPV4 channels have also been reported to play a role in flow-mediated dilation of mouse mesenteric (165) and carotid arteries (105). Shear stress induces Ca^{2+} influx through TRPV4 channels in human coronary arteries resulting in dilation; this dilation was blunted by ruthenium red, the TRPV4 antagonist RN-1732 (252) and TRPV4 downregulation (41). Additionally, TRPV4 channel overexpression restores impaired flow-induced dilation of mesenteric arteries with aging (54). In cremaster arterioles, shear stress increases endothelial TRPV4 channel sensitivity to the agonist GSK1016790A and links the TRPV4 activation to muscarinic receptor mediated dilation (38).

It is still unclear if TRPV4 channels are directly activated by shear stress or respond to signaling pathways activated by shear stress. There is some evidence that TRPV4 channels can be activated by metabolites produced by shear stress. For example, CYP2C9 inhibition blocks TRPV4 activation and attenuates flow-induced dilation of mouse carotid arteries (151). Inhibition of phospholipase A_2 (PLA_2) also impaired the flow-induced dilation of mouse carotid arteries. PLA_2 stimulated TRPV4 activation in rat MCA (155). These studies supported the hypothesis that shear stress leads to the conversion of PLA_2 to EETs by CYP2C9 and this indirectly activates TRPV4 channels.

TRPV4 channels are also involved in endothelium-dependent dilation of systemic and cerebral arteries in response to muscarinic and purinergic receptor agonists. Carbachol (CCh) and acetylcholine mediated dilation is impaired in the mesenteric arteries of $TRPV4^{-/-}$ mice (250, 276). Ca^{2+} influx and NO production in endothelial cells were also reduced in these $TRPV4^{-/-}$ mice (276). Uridine-5'-triphosphate (UTP) induces Ca^{2+} influx through TRPV4 channels and causes cerebral artery dilation via an EDH-dependent mechanism (155). The TRPV4 agonist GSK1016790A also caused dilation in isolated renal and pulmonary arteries (58). Endothelial TRPV4 channel activation results in Ca^{2+} influx and TRPV4 sparklets (241) and 4α- PDD, 11,12-EET, or GSK1016790A stimulate TRPV4 sparklets in the endothelium of mesenteric arteries. A Ca^{2+} sparklet is a Ca^{2+} influx event through a Ca^{2+}-permeable plasma membrane ion channel such as the L-type voltage gated Ca^{2+} channel (5). The amplitude of individual TRPV4 sparklets is large but the total number of active sites per cells is low; only a few (~3-8 sites) TRPV4 sparklets are active in individual endothelial cells during a maximal dilation (234, 241). These sparklets activate IK_{Ca}/SK_{Ca} channels hyperpolarizing the endothelial cells. This hyperpolarization moves to the SMC via the MEJ, hyperpolarizes the SMC and causes dilation of the arteries (Figure 1.10).

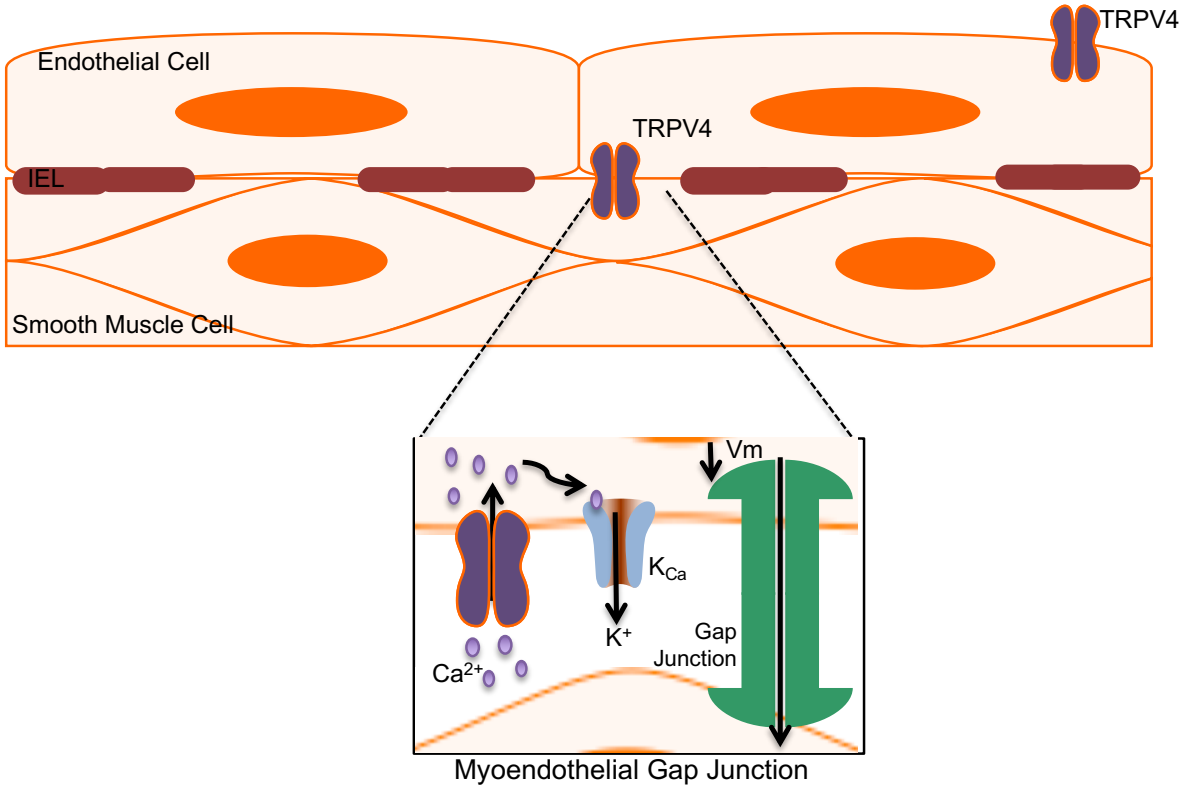

Figure 1.10 TRPV4 channels in cerebral artery dilation. TRPV4 channels are expressed in smooth muscle cells and endothelial cells. Activation of TRPV4 in endothelial cells leads to Ca^{2+} influx and activates IK_{Ca}/SK_{Ca} channels to allow for K^+ efflux. The efflux of K^+ changes membrane potential in the endothelial cells and this travels to the smooth muscle via myoendothelial gap junctions, hyperpolarizes the smooth muscle and results in dilation.

Ca^{2+} influx through TRPV4 channels could also trigger Ca^{2+} induced- Ca^{2+} release (CICR) in the endothelium (10). In mesenteric arteries, TRPV4 channels are clustered in MEPs and spatially coupled with Ca^{2+} pulsar sites. Ca^{2+} pulsars resemble Ca^{2+} sparks but instead of originating from the endoplasmic reticulum ryanodine receptors, Ca^{2+} pulsars originate from the IP_3 receptors in the endoplasmic reticulum (246). The Ca^{2+} pulsar activity in the mesenteric arteries was greater at lower intralumenal pressures (5mmHg) and higher at more physiological pressures (80mmHg) (10). However, in the internal elastic lamina of uterine arteries from rats, TRPV4 channels were not found suggesting that TRPV4 expression may be different between vascular beds (58). Recent studies have also shown that hydrogen peroxide induces endothelium-dependent dilation of mesenteric arteries through endothelial TRPV4 channels (173). Other studies have also shown that NOS inhibition attenuates the TRPV4-induced dilation through a thromboxane A_2 pathway in the rat pulmonary artery (1).

Some studies have suggested that TRPV4 channels are involved in pulmonary and systemic hypertension. Chronic hypoxia-induced pulmonary hypertension upregulated TRPV4 channel expression in rat pulmonary arteries that enhanced pulmonary vascular contractility and pulmonary hypertension (407). Other studies have shown that TRPV4 channels play a minor role in blood pressure regulation in L-NAME but not AngII-induced hypertension (179). However, TRPV4 channels are involved in AngII-induced impaired mesenteric artery endothelial dysfunction. In a mouse model of AngII-hypertension, the coupling efficiency of TRPV4 channels and EDH-mediated dilation of mesenteric arteries was impaired. This was linked to a decrease in PKC-anchoring protein AKAP150 in the

MEJ (325). TRPV4 channels are also involved in the regulation of carotid artery endothelial function in rat model of DOCA-induced hypertension. This recent study showed that the TRPV4 agonist GSK1016790A-induced dilation in the carotid artery was impaired in DOCA hypertensive rats (39). Studies from our lab showed that TRPV4 channel activation with GSK1016790A induced PA dilation in normotensive WKY rats (157). This PA TRPV4-mediated dilation was impaired in hypertensive rats and in a rat model of chronic cerebral hypoperfusion (156, 157). Another study also showed that TRPV4 channels mediate PA dilation in a mouse model of chronic hypoperfusion (27). In this study they also showed that TRPV4 channels mediate PA remodeling in chronic hypoperfusion but did not change baseline PA structure (27). TRPV4-mediated dilation in the MCAs is impaired in mouse models of cerebrovascular pathologies related to Alzheimer's disease (277).

Many of the studies performed to show the expression and function of TRPV4 channels in the vasculature used ex vivo preparations and these data are at odds with in vivo studies. Two in vivo studies showed that systemic administration of GSK1016790A caused a significant drop in mean arterial blood pressure (MAP) independent of changes in cardiac output suggesting that loss of the channel activity should increase MAP (257, 389). However, administration of the TPRV4 antagonists GSK2193874 or GSK2263095 had no effect on basal MAP or heart rate (350). Additionally, GSK2193874 had no effect on MAP after intravenous administration of acetylcholine (257). These studies suggested that TRPV4 channels do not play a role in cardiovascular regulation in healthy animals.

The role of MR signaling in the TRPV4-mediated dilation of PAs has not been explored. In the current study I will examine the role of EC-MR activation during AngII-hypertension in the TRPV4-mediated dilation of PAs.

1.6 – Vascular Cognitive Impairment and Dementia

Dementia is an umbrella term used to describe a range of symptoms associated with cognitive dysfunction. This umbrella includes Alzheimer's disease, vascular cognitive impairment and dementia (VCID), Lewis body dementia and frontotemporal dementia. VCID is a spectrum of cognitive deficits with a cerebrovascular origin and accounts for 20-30% of all dementia cases. It is the second most common cause of dementia and it exacerbates other forms of dementia including Alzheimer's disease. This a growing public health issue that affects approximately 47.5 million people and these numbers are expected to triplicate by 2050. This section will summarize clinical perspectives, risk factors, and current therapies available for VCID.

1.6.1 – Clinical perspectives

There are several forms of VCID including: vascular dementia, vascular cognitive impairment, post-stroke dementia, multi-infarct dementia, cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), subcortical vascular dementia, and cerebral amyloid angiopathy. The term vascular cognitive impairment refers to all the cognitive deficits of cerebrovascular disease while vascular dementia is the most extreme form of vascular cognitive impairment, where the daily activities are affected (95). VCID is progressive loss of memory and cognition caused by cerebrovascular injury and disease. The symptoms are sometime difficult to distinguish from Alzheimer's disease. Patients with VCID have problems with attention, organization,

problem solving and memory loss (224). Post-stroke dementia can occur after a single large stroke but only 30% of the patients will develop VCID (166), whereas multi-infarct dementia is the result of several mini-strokes that occur over time. CADASIL is a genetic disorder caused by hypertrophy of the arterioles that reduces cerebral blood flow and is associated with multi-infarcts and strokes.

Vascular risk factors such as hypertension, obesity, diabetes, and aging increase the risk for the development of VCID (50). This thesis will be focused on hypertension as a risk factor for VCID development. Differentiating the genetic versus cardiovascular risk factors for dementia development is difficult due to the presence of co-morbidities and frequent overlap with Alzheimer's disease and other neurodegenerative diseases (95). Some of the lesions in the brain that contribute to all forms of VCID include microbleeds, microinfarcts, lacunar infarcts, arteriosclerosis and small vessel disease (Figure 1.11) (110).

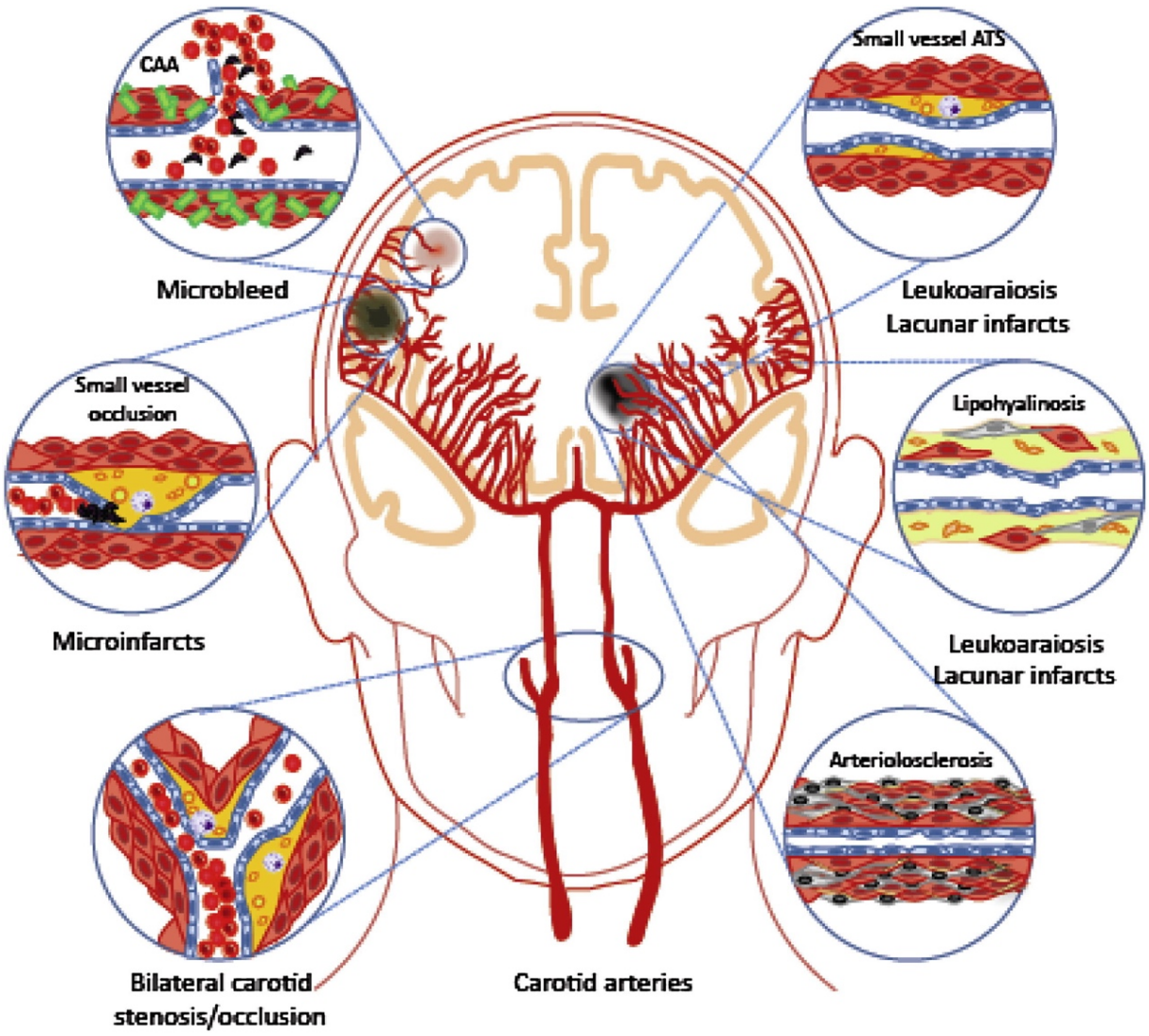

Figure 1.11 Vascular lesions leading to vascular cognitive impairment and dementia. Alterations to the function of the cerebral vasculature may compromise blood flow and increase the risk for cognitive decline. Some of the lesions that increase this risk include microbleeds, microinfarcts, carotid stenosis/occlusion, arteriosclerosis, lacunar infarcts, and cerebral small vessel disease. With permission from (110).

The NVU plays a major role in the development of VCID because it controls the regulation of cerebral perfusion. Dysfunction of the NVU can increase the risk for microinfarcts in brain regions important for cognition and can lead to cognitive impairment. When these microinfarcts occur in more than one brain region results in multi-infarct dementia. VCID risk factors can also lead to cSVD. cSVD cause cerebral artery and arteriole remodeling and endothelial dysfunction; this accounts for 45% of VCID cases (95, 186).

1.6.2 – Hypertension as a risk factor for vascular dementia

Hypertension is considered the leading risk factor for VCID. In the United States, 18 million hypertensive patients are expected to develop VCID in the next three decades (86). In a 40-year longitudinal study, mid-life systolic arterial pressure co-existed with cardiovascular risk factors and potentiated the risk of developing VCID (217). Also, uncontrolled mid-life hypertension has been shown to increase the risk for cognitive impairment (228). However, the influence of late-life hypertension on cognitive impairment is less clear. Some studies show that in an aged population low systolic blood pressure was associated with cognitive decline suggesting that the maintenance of optimal blood pressure with aging is important for cognitive function (81). As discussed earlier, hypertension increases the risk for macro- and micro-bleeds, ischemia and cognitive impairment. The cSVD associated with hypertension induces pathological changes in the PAs and leads to white matter lesions that decrease cognitive function (237). Also, hypertension-associated brain atrophy has been observed in the prefrontal cortex, inferior temporal cortex and hippocampus (94), which are all regions important for cognitive function.

Increased plasma aldosterone in patients with essential hypertension was associated with a decrease in cognitive function and MR blockade with EPL improved cognition (265). However, in healthy adults, MR blockade with SPIR resulted in impaired spatial and working memory suggesting that MR signaling plays an important role in cognitive function (261). This is further supported by studies showing that the MR is highly expressed in the hippocampus and prefrontal cortex (244). In fact, in healthy adults, MR stimulation with the agonist fludrocortisone improved visuospatial memory, learning and working memory (107). All these studies suggest that in normal conditions, MR signaling plays and important role in proper cognitive function. However, as I discussed earlier, when hypertension is present MR activation leads to cerebrovascular dysfunction and VCID.

1.6.3 – Current therapies and experimental models

Currently no therapies to treat VCID are available (21). Anti-hypertensive treatments have been used to improve cognitive function in middle-aged and elderly subjects, but these improvements in cognition were not consistent between subjects (258). From the clinical trials using anti-hypertensive drugs it was concluded that the evidence for improved cognition with anti-hypertensive therapy is weak and requires more investigation. Similarly, drugs with anti-oxidant capacity, anti-inflammatory, or drugs that increase cerebral perfusion showed inconsistent on the improvement of cognition (21). Perhaps, drugs that act directly on the vasculature such as MR antagonists could be a potential therapeutic approach. Currently, there are some on-going clinical trials testing if cholinergic drugs, vasodilators, and platelet aggregation inhibitors are effective treatments for VCID. There is also increasing evidence suggesting that a good approach

to treat VCID could just be reducing the severity of the risk factors using a multi-pronged approach. A study using hypertensive adults that were on anti-hypertensive drugs along with exercise and a healthy diet had improved cognition (229).

The lack of suitable animal models has made it difficult to identify the underlying causes of VCID and have prevented the development of effective therapies. The most widely accepted animal model for VCID is chronic hypoperfusion that is induced by interrupting blood flow through the common carotid arteries. There are several variations of the chronic hypoperfusion models that include complete occlusion of both common carotid arteries (BCAO), stenosis of both common carotid arteries (BCAS) or occlusion of one carotid artery (UCAO). However, the BCAO model can have a high mortality rate in rats (50-60%) (226). There are several hypertensive models that exhibit cognitive decline. The SHRSP is a polygenic model that mimics essential hypertension in humans and exhibits features of cSVD and cognitive decline. There are also genetic mouse models of hypertension such as the BPH/2J that also have impaired blood flow and cognitive impairment. Recent studies show that AngII-induced hypertension in mice causes cerebrovascular dysfunction and cognitive decline (71). In the current study I will explore if MR signaling mediates the cognitive dysfunction in AngII-hypertension.

There are several cognitive tests to assess vascular cognitive impairment, but the discussion here will be limited to the tests that will be used in later chapters (Figure 1.12). The first cognitive test I used was the novel object recognition test (NORT). NORT is an assessment of non-spatial short-term memory that utilizes the mouse's innate exploratory behavior in the absence of external reinforcements (6, 32). NORT can be used to assess both hippocampal and cortical lesions, highlighting that different brain regions play

different roles in memory (31). In this test the mice are acclimatized to a box with dark walls for 3 days (10 minutes each day). On the fourth day, the mouse is first place in the box with two identical objects and allowed to explore for 10 minutes, the mouse is then removed from the box. After a retention time of 60 minutes the mouse is placed back in the box with one of the familiar objects and a novel object, a mouse that is cognitively impaired will not remember the familiar object and will spend less time exploring the novel object. This test has been used in different studies to show that AngII impairs non-spatial memory in mice (32, 35, 69, 71, 144, 163). It has also been used in mouse models of aging and Alzheimer's Disease (35, 140).

I also assessed spatial memory using the Barnes maze which is similar to the Morris water maze. However, it is less stressful for the animal than Morris water maze because the mouse or rat does not have to swim. Unlike NORT, Barnes maze uses external reinforcements (9, 280, 281). A weak aversive stimulation, such as buzzer or light, is applied to motivate the animals to escape the maze (205). In this test the mice are trained to learn the location of the target hole in the maze using visual cues (9, 280). The mice are trained for 4 days, each day the mice underwent 3 trials to learn the location of the target hole. A 4000HZ sound acted as the external reinforcement and this was removed when the mouse found the target hole. The escape hole was always kept in the same location with visual cues around the room. On the fifth day (testing), the escape hole was covered but kept in the same location and the movement of the mice across the maze was tracked. A mouse with cognitive impairment will take longer to find the escape hole and it will spend less time around the target hole (9). The Barnes maze test has been

used in many studies to show that hypertension impairs spatial memory (69, 71, 137, 260). It has also been used in mouse of Alzheimer's disease (136).

I also assessed nesting behaviors because these are altered by cognitive dysfunction (46). Nest behavior is important in heat conservation, reproduction and shelter (46). This test does not use any external aversive stimuli and it is easily measured in the mice home cages (46). Mice were given a new nestlet and allowed to build a nest overnight. The next day, the nest was score on a scale from 0-5, with 0 being the worst score. A second test of nesting is to give them a cotton square nestlet for 1 hour and calculate the percentage shredded in that hour (46, 257). A mouse with cognitive dysfunction is going to shred less of the nestles as previously shown in model of hypertension (69, 71). This test has also been used to assess cognitive function in mouse models of Alzheimer's disease (73).

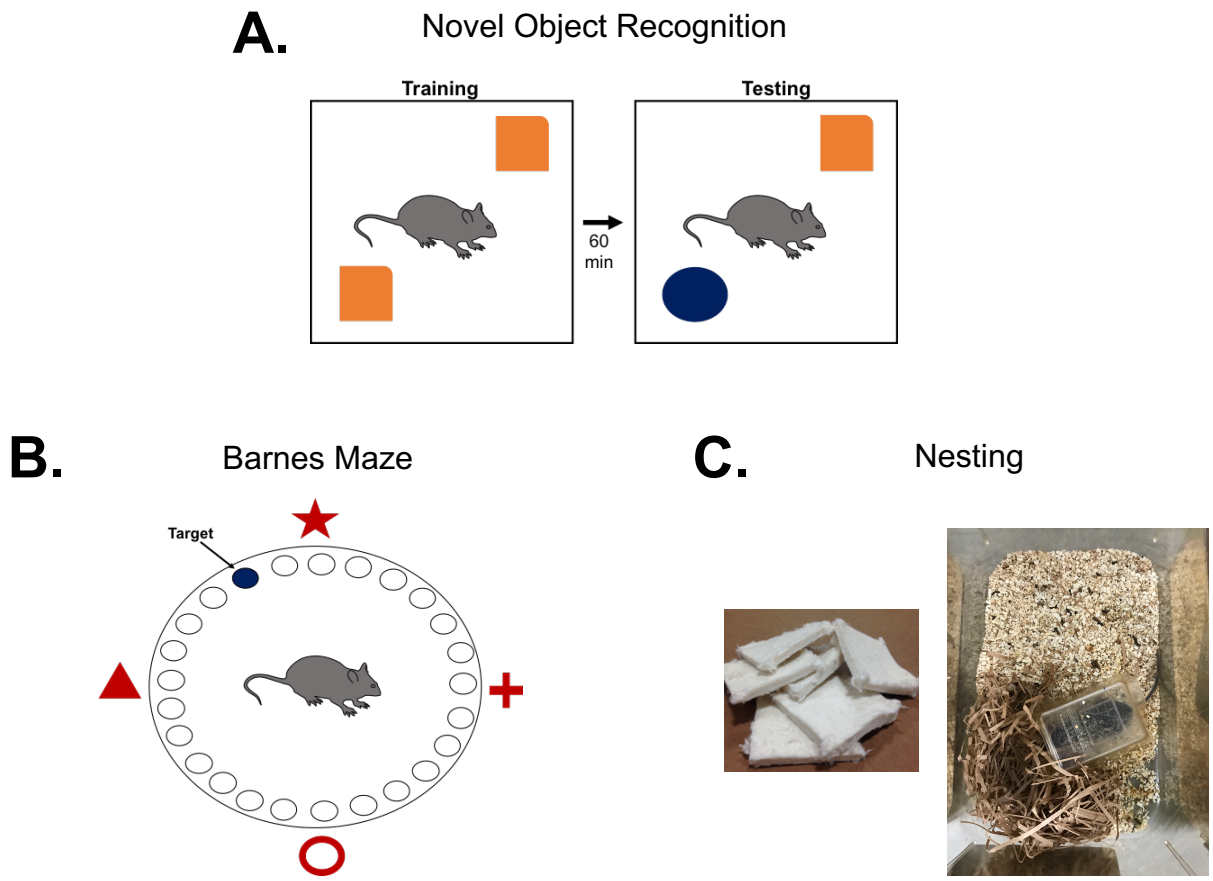

Figure 1.12 Cognitive assessments. A. The novel object recognition test is used to assess non-spatial memory where the animal is allowed to explore a familiar object versus a novel object; B. The Barnes maze is used to assess spatial memory. In this test the animals are trained to use visual cues to learn the location of the escape hole in the maze; C. Nesting behavior is another measurement of cognitive function and assessed by calculating the percentage of the cotton nestlet shredded and the score of the nest formed.

1.7 – Scope of this project

Based on the knowledge gaps in the literature and the rationale provided in the introduction, the central hypothesis for this project is that in hypertension EC-MR activation results in PA remodeling, impaired endothelium-dependent dilation and cognitive decline (Figure 1.12). This hypothesis was tested by the following experimental protocols:

1.7.1 – EC-MR signaling in cerebral artery remodeling in AngII-hypertension

As previously mentioned, AngII-hypertension is a well-established mouse model. This study predicted that EC-MR activation in AngII-hypertension will be associated with inward PA remodeling and reductions in cerebral blood flow. All the studies presented are twofold. I first evaluated the role of MR signaling using a pharmacological approach with the MR antagonist, EPL. Then, I used a genetic approach to explore the specific role of the EC-MR in the hypertension-associated changes to the PAs. For these studies I used an ECMRKO mouse.

1.7.2 – MR signaling in TRPV4-mediated dilation and cognitive function

This study was designed to investigate the role of MR signaling in the TRPV4-mediated dilation of PAs in AngII-hypertension. It also sought to assess if the MR is a druggable target to improve cognitive function in hypertension. The hypothesis was that MR antagonism with EPL would prevent the impaired TRPV4-mediated dilation of PAs in AngII-hypertension. Also, that it would improve cognitive function in AngII-hypertensive mice.

1.7.3 – EC-MR signaling in TRPV4-mediated dilation and cognitive function

This study aimed to investigate the role of EC-MR signaling in PA TRPV4-mediated dilation in AngII-hypertension. I tested the hypothesis that EC-MR mediates PA impaired TRPV4-mediated dilation. I also tested the hypothesis that EC-MR activation impairs cognitive function in AngII-hypertensive mice.

1.7.4 – Global TRPV4 deletion

This study aimed to investigate the role of TRPV4 channels in cognitive function and PA structure and function. I used a global TRPV4 knockout model to test the hypothesis that TRPV4 channels are important regulators of – 1. PA structure and endothelium-dependent dilation and 2. Cognitive function.

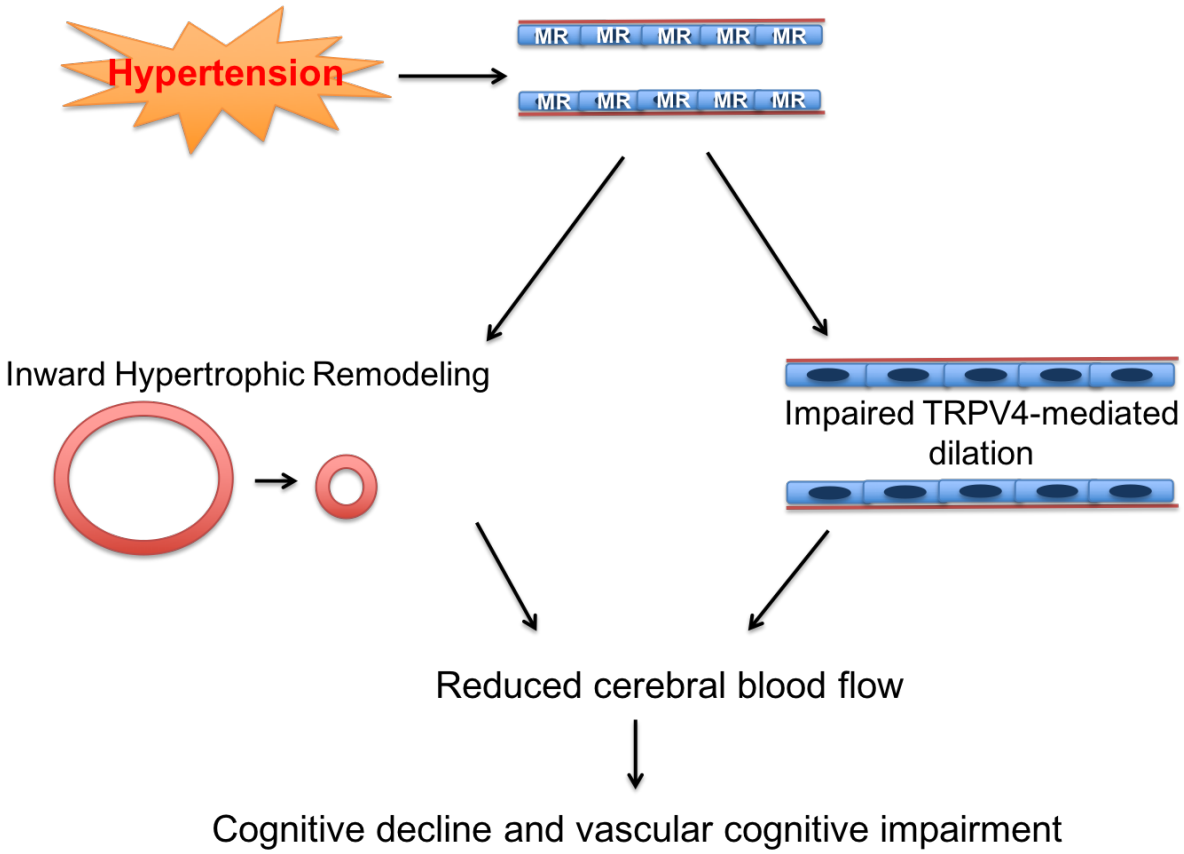

Figure 1.13 Overarching hypothesis for the current project. Hypertension causes endothelial cell mineralocorticoid receptor activation, which leads to parenchymal arteriole remodeling, impaired TRPV4-mediated dilation and subsequent reduction in cerebral blood flow. These changes will increase the risk for cognitive decline and vascular cognitive impairment.

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APPENDIX C: Aging is associated with changes to the biomechanical properties of the posterior cerebral artery and parenchymal arterioles

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Am J Physiol Heart Circ Physiol. 2016 Feb 1; 310(3): H365–H375.
Published online 2015 Dec 4.
doi: [10.1152/ajpheart.00562.2015: 10.1152/ajpheart.00562.2015]
Small Vessels–Big Problems: Novel Insights into Microvascular Mechanisms of Diseases

PMCID: PMC4796626
PMID: 26637558

Aging is associated with changes to the biomechanical properties of the posterior cerebral artery and parenchymal arterioles

Janice M. Diaz-Otero,✉ Hannah Garver, Gregory D. Fink, William F. Jackson, and Anne M. Dorrance

Department of Pharmacology and Toxicology, Michigan State University, East Lansing, Michigan
✉Corresponding author.
Address for reprint requests and other correspondence: J. M. Diaz-Otero, Dept. of Pharmacology and Toxicology, B340 Life Science Bldg., Michigan State Univ., East Lansing, MI 48824 (e-mail: diazjani@msu.edu).

Received 2015 Jul 17; Accepted 2015 Dec 2.

Abstract

Artery remodeling, described as a change in artery structure, may be responsible for the increased risk of cardiovascular disease with aging. Although the risk for stroke is known to increase with age, relatively young animals have been used in most stroke studies. Therefore, more information is needed on how aging alters the biomechanical properties of cerebral arteries. Posterior cerebral arteries (PCAs) and parenchymal arterioles (PAs) are important in controlling brain perfusion. We hypothesized that aged (22–24 mo old) C57bl/6 mice would have stiffer PCAs and PAs than young (3–5 mo old) mice. The biomechanical properties of the PCAs and PAs were assessed by pressure myography. Data are presented as means ± SE of young vs. old. In the PCA, older mice had increased outer (155.6 ± 3.2 vs. 169.9 ± 3.2 μm) and lumen (116.4 ± 3.6 vs. 137.1 ± 4.7 μm) diameters. Wall stress (375.6 ± 35.4 vs. 504.7 ± 60.0 dyn/cm^2) and artery stiffness (β-coefficient: 5.2 ± 0.3 vs. 7.6 ± 0.9) were also increased. However, wall strain (0.8 ± 0.1 vs. 0.6 ± 0.1) was reduced with age. In the PAs from old mice, wall thickness (3.9 ± 0.3 vs. 5.1 ± 0.2 μm) and area (591.1 ± 95.4 vs. 852.8 ± 100 μm^2) were increased while stress (758.1 ± 100.0 vs. 587.2 ± 35.1 dyn/cm^2) was reduced. Aging also increased mean arterial and pulse pressures. We conclude that age-associated remodeling occurs in large cerebral arteries and arterioles and may increase the risk of cerebrovascular disease.

Keywords: aging, cerebrovascular circulation, remodeling, vasculature

NEW & NOTEWORTHY

Aging is associated with changes to the biomechanical properties of parenchymal arterioles and posterior cerebral arteries; this could compromise cerebrovascular health and increase the risk of stroke and dementia. Our studies are novel because of the advanced age of the mice studied and the analysis of the parenchymal arterioles.

AGING is characterized by a decline in many physiological and vascular functions (5). Artery dysfunction (23) is an important factor in cardiovascular diseases such as hypertension, atherosclerosis, and cerebral artery disease, which are major causes of mortality in the elderly (36). The incidence of cardiovascular and cerebrovascular disease increases significantly with age; this is especially true for stroke and heart failure (24, 25). The remodeling of arteries that occurs with age may contribute to this association between age and cardiovascular disease (31). The term "artery remodeling" refers to stable changes in artery diameter and wall structure; inward remodeling is a reduction in lumen diameter while outward remodeling refers to an increase in lumen diameter. Hypertrophic remodeling occurs when wall area is increased, while hypotrophic remodeling is a reduction in wall area (41, 59). Age-related cerebral artery remodeling could increase the risk of cerebrovascular accidents especially in situations where other risk factors, such as hypertension, are present (48). Therefore it is important to fully understand the effects of aging on cerebral artery structure.

The Stroke Treatment Academic Industry Roundtable (12a) recommendations for preclinical testing state that potential neuroprotective agents should be tested in aged animals. The effects of aging on peripheral arteries have been documented (25, 26). Aged atherosclerotic mice exhibit outward remodeling of the aorta compared with young mice (39). Artery stiffness increases with age in the rat aorta and small mesenteric arteries (27, 31). Hypertrophy of the artery wall has also been observed in small mesenteric arteries from aged rats (1, 27, 35). Aging also causes endothelial dysfunction in arteries from different vascular beds. Endothelial function is impaired in aorta, carotid, and basilar arteries from 18- and 24-mo-old mice (6, 10, 34). Interestingly, the basilar artery had the most impaired function and the authors attributed this to increased reactive oxygen species production and oxidative stress (34, 47). These studies suggest that the effects of aging on the peripheral and cerebral circulation are different; therefore we cannot assume that the effects of aging in the periphery will translate to the brain.

Cerebral artery autoregulation is an important mechanism to maintain cerebral blood flow within a normal range. The effects of aging on autoregulation are controversial. Recent studies in 24 mo C57Bl/6 mice show that aging impairs the ability of the cerebral arteries to autoregulate (52). This has also been observed in clinical studies (8). However, other studies suggest aging has no effect on autoregulation. A recent study in elderly people with mild cognitive impairment showed that low blood pressure was not associated with reduced cerebral blood flow (15). This suggests that in these patients autoregulation is normal. Similar findings have also been made in a younger population (54).

Cerebral arterioles interact with neurons, astrocytes, and glial cells to form the neurovascular unit, which coordinates coupling between neural activity and local cerebral flow. Therefore cerebral arteries may behave differently from arteries in the peripheral circulation (28). The goal of our study was to characterize the effects of aging on the biomechanical properties of the posterior cerebral artery (PCA) and parenchymal arterioles (PAs) and to assess the differential effects of aging on the microcirculation and the large pial arteries. We hypothesized that aging would impair the biomechanical properties of the PCA and the PAs resulting in outward artery remodeling and increased artery stiffness. The PCA regulates blood flow and pressure to the posterior cerebral circulation. The PCAs arise from the basilar artery and supply the midbrain, basal nucleus, and thalamus, among other structures (22). The PCA is used as a model of a large pial artery. It is frequently studied in mice because it is more amenable to pressure myography studies than the middle cerebral artery (MCA), which is highly branched. The PAs arise from the pial arteries, via the penetrating arterioles, which are located in the Virchow-Robin space. PAs studied were branching off the MCA and were 1–2 mm downstream the Virchow-Robin space; these arterioles are in direct contact with the brain parenchyma. Unlike the MCA or PCA, the PAs have few branches. The PAs play a critical role in controlling blood flow and pressure in the cerebral microcirculation and are important in determining overall cerebrovascular resistance (12). PAs are composed of endothelial cells and smooth muscle cells, but they are different from pial arteries and arteries in peripheral vascular beds in that they lack extrinsic innervation (9).

MATERIALS AND METHODS

Animal model. All experimental protocols were approved by the Michigan State University Animal Care and Use Committee and were performed in accordance with the American Physiological Society's Guiding Principles in the Care and Use of Animals. Male C57Bl/6 mice purchased from the National Institute of Aging at Charles River Laboratories were housed on 12:12-h light/dark cycle with food and water ad libitum. Mice were studied at 3–5 mo (young) and 22–24 mo (old) of age. For pressure myography studies, mice were euthanized by CO_2 asphyxiation followed by cervical dislocation and decapitation.

Telemetry. Blood pressure was measured by telemetry as described previously (62). Mice were anesthetized with 3% isoflurane/1% oxygen for implantation of a catheter attached to a radiotelemetry transmitter (Data Sciences International, St Paul, MN) in the abdominal aorta via the femoral artery; the transmitter body was placed subcutaneously. Mice were allowed to recover for 3 days and then mean, systolic, and diastolic arterial pressures were measured continuously (10 s averages collected every 10 min, 24 h/day). Data were expressed as the 1- or 24-h averages of systolic, diastolic, mean arterial pressure, and pulse pressure (systolic pressure − diastolic pressure). We report the latter because it is an independent risk factor for cardiovascular disease.

Pressure myography. The brain was collected at euthanasia and the biomechanical properties of isolated PCA and PAs were assessed by pressure myography as described previously (42–45). To dissect the PAs, a 5 × 3 mm section of brain containing the MCA was isolated. Then the pia with the MCA was separated from the brain and the PAs branching from the MCA were used for experiments (43). The PAs we studied were branching off the MCA and were located 1–2 mm downstream the Virchow-Robin space. PCAs and PAs were mounted between two glass micropipettes in a custom-made cannulation chamber (61). A servo-null system was used to pressurize the arteries and arterioles. Arteries and arterioles were equilibrated in physiological salt solution (PSS) containing (in mM) 141.9 NaCl, 4.7 KCl, 1.12 KH_2PO_4, 1.7 $MgSO_4 \cdot 7H_2O$, 10 HEPES, and 5 dextrose under zero-flow conditions at 37°C. Ethylene glycol tetraacetic acid (EGTA; 2 mM) and sodium nitroprusside (SNP; 10^{-5} M) were added to maintain the smooth muscle in a relaxed state. A leak-test was performed prior to each experiment; any artery that could not maintain its intraluminal pressure (60 mmHg for the PCA and 40 mmHg for the PAs) was discarded. A pressure-response curve was constructed by increasing the intraluminal pressure from 0 to 120 mmHg at 20-mmHg increments. The PCAs and PAs were equilibrated at each pressure for 5 min, then lumen and outer diameters were measured using a 10× objective (Nikon Plan objective; Numerical Aperture: 0.25) with a Nikon Eclipse TS100 microscope. The average of the outer and lumen diameter at each pressure was recorded using MyoVIEW II 2.0 software (Danish Myo Technology, Aarhus, Denmark). These measures were compared and used to calculate wall thickness (outer diameter − lumen diameter). Wall cross-sectional area was calculated using the formula "artery area − lumen area". The wall-to-lumen ratio, wall stress, strain, and distensibility were calculated as described previously (4). Wall stiffness was quantified using the β-coefficient calculated from the individual stress-strain curves using the model ($y = ae^{\beta x}$), where y is wall stress, x is wall strain, a is the intercept and β is the slope of the exponential fit; a higher β-coefficient represents a stiffer vessel.

Immunofluorescence. Quantification of artery and capillary numbers in young and aged mice was performed by immunofluorescence (IF) staining of the endothelial cell marker isolectin GS-IB_4. Mice were transcardially perfused with 100 ml of PSS containing 2.8 mM calcium plus 1,000 UI/ml heparin sodium salt, 10^{-4} M SNP, and 10^{-5} M diltiazem to maximally dilate the cerebral vasculature. The perfusion pressure was maintained at 60 mmHg. Following perfusion with PSS, mice were perfusion-fixed with 60 ml of 4% formaldehyde. Brains were removed and postfixed in 4% formaldehyde for 48 h. Brains were then washed twice in 0.01 M phosphate-buffered saline (PBS) and placed in 20% sucrose-PBS solution for cryosectioning. Cryosections (20 μm thick) were incubated overnight in 0.01 mg/ml isolectin GS-IB4 Alexa Fluo-568 conjugate (Invitrogen, Cambridge, CA) at 4°C. This is a conjugated lectin;

therefore incubation with a secondary antibody was not necessary. The next day, sections were washed 4× in 0.01 M PBS (5 min each wash) and coverslips were mounted using Prolong antifade reagent (Invitrogen, Carlsbad, CA) (21). Two fields of the premotor cortex, one in each hemisphere, more specifically in the second and third layers of the neocortex, were acquired using a 20× objective (UPLSAPO 20X NA: 0.75) coupled to an inverted Olympus Confocal Laser Scanning microscope (Olympus America, Central Valley, PA) with Olympus Fluoview FV1000 (Olympus America). All images were acquired using the red fluorescent dye Alexa Fluor 568 that has an excitation wavelength of 578 nm and an emission wavelength of 603 nm. Sections without the isolectin served as negative controls. For the quantification of the vessel density 3D volume reconstruction of the z-stacks were made. We rotated the 3D volume reconstruction to better visualize when a vessel started and where it ended to make sure we were not counting the same vessel twice. We also used a grid to make sure we were counting the vessel correctly and not twice. We did not have software available to do the quantification of the vessels; therefore all the quantifications were done manually by the investigator using ImageJ (46).

Calcium and collagen staining. The Investigative Histology Laboratory at Michigan State University performed staining for calcium and collagen in the cerebral arteries. Calcification of the intraparenchymal arteries was assessed using the Von Kossa stain (33). Six random fields were acquired to count the number of positive vessels that contained calcium deposits. Masson's Trichrome stain was used to stain collagen in the cerebral arteries (7). Six fields were acquired to quantify the amount of collagen deposition in the vessels. Images were acquired using an Axioskop 40 (Carl Zeiss, Gottingen, Germany) coupled to a camera (AxioCam MRc5 Carl Zeiss) with the AxioVision Rel 4.6 software (Carl Zeiss Imaging Solutions, Gottingen, Germany). A blinded investigator analyzed images.

Statistical analyses. All data are presented as means ± SE. Body weight, blood pressure, calcium and collagen deposition, and vessel quantification data were analyzed by Student's t-test. For analysis of artery structure, two-way analysis of variance was utilized followed by Bonferroni t-test for post hoc comparison of the means. All statistical analyses were performed using GraphPad Prism 6.0 software (GraphPad, San Diego, CA). In all cases statistical significance was denoted by $P < 0.05$.

Drugs and chemicals. All drugs and chemicals were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise stated.

RESULTS

Physiological measures. Old mice were significantly heavier than the young mice (31.02 ± 1.57 vs. 34.79 ± 1.09 g; young vs. old). Blood pressure, measured by telemetry, showed that in our cohort of mice, advanced age was associated with higher systolic, diastolic, mean, and pulse pressures (Fig. 1, A–D). However, we observed no significant effect of age on heart rate or activity (Fig. 1, E and F). The higher blood pressure in older mice resulted mainly from substantial differences in blood pressure during the night-time when the animals are most active (Fig. 2).

Biomechanical properties of the posterior cerebral artery and penetrating arterioles. Older mice had increased PCA outer and lumen diameter (Fig. 3, A and B). Lumen cross-sectional area was also larger in the old mice (Fig. 3C). No significant differences between young and old mice were observed in the wall thickness and cross-sectional area (Fig. 3, D and E). Older mice showed decreased wall-to-lumen ratio (Fig. 3F). The mechanical properties of the arteries differed with age. Wall stress was higher in old mice (Fig. 4A) while wall strain, distensibility, and stress vs. strain were lower in PCAs from old mice (Fig. 4, B–D).

Aging was also associated with PA remodeling (Figs. 5 and 6). Wall thickness, cross-sectional area, and wall-to-lumen ratio were larger in PAs from old mice compared with young. No other significant differences in artery structure were observed. Older mice had greater wall stiffness in the PCAs (Fig. 7A) but not the PAs (Fig. 7B).

We compared arterial stiffening between the PCA and PAs in young and old mice separately. In the young mice, the PAs were stiffer than the PCAs (Fig. 7C). No differences in stiffness were observed between the PCAs and PAs from the old mice (Fig. 7D).

Calcium and collagen in the arterial wall. In a small cohort of mice, we observed that older mice did not have a significantly greater number of cerebral arteries with increased calcium deposits in the wall ($P = 0.3$) (Fig. 8A). However, as shown in the representative images, it appears that the percentage of calcification in the individual cerebral arteries from old mice is greater (Fig. 8, C and D). Arteries from old mice had more collagen deposition (Fig. 9A). Representative images are shown (Fig. 9, B–D).

Artery and capillary density in the cerebral cortex. Artery and capillary density was quantified using the endothelial cell marker Isolectin IB-4. Two fields of the neocortex, one in each hemisphere, were acquired. In a small cohort of mice, we observed that old mice had significantly fewer arteries and capillaries in the cerebral cortex (Fig. 10).

DISCUSSION

The novel finding of our study is that aging is associated with changes in the biomechanical properties of the PCA and PAs. The effects of aging on the biomechanical properties of the posterior cerebral circulation and smaller cerebral arterioles have not been widely characterized (29). The PCA was utilized as a model of a large pial artery. The PCA is important for regulating the blood flow to the posterior cerebral circulation. The PAs serve as a bottleneck for perfusion of the neocortex (41). PAs also play an important role in determining the outcome of ischemia; however, these arterioles have not been well characterized (9, 21, 43) and the effects of aging have not been assessed. In the PCAs, aging was associated with an increase in the outer and lumen diameter and a decrease in wall-to-lumen ratio. Aging was also associated with increased wall stress and stiffness. However, wall strain and distensibility were decreased with age in the PCAs. In the PAs, no changes in the size of the artery were observed but aging was associated with changes to the wall structure. The wall area, wall thickness, and wall-to-lumen ratio of the PAs were increased with age while wall stress was reduced.

The increase in the lumen diameter of the PCA we observed with age could increase cerebral blood flow and cause hyperemia. This could be compensated for by increased myogenic tone. We did not measure myogenic tone in this study, but studies in aged mice treated with angiotensin II show age, combined with hypertension, causes a loss of myogenic tone and autoregulation in the MCA (52). Loss of myogenic tone in a large artery such as the MCA increases the risk of rupture of the PAs with fluctuations in blood pressure. In the PAs, the increased wall thickness without changes in wall stress we observed could be a positive adaptation to protect these arterioles from rupture and vascular damage.

One of the strengths of our study is the advanced age of the mice in the aged group. At 24 mo old the mice used in this study were close to the end of their natural lifespan; therefore these mice truly model the geriatric population. The use of telemetric blood pressure recording in this study also is a strength because it is a more accurate technique to measure blood pressure than tail-cuff plethysmography. We also avoided the carotid catheterization approach used in many mouse telemetry studies because it may artificially alter blood pressure by affecting baroreflex function. Our studies show that mean arterial pressure and pulse pressure were increased with age. This is in contrast to studies using tail-cuff plethysmography which suggest that aging does not affect (52) or reduces (19) blood pressure. However, our studies are in agreement with clinical studies showing that blood pressure increases with age (20, 40, 58, 55). Pulse pressure was markedly increased in the aged mice; this could lead to vascular cognitive impairment (17, 32, 56).

The higher day-night blood pressure ratio that we observed in older mice (Fig. 2) is notable because this ratio is known to be an independent predictor of all-cause mortality and cardiovascular events in humans even after adjustment for 24 h average blood pressure (11). A caveat to our study is that we do not know

when blood pressure became elevated in the aged mice because blood pressure was only measured at the 24-mo time point. Age alters the ability of cerebral arteries to adapt to hypertension. Cerebral arteries from young mice have the ability to functionally and structurally adapt to hypertension (38, 52). However, the MCAs from 24-mo-old mice have an impaired ability to respond to hypertension (51). Our studies show that age results in high blood pressure. Therefore, it is possible that the ability of the PCA and PAs from old mice to adapt to hypertension is impaired. It is also important to note that in our study we observed outward remodeling which is the opposite of what we would expect with hypertension (41). It should be noted that angiotensin II-induced hypertension is likely to have a more rapid onset than an aging-associated blood pressure change; with a more gradual increase in blood pressure the mechanisms of artery remodeling may be different.

Our preliminary studies of a small cohort of mice suggest that the ageing process also resulted in artery rarefaction, that is, a decrease in the vessel density in the brain. Cerebral artery rarefaction has been observed in some models of hypertension (37, 49) and aging (52). A reduction in the number of vessels in the brain could lead to chronic hypoperfusion (28). We also show that aging increases pulse pressure and this has been associated with artery rarefaction (50, 52). However, we do not know if the changes in PCA and PA structure observed are a causative factor in the artery rarefaction or if remodeling and rarefaction occur independent of each other.

We observed increased stiffness in the PCA but not in the PAs, suggesting that age-associated changes in stiffness are different in small arterioles and large cerebral arteries. The increases in stiffness in the PCA could have been a result of higher mean arterial pressure or pulse pressure in the older mice rather than aging per se. In rat models of essential hypertension, the large cerebral arteries remodel first; this presumably serves to protect the smaller downstream arteries from the increased pressure (28). The small arteries remodel after a prolonged period of hypertension (28). It is possible that the same pattern of remodeling occurs with aging and that the cerebral artery remodeling we observed was a consequence of both aging and increased blood pressure. Further studies will be required to determine if cerebral artery stiffness in the aged mice is caused by aging itself or by increased blood pressure. Increased arterial stiffness is a hallmark of artery dysfunction and an independent predictor of cardiovascular disease (16). In peripheral arteries, aging has been associated with changes in the composition and organization of the arterial wall that increase artery stiffness (13). We observed an increase in stiffness of the PCA without an increase in wall thickness suggesting that increased stiffness is the result of changes in extracellular matrix composition.

Collagen and elastin are important components of the extracellular matrix (14), and they play key roles in maintaining the strength and elasticity of the arterial wall. With normal aging, collagen and elastin expression is differentially regulated (13) such that the increased stiffness in large arteries is associated with increased collagen and reduced elastin deposition (30, 53). The increase in collagen deposition could alter the mechanical properties of the artery wall resulting in stiffening. Mandala et al. (29) showed that in adult normotensive rats the amount of elastin in the PCA was reduced compared with young rats but no changes in collagen were observed. Our findings suggest that collagen deposition is increased with aging in the penetrating arterioles. The discrepancy between these studies can be attributed to the age and strain of the animals; Mandala et al. studied 11- to 12-mo-old Sprague Dawley rats, while we studied mice that better represent the geriatric population (22–24 mo old). The elastin fragmentation that occurs with increased age is associated with increased expression of the matrix metalloproteases (MMP) (18, 53, 57). MMP-2 and -9 in particular have been associated with elastin calcification (2, 3). The ageing process also increases the amount of calcium and phosphate in the wall (63, 64). This is associated with the calcification of the elastic fibers and could contribute to artery wall stiffness. We found that the amount of calcium in the wall and the number of arteries with calcium deposits might be increased with ageing but our data did not reach statistical significance. We recognize that these studies were underpowered (n = 3) but the number of aged mice was limited; therefore further studies will be required.

Liu et al. (28) showed that aged mice have smaller infarct size after an ischemic stroke than young mice. Interestingly, however, the functional impairments in old mice post-stroke were much worse. Post-stroke the cerebral arteries are maximally dilated such that flow is proportional to the lumen diameter. Our studies showed that advancing age is associated with increased lumen and artery cross-sectional area of the PCA. This could be the cause of the smaller infarct observed in aged rats. The magnitude of the increase between the outer and lumen diameter was different. The change with aging was greater for the outer diameter of the PCA and this is likely the cause of the increase in artery stiffness. Wall thickness of the PCA was not changed with age but it was increased in the PAs. This difference implies that missing protection from high intraluminal pressure in pial arteries is associated with increased wall thickness of the downstream arterioles. In the PAs, wall thickening with age is probably a result of smooth muscle cell hypertrophy (30). Our results are consistent with previous findings showing that in human conduit arteries such as the aorta, iliac arteries and carotid arteries, aging is associated with increased lumen area and wall thickening (53). Our studies also show that aged mice have higher wall cross-sectional area in the PCA and PAs than do young mice. In contrast, in aged Fischer rats the wall cross-sectional area of the pial arteries was less than in young animals (19). Aging also altered the mechanical properties of the PCA resulting in a less distensible artery and decreased wall strain. This is consistent with the work of Hadju et al. (19), in the pial arteries of 24-mo-old Fischer rats. However, in another study the distensibility of the MCA from 24-mo-old mice was not changed (50). The reduced distensibility we observed may be also associated with alterations of the artery stiffness.

A limitation to our study is that we did not study the development of spontaneous myogenic tone or endothelial dysfunction with age. However, it is known that aging is associated with endothelial dysfunction in the basilar artery through a reactive oxygen species-dependent mechanism (34, 47). Aging also impairs the ability of the MCA to generate tone in response to static (52) and pulsatile pressure (50). Another limitation of our studies is that we did not assess the mechanism of artery remodeling; this is a topic for future study. Possible mechanisms involve changes in the arrangement of the smooth muscle cells, increases in the expression of MMPs such as MMP-2 and -9, and elastin fragmentation. We did show that the cerebral arteries of aged mice have increased collagen that could have an important role in the changes in the artery wall observed.

In summary, aging is associated with structural changes that increase the wall stiffness of the PCA and wall stress and wall thickness of the PAs; combined, these changes could result in a dysregulation of cerebral blood flow that would increase the risk of stroke and dementia. The vasculature is a potential therapeutic target for stroke, and potential neuroprotective or neurorestorative therapies need a functioning vasculature to deliver the drug to the site of injury. Therefore, it is important to fully understand the mechanisms of age-associated cerebral artery remodeling to improve cerebrovascular health. For practical reasons all of our studies were conducted in male mice; therefore, further studies should be conducted in female mice to evaluate sex differences. In future studies we should assess if aging impairs vascular tone of the cerebral arteries and if the age-associated changes in cerebral artery structure are caused by the increased blood pressure or aging itself.

GRANTS

This study was supported by National Heart, Lung, and Blood Institute Grant PO1-HL-070687 to G. D. Fink and W. F. Jackson and American Heart Association Grant 13GRNT17210000 to A. M. Dorrance. J. M. Diaz-Otero was supported by National Institutes of Health Grant 5T32-GM-092715-04.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: J.M.D.-O. and H.G. performed experiments; J.M.D.-O. and W.F.J. analyzed data; J.M.D.-O., G.D.F., W.F.J., and A.M.D. interpreted results of experiments; J.M.D.-O. and W.F.J. prepared figures; J.M.D.-O. drafted manuscript; J.M.D.-O., G.D.F., W.F.J., and A.M.D. edited and revised manuscript; J.M.D.-O., H.G., G.D.F., W.F.J., and A.M.D. approved final version of manuscript; A.M.D. conception and design of research.

ACKNOWLEDGMENTS

We acknowledge the Investigative Histology Laboratory at Michigan State University for technical support.

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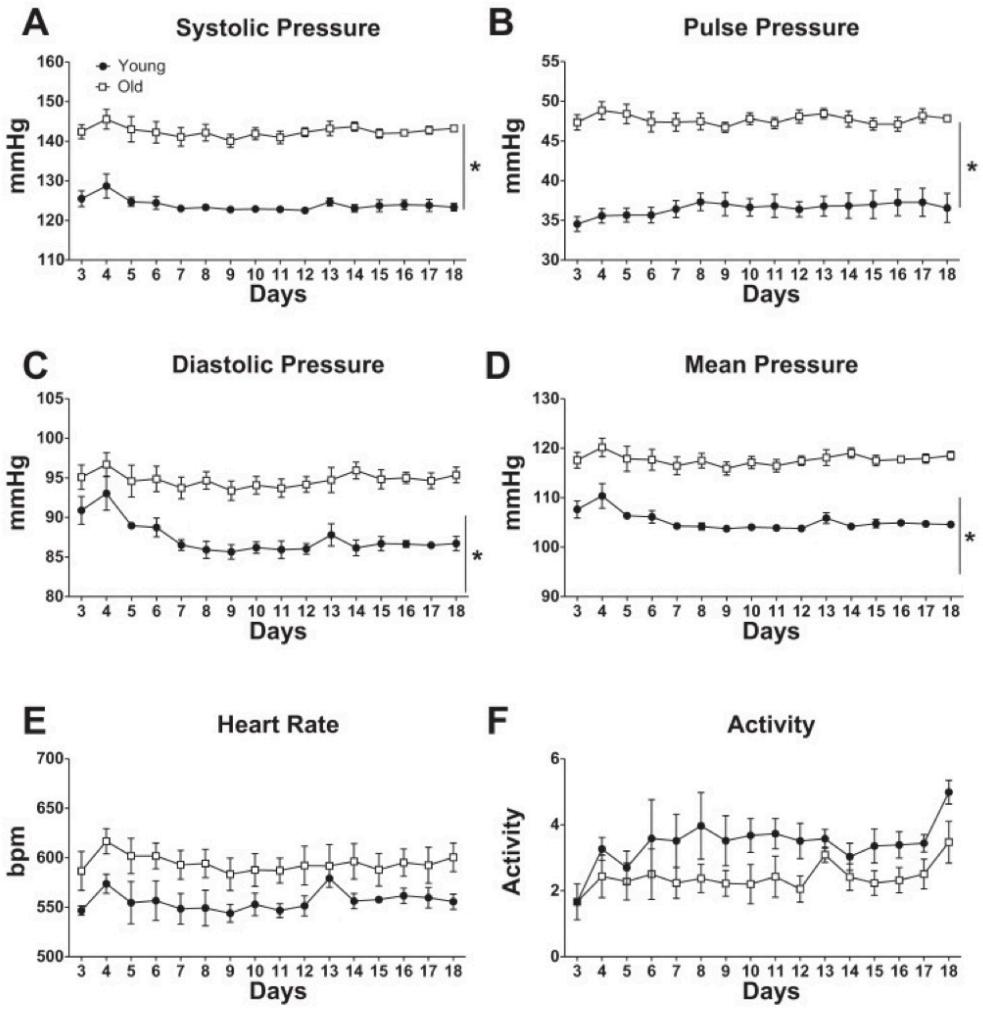
Figures and Tables

Fig. 1.



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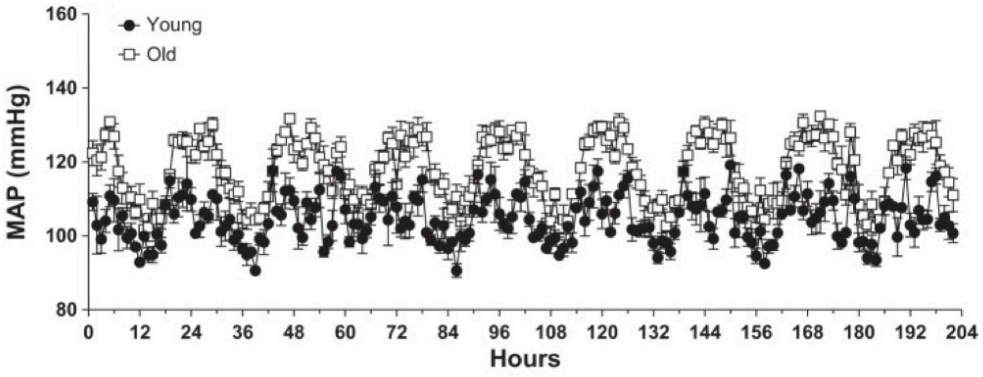
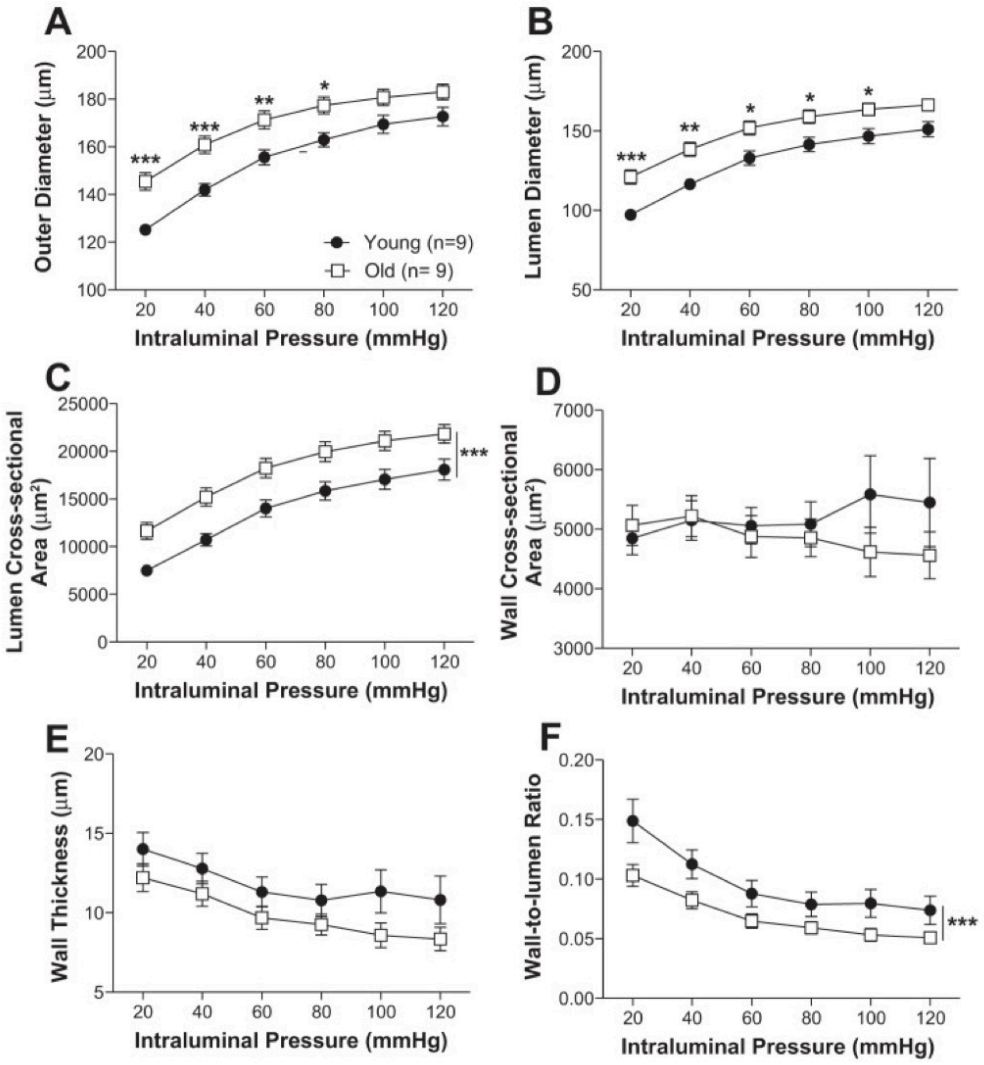
Aging increases blood pressure with no change in heart rate. Data are means ± SE (n = 4 for Young and n = 3 for Old) of 24-h averages of blood pressure or heart rate, as indicated, measured by telemetry. Two-way ANOVA indicated significant effects of age for all blood pressures (A–D) (*$P < 0.05$), but not for heart rate (E) or activity (F) ($P > 0.05$). Blood pressure data recording started on day 3 after telemeter implantation.

Fig. 2.



Night-time blood pressures are elevated in aged mice. Data are means ± SE (n = 4 for Young and n = 3 for Old) of 1-h averages of mean arterial pressure (MAP) for the last 8 days of the data shown in Fig. 1 showing substantially elevated night-time blood pressures in the aged mice. The 0-h time point represents the first midnight of the time period shown. Two-way ANOVA indicated significant effects of time and age, with a significant interaction term ($P < 0.05$ for each).

Fig. 3.



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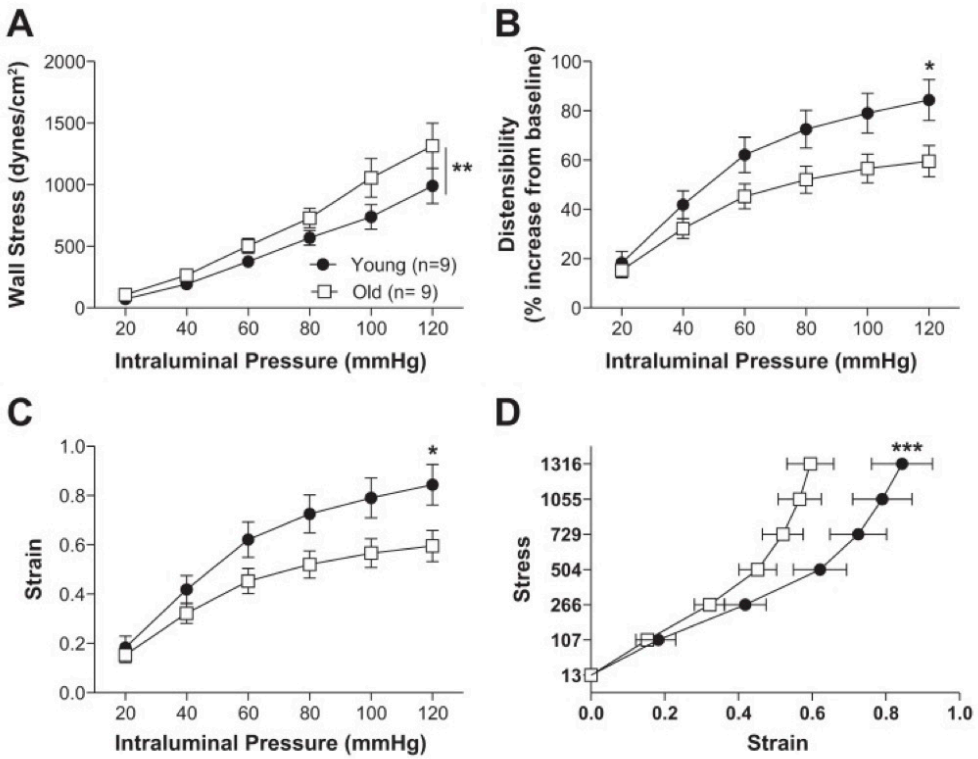
Aging results in posterior cerebral artery remodeling. Outer diameter (A), lumen diameter (B), and lumen area (C) were increased with age. Wall cross-sectional area and thickness were not changed (D, E). Aging did decrease wall-to-lumen ratio (F). Data are presented as means ± SE. ***P < 0.001, **P < 0.01, *P < 0.05, 2-way ANOVA followed by Bonferroni for post hoc comparisons.

Fig. 4.



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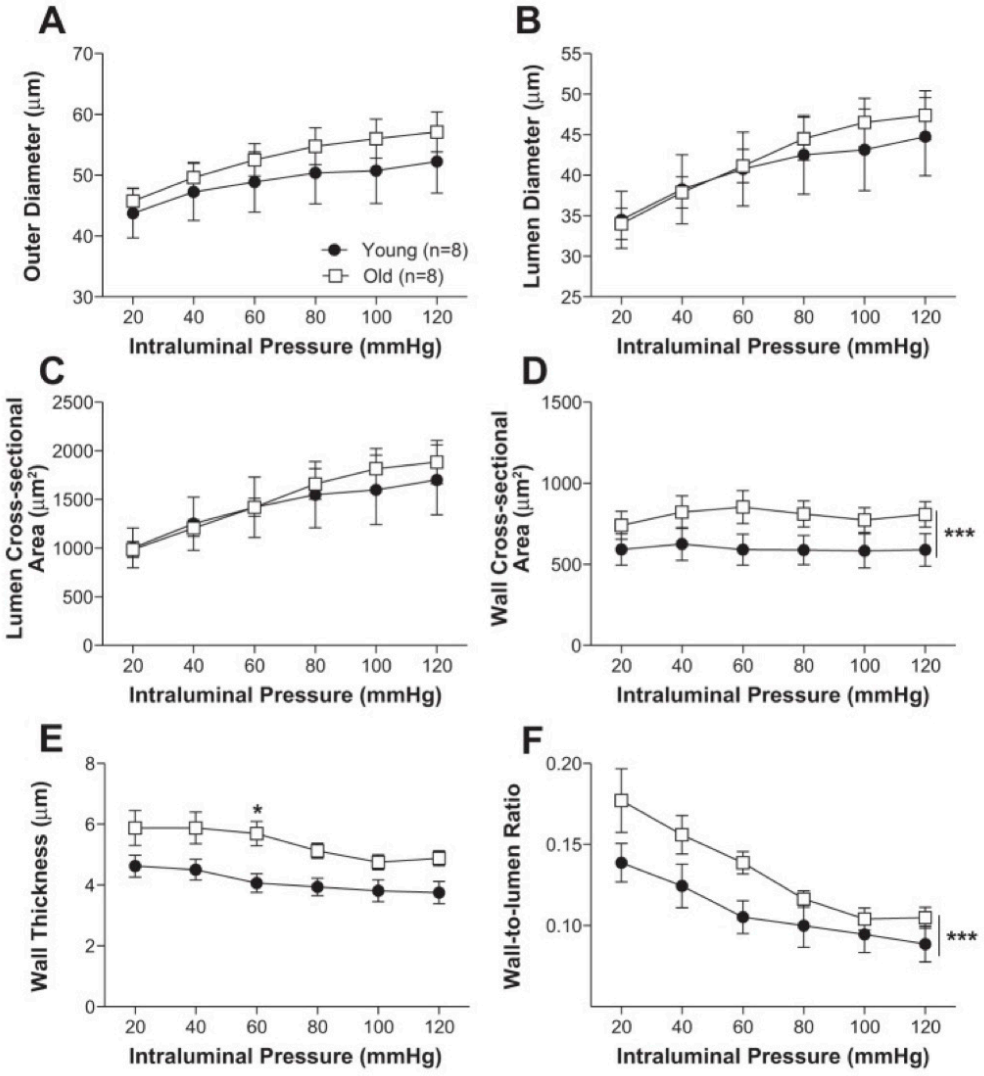
Posterior cerebral artery (PCA) mechanical properties were changed with aging. Wall stress was increased (A) with age. Aging also decreased strain in the PCA (C). The PCA was less distensible with age (B). Wall stress vs. strain was reduced in the older mice (D). Data are presented as mean ± SE; ***P < 0.001, **P < 0.01, *P < 0.05, 2-way ANOVA followed by Bonferroni for post hoc comparisons. Data from Fig. 3 were used to calculate the mechanical properties of the PCA.

Fig. 5.



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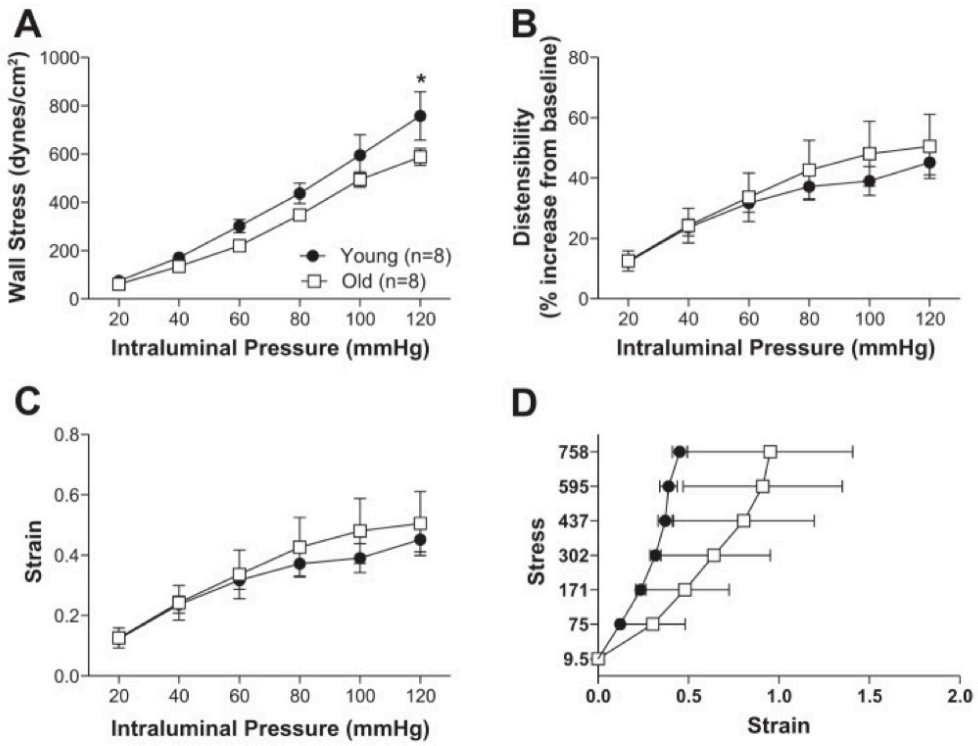
Aging increases wall thickness in the penetrating arterioles. Outer diameter (A), lumen diameter (B), and lumen area (C) of the penetrating arterioles were not significantly changed with aging. Wall cross-sectional area (D), wall thickness (E), and wall-to-lumen ratio (F) were increased with age. Data are presented as means ± SE. ***P < 0.001, *P < 0.05, 2-way ANOVA.

Fig. 6.



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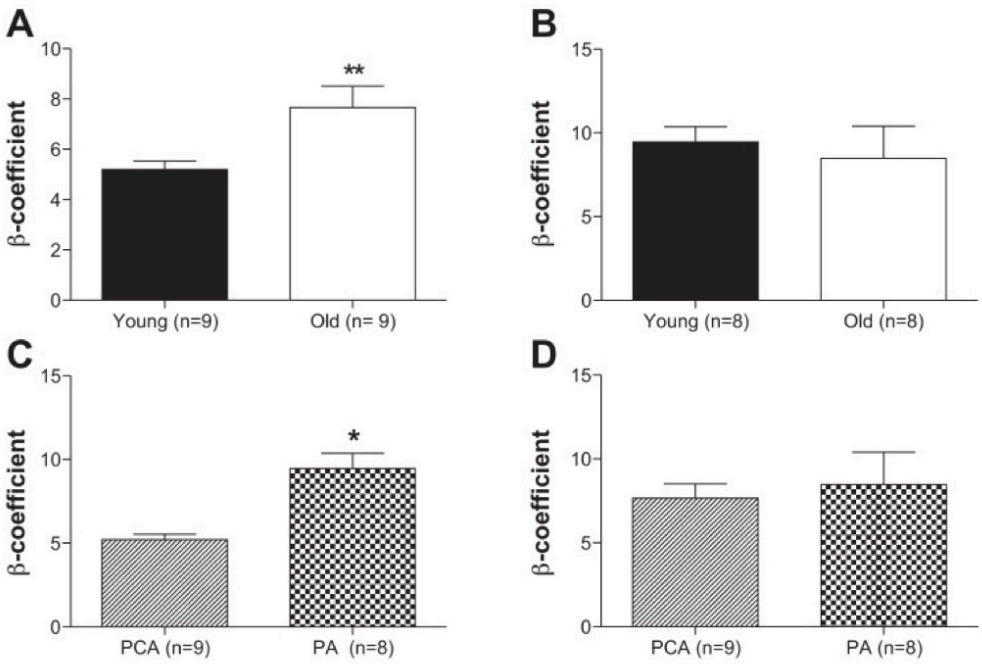
Aging resulted in changes to the mechanical properties of the penetrating arterioles. At 120 mmHg, wall stress was increased with age (A). Wall strain (C), distensibility (B), and stress-strain (D) were unchanged. Data are presented as means ± SE. *P < 0.05, 2-way ANOVA followed by Bonferroni for post hoc comparisons. Data from Fig. 5 were used to calculate the mechanical properties of the PCA.

Fig. 7.



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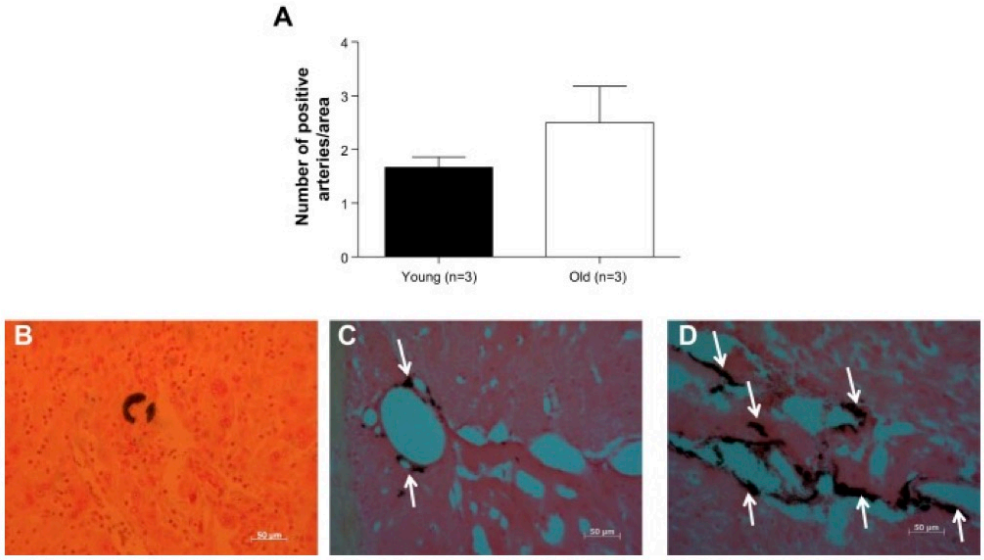
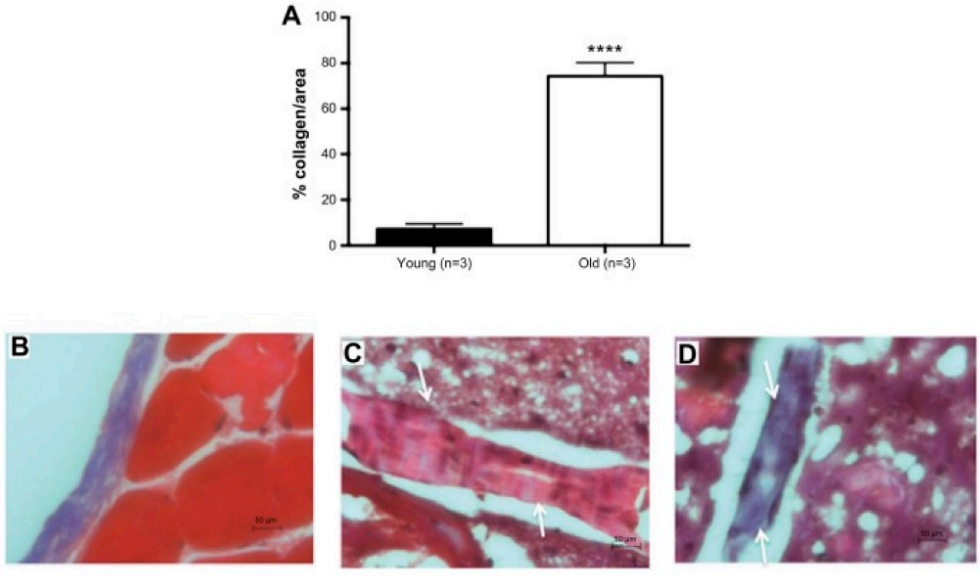
Aging increases vascular stiffness in the posterior cerebral artery, and vascular stiffness is different depending on the type of artery. Wall stiffness is increased with aging in the posterior cerebral artery (A) but not in the penetrating arterioles (B). An increased β-coefficient represented increased wall stiffness. In young mice vascular stiffness is increased in the small arteries compared with large arteries (C). This was not the case in old mice (D). PA, parenchymal arterioles. Data are presented as means ± SE. *P < 0.05, **P < 0.01, Student's t-test. Data from Fig. 4 and 7 were used to calculate the mechanical properties of the PCA.

Fig. 8.



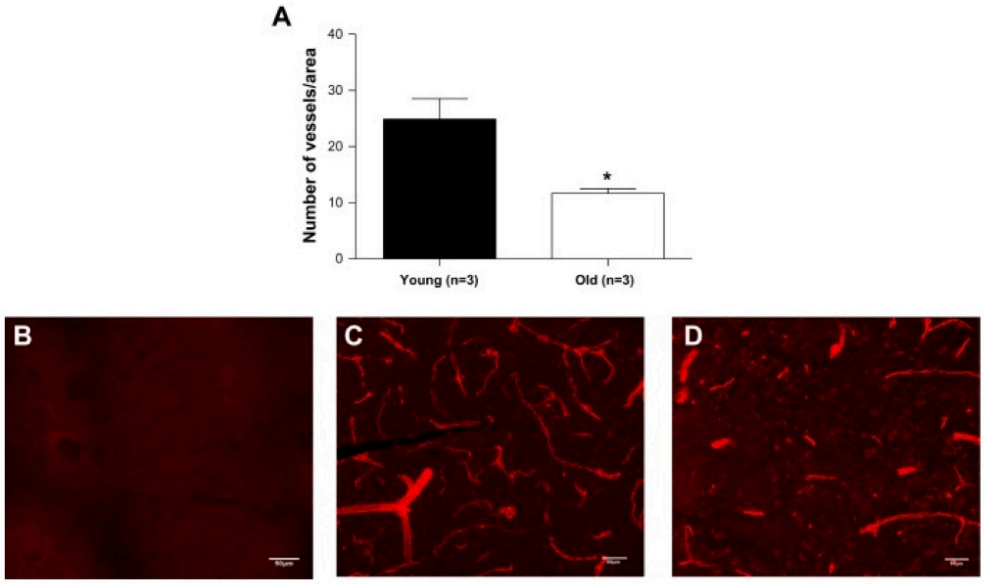
Aging may increase calcium content in the wall of cerebral arteries. In a small cohort of mice, arteries with calcium deposits were counted in young and old mice. The amount of vessel with calcium deposits or the increase in the percentage of calcification (data not shown) was not significantly different between both groups (A). Representative images at 40× magnification are shown (B: positive control; C: young mouse; D: old mouse). The arrows indicate the arteries. Data are presented as means ± SE. $P = 0.3$ by Student's t-test; $n = 3$.

Fig. 9.



Aging increases the percent of collagen deposition in cerebral arteries. In a small cohort of mice, the percentage of collagen deposits in the wall of young and old mice was quantified. Aging resulted in a significant increase in collagen deposition (A). Representative images at 40× magnification are shown (B: positive control; C: young mouse; D: old mouse). The arrows indicate the artery. An artery with increase collagen content will be stained purple. Data are presented as means ± SE. ****$P < 0.001$ by Student's t-test; $n = 3$.

Fig. 10.



Aging decreases artery density. In a small cohort of mice, the amount of arteries and capillary number was quantified using Isolectin-IB4. In each animal, two images were acquired in the neocortex, one per hemisphere. Representative images are shown above. B: control; C: young; D: old. Data are presented as means ± SE. *P < 0.05, Student's t-test; n = 3.

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CHAPTER 2

The Endothelial Mineralocorticoid Receptor Mediates Parenchymal Arteriole and Posterior Cerebral Artery Remodeling during Angiotensin II-induced Hypertension

2.1 – Abstract

The brain is highly susceptible to injury caused by hypertension because the increased blood pressure causes artery remodeling that can limit cerebral perfusion. MR antagonism prevents hypertensive cerebral artery remodeling, but the vascular cell types involved have not been defined. In the periphery, the EC-MR mediates hypertension-induced vascular injury, but cerebral and peripheral arteries are anatomically distinct; thus, these findings cannot be extrapolated to the brain. The PAs determine cerebrovascular resistance. Determining the effects of hypertension and MR signaling on these arterioles could lead to a better understanding of cSVD. I hypothesized that EC-MR signaling mediates inward cerebral artery remodeling and reduced cerebral perfusion during AngII-hypertension. The biomechanics of the PAs and PCAS were studied in male C57Bl/6 and ECMRKO mice and their appropriate controls using pressure myography. AngII increased plasma aldosterone and decreased cerebral perfusion in C57Bl/6 and MR-intact littermates. EC-MR deletion improved cerebral perfusion in AngII treated mice. AngII-hypertension resulted in inward hypotrophic remodeling; this was prevented by MR antagonism and EC-MR deletion. My studies suggest that EC-MR signaling mediates hypertensive remodeling in the cerebral microcirculation and large pial arteries. AngII-induced inward remodeling of cerebral arteries and arterioles was associated with a reduction in cerebral perfusion that could worsen the outcome of stroke or contribute to VCID.

2.2 – Introduction

Hypertension is a modifiable risk factor for cSVD (10, 26, 38). In animal models of hypertension, remodeling occurs in the MCA (32) and PAs (28), producing arteries with smaller lumens and thicker walls and the same effect is observed in patients with hypertension. However, our understanding of the mechanisms responsible for these changes in the cerebral circulation remains incomplete. My studies focus on two artery types: PAs and PCAs. The PAs have a limited number of anastomoses and arise from pial arteries to perfuse the cerebral microcirculation. These arterioles play a critical role in the outcome of ischemia and the development of VCID (5, 17, 28). The PCA, a large pial artery, regulates perfusion of the hippocampus, the temporal cortex and parts of the parieto-occipital cortex (16) which are associated with memory formation.

Aldosterone and MR activation have been linked to vascular damage in hypertension (13, 28). However, the specific cell type involved in MR-mediated artery remodeling in the brain has not been defined. Identifying the cell type that drives the MR-mediated remodeling will allow us to define the specific cellular pathways activated by the MR, and this may allow for the development of more specific therapies to prevent or reverse cerebral vascular remodeling in hypertensive patients. Cell-specific MR knockout mice have been developed to facilitate studies defining the role played by the EC-MR. Mueller *et al.*, utilized a specific EC-MR knockout driven by the VE-cadherin promoter to show that the EC-MR regulates mesenteric artery vasodilation but not coronary artery function in response to hypertension (22). These studies suggest that EC-MR activation is an important mediator of hypertensive vascular injury. These studies also highlight the need to study the cerebral vasculature separately, not only

because it is anatomically unique but also because it is clear that different vascular beds have varying sensitivities to EC-MR signaling.

In this study, I used a pharmacological and genetic approach to test the hypothesis that the EC-MR activation is required for AngII-hypertensive cerebral artery remodeling. I used EPL (100mg/kg/day) (28) to pharmacologically inhibit the receptor to confirm that MR signaling mediates hypertensive remodeling of PAs and PCAs. ECMRKO mice generated using the VE-cadherin promoter were used to determine if EC-MR is involved in cerebral artery remodeling in AngII-hypertension. PAs were used as a model of the cerebral microcirculation and the PCA as a model of a large cerebral resistance artery.

2.3 – Materials and Methods

2.3.1 – Experimental models

All experimental protocols were approved by the Michigan State University Animal Care and Use Committee and were performed in accordance with the American Physiological Society's *Guiding Principles in the Care and Use of Animals.* C57Bl/6 mice (n=6-8 per group) were purchased from Charles River Laboratories. ECMRKO mice (n=6-8 per group) were generated by Dr. Iris Z. Jaffe at Tufts Medical Center by crossing VE-cadherin Cad-Cre^{+} mice with MR$^{f/f}$ mice as described previously (22). MR$^{f/f}$ mice had exons 5 and 6 of the MR gene flanked by loxP sites (22). These EC-MR knockouts have been shown to be specific for the endothelium with MR recombination confirmed in endothelial cells from all vascular beds tested including lung, heart, and aorta without MR recombination in splenic leukocytes or lymph nodes and with intact MR mRNA expression in isolated leukocytes (22, 36). MR-intact littermates were used as controls.

All mice studied were males and housed on 12h: 12h light/dark cycle with food and water ad libitum.

2.3.2 – Animal treatment

AngII (800ng/kg/min) was administered subcutaneously for 4 weeks via osmotic minipumps (Alzet model 1004, Durect Corp, Cupertino CA). To implant the minipumps, mice were anesthetized with 3% isoflurane in oxygen and body temperature was maintained at 37°C. Mice were given a single dose of the antibiotic Baytril (5mg/kg, intramuscular (IM)) and the analgesic Rimadyl (5mg/kg, subcutaneous (SQ)) prior to surgery. Sham-operated mice underwent anesthesia, an incision subcutaneous pocket was made but mini-pumps were not inserted. Mice were 16-17 weeks old when the treatment began and they were euthanized at 20-21 weeks (after 4 weeks of treatment). A group of C57Bl/6 mice were treated with the MR antagonist, EPL (8, 28), given orally suspended in peanut butter; control mice received peanut butter alone during the 4 weeks of the AngII infusion. The dose of EPL used is higher than what normally administered in humans because the pharmacokinetic profile of the drug is different in rodents. No toxicity or carcinogenesis have been observed after EPL treatment at the dose used. C57Bl/6 mice treated with AngII, AngII+EPL, EPL alone or vehicle were compared to Sham C57Bl/6 mice. ECMRKO with/without AngII were compared to their MR-intact littermates with/without AngII.

2.3.3 – Tail-cuff plethysmography

Animals were allowed to recover for 3 days after the surgical implantation of the osmotic minipump. Blood pressure was measured in conscious mice twice a week during the 4

weeks of the AngII treatment by tail-cuff plethysmography using a RTBP1001 tail-cuff blood pressure system (Kent Scientific, Torrington, CT).

2.3.4 – Scanning Laser Doppler

Pial artery blood flow was measured by scanning laser Doppler flowmetry (Perimed, Ardmore, PA) using the LDPIwin 3.1 software. Prior to euthanasia, mice were anesthetized in 3% isoflurane in oxygen and body temperature was maintained at 37°C. A midline incision was made in the skin and the skull was exposed and cleaned. The laser Doppler was positioned ~14-15cm above the skull and a total of 3 consecutive scans were performed. Mean perfusion in both hemispheres was calculated; perfusion units are arbitrary. The penetration of the laser used in these studies was between 0.5 and 1mm. Thus, with the skull intact the assessment of blood flow are primarily at the level of the pial arteries.

2.3.5 – Plasma aldosterone and corticosterone

Blood was collected by cardiac puncture prior to euthanasia in anesthetized mice. Plasma aldosterone and corticosterone levels were measured by ELISA (Enzo Life Sciences).

2.3.6 – Pressure Myography

The structure and biomechanical properties of the PCA and PAs were assessed by pressure myography as described previously (13, 28). To isolate PAs, a 5x3mm section of the brain containing the MCA was dissected, then the pia with the MCA was separated from the tissue and the PAs branching from the MCA were used for experiments. Isolated PCAs and PAs were mounted in two glass micropipettes in a custom-made cannulation chamber. A servo-null system was used to pressurize the

arteries and arterioles and a leak test performed prior to each experiment. Any artery or arteriole that could not maintain its physiological intraluminal pressure (60mmHg for the PCA and 40mmHg for the PAs) were discarded. Arteries and arterioles were equilibrated in physiological salt solution (PSS) containing 141.9mmol/L NaCl, 4.79mmol/L KCl, 1.12mmol/L KH_2PO_4, 1.79mmol/L $MgSO_4 \bullet 7H_2O$, 109mmol/L HEPES, and 59mmol/L Dextrose under zero-flow conditions at 37°C. Ethylene glycol tetraacetic acid (EGTA; 29mmol/L) and sodium nitroprusside (SNP; 10^{-5}mmol/L) were added to the bath to maintain the smooth muscle in a relaxed state. A pressure-response curve was constructed by increasing the intraluminal pressure from 0-120mmHg at 20mmHg increments. The PCAs and PAs were equilibrated at each pressure for 5 minutes then lumen and outer diameters were measured using a 10X objective (Nikon Plan objective; Numerical Aperture: 0.25) with a Nikon Eclipse TS100 microscope. The average of the outer and lumen diameter at each pressure was recorded using MyoVIEW II 2.0 software (Danish Myo Technology, Aarhus, Denmark). These measures were compared and used to calculate wall thickness (outer diameter – lumen diameter). Wall cross-sectional area was calculated using the formula “artery area – lumen area”. The wall-to-lumen ratio, wall stress, strain, and distensibility were calculated as described previously (27). Wall stiffness was quantified using the β-coefficient calculated from the individual stress-strain curves using the model ($y=ae^{\beta x}$); y wall stress, x is wall strain, a is the intercept and β is the slope of the exponential fit; a higher β-coefficient represents a stiffer vessel (9).

2.3.7 – qRT-PCR

RNA was extracted from isolated PCAs for qRT-PCR analysis using Trizol. RNA was reverse transcribed using VILO reverse transcriptase (Invitrogen, Carlsbad, CA). TAQMAN-specific probes were used for the PCR to assess the mRNA expression of MR, MMP-2, MMP-9, MMP-12, MCP-1, TNFα, IL-6, Nox2, Nox4, Collagen (Col) 2a1, Col3a1, agt1r and YM1. mRNA expression is expressed as the fold change from control using the $2^{-\Delta\Delta Ct}$ method[12]. β2-microglobulin was used for normalization.

2.3.8 – Drugs and chemicals

All drugs and chemicals were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise stated.

2.3.9 – Statistical analyses

All data are presented as mean ± standard error of the mean (SEM). Blood pressure, plasma aldosterone, mRNA expression and cerebral perfusion data were analyzed by one-way analysis of variance. For analysis of artery structure, two-way analysis of variance with repeated measures in one factor (pressure) was utilized followed by Bonferroni- adjustments for post-hoc comparison. All statistical analyses were performed using GraphPad Prism 7.0 software (GraphPad, San Diego, CA). In all cases statistical significance was denoted by $p<0.05$.

2.4 – Results

2.4.1 – MR antagonism does not change PA and PCA structure under control conditions.

To test the possibility that under normal conditions MR activation regulates artery structure, Sham-operated mice were treated with EPL. MR inhibition in normotensive

mice did not cause PA or PCA remodeling as evidenced by the lack of difference in the outer diameter, lumen diameter, or wall cross-sectional area when compared to Sham mice (Figure 2.1). Measurements are presented at the physiological intralumenal pressure of 40mmHg for the PAs and 60mmHg for the PCA. As previously demonstrated in the aorta (10), I confirmed that the ECMRKO mice also had decreased MR mRNA expression in the PCA when compared to the MR-intact littermates (Figure 2.2). There was still some MR mRNA expression in the PCAs from the ECMRKO mice because the vascular SMC also express the receptor. While I expect MR gene recombination in all ECs, due to insufficient tissue from the PAs, I could not measure changes in gene expression of the MR. Specific EC-MR deletion did not change the structure of the PAs or PCAs under normotensive conditions. Outer diameter, lumen diameter and wall area were not changed when compared to MR-intact littermates (Figure 2.3). MR inhibition and specific EC-MR deletion also did not alter cerebral perfusion as shown in the C57Bl/6 mice treated with EPL and the ECMRKO mice (Figure 2.4).

2.4.2 – AngII increases systolic blood pressure and plasma aldosterone.

2.4.2.1 – C57Bl/6 mice

To assess the role of the MR in the genesis of AngII-hypertension, C57Bl/6 mice were treated with EPL during the 4 weeks of AngII infusion (800ng/kg/min). AngII treatment increased systolic blood pressure (Table 2.1) but EPL did not reduce blood pressure suggesting that MR activation is not the major driving force for the elevation in blood pressure caused by AngII administration. As expected, due to its role in activating adrenal mineralocorticoid production, AngII infusion increased plasma

aldosterone levels. This was not altered by EPL treatment (Figure 2.5 A). The mice treated with AngII and the vehicle for EPL had a similar increase in aldosterone (Table 2.2). Plasma corticosterone levels remained unchanged in all groups (Figure 2.5 C).

2.4.2.2 – ECMRKO mice

As previously reported (22), EC-MR specific deletion is not associated with changes in baseline blood pressure (Table 2.1). ECMRKO mice were infused with AngII and compared to MR-intact littermates with AngII treatment or Sham surgery. AngII increased systolic blood pressure to the same extent in the MR-intact and ECMRKO mice when compared to untreated MR-intact and ECMRKO mice (Table 2.1). Plasma aldosterone levels were not different between control MR-intact and ECMRKO mice and AngII treatment increased plasma aldosterone to the same degree in both mouse strains (Figure 2.5 B). Plasma corticosterone was not changed in any group (Figure 2.5 D).

2.4.3 – Specific deletion of the endothelial MR attenuated the reduction in cerebral perfusion induced by AngII-hypertension.

I next determined the effect of AngII-hypertension on cerebral perfusion. As expected, AngII-hypertension reduced cerebral perfusion. I cannot confirm that EPL treatment improved perfusion because the vehicle for EPL (Figure 2.4 C) also had a protective effect (Table 2.2). However, the changes in perfusion were prevented by EC-MR deletion (Figure 2.4 D), which suggests that MR signaling in EC is involved in the process independent of changes in blood pressure. As the improvement in cerebral perfusion could be in part due to alterations in artery remodeling, I next examined the effect of MR inhibition on cerebral vascular structure in response to AngII-hypertension.

2.4.4 – MR antagonism prevents PA and PCA AngII-hypertensive remodeling in C57BL/6 mice.

As expected, AngII-hypertension caused inward hypotrophic remodeling of the PAs and PCA as evidenced by smaller outer diameters (Figure 2.6 A, 2.8 A), lumen diameters (Figure 2.6 B, 2.8 B) and wall areas (Figure 2.6 C, 2.8 C) in the AngII-treated mice when compared to Sham C57BL/6 mice. Wall-to-lumen ratio was increased by AngII-hypertension in the PAs but not in the PCAs (Figure 2.6 D, 2.8 E). In both artery types, artery cross sectional and lumen cross sectional area were reduced by AngII treatment, and MR antagonism prevented this reduction (Figure 2.7, 2.9). The changes in the mechanical properties of the PAs and PCAs in response to AngII-hypertension were distinct suggesting that the cerebral arteries and arterioles respond differently to the effects of AngII infusion and hypertension. The distensibility of the PAs and PCAs was not changed by AngII-hypertension nor by addition of EPL (Figure 2.7, 2.9). Conversely, AngII-hypertension reduced wall stress in the PAs (Figure 2.6 E), but not in the PCA (Figure 2.8 E); EPL treatment prevented the changes in PA wall stress. Wall stiffness was not changed by AngII-hypertension in either vessel type (Figure 2.6, 2.8 F). My data shows that MR inhibition prevented inward remodeling (Figure 2.6, 2.8) without changing blood pressure (Table 2.1) suggesting that the MR contributes directly to the process of vascular remodeling in response to AngII-hypertension independent of changes in blood pressure. The vehicle for EPL treatment did not improve AngII-induced artery remodeling (Figure 2.10).

2.4.5 – Endothelial MR signaling mediates PA and PCA remodeling in AngII-hypertension.

The benefits of MR antagonism were independent of blood pressure suggesting a potential direct effect on the vasculature. To explore this possibility, I determined the role of EC-MR signaling in the AngII-induced remodeling observed in the PAs and PCA. In the PAs, deletion of the MR, specifically from endothelial cells, prevented the inward remodeling as ECMRKO mice treated with AngII had increased outer diameter (Figure 2.11 A), lumen diameter (Figure 2.11 B), vessel area (Figure 2.12), lumen area (Figure 2.12), and wall area (Figure 2.11 C) when compared to MR-intact+AngII mice. As a result, there were no significant differences in cerebral vessel structure between MR-intact sham-treated mice and ECMRKO littermates treated with AngII. EC-MR specific deletion also prevented the change in wall-to-lumen ratio caused by AngII infusion (Figure 2.11 D). However, the decreased wall stress caused by AngII-hypertension in the MR-intact+AngII was not reversed by EC-MR deletion in the ECMRKO+AngII mice (Figure 2.11 E). This was contrary to the EPL study suggesting that changes in wall stress are mediated by MR outside the endothelial cells, maybe in the SMCs. As in the EPL study, wall stiffness (Figure 2.11 F) and artery distensibility were not significantly different in either group (Figure 2.12 C).

In the PCA, EC-MR deletion also prevented the AngII-induced remodeling as evidenced by the increase in the outer diameter (Figure 2.13 A), lumen diameter (Figure 2.13 B), vessel area (Figure 2.14 A), lumen area (Figure 2.14 B) and wall area (Figure 2.13 C) but the changes were not as marked as in the PAs. In the PCA, EC-MR deletion did not prevent the increase in wall-to-lumen ratio caused by AngII-hypertension (Figure

2.13 D) as it had in the PA. Distensibility (Figure 2.14 C) and artery wall stiffness (Figure 2.13 F) were not significantly changed by EC-MR deletion. EC-MR deletion did not reverse the reduced wall stress that was observed in MR-intact+AngII mice (Figure 2.13 E).

2.4.6 – AngII-hypertension and MR antagonism change the mRNA expression of MCP-1, IL-6 and MMP-2 in the PCA.

To explore potential mechanisms by which the MR contributes to cerebral artery remodeling, I assessed the PCA mRNA expression for markers of inflammation, macrophage infiltration, oxidative stress, and MMP which have been previously associated with AngII-induced vascular remodeling. Importantly, MR and AT_1Ra mRNA expression were unchanged in the PCA in response to AngII-hypertension and EPL treatment (Figure 2.15 A, M). Previous studies have shown that AngII-hypertension increases the expression of MMP-2 in the aorta and in SMC and that this is important for the process of arterial remodeling (33, 41). Consistent with these previous findings, AngII treatment significantly increased the mRNA expression of MMP-2 in the PCA and this appears to be mediated, at least in part, by MR signaling as EPL prevented the increase (Figure 2.15 B). AngII was also shown to increase the expression of MMP-9 in the aorta. However, in the PCA, MMP-9 mRNA level was not significantly changed by AngII or EPL treatment (Figure 2.15 C). MMP-12, produced by macrophages, may also play an important role in flow-induced artery remodeling (25). In this study, I show that AngII-hypertension increases the expression of MMP-12 mRNA in the PCA, but this does not appear to be mediated by MR signaling as MMP-12 expression was unchanged by EPL treatment (Figure 2.15 D).

AngII has also been shown to increase the production of NOX2 (7) as well as NOX4 which has been shown to be regulated by the MR in human coronary arteries ECs (3). However, in the PCA the mRNA expression of NOX2 and NOX4 genes were not changed in response to AngII-hypertension or MR inhibition (Figure 2.15 E, F). Inflammatory markers including monocyte chemoattractant protein 1 (MCP-1) and IL-6 were increased in the PCA in response to AngII (Figure 2.15 G, H). Other inflammatory markers including TNFα and the alternative macrophage marker YM1 tended to increase, but these changes were not statistically significant (Figure 2.15 I, J). The mRNA expression of two collagen genes, Col2a1 and Col3a1, were not significantly changed by AngII-hypertension or MR antagonism (Figure 2.15 K, L). In summary, MR antagonism did not change the degree of AngII induction of inflammatory genes nor did AngII change the mRNA expression of Nox2. However, AngII significantly increased mRNA expression of MMP-2 and this was prevented by EPL.

Treatment Group	Systolic blood pressure (mmHg)
Sham	147.2 ± 3.7
AngII	177.0 ± 3.9*
AngII+EPL	182.2 ± 6.0 *
MR-intact	141.9±1.5
ECMRKO	153.0±1.2
MR-intact + AngII	193.4±2.2#
ECMRKO + AngII	188.7±2.7#

Table 2.1 Blood pressures for C57bl/6 mice and ECMRKO mice. In C57bl/6 mice, AngII significantly increased systolic blood pressure compared to Sham; EPL treatment did not cause a further increase in blood pressure. There was no difference in systolic blood pressure between the MR-intact and ECMRKO mice. AngII increased systolic blood pressure in the MR-intact and ECMRKO mice when compared to untreated MR-intact and ECMRKO mice. Data are presented as mean ± SEM. *$p<0.05$ from Sham; #$p<0.05$ from MR-intact or ECMRKO mice by one-way ANOVA.

Parameter Measured	AngII + Peanut Butter (n=5)	Eplerenone (n=6)
Cerebral blood flow	866.4 ± 17.9 perfusion units	946.00 ± 17.18 perfusion units
Plasma aldosterone	241.8 ± 41.93pg/mL	

Table 2.2 Eplerenone and vehicle treated C57bl/6 mice. EPL treatment under normal conditions did not significantly change Sham C57Bl/6 mice. Vehicle treatment in AngII infused mice did not significantly change cerebral perfusion or plasma aldosterone compared to AngII treated C57bl/6 mice. Data are presented as mean ± SEM.

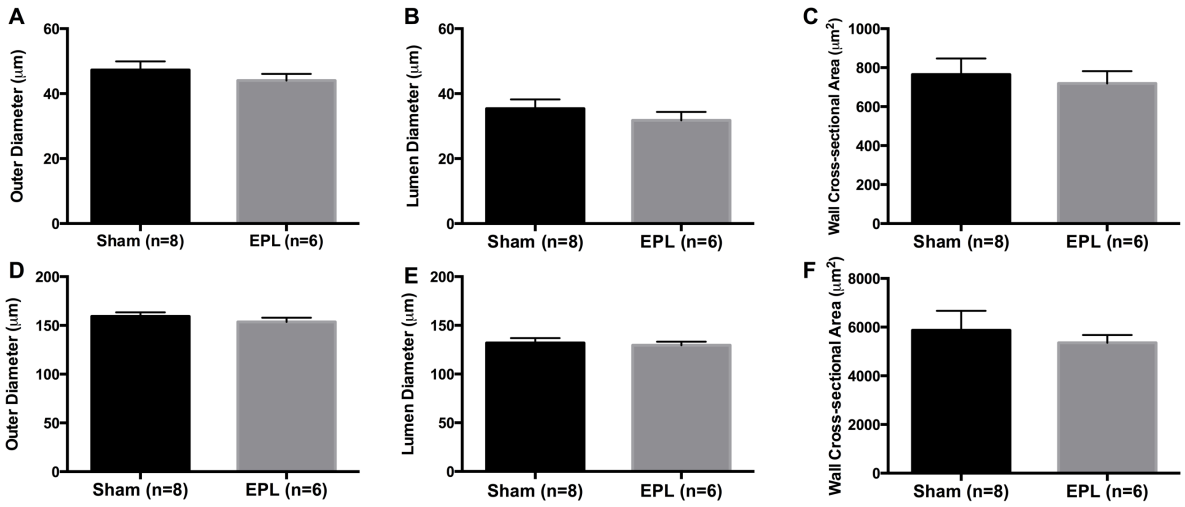

Figure 2.1 MR antagonism under control conditions does not change baseline artery structure. Structure was assessed in isolated arteries using pressure myography. EPL treatment alone did not change the PA (A) outer diameter, (B) lumen diameter, (C) wall area or PCA (D) outer diameter, (E) lumen diameter, (F) wall area. Data for the PAs are presented at 40mmHg intralumenal pressure and for the PCA at 60mmHg. No changes were observed at any other intralumenal pressure. Data are presented as mean ± SEM. $p>0.05$ for all by Student's t-test.

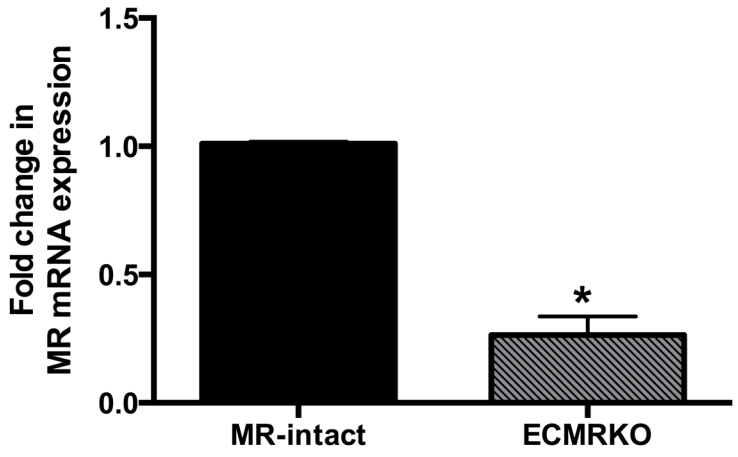

Figure 2.2 Gene expression of the MR in the PCA. The PCAs from ECMRKO mice have decreased mRNA expression of the MR compared to the MR-intact littermates. Data are presented as mean ± SEM n=3 in each group. *$p<0.05$ compared to MR-intact by Student's t-test.

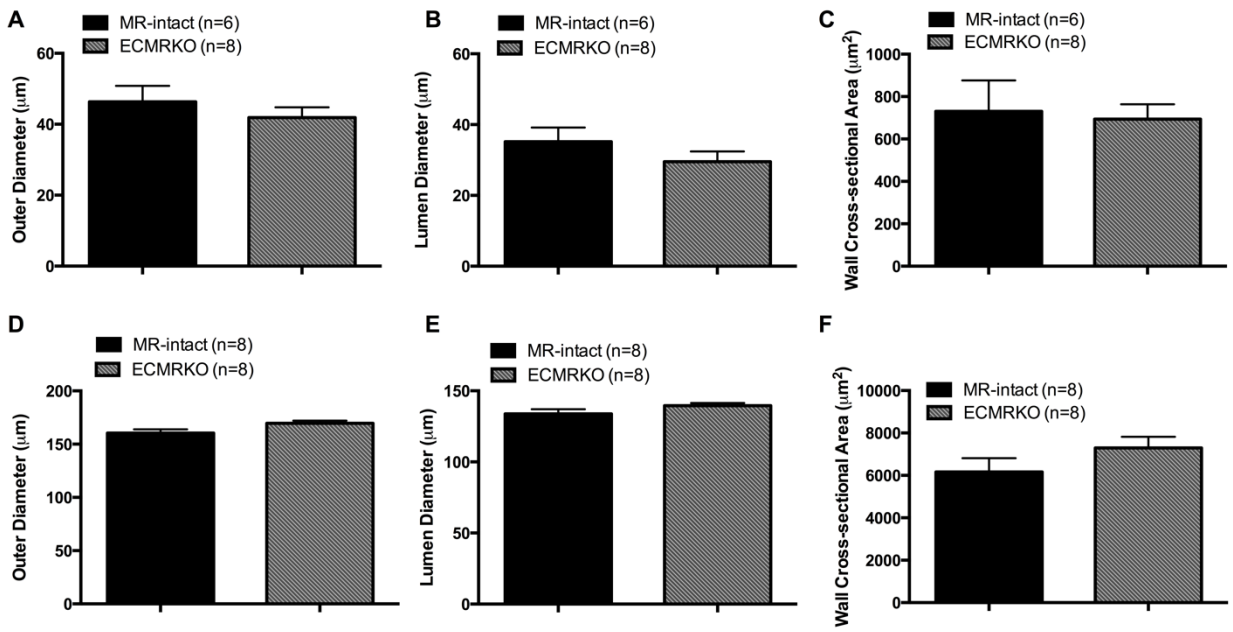

Figure 2.3 Endothelial MR deletion does not change PA or PCA structure under normotensive conditions. Structure was assessed in isolated arteries using pressure myography. Data are presented as mean ± SEM. In the PAs, EC-MR deletion did not cause any changes in the (A) outer diameter, (B) lumen diameter, or (C) wall area. Data at 40mmHg intralumenal pressure are shown. However, no changes were observed at any other intralumenal pressures (data not shown). In the PCA, EC-MR deletion did not change the (D) outer diameter, (E) lumen diameter, or (F) wall area. Data are shown at 60mmHg intralumenal pressure and no changes were observed at any other intralumenal pressures (data not shown). *$p<0.05$ vs MR-intact by Student's t-test.

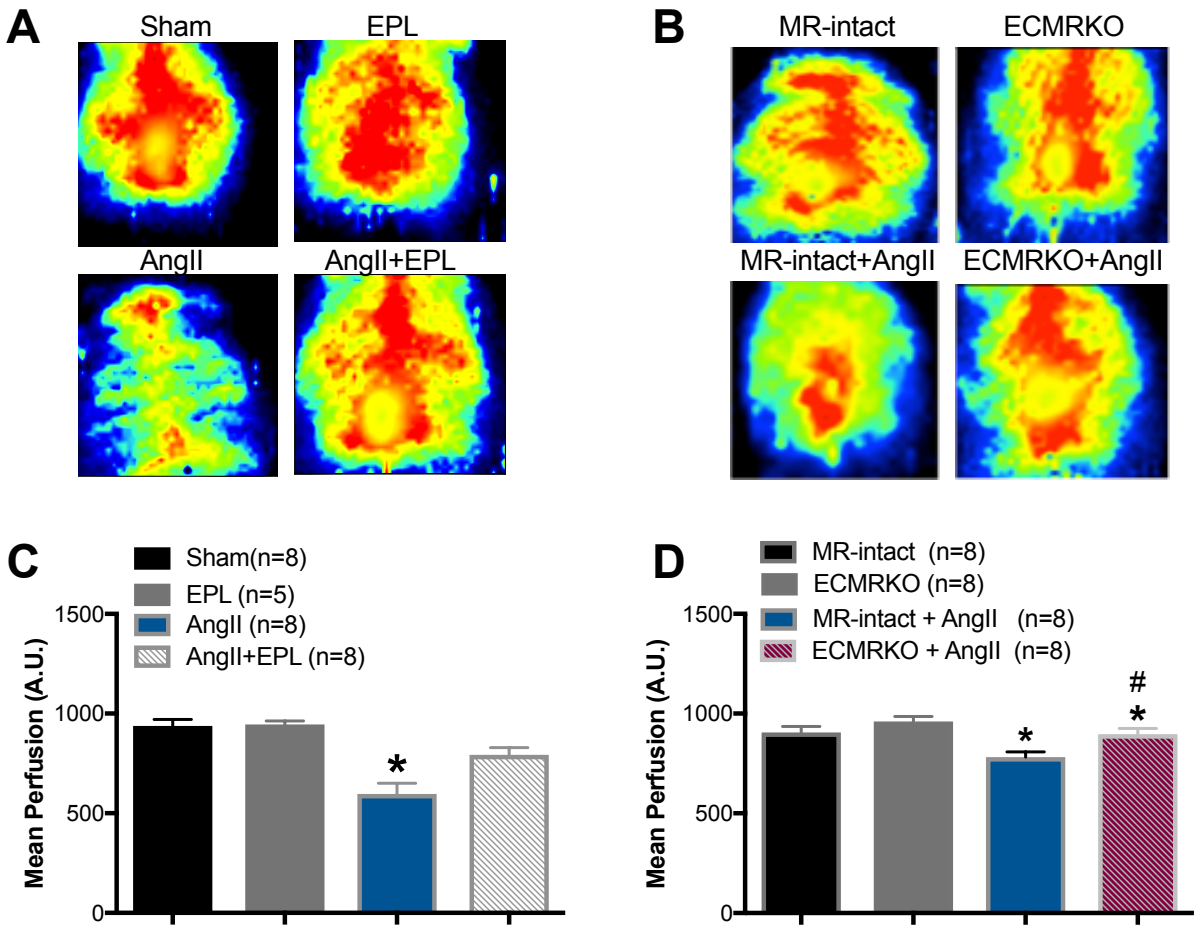

Figure 2.4 Endothelial MR deletion prevents a reduction in cerebral perfusion with AngII-hypertension. Cerebral perfusion was measured prior to euthanasia in anesthetized mice using scanning laser Doppler. (A, B) Representative images of the scanning laser Doppler are shown. In Figures C and D, data are presented as mean ± SEM. (C) Mean cerebral perfusion was reduced in AngII treated mice compared to Sham. Cerebral perfusion was improved in the AngII+EPL treated C57Bl/6 mice. (D) EC-MR deletion did not change baseline perfusion compared to MR-intact. AngII-hypertension decreased cerebral perfusion in MR-intact mice. EC-MR deletion in the ECMRKO+AngII significantly increased perfusion compared to the MR-intact+AngII mice. *p<0.05; vs. Sham, MR-intact or ECMRKO. #p<0.05 AngII vs. AngII+EPL or MR-intact+AngII vs. ECMRKO+AngII.

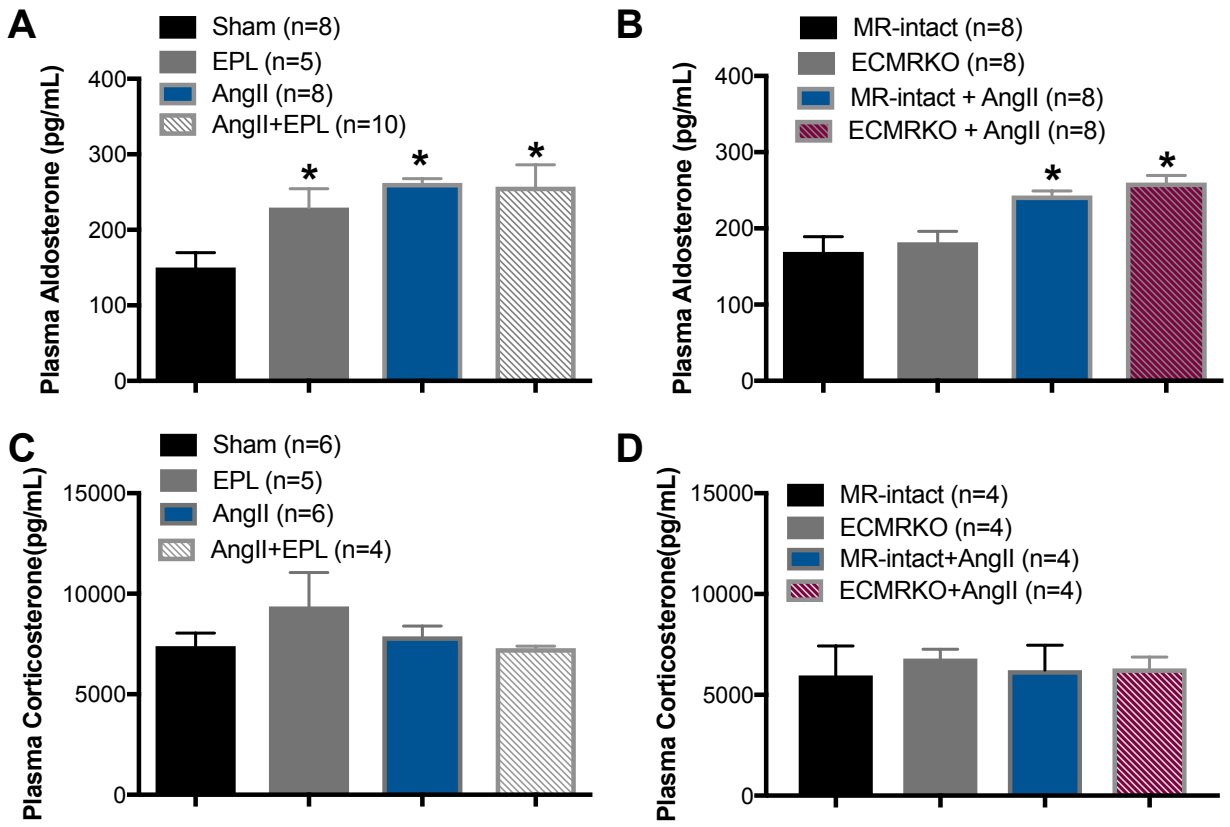

Figure 2.5 AngII-hypertension increases plasma aldosterone in C57bl/6 and EMCRKO mice. Plasma aldosterone and corticosterone were measured after 4 weeks of AngII or vehicle treatment. (A) EPL treatment increased plasma aldosterone levels when compared to Sham C57bl/6 mice. AngII-hypertension also increased plasma aldosterone, AngII+EPL treatment did not further increase aldosterone levels in C57Bl/6 mice. (B) EC-MR deletion did not alter plasma aldosterone levels when compared to MR-intact littermates in the absence of hypertension. AngII infusion significantly increased aldosterone levels in MR-intact and ECMRKO mice. (C-D) AngII-induced hypertension did not change plasma corticosterone levels. Data are presented as mean ± SEM. *$p<0.05$ vs Sham or MR-intact and ECMRKO.

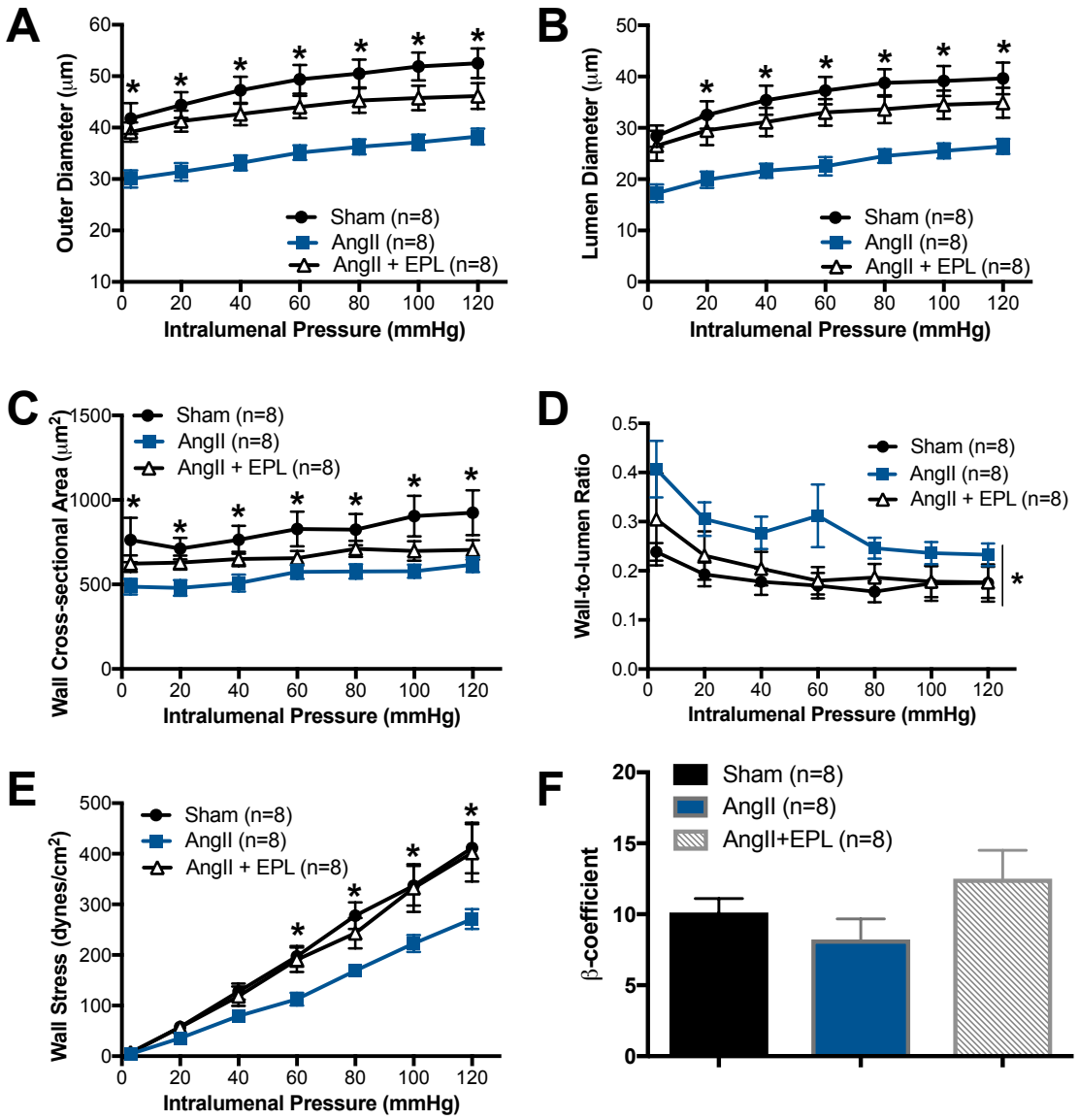

Figure 2.6 MR antagonism prevents PA inward hypotrophic remodeling with AngII-hypertension. The biomechanical properties were assessed in isolated PAs using pressure myography. AngII infusion resulted in a reduced (A) outer diameter, (B) lumen diameter, and (C) wall-cross sectional area. (D) Wall-to-lumen ratio was increased in the AngII treated mice. EPL treatment prevented these changes. (E) Wall stress was significantly reduced. However, (F) artery wall stiffness was not changed. Data are presented as mean ± SEM. *p<0.05 vs Sham or AngII+EPL.

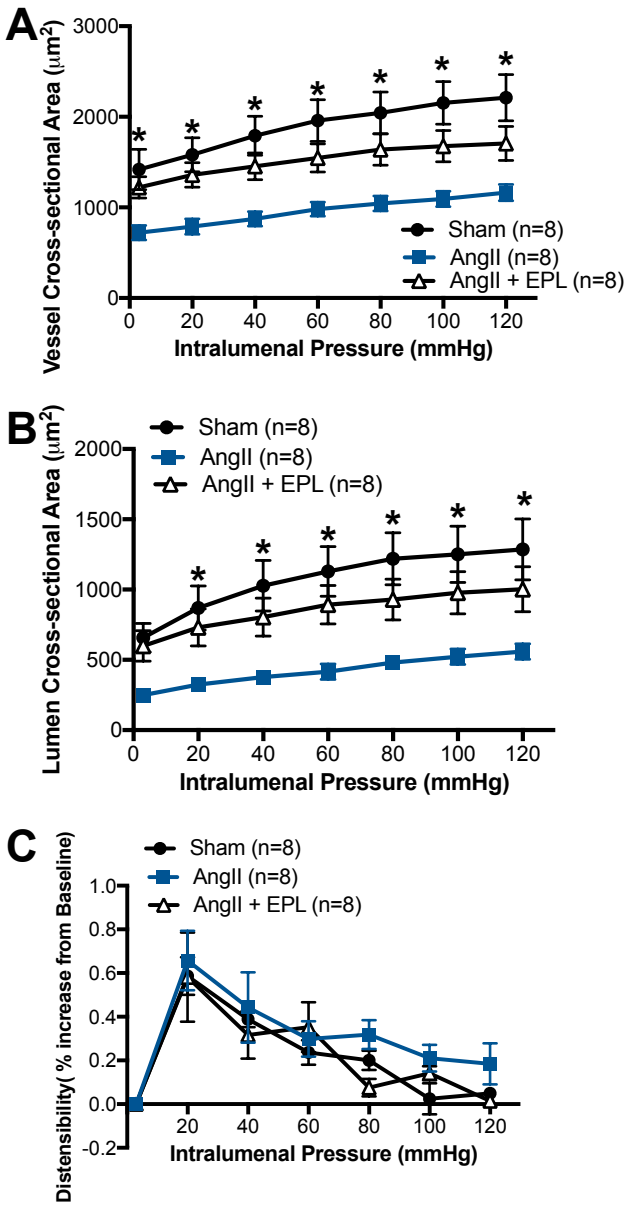

Figure 2.7 MR antagonism prevents PA inward hypotrophic remodeling with AngII-hypertension. The biomechanical properties were assessed in isolated PAs using pressure myography. Data are presented as mean ± SEM. AngII infusion resulted in a reduced (A) vessel area and (B) lumen area. EPL treatment prevented these changes. (C) Distensibility was not changed. *p<0.05 vs Sham or AngII+EPL by two-way ANOVA.

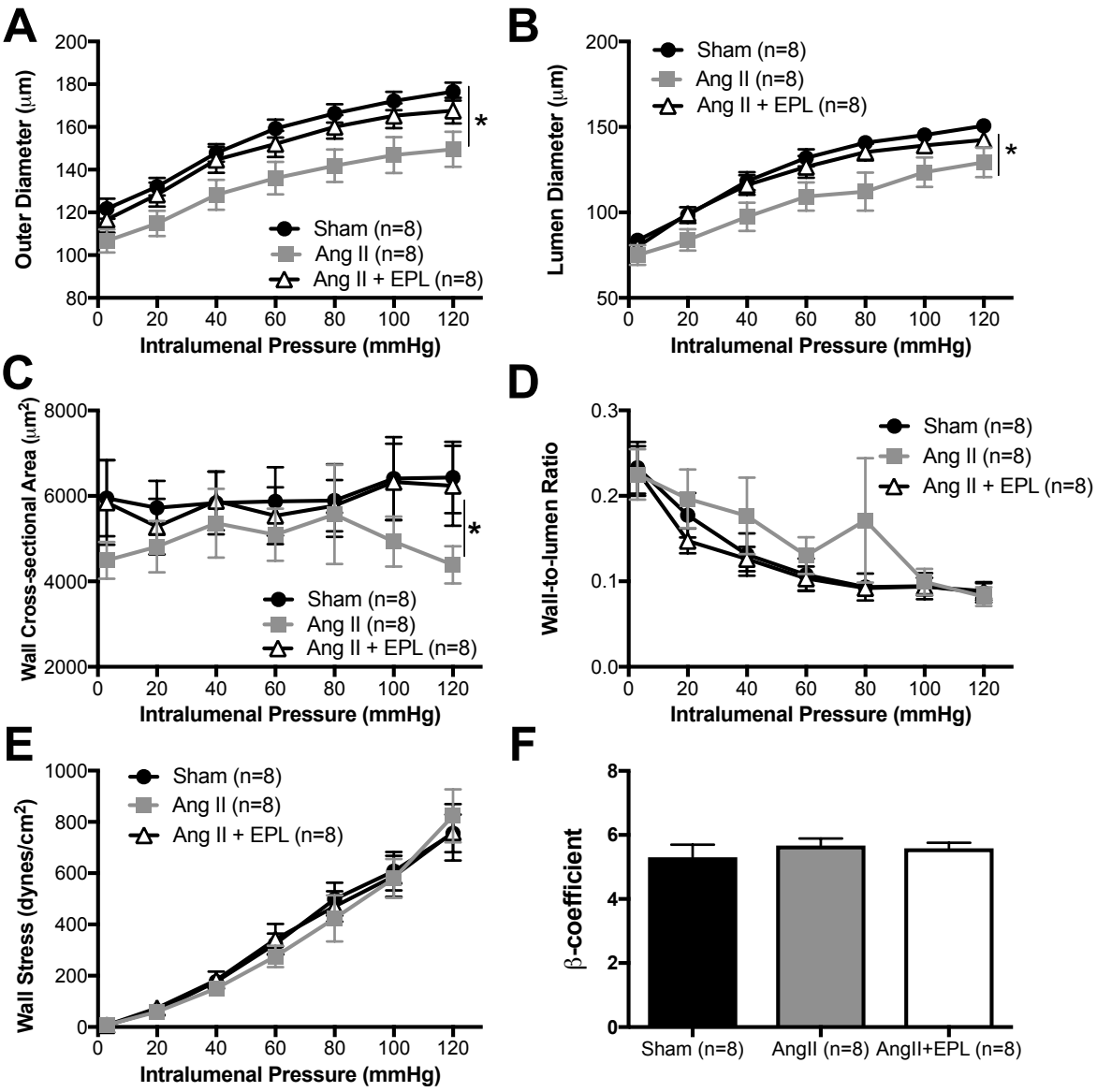

Figure 2.8 MR antagonism prevents inward remodeling of the PCA during AngII-hypertension. Structure was assessed in isolated PCAs using pressure myography. The (A) outer and (B) lumen diameter were reduced in AngII treated mice. (C) Wall cross sectional area was reduced at 120mmHg. (D) Wall-to-lumen ratio was not changed. EPL treatment prevented the inward remodeling of the PCA. (E) Wall stress and (F) wall stiffness remained unchanged. Data are presented as mean ± SEM. *p<0.05 vs Sham or Ang+EPL.

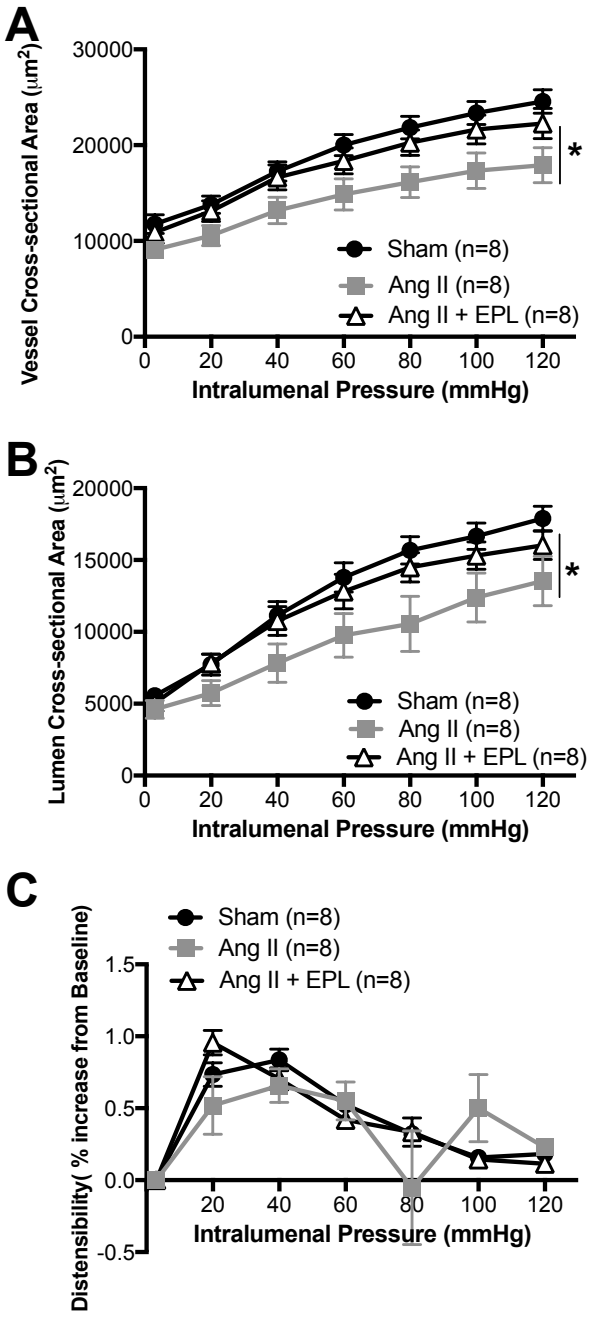

Figure 2.9 MR antagonism prevents inward remodeling of the PCA during AngII-hypertension. Structure was assessed in isolated PCAs using pressure myography. Data are presented as mean ± SEM. The (A) vessel area and (B) lumen area were reduced in AngII treated mice; EPL treatment prevented this. (C) Artery distensibility was not significantly changed. *p<0.05 vs Sham or Ang+EPL by two-way ANOVA.

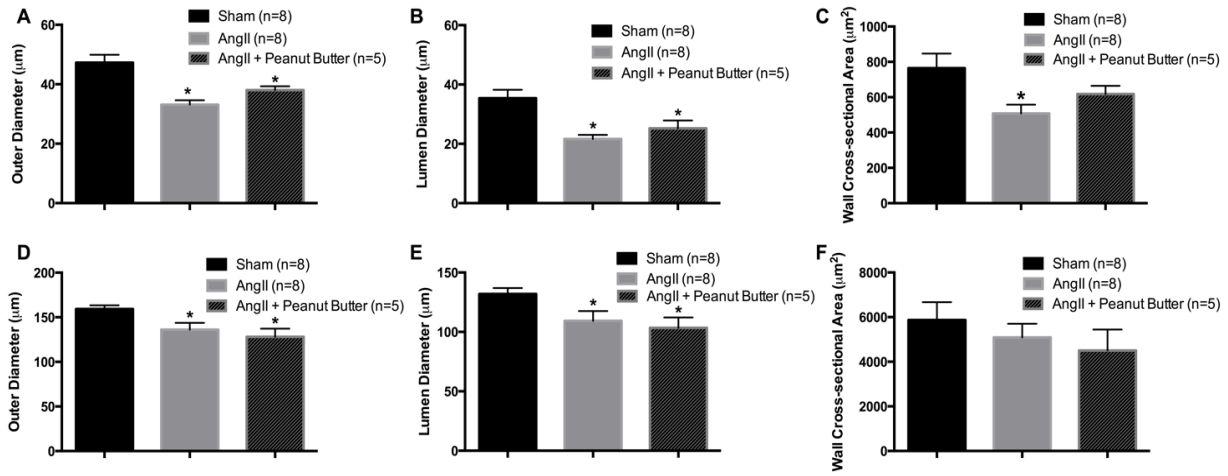

Figure 2.10 Treatment with the vehicle for eplerenone does not improve PAs or PCA remodeling. Artery structure was assessed in isolated arteries using pressure myography. Vehicle, peanut butter, treatment did not improve (A-C) PA or (D-F) PCA inward remodeling. Data are shown at the physiological pressure of 40mmHg for PAs and 60mmHg intralumenal pressure for PCA; no changes were observed at any other intralumenal pressures (data not shown). Data are presented as mean ± SEM. *$p<0.05$ vs Sham by one-way ANOVA.

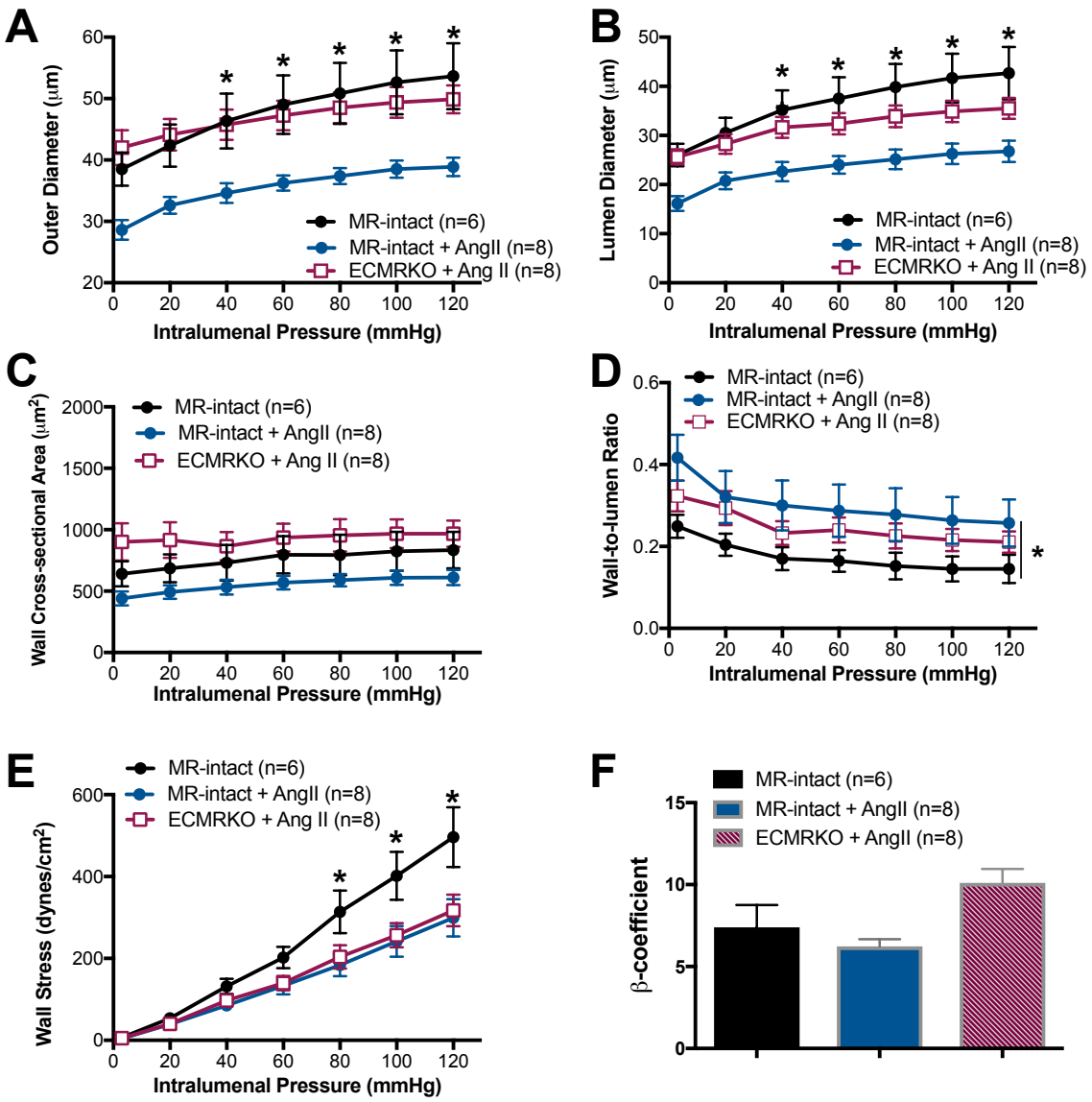

Figure 2.11 Endothelial MR signaling mediates PA remodeling during AngII-hypertension. AngII-hypertension significantly reduced the (A) outer diameter, (B) lumen diameter, and (C) wall area in MR-intact+AngII compared to MR-intact mice. (D) Wall-to-lumen ratio was increased in MR-intact mice. (E) Wall stress was also changed, but (F) wall stiffness was not significantly changed. EC-MR deletion prevented the inward hypotrophic remodeling, but it did not increase the wall stress. Data are presented as mean ± SEM. *p<0.05 vs MR-intact or ECMRKO+AngII.

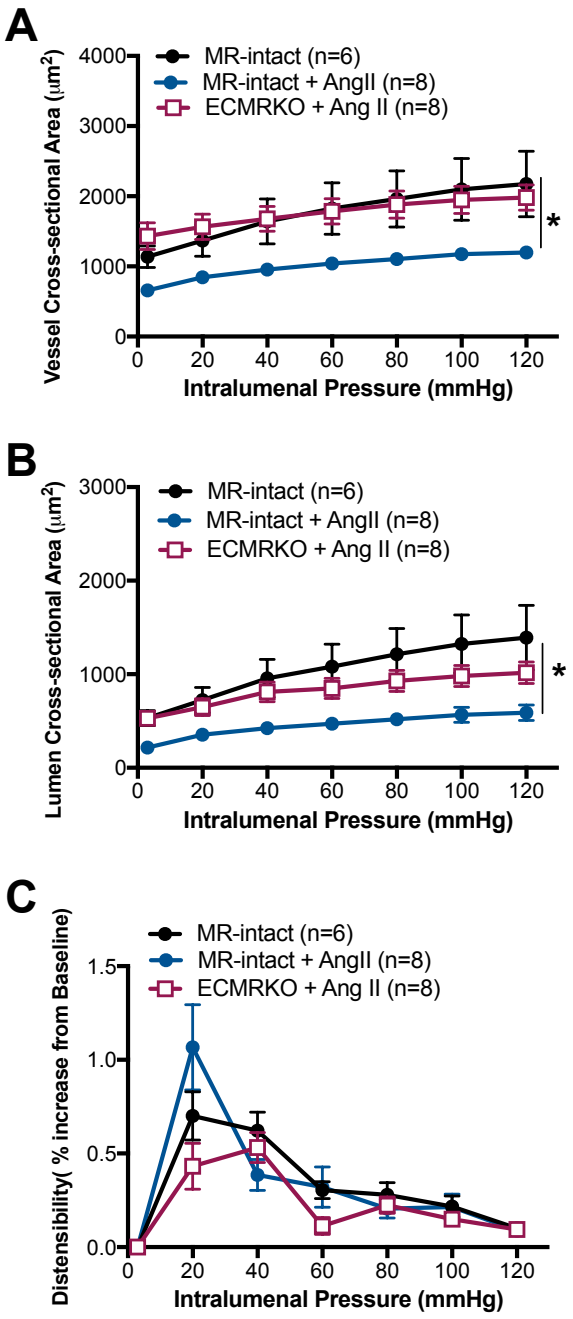

Figure 2.12 Endothelial MR signaling mediates PA remodeling during AngII-hypertension. Data are presented as mean ± SEM. AngII-hypertension significantly reduced the (A) vessel area and (B) lumen area in MR-intact+AngII compared to MR-intact mice. The mechanical property (C) distensibility was not changed. EC-MR deletion in the ECMRKO+AngII mice prevented the remodeling. *$p<0.05$ vs MR-intact or ECMRKO+AngII by two-way ANOVA.

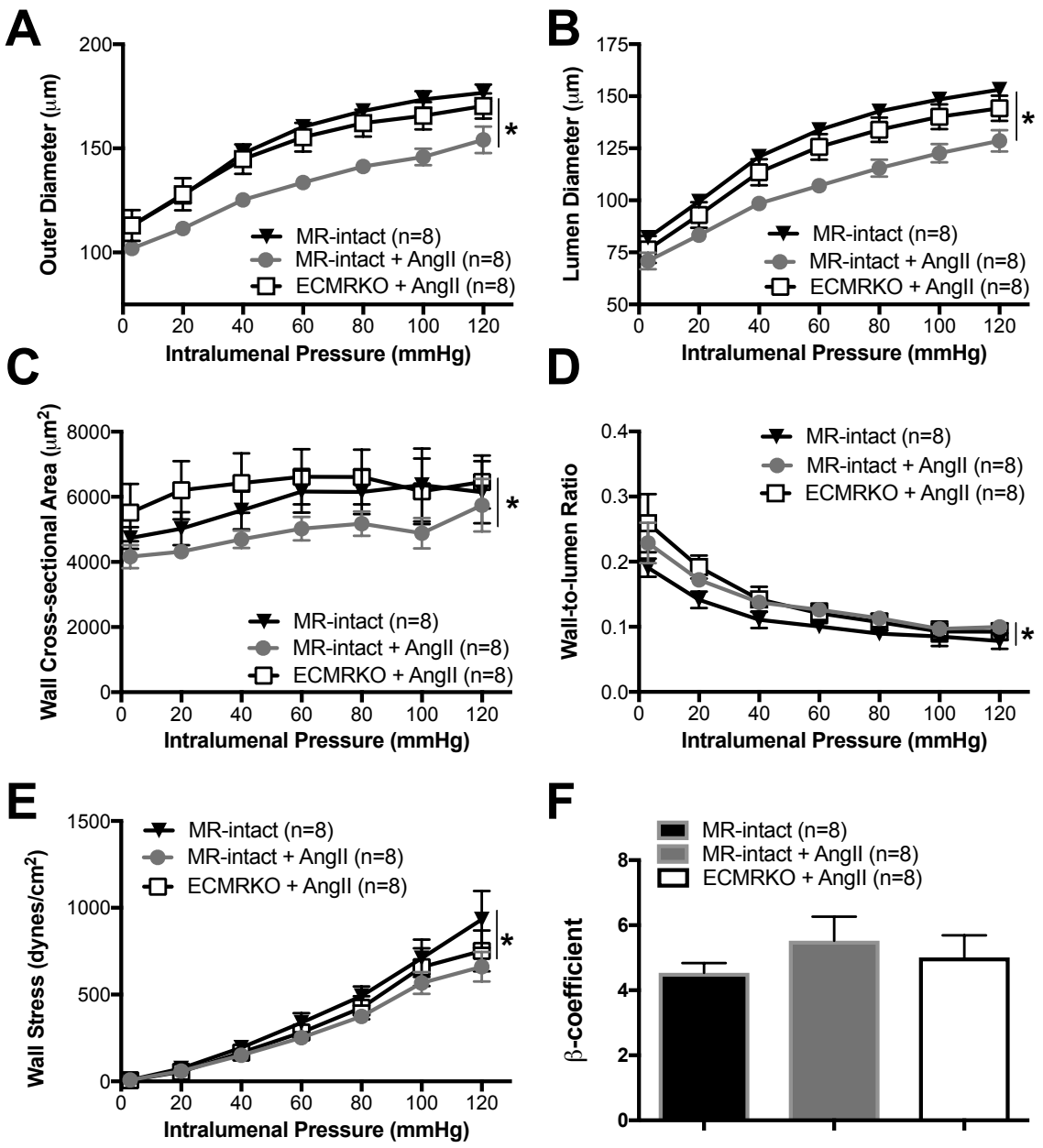

Figure 2.13 Endothelial MR deletion prevents PCA remodeling during AngII-hypertension. Structure was assessed in isolated arteries using pressure myography. AngII infusion resulted in inward remodeling evidenced by the reduced (A) outer, and (B) lumen diameter in the MR-intact+AngII compared to MR-intact mice. EC-MR deletion prevented the inward remodeling in the ECMRKO+AngII mice. (C) Wall area was not significantly changed in the MR-intact+AngII compared to MR-intact, but it was significantly increased in the ECMRKO+AngII compared to MR-intact+AngII mice. (D) Wall-to-lumen ratio did not change. (E) Wall stress was reduced in the MR-intact+AngII and ECMRKO+AngII compared to MR-intact. (F) Artery wall stiffness was not changed. Data are presented as mean ± SEM. *p<0.05 vs MR-intact or ECMRKO+AngII; #p<0.05 MR-intact+AngII vs ECMRKO+AngII.

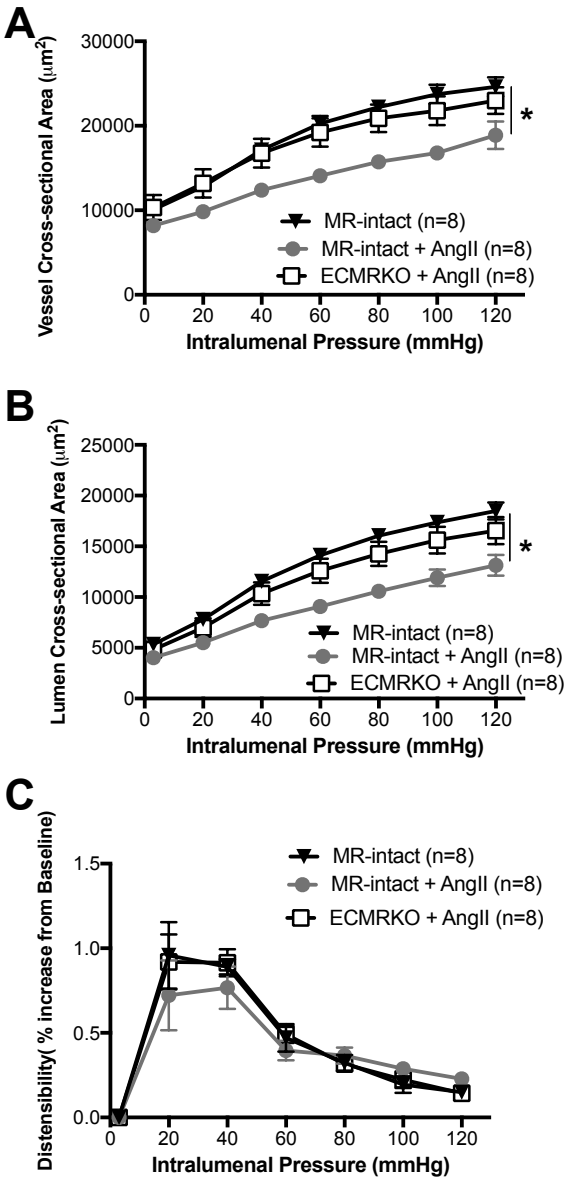

Figure 2.14 Endothelial MR deletion prevents PCA remodeling during AngII-hypertension. Structure was assessed in isolated arteries using pressure myography. Data are presented as mean ± SEM. AngII infusion resulted in inward remodeling evidenced by the (A) vessel area, and (B) lumen area in the MR-intact+AngII compared to MR-intact mice. EC-MR deletion prevented the inward remodeling in the ECMRKO+AngII mice. (C) Artery distensibility was not changed with AngII-hypertension. *p<0.05 vs MR-intact or ECMRKO+AngII by two-way ANOVA.

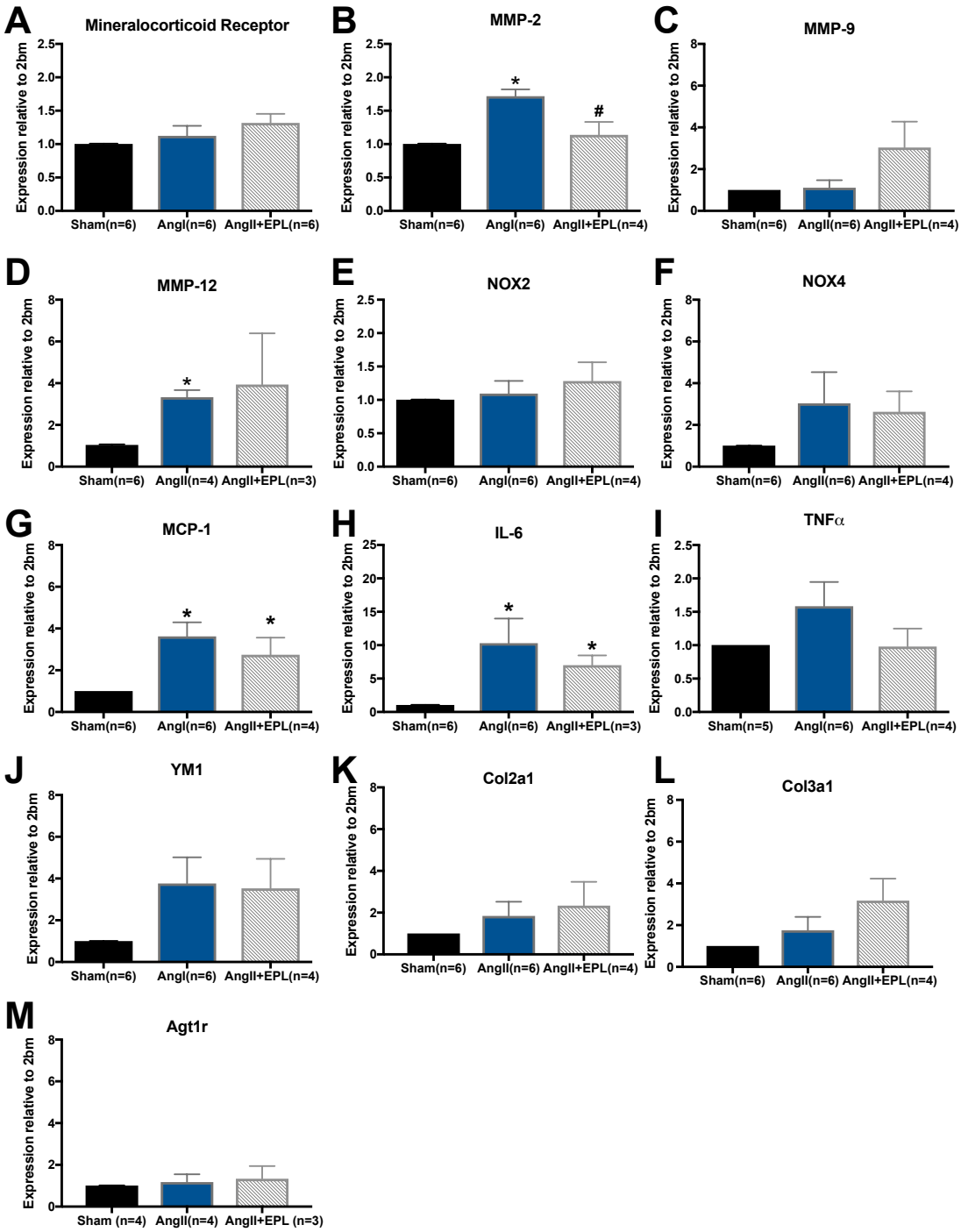

Figure 2.15 AngII-hypertension and MR antagonism change the mRNA expression of MCP-1, IL-6 and MMP-2. The mRNA expression of the (A) MR, (C) MMP-9, (E) NOX2, (F) NOX4, (I) TNFα, (J) YM1, (K) Col2a1, (L) Col3a1 or (M) AT_1R was not significantly changed by AngII infusion or EPL treatment when compared to Sham. (B) AngII increased the mRNA expression of MMP-2 and MR antagonism decreased this. (C,D) The mRNA expression of MMP-12, MCP-1 and IL-6 was increased with AngII infusion. *p<0.05 vs Sham; #p<0.05 AngII vs AngII+EPL by one-way ANOVA.

2.5 – Discussion

The novel findings in my study are that: 1) in the absence of hypertension, cerebral artery structure does not depend on MR activation; 2) the protective effect of MR inhibition in the cerebral circulation could be almost completely reproduced by EC-specific MR deletion suggesting that during AngII-hypertension, EC-MR is necessary for hypertensive vascular remodeling and that cerebrovascular protection by MR inhibition is mediated by EC-MR. These findings enhance our understanding of the role of vascular MR signaling in cerebral artery remodeling.

AngII promotes the secretion of aldosterone by activation of the AT_1R in the adrenal gland and animal studies have shown that increases in circulating levels of AngII or aldosterone are associated with adverse vascular remodeling. As expected, AngII-hypertension resulted in PA and PCA inward remodeling. The PAs also exhibited an increased wall-to-lumen ratio which has been shown to predict end organ damage. Despite the increase in wall-to-lumen ratio, the wall area was reduced in the AngII-treated mice; this hypotrophic remodeling was an unexpected finding that will be discussed in more detail later. The AngII-hypertension-associated changes in the mechanical properties were different in the two artery types supporting the need to study changes in the macro- and microcirculation separately. In the PAs, wall stress was decreased, but the same was not observed in the PCA. These differences could be associated with the location and innervation of these arteries and arterioles. To the best of my knowledge this is the first time that AngII-dependent artery remodeling has been studied in the PAs and PCAs from the same animal; this is a strength of my study because all of the effects of circulating factors are the same.

My data further support previous studies (12, 28, 32) indicating that MR activation contributes to inward hypotrophic remodeling in the PAs and PCAs during AngII-hypertension. However, those previous studies did not identify the cell type involved in MR-mediated cerebral artery remodeling. In the current study, I show that EC-MR is necessary for the AngII-dependent hypertensive cerebral artery remodeling. The role of EC-MR was independent of changes in blood pressure or basal cerebral vessel structure prior to exposure to AngII-hypertension suggesting a direct role for EC-MR in the response of the cerebral vasculature to hypertension induced by AngII. Whether EC-MR contributes to cerebral remodeling in response to hypertension caused by a distinct mechanism remains to be explored. Since hypertension is a disorder of the elderly and AngII signaling increases with aging and contributes to the rise in blood pressure with aging (42), this mechanism has important implications for the growing aging population in which dementia and stroke are important causes of morbidity and mortality.

This study implicates an important role for EC-MR in cerebral artery remodeling however, the mechanisms downstream of MR activation have not been identified. Additional in vivo studies that are beyond the scope of this study will be required to identify the mechanism because the remodeling process likely requires exposure to hypertension and circulating factors and hence cannot be reproduced in vitro. Previous studies have implicated oxidative stress, inflammation, and extracellular matrix remodeling in AngII induced artery remodeling (2, 4, 7, 18, 20, 21). In the PCAs I found that AngII-hypertension increases the mRNA expression of MCP-1 and IL-6. However, these changes in cytokine mRNA were not mediated by MR activation, because EPL

did not reduce MCP-1 or IL-6 mRNA levels. I did not observe any changes in the gene expression of collagen, but AngII-hypertension increased the mRNA expression of MMP-2 that was prevented by EPL treatment. The increase in MMP-2 mRNA appears to be specific, because no changes in MMP-9 and -12 mRNA were observed. These observations suggest the possibility that changes in MMP-2 induced by AngII-hypertension could involve MR signaling and contribute to vascular remodeling during AngII-hypertension, however additional studies are needed to confirm this potential mechanism. Although previous studies link aldosterone production to MMP-2 activity (34), other studies have also shown that AngII increased MMP-9 production to mediate pial arteriole remodeling (39) confirming regional heterogeneity in remodeling mechanisms, that the regulation of the MMPs is complex, and that MMP-2 might not be the only MMP regulating artery wall structure (14). Studies from our lab support a role for MMP-2 in the hypertensive cerebral artery remodeling process as doxycycline, an MMP-2 inhibitor, prevented cerebral artery remodeling in hypertensive rat (29). A limitation in my study is that I could only measure changes in mRNA expression in the PCA, but not in PA due to limited tissue availability.

The mechanical properties of the PAs and PCAs were differentially affected by AngII-hypertension. In the PAs, the decrease in diameter was accompanied by a reduction in wall stress that suggests that these arterioles remodeled to maintain wall stress within a normal range during hypertension. The changes in wall stress were prevented in the hypertensive mice by EPL treatment, but not by EC-MR deletion; this suggests that the effect of EPL on wall stress might be mediated by MR in SMC, or

some other cell type. However, this is speculative and additional studies with SMC-specific MR deletion will need to be conducted to confirm this possibility.

EC-specific MR deletion enhanced cerebral blood flow in mice with AngII-hypertension suggesting that MR inhibition in the endothelium is sufficient to protect from impaired cerebral blood flow in this hypertension model. Previous studies have shown that artery structure changes are associated with changes in perfusion (43). Although the changes in artery structure correlate well with the reduction in blood flow, it is important to note that AngII could also negatively impact endothelium-dependent vasodilation and that, and other factors could also contribute to decreased blood flow (1, 6, 19, 23, 40). Previous studies demonstrated that EC-MR deletion protects the mesenteric vasculature from endothelial dysfunction caused by AngII-hypertension (22). Future studies will examine whether this is also occurring in the cerebral vasculature in addition to the structural benefits of EC-MR deletion. Since EC-MR deletion preserved cerebral blood flow in the setting of AngII-hypertension and AngII is an important driver of hypertension in the elderly (11, 42), future studies could explore whether this could have implications for improving cognitive function and stroke outcomes.

Some limitations of my study must be acknowledged. First, VE-Cadherin is also expressed in a small compartment of hematopoietic cells. All of the studies were done in male mice, so I did not assess possible sex differences. Although I implicate EC-MR signaling in cerebral artery remodeling in AngII-hypertension, I have not determined the detailed molecular mechanism. My studies suggest IL-6, MMP-2, -9 are in involved in the AngII-induced hypertensive remodeling process and the data I have thus far suggest that MMP-2 could be a critical mediator of the MR dependent remodeling, but

additional studies are required to verify this. Another limitation is that the vehicle for EPL improved cerebral blood flow; however, it did not alter artery structure. This disconnect between structure and flow could be a consequence of the technique used to assess blood flow. The laser in the Pim3 system I used has a penetration of approximately 1mm, with the skull intact it is likely that I was measuring only the blood flow on the surface of the brain, and not the flow in the PAs.

I have not studied remodeling of the pial arteries, so it is unclear if changes in their structure can explain the differences in cerebral blood flow. However, the fact that in the AngII-treated ECMRKO mice the perfusion was improved suggests that EC-MR signaling is involved in the changes in perfusion. Cerebral blood flow was measured in mice anesthetized with isoflurane which is a vasodilator (37), I also did not measure blood gasses while the animals were under anesthetic, which is a limitation. However, the comparisons were made with appropriate controls also exposed to isoflurane. Nonetheless, additional studies using alternative methods are warranted. I recognize differences in dilator responses to isoflurane are possible between the groups, but this is a limitation of all studies of this type.

I only assessed changes in gene expression of the PCA because the limited tissue from the PAs did not allow accurate measurement of changes in gene expression. I measured blood pressure using tail-cuff plethysmography; I recognize that telemetry blood pressure measurements are generally considered to be more accurate than tail-cuff. The increase in blood pressure after AngII infusion was consistent in every mouse and, recent studies show that tail-cuff blood pressure measurements in AngII treated mice are very similar to those obtained by telemetry in an undisturbed mouse.

The role of the MR in SMC in hypertensive artery remodeling should also be considered as SMC MR contributes to myogenic tone and arterial remodeling in response to carotid injury and to vascular stiffness in response to aldosterone-salt induced hypertension (15, 30). Future studies will also consider whether there are EC-MR-mediated changes in endothelium-dependent vasodilation and myogenic tone in cerebral vessels in response to hypertension. Previous studies have shown that PAs and PCA from hypertensive rats have increased myogenic tone compared to normotensive WKY rats (24).

My study fills a gap in our knowledge of how MR signaling mediates hypertensive remodeling in arterioles in the microcirculation and arteries in the pial circulation in the brain. Hypertension has been linked to cerebrovascular diseases such as ischemic stroke and the development of VCID (31, 35). I utilized ECMRKO mice to show, for the first time, the role-played by the EC-MR in cerebral artery remodeling and that MR inhibition or EC-MR deletion improved cerebral blood flow without changing blood pressure. Identification of the cell specific actions of aldosterone and MR signaling could allow us to better define the downstream mechanisms of MR-mediated cerebral artery remodeling in hypertension. This could contribute to the development of better therapeutic approaches to improve cerebrovascular health in hypertensive patients, a rapidly growing concern in our aging population.

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CHAPTER 3

Mineralocorticoid Receptor Antagonism Improves Parenchymal Arteriole Dilation Via a TRPV4-Dependent Mechanism and Prevents Cognitive Dysfunction in Angiotensin II-Hypertension

3.1 – Abstract

Hypertension and MR activation cause cerebral parenchymal arteriole remodeling; this can limit cerebral perfusion and contribute to cognitive dysfunction. I utilized a mouse model of AngII-induced hypertension to test the hypothesis that MR activation impairs both TRPV4-mediated dilation of cerebral PAs and cognitive function. 16-18-week-old male C57bl/6 mice were treated with AngII (800 ng/kg/min) ± the MR antagonist, EPL (100mg/kg/day) for 4 weeks; sham mice served as controls. Data are presented as mean ± SEM; n=5-14 per group. EPL prevented the increased PA myogenic tone and impaired CCh-induced (10^{-9}-10^{-5}mol/L) dilation observed during hypertension. The CCh-induced dilation was EDH mediated because it could not be blocked by L-NAME (10^{-5}mol/L) and indomethacin (10^{-4}mol/L). I used GSK2193874 (10^{-7}mol/L) to confirm that in all groups this dilation was dependent on TRPV4 activation. Dilation in response to the TRPV4 agonist GSK1016790A (10^{-9}-10^{-5}mol/L) was also reduced in the hypertensive mice and this defect was corrected by EPL. In the hypertensive and EPL treated animals, TRPV4 inhibition reduced myogenic tone, an effect that was not observed in arterioles from control animals. EPL treatment also improved cognitive function and reduced microglia density in the hypertensive mice. These data suggest that the MR is a potential therapeutic target to improve cerebrovascular function and cognition during hypertension.

3.2 – Introduction

MR signaling causes cerebral artery and arteriole remodeling in rodent models of hypertension (13, 33, 37). The studies in Chapter 2 showed that MR signaling at the level of the endothelium, mediates hypertensive inward cerebral artery remodeling that results in a reduction of artery lumen diameter and reduced cerebral perfusion (13). These changes in artery structure, particularly of the PAs, could increase the risk for cSVD. The PAs regulate blood flow to the microcirculation and the occlusion of a single PA can result in microinfarcts and cognitive dysfunction (40, 43). Cerebral perfusion is regulated by a combination of artery structure and function; the latter includes the basal tone and the dilator capacity of arteries and arterioles. Impaired artery function could have detrimental effects on perfusion and neuronal and cognitive function.

We know that MR activation impairs dilation of arteries where the primary dilator pathway is NO (6, 36). However, we know less about the effects of MR activation in vessels, such as the PAs, where EDH is an important dilator pathway (7, 28). In addition, MR activation can contribute to changes in memory and cognition (22). The changes in cognitive function could be the result of reduced cerebral perfusion caused by artery remodeling and MR activation.

There is emerging evidence that aldosterone and MR activation also impair ion channel function in peripheral arteries (15, 50). I am particularly interested in TRPV4 channels because of its role in PA endothelium-dependent dilation in normotensive and hypertensive rats (27, 28). TRPV4 channels are Ca^{2+}-permeable, non-selective cation channels that mediate acetylcholine-induced, EDH-mediated dilation in mouse MCAs as a result of IK_{Ca}/SK_{Ca} activation in endothelial cells (7, 45). The role of MR signaling in

TRPV4-mediated dilation has not been studied. My previous studies show that MR antagonism improves cerebral perfusion in hypertensive mice (13) and this increased perfusion could possibly be a result of improved cerebral artery dilation. Therefore, I tested the hypothesis that MR activation impairs TRPV4-mediated dilation in PAs and impairs cognitive function during hypertension. I used a pharmacological approach with the MR antagonist EPL (100mg/kg/day) (13) to test this hypothesis in a mouse model of AngII-dependent hypertension. I chose to perform my studies using the AngII-dependent model of hypertension. AngII is the active component of the RAAS and it has been shown to have wide ranging detrimental effects of the cerebral vasculature (32).

3.3 – Materials and Methods

3.3.1 – Experimental model and treatment

All experimental protocols were approved by the Michigan State University Animal Care and Use Committee and were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. C57Bl/6 mice (n=5-14 per group) were purchased from Charles River Laboratories. All animals studied were male and housed on 12h:12h light/dark cycle with food and water ad libitum.

3.3.2 – AngII infusion

To induce hypertension in the C57Bl/6 mice, AngII (800ng/kg/min) was infused subcutaneously via osmotic minipumps (Alzet model 1004, Durect Corp, Cupertino CA). Mice were anesthetized with 3% isoflurane in oxygen and body temperature was maintained at 37°C. A single dose of the analgesic Rimadyl (5mg/kg, SQ) and the antibiotic Baytril (5m/kg, IM) were administered immediately prior to the surgical procedure. A subcutaneous pocket was made, and mini-pumps were inserted; Sham-

operated mice underwent anesthesia, and a subcutaneous pocket was made, but mini-pumps were not inserted. Mice were 16-18 weeks old when the treatment began, and they were euthanized at 20-22 weeks (after 4 weeks of treatment). I have previously shown that this dose of AngII increases blood pressure and the effect is not blunted by EPL treatment (Sham: 147.2 ± 3.7; AngII: 177.0 ± 3.9; AngII+EPL: 182.2 ± 6.0mmHg) (13). A group of C57Bl/6 mice was also treated with the MR antagonist EPL (100mg/kg/day) (13, 33), given orally suspended in peanut butter for 4 weeks.

3.3.3 – Pressure Myography

The endothelial function of PAs was assessed by pressure myography (13, 28, 33). To isolate the arterioles, a 5 x 3 mm section of the brain containing the MCA was dissected, then the pia with the MCA were separated from the brain and the PAs branching off the MCA were used for experiments. Isolated arterioles were cannulated with two glass micropipettes in a custom-made cannulation chamber. Arterioles were equilibrated at 37°C in artificial cerebrospinal fluid (aCSF) containing 124mmol/L NaCl, 3mmol/L KCl, 2mmol/L $CaCl_2$, 2mmol/L $MgCl_2$, 1.25mmol/L NaH_2PO_4, 26mmol/L $NaHCO_3$ and 4mmol/L Glucose. A servo-null system was used to pressurize the arterioles, and a leak test was performed prior to each experiment. Arterioles were pressurized to 40mmHg (13) until the development of stable myogenic tone (% tone = [1-(active lumen diameter/passive lumen diameter)] x 100. The diameter of the arterioles was recorded using MyoView 2.0 software (Danish Myo Technology, Aarhus, Denmark).

3.3.4 – Parenchymal arteriole dilation

After the generation of myogenic tone, endothelium-dependent vasodilation was assessed by incubating the arterioles with increasing concentrations of the muscarinic receptor agonist CCh (10^{-9}-10^{-5}mol/L) in the bath. To assess the role played by EDH factors in the CCh-induced dilation, arterioles were incubated with the COX inhibitor indomethacin (indo; 10μmol/L) and the NOS inhibitor L-NAME (100μmol/L) for 30 minutes prior the development of myogenic tone, then CCh-induced dilation was assessed. To assess the role played by the TRPV4 channels in PA dilation, arterioles were incubated with the TRPV4 channel antagonist GSK2193874 (10^{-7}mol/L) for 10 minutes after the development of stable myogenic tone and then a CCh-induced dilation (10^{-9}-10^{-5}mol/L) was assessed. A separate group of PAs were incubated with increasing concentrations of the TRPV4 channel agonist GSK1016790A (10^{-9}-10^{-5}mol/L). To assess the role played by IK_{Ca}/SK_{Ca} channels, PAs were incubated with increasing concentrations of the agonist NS309 (10^{-9}-10^{-5}mol/L).

To assess the role of endothelial TRPV4 channels in the maintenance of myogenic tone, the endothelium of PAs was removed by passing an air bubble through the lumen of the arteriole. The PAs were then pressurized and after the development of stable myogenic tone I confirmed endothelium denudation by incubating the arterioles with CCh (10^{-4}mol/L) for 10 min. Successfully denuded arterioles did not dilate in response to CCh. PAs without endothelium were incubated with 10^{-7}mol/L GSK2193874 for 10 minutes and changes in myogenic tone were recorded. At the end of each experiment Ca^{2+} free buffer containing EGTA (29mmol/L) and SNP (10^{-5}mol/L) were added to the bath to maximally relax the smooth muscle. Dilation was calculated using the following formula: [lumen

diameter at drug concentration – baseline lumen diameter)/ (passive lumen diameter – baseline lumen diameter)] x 100.

3.3.5 – qRT-PCR

RNA was extracted from isolated PCAs and brain for qRT-PCR analysis using Trizol. RNA was reverse transcribed using VILO reverse transcriptase (Invitrogen, Carlsbad, CA). TAQMAN-specific probes were used for the PCR to assess the mRNA expression of TRPV4, IK_{Ca}, and SK_{Ca} in the PCAs. The mRNA expression of brain-derived neurotrophic factor (BDNF), synaptophysin (SYP), intracellular adhesion molecule-1 (ICAM-1), or doublecortin was assessed in mice brains. mRNA expression is expressed as the fold change from control using the $2^{-\Delta\Delta Ct}$ method. β2-microglobulin was used for normalization in both types.

3.3.6 – Novel object recognition

Mice were acclimated over a course of three days for 10 minutes per day in the arena (an open box with dark walls). On the testing day, mice were placed facing away from the objects and allowed to explore the arena for 10 minutes with two identical objects. After a retention time of 60 minutes, mice were returned to the arena in a similar manner and allowed to explore one familiar and one novel object for 3 minutes while tracked using EthoVision XT software. Exploration time of novel object and total time exploring were calculated (2).

3.3.7 – Barnes Maze

The training period occurred over 4 days. Each day, the mice underwent 3 trials (3min each) to learn the location of the escape hole. A 4000 Hz sound acted as an aversive stimulus while the mice explored the table. The aversive stimulus was removed when the

mice entered the escape hole. Mice were left in the escape box for 1 minute and then returned to the home cage. The escape hole was kept in the same location with sufficient visual cues around the room. On the fifth (probe) day, the escape hole was covered but kept in the same location, and movement of the mice was tracked for 90 seconds using EthoVision XT software (3).

3.3.8 – Nesting

Mice were given a fresh cage with a paper nestlet and allowed to build a nest overnight. The next day, the nest was scored on a scale of 0 though 5. The initial weight of a cotton nestlet was weighed before being placed in the cage. The time it took to integrate new material to the nest was calculated (11).

3.3.9 – Immunofluorescence

Mice were transcardially perfused with 60mL Ca^{2+} PSS containing 10^{-5}mol/L diltiazem and 10^{-4}mol/L heparin sodium salt followed by 60mL of 4% formaldehyde. 40µm sections of the cortex and hippocampus were made. For microglia quantification, free-floating brain sections were blocked and permeabilized in 0.1% Triton-X with 10% donkey serum PBS, then incubated in 1:200 rabbit Anti-IBA1 (catalog number 019-19741, Wako, Richmond, VA) overnight at 4°C. After washing in 1X PBS, sections were incubated in secondary AlexaFluor 488 donkey anti-rabbit antibody (catalog number ab150073, Abcam, Cambrige, UK) for one hour. For artery and capillary quantification, 40µm cryosections were incubated overnight in 0.01mg/ml isolectin GS-IB4 Alexa Fluo-568 conjugate (catalog number I21412, Invitrogen, Carlsbad, CA) at 4°C. The next day, sections were washed 4x in 1X PBS (5 min each wash) and coverslips were mounted using Prolong antifade reagent (Invitrogen, Carlsbad, CA) (23).

Two fields of the cortex and hippocampus, one in each hemisphere, were acquired using a 40X objective coupled to an inverted Olympus Confocal Laser Scanning microscope (Olympus America Inc, Central Valley, PA) with Olympus Fluoview FV1000 (Olympus America Inc, Central Valley, PA). Sections without the isolectin or IBA-1 served as negative controls. All the quantification were done manually by a blinded investigator using Image J (38).

3.3.10 –Drugs and chemicals

GSK1016790A and GSK2193874 were purchased from Cayman Chemicals (Ann Arbor, MI). NS309 was purchased from Tocris (Pittsburg, PA). All other drugs and chemicals were purchased from Sigma-Aldrich unless otherwise specified.

3.3.11 – Statistical analysis

All data are presented as mean ± SEM. Myogenic tone, mRNA expression, behavioral, and immunofluorescence data were analyzed by one-way analysis of variance. For analysis of artery vasodilation, two-way analysis of variance with repeated measures in one factor (drug concentration) was utilized followed by Bonferroni-adjusted t-tests for post-hoc comparisons. All statistical analyses were performed using GraphPad Prism 7.0 software (GraphPad, San Diego, CA). In all cases statistical significance was denoted by $p<0.05$.

3.4 – Results

3.4.1 – Mineralocorticoid receptor antagonism prevents increased parenchymal arteriole myogenic tone during hypertension.

The role of MR activation in the generation of myogenic tone of PAs was assessed. The PAs from AngII mice had increased myogenic tone when compared to the sham-

operated mice and this increased tone was prevented by EPL treatment (Figure 3.1 A), suggesting that MR activation enhances myogenic tone during AngII-hypertension. EPL did not change myogenic tone in normal conditions.

3.4.2 – Mineralocorticoid receptor antagonism prevents impaired endothelium-dependent dilation during hypertension.

As expected, PA endothelium-dependent dilation was impaired in the AngII-hypertensive mice. The impaired PA dilation was prevented by EPL treatment (Figure 3.1 B). EPL treatment did not change baseline PA endothelium-dependent dilation in normal mice (Figure 3.1 B). Previously, we have shown in SHRSP that when we inhibited NOS with L-NAME and COX with indomethacin the PA dilation was not altered suggesting that EDH factors contribute to the dilation (27). I confirmed this finding in the PAs from mice and explored if MR antagonism would reverse the impaired dilation. My data show that in mouse PAs, EDH is an important mediator of dilation irrespective of blood pressure or MR antagonism (Figure 3.2).

3.4.3 – TRPV4 channels are key regulators of parenchymal arteriole endothelium-dependent vasodilation and these are regulated by mineralocorticoid receptor activation.

To further explore the role of MR signaling in EDH-mediated dilation, I assessed TRPV4-dependent dilation in PAs. TRPV4 channel blockade with GSK2193874 (10^{-7} mol/L) inhibited CCh-induced dilation of PAs in all groups of mice (Figure 3.3 A-D). Conversely, activation of TRPV4 with GSK1016790A resulted in a robust dilation of the PAs from Sham (Figure 3.3 E). Hypertension impaired TRPV4 dilation and was prevented by EPL (Figure 3.3 E). I also confirmed that GSK2193874 was an effective inhibitor of TRPV4 (Figure 3.3 F). To show a role for EDH in PA dilation, I assessed IK_{Ca}/SK_{Ca} dilation

using the IK_{Ca}/SK_{Ca}-agonist NS309. My data show that the NS309 dilation is impaired in the AngII mice and this is prevented in the EPL treated group (Figure 3.4).

3.4.4 – MR antagonism prevents changes cerebral artery TRPV4 and IK_{Ca}/SK_{Ca} mRNA expression in AngII-hypertension.

The PCAs were isolated and TRPV4, IK_{Ca}, and SK_{Ca} mRNA expression was assessed. My data suggest that AngII mice had reduced TRPV4 and IK_{Ca} mRNA expression and this was prevented by EPL (Figure 3.5 A-B). The SK_{Ca} mRNA expression was increased in AngII mice and this was prevented by EPL (Figure 3.5 C).

3.4.5 – Endothelial TRPV4 plays a role in parenchymal arteriole myogenic tone.

My studies utilizing GSK2193874 to inhibit TRPV4 led to an unexpected finding; TRPV4 inhibition reduced myogenic tone in the PAs from AngII and AngII+EPL treated mice, but not in the sham-operated mice (Figure 3.6). To further explore if endothelial TRPV4 was involved in the changes in myogenic tone, PAs from AngII hypertensive mice were de-endothelialized. When PAs without endothelium were incubated with GSK2193874, there was no significant loss of myogenic tone suggesting that endothelial TRPV4 channels are involved in the modulation of myogenic tone in AngII mice (Figure 3.6).

3.4.6 – MR antagonism improves cognitive function in AngII-hypertensive mice.

MR activation during hypertension causes cerebral artery remodeling and impaired endothelium-dependent dilation that are associated with changes in brain perfusion (13). Therefore, I evaluated whether these vascular changes were associated with cognitive dysfunction in hypertensive mice. I first evaluated non-spatial, short-term memory using the novel object recognition test (8). The AngII mice spent less time exploring the novel

object, when compared to the sham-operated mice, and this was improved in the AngII+EPL treated mice (Figure 3.7 A). There was no statistically significant difference in the total time of exploration among all groups (Figure 3.7 B). I also evaluated changes in nest building as it is an important behavior that is altered by cognitive dysfunction (11). The AngII mice had a decreased nest score (Figure 3.7 C) and an increased time to integrate new material into nests (Figure 3.7 D) compared to sham; EPL treatment prevented these differences.

Spatial memory or executive function was evaluated using Barnes maze (4). The maze was divided into four quadrants: target, opposite, negative and positive quadrants (Figure 3.7 E). The escape hole was in the target quadrant. The AngII mice spent less time in the target quadrant and significantly more time in the opposite and positive quadrants. AngII mice treated with EPL behaved similarly to sham-operated normotensive mice (Figure 3.7 F). The AngII treated mice also visited the target quadrant fewer times compared to the sham and EPL treated mice (Figure 3.7 G). Together these data suggest that MR antagonism improves cognitive function in AngII-hypertensive mice.

3.4.7 – MR antagonism reduces the number of microglia in AngII-hypertension.

To explore possible mechanisms for the cognitive dysfunction, I counted microglia in the brain as a measure of inflammation (24) and assessed plasma protein oxidation as an index of oxidative stress (26). I used an Iba-1 antibody to label activated microglia, however, this antibody can also detect PVMs (39). Thus, we cannot rule out that some PVMs were also detected. My data indicate that AngII increased the density of Iba-1 labeled cells in the cortex (Figure 3.8 B) and hippocampus (Figure 3.8 C) suggesting an increase in the number of microglial in these regions. However, EPL treatment only

prevented the increased in microglia density within the cortex. There were no significant changes in plasma protein carbonyl content (Figure 3.9).

3.4.8 – AngII-hypertension reduces the mRNA expression of genes for neuronal support and synapse proteins.

I also evaluated changes in mRNA expression of markers for neuronal support in brain tissue. AngII reduced the mRNA expression of BNDF, SYP, doublecortin and ICAM-1; the reductions in BDNF, ICAM-1 and SYP were prevented by EPL (Figure 3.10). These data suggest that AngII-hypertension disrupts expression of genes involved in neuronal support and synapse formation that depends on MR activity.

3.4.9 – AngII-hypertension does not result in artery rarefaction.

I also evaluated if AngII-hypertension resulted in vascular rarefaction, because a reduced density of microvessels can also reduce perfusion and subsequently, neuronal health (32). There was no difference in the number of vessels between the AngII and sham-operated mice (Figure 3.11) suggesting that the changes in cognition were not associated with vessel rarefaction.

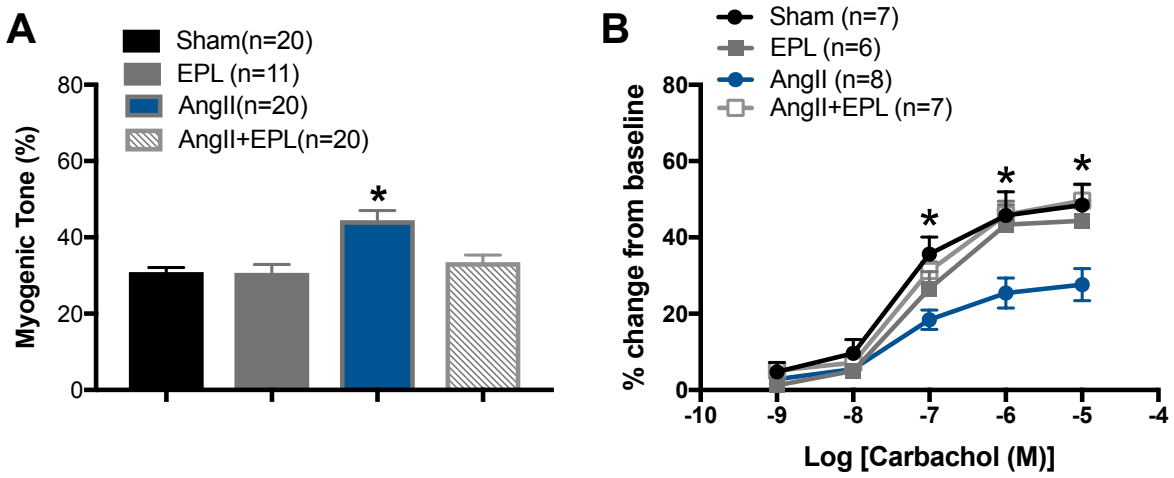

Figure 3.1 MR antagonism improves endothelium-dependent dilation and myogenic tone generation during hypertension. Myogenic tone and endothelium-dependent dilation in isolated PAs were assessed by pressure myography. (A) EPL did not change PA myogenic tone in normotensive sham mice. EPL prevented the increase in myogenic tone in the AngII-hypertensive mice. (B) EPL treatment did not change PA dilation in normotensive sham mice. AngII impaired PA dilation and was prevented by EPL. *$p<0.05$ by one-way or two-way ANOVA.

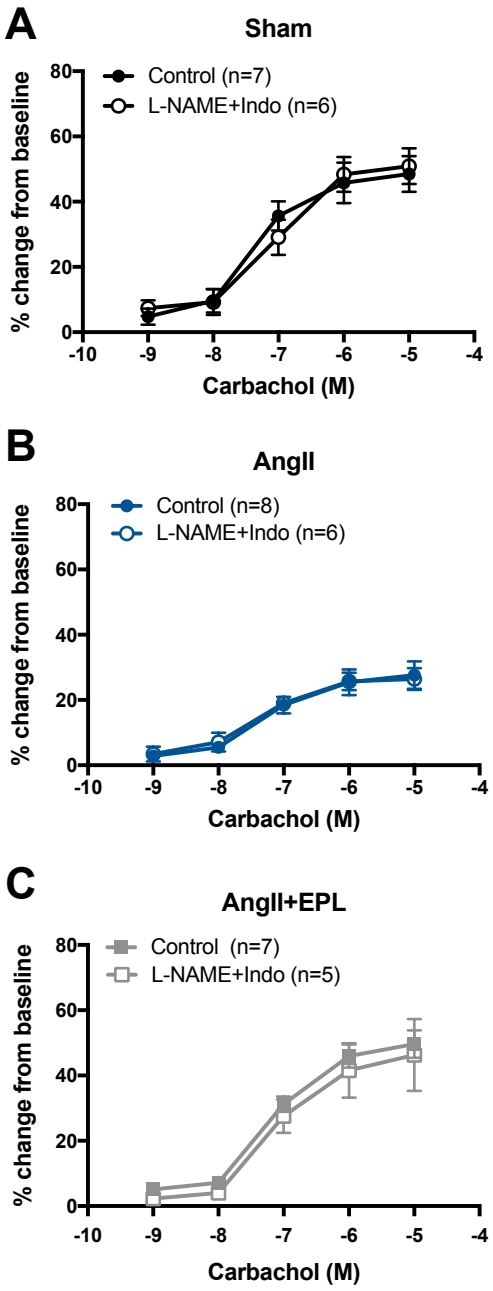

Figure 3.2 Endothelium-derived hyperpolarization mediates the PA vasodilation. PA endothelium-dependent dilation was assessed by pressure myography. The carbachol-mediated dilation in (A) Sham, (B) AngII, and (C) AngII+EPL was not altered by L-NAME (100μmol/L) + Indo (10μmol/L). $p>0.05$ by two-way ANOVA.

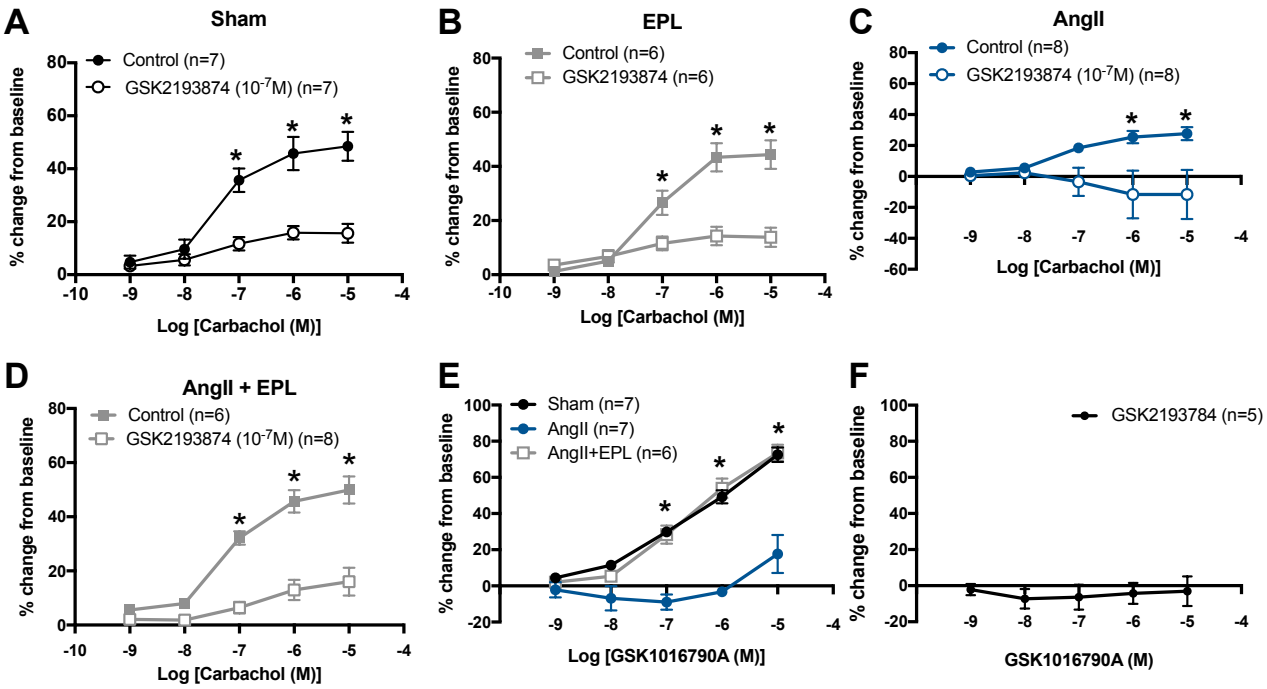

Figure 3.3 MR antagonism prevents the impaired TRPV4-mediated dilation of PAs during hypertension. PA endothelium-dependent dilation was assessed by pressure myography. TRPV4 inhibition with GSK2193874 prevented the CCh-mediated dilation in (A) Sham, (B) Sham+EPL, (C) AngII and (D) AngII+EPL treated mice. (E) GSK1016790A caused a dilation in the PAs from Sham mice. This dilation was impaired in the AngII-hypertensive mice; this reduced dilation was prevented by EPL treatment. (F) The TRPV4 antagonist, GSK2193874, effectively inhibits the GSK1016790A-mediated dilation. *p<0.05 by two-way ANOVA.

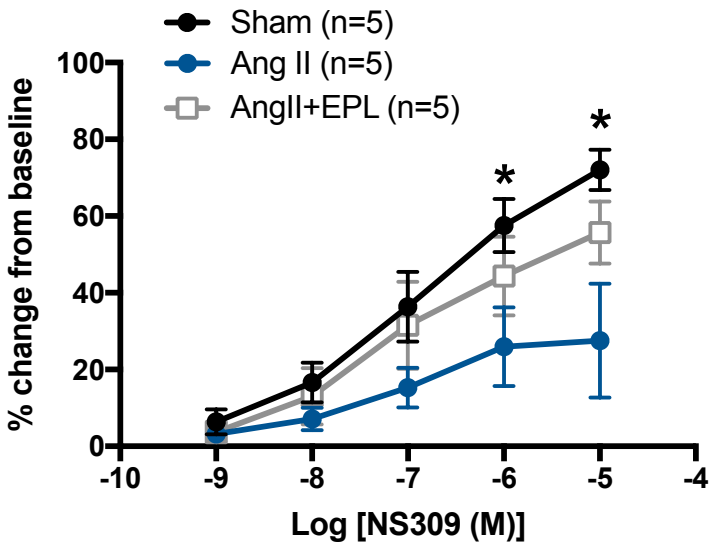

Figure 3.4 Intermediate and small conductance calcium activated potassium channel mediated dilation is reduced in AngII mice. The role of IK_{Ca}/SK_{Ca} in PA dilation was assessed using the agonist NS309 by pressure myography. The AngII mice had impaired dilation and this was prevented in the EPL treated group. *$p<0.05$ by two-way ANOVA.

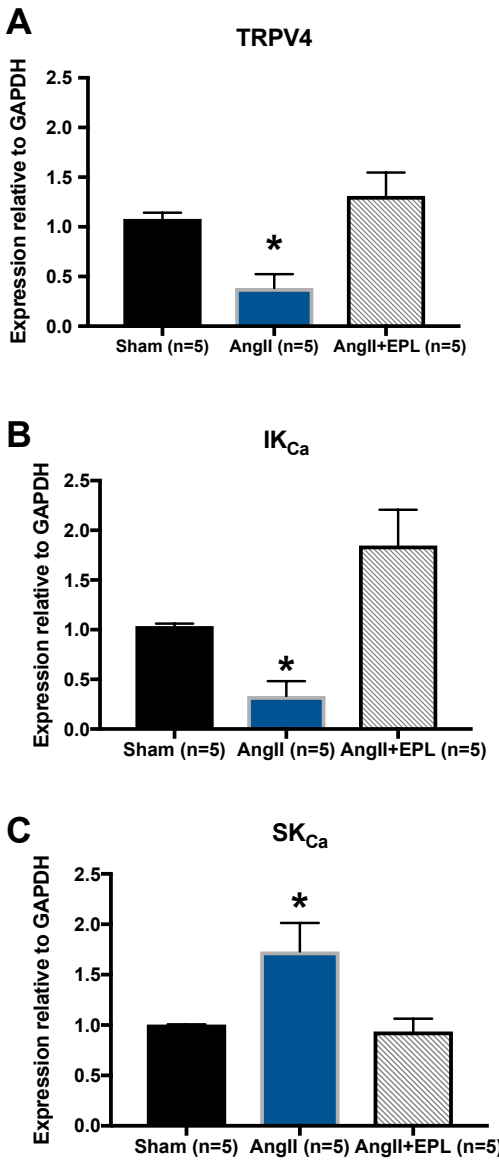

Figure 3.5 MR antagonism prevents changes in TRPV4 and IK_{Ca}/SK_{Ca} mRNA expression. The gene expression of TRPV4, IK_{Ca} and SK_{Ca} channels was assessed in isolated cerebral arteries. AngII resulted in a significant reduction in the mRNA expression of (A) TRPV4 and (B) IK_{Ca} and this was prevented by EPL treatment. (C) AngII resulted in a significant increase in the mRNA expression of SK_{Ca} channels and the changes were prevented by EPL. *$p<0.05$ by one-way ANOVA.

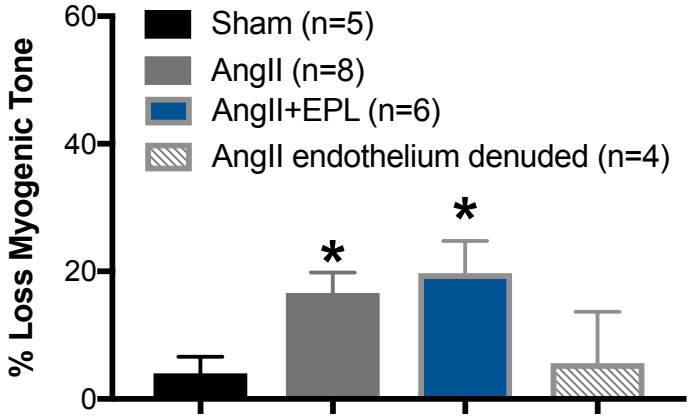

Figure 3.6 Endothelial TRPV4 channel modulate PA myogenic tone generation in mice. The role of TRPV4 channel in myogenic tone generation was assessed by pressure myography. Inhibiting TRPV4 with GSK2193874 did not change myogenic tone in the PAs from Sham mice. However, TRPV4 inhibition resulted in a significant loss of myogenic tone in the PAs from AngII and AngII+EPL mice. Denuding the endothelium of PAs from AngII prevented the significant loss in myogenic tone after TRPV4 inhibition. *$p<0.05$ by one-way ANOVA.

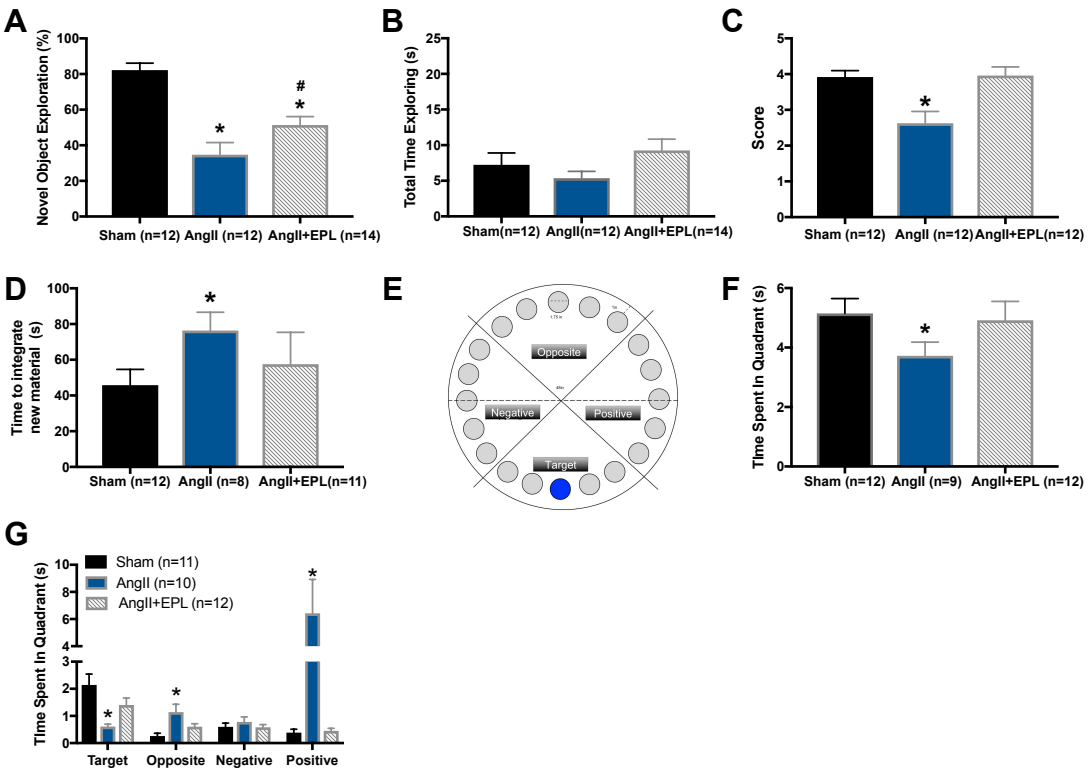

Figure 3.7 MR antagonism improves cognitive function in AngII-hypertensive mice. Novel object recognition, Barnes maze testing and nest building were used to assess changes in cognition. (A) The AngII mice spent less time exploring the novel object compared to the sham; memory function was improved by EPL treatment. (B) There was no significant difference in the total exploration time between the groups. (C) AngII mice had decreased mean nest score (D) and increase in the time it took for mice to integrate new nesting material to their nests; these changes were also prevented by EPL treatment. (E) Schematic of the Barnes maze. (F) AngII mice spent significantly less time in the target quadrant but more time in the opposite and positive quadrants. These changes were prevented in the EPL treated mice. (G) The AngII mice visit significantly less the target quadrant and this is prevented in the EPL treated mice *p<0.05 vs sham by one-way ANOVA; #p<0.05 vs AngII by one-way ANOVA.

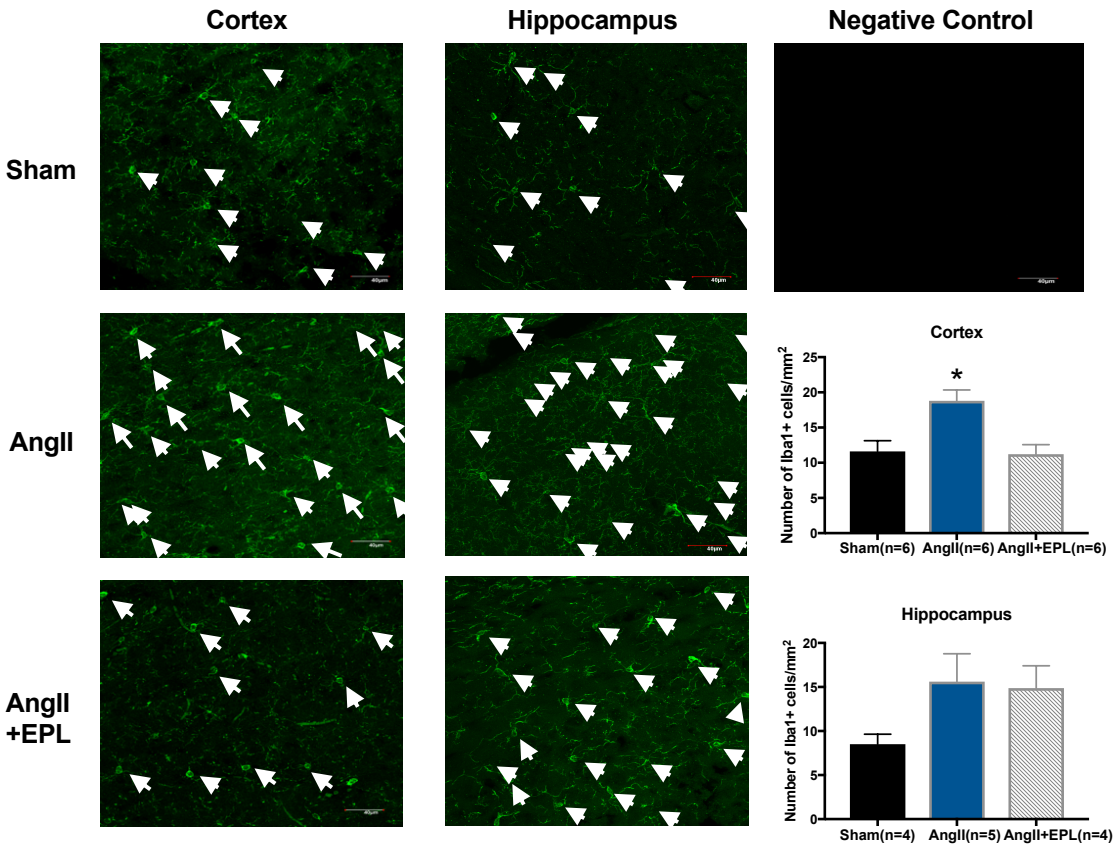

Figure 3.8 MR antagonism prevents increased microglia density during AngII-hypertension. The number of IBA-1 positive microglia were quantified in the cortex and hippocampus. (A) Representative images are shown. (B) Iba-1 positive cells were increased in the cortex of AngII-hypertensive mice and this was prevented by EPL. (C) Iba-1 positive cells were increased in the hippocampus of AngII-hypertensive mice, but this was not prevented by EPL. *$p<0.05$ vs sham by one-way ANOVA.

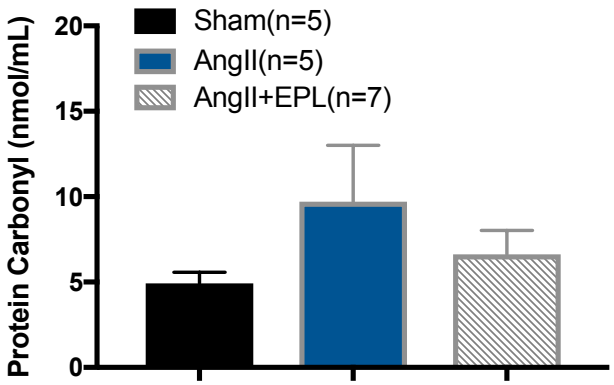

Figure 3.9 AngII-hypertension or eplerenone treatment does not change plasma oxidized proteins. The protein carbonyl content was assessed by ELISA. The protein carbonyl content was not significantly changed in the AngII mice or in the mice treated with EPL. $p>0.05$ by one-way ANOVA.

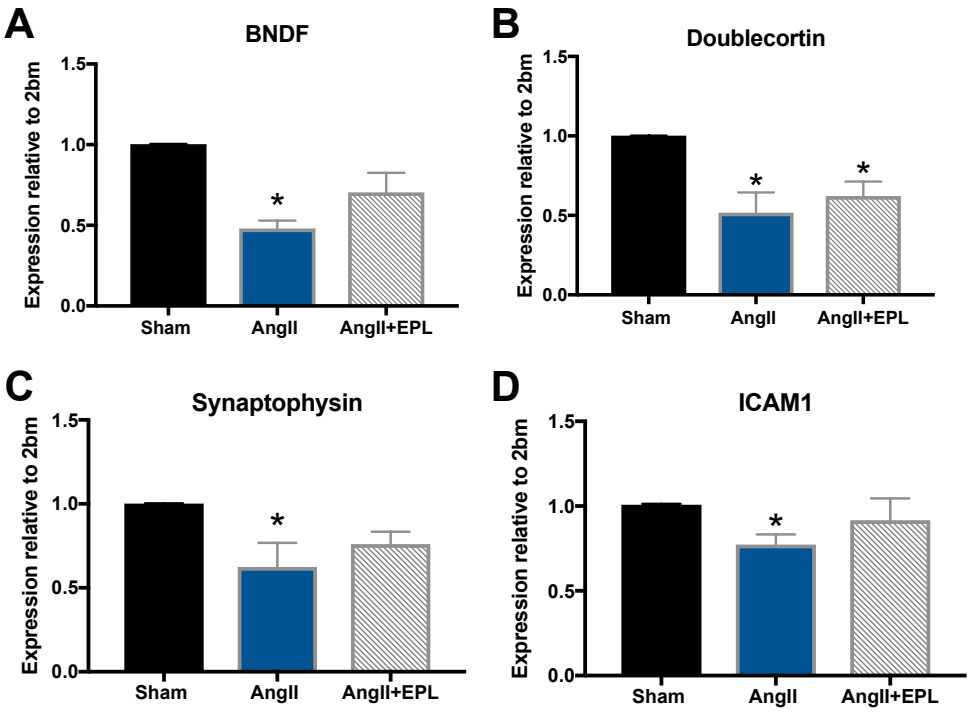

Figure 3.10 AngII-hypertension creates an environment where the mRNA expression of markers for neuronal support and synapse proteins are reduced. The brain mRNA expression of markers for neuronal support and synapse proteins were assessed by qRT-PCR. AngII reduced the mRNA expression of (A) BDNF, (B) doublecortin, (C) SYP and (D) ICAM-1; the reduction in BDNF, ICAM-1 and SYP were prevented by EPL. *$p<0.05$ vs Sham by one-way ANOVA.

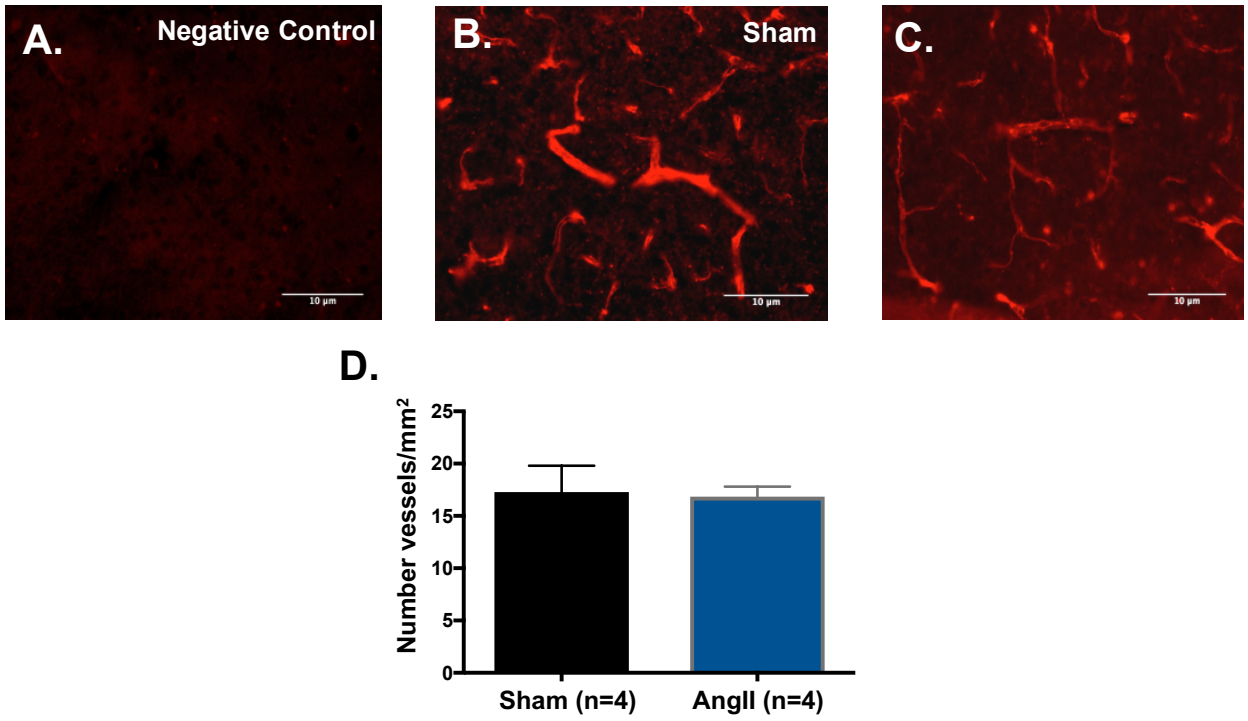

Figure 3.11. AngII-hypertension does not result in vessel rarefaction. Quantification of artery and capillary numbers was performed by immunofluorescence staining of the endothelial cell marker isolectin GS-IB_4. (A-C) Representative images are shown. (D) The number of vessels were not reduced in the AngII mice. $p>0.05$ by Student's t-test.

3.5 – Discussion

The novel findings of this study are that: 1). MR antagonism prevents the impaired endothelium-dependent dilation and increased myogenic tone in PAs observed during AngII-hypertension; 2). TRPV4 channels, which are important regulators of PA endothelium-dependent dilation, are regulated by MR signaling; 3). TRPV4 channels play a role in the regulation of myogenic tone in AngII-hypertension; 4). MR blockade improves cognition in AngII-hypertension and is associated with reduced cortical microglia density. These findings improve our understanding of the role of vascular MR signaling in cerebral arteriolar dilation. This is important because PA reactivity tightly regulates cerebral perfusion to maintain proper neuronal function, and dysregulation of PA function could

increase the risk of cognitive decline. To the best of omy knowledge, these are the first studies to show a role for MR signaling in PA dilation during AngII-hypertension. The therapeutic potential of MR antagonists is broadened by studies showing that blocking the MR can be beneficial even when aldosterone levels are not increased (35).

The effects of MR activation on peripheral artery function in health and disease have been previously explored. These studies suggest that the effects of MR activation are vascular bed-specific and that we need to study different vascular beds separately. My data suggest that MR signaling regulates myogenic tone generation and impaired endothelium-dependent dilation in response to hypertension. I recognize that the current study is at odds with our previous study using rats with polygenic hypertension, the SHRSP model (33), where EPL had no effect on myogenic tone. The different model and species used in the current study could explain the different results. That study also showed that EPL did not alter the PA responsiveness to the L-type Ca^{2+} channel blocker, nifedipine. In the current study, I used the muscarinic receptor agonist, CCh which is an endothelium-specific dilator; my data suggest that MR activation impairs the CCh-induced dilation of PAs. Future studies beyond the scope of the present investigation are required to fully understand the observed differences in myogenic tone across species. In the current study, I focused on the role of MR activation in PA dilation.

Consistent with previous studies (27), inhibition of the NO and COX pathways did not alter PA dilation in sham-operated mice suggesting that EDH factors mediate most of the dilation in PAs. Hypertension or EPL treatment did not change this dependence. However, recent studies from De Silva *et al.,* show that NO also plays an important role in mice PA dilation. I currently cannot explain these conflicting findings but a possibility is

the use of different inhibitors (L-NAME+Indomethacin vs LNNA) for the NO-mediated pathway as well as different endothelium-dependent dilators (CCh vs acetylcholine). There are also studies in cerebral arterioles from humans showing that NO is an important dilator of the arterioles, however, the samples were from the temporal and frontal cortex which could account for the different results (18). De Silva *et al.* also show that activation of IK_{Ca}/SK_{Ca} with NS309 also causes PA dilation suggesting there is an important EDH component (10). In the current study, I confirmed the role of EDH in the PA dilation using the IK_{Ca}/SK_{Ca} agonist NS309.

To further investigate the mechanisms responsible for PA dilation, I explored the role played by the TRPV4 channels in endothelium-dependent vasodilation. TRPV4 is a non-selective cation channel mainly expressed in endothelial cells, but expression has also been observed in the SMC of cerebral arteries (16, 41). In pial arteries, endothelial TRPV4 activation, via a PKC-dependent mechanism leads to the production of Ca^{2+} sparklets that activate SK_{Ca}/IK_{Ca} channels and initiate EDH-mediated vasodilation (17, 41). My data indicate that TPRV4 activation is a key determinant of PA dilation and that hypertension impairs TRPV4-mediated dilation. Studies have also shown that hypertension impairs Ca^{2+} signaling, the coupling of TRPV4 with the anchoring protein AKAP-150, and TRPV4-mediated dilation in mesenteric arteries (42). Although I do not have data to support this mechanism, it is possible that MR activation impairs the TRPV4 signal transduction pathway. There is also a possible role for mitogen-activated protein kinase/extracellular signal-regulated kinase-dependent pathways (30), but I think that in this model this is less likely. My data suggests that MR blockades prevents the reduced mRNA expression of TRPV4 channels in AngII-hypertension. The mRNA expression of

SK_{Ca} was reduced in AngII mice, however, IK_{Ca} mRNA expression was increased in AngII mice. This was unexpected but suggest that maybe there is a compensation after the reduced SK_{Ca} expression. My data also suggests that MR blockade prevents the changes in the IK_{Ca}/SK_{Ca} and function. Future studies beyond the scope of the current manuscript are required to understand these changes. Interestingly, when I inhibited TRPV4 channels and dilated the murine PAs with CCh, there was a residual dilation that could not be blocked by GSK2193874. This presents the possibility that another TRP channel such as TRPA1 or TRPV3 (34), or other dilator pathways such as NO, might also be involved in PA dilation.

My findings implicate MR signaling in the regulation of TRPV4-mediated dilation in PAs, though the specific mechanisms behind this have yet to be identified. Nonetheless, my data suggest that MR blockade prevents the reduced mRNA expression of TRPV4 channels in AngII-hypertension. It is well known that AngII induces oxidative stress that contributes to vascular dysfunction (9, 25). Thus, it is possible that increased oxidative stress is the link between MR activation and inhibition of TRPV4-mediated dilation. This oxidative stress may impair the function of TRPV4 channels (46). Zhang *et al.*, demonstrated that the function of endothelial TRPV4 channels in PCAs is impaired by oxidative stress and that this impairment could be rescued by antioxidant therapy (48). Aldosterone and MR activation also induces inflammation in the brain (14) that could mediate the impaired endothelium-dependent dilation. Innate immune cells, including macrophages, have been implicated in the hypertension-associated pathology through the production of cytokines and ROS (31). A recent study showed that AngII crosses the BBB and interacts with PVMs to promote the production of ROS, through activation of

NOX2, leading to cognitive dysfunction (20). This study highlights the possibility of a detrimental direct effect of macrophages on susceptible cortical and hippocampal neurons contributing to vascular and cognitive dysfunction. In the present study, the hypertensive mice had an increased microglia density, the resident macrophages of the brain; this was prevented by EPL treatment. However, EPL only prevented the increase in cortical microglia. Hippocampal microglia density was not changed by EPL.

Cerebral blood flow autoregulation is also modulated by changes in myogenic tone and the endothelium can also release contracting factors to regulate arteriole tone (1, 21). Although TRPV4 activation is generally known to modulate endothelium-dependent dilation, a recent study showed that endothelial TRPV4 activation with GSK1016790A produces a contraction of the aorta from hypertensive mice (49). In this study, I also observed a small contraction in the PAs from AngII-hypertensive mice when TRPV4 was activated. Although vascular SMCs are responsible for the generation of myogenic tone, endothelial cells produce substances that modulate tone (32). My data also suggests that endothelial TRPV4 channels are capable of modulating myogenic tone only when hypertension is present, and this regulation appears to be independent of MR signaling. These data present the possibility that under pathological conditions TRPV4 could activate different pathways such as cytosolic PLA_2 (49) to modulate vascular tone. It is important to note that the putative constrictor effects of TRPV4 appears to only be apparent in the absence of the ligands that activate TRPV4. This was an unexpected finding and the mechanisms by which endothelial TRPV4 channel modulates myogenic tone in hypertension remains to be explored.

The artery remodeling (13), impaired endothelium-dependent dilation, and reduced perfusion (13) in AngII-hypertension could cause cognitive dysfunction (19, 29). I explored if MR blockade could be a potential therapeutic approach to improve cognitive function in hypertension. I first assessed non-spatial memory using the novel object recognition test (8). I found that AngII mice spent less time exploring the novel object than sham-operated mice and that MR blockade improved cognitive function. I also used Barnes maze to assess spatial memory (4) and showed that AngII mice have impaired executive function that was improved by EPL treatment. Changes in nest building are associated with cognitive decline (11, 19); nest building was impaired in AngII mice and this deficit was prevented by EPL. The changes in cognitive function could also be associated with altered mRNA expression of BDNF, a neurotrophin expressed in the hippocampus, which is important for neuronal function, synaptic plasticity, and memory (5, 44). The changes in cognition could also be associated with the reduced mRNA expression of SYP, an important synapse protein. Recent studies show a correlation between the degree of dementia in Alzheimer's disease and reduced SYP levels (47). These observations suggest that AngII-hypertension disrupts markers for neuronal function and synapses that may lead to impaired cognition. A strength in my study is that I used several behavioral and biochemical approaches to show that MR blockade could also be beneficial to reduce cognitive dysfunction associated with vascular deficits observed in models of hypertension.

Some limitations in my study must be acknowledged. First, all of my studies were conducted in male mice to match previous studies in our lab on the role of MR signaling in the cerebral vasculature, thus I did not explore possible sex differences. When these

studies began I was interested in ischemic stroke where there are significant sex effects, thus I wanted to study males first and then expand my studies. I acknowledge that I did not administer saline to the sham control group. However, I have previously shown that the dose of AngII given causes hypertension in the mice and that the sham-operated mice were a suitable control. Also, I used a dose of EPL that is higher than used therapeutically in humans. However, it is known that rodents metabolize/excrete EPL at a higher rate than humans (12, 51). Thus, higher doses are required in mice to produce the similar levels of MR antagonism. It is unlikely that the dose/duration of the treatment used will cause off-target effects or toxicity. Toxicity studies using similar dose did not show toxicity or carcinogenicity in mice. The small size of PAs prevented me from assessing changes in mRNA in the PAs. I also have not measured changes in TRPV4 protein expression. In the future, I hope to identify the cell-specific actions of MR signaling in PA myogenic tone and vasodilation.

In summary, PAs are the bottleneck to perfusion of the cerebral microcirculation and they are important for the development of cerebral small vessel disease. My data suggest that TRPV4 may be an important determinant of the hypertension-associated changes in cerebral arteriolar function. MR activation increases PA myogenic tone and impairs endothelium-dependent dilation during hypertension. TRPV4 activation mediates PA endothelium-dependent vasodilation and appears to be regulated by MR activation. AngII-hypertension results in an increased number of immune cells and creates an environment where the expression of genes for markers of neuronal support and synapse proteins are disrupted. These changes are regulated by MR signaling and may be associated with the observed declined in cognitive function. Impairments in vasodilation

and alterations in myogenic tone may reduce cerebral blood flow during hypertension and increase the risk of cerebrovascular diseases. The MR could be potential therapeutic target to improve vascular and cognitive function during hypertension.

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CHAPTER 4

Endothelial Mineralocorticoid Receptor Mediates Cerebrovascular Dysfunction in Parenchymal Arterioles during Angiotensin II-Hypertension

4.1 – Abstract

MR activation causes hypertension-associated PA remodeling, impaired endothelium-dependent dilation and cognitive dysfunction. A mouse model of AngII-induced hypertension was used to test the hypothesis that EC-MR activation impairs PA TRPV4-mediated dilation and cognitive function. 16-18-week-old MR-intact and ECMRKO mice were treated with AngII (800ng/kg/min) for 4 weeks; sham operated mice served as controls. Data are presented as mean ± SEM; n=5-14 per group. EC-MR deletion prevents the impaired PA endothelium-dependent dilation in hypertension but not the increased myogenic tone. Dilation in response to the TRPV4 agonist GSK1016790A (10^{-9}-10^{-5}mol/L) and IK_{Ca}/SK_{Ca} agonist NS309 (10^{-9}-10^{-5}mol/L) were also reduced in the hypertensive mice and this was corrected by EC-MR deletion. Hypertensive mice had impaired cognitive function, interestingly this was not corrected by EC-MR deletion. Plasma protein oxidation was not altered by EC-MR deletion or AngII treatment. The mRNA expression of markers for neurogenesis and synapse proteins, doublecortin and SYP and were reduced by AngII; EC-MR deletion prevented the reduced mRNA expression. My data shows that EC-MR signaling mediates PA endothelium-dependent dilation but not cognitive function and suggests that the MR is a potential therapeutic target to improve cerebrovascular function during hypertension.

4.2 – Introduction

Hypertension is a primary risk factor for cSVD and VCID, both growing public health issues that lack effective treatments. Maintenance of constant blood flow to the brain and the ability to match flow to metabolic demands are critical for cognitive function. The constant blood flow is maintained by cerebrovascular autoregulation and NVC. Hypertension impairs cerebral blood flow autoregulation that is partially controlled by arteriolar structure. The increased blood pressure also impairs the structure and function of cerebral arteries and PAs. The PAs perfuse the cerebral microcirculation and are considered the weak link in perfusion because these are not connected to each other by collaterals. More importantly, occlusion of a single PA causes a microinfarct and cognitive impairment (34). NVC, or functional hyperemia, links the metabolic demands of the neurons to cerebral blood flow by integrating signaling between all the cells in the unit (12, 13). NVC is impaired in hypertensive rats and mice and this is not prevented by drugs that lower blood pressure (4). Since NVC and cerebral artery autoregulation work together to regulate perfusion to the brain, alterations in the components of these can cause cerebral hypoperfusion and cognitive decline.

I am interested in the role of TRPV4 channels in cerebrovascular function. TRPV4 channels are Ca^{2+} permeable non-selective cation channels that mediate endothelium-dependent dilation in PAs. My previous studies show that MR activation impairs the TRPV4-mediated dilation of PAs and cognitive function during AngII-hypertension (8). However, those studies did not identify the specific cell type involved in the MR-mediated impaired PA dilation and cognitive dysfunction. Cell-specific MR knockout models have been developed to facilitate studies defining the role played by the EC-MR. My studies in

Chapter 2 show that the EC-MR regulates PA inward remodeling and reduced cerebral perfusion. Futhermore, studies in the peripheral circulation have demonstrated a role for the EC-MR in endothelial function, dependent on the vascular bed, but none have studied the role of EC-MR in PA endothelial function (1, 14, 17, 25, 26, 29, 32). The studies in the peripheral vasculature highlight the need to study the cerebral vasculature separately because- 1). it is anatomically unique; 2). it is clear that different vascular beds have varying sensititivies to EC-MR signaling.

In the current study, I used a ECMRKO mouse to test the hypothesis that EC-MR signaling impairs TRPV4-mediated dilation in PAs, and cognitive function in Ang-II dependent hypertension. Identifying the cell type that drives the actions of the MR will allow us to define the specific cellular pathways activated by the MR, may allow for the development of more specific therapies to prevent cerebrovascular diseases and VCID.

4.3 – Materials and Methods

4.3.1 – Experimental model and treatment

All experimental protocols were approved by the Michigan State University Animal Care and Use Committee and were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. ECMRKO mice (n=5-10) were generated by Dr. Iris Z. Jaffe at Tufts Medical Center by crossing ve-cadherin Cad-Cre^{+} mice with MR$^{f/f}$ mice as described previously (25). MR$^{f/f}$ mice had exons 5 and 6 of the MR gene floxed by loxP sites. MR-intact littermates were used as controls. All mice studied were 20-22 weeks old males and housed in 12h: 12h light/dark cycle with food and water ad libitum.

4.3.2 – AngII infusion

16-18 week old MR-intact and EMCKRO mice were infused with AngII (800ng/kg/min) subcutaneously via osmotic minipumps (Alzet model 1004, Durect Corp, Cupertino CA). Mice were anesthetized with 3% isoflurane in oxygen and body temperature was maintained at 37°C. Single doses of the analgesic Rimadyl (5mg/kg, SQ) and the antibiotic Baytril (5m/kg, IM) were administered immediately prior to the surgical procedure. A subcutaneous pocket was made, and mini-pumps were inserted; Sham-operated mice underwent anesthesia, and a subcutaneous pocket was made, but mini-pumps were not inserted. Mice were treated for 4 weeks.

4.3.3 – Pressure Myography

The endothelial function of PAs was assessed by pressure myography (7, 19, 27). To isolate the arterioles, a 5 x 3 mm section of the brain containing the MCA was dissected, then the pia with the MCA was separated from the brain and the PAs branching off the MCA were used for experiments. Isolated arterioles were cannulated with two glass micropipettes in a custom-made cannulation chamber. Arterioles were equilibrated at 37°C in aCSF containing 124mmol/L NaCl, 3mmol/L KCl, 2mmol/L $CaCl_2$, 2mmol/L $MgCl_2$, 1.25mmol/L NaH_2PO_4, 26mmol/L $NaHCO_3$ and 4mmol/L Glucose. A servo-null system was used to pressurize the arterioles, and a leak test was performed prior to each experiment. Arterioles were pressurized to 40mmHg (7) until the development of stable myogenic tone (% tone = [1-(active lumen diameter/passive lumen diameter)] x 100. The diameter of the arterioles was recorded using MyoView 2.0 software (Danish Myo Technology, Aarhus, Denmark).

After the generation of myogenic tone, endothelium-dependent dilation was assessed by incubating the arterioles with increasing concentrations of the muscarinic receptor agonist CCh (10^{-9}-10^{-5}mol/L) in the bath. To assess the role played by the TRPV4 channels in PA dilation, arterioles were incubated with the TRPV4 channel antagonist GSK2193874 (10^{-7}mol/L) for 10 minutes after the development of stable myogenic tone and then CCh-induced dilation (10^{-9}-10^{-5}mol/L) was assessed. A separate group of PAs were incubated with increasing concentrations of the TRPV4 channel agonist GSK1016790A (10^{-9}-10^{-5}mol/L). To assess the role played by IK_{Ca}/SK_{Ca} channels, PAs were incubated with increasing concentrations of the agonist NS309 (10^{-9}-10^{-5}mol/L). At the end of each experiment Ca^{2+} free buffer containing EGTA (29mmol/L) and SNP (10^{-5}mol/L) were added to the bath to maximally relax the smooth muscle. Dilation was calculated using the following formula: [lumen diameter at drug concentration – baseline lumen diameter)/ (passive lumen diameter – baseline lumen diameter)] x 100.

4.3.4 – Novel object recognition

Mice were acclimated over three days for 10 minutes per day in the arena (an open box with dark walls). On the testing day, mice were placed in the arena and allowed to explore two identical objects for 10 minutes. After a retention time of 60 minutes, mice were returned to the arena and allowed to explore one familiar object and a novel object for 5 minutes and tracked using the software EthoVision XT. A blinded investigator calculated the percentage time of novel object exploration (2).

4.3.5 – qRT-PCR

RNA was extracted from brain for qRT-PCR analysis using Trizol. RNA was reverse transcribed using VILO reverse transcriptase (Invitrogen, Carlsbad, CA). TAQMAN-specific probes were used for the PCR to assess the mRNA expression of BDNF, SYP, and doublecortin. mRNA expression is expressed as the fold change from control using the $^{-\Delta\Delta Ct}$ method. β2-microglobulin was used for normalization.

4.3.6 – Plasma protein oxidation

Blood was collected by cardiac puncture prior to euthanasia in anesthetized mice. Plasma protein carbonyl was quantified using an ELISA assay (Cayman Chemicals, Ann Arbor, MI) according to the manufacturer's instructions.

4.3.7 – Drugs and chemicals

GSK1016790A and GSK2193874 were purchased from Cayman Chemicals (Ann Arbor, MI). NS309 was purchased from Tocris (Pittsburg, PA). All other drugs and chemicals were purchased from Sigma-Aldrich unless otherwise specified.

4.3.8 – Statistical analysis

All data are presented as mean ± SEM. Myogenic tone, mRNA expression, behavioral, and immunofluorescence data were analyzed by one-way analysis of variance. For analysis of artery vasodilation, two-way analysis of variance with repeated measures in one factor (drug concentration) was utilized followed by Bonferroni-adjusted t-tests for post-hoc comparisons. All statistical analyses were performed using GraphPad Prism 7.0 software (GraphPad, San Diego, CA). In all cases statistical significance was denoted by $p<0.05$.

4.4 – Results

4.4.1 – EC-MR deletion does not prevent increased parenchymal arteriole myogenic tone in AngII-hypertension.

The role of EC-MR activation in the generation of myogenic tone of PAs was assessed. EC-MR deletion did not change PA myogenic tone in sham mice. The PAs from MR-intact+AngII mice had increased myogenic tone when compared to the sham-operated mice and this increase was not prevented by EC-MR deletion (Figure 4.1), suggesting that EC-MR activation does not enhance myogenic tone during AngII-hypertension.

4.4.2 – EC-MR deletion prevents impaired endothelium-dependent dilation during hypertension.

Next, I assessed PA endothelium-dependent dilation. EC-MR deletion did not change PA endothelium-dependent dilation in response to CCh in sham mice. As expected, PA dilation was impaired in the AngII-hypertensive mice (8); this impairment was prevented by EC-MR deletion (Figure 4.2). Previously, I showed that PA TRPV4-mediated dilation is impaired in AngII-hypertension and this was corrected by MR antagonism with EPL (8). Therefore, I next sought to determine if EC-MR was involved in the MR mediated effects. PA dilation in response to the TRPV4 agonist, GSK1016790A, was impaired in the MR-intact+AngII mice and this impaired dilation was prevented by EC-MR deletion. EC-MR deletion did not change the baseline response to the TRPV4 agonist in sham mice (Figure 4.3). I confirmed that TRPV4 channels are important mediators of the CCh-mediated dilation of PAs using the TRPV4 antagonist GSK2194874. TRPV4 antagonism blunted the CCh-mediated dilation of PAs in all groups

(Figure 4.4). To further explore the role of EC-MR signaling in the EDH-mediated dilation of PAs, I incubated the arterioles with increasing concentrations of the IK_{Ca}/SK_{Ca} agonist, NS309. In our previous study, I showed that AngII-hypertension impairs IK_{Ca}/SK_{Ca}-mediated dilation of PAs and this impaired dilation was prevented by EPL treatment (8). In the current study, I show that the effects of MR activation on IK_{Ca}/SK_{Ca} dilations are mediated by the EC-MR (Figure 4.5).

4.4.3 – EC-MR deletion does not improve cognitive function in AngII-hypertensive mice.

EC-MR activation mediates the hypertension-associated PA-remodeling (7), impaired dilation and reduced cerebral blood flow. I have previously shown that MR blockade with EPL improves cognition in AngII-hypertension (8). Therefore, I evaluated whether EC-MR signaling is involved in the MR-mediated effects. I evaluated non-spatial, short-term memory using the novel object recognition test (6). As expected, the MR-intact+AngII treated mice spent less time exploring the novel object when compared to the MR-intact and ECMRKO sham-operated mice. However, EC-MR deletion did not improve cognition in the hypertensive mice. EC-MR deletion in baseline normotensive conditions does not change short-term memory as evidenced by the lack of change compared to the sham mice (Figure 4.6).

4.4.4 – Possible mechanisms for the impaired cerebrovascular and cognitive function.

To explore possible mechanisms for the impared cerebrovascular and cognitive function I assessed plasma protein oxidation as an index of oxidative stress (18). No significant changes were observed by EC-MR deletion or AngII treatment. (Figure 4.7). Then changes in mRNA expression of markers for neuronal support and new neuron formation in brain tissue were assessed. AngII did not change the mRNA expression of

the marker for synapses, SYP (Figure 4.8 A) or the mRNA expression of BNDF (Figure 4.8 B). The mRNA expression of doublecortin, a marker for new and immature neurons, was reduced in the AngII treated mice and this was prevented by EC-MR deleltion (Figure 4.8 C). These data suggest that AngII-hypertension disrupts expression of genes involved in neuronal support and synapse formation.

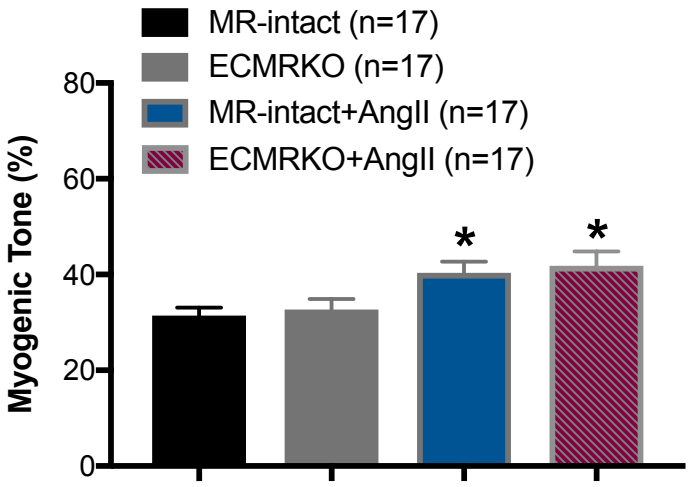

Figure 4.1 EC-MR deletion does not prevent the increased myogenic tone in AngII-hypertension. PA myogenic tone was assessed by pressure myography. EC-MR deletion does not change PA myogenic tone under normotensive conditions. AngII-hypertension increases PA myogenic tone, and this is not prevented by EC-MR deletion. *p<0.05 by one-way ANOVA.

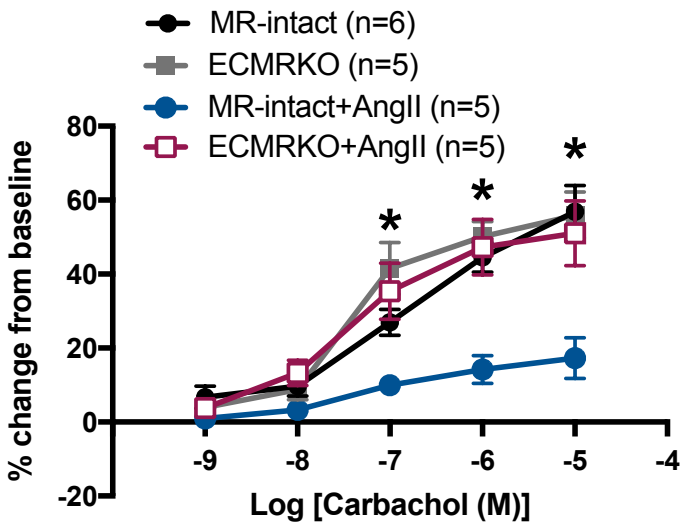

Figure 4.2 EC-MR prevents impaired PA endothelium-dependent dilation. PA endothelium-dependent dilation in response to CCh was assessed by pressure myography. EC-MR deletion does not change PA dilation in response to CCh in sham mice. AngII impaired the PA dilation and this was prevented by EC-MR deletion. *p<0.05 by two-way ANOVA.

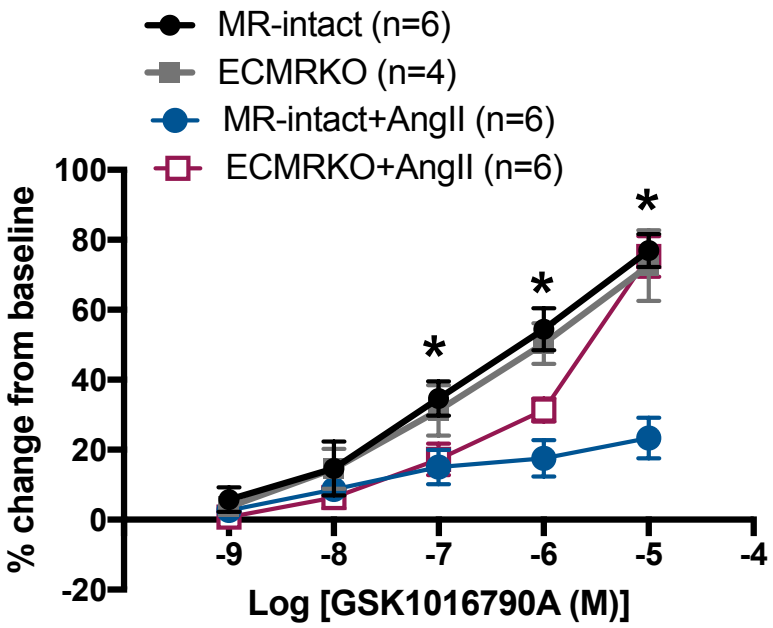

Figure 4.3 EC- MR deletion prevents the impaired TRPV4-mediated dilation of PAs during hypertension. PA endothelium-dependent dilation was assessed by pressure myography using the TRPV4 agonist GSK1016790A. EC-MR deletion does not alter the TRPV4-mediated dilation in sham mice. The GSK1016790A-induced dilation was impaired in the AngII mice and this was prevented by EC-MR deletion. *p<0.05 by two-way ANOVA.

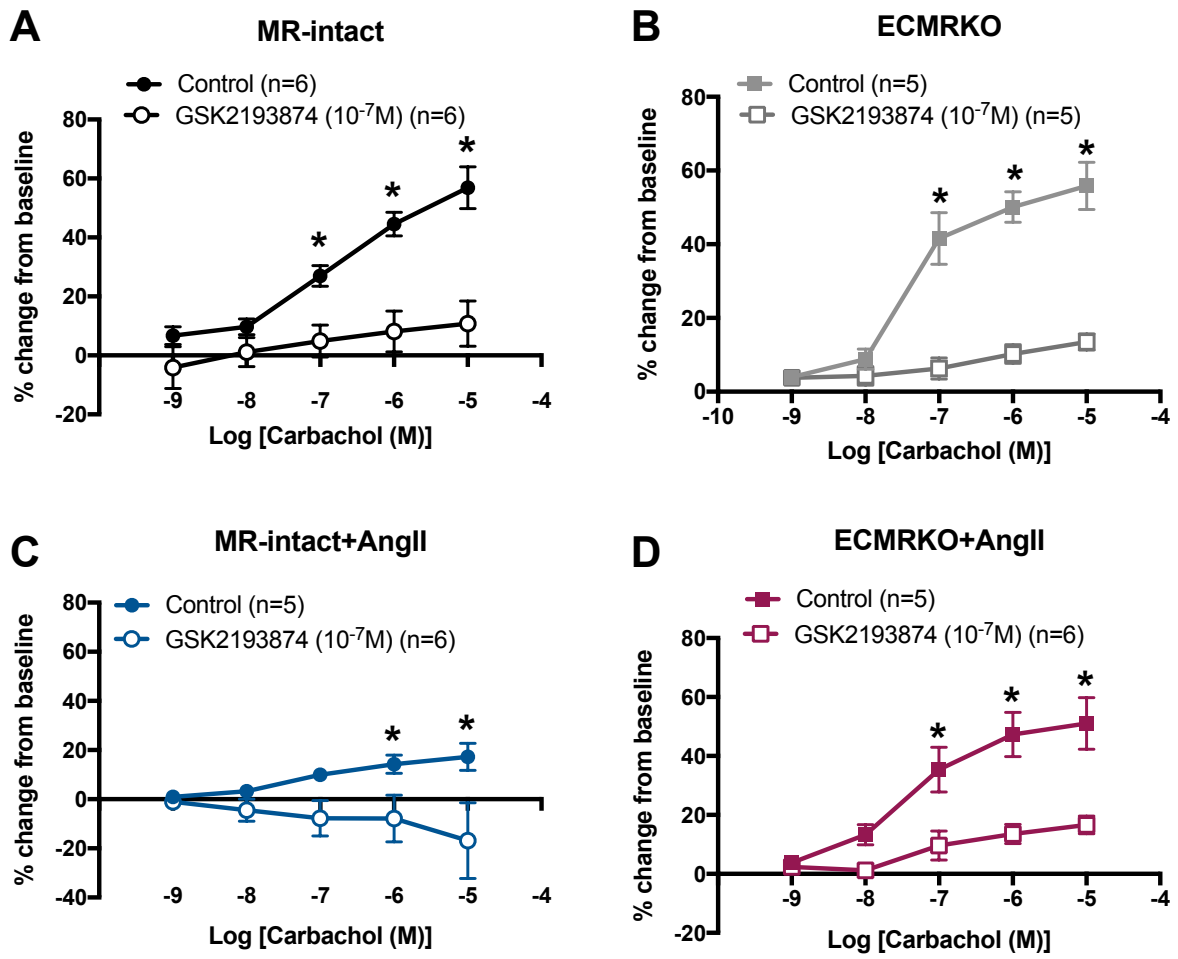

Figure 4.4 TRPV4 antagonism blunts the carbachol-mediated dilation in PAs. PA endothelium-dependent dilation was assessed by pressure myography. TRPV4 inhibition with GSK2193874 prevented the CCh-mediated dilation in (A) MR-intact, (B) ECMRKO, (C) MR-intact+AngII, and (D) ECMRKO+AngII treated mice. *p<0.05 by two-way ANOVA.

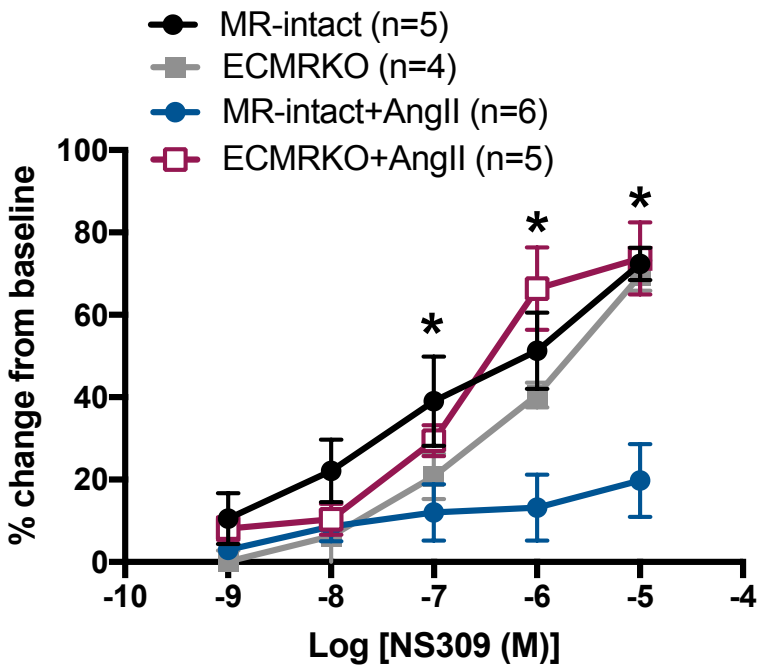

Figure 4.5 EC-MR deletion prevents the reduced IK_{Ca}/SK_{Ca}-mediated dilation in PAs during hypertension. IK_{Ca}/SK_{Ca}-mediated PA dilation was assessed using the agonist NS309 by pressure myography. EC-MR deletion did not change the IK_{Ca}/SK_{Ca}-mediated dilation in sham mice. The AngII mice had impaired dilation and this was prevented by EC-MR deletion. *p<0.05 by two-way ANOVA.

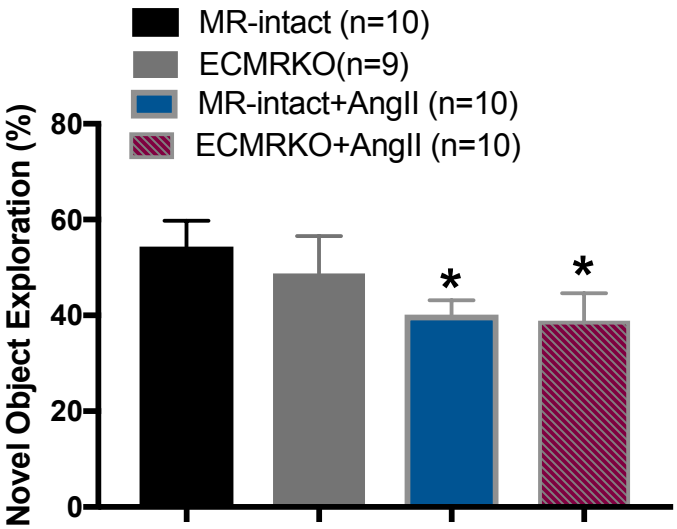

Figure 4.6 EC-MR deletion does not improve cognitive function in AngII-hypertensive mice. Novel object recognition testing was used to assess changes in non-spatial short-term memory. EC-MR deletion did not change cognition in sham mice. The AngII mice spent less time exploring the novel object compared to the MR-intact but this was not improved by EC-MR deletion. *$p<0.05$ vs sham by one-way ANOVA.

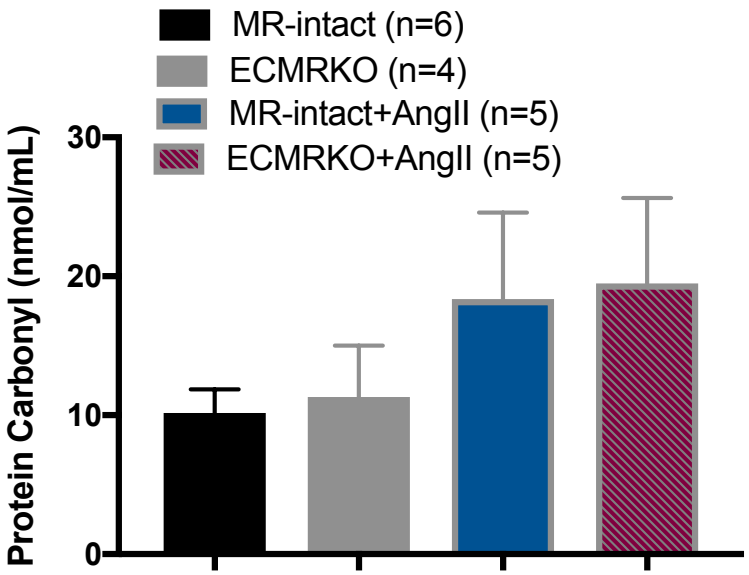

Figure 4.7 AngII or EC-MR deletion does not change plasma oxidized proteins. The protein carbonyl content was assessed by ELISA as index of oxidative stress. The protein carbonyl content was not significantly changed in ECMRKO, MR-intact+AngII, or ECMRKO+AngII mice. $p>0.05$ by one-way ANOVA.

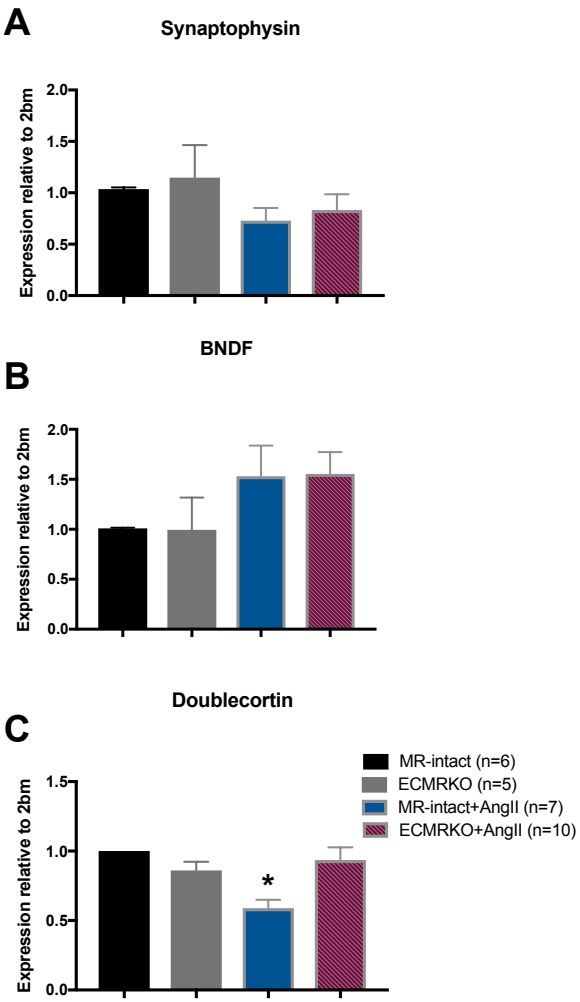

Figure 4.8 AngII-hypertension reduces the mRNA expression of doublecortin. The brain mRNA expression of markers for neuronal support and synapse proteins were assessed by qRT-PCR. (A) AngII caused a small but not statiscally significant reduction in the mRNA expression of synaptophysin; (B) AngII did not significantly change the mRNA expression of BDNF; (C) the mRNA expression of doublecortin was also reduced in the AngII treated mice. EC-MR deletion prevented the changes doublecortin. *$p<0.05$ vs MR-intact by one-way ANOVA.

4.5 – Discussion

The novel findings of this study are that: 1). EC-MR deletion prevents the impaired endothelium-dependent dilation of PAs in AngII-hypertension; 2). EC-MR deletion prevents the impaired TRPV4- and IK_{Ca}/SK_{Ca}-mediated dilation of PAs in AngII-hypertension; 3). EC-MR deletion does not prevent the increased myogenic tone of PAs in AngII-hypertension; 4). EC-MR deletion does not improve cognitive function in AngII-hypertension. My previous studies show that MR activation was associated with impaired TRPV4-mediated dilation of PAs in AngII-hypertension. Here, I further demonstrate that EC-MR signaling is involved. These findings improve our understanding of the role of endothelial cell specific MR signaling in cerebral arteriolar dilation and cognition. My studies are important because PAs regulate perfusion to the microcirculation and dysfunction of these arterioles causes microinfarcts and increases the risk of VCID. To the best of my knowledge, these are the first studies to assess the role of EC-MR signaling in cerebrovascular and cognitive function. These are also the first to explore the role of EC-MR signaling in the TRPV4-mediated dilation in cerebral arterioles.

The effects of EC-MR activation in peripheral arteries have been previously explored, but less is known about the effects in cerebral arteries (15–17, 25, 26, 29, 32, 33). The anatomical differences between cerebral and peripheral arteries prevents the extrapolation of findings from the periphery to the brain. I have previsouly shown that EC-MR activation mediates cerebral artery remodeling (7), but no studies have explored endothelium-dependent dilation in cerebral arteries. In control mice EC-MR activation in aorta or mesenteric arteries does not impair endothelium-dependent vasodilation (25, 29). In the current study, I also show that in normotensive mice EC-MR activation does not

impair or enhance PA endothelium-dependent dilation or the generation of myogenic tone. However, in hypertensive mice enhanced EC-MR signaling impairs endothelial function in the thoracic aorta but not in mesenteric arteries (25). This suggests that different vascular beds have different sensitivities to MR activation. We have shown negative effects of MR activation and positive effects of MR antagonism with EPL or SPIR on cerebral artery structure and endothelium-dependent dilation (8, 20, 27, 28, 30, 31). However, those studies did not identify the specific cell type involved in the MR-mediated effects. In the current study I explored the specific role of EC-MR activation on PA endothelium-dependent dilation and myogenic tone in AngII-hypertension.

My previous study shows that MR antagonism with EPL prevents the increased myogenic tone and impaired endothelium-dependent dilation of PAs in AngII-hypertension (8). The increased myogenic tone does not appear to be mediated by the EC-MR because in the current study EC-MR deletion did not change the increased myogenic tone in hypertension suggesting that the changes in tone are likely mediated by the SMC-MR. In fact, resistance arteries from SMC-MR knockout mice have reduced myogenic tone. The same study showed the expression and activity of the L-type Ca^{2+} channels in the SMC-MR knockout mice was reduced, suggesting that SMC-MR regulates Ca^{2+} signaling and myogenic tone (21). Additional studies are needed to confirm the role of SMC-MR in the regulation of PA myogenic tone.

Next, I explored the role of EC-MR signaling in the EDH mediated dilation of PAs using the IK_{Ca}/SK_{Ca} agonist, NS309. Previous studies suggested that MR antagonism prevents the reduced IK_{ca}/SK_{ca}-mediated dilation of PAs and in this study I show that this is mediated by EC-MR signaling. This is supported by studies in peripheral arteries where

EC-MR signaling regulates endothelium-dependent dilation through IK_{Ca}/SK_{Ca} channels (22). IK_{Ca}/SK_{Ca} are expressed in the cerebral arteries and their gene expression is altered in AngII-hypertension (5, 8). In PCAs, MR activation increases the mRNA expression of IK_{Ca} whereas SK_{Ca} expression is reduced (8). To further investigate the mechanisms responsible for PA dilation, I explored the role played by the TRPV4 channels in endothelium-dependent dilation. TRPV4 channels are non-selective cation channels that allow Ca^{2+} influx into the endothelium to activate IK_{Ca}/SK_{Ca} channels (3). MR activation impairs the TRPV4-mediated dilation of PAs during AngII-hypertension. My current data indicates that this is mediated by MR signaling at the level of the endothelium.

The mechanisms by which EC-MR exerts effects on PA function remains to be explored. It is possible that EC-MR activation impaired the TRPV4 channel signal transduction pathway. This concept is based on studies in mesenteric arteries where hypertension impaired Ca^{2+} signaling, the coupling of TRPV4 channels with the anchoring protein AKAP150, and the TRPV4-dilation (35). It is also possible that oxidative stress is the link between EC-MR signaling and impaired TRPV4-mediated dilation (36). Studies by Zhang *et al.,* show that oxidative stress impairs the function of endothelial TRPV4 channels in PCAs (37). EC-MR signaling stimulates oxidative stress in the aorta of female mice fed a Western diet as shown by an increase in NOX2 and NOX4 expression (15). These mice also had increased macrophage M1/M2 polarization as evidenced by the increased expression of proinflammatory M1 macrophage markers CD86 and CD11c (15). However, in this study no changes in the mRNA expression of NOX2 or NOX4 in the PCAs in AngII treated C57bl/6 mice were observed (7). Also, in the current study and in the studies in Chapter 3 I did not observe changes in oxidized proteins suggesting that

perhaps inflammation mediated the impaired endothelium-dependent dilation. It is known that MR activation induces inflammation in the brain (9) and that innate immune cells are involved in the hypertension-associated pathology through the production of cytokines (24). In Chapter 3 I showed that AngII increases the number of activated microglia and this is prevented by MR antagonism suggesting this might be a potential mechanism for the impaired dilation. However, further studies beyond the scope of the current study are required to fully determine the mechanism for the EC-MR mediated effects on PA dilation.

The PA remodeling (7), impaired endothelium-dependent dilation, and reduced perfusion (7) in AngII-hypertension could be the cause cognitive impairment observed (10, 23). This cognitive impairment observed in AngII hypertensive mice is improved by EPL treatment which also corrects all the reported vascular dysfunction (8). However, EC-MR deletion did not improve cognitive function in the AngII mice suggesting that the effects of EPL may also involve glia, neuronal activity or the SMC or macrophage MR. In fact, EPL can cross the BBB and get into the brain to have an effect on neurons and astrocytes (11). The changes in cognitive function have been associated with changes in the gene expression of markers for neuronal support and synapse proteins. AngII-hypertension reduced the mRNA expression doublecortin, a marker of new and immature neurons. These studies suggest that EC-MR deletion restores PA endothelium-dependent dilation but not cognitive function. This suggests that the improved PA endothelium-dependent dilation is not enough to reverse the cognitive impairment and perhaps the capillaries may play an important role as well.

Some limitations in the study must be acknowledged. First, all of the studies were conducted in male mice to match previous studies in our lab on the role of MR signaling in PA remodeling in AngII-hypertension, thus we did not explore possible sex differences. I acknowledge that I did not administer saline to the sham control group. However, I have previously shown that the dose of AngII given causes hypertension in the mice and that the sham-operated mice were a suitable control. The small size of PAs prevented me from assessing changes in mRNA in the PAs. I also have not measured changes in MR and TRPV4 protein expression. In the future, I hope to identify the mechanism for the actions of EC-MR in PA dilation.

In summary, our studies fill a gap in our knowledge of how MR signaling mediates PA endothelial dysfunction in hypertension. I utilized an EC-MR knockout mouse model to show, for the first time, the role of EC-MR in cerebral artery EDH-dilation and particularly TRPV4-mediated dilation. These data support the idea that EC-MR have a minimal role in artery function under normal control conditions, but that EC-MR signaling mediates endothelial dysfunction in response to cardiovascular risk factors such as AngII-induced hypertension. My data suggest that MR signaling at the level of the endothelium regulates the TRPV4-mediated dilation of PAs in hypertension. I also show that EC-MR signaling does not regulate the increased myogenic tone in PAs during AngII-hypertension suggesting a possible role of SMC-MR in PA myogenic tone. AngII creates an environment where the expression of genes for markers of neurogenesis and synapse proteins are disrupted. These changes are regulated by MR signaling but not specifically the EC-MR signaling. Aditionally, EC-MR signaling is not involved in the improved cognitive function in AngI-hypertension observed with MR antagonism. Impairment of

vasodilation and increased myogenic tone may reduce cerebral blood flow during hypertension and increase the risk of VCID.

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CHAPTER 5

Transient Receptor Potential Vanilloid 4 Channels are Important Regulators of Parenchymal Arteriole Dilation and Cognitive Function

5.1 – Abstract

Hypertension-associated PA dysfunction reduces cerebral perfusion and impairs cognition. This is associated with impaired TRPV4-mediated PA dilation; therefore, I tested the hypothesis that TRPV4 channels are important regulators of cerebral perfusion, PA structure and dilation, and cognition. 10-12-month-old male TRPV4 knockout (WKY-*Trpv4*^{em4Mcwi}) and age-matched control WKY rats were studied. Cerebral perfusion was measured by MRI with arterial spin labeling. PA structure and function were assessed using pressure myography and cognitive function using the novel object recognition test. Cerebral perfusion was reduced in the WKY-*Trpv4*^{em4Mcwi} rats. This was not a result PA remodeling because TRPV4 deletion did not change PA structure. TRPV4 deletion did not change PA myogenic tone development, but PAs from the WKY-*Trpv4*^{em4Mcwi} rats had severely blunted endothelium-dependent dilation. The WKY-*Trpv4*^{em4Mcwi} rats had impaired cognitive function and exhibited depressive-like behavior. The WKY-*Trpv4*^{em4Mcwi} rats also had increased microglia activation, and increased mRNA expression of GFAP and TNFα suggesting increased inflammation. My data indicate that TRPV4 channels play a critical role in cerebral perfusion, PA dilation, cognition and inflammation. Impaired TRPV4 function in diseases such as hypertension may increase the risk for the development of vascular dementia.

5.2 – Introduction

cSVD is a growing public health issue that lacks effective treatments. cSVD is described as an impairment in the structure and function of small arteries and arterioles, including PAs, capillaries, and venules with a lumen diameter <100μm (15). PAs are critical regulators of blood flow to the cerebral microcirculation and contribute to vascular resistance (22). These arterioles are not connected to each other by collaterals, thus they are considered the weak link or a bottle-neck in the perfusion of the cerebral microcirculation. Importantly, occlusion of a single PA produces a microinfarct and cognitive dysfunction (22). Since cerebral perfusion is regulated by a combination of artery structure and function, alterations in these arterioles can cause cerebral hypoperfusion which leads to the development of vascular cognitive impairment and exacerbation of other forms of dementia. In fact, cSVD accounts for about 50% of all dementia cases including Alzheimer's disease (12). Hypertension is a risk factor for cSVD and vascular dementia development. We have previously shown that aging is associated with remodeling of PAs (6). Also, that hypertension causes inward hypotrophic remodeling (5) and impairs endothelium-dependent dilation of PAs; these changes were associated with reduced cerebral perfusion and impaired cognitive function (7).

I am interested in the role of the TRPV4 channels in cSVD and vascular cognitive impairment. TRPV4 channels are non-selective Ca^{2+} permeable channels expressed in endothelial cells, SMCs, astrocytes and neurons (14). All of these cell types are included in the NVU and act together to regulate cerebral perfusion (13). In endothelial cells, TRPV4 channel activation allows for Ca^{2+} influx that activates IK_{Ca}/SK_{Ca} to elicit EDH dilation in peripheral arteries and large pial cerebral arteries (4, 26). TPRV4 channels are

critical regulators of PA endothelium-dependent dilation in normotensive and hypertensive rats and mice (7, 17, 18, 28). AngII-hypertensive mice have impaired TRPV4-mediated dilation and cognition, and treatments that improve TRPV4 mediated dilation also correct the cognitive dysfunction (7). The link between TRPV4 mediated dilation and cognitive function is further strengthened by studies showing that TRPV4-mediated dilation in PAs is impaired in other models of cognitive decline associated with chronic cerebral hypoperfusion (17). Therefore, I hypothesized that genetic disruption of TRPV4 channel signaling would impair endothelium-dependent dilation and cognitive function using middle-aged WKY-*Trpv4*^{em4Mcwi} rats and their appropriate controls.

5.3 – Materials and Methods

5.3.1 – Experimental model

All experimental protocols were approved by the Michigan State University Animal Care and Use Committee and were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. These studies were performed on 10-12-month-old male rats. Control WKY rats were purchased from Envigo. WKY-*Trpv4*^{em4Mcwi} were generated by CRISPR/Cas9 resulting in a 4 base pair deletion in Exon 4 of the Trpv4 gene, these rats were obtained from the Gene Editing Resource Center at the Medical College of Wisconsin. All rats were housed on 12h:12h light/dark cycle with food and water ad libitum.

5.3.2 – Tail-cuff plethysmography

Blood pressure was measured in conscious rats by tail-cuff plethysmography using a RTBP1001 tail-cuff blood pressure system (Kent Scientific, Torrington, CT) as described previously (5, 20, 24).

5.3.3 – Pressure Myography

The endothelial function of PAs was assessed by pressure myography (5, 18, 20). To isolate the arterioles, a 5 x 3 mm section of the brain containing the MCA was dissected, then the pia with the MCA was separated from the brain and the PAs branching off the MCA were used for experiments. Isolated arterioles were cannulated with two glass micropipettes in a custom-made cannulation chamber. Arterioles were equilibrated at 37°C in PSS containing 141.9mmol/L NaCl, 4.79mmol/L KCl, 1.12mmol/L KH_2PO_4, 1.79mmol/L $MgSO_4 \bullet 7H_2O$, 109mmol/L HEPES, and 59mmol/L Dextrose. A servo-null system was used to pressurize the arterioles, and a leak test was performed prior to each experiment. Arterioles were pressurized to 60mmHg (5) until the development of stable myogenic tone (% tone = [1-(active lumen diameter/passive lumen diameter)] x 100. The diameter of the arterioles was recorded using MyoView 2.0 software (Danish Myo Technology, Aarhus, Denmark). After the generation of stable myogenic tone, endothelium-dependent dilation was assessed by incubating the arterioles with increasing concentrations of the muscarinic receptor agonist CCh (10^{-9}-10^{-5}mol/L) or the TRPV4 agonist, GSK1016790A (10^{-9}-10^{-5}mol/L).

To assess structure and biomechanics, the arterioles were incubated in Ca^{2+} free-PSS and a pressure response curve was constructed. Intralumenal pressure was increased from 3-120mmHg in 20mmHg increments, at each pressure outer and lumen diameter were recorded. The measurements for diameter were used to calculate wall thickness, wall stress, strain and stiffness.

5.3.4 – Novel object recognition

Rats were acclimated over a course of three days for 10 minutes per day in the empty arena (an open box with dark walls). On the testing day, rats were placed facing away from the objects and allowed to explore the arena for 10 minutes with two identical objects. After a retention time of 90 minutes, rats were returned to the arena in a similar manner and allowed to explore one familiar and one novel object for 5 minutes and tracked using EthoVision XT software. Exploration time of novel object and total time exploring were calculated (1).

5.3.5 – Porsolt swim test

Rats were pre-exposed to the test condition 24 hours before the test session. A 30cm swim cylinder was filled with water (23-25°C), the rat was then place into the swim cylinder and allowed to swim for 15 minutes and tracked using the software EthoVision XT. The amount of time the rats spent moving and the time immobile was calculated. All rats were monitored by the investigator to ensure that they did not drown in the event that they stopped swimming.

5.3.6 – Immunostaining

Brain section were fixed for 48hrs, then washed twice in 1X PBS (24 hrs each) and stored in 20% sucrose-PBS until section. 40µm cryosections of the cortex were made. For microglia quantification, free-floating brain sections were blocked and permeabilized in 0.1% Triton-X with 10% donkey serum PBS, then incubated in 1:200 rabbit Anti-IBA1 (catalog number 019-19741, Wako, Richmond, VA) overnight at 4°C. After washing in 1X PBS, sections were incubated in secondary AlexaFluor 488 donkey anti-rabbit antibody (catalog number ab150073, Abcam, Cambrige, UK) for one hour. For artery and capillary quantification, 40µm cryosections were incubated overnight in 0.01mg/ml isolectin GS-IB_4 Alexa Fluo-568 conjugate (catalog number I21412, Invitrogen, Carlsbad, CA) at 4°C. The next day, sections were washed 4x in 1X PBS (5 min each wash) and coverslips were mounted using Prolong antifade reagent (Invitrogen, Carlsbad, CA) (14).

5.3.7 – qRT-PCR

RNA was extracted from brain sections, near the MCAs, for qRT-PCR analysis using Trizol. RNA was reverse transcribed using VILO reverse transcriptase (Invitrogen, Carlsbad, CA). TAQMAN-specific probes were used for the PCR to assess the mRNA expression of TRPV4, glial fibrillary acidic protein (GFAP), IL-6, TNFα, SYP, and doublecortin. mRNA expression is expressed as the fold change from control using the $2^{-\Delta\Delta Ct}$ method. β2-microglobulin was used as control for normalization.

5.3.8 – Plasma protein oxidation

Blood was collected by cardiac puncture immediately prior to euthanasia in anesthetized mice. Plasma oxidized proteins were measured by ELISA (Cayman Chemical, Ann Arbor, MI) following the manufacturer instructions.

5.3.9 – Magnetic Resonance Imaging

Cerebral perfusion was measured by continuous arterial spin labeling (CASL) using a Bruker 7T BioSpec 70/30 USR housed in the Department of Physiology. Rats were initially anesthetized at 3-5% isoflurane in 100% oxygen and maintained at 1-2% throughout experimentation. Temperature was monitored (SA Instruments) and maintained at 37°C using a water bath. Breathing was monitored (SA Instruments) and maintained between 45-60 breaths per minute by changing the % of isoflurane. The anatomical slice of interest was determined using Turbo-RARE sequence with an echo time of 33msec, 11msec spacing and RARE factor of 8. Repetition time was 3.8sec using 1mm slice thickness, 35mm x 35mm FOV and 256x256 pixel resolution which yielded voxel sizes of 137 x 137microns. T1 values for the region of interest were determined by generating a T1 mapping sequence (TE=7msec, 7msec echo spacing, RARE factor 2, 6T1s) using a 35x35mm FOV and 128x128 pixels. The true in plane resolution for the T1 map was 273 x 273microns and was kept during the spin labeling experiment. CASL perfusion measurements were performed with the following parameters: TE= 12msec, repetition time 2040 msec, 16 averages. The labeling slice was maintained 25mm from the measuring slice and over the carotids (2sec saturation time, 2.5W power). Using the T1 map, control, and labeled brain slice acquisitions the perfusion was calculated according to:

$\frac{0.9}{T1} \times \frac{Control-Label}{2 \times Control}$ (25).

5.3.10 – Drugs and chemicals

GSK1016790A was purchased from Cayman Chemicals (Ann Arbor, MI). All other drugs and chemicals were purchased from Sigma-Aldrich unless otherwise specified.

5.3.11 – Statistical analysis

All data are presented as mean ± SEM. Myogenic tone, physiological characteristics, blood pressure, mRNA expression, behavioral, and immunofluorescence data were analyzed by Student's t-test. For analysis of artery vasodilation and structure, two-way analysis of variance with repeated measures in one factor was utilized followed by Bonferroni-adjusted t-tests for post-hoc comparisons. All statistical analyses were performed using GraphPad Prism 7.0 software (GraphPad, San Diego, CA). In all cases, statistical significance was denoted by $p<0.05$.

5.4 – Results

5.4.1 – TRPV4 channel deletion reduced cerebral perfusion but does not alter blood pressure.

Cerebral perfusion was assessed using MRI with continuous arterial spin labeling. The WKY-*Trpv4*^{em4Mcwi} had reduced cerebral perfusion (Figure 5.1). WKY-*Trpv4*^{em4Mcwi} had increased spleen and kidney weights (Figure 5.2 D-E) but no other changes in physiological characteristics were observed (Figure 5.2 A-C). Deletion of TRPV4 channels did not alter systolic blood pressure (Figure 5.2 F).

5.4.2 – TRPV4 channels regulate parenchymal arteriole endothelium-dependent dilation.

The role of TRPV4 channels in the generation of myogenic tone and endothelium-dependent dilation of PAs was assessed by pressure myography. No significant differences were observed in myogenic tone suggesting that TRPV4 channels do not

contribute to tone at the physiological intralumenal pressure of 60mmHg (Figure 5.3 A). Then endothelium-dependent dilation in response to CCh was assessed. WKY-*Trpv4*^{em4Mcwi} rats had severely impaired CCh-mediated dilation of PAs (Figure 5.3 B). Impaired dilation was also observed in response to the TRPV4 agonist, GSK1016790A (Figure 5.3 C).

5.4.3 – TRPV4 channel deletion does not affect parenchymal arteriole structure or cause cerebral artery rarefaction.

Next, the structure and biomechanical properties of PAs were assessed under Ca^{2+} free conditions. The outer diameter (Figure 5.4 A), lumen diameter (Figure 5.4 B) and wall thickness (Figure 5.4 C) were not changed. Wall stress was significantly increased at 120mmHg intralumenal pressure (Figure 5.4 D). Wall strain (Figure 5.4 E) and artery wall stiffness remained unchanged (Figure 5.4 F). These data suggest that WKY-*Trpv4*^{em4Mcwi} rats do not exhibit significant PA remodeling. The number of vessels in the cortex were then quantified using an endothelial cell marker. No changes in the number of vessels were observed between WKY-*Trpv4*^{em4Mcwi} rats and the controls (Figure 5.5).

5.4.4 – TRPV4 knockout rats have impaired cognitive function and depressive-like behavior.

TRPV4 channel deletion severely impairs PA endothelium-dependent dilation and reduces brain perfusion. Therefore, I evaluated whether these vascular changes are associated with cognitive dysfunction. I evaluated non-spatial, short-term memory using the novel object recognition test. The WKY-*Trpv4*^{em4Mcwi} spent less time exploring the novel object (Figure 5.6 A). There was also an insignificant reduction in the total time

exploring both objects (p=0.15, Figure 5.6 B). The reduction in time spent exploring the novel object was not the result of reduced movement in the WKY-*Trpv4*^{em4Mcwi} rats, because no changes were observed in total distance moved (Figure 5.6 C) or the velocity of movement (Figure 5.6 D). To explore if the WKY-*Trpv4*^{em4Mcwi} rats are less motivated and have depressive-like behaviors, I used the Porsolt swim test. The WKY-*Trpv4*^{em4Mcwi} rats spent less time moving compared to the controls (Figure 5.7). Together these data suggest that TRPV4 channel deletion results in cognitive dysfunction and depressive-like behaviors.

5.4.5 – WKY-*Trpv4*^{em4Mcwi} rats have increased cerebral inflammation but not systemic oxidative stress.

To explore mechanisms associated with the vascular and cognitive changes, I counted activated microglia in the brain as a marker of inflammation and assessed plasma oxidation as an index of oxidative stress. My data show that WKY-*Trpv4*^{em4Mcwi} rats have increased number of activated microglia suggesting increased inflammation (Figure 5.8). However, there was no change in plasma oxidized proteins (Figure 5.9).

5.4.6 – TRPV4 channel deletion increases the mRNA expression of markers for inflammation.

The gene expression of TRPV4 channels was significantly reduced in the brain of TRPV4 knockout rats, as expected (Figure 5.10 A). I then evaluated the mRNA expression of GFAP in the brain to evaluate changes in astrocytes. The gene expression of GFAP was increased in WKY-*Trpv4*^{em4Mcwi} rats (Figure 5.10 B) suggesting astrogliosis that could contribute to vascular and cognitive dysfunction. Activated microglia can release several inflammatory substances including TNFα and IL-6. WKY-*Trpv4*^{em4Mcwi} rats

have a significant increase in the mRNA expression of TNFα (Figure 5.10 C) and a small, but not significant, increase in IL-6 (Figure 5.10 D). I also evaluated changes in the mRNA expression of markers for neuronal support. There were no significant changes in the mRNA expression of SYP (Figure 5.10 E). The mRNA expression of doublecortin was significantly increased in the WKY-*Trpv4*^{em4Mcwi} rats (Figure 5.10 F).

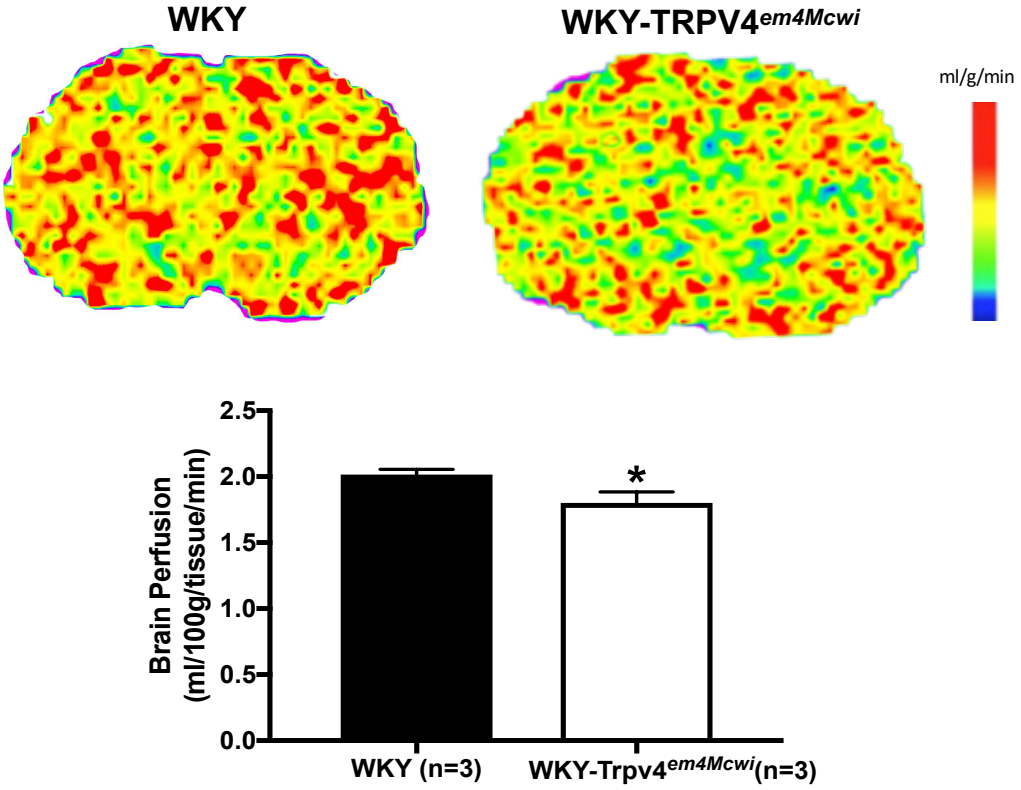

Figure 5.1 TRPV4 channel deletion decreases cerebral perfusion. The role of TRPV4 channels in cerebral perfusion was assessed using MRI with arterial spin labeling. Representative perfusion maps are shown. WKY-*Trpv4*^{em4Mcwi} rats have reduced cerebral perfusion. Data are presented as mean ± SEM. *p<0.05 by Student's t-test.

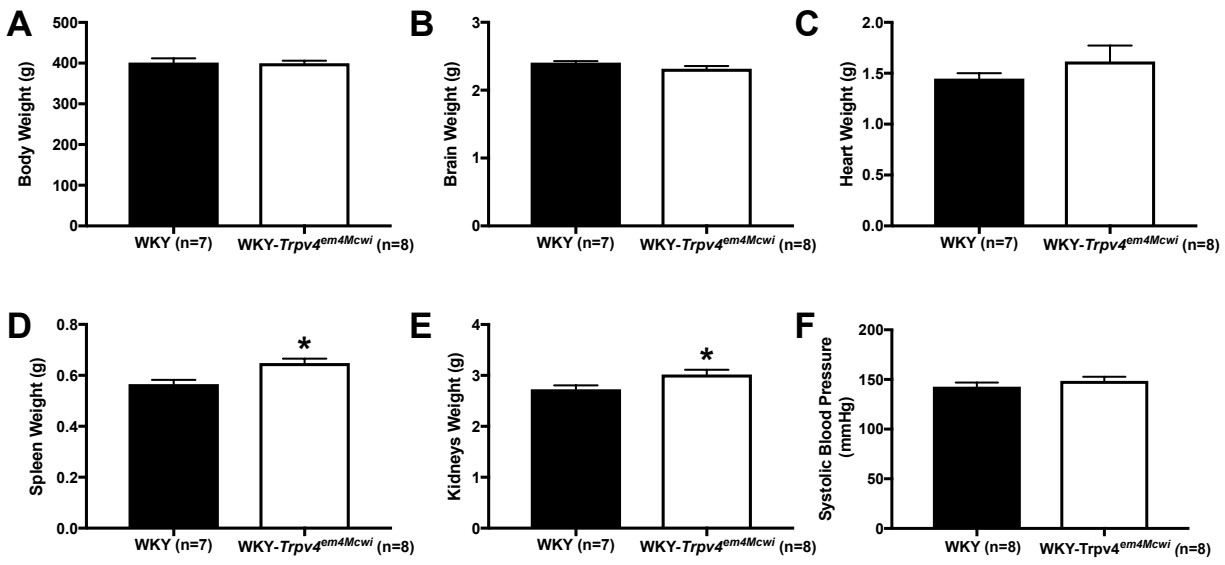

Figure 5.2 TRPV4 channel deletion does not alter blood pressure. The physiological characteristic and blood pressure were assessed. (A) Body weight, (B) brain weight, (C) heart weight were not changed. WKY-*Trpv4*^{em4Mcwi} rats have increased (D) spleen and (E) kidneys weight. (F) Systolic blood pressure was not altered by TRPV4 deletion. Data are presented as mean ± SEM. *$p<0.05$ by Student's t-test.

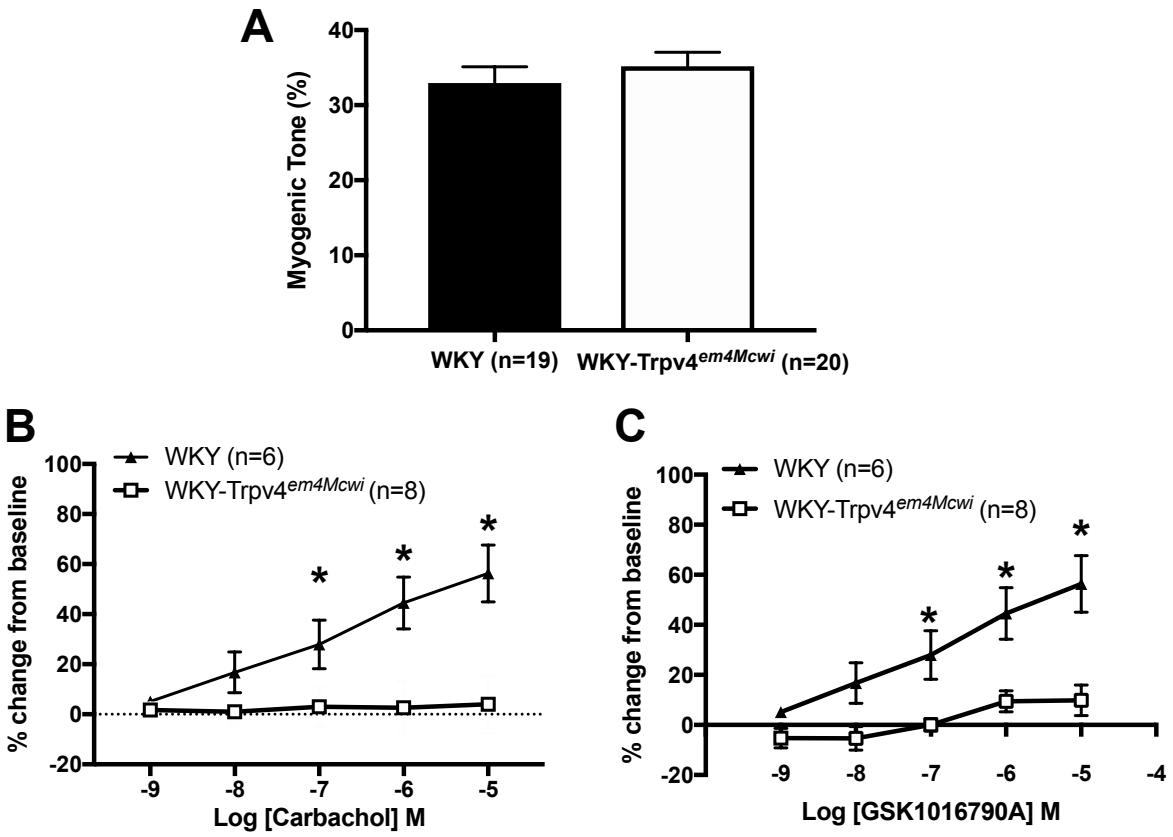

Figure 5.3 TRPV4 channel deletion severely impairs endothelium-dependent dilation but does not alter myogenic tone. The role of TRPV4 channel in PA myogenic tone and endothelium-dependent dilation was assessed using pressure myography. (A) No changes in PA myogenic tone were observed at 60mmHg intralumenal pressure. WKY-*Trpv4*^{em4Mcwi} rats did not dilate in response to (B) CCh or (C) the TRPV4 agonist, GSK1016790A. Data are presented as mean ± SEM. *p<0.05 by Student's t-test or two-way ANOVA.

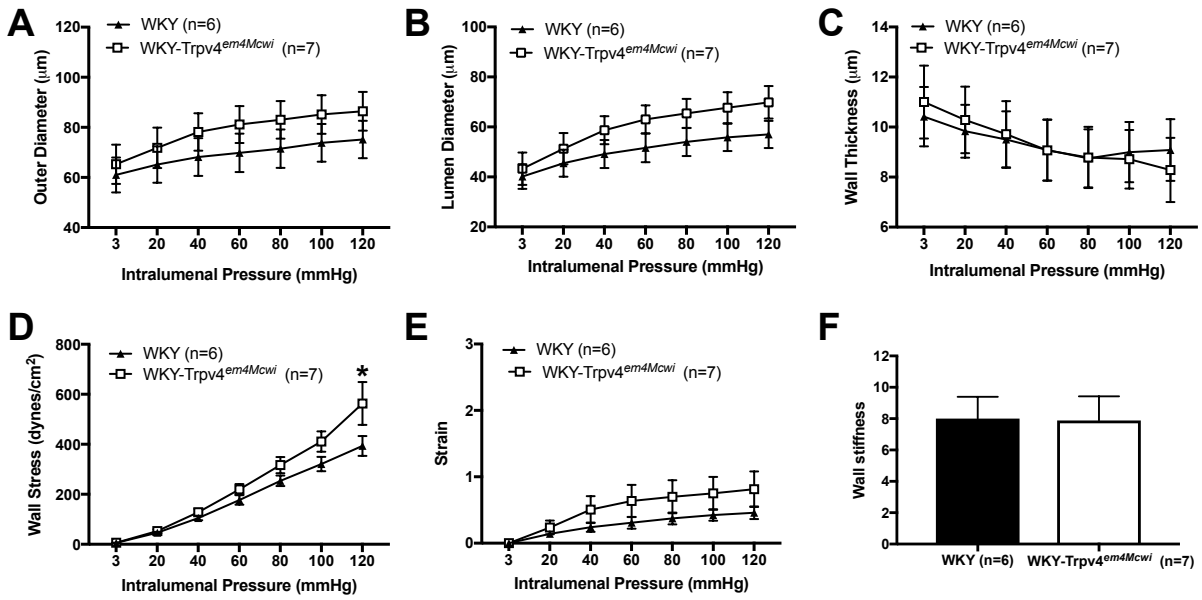

Figure 5.4 TRPV4 channel deletion does not alter PA structure. The role of TRPV4 channels in PA structure was assessed using pressure myography. The (A) outer diameter and (B) lumen diameters, and (C) wall thickness were not changed by TRPV4 deletion. (D) Wall stress was significantly increased in WKY-*Trpv4*^{em4Mcwi} rats at 120mmHg intralumenal pressure. (E) Wall strain and (F) artery wall stiffness were not changed. Data are presented as mean ± SEM. *p<0.05 by two-way ANOVA.

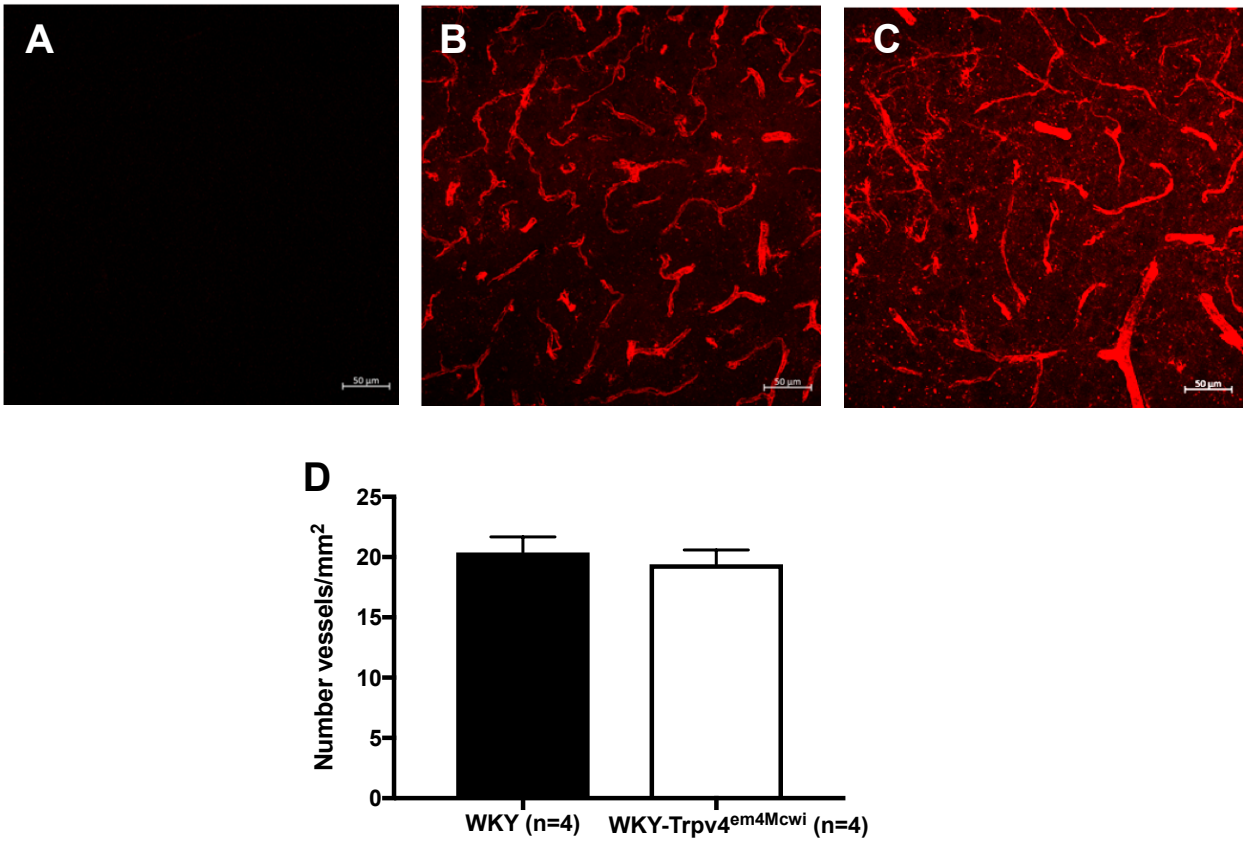

Figure 5.5 TRPV4 channel deletion does not cause cerebral artery rarefaction. The number of vessels in the cortex was quantified using the endothelial cell marker isolectin IB-4. (A-C) Representative images are shown. (D) WKY-*Trpv4*^{em4Mcwi} rats do not have artery rarefaction. Data are presented as mean ± SEM. $p>0.05$ by Student's t-test.

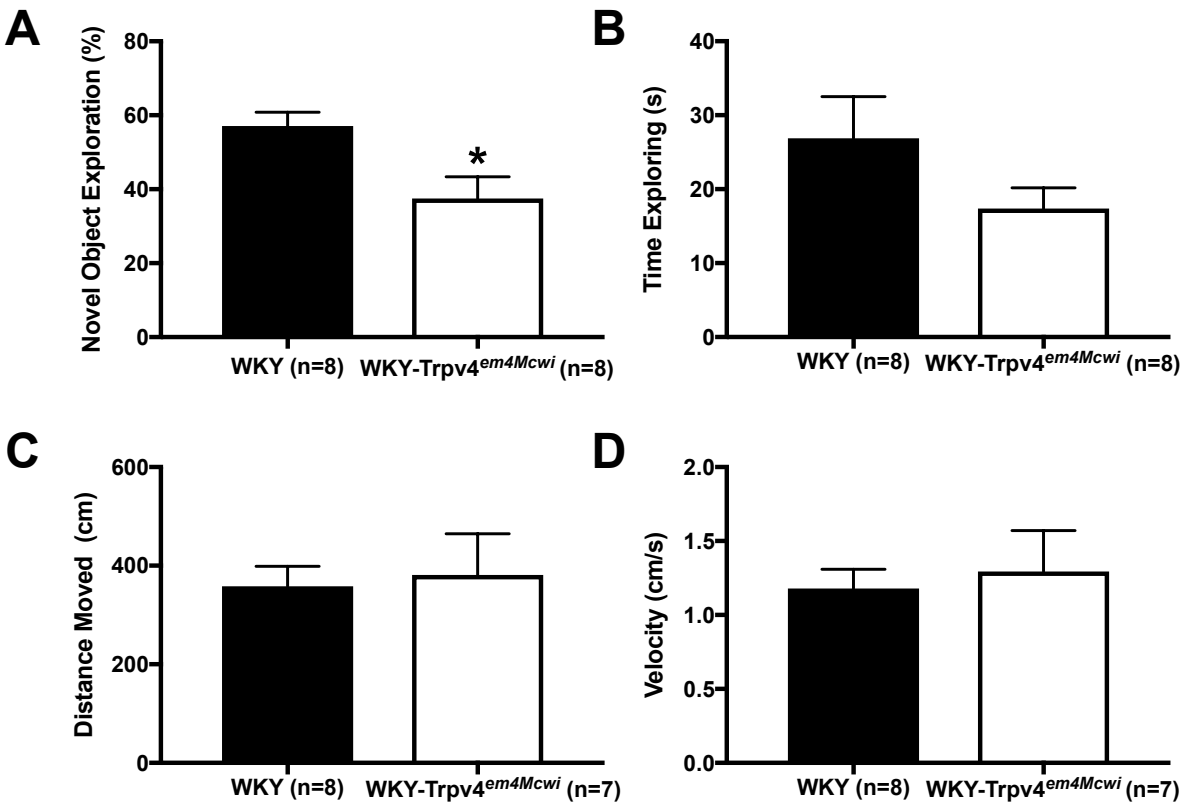

Figure 5.6 TRPV4 channel deletion impairs cognitive function. Non-spatial, short-term memory was assessed using the novel object recognition test. (A) WKY-*Trpv4*^{em4Mcwi} rats spent less time exploring the novel object. (B) There was a trend in reduced total time of exploration in WKY-*Trpv4*^{em4Mcwi} rats, but the changes were not statistically significant. (C) total distance moved and (D) velocity were not significantly changed. Data are presented as mean ± SEM. *p<0.05 by Student's t-test.

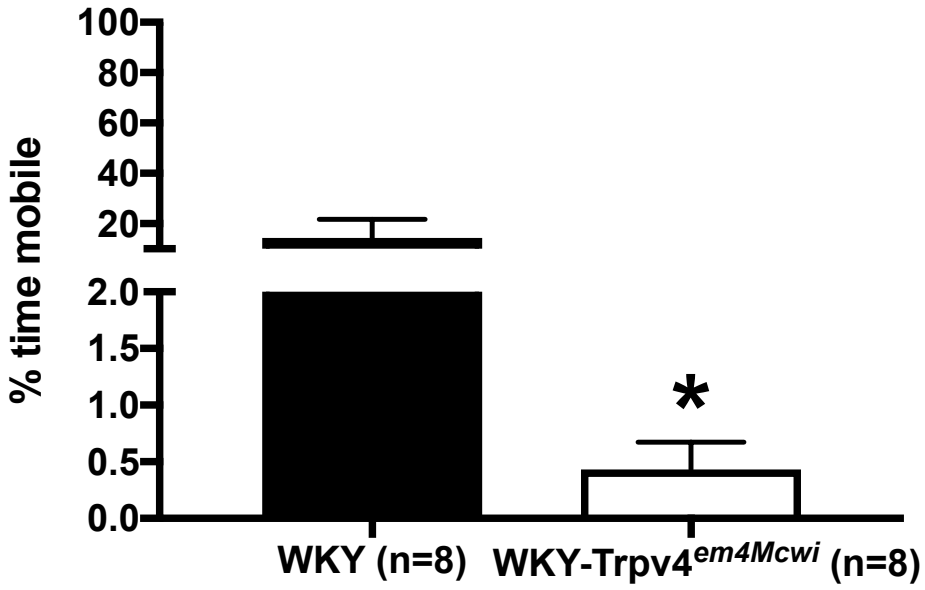

Figure 5.7 TRPV4 channel deletion increases depressive-like behaviors. The Porsolt swim test was used to assess depressive-like behaviors. WKY-*Trpv4*^{em4Mcwi} rats spent less time swimming compared to control. Data are presented as mean ± SEM. *p<0.05 by Student's t-test.

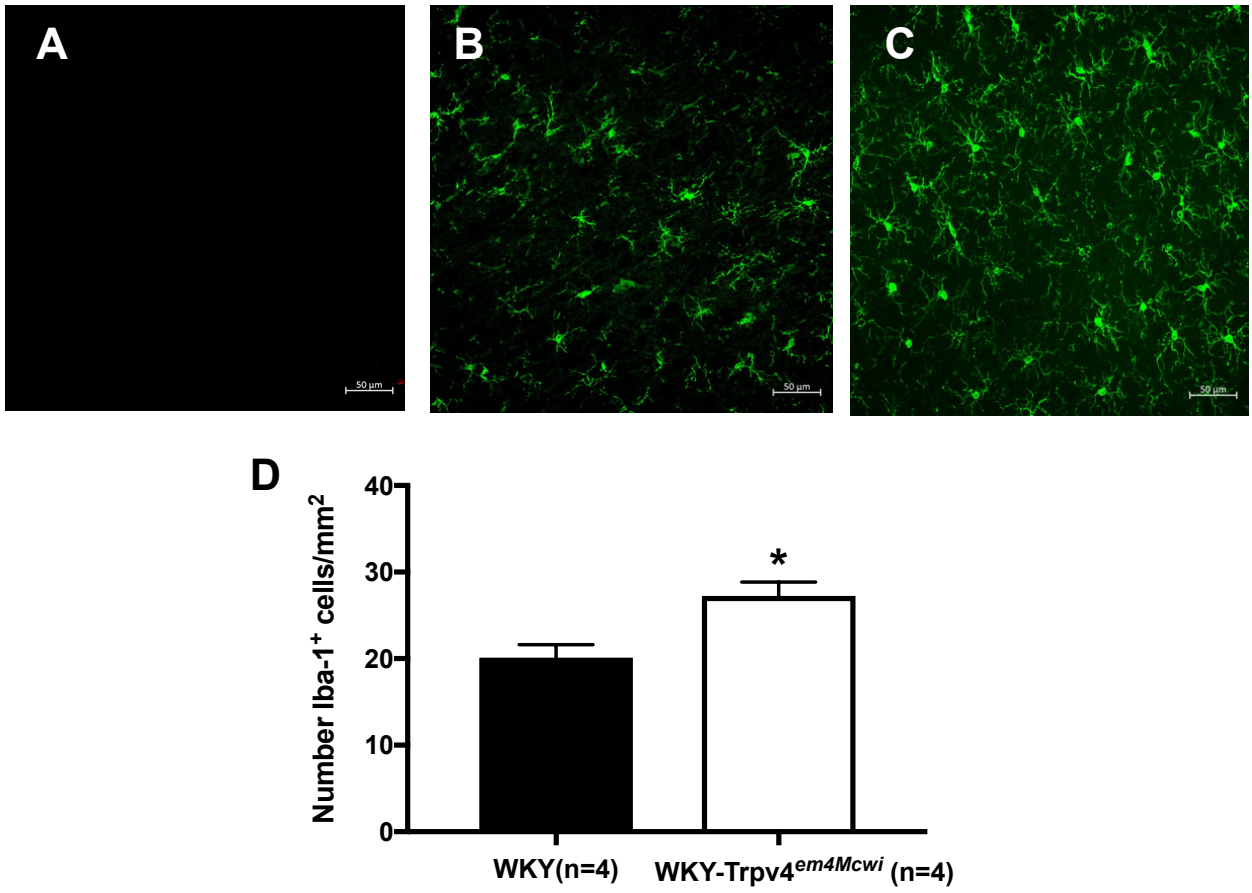

Figure 5.8 WKY-*Trpv4*^{em4Mcwi} rats have increased microglia. The number of activated microglia were quantified using Iba-1. (A-C) Representative images are shown. (D) WKY-*Trpv4*^{em4Mcwi} rats have increased number of activated microglia. Data are presented as mean ± SEM. *p<0.05 by Student's t-test.

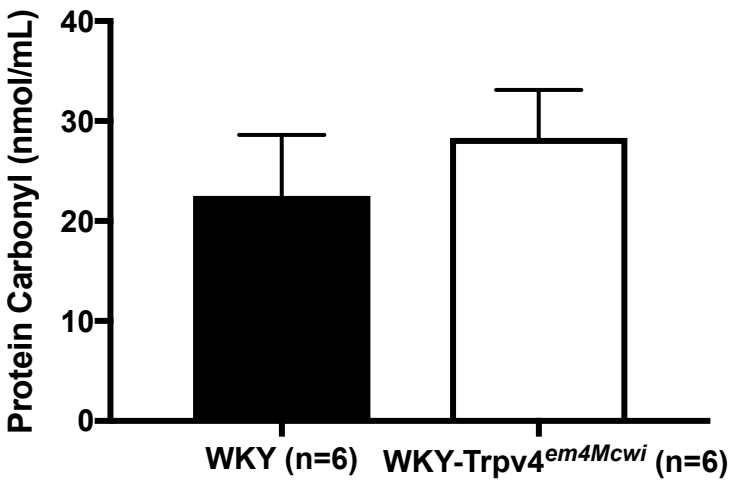

Figure 5.9 WKY-*Trpv4*^{em4Mcwi} rats do not have increase plasma protein oxidation. The protein carbonyl content was assessed by ELISA. No changes in plasma oxidized proteins were observed. Data are presented as mean ± SEM. *p<0.05 by Student's t-test.

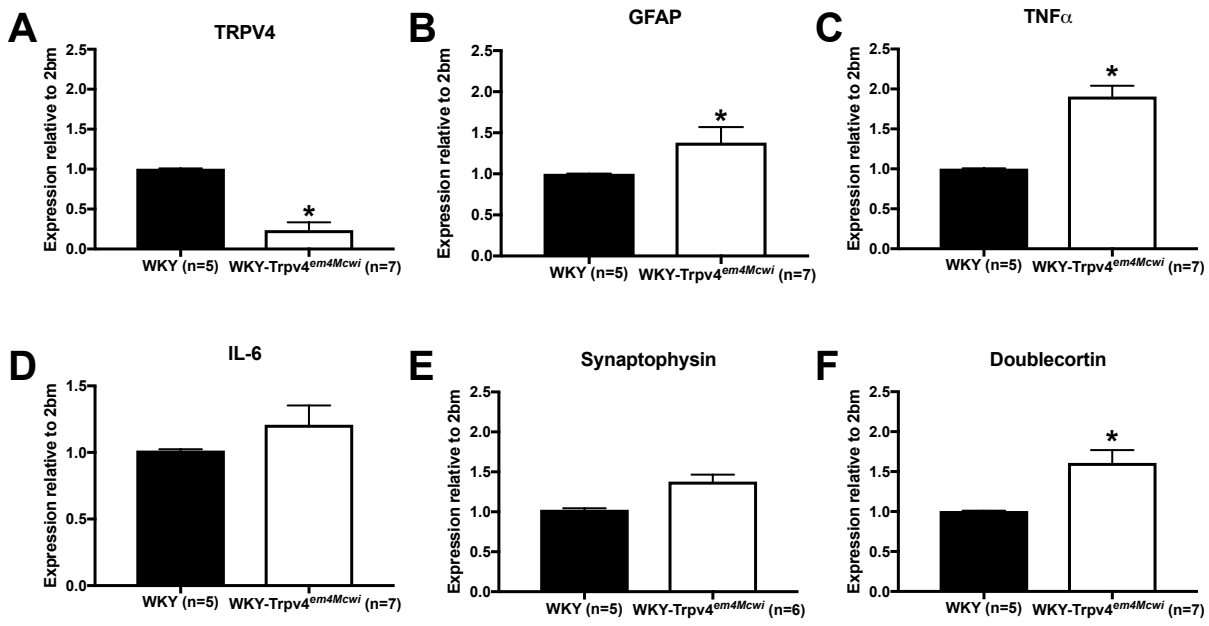

Figure 5.10 WKY-*Trpv4*^{em4Mcwi} rats have increased mRNA expression of inflammatory markers. The brain mRNA expression of markers for inflammation and neuronal support were assessed by qRT-PCR. (A) TRPV4 gene was significantly reduced. (B) GFAP and (C) TNFα were significantly increased in WKY-*Trpv4*^{em4Mcwi} rats. (D) IL-6 and (E) SYP were not significantly changed. (F) Doublecortin was significantly increased in WKY-*Trpv4*^{em4Mcwi} rats. Data are presented as mean ± SEM. *p<0.05 by Student's t-test.

5.5 – Discussion

The novel findings in this study are that TRPV4 channel deletion: 1). reduces cerebral perfusion; 2). impairs cognitive function and increases depressive-like behaviors; 3). causes impaired PA endothelium-dependent dilation without affecting artery structure; 4). causes cerebral inflammation including increased microglia activation; 5). does not alter blood pressure. One strength of this study is that middle-aged rats were used. These studies improve our understanding on the role of TRPV4 channels in cognition and PA structure and function. This is critical because PA remodeling and impaired dilation with hypertension alters neuronal health and increases the risk of vascular dementia development.

TRPV4 channels are expressed in endothelial cells where they regulate vasodilation, but these channels aid other cell types such as SMC, glia and neurons to control vascular tone and cerebral blood flow (9). TRPV4 channel deletion did not alter blood pressure consistent with previous studies in TRPV4$^{-/-}$ mice (10). This lack of change in blood pressure fits with the absence of change in heart weight. Other studies have shown that TRPV4 channels play a role in L-NAME-induced hypertension but not in AngII-hypertensive in mice (19). In that study, TRPV4$^{-/-}$ mice were given L-NAME (7 days) or AngII (14 days) and blood pressure was measured. There were no differences in blood pressure in the AngII treated control and TRPV4$^{-/-}$ mice. However, TRPV4$^{-/-}$ mice treated with L-NAME had a significantly higher mean arterial blood pressure than controls. The AngII treated TRPV4$^{-/-}$ and control mice had similar increases in blood pressure (19). That study suggests that TRPV4 channels play a minor role in L-NAME induced hypertension

but not in AngII. However, TRPV4 channels play an important role in endothelial dysfunction in AngII-hypertension (7).

Vascular and cognitive dysfunction could be associated with reduced cerebral perfusion, therefore I examined cerebral perfusion in the WKY-*Trpv4*^{em4Mcwi} rats using MRI with continuous arterial spin labeling. My data shows that the TRPV4 deficient rats have reduced cerebral perfusion. TRPV4 channels are important in amplifying the responses in NVC, thus their absence could change cerebral perfusion (8, 11).

To better understand the reduced perfusion in the WKY-*Trpv4*^{em4Mcwi} rats I first evaluated PA function. TRPV4 channel deletion did not change the generation of myogenic tone but resulted in a severe impairment in PA dilation suggesting a critical role for this channel in cerebral arteriolar dilation. This is consistent with previous studies in TRPV4$^{-/-}$ mice (3) and with my previous studies in C57bl/6 mice where inhibition of TRPV4 channels with the pharmacological antagonist GSK2193874 severely blunted PA dilation (7). However, TRPV4 channels may regulate myogenic tone in pathological conditions. My previous studies show that TRPV4 inhibition with GSK2193874 in normotensive mice did not change myogenic tone of PAs but it resulted in a significant loss of myogenic tone in the PAs from hypertensive mice (7). Preliminary studies suggest that GSK2193874 results in a modest loss of myogenic tone in the WKY-*Trpv4*^{em4Mcwi} rats (approximately 8% loss of myogenic tone).

Contrary to recent findings in TRPV4$^{-/-}$ mice where the PAs were outward remodeled (3), the structure of the PAs from WKY-*Trpv4*^{em4Mcwi} rats were not changed. The different findings could be explained by the different species used and the age of the animals. My studies were conducted in middle aged rats while the TRPV4$^{-/-}$ mice in the

other study were young adults. I also evaluated cerebral artery rarefaction as a possible cause of the reduced cerebral perfusion. My data suggests that the TRPV4 deficient rats do not exhibit rarefaction in the cerebral cortex. Therefore, the reduction in blood flow could be explained by the severely blunted PA endothelium-dependent dilation observed.

The impaired endothelium-dependent dilation could compromise regulation of cerebral blood flow and cause cognitive dysfunction. I assessed non-spatial, short-term memory using the novel object recognition test. My data shows that TRPV4 channel deletion impairs short-term memory as shown by the reduction in the time spent exploring the novel object. No changes were observed in the velocity or the total distance moved between both groups. Although it was not statistically significant, there was a reduction in the total time of exploration in the WKY-*Trpv4*^{em4Mcwi} rats. This presented the possibility that the WKY-*Trpv4*^{em4Mcwi} rats might be less motivated than the controls. Therefore, I also assessed depressive-like behaviors using the Porsolt swim test and my data suggests that the WKY-*Trpv4*^{em4Mcwi} rats have increased depressive-like behaviors. My data are at odds with studies in the TRPV4$^{-/-}$ mice where no changes in memory were observed and also where the knockout mice had a reduction in depressive-like behaviors (21). The differences could be explained by the different species used between both studies and also by the age of the animals. Shibasaki *et al.,* used young adult mice (18-24 weeks of age) whereas in my study I used middle age rats (40-48 weeks of age) (21).

My data also suggests that the WKY-*Trpv4*^{em4Mcwi} rats have increased inflammation as shown by the increased number of activated microglia. These activated microglia can release pro-inflammatory cytokines such as IL-6 and TNFα and the mRNA expression of TNFα was increased in the WKY-*Trpv4*^{em4Mcwi} rats. Astrocytes can also

play an important role in inflammation and NVC. Importantly, astrocytes express TRPV4 channels. My data shows that the WKY-*Trpv4*^{em4Mcwi} rats have increased mRNA expression of the astrocytic marker, GFAP, suggesting there might be astrogliosis. Similar increases in microglia activation, astrogliosis, IL-6 and TNFα production have been shown to be involved in the pathogenesis of cSVD. Therefore, these pro-inflammatory markers observed in the WKY-*Trpv4*^{em4Mcwi} rats could be mediating the cognitive decline.

The altered cognition could be associated with changes in the expression of neuronal markers such as SYP, a marker for neuronal function and synaptic plasticity and doublecortin, a marker for new and immature neurons (2, 23). Recent studies show reduced mRNA level of these in AngII-hypertensive mice with vascular cognitive impairment (7). Studies in old rats also show a correlation between reduction in SYP levels and the degree of dementia development (27). However, in the current study the WKY-*Trpv4*^{em4Mcwi} rats have increased mRNA expression of SYP and doublecortin. It is possible that the WKY-*Trpv4*^{em4Mcwi} rats I used in the current study are at the beginning of the slope of cognitive decline and the increase in the mRNA expression in the markers for neuronal support might be a compensatory response for the reduced perfusion. I also examined protein carbonyl content as a marker for oxidative stress but did not observe any differences. I recognize this is not a direct measure of oxidative stress, but this is relevant to my studies because oxidized plasma proteins have been observed in the plasma of Alzheimer's patients (16).

Some limitations in my study must be acknowledged. First, all of my studies were conducted in male rats to match previous studies in our lab on the role of MR signaling in the cerebral vasculature, thus I did not explore sex differences. I have only studied middle

aged rats; thus, more studies should be conducted in younger rats to assess age-associated changes. Also, the MRI studies were performed in isoflurane-anesthetized rats that is vasodilator. In the future, I would also like to expand my studies to hypertensive rats and further explore the role of TRPV4 channels in NVC.

In summary, TRPV4 channels are important regulators of cerebral perfusion and PA endothelium-dependent dilation. TRPV4 channels also are important regulators of cognitive function. Impaired TRPV4 channel function in diseases like hypertension could increase the risk for the development of VCID.

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CHAPTER 6

Endothelial Mineralocorticoid Receptors in Cerebrovascular and Cognitive Function in Angiotensin II-Hypertension

6.1 – General conclusions

The data presented in this dissertation shows a link between EC-MR signaling and PA structure and function in male mice with AngII-hypertension. The studies described support the overall hypothesis that EC-MR activation in hypertension mediates the PA remodeling and impaired endothelium-dependent dilation that are associated with reduced cerebral perfusion. The hypothesis was tested through three specific aims.

The first aim was designed to assess the role played by the EC-MR in PA remodeling and changes in cerebral blood flow in AngII-induced hypertension. Prior to beginning this aim I verified that AngII infusion increased plasma aldosterone levels in male mice. In this aim I used a pharmacological approach with EPL, and a genetic approach with ECMRKO mice. I first assessed the possibility that under control conditions MR activation causes PA remodeling. My studies show that in the absence of a hypertensive challenge MR antagonism or EC-MR deletion does not change blood pressure, cerebral blood flow or PA structure. However, when cardiovascular risk factors, such as hypertension, are present MR activation negatively alters the vasculature. AngII infusion increased blood pressure but this was not changed by either EPL treatment or EC-MR deletion. As expected AngII infusion reduced cerebral perfusion, and MR antagonism and EC-MR deletion improved cerebral perfusion in the AngII mice.

To better understand the potential causes of the reduced cerebral perfusion, the structure of the PAs was assessed. AngII-hypertension resulted in inward remodeling of the PAs; this was prevented by MR antagonism and EC-MR deletion. The same was true

in PCAs. The remodeling observed could be associated with increased inflammation and MMP expression. AngII-hypertension increased the mRNA expression of the proinflammatory markers MCP-1 and IL-6, but these do not appear to be involved in the remodeling process because when the remodeling was corrected with EPL the mRNA levels of the cytokines did not change. A previous study from our lab showed that remodeling could also be mediated by TNFα release through PVMs in the MCAs from SHRSP (24). In my studies there was a trend in the increase of TNFα mRNA expression in the PCA but this increase did not reach statistical significance. The mRNA expression of MMP-2 was increased in AngII mice and this was prevented by MR antagonism. This is in agreement with previous studies showing that MMPs are involved in the MCA remodeling during hypertension (26). Aldosterone also induces MMP-2 expression in cardiomyocytes (29).

AngII and aldosterone increase the production of ROS (2, 4). NOX2-containing NADPH oxidase is important in the AngII-mediated inward remodeling in pial arterioles (4), and NOX4 gene expression is upregulated by MR signaling (2). In my studies, neither AngII or MR antagonism significantly changed the mRNA expression of NOX2 and NOX4 in PCAs. I have not directly measured changes in the NOX2 or NOX4 protein or ROS production, thus I cannot rule out their involvement in the remodeling process. Very interestingly and unexpected, the PAs had hypotrophic remodeling instead of the wall hypertrophy usually observed in hypertension; this hypotrophic remodeling could increase the risk for hemorrhagic stroke. The benefits of MR antagonism were independent of blood pressure suggesting a potential direct effect on the vasculature. The results of the studies conducted for the first aim suggest that EC-MR mediates

hypertensive remodeling in the cerebral microcirculation and large pial arteries. Cerebral blood flow is regulated by artery structure and the dilator capacity of the arterioles. Therefore, next, I wanted to explore the effects of EC-MR in PA endothelium-dependent dilation in AngII-hypertension.

My second aim was designed to assess the role played by the MR in PA endothelium-dependent dilation, specifically in the TRPV4-mediated dilation of the arterioles in AngII-induced hypertension. In this aim I also wanted to assess the role of MR signaling in cognitive impairment in AngII-induced hypertension. Several studies show a protective role of MR activation on endothelial function in healthy populations. In healthy men, short-term aldosterone infusion and MR activation improved endothelial function in the forearm vasculature (21), and acute MR antagonism with EPL impaired brachial artery endothelium-dependent dilation (11) showing a direct rapid effect of aldosterone in the vasculature. However, in the presence of cardiovascular risk factors such as hypertension, MR activation negatively alters the vasculature (13, 18, 31). Therefore, I assessed the chronic effects of MR signaling on PA function in AngII-hypertension. First, the role of MR activation in the generation of myogenic tone of PAs was assessed. EPL prevented the increased myogenic tone observed in PAs from AngII-hypertensive mice. Next the role of MR signaling in PA endothelium-dependent dilation was assessed. EPL treatment prevented the impaired endothelium-dependent dilation of PAs in AngII-hypertension. The combination of increased myogenic tone and impaired endothelium-dependent dilation I observed are likely to markedly reduce cerebral perfusion.

There is emerging evidence that MR activation impairs ion channel function. IK_{Ca}/SK_{Ca} are important components of EDH and MR activation impairs their function in the rat choroidal arteries (8). My studies also show that EDH is an important mediator of PA dilation in mice irrespective of blood pressure or MR activation status. To further explore the role of MR signaling in EDH-mediated dilation, I assessed TRPV4-dependent dilation in PAs. We have previously shown that TRPV4 channels are important regulators of PA endothelium-dependent dilation in normotensive WKY rats and SHRSP (16, 17).

In the current study, I show that AngII-hypertension impairs TRPV4-mediated dilation in PAs from mice. MR antagonism prevented the impaired TRPV4-mediated dilation in PAs from AngII-hypertensive mice and was associated with increased TRPV4 mRNA expression in the EPL treated mice. It is also possible that MR signaling negatively alters the coupling of TRPV4 channels to the anchoring protein AKAP-150 and that this impairs the channel activity (20, 32). This is based on studies in the mesenteric arteries where AngII impairs the coupling of AKAP150 to TRPV4 channels and reduces its activity (20, 32). Also, studies have shown that aldosterone downregulates other anchoring proteins such as AKAP12 (12), therefore it is possible that aldosterone and MR activation downregulate AKAP150 as well. My studies also show that IK_{Ca}/SK_{Ca} channel-mediated dilation is impaired in PAs from AngII mice and this was also improved by MR antagonism. EPL also prevented the changes in mRNA expression of the IK_{Ca}/SK_{Ca} channels. Dysfunction in the TRPV4 and IK_{Ca}/SK_{Ca} channels are linked to cardiovascular disease (1, 8, 9), and these channels are emerging as therapeutic targets.

My studies show that MR activation during hypertension causes cerebral artery remodeling and impaired endothelium-dependent dilation that are associated with reduced brain perfusion (6) and this could cause cognitive impairment in AngII-hypertension. In this aim, I explored if MR antagonism could be a therapeutic approach to improve cognitive function in AngII-hypertension and I show that MR blockade does improve cognitive function. I also show that AngII-hypertension increases the number of activated microglia. The activated microglia can release a number of proinflammatory cytokines leading to inflammation. AngII-hypertension also disrupts the expression of genes for markers of neuronal support and synapse proteins such as BDNF, SYP and doublecortin. These changes may be associated with the cognitive impairment and appear to be regulated by MR signaling. However, MRs are expressed in different cell types in the arterioles. I focused on the EC-MR because it is critical for BBB and is the only cell type present in all segments in the vasculature.

The third aim was designed to assess the specific role played by the EC-MR in TRPV4-mediated dilation of PAs and in cognitive function in AngII-hypertension. The studies in this aim show that EC-MR signaling regulates the impaired endothelium-dependent dilation of PAs in AngII-hypertension, and that EC-MR regulates the TRPV4-mediated dilation of the PAs. However, the increased myogenic tone observed with AngII-hypertension does not appear to be mediated by the EC-MR and is more likely mediated by the SMC-MR. Other studies suggest this is a possibility because SMC-MR regulates tone in peripheral resistance arteries (19). Several molecular mechanisms have been proposed for the actions of the EC-MR in endothelial function. First, in cerebral arteries the EC-MR contributes to vascular oxidative stress by increasing eNOS uncoupling, this

occurs because of decreased expression of the chaperon HSP90 and availability of tetrahydrobiopterin resulting in increased ROS production (7). EC-MR deletion also results in enhanced eNOS Ser1177 phosphorylation leading to eNOS activation and improves endothelial function in the aorta of mice (14). Although my studies suggest that plasma oxidized proteins are not increased in the AngII mice I have not assessed ROS production, NO availability or other indices of oxidative stress. Therefore, I cannot rule out the possibility that EC-MR activation during hypertension induces oxidative stress that is associated to the cerebrovascular dysfunction. EC-MR activation also increases the expression of ENaC and this increased expression is associated with stiffening in the aorta of obese mice. However, I did not observe stiffening of the wall in the PAs or PCAs in my studies. Numerous studies support a role of EC-MR in vascular inflammation. In human coronary endothelial cells, EC-MR promotes leukocyte adhesion (2). Also, in Western diet-induced obese female mice, EC-MR deletion reduces inflammatory cytokine levels and increases the anti-inflammatory cytokine IL-10 (14, 31). In contrast, in obese male mice EC-MR deletion does not prevent the increased in inflammatory cytokines and T-cell recruitment in response to pressure overload-induced heart failure (30).

I also explored the role of EC-MR in cognitive function in AngII-hypertension. Despite restoring cerebral blood flow and vascular reactivity, EC-MR deletion could not improve cognition. This suggests that the remodeling and impaired PA endothelium-dependent dilation are not the only thing that could be associated with the cognitive impairment. Therefore, the improved cognition with EPL treatment observed in the second aim may be associated with MR-mediated effects on glia or neuronal activity. The fact that EPL crosses the BBB presents the possibly that it can have direct effects on the

neurons and other cells in the brain (10). The data in the third aim shows that EC-MR signaling mediates PA endothelium-dependent dilation but not cognitive function. In the future, it will also be important to consider the role of MR signaling in the SMC and macrophages as there could be some crosstalk between the different cell types.

Lastly, I explored the role of TRPV4 channels in cognitive function. These studies were not part of my original hypothesis, but the data presented in Aims 2 and 3 highlighted the fact that TRPV4 channels are critical regulators of PA endothelium-dependent dilation in normotensive and AngII-hypertensive mice. Therefore, assessing TRPV4 channels in cognitive function was a logical extension of my first aims. TRPV4 channels are cation channels that are expressed in endothelial cells, SMC, astrocytes, neurons among other cell types. For these studies, I used a middle aged TRPV4 deficient rats to show that TRPV4 channels are critical regulators of cerebral perfusion, PA endothelium-dependent dilation, and cognitive function. These studies suggest that impaired TRPV4 channel function in hypertension may increase the risk for the development of VCID. However, it is important to acknowledge that this is a global knockout therefore there could be direct neuronal effects. In the future, studies should be done using this TRPV4 deficient rat treated with Angiotensin II to induce hypertension.

Taken together, the studies presented in this thesis show for the first time that MR signaling at the level of the endothelium regulates PA structure and function. They also show that MR antagonism is a potential therapeutic target to improve cognitive function in hypertension. However, my studies raised an important question. The cognitive dysfunction observed does not appear to be associated to the PA dysfunction. My studies suggest that MR blockade fully prevented the PA remodeling and impaired endothelium-

dependent dilation, but it did not fully reverse the cognitive dysfunction. This raises the question of what other signaling pathways could lead to the impaired cognitive function. A possibility is that other segments of the microcirculation such as the capillaries could be involved in the regulation of cognitive function. It also presents the possibility that the MR-mediated effects occur beyond the vasculature. EPL can cross the BBB therefore it could have a direct impact in the brain parenchyma and positively regulate other components of the NVU such as astrocytes, PVMs or neurons. All the components of the NVU are important in the regulation of NVC and disrupted functional connectivity and impaired NVC are found in early stages of VCID (15). In the future, the role of MR signaling in NVC should be explored. More studies should also be conducted to examine the effects of EPL treatment in neuronal function and astrocytes, this might help better understand the improved cognitive function observed in the AngII-hypertensive mice treated with EPL. My studies also highlight that TRPV4 channels could be another therapeutic target to improve cognitive function, however, this treatment would be difficult because it could reduce blood pressure.

6.2 – Considerations of the model and treatment regimens used

I chose to perform my studies using the AngII-dependent model of hypertension. This is a very well-established model of hypertension in mice and it is easily adapted to knockout mouse models. AngII is one of the active components of the renin angiotensin aldosterone system and has been shown to have wide ranging detrimental effects of the cerebral vasculature (23). Most humans do not have high AngII levels, but they respond to ACE inhibitors, thus the AngII treated mouse is clinically relevant model (22, 33, 34).

The doses of EPL used in my studies are higher than those used therapeutically in humans. This treatment regime has been used by our laboratory for EPL (25) and other MR antagonists (27, 28) without any adverse effects. The dose of EPL was chosen from previous studies in the literature (35). However, it is known that rodents metabolize/excrete EPL at a higher rate than humans (5, 36). Thus, higher doses are required in mice to produce the similar levels of MR antagonism. It is unlikely that the dose/duration of the treatment we used will cause off-target effects. Toxicity studies using similar dose did not show toxicity or carcinogenicity in mice (3, 5, 36).

6.3 – Novel findings

My studies are the first to show a link between EC-MR and PA remodeling and endothelium-dependent dilation. I also show a link between MR signaling, TRPV4 channels and cognitive function. These are the first studies to show a link between EC-MR signaling and TRPV4 channels in the cerebral vasculature. The findings were as follows (Figures 6.1):

6.3.1 – The role of EC-MR signaling PA remodeling during AngII-hypertension.

- This study shows, for the first time, the role of EC-MR signaling in cerebral artery remodeling and cerebral perfusion during AngII-hypertension.
- In the absence of hypertension, cerebral artery structure does not depend on MR activation.
- The protective effect of MR inhibition in the cerebral circulation could be almost completely reproduced by EC-specific MR deletion suggesting that during AngII-hypertension, EC-MR is necessary for hypertensive vascular remodeling and that cerebrovascular protection by MR inhibition is mediated by EC-MR.

- These findings enhance our understanding of the role of vascular MR signaling in cerebral artery remodeling.

6.3.2 – The role of MR signaling in PA endothelium-dependent dilation and cognitive function.

- MR antagonism prevents the impaired endothelium-dependent dilation and increased myogenic tone in PAs observed during AngII-hypertension.
- TRPV4 and IK_{Ca}/SK_{ca} channels, which are important regulators of PA endothelium-dependent dilation, are regulated by MR signaling.
- Endothelial TRPV4 channels play a role in the regulation of myogenic tone in AngII-hypertension.
- MR blockade improves cognition in AngII-hypertension and is associated with reduced cortical microglia density.
- These findings improve our understanding of the role of vascular MR signaling in PA dilation.

6.3.3 – The role of EC-MR signaling in PA endothelium-dependent dilation and cognitive function.

- EC-MR deletion prevents the impaired endothelium-dependent dilation of PAs in AngII-hypertension.
- EC-MR deletion does not prevent the increased myogenic tone of PAs in AngII-hypertension.
- EC-MR deletion prevents the impaired TRPV4- and IK_{Ca}/SK_{ca}-mediated dilation of PAs in AngII-hypertension.
- EC-MR deletion does not improve cognitive function in AngII-hypertension.

- Cerebral blood flow, PA structure and function are dissociated from cognition.
- These findings improve our understanding of the role of EC-MR signaling in PA dilation.

6.3.4 – The role of TRPV4 channels in cerebral perfusion and cognitive function.

TRPV4 channel deletion

- reduces cerebral perfusion.
- impairs cognitive function and increases depressive-like behaviors.
- causes impaired PA endothelium-dependent dilation without affecting arteriolar structure.
- causes cerebral inflammation including increased microglia activation.
- does not alter blood pressure.
- These studies improve our understanding on the role of TRPV4 channels in cognition and PA structure and function.

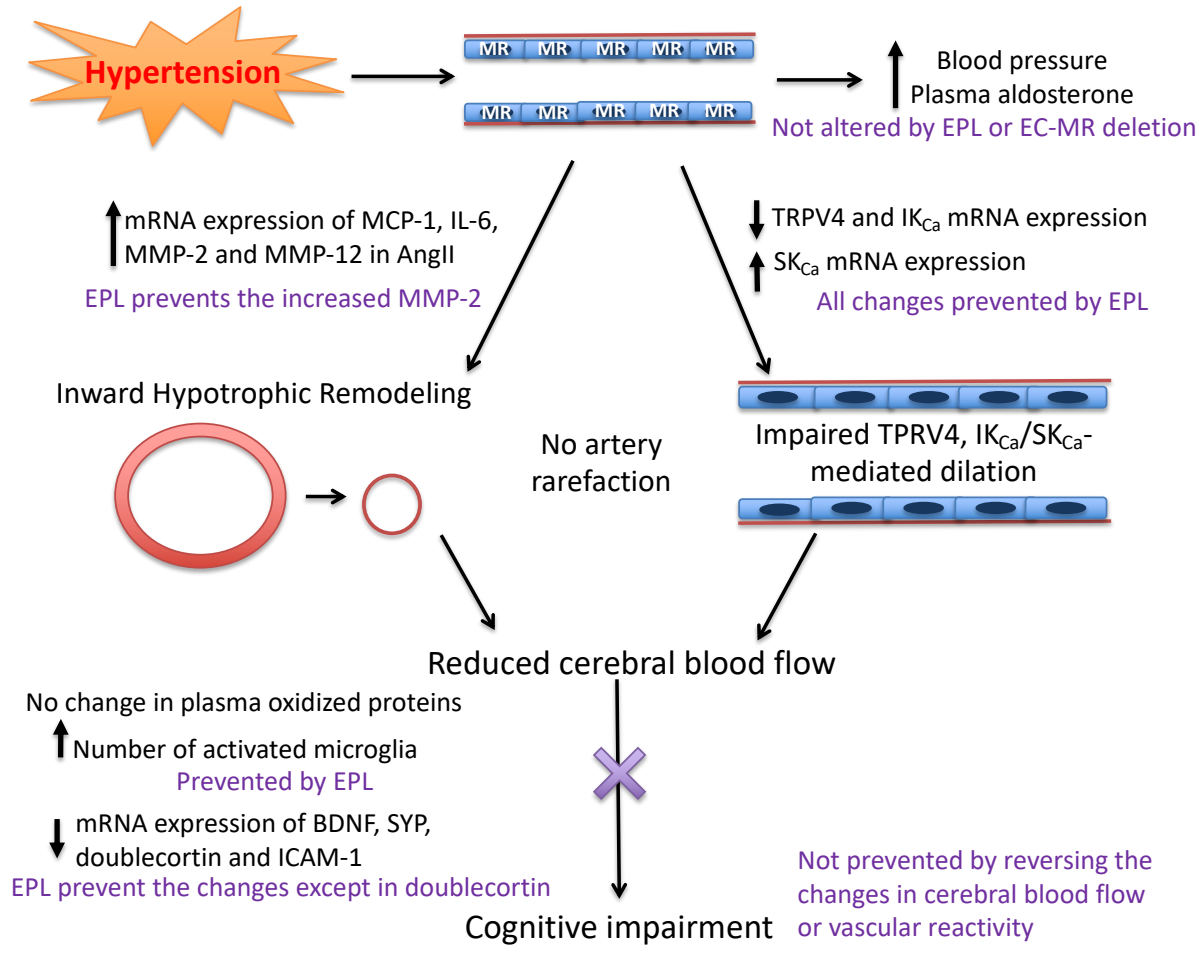

Figure 6.1 Summary of findings. EC-MR signaling mediates PA inward hypotrophic remodeling, impaired endothelium-dependent dilation and reduced cerebral perfusion.

6.4 – Limitations

- Some limitations in the animal model must be acknowledged. The mice used were relatively young (20-22 weeks of age) and were not the most clinically representative model since increased age is a major risk factor for VCID.
- Mice were treated with AngII for 4 weeks which represents years of hypertension in humans.
- Pressure myography is a widely used *ex vivo* technique to assess vascular structure and function. Pressurized arterioles are flushed with and bathed in aCSF, thus the effects of blood viscosity and flow are lost.
- Scanning laser Doppler was used to assess cerebral perfusion. The laser has a depth of penetration of approximately 1 to 2 mm and thus only assessed perfusion in the pial circulation. Also, the mice were anesthetized with isoflurane that is a potent vasodilator. However, I used the appropriate controls in all of my studies.
- I have not assessed sex-differences in any of our studies.

6.5 – Perspectives

VCID is growing public health issue that lacks effective treatments. Hypertension, a leading risk factor for VCID, changes the structure and function of the cerebral arteries and the PAs and increases the risk of cognitive impairment. Clinically, it is relevant to develop treatments that reverse existing artery remodeling and impaired vasodilation in hypertensive patients. Therefore, it is important to identify treatments that can impact the structural and functional impairments caused by hypertension. The MR is a potential therapeutic approach because MR antagonism prevents the hypertension-associated changes in the cerebral arteries. However, we do not fully understand the signaling

mechanisms downstream from the MR and this is in part because we do not know the specific cell type involved in the MR signaling. Defining which cell type drives the MR mediated remodeling will allow us to identify specific cellular pathways activated by the MR and this may allow for the development of more specific therapies. I proposed that EC-MR activation would cause PA remodeling and impaired endothelium-dependent dilation in AngII-hypertension. I also proposed that the remodeling and impaired dilation would reduce cerebral perfusion and lead to vascular cognitive impairment. My studies show that EC-MR activation during hypertension causes PA inward remodeling and impairs TRPV4-mediated dilation; these changes resulted in reduced cerebral blood flow. I also show that TRPV4 channels are critical regulators of cerebral blood flow, PA endothelium-dependent dilation and cognitive function; the data presented here suggest TRPV4 channel expression is regulated by the MR. Also, that MR antagonism improves cognitive function in AngII-hypertension. Interestingly, improving PA structure and function as well as cerebral perfusion with EPL or EC-MR deletion did not prevent the impaired cognition. This presents the possibility that MR signaling mediates effects beyond the vasculature to impair cognitive function. In conclusion, MR blockade and TRPV4 channels might be potential therapeutic approaches to improve cerebrovascular function in hypertensive patients.

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