INFLUENCE OF ENDOTHELIAL CANNABINOID 1 RECEPTOR ACTIVATION ON CEREBROVASCULAR REGULATION IN SWINE

A Thesis Submitted to the
College of Graduate and Postdoctoral Studies
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in the Western College of Veterinary Medicine,
University of Saskatchewan,
Saskatoon.

By

Cameron J. Morse

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ABSTRACT

Cannabinoid receptors 1 & 2 (CB1R & CB2R) are present throughout the cardiovascular system and evidence indicates that CB1R activation causes vasodilation in peripheral vascular beds. However, it remains unclear what the direct effects of CB1R or CB2R activation are in cerebral arteries. The present study tested the hypothesis that CB1R and not CB2R receptor activation elicits endothelial-dependent vasorelaxation in the cerebrovasculature. Female Landrace pigs (age=2 months; N=16) were euthanized, their brains were harvested, and pial arteries branching from the middle cerebral artery were isolated for wire myography. Arteries were pre-contracted with a thromboxane A_2 analogue (U-46619; $1x10^{-6}$ M to $1x10^{-4}$ M). Thereafter, vasorelaxation in response to a dual CB1R & CB2R receptor agonist CP55940 ($3x10^{-11}$ M to $1x10^{-6}$ M; half log doses) was examined under the following conditions: 1) untreated; 2) CB1R blockade (AM251; $1x10^{-7}$ M); or 3) CB2R receptor blockade (AM630; $1x10^{-7}$ M). The data revealed that CP55940 elicits a CB1R-dependent relaxation in cerebral arteries. Subsequently, to determine the role of different endothelial-dependent pathways, CB1R-mediated vasorelaxation was examined under the following conditions: 1) inhibition of nitric oxide synthase (NOS) using L-NAME ($3x10^{-4}$ M; reveals contributions from nitric oxide); 2) inhibition of in a cyclooxygenase (COX) using Naproxen ($3x10^{-4}$ M; reveals contributions from prostaglandins); 3) combined NOS and COX inhibition (reveals contributions from endothelial hyperpolarizing factor); and 4) endothelial removal (denudation of the artery; reveals total contribution of the endothelium). These data demonstrated that CB1R-mediated vasorelaxation was attenuated by NOS inhibition and the magnitude of this effect was increased with the combination of NOS + COX inhibition as well as endothelial removal ($P<0.05$). However, reductions in vasorelaxation during COX inhibition alone only approached significance ($P=0.07$). Overall, the data indicate CB1R-mediated vasorelaxation is endothelial-dependent and involves contributions from multiple dilatory pathways. Understanding how the CBRs modulate cerebrovascular function is critical when evaluating the short-term and long-term physiological effects, safety and prescriptive use of cannabis and cannabinoids in any clinical context.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisor Dr. T. Dylan Olver for taking me on as one of his first graduate students. Dr. Olver spent many tireless nights helping me develop as a young science. Though I may not have appreciated it at first, this thesis is a testament his hard work and dedicated support throughout the past two years. Needless to say, M.Sc. would not have been possible without his passion, guidance, and patience.

I would also like to thank my co-supervisor Dr. Darrell Mousseau for all of his support throughout my M.Sc. Dr. Mousseau's vast expertise and knowledge always provided me with new insights into my research.

I owe thanks to everyone in our lab that has supported me throughout my degree. I would like to give particular thanks to Dr. Jude Morton. She spent many long hours patiently training me on how to conduct myography experiments.

I would like to thank my brother, parents, and grandmothers for their continued support and interest in my academic pursuits. Your lifelong dedication to my continued growth is why I am who I am.

DEDICATION

I would like to dedicate my thesis to my brother, Alexander Morse, my parents, Kim and Jennifer Morse, my grandmother Lydia Fanning, and my late grandmother Bernice Morse.

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ABBREVIATIONS

Abbreviation	Definition
2-AG	2- Arachidonoyl Glycerol
ACA	Anterior Cerebral Artery
AEA	Anandamide
ATP	Adenosine Triphosphate
Ca^{++}	Calcium
cAMP	cyclic Adenosine Monophosphate
CB1R	Cannabinoid 1 Receptor
CB2R	Cannabinoid 2 Receptor
CBD	Cannabidiol
CBF	Cerebral Blood Flow
CBR	Cannabinoid Receptor
CCAC	Canadian Council on Animal Care
cGMP	cyclic Guanosine Monophosphate
CNS	Central Nervous System
CO_2	Carbon Dioxide
COX	Cyclooxygenase
COX 1	Cyclooxygenase 1
COX 2	Cyclooxygenase 2
DAG	Diacylglycerol
DAGL	Diacylglycerol Lipase
EDHF	Endothelium Derived Hyperpolarizing Factor
EET	Epoxyeicosatrienoic Acid
eNOS	Endothelial Nitric Oxide Synthase
GMP	Guanosine Monophosphate
GPCR	G Protein-coupled Receptor
GTP	Guanosine Triphosphate
IKCa	Intermediate Conductance Calcium-activated Potassium Channel
IP_3	Inositol 1,4,5-trisphosphate
IPR	Prostacyclin Receptors
K^+	Potassium
L-NAME	N(G)-Nitro-L-arginine Methyl Ester
MCA	Middle Cerebral Artery
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NArPE	N-arachidonoyl Phosphatidylethanolamine
nNOS	Neuronal Nitric Oxide Synthase
NO	Nitric Oxide
NOS	Nitric Oxide Synthase

NSAID	Nonsteroidal anti-inflammatory drug
NVC	Neurovascular Coupling
NVU	Neurovascular Unit
O_2	Oxygen
PCA	Posterior Cerebral Artery
PGD_2	Prostaglandin D2
PGE1	Prostaglandin E1
PGE_2	Prostaglandin E2
PGF1	Prostaglandin F1
PGF_{2a}	Prostaglandin F2alpha
PGG_2	Prostaglandin G2
PGH_2	Prostaglandin H_2
PGI_2	Prostacyclin
PI	Phosphatidylinositol
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PNS	Peripheral Nervous System
PSS	Physiological Saline Solution
SKCa	Small Conductance Calcium-activated Potassium Channel
THC	Δ^9-tetrahydrocannabinol
TX	Thromboxane A_2
TXR	Thromboxane A_2 receptors
VSM	Vascular Smooth Muscle

CHAPTER 1

1. INTRODUCTION

Cannabis is one of the most widely consumed drugs, with approximately half of Canadians over the age of 15 reported to have consumed cannabis at least once in their lifetime. Reports indicate that since the legalization of cannabis in Canada, consumption has increased.[1] Among those who have consumed cannabis, adolescents and young adults are reported to have the highest rates of use within the past year.[1] Despite being widely consumed by Canadians, there are many knowledge gaps regarding the physiological effects of cannabis consumption. Although limited cannabis use is considered safe and even therapeutic for many individuals, a recent position paper from the American Heart Association has highlighted potential adverse effects of cannabis on the cardiovascular system, including increased risk of an adverse cerebral event (e.g. transient ischemic attacks secondary to altered cerebrovascular vasomotor function and stroke).[2] Presently, evidence supporting the link between cannabis use and abnormal cerebrovascular regulation or adverse cerebral events is limited and relies predominantly on observational data. Few studies have investigated the direct effects of cannabis use on cerebrovascular function. This highlights an urgent need to better understand mechanisms underlying how cannabis influence arteries in the brain.

Cannabis is composed of a collection of more than 100 different cannabinoids.[3] Cannabinoids mediate physiological effects through the actions of two main cannabinoid receptors (CBRs): the cannabinoid 1 receptor (CB1R) and the cannabinoid 2 receptor (CB2R).[4] Whereas CB1Rs are located primarily in the central and peripheral nervous tissue, CB2Rs are located primarily in immune tissues such as the tonsils and the spleen. Of interest, CB1Rs are responsible for mediating the psychotropic effects of Δ^9-tetrahydrocannabinol (THC), the primary psychoactive cannabinoid in cannabis.[5] It is important to note, however, that a multitude of different ligands act on CB1Rs, including phytocannabinoids, synthetic cannabinoids, and endocannabinoids (i.e., cannabinoids produced within the body), as well as pharmacological

agents.[5,6] Furthermore, there is a growing body of evidence that indicates CB1Rs are expressed throughout the cardiovascular system, including the cerebrovasculature, and mediate vasoactive events.[7–10] This raises the important prospect that endogenously produced cannabinoids, those that are ingested and other associated pharmacological derivatives influence general cardiovascular and cerebrovascular function through the CB1R.

The cardiovascular system assists in regulating the microenvironment of tissues. Specifically, it is involved in regulating pH and temperature as well as facilitating nutrient and oxygen delivery and disposal of metabolic by-products.[11] The cardiovascular system is comprised of three main components: heart, blood, and the vasculature. The focus of this work is on the vasculature, with a particular emphasis on the cerebrovasculature. In general, the vasculature consists of arteries and arterioles, which distribute blood to and within organs throughout the body; capillaries, the site of gas and nutrient exchange; and post-capillary venules and veins, which collect and return blood to the heart.[12] Evidence indicates that cannabinoids mediate certain cardiovascular effects by acting on CB1Rs located in arteries and arterioles.[13–16] For example, in arteries supplying the gastrointestinal tract and the heart, activation of CB1Rs elicits arterial relaxation.[13] The pial vasculature refers to the arterial network on the surface of the brain and it contributes significantly to blood flow distribution within the brain. How CB1R and CB2R influences pial arteriolar function is not well understood.

Since the legalization of cannabis in 2018, both medical and recreational uses of cannabis and related products in Canada have increased. Evidence indicates that the cannabinoids in cannabis mediate cardiovascular effects through CB1Rs but also potentially CB2Rs; however, information on how these receptors influence cerebrovascular physiology is both lacking and unclear.[14] Given that the use of cannabis has been associated with altered cerebral blood flow and increased risk of cerebrovascular events, understanding how CB1Rs and CB2Rs influence cerebrovascular function is of the utmost importance. Technical and ethical limitations make it difficult to study mechanisms involved in CBR modulation of cerebrovascular function in humans. As a result, experiments in this field of research are often conducted using animal models. The present work utilized a swine model to study cerebrovascular regulation, because of the structural and functional similarities of the cerebrovasculature with humans. The aim is to provide a basic, translational framework for understanding how CBRs influence cerebral vasomotor control.

CHAPTER 2

2. REVIEW OF LITERATURE

2.1 Cerebral circulation

At rest, the brain is responsible for approximately 25% of the body's total oxygen consumption. To achieve this level of oxygenation, the brain is perfused by approximately 50 ml/100 g/min continuously. This reflects ~15% of cardiac output at rest.[17,18] With no stores of oxygen and glucose, interruptions of only a few minutes can lead to permanent damage. Blood is supplied to the brain by four large, extra-cranial arteries: two internal carotid arteries and two vertebral arteries. The right and left internal carotid arteries originate from the common carotid arteries and ascend through the carotid canal, ultimately piercing the dura mater, supplying an anastomotic ring at the base of the brain referred to as the circle of Willis. The right and left vertebral arteries originate from the subclavian arteries and ascend through the foramina transversaria of the cervical vertebra and merge between the medulla and the pons in the brain stem to form the basilar artery. Similar to the internal carotid arteries, the basilar artery likewise supplies the circle of Willis.[19,20]

The circle of Willis is a large circular anastomosis at the base of the brain.[20] It connects to three sets of major arteries to supply the surface of the brain; these are the middle cerebral arteries (MCAs), anterior cerebral arteries (ACAs), and posterior cerebral arteries (PCAs). The MCAs supply large parts of the cortex, including the somatosensory area and the motor cortex, as well as subcortical regions of the brain. The ACAs travels along the olfactory tract and supply both cortical and subcortical regions of the brain. The PCAs perfuse the posterior regions of the brain that include the occipital lobe and midbrain.[19] The MCAs, ACAs and PCAs are considered intracranial arteries and are referred to as pial arteries. Pial arteries run superficially along the surface of the brain, branching several times before ultimately penetrating into the brain. Arteries

that penetrate the surface of the brain are referred to as penetrating arteries, eventually becoming parenchymal arteries once they are completely positioned within the brain.[21] Parenchymal arteries branch further, ultimately forming capillaries, allowing gas and nutrient exchange within the brain. Pial arteries are the focus of this work.

Pial arteries consist of three concentric layers: i) the tunica intima, the inner layer, which is comprised primarily of endothelial cells and the internal elastic lamina; ii) the tunica media, the middle layer, which is comprised primarily of vascular smooth muscle (VSM) cells, with collagen and elastin; and iii) the tunica adventitia or externa, the outer layer, which consists primarily of collagen fibers and perivascular nerves.[19] Although, extracranial arteries supplying the brain can have as many as 20 layers of VSM cells, smaller pial arteries often contain only two to three layers of VSM cells.[19] Multiple mechanisms act jointly on the VSM, causing it to contract or relax (Figure 2.1), which contribute to the gross perfusion as well as the distribution of blood flow in the brain.[22–25] The following will expand on these mechanisms.

2.1.1 Cerebrovascular regulation

A feature of cerebral arteries is vascular tone, which is characterized as a base level of constriction relative to the maximally dilated state. Physiologically, vascular tone dictates arterial resistance to flow and can be thought of as the "dilatory reserve" of an artery.[26,27] The greater the vascular tone, the greater the vascular resistance, and the greater the dilatory capacity and ability to increase perfusion when required. Based on examination of the relationship between arterial pressure and arterial diameter in cerebral arteries along the cerebrovascular tree in anesthetized cats, Stromberg and Fox estimated that pial arterial tone represents approximately 40% of cerebrovascular resistance.[28] The focus of the present work is on pial arteries, so the mechanisms that influence pial vasomotor control will be discussed.

In general, there are four main mechanisms that act in concert, with a high level of redundancy, to regulate brain blood flow (Figure 2.1): myogenic response, metabolic hyperemia, neurogenic response and endothelial control.[29] The following discussion will explore each mechanism briefly. The focus of this thesis is on the endothelial control of the vasculature; therefore, it will be discussed in the greatest detail.

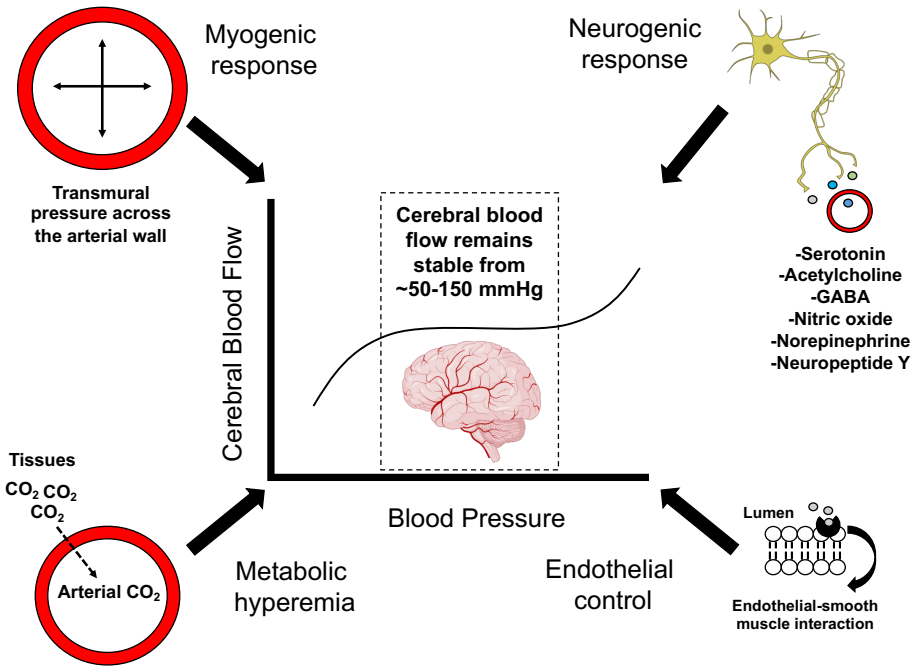


Figure 2.1 Cerebrovascular regulation. In the brain, blood flow is controlled through four primary mechanisms: myogenic response, metabolic hyperemia, neurogenic response and endothelial control. The myogenic response describes the contractile and relaxing properties of the VSM in response to changes in intramural pressure. The neurogenic response is characterized by the vasoactive neurotransmitters, neuropeptides, and small molecules released by neurons that control local blood flow. Metabolic hyperemia describes by the increase in blood flow that results from the release metabolites that dilate blood vessels. Endothelial control is exerted through the production of a wide range of vasoactive factors from the endothelium that exert either a relaxing or contractile influence directly on the VSM. These four mechanisms act in concert to provide consistent CBF, and allows for local regulation. Adapted from Silverman and Petersen.[30]

2.1.2 Myogenic response

While it took several decades to define the physiological basis of the myogenic response, the initial experiment that laid the groundwork for this field of study was conducted in the early 19th century. William Bayliss described the inherent capacity of the vasculature in the rabbit hindlimb to respond to changes in arterial blood pressure independently of neuronal input.[31] The myogenic response describes the arterial reaction whereby increases in transmural pressure are met with vasoconstriction or increases in vascular (myogenic) tone.[29] Alternatively, decreases in transmural pressure are met with vasodilation or decreases in myogenic tone (Figure 2.1). More specifically, increases in transmural pressure engage mechanically sensitive ion channels in the arterial wall, leading to VSM depolarization and contraction.[27] Since the discovery of the myogenic response, this phenomenon has been observed in numerous vascular beds, such as in the renal, skeletal, coronary, and cerebral circulation.[29] At a physiological level, researchers believe the myogenic response protects against fluctuations in cerebral perfusion during dynamic changes in blood pressure.[29] When perfusion pressure increases abruptly, cerebral arteries constrict and myogenic tone increases, limiting hyperperfusion. In contrast, when perfusion pressure decreases abruptly, cerebral arteries dilate and myogenic tone decreases, limiting hypoperfusion.[29] Evidence suggests myogenic tone assists in maintaining adequate cerebral perfusion across a blood pressure range from approximately 50-150 mmHg.[27] Although there is some evidence for the involvement of the endothelium, when blood pressure is between 50-150 mmHg, the myogenic response is believed to operate largely independent from other mechanisms of cerebral blood flow control, such as cerebral vasoreactivity and neurogenic input.[29]

2.1.3 Metabolic hyperemia

The concept of metabolic hyperemia was first introduced in the 1890s by Roy and Sherrington.[32] The authors injected a living dog with a solution of homogenized brain from a dog who had died a few hours prior because of hemorrhagic shock. The resulting increase in cerebral blood flow was thought to be the result of metabolic substances generated from the hemorrhagic shock the dog had suffered. Since then, many metabolic vasodilators have been discovered. As a principle metabolite of oxidative metabolism, carbon dioxide (CO_2) is perhaps the best studied in the cerebral circulation (Figure 2.1).[33] Any increases in brain metabolism augments local CO_2

resulting in cerebral vasodilation. Concerning the latter point, it is estimated that for each mmHg increase in arterial CO_2 concentrations, cerebral blood flow increases by up to 4%.[34] Hypoventilation leading to hypoxia will likewise increase systemic CO_2 resulting in cerebral vasodilation. Thus, CO_2 reactivity serves to couple cerebral perfusion and oxygenation to local metabolism. In addition to metabolic hyperemia or CO_2-mediated vasodilation, endothelial control and the neurogenic response also assist in coupling local neuronal activity with cerebral perfusion.[35]

2.1.4 Neurogenic response (extrinsic and intrinsic)

There are multiple neurological control mechanisms that regulate perfusion in the brain through the release of vasoactive neurotransmitter (norepinephrine, serotonin, acetylcholine, glutamate), neuropeptides (neuropeptide Y), and vasoactive small molecules (K^+, NO, H^+) (Figure 2.1).[35,36] These control mechanisms can be broadly assigned to two groups: extrinsic control by the peripheral nervous system (PNS) and intrinsic control by the central nervous system (CNS).[36]

Generally, large extracerebral arteries are densely innervated by the PNS. These perivascular neurons release neurotransmitters and neuropeptides, such as norepinephrine and neuropeptide Y which act directly on the vasculature. However, in intracranial arteries, the density of perivascular nerves decreases gradually as arteries branch along the surface of the brain and ultimately disappear upon penetration into the brain. Pial arteries derive sympathetic innervation from the superior cervical ganglion, parasympathetic innervation from the sphenopalatine and optic ganglia, and sensory innervation from the trigeminal ganglion. Both dilatory and constrictor roles for the PNS have been reported and currently there is no consensus regarding the role of PNS in cerebrovascular regulation.[19,29,36,37]

In addition to extrinsic innervation from the PNS, pial arteries also receive intrinsic neural input from the CNS.[35] Furthermore, once the pial arteries branch and dive into the parenchyma of the brain (i.e. parenchymal arterioles), extrinsic innervation arising from the PNS ceases completely and intrinsic control becomes the sole neurogenic input. A wide range of vasoactive neurotransmitters are released from both cortical neurons (e.g. gamma-Aminobutyric acid and acetylcholine) and subcortical neurons (e.g. afferents from the ralph nucleus release serotonin) that are important for controlling CBF. The neurovascular unit (NVU) is the

anatomical substrate for intrinsic neuronal control, and reflects the neurovascular interaction between neurons, interneurons and glia originating within the brain and the surrounding cerebrovasculature. Briefly, neurotransmitters released from the neurons, interneurons and glia in accordance with their activity, mediate vasodilation or vasoconstriction in the surrounding vasculature.[37–40] This intimate anatomical and functional connection facilitates the dynamic distribution of blood flow within the brain to achieve a tight coupling between local neuronal activity and blood flow. Of note, although the neurogenic responses reflect distinct features of cerebrovascular regulation, both extrinsic and intrinsic neurogenic responses are influenced by the endothelium.[36]

2.1.5 Endothelial control

The endothelium is a single cell layer that lines the entire cardiovascular system, from the heart to capillaries. The endothelium was long thought to be a passive barrier to the VSM; however, it is now recognized that it produces factors that regulate vascular tone. The primary function of the endothelium is to integrate mechanical stimuli (e.g. shear stress) as well as local and systemic chemical (e.g., acetylcholine, bradykinin, adenosine, adenosine triphosphate (ATP)) signals and relay those to the VSM to mediate a constriction or dilation response (Figure 2.1).[19,26,27] The following will expand on the three primary pathways[19] involved in endothelial-dependent VSM vasorelaxation:

Nitric oxide (NO): endothelial nitric oxide synthase (eNOS) activation leads to the production of NO. Once NO diffuses to the VSM, it leads to the production of cyclic guanosine monophosphate (cGMP) which inhibits contraction (Figure 2.1).[41]

Prostaglandins: Prostaglandins are produced from arachidonic acid by cyclooxygenase 1 & 2 (COX 1 & 2). They cause dilation by increasing levels of cyclic adenosine monophosphate (cAMP) in the VSM, which inhibits contraction (Figure 2.1).[42]

Endothelium derived hyperpolarizing factor (EDHF): EDHF is proposed to function by polarizing the VSM membrane which prevents contraction (Figure 2.1).[43]

2.1.6 Nitric oxide

The dual role of acetylcholine as a vasoconstrictor and vasodilator in seemingly identical preparations remained a point of contention until the 1980s.[44–46] In 1981, Robert F. Furchgott detailed the obligate role of the endothelium in acetylcholine-mediated dilation.[47] Furchgott described how careless interaction with the lumen during experimental preparations easily damaged and stripped away endothelial cells. This was of particular importance, because the stripping of the endothelium resulted in the complete loss of an arterial dilator response to acetylcholine. This provided direct evidence that endothelial cells were not merely an idle barrier between the blood and VSM, but actively influenced the VSM in response to the luminal environment. Subsequent research, presented in 1986, demonstrated that NO was a primary factor responsible for mediating endothelial-dependent dilation.[46] Further, this work indicated that endothelial NO production resulted in cGMP production in the VSM, which ultimately led to relaxation.[46] Furchgott's description of the role of NO on the vasculature won him the 1998 Nobel Prize in Physiology or Medicine.[48] Since the identification of NO, it has been described as one of the fundamental endothelium-dependent dilators, mediating the response of a wide range of chemicals and mechanical stimuli. In the brain, there are both endothelial and non-endothelial sources of NO, of which the former is more critical to the current work.

NO is a potent paracrine factor, whose proper function in the cardiovascular system is critical to health.[22] NO is produced from a family of enzymes, encoded by separate genes, called nitric oxide synthase (NOS). The two NOS isoforms that appear to serve central function in cerebrovascular regulation are neuronal NOS (nNOS) and eNOS.[49] Whereas nNOS is expressed primarily in neurons and involved in the neurogenic response, eNOS is expressed predominantly in endothelial cells and involved in endothelial-mediated cerebrovascular responses.[35,36] Regarding the latter, eNOS is constitutively expressed and evidence indicates it serves a central role in pial artery vasomotor control.[22]

Although eNOS is active under basal conditions, activity can increase dramatically in response to increases in intracellular calcium (Ca^{++}) .[50–53] The endothelial response to acetylcholine provides a prototypical example of Ca^{++}-dependent NO production. Acetylcholine is an agonist for the endothelial muscarinic M5 G protein-coupled receptor (GPCR).[54] Although a mechanistic discussion of GPCR function will be addressed further in this thesis, muscarinic M5 receptor function will be described now to illustrate the role of NO in vasodilation. GPCRs

are a class of trimeric metabotropic receptors that signal through the production or inhibition of second messengers. muscarinic M5 receptor are bound to a G_q unit. Upon binding to the endothelial muscarinic M5 receptor, acetylcholine activates phospholipase C (PLC), which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2), generating diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). IP_3 quickly diffuses through the cytoplasm, where it acts on the IP_3 receptor, which is a Ca^{++} channel on the endoplasmic reticulum. Upon binding, the Ca^{++} channel opens, releasing Ca^{++} into the cytoplasm.[55] When Ca^{++} binds to the calmodulin subunit of eNOS, eNOS catalyzes the conversion of L-arginine and O_2 to produce NO and L-citrulline, with the following co-factors: nicotinamide adenine dinucleotide phosphate, tetrahydrobiopterin, heme, flavin adenine dinucleotide, and flavin mononucleotide.[49] NO is a highly lipophilic, short lived, gaseous mediator that readily diffuses to the surrounding tissues, where it exerts its effects. NO has an extremely high affinity for heme. When NO diffuses into the blood it is quickly oxidized by the heme in red blood cells and cleared. The NO that diffuses from the endothelium into the VSM binds to the heme moiety sGC resulting in its activation. sGC catalyzes the dephosphorylation of guanosine triphosphate (GTP) to cGMP, causing relaxation through several mechanisms. These include through activation of myosin light chain phosphatase resulting in dephosphorylation of the myosin light chain, and activation of Ca^{++} ATPase resulting in Ca^{++} efflux and a resultant hyperpolarizing effect. The net result is VSM relaxation or vasodilation (Figure 2.1).[19,22,41,46,56]

It has been established that NO is a major regulator of cerebrovascular tone.[41] Experimental approaches have shown that administration of N(G)-Nitro-L-arginine methyl ester (L-NAME), a non-specific inhibitor of NOS, reduces cerebral blood flow *in vivo* and increases vascular tone in isolated pial arteries *ex vivo*. However, L-NAME does not distinguish the source of NO, making it difficult to determine whether the effects of L-NAME are mediated by nNOS or eNOS. Nevertheless, given the effect of L-NAME is observed in isolated arteries void of neural input and this effect can be abolished by removal of the endothelium, suggests a key role for eNOS. Supporting a role for eNOS, *ex vivo* experiments on isolated arteries reveal that VSM cGMP levels, a key mediator of NO-induced dilation, are lower in arteries with a damaged endothelium.[57,58] It has been suggested that eNOS is constitutively active and NO is produced continuously to limit off-target effects of locally produced compounds like serotonin and norepinephrine that are produced readily in the brain and mediate vasoconstrictor effects in the

cerebral circulation.[59,60] Moreover, eNOS activity, and by extension NO production, is regulated by mechanical and chemical stimuli to regulate blood flow to specific regions of the brain as required. Thus, eNOS-dependent NO production serves an important role in cerebrovascular regulation, influencing gross perfusion as well as blood flow distribution within the brain.

2.1.7 Prostaglandins

The study of endothelial control of the vasculature began in earnest with Furchgott's description of the mandatory role of the endothelium for relaxation in response to acetylcholine.[47] Although unknown at the time, the first true endothelium-dependent dilators were described in the 1930s, by Maurice W. Goldblatt[61] and Ulf von Euler.[62] Indeed, both physiologists independently reported vasoactive properties of unknown compounds in seminal fluids. The term prostaglandin was soon coined for these compounds. It was several decades before the identity of prostaglandins were clarified. The first prostaglandins to be isolated were identified as prostaglandin E_1 (PGE_1) and prostaglandin F_1 (PGF_1).[63–66]

Prostaglandins are a large family of lipid mediators called eicosanoids. Eicosanoids are lipids structurally related to the precursor arachidonic acid. Arachidonic acid is a polyunsaturated fatty acid that is stored in the phospholipid membrane of cells. Arachidonic acid is released from the cellular membrane through the actions of phospholipase A_2 (PLA_2). Traditionally, upon activation, PLA_2 breaks down phosphatidylinositol (PI) in the membrane releasing arachidonic acid into the cells. Arachidonic acid can be metabolized by several enzymes including lipoxygenases, cytochrome P450 monooxygenases as well as COX.[55] Concerning the cerebral circulation, arachidonic acid is the primary substrate for COX, which produces vasoactive prostaglandins.[42]

There are two structurally related, constitutively expressed isoforms of COX in the endothelium: COX 1 and COX 2.[42] Although there are slight differences in substrate affinity and enzymatic activity, both COX 1 and COX 2 metabolize arachidonic acid readily and mediate the production of prostaglandins. More specifically, COX proteins contain two enzymatically active subunits: a site for COX activity and a site for peroxidase activity. Once activated, catalytic COX activity leads to the formation of PGG_2. Subsequently, the peroxidase subunit catalyzes the reduction of PGG_2 to PGH_2. PGH_2 is a short-lived substrate critical to the formation of biologically active forms of prostaglandins.[67] The five primary prostaglandins generated from

PGH_2 are: PGD_2, PGE_2, PGF_{2a}, PGI_2 and thromboxane A_2. Ultimately the production of PGH_2 is the rate limiting step for the production of vasoactive prostaglandins, making COX a key enzymatic pathway of prostaglandin physiology.[68]

Vasoactive prostaglandins are extremely lipophilic, and once produced, diffuse quickly across the endothelial cell membrane to act upon the VSM. The net effect ultimately depends upon the prostaglandins produced in the endothelium and receptors present in the VSM. Of importance, in the cerebral circulation, prostaglandins can produce dilation through the actions of PGI_2 on prostacyclin receptors (IPR) or constriction through the actions of thromboxane A_2 receptors (TXR).[42]

Similar to NO, acetylcholine provides an excellent example for the endothelial-dependent production of dilatory prostaglandins.[69] As discussed previously, acetylcholine is a potent endothelial-dependent dilator and acts by increasing intracellular endothelial Ca^{++} concentration. In addition to the activation of eNOS, increases in intracellular Ca^{++} augment PLA_2 activity, promoting arachidonic acid release from the cell membrane and subsequent metabolism by COX.[70] With respect to endothelial muscarinic M5 receptors, or endothelium-dependent dilation in general, the main prostaglandin produced is PGI_2, which diffuses into surrounding VSM and binds to IPRs.[42] Briefly, IPRs are GPCRs bound to the G_s subunit. Once engaged, the G_s subunit activates adenylate cyclase (AC), which catalyzes the conversion of ATP to cyclic cAMP. In VSM, cAMP inhibits myosin light chain kinase, resulting in attenuated phosphorylation of the myosin light chain, and activates Ca^{++} channels resulting in Ca^{++} efflux and a resultant hyperpolarizing effect. The cumulative effect is VSM relaxation or vasodilation (Figure 2.1).[19]

Like eNOS, COX, and particularly COX 1, appear to be constitutively active in the cerebrovasculature; and thus, involved in the regulation of cerebrovascular tone. Indeed, experimental approaches have shown that administration of Indomethacin, a non-specific inhibitor of COX, reduces cerebral blood flow *in vivo* and increases vascular tone in isolated pial arteries *ex vivo*.[71] However, the magnitude of the effect in isolated arteries, when compared with NO, appears to be smaller.[72] The effect of COX inhibition on cerebral vasomotor control appears to be more pronounced during developmental stages, prior to maturation, suggesting basal PGI_2 serves a greater dilatory role in children than adults.[73] Given similar stimuli (mechanical and chemical) activate both eNOS and COX, PGI_2 may serve a similar, though less pronounced, role as NO in cerebrovascular regulation.

2.1.8 Endothelial-dependent hyperpolarizing factor

Although NO and PGI_2 serve central roles in endothelial-mediated responses, even when eNOS and COX pathways are pharmacologically inhibited, endothelium-dependent vasodilation often persists, though to a lesser degree.[12,19] As a result, this elusive, persistent dilatory influence, referred to as EDHF, was originally defined as the sustained dilation after both NOS and COX inhibition. Although EDHF remains to be characterized fully, as its name suggests, evidence indicates it involves the spreading of hyperpolarization from the endothelium to VSM resulting in VSM relaxation.[25]

Interestingly, acetylcholine has been shown to produce vasodilation in the presence of combined COX and NOS inhibition, indicating a portion of endothelial muscarinic M5 receptor-mediated vasodilation is the result of EDHF.[74] As previously described, muscarinic M5 receptor activation results in the increase in intracellular Ca^{++}. Concerning EDHF, this has both direct and indirect implications. First, increased Ca^{++} activates both the small-conductance (SKCa) and intermediate-conductance (IKCa) calcium-activated potassium (K^+) channels present on the endothelium, resulting in endothelial hyperpolarization.[75] In addition to K^+ efflux, this initiates the opening of myoendothelial gap junctions, which allows the hyperpolarization to "transfer" to the VSM.[22,76] Conjunctional with the transfer, VSM voltage-gated Ca^{++} close and Ca^{++} ATPase channels as well as K^+ channels open resulting in decreased VSM intracellular Ca^{++} concentrations and resultant hyperpolarization.[76] Secondly, and concurrently, increased endothelial Ca^{++} stimulates PLA_2, which initiates the breakdown of membrane phospholipids into arachidonic acid. As described earlier, in addition to COX, arachidonic acid can be metabolized by cytochrome P450 monooxygenases, which, in turn, produce epoxyeicosatrienoic acid (EET). Importantly, EET are believed be chemical mediators of EDHF and, like Ca^{++}, initiate an endothelial hyperpolarization effect.[19] Furthermore, evidence indicates EET may diffuse into VSM and mediate VSM hyperpolarization directly. The net result of endothelial Ca^{++} and EET signaling, that occurs independent of both eNOS and COX pathways, collectively termed EDHF, is decreased VSM intracellular Ca^{++} concentrations, hyperpolarization and subsequent VSM relaxation (Figure 2.1).[27]

EDHF is a complimentary dilator to NO. That is, evidence indicates EDHF and NO respond to similar stimuli, but as pial arteries branch and eventually become penetrating arteries, the contribution of NO to vascular tone decreases in favour of EDHF.[76] Although this may not

hold true in all instances, there is compelling evidence that this paradigm is applicable to certain chemical stimuli. For example, intraluminal ATP can cause cerebral vasodilation through its actions on endothelial P2 purinergic receptors. Whereas ATP-induced vasodilation can be blocked by L-NAME (NOS inhibition) in the MCA, neither L-NAME nor combined L-NAME and Indomethacin (combined NOS and COX inhibition) blocks ATP-induced vasodilation in penetrating arteries. Furthermore, removal of the endothelium blocks ATP-induced vasodilation in penetrating arteries. Thus, in penetrating arteries, endothelial-dependent, ATP-induced vasodilation appears to be mediated by EDHF (defined as sustained dilation after both NOS and COX inhibition). Similar observations have been reported concerning other components of EDHF-mediated vasodilation. That is, whereas pharmacological inhibition of IKCa channels, a key mediator of the EDHF response, has little effect on vascular tone in larger cerebral arteries, it increases vascular tone in smaller cerebral arteries.[75] Furthermore, in contrast to the role of PGI_2, evidence suggests EDHF regulation of cerebrovascular tone increases with age, perhaps acting as a compensatory dilator when the role of other endothelial-dependent dilators becomes blunted or impaired.[77] Thus, regarding the pial circulation, EDHF may serve a more significant role in the regulation of terminal branches (i.e., smallest pial arteries), with this role increasing with ageing.

2.2 Endocannabinoid system

2.2.1 General introduction to the endocannabinoid system

The endocannabinoid system is comprised of a group of receptors and ligands that are involved in the regulation of numerous physiological and behavioural processes, including cognitive functioning, mood, appetite, pain sensation and fertility.[5] More recently, a role for the endocannabinoid system in cardiovascular control has emerged.[10] There are two primary receptors in this system - the CB1R and CB2R. Each receptor has a distinct role and is produced at different levels in different tissues. There are >100 endocannabinoids (endogenously produced, fatty acid-based cannabinoids) that elicit physiological effects through the actions of the CB1R, CB2R, or both. The two principal endocannabinoids are anandamide (AEA)[78] and 2-arachadonyl glycerol (2-AG),[79] which produce local and systemic effects based on the local production and circulating plasma concentrations of these endocannabinoids as well as the expression of either CB1R and CB2R in the surrounding tissue or target tissue of interest. The

CBR distribution and the effects of CBR ligands, either endogenous or exogenous, have yet to be well characterized in the vascular system, and, in particular, in the cerebrovasculature.

2.2.2 Cannabinoids and their receptors

The term 'endocannabinoid' refers to a collection of fatty-acid ligands for the CBR.[6] More specifically, they are eicosanoids that activate one or both of the CB1R and CB2R. Two endocannabinoids that are produced in relatively larger quantities and better studied are AEA and 2-AG. Both are synthesized from arachidonic acid – a molecule generated during the breakdown of membrane bound or intracellular phospholipids. This is usually initiated following an elevation in intracellular Ca^{++}. AEA is generated from the lipolysis of phospholipid N - arachidonoyl-phosphatidylethanolamine (NArPE). 2-AG is produced from the hydrolysis of the arachidonic acid containing DAG with diacylglycerol lipase (DAGL). Factors that stimulate increases in intracellular Ca^{++} and increase the breakdown of phospholipids, resulting in an accumulation of arachidonic acid, such as activation of certain classes of GPCRs, are important for the production endocannabinoids.[1,5]

Both CB1R and CB2R are GPCRs.[6] GPCRs contain a membrane bound receptor which is coupled to an intracellular trimeric G protein (contains an α, β, and γ subunit) which relays the signal. The G proteins can be broadly grouped into three main classes based on their signaling pathway.

G_s: Stimulation of AC leading to the production of cAMP [55]

$G_{i/o}$: Inhibition of AC leading to the reduction of cAMP [55]

G_q: Stimulation of PLC leading to the hydrolysis of PIP_2. This generates two second messengers: DAG and IP_3 [55]

Ultimately, the effects of receptor stimulation depend on the type of subunit bound and the net influence of the downstream signaling cascades. Both CB1Rs and CB2Rs bind the $G_{i/o}$ subunit leading to a reduction in cAMP.[5] Of note, cAMP is a second messenger involved in mediating intracellular signal transduction. Briefly, AC catalyzes the conversion of ATP to cAMP, which activates intracellular protein kinases (e.g., protein kinase A) or acts directly on

Ca^{++} channels and potentially K^{+} channels producing a hyperpolarizing effect.[80] By inhibiting cAMP production, activation of the CBR $G_{i/o}$ leads to an increase in intracellular Ca^{++}. Concurrently, activation the CBR $G_{i/o}$ has been shown to induce the phosphoinositide 3-kinases/Akt pathway, which initiates an IP_3-dependent increase in intracellular Ca^{++}. Thus, the net effect of CBR $G_{i/o}$ signaling is an increase in intracellular Ca^{++} concentration.[55] However, it is important to note, although the CB1R and CB2R preferentially bind the $G_{i/o}$ subunit, other classes of GPCRs may also be involved in physiological effects of CBR activation.

In addition to the $G_{i/o}$ subunit, emerging evidence reveals CB1Rs may bind the G_s subunit.[81] In contrast to the $G_{i/o}$ subunit, the G_s subunit stimulates AC thereby increasing production of cAMP leading to a reduction in intracellular Ca^{++}. Additional evidence reveals, the CB1Rs may also bind the G_q subunit, mediating a PLC dependent increase in IP_3, leading to an IP_3 mediated increase in intracellular Ca^{++}. Given the access to distinct populations of GPCRs, the net effect of CB1R activation will depend on the class of G protein bound and the net effect of downstream signaling cascades. Whether activation of multiple classes of GPCRs occurs simultaneously, competitively, or in a biphasic manner, and how this influences the physiological effects of CB1R activation remains to be elucidated fully.

The first receptor discovered to bind THC, the psychoactive component in cannabis, was the CB1R.[3] The function of CB1Rs is best studied in the brain, and is the most highly expressed GPCRs in the brain.[82] CB1R signaling is involved in neurodevelopment where it controls neuronal activity and circuity. Mechanistically, the CB1R controls neuronal circuitry through the negative regulation of synaptic activity. For example, after the release of neurotransmitters that have G_q activity, there is an IP_3-dependent increase in cellular Ca^{++} and an increase in the levels of DAG. Subsequent hydrolysis of DAG leads to the production of 2-AG, which reduces neuronal activity by acting on pre-synaptic CB1R.[83–85] More specifically, this enhances $G_{i/o}$ activity, which reduces intracellular cAMP and protein kinase A activity resulting in an increase in intracellular Ca^{++} and ensuing depolarization-induced attenuation of neuronal activity.[5] Although CB1R expression is highest in the brain, there are several tissues with functional levels of CB1R, including the vascular system (Figure 2.2).[10] Owing to the hydrophobic nature of 2-AG and AEA, they are easily distributed throughout the surrounding tissue. Thus, 2-AG and AEA produced in neurons may mediate local CB1R-dependent effects in the surrounding vasculature. Moreover, there is evidence that endothelial cells themselves can produce

endocannabinoids.[86] Although cerebral arteries appear to express functional levels of the CB1R, and are located in close proximity to neurons that produce endocannabinoids, the physiological role of cerebrovascular CB1Rs has yet to be well characterized.

CB2Rs are present at high concentrations in immune tissue such as the tonsils and the spleen. Activation of CB2Rs have been shown to supress the release of inflammatory cytokines and the migration of leukocytes. However, CB2Rs are also expressed at lower concentrations in non-immune tissue such as the heart and cerebrovasculature. The physiological significance of CB2Rs in the heart or elsewhere in the cardiovascular system, such as the cerebrovasculature, remains relatively unknown.[10]

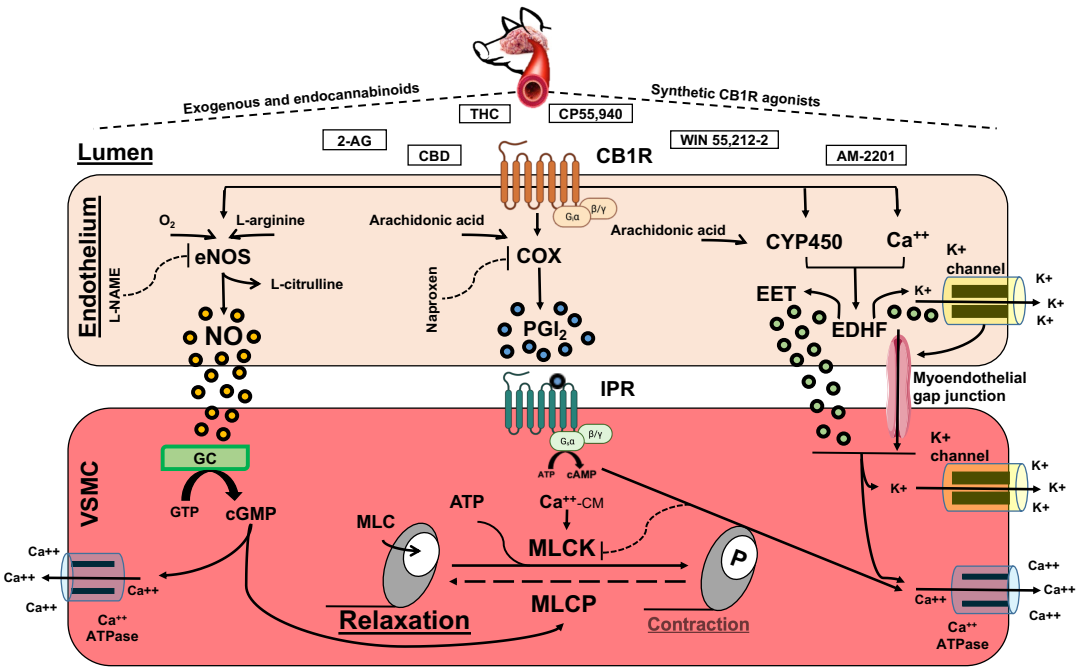


Figure 2.2 Proposed endothelial actions of cannabinoids on the cerebrovasculature of swine. eNOS, COX and EDHF are the three principle vasodilator pathways. Cannabinoids such as THC, CBD, 2-AG, AEA, CP55940, WIN55212-2 and AM-2201 bind to CB1R, which recruits a combination of one of these three pathways. CB1R activation results in either elevated cytosolic Ca^{++} or increased arachidonic acid. Increased Ca^{++} levels activate eNOS, which catalyzes the transformation of L-arginine and O_2 to L-Citrulline and NO. NO freely diffuses to the VSM where it activates sGC that increases cytosolic cGMP. cGMP activates myosin light chain phosphatase, which results in VSM relaxation. Liberated arachidonic acid is actively used for COX metabolism. COX transforms arachidonic acid initially into PGH_2 and ultimately into PGI_2. PGI_2 act on the VSM IPRs, which activate AC and, in turn, catalyzes the conversion of ATP to cAMP. cAMP leads to decreased intracellular Ca^{++}. Decreases in Ca^{++} in the VSM leads to inhibition of myosin light chain kinase, which ultimately inhibits VSM contraction. Arachidonic acid can also be used to generate EET, initiating an endothelial and VSM hyperpolarization effect. Independently, Ca^{++} can also cause endothelial hyperpolarization. Endothelial hyperpolarization travels through myoendothelial gap junctions, results in VSM muscle hyperpolarization. The net effect of hyperpolarization, is reduced VSM Ca^{++}. Consequently, the decreased VSM intracellular Ca^{++} concentrations and hyperpolarization results in VSM relaxation.

2.2.3 Cannabinoid receptors and cerebrovascular function

Both endogenous and exogenous cannabinoids can produce vasodilatory effects in the peripheral vasculature. In rat mesenteric arteries, AEA-induced vasodilation is blunted by the removal of the endothelium as well as the CB1R antagonist AM251, but is unaffected by the CB2R antagonist AM630.[15] These data indicated AEA-induced vasodilation, is endothelial-dependent and mediated by the CB1R. Vascular CB1Rs have been documented in many mammalian species including human mesenteric arteries, implicating endothelial-dependent CB1R-mediated vasodilation as a conserved vascular response.[14,15,87] Furthermore, evidence suggests CB1R agonism also elicits a cerebral vasodilatory effect.[86] Nevertheless, the functional significance of this phenomenon and the mechanisms responsible for the vasodilatory effect remain to be fully explained.

The brain is a major site of endocannabinoid action.[5,88] In addition to the presence of CB1R and CB2R, the brain and the cerebrovasculature possess the machinery involved in endocannabinoid production. With respect to cerebrovascular regulation, Rademacher and colleagues propose that endogenously produced endocannabinoids serve a role in buffering against vasoconstriction (e.g., maintenance of vascular tone).[86] This is based on experiments in rat cerebral arteries demonstrating that endothelial production of both AEA and 2-AG are increased in the presence of thromboxane analogue U-46619, a potent TXR agonist/vasoconstrictor. Furthermore, when pre-incubated with the CB1R antagonist SR-141716, to block the vascular actions of AEA and 2-AG, rat pial arteries exhibit increased vasoconstriction in response to U-46619.[86] Thus, TXR agonism increases the endothelial production of AEA and 2-AG, which subsequently attenuates TRX-induced vasoconstriction by engaging CB1R dilatory pathways. Concerning exogenous cannabinoids, AEA infusion stimulates cerebral vasodilation in rats, which is likewise abolished with the CB1R antagonist SR-141716. To date, there is no evidence to suggest CB2R mediates direct cerebral vasorelaxation;[14] however, a recent study in rats highlighted that CB2R agonist JWH-33 potentiated endothelial-dependent cerebral vasodilation. Taken together, both endogenous and exogenous cannabinoids elicit a cerebral vasodilatory effect; whereas the former appears to reduce off target constrictor effects of TXR agonism (perhaps from prostaglandin production from the CNS or endothelial cells), the latter appears to stimulate an actual dilation. Furthermore, although the vasodilatory effect of cannabinoids is mediated in large part by the CB1R, there is

an emerging interactive role for CB2R. The dependency of the vascular actions of CB1R and CB2R agonism on the endothelium and the independent roles of NO, prostaglandins and EDHF is a current area of inquiry.[89]

Evidence indicates cannabinoids mediate dilation through multiple pathways. Although the precise mechanism is unclear, either through $G_{i/o}$ or G_q activity, it is well accepted that CB1R agonism results in the mobilization of endothelial cytosolic Ca^{++} from intracellular stores, which results in the activation of vasodilatory proteins associated with NO production, prostaglandin production and EDHF (Figure 2.2).[90,91] Concerning NO, work from O'Sullivan and colleagues reveals eNOS does not contribute to AEA-induced dilation in either the superior mesenteric artery or third order branches in rodents.[15] In a another study, although cannabidiol (CBD) increased eNOS activation in human aortic endothelial cells, CBD-induced dilation in human mesenteric arteries was not attributable to eNOS. In contrast to this work, subsequent research by the same group demonstrated that AEA-induced dilation in human mesenteric arteries was eNOS-dependent.[92] Thus, the role of eNOS in cannabinoid-mediated dilation remains unclear, but may be both cannabinoid- and species-specific. Importantly, the independent role of endothelial NO on cerebrovascular regulation has not yet been studied.

Regarding the independent roles of COX-mediated production of prostaglandins and EDHF, evidence reveals potential roles for both in mediating the vasorelaxation effects of CB1R agonism. Indeed, cannabinoids have been shown to increase the cytosolic pool of arachidonic acid, which can be metabolized by COX to form prostaglandins as well as by cytochrome P450 to form EETs (chemical mediator of EDHF).[93] Furthermore, cell culture experiments reveal that AEA directly increases the levels of PGE_2 in cerebral endothelial cells[94] and the dilatory actions of AEA or THC on pial vessels *in vivo* are blunted by COX inhibitor Indomethacin.[87] With respect to EDHF, various cannabinoids that act on CB1R have been shown to activate inward rectifying K^+ currents initiating a hyperpolarization effect.[95,96] Additionally, in the presence of high K^+ physiological saline (which abolishes membrane hyperpolarization) or when myoendothelial gap junctions are inhibited (preventing the transfer of hyperpolarization from the endothelium to the VSM), mesenteric arteries lose much of their dilatory response to cannabinoids. Granted, like eNOS, these observations are from mesenteric arteries, this raises the possibility of EDHF involvement in CBR-modulation of cerebral vascular tone. Despite the lack of systematic characterization in the cerebral circulation, it appears cannabinoid-mediated

vasodilation likely involves CB1R activation and prostaglandin production, with possible contributions from endothelial derived NO, and EDHF. Given other endothelial GPCRs (e.g., muscarinic, and ATP-purinergic receptors) recruit these same dilatory pathways, but at varying degrees across different vascular beds as well as across different branch orders within the same vascular bed, it is likely CB1R recruitment of these pathways in the cerebral circulation is specific to the cerebral circulation and even the arteries being studied (e.g., MCAs vs. PCAs or pial vs. parenchymal etc.).

Although the majority of evidence supports a role for the CBRs, it is important to note that endocannabinoids and phytocannabinoids may exert a dilatory effect that operates independently from either CB1R or CB2R.[97] Some studies have attributed this latter effect to a yet unknown receptor, termed the endothelial cannabinoid receptor. Others have provided evidence that cannabinoids act on a broad range of GPCRs and ion channels outside of the classical CB1R and CB2R receptors, such as orphaned receptors (GPR18 and GPR55), and ion gated channels (e.g., 5-HT3, nicotinic acetylcholine, and glycine receptors, and Transient Receptor Potential Channels).[5] There are several studies that indicate that these non-CBR targets serve a role in modulating vascular tone through endothelial-dependent and independent mechanisms. For example, one study reported THC can induce rat aorta and mesenteric artery relaxation by increasing endothelial NO bioavailability through peroxisome proliferator-activated receptor-dependent activation of superoxide dismutase (SOD).[98] Another study demonstrated that AEA stimulates relaxation in isolated basilar arteries from guinea pigs by activating vanilloid receptors on perivascular sensory nerves, causing the release of calcitonin gene related peptide, a potent NO-independent vasodilator.[99] Given many cannabinoids, such as THC, AEA, 2-AG and CBD, may serve as ligands for CBR as well as a broad range of GPCRs and ion channels, to better understand CBR modulation of vascular control, it is critical that future studies take advantage of emerging synthetic ligands that have higher binding affinities for the CB1R and CB2R and fewer off target effects. Owing to the limitation of using traditional endocannabinoids or phytocannabinoids, and not synthetic ligands, the independent roles of CB1R and CB2R and downstream mediators have yet to be isolated.

2.3 Objective

The goal of this work is to provide a basic, translational framework for understanding how CBRs influence cerebral vasomotor control.

2.4 Purpose

The central purpose of this thesis is to examine the independent roles of CB1R and CB2R agonism and downstream signaling on cerebral vasomotor control in swine.

2.5 Hypothesis

It is hypothesized that CB1Rs mediate pial artery vasorelaxation in an endothelial-dependent manner, with potential independent contributions from the COX, NOS and EDHF pathways.

CHAPTER 3

3. METHODS

3.1 Ethical approval

This study was conducted at the University of Saskatchewan, Western College of Veterinary Medicine. All study procedures were in accordance with the guidelines of the Canadian Council on Animal Care (CCAC) and the ARRIVE guidelines, and were approved by the Animal Research Ethics Board of the University of Saskatchewan (Animal Use Protocol #20190036).

3.2 Animals

To minimize potential influences of sex, sex hormones and ageing, all of which may affect CBR expression and/or activity,[100,101] sexually immature female swine were selected as a translationally relevant model organism for the current research. Furthermore, this model (e.g., sexually immature females) was selected because of their relatively low cost, size and ease to work with. Importantly, as it pertains to the present work, although there are slight anatomical differences, the brain and cerebral architecture are remarkably similar between species. For example, like humans, swine contain internal carotid arteries and a basilar artery that converge with the circle of Willis, and they likewise possess ACAs, MCAs and PCAs that share structural and functional similarities with human cerebral arteries.[72,102] Swine also provide an ideal candidate as they display greater similarity in arterial size and cerebrovascular volume compared with lower order species (e.g. murine models). Furthermore the brain of swine are gyroencephalic and contain >60% white matter, which is similar to that of humans.[72,103] As grey-matter and white-matter have substantially different metabolic requirements (grey matter requires 3-5 times as much vascularization as white matter),[104] using models with similar ratios of grey to white matter may provide greater translational relevance and set the stage for future preclinical studies.

Female Landrace swine (N=16; mass=35±2 kg; mean±S.E.M.) were obtained from Prairie Swine. No similar studies have been conducted; therefore, a power calculation was not performed. The number of swine was chosen based on achieving an experimental sample of n=6-10 per condition, which is consistent with previous work examining vasomotor actions of CB1R.[105,106] The vendor guaranteed that the animals were virus-, bacteria-, and parasitic pathogen-free. The swine were acclimatized for a minimum of seven days prior to experimentation and were housed in a temperature (21°C) and humidity (<65%)-controlled room with an automated 12-hour, light/dark cycle. The animals had free access to water and food (Whole Earth Swine Pig Starter, CO-OP AGRO, Saskatoon, SK, CAN). Following an overnight fast, swine were anaesthetized with an IM injection of ketamine (20-30 mg/kg) and euthanized with an overdose of inhaled isoflurane (1 L/min O_2, 5% isoflurane) followed by exsanguination.

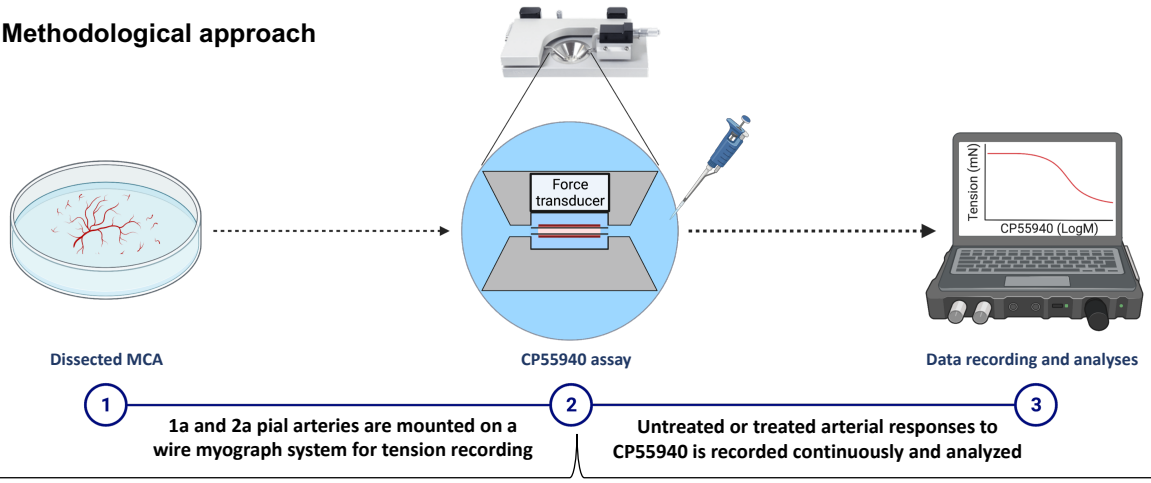
3.3 Isolated arterial experiments

The methodology used to study pial artery vasomotor control in the current study was wire myography (Figure 3.1). This technique involves threading two fine wires through the lumen of an artery, attaching those wires to a force transducer, and recording the changes in isometric tension resulting from arterial contractions and relaxations in response to pharmacological stimuli (e.g., receptor agonist, antagonists and enzyme inhibitors used alone or in combination with one another). As the arteries are separated from the surrounding tissue and many factors that contribute to vasomotor function can be controlled for (e.g., neurogenic input, tension, pH, temperature, O_2 and CO_2 levels, etc.), it is considered a gold standard method for isolating and characterizing the vasoactive properties of the endothelium and VSM.

Following euthanasia, a portion of the brain containing the MCA and downstream branches was carefully excised and transferred to a dissection dish containing an ice-cold physiological saline solution (PSS); in mM: 131.5 NaCl, 5.0 KCl, 2.5 $CaCl_2$, 1.2 NaH_2PO_4, 1.2 $MgSO_4$, 5.5 Glucose, and 25 $NaHCO_3$; pH 7.4. First-order branches of the MCA were dissected, cut into segments (2 mm) and mounted on 40 μm wires attached to a Multi Wire Myograph System (DMT 610M, Copenhagen, SV, Denmark) containing warm PSS to allow for isometric tension readings at 37°C. Data was acquired continuously and extracted using the data acquisition and analysis software LabChart (8.1.16)(Figure 3.1). Artery diameters for

optimal resting tension were normalized to 0.9 of internal circumference at 60 mmHg (IC_{60}; the IC_{60} produces a standardized, resting tension at a transmural pressure of 0.9 of 60 mmHg by a series of stepwise increments in diameter). Following a 40-minute stabilization, arteries were exposed to 80 mM KCl. In response to KCl, VSM becomes depolarized and a contraction is initiated. A contractile response to KCl is used to determine VSM integrity, and confirm functionality of the artery following dissection and mounting. Arteries that generated less than a 3 mN increase in tension in response to KCl were excluded. Thereafter, arteries were allotted an additional 40 minutes to achieve a stable baseline tension prior to experimentation.

A) **Methodological approach**



B) **Experimental conditions**

	Untreated	CB1R inhibition (AM251)	CB2R inhibition (AM630)	COX inhibition (Naproxen)	NOS inhibition (L-NAME)	Denuded
1	+	-	-	-	-	-
2	-	+	-	-	-	-
3	-	-	+	-	-	-
4	-	-	+	+	-	-
5	-	-	+	-	+	-
6	-	-	+	+	+	-
7	-	-	+	-	-	+

Figure 3.1 Methodological approach and experimental conditions. A) To determine the effects of CP55940 on the cerebrovasculature, pial arteries downstream from the MCA were dissected and mounted on a wire myograph. Once mounted, arterial contraction and relaxation responses were recorded continuously using Labchart software. Subsequently, data were extracted and analyzed. B) To study the effects CB1R and CB2R activation, vasoreactivity in response to CP55940 was examined in untreated, CB1R inhibition and CB2R inhibition conditions. To study CB1R-mediated, endothelial-dependent, and independent vasorelaxation, vasoreactivity in response to CP55940 was examined in the setting of CB2R inhibition combined with NOS inhibition, COX inhibition, combined NOS and COX inhibition and in denuded arteries.

Role of CB1R and CB2R: Arteries were treated with vehicle or pre-incubated with either the CB1R inverse antagonist AM251 ($1x10^{-7}$ M; n=8) or the CB2R inverse antagonist AM630 ($1x10^{-7}$ M; n=11) for 20 minutes. AM251 and AM630 display high selectivity for their respective receptors in the nanomolar range,[107] and the chosen concentrations were selected because they have been used previously in vascular preparations to isolate the contributions of either CB1R or CB2R.[89] Thereafter, they were pre-contracted with the thromboxane A_2 receptor agonist U-46619 (from $1x10^{-6}$ M to $1x10^{-4}$ M) and vasoreactivity in response to CP55940 (CB1R and CB2R receptor agonist; $1x10^{-9}$ M to $1x10^{-6}$ M; half-log doses) was assessed (Figure 3.1). CP55940 is a synthetic cannabinomimetic that is a selective agonist and displays high potency for the CB1Rs and CB2Rs.[108] Thus, it is an ideal drug to isolate the roles of CB1R and CB2R on cerebral vasomotor control (Figure 3.1).

Establishing the signaling mechanisms underlying CB1R-mediated relaxation: Vasoreactivity to CP55940 was examined in arterial segments pre-incubated for 20 minutes with AM630 to block any contribution of CB2R signaling as well as the following experimental conditions: 1) COX 1 & 2 inhibitor Naproxen ($3x10^{-4}$ M; n=7; Figure 2.2 and 3.1); 2) non-specific NOS inhibitor L-NAME ($3x10^{-4}$ M; n=7; Figure 2.2 and 3.1); 3) combined COX and NOS inhibition (L- NAME + Naproxen; reveals EDHF contribution, as EDHF is defined as the non-NO and non-prostaglandin contribution to relaxation; n=7; Figure 2.2 and 3.1); 4) denuded arteries (reveals endothelial-independent relaxation; n=6; Figure 3.1). Denudation is an established method to remove the endothelium and is accomplished by rubbing a horsehair through the lumen of the arterial segment prior to mounting it in the wire myograph system. To confirm the removal of the endothelium, arteries were exposed to U-46619, followed by the endothelium-dependent vasodilator bradykinin prior to examination of vasoreactivity in response to CP55940. Successful denudation was defined as a less than 15% relaxation in response to endothelial-dependent vasodilator bradykinin ($1x10^{-6}$ M) (Figure 3.1).

3.5 Data analyses and statistics

Percent contraction in response to U-46619 was calculated as the quotient of ΔU-46619 (mN) and ΔKCl (mN), multiplied by 100% (modified from Ingram et al):[109]

$$Contraction\ (\%) = \left(\frac{U-46619}{KCl}\right) * 100\% \quad \text{.....Eq.3.1}$$

Percent relaxation at each dose was calculated as the quotient of Δtension from baseline (mN) and tension at baseline (mN), multiplied by 100% (modified from Ingram et al):[109]

$$Relaxation\ (\%) = \left(\frac{(tension\ at\ baseline - tension)}{tension\ at\ baseline}\right) * 100\% \quad \text{.....Eq.3.2}$$

Data were analyzed using GraphPad Prism (8.4). A mixed model repeated measures ANOVA was performed to examine condition x dose differences in vasoreactivity to CP55940. A *post-hoc* Sidak test was used to determine the location of significance. Physiological maximum relaxation and area under the curve (AUC) were extracted for each dose-response curve. Data for the AUC are presented as absolute values as well as normalized to control. To extract negative logarithm of the half maximal effective concentration (pEC_{50}), dose-response curves were fitted using four-parameter non-linear regression fit. As the relaxation curves do not always adequately plateau, to calculate pEC_{50} values non-linear regression-fitted curves were restrained in the following ways: the bottom plateau was constrained to be equal or greater than zero, and the top plateau was constrained to be no greater than the physiological maximum.

Role of CB1R and CB2R: It was hypothesized that CB1R inhibition would attenuate the maximal and cumulative (AUC) relaxation in response to CP55940. Likewise, it was hypothesized that CB2R inhibition would not alter vasomotor responses to CP55940. Therefore, independent one-tailed Student's t-tests were performed to compare maximal and cumulative relaxation for the following conditions: Untreated vs. CB1R inhibition (AM251) and Untreated vs. CB2R inhibition (AM630). It was uncertain how CBR inhibition would influence relative sensitivity to CP55940; thus, independent two-tailed Student's t-tests were performed to compare the pEC_{50} data under the same experimental conditions.

Signaling mechanisms underlying CB1R-mediated relaxation: It was hypothesized that CB1R-mediated relaxation would be attenuated by NOS, COX and NOS+COX inhibition as well as by arterial denudation. Therefore, independent one-tailed Student's t-tests were performed to compare the maximal and cumulative relaxation for the following conditions: control vs. COX inhibition (AM630+Naproxen); control (AM630) vs. NOS inhibition (AM630+L-NAME); control vs. combined NOS and COX inhibition (AM630+L-NAME and Naproxen); control vs. denudation. However, it was uncertain how these would influence relative CB1R sensitivity to

CP55940; thus, independent two-tailed Student's t-tests were performed to compare the pEC_{50} under the same experimental conditions. Statistical significance was set at $P \leq 0.05$ and P values approaching significance (P=0.06-0.10) were interpreted as trends. Cohen's d effect size analysis[110] was used to quantify the magnitude of attenuated cumulative vasorelaxation under the following conditions: COX inhibition, NOS inhibition, combined NOS and COX inhibition as well as denudation. All data are presented as mean ± S.E.M. Where possible the data range as well as individual data are shown.

CHAPTER 4

4. RESULTS

4.1 Cannabinoid receptor signaling

Passive tension ($P \geq 0.15$), contraction in response to KCl ($P \geq 0.11$), and U-46619 ($P \geq 0.14$) were similar across all conditions. Therefore, values were averaged across all arteries and presented as arterial characteristics in Table 4.1

In isolated pial arterial segments, the CB1R and CB2R agonist CP55940 induced vasorelaxation. Furthermore, CP55940-induced relaxation was blunted by CB1R inhibition (AM251) at $1x10^{-10.5}$, $1x10^{-9.5}$, $1x10^{-8}$, $1x10^{-7.5}$ and $1x10^{-7}$ M of CP55940. Likewise, the pEC_{50} ($P=0.001$), cumulative ($P \leq 0.001$) and maximal vasorelaxation ($P \leq 0.05$) in response to CP55940 was decreased by CB1R inhibition (Table 4.1 and Figure 4.1 A, B). In contrast, CB2R inhibition (AM630) did not affect vasomotor responses to CP55940 ($P \geq 0.50$; Table 4.1 and Figure 4.1 C, D).

4.2 CB1R-mediated vasorelaxation and endothelial signaling

In isolated pial arterial segments, COX inhibition (Naproxen) did not affect CB1R-mediated vasorelaxation at any specific dose of CP55940 ($P \geq 0.22$; Figure 4.2 A). Neither the pEC_{50} nor maximal CB1R-mediated relaxation were altered by COX inhibition ($P \geq 0.45$; Table 4.1). Reductions in the cumulative CB1R-mediated relaxation with COX inhibition approached significance ($P=0.07$; Figure 4.2 B).

CB1R-mediated vasorelaxation was blunted by NOS inhibition (L-NAME) at $1x10^{-7}$ and $1x10^{-6}$ M of CP55940 ($P \leq 0.05$; Figure 4.2 C). Furthermore, although pEC_{50} was not significantly different ($P=0.25$; Table 4.1), both the maximal and cumulative CB1R-mediated vasorelaxation were blunted by NOS inhibition ($P \leq 0.05$; Table 4.1 and Figure 4.2 D).

Reductions in CB1R-mediated vasorelaxation during combined NOS and COX inhibition (L-NAME and Naproxen) approached significance from 1x10-9.5 to 1x10-8.5 M ($P \leq 0.07$) and were

significant at 1x10-8 M of CP55940 ($P=0.02$; Figure 4.2 E). Moreover, pEC50 values ($P<0.01$), the maximal ($P=0.05$) and cumulative ($P<0.001$) CB1R-mediated vasorelaxation were decreased by combined NOS and COX inhibition (Table 4.1 and Figure 4.2 F).

CB1R-mediated vasorelaxation was blunted in denuded arterial segments from $1x10^{-9.5}$ to $1x10^{-6}$ M of CP55940 ($P\leq0.05$; Figure 4.2 G). Although the pEC_{50} values were not different ($P=0.60$), the maximal ($P<0.001$) and cumulative ($P<0.001$) CB1R-mediated vasorelaxation were blunted in denuded arteries (Table 4.1 and Figure 4.2 H).

In summary, whereas the effect of COX inhibition only approached significance ($P=0.07$), NOS inhibition, the combination of NOS and COX inhibition as well as denudation attenuated the cumulative vasorelaxation to CP55940 ($P\leq0.05$; Figure 4.1 A-H; values normalized to control are presented in Table 4.2). Regarding the magnitude of attenuated vasorelaxation, effect size analysis reveals COX inhibition had a medium negative effect, albeit non-significant, NOS inhibition had a large negative effect, and both the combination of NOS and COX inhibition as well as denudation had a very large negative effect on cumulative CB1R-dependent vasorelaxation (Table 4.2).

Table 4.1. Arterial function

Arterial characteristics		
Passive tension (mN; n=54) (range; mN)	1.2±0.2 (-0.19-2.69)	
KCl-induced contraction (mN; n=54) (range; mN)	6±2 (3-32)	
Precontraction (% KCl contraction; n=54) (range; %)	92±6 (43-220)	
	Max relaxation (%)	**pEC_{50} (-log(M))**
Untreated (n=8) (range; %)	21±2 (12-30)	9.7±0.4
CB1R inhibition (range; %) (Pretreated with AM251; n=8)	13±1* (6-24)	7.4±0.4*
CB2R inhibition/Control (range; %) (Pretreated with AM630; n=11)	23±2 (14-33)	9.4±0.4
COX inhibition (range; %) (Pretreated with AM630+Naproxen; n=7)	21±3 (6-34)	8.6±0.7
NOS inhibition (range; %) (Pretreated with AM630+L-NAME; n=6-7)	17±3* (11-28)	10.5±1.0
NOS+COX inhibition (range; %) (Pretreated with AM630+L-NAME+Naproxen; n=7)	18±3* (10-23)	7.7±0.2*
Denuded+CB2R inhibition (range; %) (Denuded pretreated with AM630; n=6)	9±2* (5-15)	9.8±0.7

Passive tension (mN), KCl-induced contraction (mN), degree of precontraction prior to the dose response curves (%KCl) for all arterial segments. Maximal relaxation and pEC_{50} data for each experimental condition. CB1R blocker (AM251) and CB2R blocker (AM630) conditions were compared with untreated conditions, and CB2R blocker with either NOS inhibition (L-NAME), COX inhibition (Naproxen), NOS+COX inhibition or denudation were compared with intact CB2R inhibition only conditions (Control). Maximal relaxation data were analyzed using a one-tailed t-test and pEC_{50} data were analyzed using a two-tailed t-test. *Significantly less than Untreated or Control ($P \leq 0.05$). Data are presented as mean ± S.E.M.

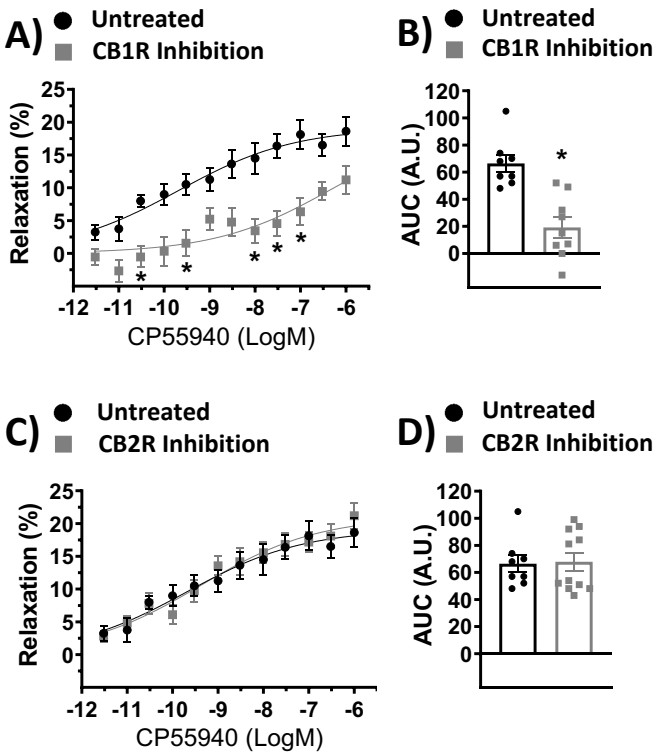


Figure 4.1. Concentration response curves and cumulative relaxation (area under the curve; AUC) in response to CP55940 in untreated (n=8) or A) and B) CB1R inhibition using AM251 (n=8); as well as C) and D) CB2R inhibition using AM630 (n=11). Dose response curves were analyzed using a mixed model repeated measures ANOVA and AUC data were analyzed using a one-tailed t-test. *Significantly less than untreated ($P \leq 0.05$). Individual data are presented in B) and D). All data are presented as mean ± S.E.M.

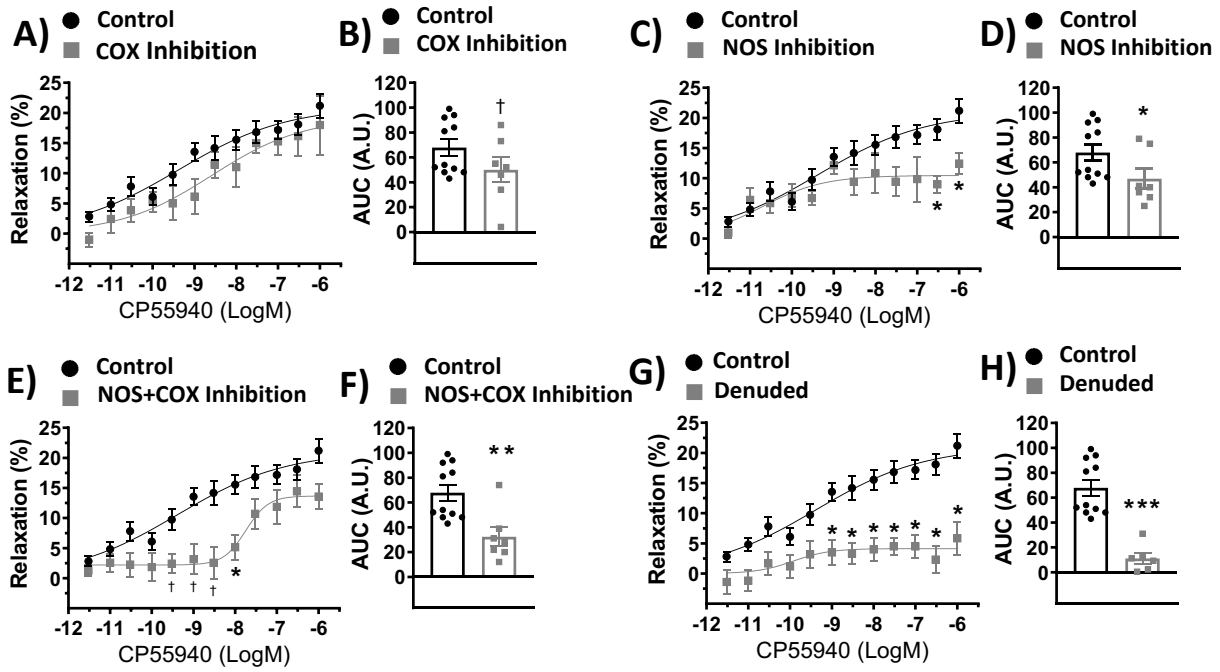


Figure 4.2. Concentration response curves and cumulative relaxation (area under the curve; AUC) in response to CP55940 in control (AM630; n=11; same data as Figure 4.1 C and D, CB2R inhibition) compared to A) and B) COX inhibition (AM630+Naproxen; n=7); C) and D) NOS inhibition (AM630+L-NAME; n=7); E) and F) NOS+COX inhibition (AM630+L-NAME+Naproxen; n=7) conditions; as well as in G) and H) denuded (AM630+denudation; n=6) arteries. Dose response curves were analyzed using a mixed model repeated measures ANOVA and AUC data were analyzed using a one-tailed t-test. Significantly less than control *($P \leq 0.05$), **($P \leq 0.01$), ***($P \leq 0.001$); approaching significance †($P = 0.06$-0.10). Individual data are presented in B), D), F), and H). Data are presented as mean ± S.E.M.

Table 4.2. Effect size analysis

Condition	Normalized AUC	Effect size
COX inhibition (range) (Pretreated with AM630+Naproxen; n=7)	0.74±0.15† (0.07-1.27)	-0.70
NOS inhibition (range) (Pretreated with AM630+L-NAME; n=7)	0.69±0.12* (0.36-1.17)	-0.86
NOS+COX inhibition (range) (Pretreated with AM630+L-NAME+Naproxen; n=7)	0.48±0.12* (0.18-1.10)	-1.28
Denuded (range) (Denuded arteries pretreated with AM630; n=6)	0.21±0.12* (0.01-0.77)	-1.59

CB2 receptor blocker (AM630) combined with either COX inhibition (Naproxen), NOS inhibition (L-NAME), or both as well as denuded arteries with CB2R inhibition were normalized and compared to intact CB2R inhibition only conditions (control). Normalized AUC data were analyzed using a one-tailed t-test. Cohen's d effect size was used to determine the magnitude and direction of effect. *Significantly less than control ($P \leq 0.05$); approaching significance †($P=0.07$). Medium effect ≥0.5-0.8; large effect ≥0.8-1.2; very large effect >1.2. Data are presented as mean ± S.E.M.

CHAPTER 5

5. DISCUSSION

The major finding of the current study was that CB1R activation induces an endothelial-dependent cerebral artery vasorelaxation. More specifically, the data indicate the pial artery vasorelaxation elicited by CP55940 is mediated by the CB1R. Given that relaxation was almost completely abolished by denudation, it appears that in this experimental paradigm, CB1R-mediated relaxation operates through an endothelial-dependent pathway. Furthermore, the cumulative relaxation response to CB1R agonism was attenuated by NOS inhibition alone, to a much lesser (non-significant) extent by COX inhibition alone, and the combination of NOS and COX inhibition enhanced the magnitude of the attenuation effect. Regarding the candidate mechanisms, these data indicate that CB1R-dependent relaxation is mediated by both NO and prostaglandins, with a larger contribution from the former. Although combined NOS and COX inhibition blunted vasoreactivity, the relaxation that was observed at higher doses of CP55940 indicating EDHF is also involved in CB1R-mediated relaxation. Collectively, these data reveal a potential role for CB1R in modulating cerebrovascular tone through multiple endothelial-dependent mechanisms (Figure 2.2). Validating these findings under *in vivo* conditions and in humans is necessary to confirm the mechanisms involved and informing on the translational relevance of these findings.

5.1 Cannabinoid receptor signaling and cerebrovascular regulation

This study demonstrated that vasorelaxation in response to CP55940 is attenuated, but not abolished by the CB1R antagonist AM251. The lack of complete blockade may be because CP55940 and AM251 bind to different sites on the CB1R. Nevertheless, these data provide evidence that CB1R serves a direct role in mediating cerebral vasorelaxation. These findings are in agreement with prior studies that have suggested endocannabinoids contribute to

CBF regulation through CB1R signaling.[111] For example, Iring and colleagues showed that endocannabinoid-induced increases in CBF are blunted by the CB1R antagonist AM251.[8] Given that CB1R is expressed in a wide array of cells in the CNS, such as neurons, astrocytes and the endothelium, this study could not determine whether the effects of endocannabinoids were owing to non-vascular or vascular CB1Rs. Endocannabinoids also mediate systemic cardiovascular effects, including reduced cardiac contractility and reduced blood pressure, which interact with cerebral autoregulatory mechanisms.[10] Therefore, the effect of CB1R agonism on CBF control *in vivo* may reflect the cumulative contributions from neurogenic and myogenic responses to alterations in systemic hemodynamics as well as endothelial-mediated responses. By using an *ex vivo/in vitro* experimental preparation, the current data excludes contributions from neurogenic or mechanical inputs (resulting from changes systemic hemodynamics). Therefore, these data advance the understanding of cannabinoids and cerebrovascular physiology and confirms the existence of an exclusively vascular component of CB1R-dependent pial artery vasorelaxation.

Vasorelaxation in response to CP55940 was unaffected by the CB2R antagonist AM630, suggesting CB2R is not involved in mediating a direct vasodilatory effect in pial arteries. CB2R has been detected throughout the vasculature, including in cerebral endothelial cells.[5,111] Although CB2R may not cause relaxation directly, there is evidence suggesting it potentiates endothelial-dependent cerebral vasodilation (e.g. CB2R agonism enhances the effect of other endothelial-dependent dilators). This prospect was not tested in the current study. Thus, although CB2R may serve a role in cerebral vasomotor control, data from this study indicate CBR-mediated vasorelaxation is a result of CB1R agonism, and not CB2R agonism.

In addition to isolating a vascular component of CB1R-dependent cerebral vasorelaxation, the present data reveal the relaxation was abolished by arterial denudation, which indicates endothelial CB1Rs mediated this response. A previous study reported that arterial denudation significantly attenuated, but did not completely block, relaxation in response to the endocannabinoid AEA.[15] Discrepancies between the present findings and the latter study may be the result of the different pharmacological approaches. In the present study, CP55940 was used to agonize CB1R and CB2R; whereas, the previous study used the endocannabinoid AEA. Given the wide array of receptors that endocannabinoids interact with, when compared to CP55940 which has a higher binding affinity for CBRs,[108] it is likely the residual dilation observed

following denudation in the prior study was the result of other GPCR and ion channels receptors on the VSM.

In the current study, the COX pathway did not emerge as a significant mediator of CB1R-dependent pial artery vasorelaxation. Although the combination of NOS+COX inhibition blunted CB1R-mediated relaxation greater than NOS inhibition alone, decreases in CB1R-dependent pial artery vasorelaxation with COX inhibition alone only approached significance. Prior data has shown that COX inhibitor Indomethacin blunts *in vivo* pial artery vasodilation in response to AEA and THC.[87] Given the present data suggest endothelial prostaglandins are likely not major contributors to CB1R-dependent vasorelaxation, potentially, in the latter study, dilation was blunted owing to reduced neuronal prostaglandin production. During neurogenic-mediated hyperemia, neuronal prostaglandin production may account for up to 50% of the increases in CBF.[112] Furthermore, considering both AEA and THC interact with a variety of other GPCR and ion channels, it is possible COX inhibition attenuated dilation by reducing non-endothelial CB1R sources of prostaglandins. Thus, although CBR agonists likely elicit a COX-mediated production of prostaglandins and subsequent cerebrovascular dilation, the present data suggest the endothelial CB1R may only serve a minor role in this vasomotor response.

Although the COX pathway did not emerge as a significant contributor to CB1R-dependent pial artery vasorelaxation in the present study, the data revealed independent roles for NO and EDHF. Specifically, the data show that in the presence of the NOS inhibition, vasorelaxation in response to CB1R agonism was blunted. Furthermore, although the degree of attenuation was amplified with combined NOS+COX inhibition, suggesting the contribution from EDHF is quite small, CB1R-mediated vasorelaxation persisted at higher concentrations of CP55940. That NOS inhibition attenuated CB1R-mediated pial artery relaxation indicates this response is partially NO-dependent. Concurrently, that CB1R-mediated pial artery relaxation persisted in the setting of combined NOS+COX inhibition indicates a portion of this response is mediated by EDHF. Indeed, previous data indicate AEA-induced dilation in human mesenteric arteries is likewise blunted by NOS inhibition.[92] Additionally, in the presence of high K^+ physiological saline (which abolishes membrane hyperpolarization) or when myoendothelial gap junctions are inhibited, AEA-induced dilation in mesenteric is likewise blunted.[15] Collectively, the data support roles for both endothelial NO and EDHF in CB1R-dependent vasorelaxation in mesenteric and cerebral arteries.

5.4 Practical and translational considerations

As medical and recreational uses of cannabis products in Canada has increased,[1] the focus of this thesis was to determine how CB1Rs influence cerebral vasomotor control. This work shows that cannabinoids can causes vasorelaxation in isolated cerebral arteries in part by acting on CB1R. However, the way an individual will respond to cannabis (medical or recreational) *in vivo* will depend on how cannabinoids affect several physiological responses, ranging from changes in blood pressure, attenuation of central nervous system activity and a subsequent reduction in neuronal metabolism. As such a practical overview of how cannabinoids may alter CBF and the translational implications is warranted.

CB1Rs are expressed throughout the cardiovascular system, where they mediate a vasorelaxation response.[89] As such, a common physiological response to cannabis consumption is systemic arterial relaxation leading to reduced blood pressure.[9] When blood pressure decreases, cerebral perfusion pressure decreases, and by extension, CBF would be expected to decrease. To prevent cerebral hypoperfusion, the reduction in blood pressure will engage the cerebral myogenic mechanism, which will mediate a local cerebral vasodilation in an effort to preserve CBF in the setting of reduced perfusion pressure. Concurrently, acting on CB1Rs in the central nervous system, cannabinoids may supress both extrinsic and intrinsic neuronal activity which will have a direct impact CBF.[10,111] Depression of the extrinsic nerves originating from the sympathetic nervous system will attenuate the release of norepinephrine and neuropeptide Y. Systemically, attenuation of sympathetic input will decrease vascular tone and contribute to the reduction in blood pressure. Locally, within the cerebrovasculature, although both sympathetic neurotransmitters can mediate vasoconstriction and dilation, the net effect of attenuated sympathetic nervous system activity is believed to be an increase in CBF. In contrast to withdrawal of sympathetic input, attenuation of intrinsic nerve activity may reduce CBF through two primary mechanisms. First, a decrease in neuronal activation will decrease the release of vasodilatory neurotransmitters from within the NVU. Secondly, reduced neuronal activity will be coupled with a decrease in the production of vasodilatory metabolites, such as CO_2. Thus, although the present work revealed endothelial CB1Rs mediate a dilatory response, the effect of cannabis use on CBF will reflect the cumulative actions of many different features of vascular regulation operating in concert. Furthermore, contribution from these factors may differentially

depend on the age, sex, health status, resting state hemodynamics and history of cannabis use of the individual.[5]

Although the cumulative effect of cannabis use on CBF is not entirely clear, emerging evidence reveals endogenous cannabinoids produced within the brain may serve a role in increasing CBF through a CB1R-dependent mechanism.[8,89] Regarding the latter phenomenon, a recent study revealed that CB1R modulates the NVC response in mice, through the production of the endocannabinoid 2-AG.[113] That is, neuronal activation increases local endocannabinoid production, which in turn mediates a CB1R-dependent cerebral vasodilation.[113] The current data demonstrate that CB1R activation mediates endothelial NO-dependent vasorelaxation. Therefore, although the authors of the latter study did not address the dilatory mechanism, endothelial-dependent production of NO is a potential candidate. Interestingly, reports indicate that NO accounts for approximately 50% of the NVC response in mice.[114] As such, CB1R-mediated endothelial NO-dependent vasodilation may serve a significant role in the vasomotor responses to both exogenous and endogenously produced cannabinoids. Given neurogenic responses may stimulate endocannabinoid production, this raises the possibility that CB1R-mediated endothelial NO-dependent vasodilation is not merely an experimental phenomenon, but involved in fundamental features of CBF control. However, this prospect is speculative and requires further study.

5.4 Clinical considerations

Although cannabis use is broadly considered safe for most individuals, the American Heart Association has highlighted that chronic cannabis can have negative effects on the cardiovascular system, with the cerebrovasculature being considered at particular risk (e.g., transient ischemic attacks secondary to altered cerebrovascular vasomotor function and stroke).[2] Kalla and colleagues assessed over 316,000 people 18–55 years of age and found that cannabis use was an independent predictor of cardiovascular events, and in particular cerebrovascular incidents.[115] Furthermore, in a cohort of approximately 7,500 Australians, Hemachandra and colleagues reported that cannabis use significantly increased the risk of transient ischemic attack and stroke in a dose-dependent manner.[116] Although these studies are not capable of dissecting the mechanistic link between chronic cannabis consumption and transient ischemic attack or

stroke, given the apparent involvement of endothelial CB1R in mediating vascular responses, it is possible endothelial dysfunction may play a role.

The interactions between cannabinoid signaling generally, and CB1R signaling specifically on the cerebrovasculature are complex. But little research has been conducted to determine the mechanistic link between chronic cannabis consumption and associated cerebrovascular pathologies. Impaired endothelial-dependent NO-mediated vasodilation is a hallmark of cerebrovascular dysfunction and is implicated in both transient ischemic attacks and stroke.[117] The data presented herein indicates that a primary mechanism of CB1R-dependent dilation is through the endothelial NO pathway. Thus, in the setting of cerebrovascular pathology, where endothelial NO signaling is impaired, the vasorelaxation effect of CB1R agonism may likewise be compromised. Furthermore, evidence reveals over-stimulation of CB1R may increase ROS, which are known to further impair NO signaling.[118] This is of particular importance, as the phytocannabinoids found in cannabis are not metabolized as quickly as endocannabinoids and likely activate CB1R beyond the normal physiological range. Thus, they may contribute to pathological CB1R signalling. Potentially, pre-existing cerebrovascular pathology coupled with persistent CB1R agonism, secondary to chronic cannabis consumption, may exacerbate cerebrovascular dysfunction and contribute mechanistically to the integrative link between cannabis use, impaired NO signaling and transient ischemic attack or stroke. However, this is based on evidence from several species (e.g. mice, rabbits, rats), and the precise mechanism remains highly speculative. To determine the underlying mechanism responsible for the link between cannabis use and cerebrovascular insults requires further study (e.g., preclinical chronic intervention studies in relevant animal models).

Another important clinical consideration arising from this work and others,[119] is the potential interactions between cannabis (or CBR agonists) and nonsteroidal anti-inflammatory drugs (NSAIDs). Whereas CBR agonist stimulate the COX pathway, NSAIDs such as Naproxen, Indomethacin and Aspirin inhibit the COX pathway. Although the present data revealed Naproxen-induced reductions in CB1R-mediated vasorelaxation only approached significance, this does not preclude the possibility of significant interaction effects under different, more physiologically relevant conditions. Possibly owing to a larger contribution from neuronal prostaglandin production, evidence shows that the COX pathway is critical to CB1R-mediated increases in CBF *in vivo*.[87] Although the effect of Naproxen was not robust in the current study,

it may be much more pronounced under normal physiological conditions where other sources of prostaglandins contribute to cerebrovascular control. Regarding the cerebrovasculature, determining how the combination of these drugs affect CBF control is essential for understanding the proper prescriptive use of NSAIDs and CBR agonists for pain management.

5.5 Limitations

There are several limitations that must be considered when interpreting the data from this study. Although a major advantage of wire myography is that it allows the contribution of the endothelium in vasomotor control to be isolated, it does not necessarily reflect CBF responses *in vivo*. Cannabinoids acting on the CB1R have been shown to alter synaptic activity and blood pressure,[8,10,111] both of which influence CBF control. Therefore, validating the involvement of the endothelial CB1R under *in vivo* conditions is critical to confirm the translational relevance of this work.

A major purpose of this research was to examine CBR modulation of cerebral vasomotor control. The synthetic cannabinoid CP55940 was used, in conjunction with AM251 and AM630, because it is a highly selective CBR agonist.[6] However, it is important to note, natural endocannabinoids that act on CB1R and CB2R often display a lower potency for CBRs and induce a wide range of effects mediated by other receptors and ion channels (e.g.: 5-HT3, nicotinic acetylcholine, glycine receptors, and transient receptor potential channels).[6] Consequently, the vasomotor effects of CP55940 should not be equated directly to those of other cannabinoids such as AEA, 2-AG and THC. The current data provide insight into CBR-modulation of cerebral vasomotor control, but additional studies are required to determine the physiological relevance of these data with respect to cannabinoid signaling in the cerebrovasculature.

In this study, sexually immature female swine were used as a model organism to study cerebral vasomotor function. These pigs were selected because of their relatively low cost, size and ease to work with. Although cardiovascular anatomy of swine this size closely resembles that of humans, the cerebrovascular anatomy varies slightly (pigs brain and their arteries are smaller)[120] and the cannabinoid system in the brain is recognized to undergo major changes during sexual development.[5] In humans, the density of CB1R in the brain gradually decreases throughout development until early adulthood.[100] Furthermore, CBR may interact with sex

hormones, including testosterone and estradiol.[101] Likewise, both ageing and sex hormones influence endothelial function.[121] As cannabis use rarely begins before the onset of puberty, and the swine in the present study were not sexually mature, the data do not account for the potential role that age or hormones may serve on CBR modulation of cerebrovascular tone. Although the present data provide proof-of-concept that endothelial CB1R activation induces cerebral vasorelaxation, subsequent studies using more translational models (e.g., older pigs, primates etc.) and humans are needed to validate the general applicability of these findings.

5.6 Conclusion

The aim of this work was to provide a basic, translational framework for understanding how CBRs influence cerebral vasomotor control. The data demonstrated that CB1R activation induces a cerebral artery vasorelaxation in swine. More specifically, the CB1R mediates vasorelaxation in swine pial arteries primarily through endothelial NO with possible smaller contributions from prostaglandins and EDHF. These data suggest exogenously consumed and endogenously produced cannabinoids as well as other associated pharmacological derivatives may influence cerebrovascular function though the endothelial CB1R. Future work using more comprehensive methodologies and performed in more translationally relevant models is warranted to elucidate the role of CBR signaling on cerebrovascular function in health and disease.

REFERENCES

1. Rotermann M. What has changed since cannabis was legalized? *Heal Reports*. 2020;31(2):11-20. doi:10.25318/82-003-x202000200002-eng
2. Page RL, Allen LA, Kloner RA, et al. Medical marijuana, recreational cannabis, and cardiovascular health: A scientific statement from the american heart association. *Circulation*. 2020:E131-E152.
3. Aizpurua-Olaizola O, Soydaner U, Öztürk E, et al. Evolution of the Cannabinoid and Terpene Content during the Growth of Cannabis sativa Plants from Different Chemotypes. *J Nat Prod*. 2016;79(2):324-331. doi:10.1021/acs.jnatprod.5b00949
4. Begg M, Pacher P, Bátkai S, et al. Evidence for novel cannabinoid receptors. *Pharmacol Ther*. 2005;106(2):133-145. doi:10.1016/j.pharmthera.2004.11.005
5. Ligresti A, De Petrocellis L, Di Marzo V. From phytocannabinoids to cannabinoid receptors and endocannabinoids: Pleiotropic physiological and pathological roles through complex pharmacology. *Physiol Rev*. 2016;96(4):1593-1659. doi:10.1152/physrev.00002.2016
6. Pertwee RG, Howlett AC, Abood ME, et al. Cannabinoid Receptors and Their Ligands : Beyond CB 1 and CB 2. *Pharmacol Rev*. 2010;62(4):588-631. doi:10.1124/pr.110.003004.588
7. Jourdan T, Park JK, Varga Z V., et al. Cannabinoid-1 receptor deletion in podocytes mitigates both glomerular and tubular dysfunction in a mouse model of diabetic nephropathy. *Diabetes, Obes Metab*. 2018;20(3):698-708. doi:10.1111/dom.13150
8. Iring A, Ruisanchez É, Leszl-Ishiguro M, et al. Role of Endocannabinoids and Cannabinoid-1 Receptors in Cerebrocortical Blood Flow Regulation. *PLoS One*. 2013;8(1). doi:10.1371/journal.pone.0053390
9. Bátkai S, Pacher P, Osei-Hyiaman D, et al. Endocannabinoids acting at cannabinoid-1 receptors regulate cardiovascular function in hypertension. *Circulation*. 2004;110(14):1996-2002. doi:10.1161/01.CIR.0000143230.23252.D2
10. Pacher P, Steffens S, Haskó G, Schindler TH, Kunos G. Cardiovascular effects of marijuana and synthetic cannabinoids: The good, the bad, and the ugly. *Nat Rev Cardiol*.

2018;15(3):151-166. doi:10.1038/nrcardio.2017.130

11. Tortora GJ, Derrickson B. *Principles of Anatomy and Physiology*. 12th ed. (Tortora G, Derrickson B, eds.). John Wiley & Sons, Inc.; 2009.
12. Touyz RM, Delles C. *Textbook of Vascular Medicine*.; 2019.
13. O'Sullivan SE, Kendall DA, Randall MD. Further characterization of the time-dependent vascular effects of Δ9-tetrahydrocannabinol. *J Pharmacol Exp Ther*. 2006;317(1):428-438. doi:10.1124/jpet.105.095828
14. Stanley CP, Hind WH, Tufarelli C, O'Sullivan SE. Cannabidiol causes endothelium-dependent vasorelaxation of human mesenteric arteries via CB1 activation. *Cardiovasc Res*. 2015;107(4):568-578. doi:10.1093/cvr/cvv179
15. O'Sullivan SE, Kendall DA, Randall MD. Heterogeneity in the mechanisms of vasorelaxation to anandamide in resistance and conduit rat mesenteric arteries. *Br J Pharmacol*. 2004;142(3):435-442. doi:10.1038/sj.bjp.0705810
16. O'Sullivan SE, Kendall DA, Randall MD. The effects of Δ 9-tetrahydrocannabinol in rat mesenteric vasculature, and its interactions with the endocannabinoid anandamide. *Br J Pharmacol*. 2005;145(4):514-526. doi:10.1038/sj.bjp.0706218
17. Lassen NA. Normal average value of cerebral blood flow in younger adults is 50 ml/100 g/min. *J Cereb Blood Flow Metab*. 1985;5(3):347-349. doi:10.1038/jcbfm.1985.48
18. Andreone BJ, Lacoste B, Gu C. Neuronal and Vascular Interactions. *Annu Rev Neurosci*. 2015;38(March):25-46. doi:10.1146/annurev-neuro-071714-033835
19. Cipolla MJ. *The Cerebral Circulation*. Vol 50. Morgan & Claypool Publishers; 2016. doi:10.1136/pgmj.50.587.566
20. D'Antoni AV. *Gray's Anatomy, the Anatomical Basis of Clinical Practice*. Forty-Firs. (Standring S, ed.). Elsevier Limited; 2016.
21. Pires PW, Dabertrand F, Earley S. Isolation and cannulation of cerebral parenchymal arterioles. *J Vis Exp*. 2016;2016(111):1-10. doi:10.3791/53835
22. Faraci FM. Regulation of the cerebral circulation by endothelium. *Pharmacol Ther*. 1992;56(1):1-22. doi:10.1016/0163-7258(92)90035-X
23. Faraci FM. Role of endothelium-derived relaxing factor in cerebral circulation: Large arteries vs. microcirculation. *Am J Physiol - Hear Circ Physiol*. 1991;261(4 30-4). doi:10.1152/ajpheart.1991.261.4.h1038

24. Faraci FM. nNOS-containing perivascular nerves: Stranger by the minute. *Circ Res*. 2002;91(1):7-8. doi:10.1161/01.RES.0000026649.69203.0A
25. Lacza Z, Puskar M, Kis B, Perciaccante J V., Miller AW, Busija DW. Hydrogen peroxide acts as an EDHF in the piglet pial vasculature in response to bradykinin. *Am J Physiol - Hear Circ Physiol*. 2002;283(1 52-1):406-411. doi:10.1152/ajpheart.00007.2002
26. Harold Laughlin M, Davis MJ, Secher NH, et al. Peripheral circulation. *Compr Physiol*. 2012;2(1):321-447. doi:10.1002/cphy.c100048
27. Hill M, Davis M. *Local Control of Microvascular Perfusion*. Vol 4.; 2012. doi:10.4199/c00061ed1v01y201206isp035
28. Stromberg DD, Fox JR. Pressures in the pial arterial microcirculation of the cat during changes in systemic arterial blood pressure. *Circ Res*. 1972;31(2):229-239. doi:10.1161/01.RES.31.2.229
29. Claassen JAHR, Thijssen DHJ, Panerai RB, Faraci FM. Regulation of Cerebral Blood Flow in Humans: Physiology and Clinical Implications of Autoregulation. *Physiol Rev*. 2021.
30. Silverman A, Petersen NH. Physiology , Cerebral Autoregulation.
31. Bayliss WM. On the local reactions of the arterial wall to changes of internal pressure. *J Physiol*. 1902;3(28):220-231. doi:10.1113/jphysiol.1902.sp000911.
32. Roy CS, Sherrington CS. On the Regulation of the Blood-supply of the Brain. *J Physiol*. 1890;11(1-2):85-158. doi:10.1113/jphysiol.1890.sp000321
33. Liu P, De Vis JB, Lu H. Cerebrovascular reactivity (CVR) MRI with CO2 challenge: A technical review. *Neuroimage*. 2019;187(February 2018):104-115. doi:10.1016/j.neuroimage.2018.03.047
34. Farkas E, Luiten PGM. *Cerebral Microvascular Pathology in Aging and Alzheimer's Disease*. Vol 64.; 2001. doi:10.1016/S0301-0082(00)00068-X
35. Iadecola C. The Neurovascular Unit Coming of Age: A Journey through Neurovascular Coupling in Health and Disease. *Neuron*. 2017;96(1):17-42. doi:10.1016/j.neuron.2017.07.030
36. Hamel E. Perivascular nerves and the regulation of cerebrovascular tone. *J Appl Physiol*. 2006;100(3):1059-1064. doi:10.1152/japplphysiol.00954.2005
37. Edvinsson L. *Perivascular Neurotransmitter Regulation of Cerebral Blood Flow*. Second

Edi. Elsevier; 2017. http://dx.doi.org/10.1016/B978-0-12-803058-5.00013-8.

38. Edvinsson L, Owman C, Siesjö B. Physiological role of cerebrovascular sympathetic nerves in the autoregulation of cerebral blood flow. *Brain Res*. 1976;117(3):519-523. doi:10.1016/0006-8993(76)90760-5
39. Edvinsson L. Characterization of the contractile effect of neuropeptide Y in feline cerebral arteries. *Acta Physiol Scand*. 1985;125(1):33-41. doi:10.1111/j.1748-1716.1985.tb07690.x
40. Edvinsson L, Owman C. Pharmacological characterization of adrenergic alpha and beta receptors mediating the vasomotor responses of cerebral arteries in vitro. *Circ Res*. 1974;35(6):835-849. doi:10.1161/01.RES.35.6.835
41. Faraci FM, Brian JE. Nitric oxide and the cerebral circulation. *Stroke*. 1994;25(3):692-703. doi:10.1161/01.STR.25.3.692
42. Félétou M, Huang Y, Vanhoutte PM. Endothelium-mediated control of vascular tone: COX-1 and COX-2 products. *Br J Pharmacol*. 2011;3(164):894–912. doi:10.1111/j.1476-5381.2011.01276.x
43. Petersson J, Zygmunt PM, Högestätt ED. Characterization of the potassium channels involved in EDHF-mediated relaxation in cerebral arteries. *Br J Pharmacol*. 1997;120(7):1344-1350. doi:10.1038/sj.bjp.0701032
44. Furchgott RF. The 1989 Ulf von Euler Lecture. Studies on endothelium-dependent vasodilation and the endothelium-derived relaxing factor. *Acta Physiol Scand*. 1990;139(2):257-270. doi:10.1111/j.1748-1716.1990.tb08923.x
45. Furchgott RF. The discovery of endothelium-derived relaxing factor and its importance in the identification of nitric oxide. *J Am Med Assoc*. 1996;276(14):1186-1188. doi:10.1001/jama.276.14.1186
46. Martin W, Furchgott RF, Villani GM, Jothianandan D. Phosphodiesterase inhibitors induce endothelium-dependent relaxation of rat and rabbit aorta by potentiating the effects of spontaneously released endothelium-derived relaxing factor. *J Pharmacol Exp Ther*. 1986;237(2):539-547.
47. Furchgott R, Zawadski J V. The obligatory role of endothelial cells in the relaxation of atrial smooth muscle. *Nature*. 1980;288(November):373-376.
48. Martin W. Editorial: Robert F. Furchgott, Nobel laureate (1916-2009) - A personal reflection. *Br J Pharmacol*. 2009;158(3):633-637. doi:10.1111/j.1476-5381.2009.00418.x

49. Förstermann U, Sessa WC. Nitric oxide synthases: Regulation and function. *Eur Heart J*. 2012;33(7):829-837. doi:10.1093/eurheartj/ehr304
50. Ungvari Z, Sun D, Huang A, Kaley G, Koller A. Role of endothelial [Ca2+]i in activation of eNOS in pressurized arterioles by agonists and wall shear stress. *Am J Physiol - Hear Circ Physiol*. 2001;281(2 50-2):606-612. doi:10.1152/ajpheart.2001.281.2.h606
51. Knot HJ, Lounsbury KM, Brayden JE, Nelson MT. Gender differences in coronary artery diameter reflect changes in both endothelial Ca2+ and ecNOS activity. *Am J Physiol - Hear Circ Physiol*. 1999;276(3 45-3):12-14. doi:10.1152/ajpheart.1999.276.3.h961
52. Falcone JC, Kuo L, Meininger GA. Endothelial cell calcium increases during flow-induced dilation in isolated arterioles. *Am J Physiol - Hear Circ Physiol*. 1993;264(2 33-2). doi:10.1152/ajpheart.1993.264.2.h653
53. Muller JM, Davis MJ, Kuo L, Chilian WM. Changes in coronary endothelial cell Ca2+ concentration during shear stress- and agonist-induced vasodilation. *Am J Physiol - Hear Circ Physiol*. 1999;276(5 45-5):1706-1714. doi:10.1152/ajpheart.1999.276.5.h1706
54. Elhusseiny A, Cohen Z, Olivier A, Stanimirović DB, Hamel E. Functional acetylcholine muscarinic receptor subtypes in human brain microcirculation: Identification and cellular localization. *J Cereb Blood Flow Metab*. 1999;19(7):794-802. doi:10.1097/00004647-199907000-00010
55. Kandel ER, Schwartz JH, Jessell TM. *Principles of Neural Science, Fourth Addition.*; 2000.
56. Ignarro LJ, Buga GM, Wei LH, Bauer PM, Wu G, Del Soldato P. Role of the arginine-nitric oxide pathway in the regulation of vascular smooth muscle cell proliferation. *Proc Natl Acad Sci U S A*. 2001;98(7):4202-4208. doi:10.1073/pnas.071054698
57. Kim P, Schini VB, Sundt TM, Vanhoutte PM. Reduced production of cGMP underlies the loss of endothelium-dependent relaxations in the canine basilar artery after subarachnoid hemorrhage. *Circ Res*. 1992;70(2):248-256. doi:10.1161/01.RES.70.2.248
58. Sugawa M, Koide T, Takato M. BY-1949 elicits vasodilatation via preferential elevation of cyclic GMP levels within the cerebral artery: possible involvement of endothelium-mediated mechanisms. *Eur J Pharmacol*. 1992;215(1):57-62. doi:10.1016/0014-2999(92)90608-7
59. Trezise DJ, Drew GM, Weston AH. Analysis of the depressant effect of the endothelium

on contractions of rabbit isolated basilar artery to 5-hydroxytryptamine. *Br J Pharmacol*. 1992;106(3):587-592. doi:10.1111/j.1476-5381.1992.tb14380.x

60. Bauknight CG, Faraci FM, Heistad DD. Endothelium-derived relaxing factor modulates noradrenergic constriction of cerebral arterioles in rabbits. *Stroke*. 1992;23(10):1522-1525. doi:10.1161/01.STR.23.10.1522
61. Goldblatt MW. Properties of human seminal plasma. 1935:208-218.
62. von Euler US. Über die Spezifische Blutdrucksenkende Substanz des Menschlichen Prostata- und Samenblasensekretes. *Klin Wochenschr*. 1935;14(33):1182-1183. doi:10.1007/BF01778029
63. Bergström S, Danielsson H, Samuelsson B. The enzymatic formation of prostaglandin E2 from arachidonic acid prostaglandins and related factors 32. *BBA - Gen Subj*. 1964;90(1):207-210. doi:10.1016/0304-4165(64)90145-X
64. Bergstroem S, Ryhage R, Samuelsson B, Sjoevall J. Prostaglandins and Related Factors. The Structures of Prostaglandin E1, F1 AND F1B. *J Biol Chem*. 1963;238(11):3555-3564. doi:10.1016/S0021-9258(19)75307-1
65. Bergström S, Sjövall J, Heath RG, Varde E, Westin G. The Isolation of Prostaglandin F from Sheep Prostate Glands. *Acta Chem Scand*. 1960;14:1693-1700. doi:10.3891/acta.chem.scand.14-1693
66. Best LC, Martin TJ, Russell RGG, Preston FE. Prostacyclin increases cyclic AMP levels and adenylate cyclase activity in platelets. *Nature*. 1977.
67. Fitzpatrick F. Cyclooxygenase Enzymes: Regulation and Function. *Curr Pharm Des*. 2005;10(6):577-588. doi:10.2174/1381612043453144
68. Woodward DF, Jones RL, Narumiya S. International union of basic and clinical pharmacology. LXXXIII: Classification of prostanoid receptors, updating 15 years of progress. *Pharmacol Rev*. 2011;63(3):471-538. doi:10.1124/pr.110.003517
69. Kellogg DL, Zhao JL, Coey U, Green J V. Acetylcholine-induced vasodilation is mediated by nitric oxide and prostaglandins in human skin. *J Appl Physiol*. 2005;98(2):629-632. doi:10.1152/japplphysiol.00728.2004
70. Wong MSK, Vanhoutte PM. COX-mediated endothelium-dependent contractions: From the past to recent discoveries. *Acta Pharmacol Sin*. 2010;31(9):1095-1102. doi:10.1038/aps.2010.127

71. Ohlsson A, Bottu J, Govan J, Ryan ML, Fong K, Myhr T. Effect of indomethacin on cerebral blood flow velocities in very low birth weight neonates with a patent ductus arteriosus. *Dev Pharmacol Ther*. 1993;20(1-2):100-106. doi:10.1159/000457546
72. Bevan RD, Vijayakumaran E, Gentry A, Wellman T, Bevan JA. Intrinsic tone of cerebral artery segments of human infants between 23 weeks of gestation and term. *Pediatr Res*. 1998;43(1):20-27. doi:10.1203/00006450-199801000-00004
73. Mirro R, Pharris LJ, Armstead WM, Shibata M, Leffler CW. Effects of indomethacin on newborn pig pial arteriolar responses to PCO2. *J Appl Physiol*. 1993;75(3):1300-1305. doi:10.1152/jappl.1993.75.3.1300
74. Chen G, Suzuki H, Weston AH. Acetylcholine releases endothelium-derived hyperpolarizing factor and EDRF from rat blood vessels. *Br J Pharmacol*. 1988;95(4):1165-1174. doi:10.1111/j.1476-5381.1988.tb11752.x
75. Cipolla MJ, Smith J, Kohlmeyer MM, Godfrey JA. SK Ca and IK Ca channels, myogenic tone, and vasodilator responses in middle cerebral arteries and parenchymal arterioles effect of ischemia and reperfusion. *Stroke*. 2009;40(4):1451-1457. doi:10.1161/STROKEAHA.108.535435
76. Golding EM, Marrelli SP, You J, Bryan RM. Endothelium-derived hyperpolarizing factor in the brain: A new regulator of cerebral blood flow? *Stroke*. 2002;33(3):661-663. doi:10.1161/str.33.3.661
77. Inokuchi K, Hirooka Y, Shimokawa H, et al. Role of Endothelium-Derived Hyperpolarizing Factor in Human Forearm Circulation. *Hypertension*. 2003;42(5):919-924. doi:10.1161/01.HYP.0000097548.92665.16
78. Devane WA, Dysarz FA, Johnson MR, Melvin LS, Howlett AC. Determination and characterization of a cannabinoid receptor in rat brain. *Mol Pharmacol*. 1988.
79. Mechoulam R, Ben-Shabat S, Hanus L, et al. Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem Pharmacol*. 1995;50(1):83-90. doi:10.1016/0006-2952(95)00109-d
80. Haspula D, Clark MA. Cannabinoid receptors: An update on cell signaling, pathophysiological roles and therapeutic opportunities in neurological, cardiovascular, and inflammatory diseases. *Int J Mol Sci*. 2020;21(20):1-65. doi:10.3390/ijms21207693
81. Glass M, Felder CC. Concurrent stimulation of cannabinoid CB1 and dopamine D2

receptors augments cAMP accumulation in striatal neurons: Evidence for a G(s) linkage to the CB1 receptor. *J Neurosci*. 1997;17(14):5327-5333. doi:10.1523/jneurosci.17-14-05327.1997

82. Herkenham M, Lynn AB, Johnson MR, Melvin LS, de Costa BR, Rice KC. Characterization and localization of cannabinoid receptors in rat brain: a quantitative in vitro autoradiographic study. *J Neurosci*. 1991;11(2):563-583. doi:10.1523/JNEUROSCI.11-02-00563.1991
83. Maejima T, Hashimoto K, Yoshida T, Aiba A, Kano M. Presynaptic inhibition caused by retrograde signal from metabotropic glutamate to cannabinoid receptors. *Neuron*. 2001;31(3):463-475. doi:10.1016/S0896-6273(01)00375-0
84. Kim J, Isokawa M, Ledent C, Alger BE. Activation of muscarinic acetylcholine receptors enhances the release of endogenous cannabinoids in the hippocampus. *J Neurosci*. 2002;22(23):10182-10191. doi:10.1523/jneurosci.22-23-10182.2002
85. Kano M, Ohno-Shosaku T, Hashimotodani Y, Uchigashima M, Watanabe M. Endocannabinoid-mediated control of synaptic transmission. *Physiol Rev*. 2009;89(1):309-380. doi:10.1152/physrev.00019.2008
86. Rademacher DJ, Patel S, Ho WSV, et al. U-46619 but not serotonin increases endocannabinoid content in middle cerebral artery: Evidence for functional relevance. *Am J Physiol - Hear Circ Physiol*. 2005;288(6 57-6):2694-2701. doi:10.1152/ajpheart.00978.2004
87. Ellis EF, Moore SF, Willoughby KA. Anandamide and Δ9-THC dilation of cerebral arterioles is blocked by indomethacin. *Am J Physiol - Hear Circ Physiol*. 1995;269(6 38-6). doi:10.1152/ajpheart.1995.269.6.h1859
88. Devane WA, Hanus L, Breuer A, et al. Isolation and Structure of a Brain Constituent That Binds to the Cannabinoid Receptor. *Science (80-)*. 1992;258:1946-1949. doi:10.1126/science.1470919
89. Stanley C, O'Sullivan SE. Vascular targets for cannabinoids: Animal and human studies. *Br J Pharmacol*. 2014;171(6):1361-1378. doi:10.1111/bph.12560
90. Chen Y, McCarron RM, Ohara Y, et al. 2-Arachidonoglycerol (Endocannabinoid) Interacts With Endothelin-1. *Circ Res*. 2000;87(4):323-327. doi:10.1161/01.res.87.4.323
91. Fimiani C, Mattocks D, Cavani F, et al. Morphine and anandamide stimulate intracellular

calcium transients in human arterial endothelial cells: Coupling to nitric oxide release. *Cell Signal*. 1999;11(3):189-193. doi:10.1016/S0898-6568(98)00060-6

92. Stanley CP, Hind WH, Tufarelli C, O'Sullivan SE. The endocannabinoid anandamide causes endothelium-dependent vasorelaxation in human mesenteric arteries. *Pharmacol Res*. 2016;113:356-363. doi:10.1016/j.phrs.2016.08.028
93. Burstein S, Hunter SA. Prostaglandins and cannabis-VI. Release of arachidonic acid from HeLa cells by δ1-tetrahydrocannabinol and other cannabinoids. *Biochem Pharmacol*. 1978;27(8):1275-1280. doi:10.1016/0006-2952(78)90463-X
94. Chen P, Hu S, Yao J, Moore SA, Spector AA, Fang X. Induction of cyclooxygenase-2 by anandamide in cerebral microvascular endothelium. *Microvasc Res*. 2005;69(1-2):28-35. doi:10.1016/j.mvr.2005.02.001
95. Mcallister SD, Griffin G, Satin LS, Abood ME. Cannabinoid receptors can activate and inhibit G protein-coupled inwardly rectifying potassium channels in a Xenopus oocyte expression system. *J Pharmacol Exp Ther*. 1999;291(2):618-626.
96. Mackie K, Lai Y, Westenbroek R, Mitchell R. Cannabinoids activate an inwardly rectifying potassium conductance and inhibit Q-type calcium currents in AtT20 cells transfected with rat brain cannabinoid receptor. *J Neurosci*. 1995;15(10):6552-6561. doi:10.1523/jneurosci.15-10-06552.1995
97. Bondarenko AI. Endothelial atypical cannabinoid receptor: Do we have enough evidence? *Br J Pharmacol*. 2014;171(24):5573-5588. doi:10.1111/bph.12866
98. O'Sullivan SE, Tarling EJ, Bennett AJ, Kendall DA, Randall MD. Novel time-dependent vascular actions of Δ9- tetrahydrocannabinol mediated by peroxisome proliferator-activated receptor gamma. *Biochem Biophys Res Commun*. 2005;337(3):824-831. doi:10.1016/j.bbrc.2005.09.121
99. Zygmunt PM, Petersson J, Andersson DA, et al. Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature*. 1999;400(6743):452-457. doi:10.1038/22761
100. Laprairie RB, Kelly MEM, Denovan-Wright EM. The dynamic nature of type 1 cannabinoid receptor CB1 transcription. *Br J Pharmacol*. 2012;167(8):1583-1595. doi:10.1111/j.1476-5381.2012.02175.x
101. Cooper ZD, Craft RM. Sex-Dependent Effects of Cannabis and Cannabinoids: A

Translational Perspective. *Neuropsychopharmacology*. 2018;43(1):34-51. doi:10.1038/npp.2017.140

102. Bevan JA, Duckworth J, Laher I, Oriowo MA, McPherson GA, Bevan RD. Sympathetic control of cerebral arteries: specialization in receptor type, reserve, affinity, and distribution. *FASEB J Off Publ Fed Am Soc Exp Biol*. 1987;1(3):193-198. doi:10.1096/fasebj.1.3.2887477
103. Imai H, Konno K, Nakamura M, et al. A new model of focal cerebral ischemia in the miniature pig. *J Neurosurg*. 2006;104(2 Suppl):123-132. doi:10.3171/ped.2006.104.2.123
104. Nonaka H, Akima M, Hatori T, Nagayama T, Zhang Z, Ihara F. Microvasculature of the human cerebral white matter: Arteries of the deep white matter. *Neuropathology*. 2003;23(2):111-118. doi:10.1046/j.1440-1789.2003.00486.x
105. Járai Z, Wagner JA, Varga K, et al. Cannabinoid-induced mesenteric vasodilation through an endothelial site distinct from CB1 or CB2 receptors. *Proc Natl Acad Sci U S A*. 1999;96(24):14136-14141. doi:10.1073/pnas.96.24.14136
106. Baranowska-Kuczko M, Kozłowska H, Kloza M, et al. Vasodilatory effects of cannabidiol in human pulmonary and rat small mesenteric arteries: Modification by hypertension and the potential pharmacological opportunities. *J Hypertens*. 2020;38(5):896-911. doi:10.1097/HJH.0000000000002333
107. Breyne J, Van De Voorde J, Vanheel B. Characterization of the vasorelaxation to methanandamide in rat gastric arteries. *Can J Physiol Pharmacol*. 2006;84(11):1121-1132. doi:10.1139/Y06-058
108. Abood M, Alexander SPH, Barth F, et al. Cannabinoid receptors (version 2019.4) in the IUPHAR/BPS Guide to Pharmacology Database. *IUPHAR/BPS Guid to Pharmacol CITE*. 2019;2019(4 SE-Summaries). doi:10.2218/gtopdb/F13/2019.4
109. Ingram DG, Newcomer SC, Price EM, Eklund KE, McAllister RM, Laughlin MH. Chronic nitric oxide synthase inhibition blunts endothelium-dependent function of conduit coronary arteries, not arterioles. *Am J Physiol - Hear Circ Physiol*. 2007;292(6):2798-2808. doi:10.1152/ajpheart.00899.2006
110. Lakens D. Calculating and reporting effect sizes to facilitate cumulative science: A practical primer for t-tests and ANOVAs. *Front Psychol*. 2013;4(NOV):1-12. doi:10.3389/fpsyg.2013.00863

111. Benyó Z, Ruisanchez É, Leszl-Ishiguro M, Sándor P, Pacher P. Endocannabinoids in cerebrovascular regulation. *Am J Physiol - Hear Circ Physiol*. 2016;310(7):H785-H801. doi:10.1152/ajpheart.00571.2015
112. Lacroix A, Toussay X, Anenberg E, et al. COX-2-derived prostaglandin E2 produced by pyramidal neurons contributes to neurovascular coupling in the rodent cerebral cortex. *J Neurosci*. 2015;35(34):11791-11810. doi:10.1523/JNEUROSCI.0651-15.2015
113. Ho WS, Patel S, Thompson JR, Roberts CJ, Stuhr KL, Hillard CJ. Endocannabinoid modulation of hyperaemia evoked by physiologically relevant stimuli in the rat primary somatosensory cortex. *Br J Pharmacol*. 2010;160(3):736-746. doi:10.1111/j.1476-5381.2010.00772.x
114. Park L, Hochrainer K, Hattori Y, et al. Tau induces PSD95–neuronal NOS uncoupling and neurovascular dysfunction independent of neurodegeneration. *Nat Neurosci*. 2020;23(9):1079-1089. doi:10.1038/s41593-020-0686-7
115. Kalla A, Krishnamoorthy PM, Gopalakrishnan A, Figueredo VM. Cannabis use predicts risks of heart failure and cerebrovascular accidents: Results from the National Inpatient Sample. *J Cardiovasc Med*. 2018;19(9):480-484. doi:10.2459/JCM.0000000000000681
116. Hemachandra D, McKetin R, Cherbuin N, Anstey KJ. Heavy cannabis users at elevated risk of stroke: Evidence from a general population survey. *Aust N Z J Public Health*. 2016;40(3):226-230. doi:10.1111/1753-6405.12477
117. Cosentino F, Volpe M. Hypertension, stroke, and endothelium. *Curr Hypertens Rep*. 2005;7(1):68-71. doi:10.1007/s11906-005-0057-5
118. Mukhopadhyay P, Pan H, Rajesh M, et al. CB 1 cannabinoid receptors promote oxidative/nitrosative stress, inflammation and cell death in a murine nephropathy model. *Br J Pharmacol*. 2010;160(3):657-668. doi:10.1111/j.1476-5381.2010.00769.x
119. Brown J, Winterstein A. Potential Adverse Drug Events and Drug–Drug Interactions with Medical and Consumer Cannabidiol (CBD) Use. *J Clin Med*. 2019;8(7):989. doi:10.3390/jcm8070989
120. Melià-Sorolla M, Castaño C, Degregorio-Rocasolano N, et al. Relevance of porcine stroke models to bridge the gap from pre-clinical findings to clinical implementation. *Int J Mol Sci*. 2020;21(18):1-30. doi:10.3390/ijms21186568
121. Parfenova H, Massie V, Leffler CW. Developmental changes in endothelium-derived

vasorelaxant factors in cerebral circulation. *Am J Physiol - Hear Circ Physiol*. 2000;278(3 47-3):780-788. doi:10.1152/ajpheart.2000.278.3.h780