THE CANNABINOID-2 RECEPTOR AGONIST O-1966 REVERSES POSTISCHEMIC LEARNING AND MEMORY DEFICITS THROUGH ANTI-INFLAMMATORY PROCESSES

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ABSTRACT

Ischemic stroke is the third leading cause of death and the leading cause of morbidity in the United States. Cognitive deficits, specifically with respect to learning and memory, are a significant contributor to morbidity in stroke patients. Unfortunately, current treatment options must be administered within a thin therapeutic window of the initial infarct. This requirement results in less than 10% of stroke patients being eligible for treatment. There are currently no treatment options that are effective in the subacute phase of the disease and no treatments that are effective in reversing postischemic learning and memory deficits. We sought to examine the potential efficacy of the anti-inflammatory Cannabinoid-2 Receptor Agonist, O-1966, in attenuating infarct expansion and reversing cognitive deficits in the subacute phase of the disease using a photothrombosis model of stroke. Additionally, we sought to characterize the inflammatory response in photothrombosis.

Mice were treated with repeated doses of O-1966 or vehicle and were sacrificed at 24 hours and 7 days to study the acute and subacute phase of the disease respectively. Learning and memory testing, immunohistochemistry, and polymerase chain reaction were used to measure the effect of O-1966 on infarct expansion, inflammatory gene expression, and cognitive function. In addition to PCR, flow cytometry was used to characterize the temporal dynamics of inflammation following photothrombosis. Our studies show that O-1966 is effective in the subacute phase in attenuating infarct expansion and proinflammatory gene expression and reversing learning and memory deficits.

To Wendy and Phil Ronca, the best mother and father in the world. And to Gloria and Richard Ronca, the best grandparents in the world.

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CHAPTER 1

STATEMENT OF SPECIFIC AIMS

Ischemic stroke is initiated by sudden, focal, hypoperfusion and progresses over several weeks to months as a result of secondary damage. This secondary damage results in death and permanent disability including focal motor deficits and cognitive impairment. Treatment is currently limited to tPa, a clot buster that must be administered within several hours after stroke and is therefore contraindicated in the vast majority of stroke patients. Thus, development of a drug effective in the subacute phase is a clinical prerogative. A major source of secondary progressive damage is inflammation, making inflammation a target for novel therapeutic drugs that are effective in the subacute phase. Achieving this goal requires the identification of modulators of secondary inflammatory damage as well as characterization of inflammatory processes in animal models. Additionally, there are currently no effective treatments that aim to improve postischemic cognitive decline. The Endocannabinoid system is a modulator of both secondary inflammatory damage as well as learning and memory processes. Activation of the Cannabinoid-2 Receptor pathway has been shown to be neuroprotective in ischemic stroke, while CB2R has not previously been studied as a modulator of learning and memory. The main hypothesis of this thesis is that CB2R activation will attenuate inflammation-mediated infarct progression and preserve learning and memory function.

The specific aims of this proposal are:

1. Characterization of the inflammatory response in a photothrombosis model of cerebral ischemia.

2. Determine whether CB2R is a modulator of inflammatory damage progression in the subacute phase of ischemic stroke and is associated with learning and memory improvement.

CHAPTER 2

MATERIALS AND METHODS

Experimental Design

O-1966 and vehicle treated control mice underwent Photothrombosis (PT) for induction of cerebral ischemia. Drug doses were administered 1 hour prior to PT, 2 days following PT, and then again on day 5. Mice were sacrificed on day 1 and day 7 post cerebral ischemia, and brain tissue at both time points was stained with Triphenyltetrazolium Chloride (TTC) for calculation of Infarct Size and then homogenized in Trizol for RT-PCR to measure expression of inflammatory genes. The mice in the 7 day group underwent autoshaping cognitive testing on day 6 to study memory acquisition and then again on day 7 to study memory retention prior to being sacrificed. In addition, these mice underwent novel object testing on day 7. Thus the effects of O-1966 treatment on infarct size, inflammatory gene expression, and memory testing could be correlated on day 1 and day 7 respectively.

Cerebral Photothrombosis

9-12 wk old male C57B/6 mice were anesthetized with an intraperitoneal injection of 1:1 Ketamine (100mg/kg) : Xylazine (20mg/kg). The scalp was excised over the skull and the periosteum removed. A marker was used to identify the sensorimotor cortex 2 mm posterior and 2 mm lateral to the bregma. 0.1 ml of 10mg/ml Rose Bengal dissolved in saline was administered i.p. and five minutes later a cold light source was

placed on the skull at the sensorimotor cortex marker and was left in place for 20 minutes (Lee et al., 2007; Watson et al., 1985). Hippocampal injury was seen on the majority of samples. Body temperature was maintained at 37.0 +/- 5 degrees Celsius by a heating pad.

Cannabinoid Agonist Preparation

The CB2R agonist O-1966 was dissolved in pure ethanol : emulphor: saline mixed solution at 1:1:18. Intraperitoneal injections were administered one hour prior to Photothrombosis induction, and then again on day 2 post ischemia and day 5 post ischemia at a concentration of 5mg/kg.

Histology:

Animals were euthanized with an overdose of pentobarbital (200mg/kg i.p) 24 hours and 7 days following photothrombosis. A thoracic incision was made and the right atrium was punctured. A pump was inserted into the left ventricle and the animal was perfused with 50 ml of ice cold PBS. The brains were removed and placed in PBS on ice for 20 minutes. For volume studies the brain was sliced into 5 2mm coronal sections using a mouse brain matrix (Zivic lab). The brains were stained in 2% Triphenyltetrazolium Chloride (TTC) (Sigma, Inc) dissolved in saline for 20 minutes at 37 degrees Celsius in the dark. For surface area studies, the intact brain was sliced sagittally into hemispheres, and each hemisphere was placed in 10 ml of 2 % TTC.

Slices and hemispheres were scanned and total stroke volume, infarct fraction, and surface area were calculated using Image J software.

Memory and Learning Testing:

Novel Object Recognition Test: Mice were placed in a cage for 5 min to explore two unfamiliar objects composed of legos. One hour later one of the original objects was replaced with a new, different object. The time spent exploring the novel object was compared to the time spent exploring the original object. Novel object testing was performed on day 7 following PT.

Autoshaping Procedure: Mice were food restricted and placed in an operant chamber for 2 hours. The time required to learn and perform 10 correct food seeking behaviors through a center food receptacle hole under a variable interval schedule was determined as a measure of acquisition. Mice were returned to the chambers 24 h later and their ability to retain the operant task was measured in an identical manner to measure retention. Autoshaping testing was performed on days 6 and day 7 following PT to test memory acquisition and memory retention respectively.

Real time RT-PCR:

RT-PCR was performed on brains on day 1 and day 7 following PT. Brains were sliced sagitally into separate hemispheres and each hemisphere homogenized in 2 ml Trizol solution. RNA was then extracted from the total homogenate with chloroform

before being washed with isopropanol and ethanol and dissolved in DEPC water. Concentration of mRNA was determined by spectrophotometric means at 260nm for each sample and samples were reverse transcribed with MMLV Reverse Transcriptase. Reverse Transcribed cDNA was analyzed with the ABI System and the mRNA quantified relative to the expression levels of the housekeeping gene GAPDH. The cycling conditions were 95°C for 30 seconds, 55°C for one minute, and 72°C for 30 seconds for 40 cycles. Primers were used to calculate mRNA expression levels of TNF-α, IL-1β, MMP-2, MMP-9, TLR-4, E-selectin, IL-6, IL-10, TGF-β, FOXP3, IFN-γ, iNOS, and GAPDH with SYBR Green RT-PCR.

The Primer sequences are as follows:

TNF-α	Sense: 5'-GACCCTCACACTCAGATCATCTTCT-3'
	Antisense: 5'CCTCCACTTGGTGGTTTGCT-3'
IL-1β	Sense: 5'-CCCTGCAGCTGGAGAGTGTGGA-3'
	Antisense: 5'-TGTGCTCTGCTTGTGAGGTGCTG-3'
MMP2	Sense: 5'CGCTCAGATCCGTGGTGAG -3'
	Antisense:5' CGCCAAATAAACCGGTCCTT-3'
MMP-9	Sense: 5'-AAAACCTCCAACCTCACGGA-3'
	Antisense: 5'-GCGGTACAAGTATCCCTCTGC-3'
TLR-4	Sense: GGACCTTACCGGGCAGAAG-3'
	Antisense: ACCCCTGGAAAGGAAGGTGT-3'
E-SELECTIN	Sense: 5'-GGGGCCAGCGCAGGTTGAAT-3'
	Antisense: 5'-GCCCTGCTGTGGCGCAGATA-3'

IL-6	Sense: 5’-TCCTCTCTGCAAGAGACTTCCATCC-3’
	Antisense: 5’-GGGAAGGCCGTGGTTGTCACC-3’
iNOS	Sense: 5’-CGCAGCTGGGCTGTACAA -3’
	Antisense: 5’-TGATGTTTGCTTCGGACATCA-3’
TGF-β	Sense: 5’-GACCTGTGGAAGTGGATC -3’
	Antisense: 5’-GAAGTTGGCATGGTAGCCCTT-3’
IL-10	Sense: 5’-CCTGGTAGAAGTGATGCCCC-3’
	Antisense: 5’-TCCTTGATTTCTGGGCCATG-3’
FOXP3	Sense: 5’-AGCTGCCTACAGTGCCCCTA-3’
	Antisense: 5’-CATTTGCCAGCAGTGGGTAG-3’
IFN-γ	Sense: 5’-AGCTCATCCGAGTGGTCCAC-3’
	Antisense: 5’-GCTTCCTGAGGCTGGATTCC-3’
CB1R	Sense: 5’-GGCCAGGCTCAACGTGACTGA-3’
	Antisense: 5’-CCTTGGCTGGGCGACAGGTG-3’
CB2R	Sense: 5’-TGAATGAGAGGCACAGG-3’
	Antisense: 5’AGAGATGTTTGCTGGGTGGC-3’
GAPDH	Sense: 5’-GGAGCGAGACCCCACTAACA-3’
	Antisense: 5’-ACATACTCAGCACCGGCCTC-3’

Fluorescence-Activated Cell Sorting:

FACS was performed on days 1, 3, and 7 following PT. Brains were removed and homogenized in 5mL HBSS (w/ Mg2+ Ca2+ -- Gibco 14025) with 1mg DNAseI (Roche 10104159001) and 0.5 mg Liberase TL (Roche 05401020001). Blocking solution

was added, the samples were centrifuged, and then resuspended in 5mL of 30-37% isotonic Percoll (made of 10x HBSS (Gibco 14185), dH20, Percoll 100%) underlayed with 5mL 70% isotonic Percoll. Cells from the interphase ring were removed and washed again. The supernatant was decanted and the pellet resuspended in FLOW buffer (2% FCS, 1mM EDTA, 0.1% NaN3). Staining was performed and the solution blocked with anti-CD16/CD32 antibody. FACS analysis was then performed on the solution initially gated for CD45, CD11b, CD11c, and then CD3, and GR1.

Statistical Analysis

Student's t-test was used to analyze differences in stroke volume, infarct fraction, infarct surface area, and mRNA expression. Bonferonni's test after two way ANOVA was used for analyzing performance in novel object recognition and autoshaping tasks, and cell number over time. One way ANOVA was used to analyze mRNA expression over time. Pearson's correlation coefficient was calculated to determine relationships between infarct size and performance on memory tasks. Data were presented as means +/- SEM. A statistically significant difference was assumed at $p<0.05$.

CHAPTER 3

BACKGROUND

Introduction to Stroke

Cerebral ischemia occurs when an occlusion causes a sudden cessation or decrease of cerebral blood flow. It occurs in two variants, ischemic stroke due to vessel occlusion, and hemorrhagic stroke due to vessel wall perforation. Ischemic stroke counts for 85% of stroke cases in comparison to hemorrhage (Rosamond et al., 1999). Cerebral ischemia, or stroke, can be focal as is the case with cardiac embolism or an atherosclerotic thrombus, or global as is the case with diffuse infarcts as commonly seen in hypertensive patients. Stroke is the leading cause of morbidity and the fourth leading cause of mortality in the United States, and these figures will only increase as the population ages ("Prevalence of disabilities and associated health conditions among adults--United States, 1999," 2001; Towfighi et al., 2011).

Cognitive impairment is a significant cause of morbidity, and is experienced by 55% of dependent living patients (Tatemichi et al., 1994). Memory deficits are a significant contributor of post stroke cognitive decline, and of all patients that survive a stroke, 20-50% report memory difficulties (Nys et al., 2005; Rasquin et al., 2002; Sorensen et al., 1982; Wade et al., 1986). In addition, having a stroke increase the risk of developing dementia by two-fold (Schneider et al., 2003). Although stroke-induced deficits are highly heterogeneous in nature the brain is highly susceptible to memory and cognitive deficits in particular. The hippocampus plays an essential role in memory consolidation (Frey et al., 1997; Sun et al., 1999). Neurons that comprise this region,

specifically CA1 pyramidal neurons, are highly vulnerable to the effects of ischemia (Sun, 1999).

Despite the significant prevalence of this disease, treatment is currently limited to tissue-plasminogen activator (tPA), which has a narrow therapeutic window of 3 hours, substantial contraindications, and controversial efficacy ("A systems approach to immediate evaluation and management of hyperacute stroke. Experience at eight centers and implications for community practice and patient care. The National Institute of Neurological Disorders and Stroke (NINDS) rt-PA Stroke Study Group," 1997; "Tissue plasminogen activator for acute ischemic stroke. The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group," 1995). Indeed, less than 10% of patients that present to the emergency room with a chief complaint of stroke are eligible for the therapy, and it is only found to be effective in one-third of these patients (Reeves et al., 2005). The societal, economic, and human burden of this disease is enormous, necessitating the study and development of more effective therapeutics.

Treatment strategies for vascular cognitive decline such as that seen after ischemic stroke is limited to prevention and rehabilitation. Prevention includes an understanding of modifiable risk factors such as diet, smoking, and exercise and non-modifiable risk factors such as education level and hyperhomocysteinemia (Dichgans et al., 2012). However, antiplatelet and anticoagulation drugs such as tPa used to reduce stroke risk have shown no effect on cognitive decline. Indeed, there are currently no treatments available that selectively target cognitive symptoms in stroke patients. The development of treatments for postischemic cognitive decline depends on the study of direct and indirect effects of potential therapeutics, as well as the creation of improved

testing paradigms in preclinical studies. In addition, therapeutics such as the CB2R activator O-1066 may act as both direct and indirect effectors, as they may directly influence memory through eCS activation as well as indirectly by attenuating infarct expansion through immunosuppression. Direct effectors may include modulators of systems such as the immune and Endocannabinoid systems that play essential roles in learning, memory, and other cognitive functions. Theses effectors are stroke size independent.

Indirect effectors may act through the preservation of cognitive function through attenuation of infarct expansion. While the neural tissue in closest proximity to the occlusion will die via necrosis in the very early stages of stroke onset, the adjacent tissue, or penumbra, is still salvageable. The penumbra was formerly defined qualitatively as a region of decreased function, but with maintained structural integrity (Astrup et al., 1981). More recent, quantitative definitions characterize the penumbra as a region of decreased protein synthesis, but with preservation of ATP production (Hata et al., 2000a, 2000b; Hossmann, 2012). Over time in the absence of an intervention, this area will coalesce into the infarct. Studies using the protein synthesis/ATP mismatch definition of the penumbra have found temporal differences in this incorporation into the infarct, with the size of the penumbra initially increasing in the transient experimental model before being incorporated into the infarct while the permanent model does not show this initial increase.

Current tests used in the assessment of postischemic cognitive decline in animals include Morris water maze, radial arm maze, and passive and active avoidance testing with the first two tests examining spatial memory and the latter testing associative

learning (DeVries et al., 2001; Morris et al., 1982; M. Okada et al., 1995; Sakai et al., 1996). However, these tests have only been employed in more severe models of ischemic stroke and may lack the necessary sensitive to distinguish differences in more mild models of ischemic injury that are more representative of clinical stroke. The radial arm maze test requires a high level of motivation, while the Morris water maze test introduces high levels of anxiety in the animal which may confound performance analysis. Passive and active avoidance testing is more commonly used, namely due to the ease of interpretation of results and the consistency in deficits seen across several different labs. However, all memory tests including passive and active avoidance have failed to show a link between histologic outcome and cognitive behavioral outcome, necessitating the implementation of new testing methods (DeVries et al., 2001). Autoshaping has not been previously used to study cognitive decline following cerebral ischemia, but may offer an attractive option as it features food delivery as motivation for animals to carry out targeted behaviors.

The pathophysiology of ischemic stroke is highly complex and involves a number of interconnected mechanisms in addition to the initial focal hypoperfusion. These include but are not limited to neuroexcitotoxicity, blood brain barrier breakdown, reactive oxygen species formation, acidosis, increased intracellular calcium, and inflammation (Durukan et al., 2007).

Inflammation in Cerebral Ischemia

Inflammation has emerged as a potential target for neurotherapeutics in cerebral ischemia, as inflammation-mediated secondary injury exacerbates stroke injury (Dawson et al., 1996; Dutka et al., 1987; Dutka et al., 1988; Dutka et al., 1989; Hallenbeck et al., 1988; Hallenbeck et al., 1986; Kochanek et al., 1987; Kochanek et al., 1988; Kochanek et al., 1992). The ischemic cascade beings with vessel occlusion leading to sudden, focal cerebral hypoperfusion. This obstruction leads to changes in sheer stress, increases in reactive oxygen species formation, and decreases in nitric oxide formation which further exacerbate the low blood flow state and initiate further deleterious events such as complement system activation, platelet aggregation, and initiation of inflammatory processes (Carden et al., 2000; Eltzschig et al., 2011; Iadecola et al., 2011; Peerschke et al., 2010; Pinsky et al., 1996; Yilmaz et al., 2010). Neurons in the ischemic core most dependent on the occluded vessel will die within hours via necrosis. Dead and dying neurons will excrete ATP and other DAMPS (Damage Associated Molecular Patterns) which react with TLRs (Toll Like Receptors) and other receptors of the innate immune system on microglia and endothelial cells (Bune et al., 2010; Burnstock, 2008; Marsh et al., 2009; Melani et al., 2005; Schock et al., 2007). Microglia become activated and begin to secrete cytokines and chemokines which activate endothelial cells and lead to the proliferation and activation of other microglia. The activated microglia will transform from its more quiescent stellate shape to an amoeboid morphology more closely resembling the classical shape of peripheral macrophages.

Activated Endothelial cells begin to secrete P-selectin containing granules to the cell membrane where P-selectin will anchor peripheral leukocytes (Yilmaz & Granger,

2010). Expression of E-selectin is increased as a result of endothelial activation, and both P-selectin and E-selectin will form transient, low affinity interactions with the carbohydrate moieties of leukocyte transmembrane glycoproteins in a step commonly referred to as "rolling" (Bevilacqua, 1993; Butcher, 1991; Patarroyo et al., 1990; R. L. Zhang et al., 1996). Rolling provides time and closer proximity for additional signaling by which the rolling leukocytes trigger firm adhesion of I-CAM and V-CAM to the endothelial cell surface where they interact with integrins (e.g. LFA-1, Mac-1, VLA-4) on the surface of rolling leukocytes. Cell adhesion molecules have been demonstrated in several different animal models of stroke (Frijns et al., 2002; Haring et al., 1996; Huang et al., 2000; Jander et al., 1995; Y. Okada et al., 1994; H. Suzuki et al., 1998; H. Suzuki et al., 1997; X. Wang et al., 1995; R. Zhang et al., 1998; R. L. Zhang et al., 1995; R. L. Zhang et al., 1996). These strong interactions lead to diapedesis of leukocytes through the intercellular space of endothelial cells toward a cytokine gradient provided by activate glial cells.

Cytokines are powerful transmembrane and soluble chemical mediators of inflammation and there is varying degrees of evidence to suggest they play an important role in clinical and experimental stroke. Tumor Necrosis Factors Alpha (TNF-α), Interleukin-1Beta (IL-1β), and Interleukin 6 (IL-6) are three of the most studied cytokines in cerebral ischemia. Each of these cytokines is produced by microglia and macrophages, and IL-6 is also produced by neurons (Buttini et al., 1994; Clausen et al., 2008; Davies et al., 1999; Lambertsen et al., 2005; S. Suzuki et al., 1999; S. Suzuki et al., 2009). Indeed, these cytokines are present at low levels during normal physiologic conditions (Breder et al., 1993; Clausen et al., 2005; Gahring et al., 1996; Gregersen et al., 2000; Hillhouse et

al., 1998; Lambertsen et al., 2012; Lambertsen et al., 2002; Lambertsen et al., 2005; Vitkovic et al., 2000). Each of these cytokines begins to increase within 6 hours post injury, but do not reach a peak until between 12 and 24 hours, far past what is commonly accepted as the "therapeutic window" of 3-4.5 hours for stroke intervention with tPa (Ginsberg, 2008; Lambertsen et al., 2012). Indeed, it has been postulated that low levels of these cytokines is sufficient to modulate the immune response in cerebral ischemia if they are released near ischemic neurons and if released glutamate levels are sufficiently high to sensitize these neurons to the effects of these cytokines (Cardenas et al., 2002; Clausen et al., 2005; Pradillo et al., 2005).

TNF-α is the most studied cytokine in the setting of stroke, and controversy exists over whether TNF-α plays a more harmful or protective role in cerebral ischemia, as data exists to support both conclusions. While animal studies using TNF-α neutralizing antibodies show decreases in infarct size, TNF-α -/- KO mice show larger infarcts (Bruce et al., 1996; Gary et al., 1998; Taoufik et al., 2007). In contrast to TNF-α, experimental studies of IL-1β have almost unanimously demonstrated the ability of this molecule to exacerbate stroke injury (Lambertsen et al., 2012). IL-1β, like TNF-α, is an extremely potent cytokine, and both are produced at early time points in the ischemic cascade by microglia and act on two different receptors (IL-1R1 and IL-1R2). However, studies utilizing recombinant human anti-IL-1β antibody (rhIL-1Ra), Il-1-converting enzyme inhibitors, and Il-1 antibodies all significantly decrease infarct size in experimental stroke (Dinarello, 1994; Schielke et al., 1998; Yamasaki et al., 1995). Consistent with these studies, intracerebroventricular administration of IL-1β is detrimental (Boutin et al., 2001; Hara et al., 1997).

Far fewer studies have looked at IL-6 as a potential target for neuroprotective strategies in an experimental stroke. Although TNF-α, IL-1β, and IL-6 are all increased in the blood and cerebrospinal fluid during the acute phase of stroke in patients, IL-6 is the only cytokine to consistently demonstrate a relationship between expression levels and injury severity (Beridze et al., 2011). A recent study shows a strong correlation between IL-6 expression levels at 6 hours following stroke and infarct size at 24 hours.

There are a number of important differences and similarities in the temporal dynamics of inflammation between humans and rodents. In humans, neutrophils are the first subtype of leukocytes to enter the infracted tissue, where they secrete soluble factors such as Interleukin-6 (IL-6) and Matrix Metalloprotease-9 (MMP-9) which further amplify the inflammatory process and further breakdown the blood brain barrier to lead to enhanced leukocyte extravasation. Beginning 3 days later, monocytes begin to enter the infracted tissue where they are activated into macrophages and begin to release their own cytokines as well as phagocytize necrotic cells. This process is very different in rodents, with studies showing that monocytes, not neutrophils, are the first cells to appear in a murine filament model of transient middle cerebral artery occlusion (Gelderblom et al., 2009). Several days later, neutrophils begin to enter the tissue, and on day 5 a much smaller population of T-cells begin to invade the CNS.

While much is known about the proinflammatory factors involved in cerebral inflammation following cerebral ischemia, comparatively little attention has been paid toward the brains endogenous anti-inflammatory process by which inflammation is attenuated at its conclusion. Proinflammatory microglia, one of the major and earliest players in the process, are commonly considered to play a detrimental role in

exacerbating secondary injury following ischemia. However, the M2 anti-inflammatory phenotype of microglia is important in resolution of the inflammatory process via powerful cytokines such as TGF-β (Transforming Growth Factor-Beta) and IL-10 (Interleukin-10). Additionally, FoxP3+ CD25+ TRegs (Regulatory T cells) are theorized to play an important role in the latter stages of the disease, as studies using a FoxP3 antibody to selectively destroy Tregs resulted in significant increases in infarct sizes as far as 7 days from the induction of permanent cerebral ischemia using a transtemporal craniotomy and direct cauterization model (Liesz et al., 2009).

In addition to variance between species, there is also significant differences in the temporal and spatial dynamics depending on the model used for stroke induction. Reports on the characterization of the inflammation in the PT (photothrombosis) model of stroke are limited. Studies in mice using CD11b antibody to stain for microglia l activation demonstrated a protracted immune response in mice that underwent this model (Schroeter et al., 2002). A small number of activated microglial cells surrounded the infarct border 24 hours post ischemia with this number greatly increasing by day 7. However, activated microglia had not invaded the infarct core until the latest time point of 28 days following ischemia. It is important to note that no time point was examined between 7 and 28 days so it is unknown how early the cells had invaded. In contrast to rat tissue that had undergone photothrombosis where CD4+ and CD8+ T cells were numerous, these cells were almost nonexistent in the phothrombosed mouse brain. Very few studies have looked at the corresponding temporal dynamics of proinflammatory cytokine expression in this model. Mirroring the differences seen in the presence of leukocyte subpopulations between mouse and rat, there were differences found in the

timing and magnitude of expression of *Tnf-α*, *Il-1β*, and *inos* with rat expression being significantly greater and peaking earlier than in mice (Schroeter et al., 2003).

Introduction to the Endocannabinoid System

One endogenous system that modulates inflammation is the Endocannabinoid System (eCs), a signaling system of lipid metabolism that performs a diverse array of physiologic functions including regulation of neurotransmission, metabolic homeostasis, and the brain's stress response (Gorzalka et al., 2008; Kunos, 2007; Shen et al., 1996). It is highly conserved throughout evolution suggesting an essential role in biological systems (Elphick et al., 2001). The system is comprised of numerous endogenous cannabinoid ligands, including 2-arachidonylglyceral (2AG) and n-arachidonyl-ethanoloamine (anandamide or AEA), their membrane bound receptors, and the enzymes responsible for their degradation.

There are two known cannabinoid receptors, the cannabinoid-1 receptor (CB1R) and the cannabinoid-2 receptor (CB2R). Both types of receptors are G-protein coupled receptors that inhibit production of cAMP (Devane et al., 1988; Howlett et al., 1990). CB1R is found in a number of different organs including the testes, microcirculation, and some glandular systems but is predominantly expressed in the central nervous system specifically on presynaptic inhibitory interneurons (Batkai et al., 2001; Devane et al., 1988; Herkenham et al., 1991; Howlett et al., 1990; J. A. Wagner et al., 1997). Signaling through CB1R has an inhibitory effect at the axonal junction and is important in modulation of neural messaging. In contrast, CB2R is mainly expressed on cells of the immune system, both in the brain's own resident microglial as well as peripheral leukocytes: (in order of decreasing expression level)(B-cells > Monocytes and Neutrophils> T-cells) (Galiegue et al., 1995; Munro et al., 1993; Piomelli, 2003). Signaling through CB2R has largely been shown to have immunosuppressive properties

including downregulation of macrophage proliferation, release of cytokines, phagocytosis, neutrophil migration, and alteration of T-cell polarity toward anti-inflammatory Th2 (Ashton et al., 2007; Chuchawankul et al., 2004; Nilsson et al., 2006; Ziring et al., 2006). Several endocannabinoids are also able to act on ion channels such as vanilloid TRPV receptors, T-type calcium channels, and serotonin receptors (Akerman et al., 2004; Baker et al., 2004; Barann et al., 2002; Chemin et al., 2001; Dannert et al., 2007).

Endogenous cannabinoids are produced on demand from phospholipid precursors in response to Calcium-dependent membrane depolarization (Stella et al., 2001). AEA is produced via Phospholipase D hydrolase of N-acyl-phophatidylet hanolamines (NAPEs), which itself a product of phospholipid metabolism. 2-AG is produced in larger quantities in the brain than AEA and is itself a product of phospholipid metabolism (Stella et al., 1997; Sugiura et al., 1995). AEA has much higher affinity for CB1R than 2-AG, while 2-AG is a full agonist at the CB2R (Hillard et al., 1999; Howlett et al., 2002; Stella et al., 1997). Their levels are regulated through cellular intake mechanisms but the specific transporters of these ligands have yet to be identified (Beltramo et al., 1997).

Cannabinoid-2 Receptor and Ischemic Stroke

The anti-inflammatory properties of CB2R activation have been shown to result in neuroprotection in animal models of ischemic stroke. Zhang et al was the first to examine the potential neuroprotective effects of a selective CB2R agonist in a mouse Middle Cerebral Artery Occlusion/Reperfusion (MCAO/R) model (M. Zhang et al., 2007). Intravenous injections of 1mg/kg O-3853 and O-1966 1 hour prior to and 1 hour post 60 minute MCAO occlusion resulted in significantly decreased infarct volume at 24 hours post ischemia. These results correlated with improvement in neurological score on a 4-point scoring system. Our lab hypothesized that the anti-inflammatory properties of CB2 agonists as seen in both in vitro and in vivo models of other diseases such as Alzheimer's disease, Multiple Sclerosis, and Traumatic Brain injury may be effective in preventing the secondary inflammatory damage associated with cerebral ischemia (Croxford, 2003; Grundy et al., 2001; Molina-Holgado et al., 2002; Ni et al., 2004; Ramirez et al., 2005). Indeed, studies using intravital microscopy and a cranial window showed significant decreases in both rolling and adhesion of leukocytes on cerebral arterioles and venules as early as 1 hour post ischemia and lasting until the final time point of 24 hours (M. Zhang et al., 2009). The specific mechanisms by which rolling and adhesion was attenuated remained to be elucidated.

Further Studies by Zhang explored the effects of modulating the balance between CB1R and CB2R activation in cerebral ischemia (M. Zhang et al., 2008). Using the CB1R antagonist SR141716, the CB2R antagonist SR144528 and the CB2R agonist O-1966 alone and in combination, it was shown that CB1 antagonism and CB2 agonism alone showed significant decreases in infarct size. Administration of the CB2R

antagonist resulted in exacerbation of CNS injury and an increase in infarct size. Most striking, however, was the finding that the combination therapy of the CB1R antagonist and the CB2R agonist resulted in the largest decease in infarct size as well as a synergistic increase in regional cerebral blood flow. No other single treatment group showed any significant change in blood flow. The infarct size results corresponded with neurologic score, as the combined therapy showed the greatest improvement in neurologic function following MCAO/R.

Neutrophils have emerged as a potential suspect involved in the neuroprotective effects of CB2R agonist in cerebral ischemia. Using a craniectomy with direct MCA cauterization method to model permanent cerebral ischemia in a mouse, the CB2R selective agonist, JWH-133 significantly decreased infarct volume 2 days following surgery in a neutrophil dependent manner (Murikinati et al., 2010). JWH-133 was originally reported to have a 200 fold higher affinity for CB2R than CB1R, similar to the O-1966 and O-3853. However, this value was recently reported to be 40 times higher for CB2R. Despite the lower selective affinity, the decreases in infarct size seen with JWH-133 were abolished in CB2-/- mice but still present in CB1-/- validating the CB2 dependent mechanism. Indeed, an inverted bell shaped dose response curve was seen with a peek at a middle dose of 1mg/kg with respect to infarct size. This is consistent with previous studies, suggesting that higher doses are less effective possibly due to nonspecific effects.

The effects of JWH-133 were found to be mediated by peripheral recruitment of neutrophils, as bone marrow depleted mice reconstituted with CB2-/- leukocytes lost the beneficial effects of JWH-133 treatment. Reconstituting CB2-/- mice with wild type

bone marrow resulted in a return of the CB2R protective effect. The beneficial effects of the compound were also abolished with neutrophil ablation and FACS analysis of infarcted brain tissue demonstrated significant decreases in GFP-labeled neutrophils with JWH-133 treatment. In vitro studies demonstrated significant decreases in CXCL-2 mediated chemotaxis, operating through a p38 dependent mechanism, but treatment did not result in decreases in the expression level of the ligand of CXCL-2, CXCR2 or fMLP mediated chemotaxis.

The first evidence of the effect of CB2R agonist on the brain's own resident immune cells, microglia, was discovered by Zarruk et al (Zarruk et al., 2012). Using JW-133 they demonstrated a CB2R-dependent decrease in infarct size and neurologic impairment at 48 hours post ischemia when 1.5 mg/kg was administered i.p 10 minutes or 3 hours following occlusion. Interestingly, these results corresponded with decreases in IBA-1 positive cells and activated microglia morphology. Furthermore, JW-133 administration resulted in decreases in the expression of several markers of classical pro-inflammatory microglial activation including IL-6, IL-12/Il-23p40, MCP-1, MIP-1a, RANTES and iNOS. TNF-α, Il-1β expression was not decreased, nor was the expression of Myeloperoxidase (MPO) which conflicts with the results obtained by Murikinati. In addition, no change was seen in neutrophil invasion upon staining. The author postulates that the reason for the inhibition of microglial activation and processes without an effect on neutrophils may be due to difference in the disease model used, the doses, and the time of administration. Surprisingly, alternative, anti-inflammatory markers of microglial activation were also decreased upon JWH-133 administration, including TGF-β, Tm1,

and IL-10, suggesting that rather than altering the polarity of microglia in the setting of ischemic stroke, CB2R activation reduces these cells to overall quiescent state.

Recent evidence has emerged implicating neurons as the target by which cannabinoids exert their neuroprotective properties in cerebral ischemia. The combined CB1/CB2 agonist KN38-72717 was administered to Sprague-Dawley rats that underwent transient endothelin middle cerebral artery occlusion (eMCAO) (Schmidt et al., 2012). Immunohistochemical staining demonstrated CB2R expression on neurons 24 hours following cerebral ischemia, while CB2R expression was not evident on microglia until day 3. Thus, neurons may be the target of cannabinoid activation in this model of cerebral ischemia. Consistent with several previous reports, a dose response curve demonstrated an inverted U-shaped curve with an intermediate dose providing the largest decrease in infarct size at 7 days. This study was also the first to demonstrate the efficacy of a repeated dose cannabinoid administration protocol. Several different repeated dose protocols were used, with the combined pre and post treatment protocol showing the greatest decrease in infarct size. However, the effect was preserved in mice who received no pretreatment and rather received their first dose 4 hours after ischemia, demonstrating the efficacy of cannabinoid treatment in a more clinically relevant time window. Furthermore, the decreases in infarct size were accompanied by corresponding improvement in sensorimotor behavior on a ladder rung test, and both behavioral and histologic effects were preserved as far as 21 days post ischemia.

Consistent with previous studies, the expression of CB1R and CB2R were found to decrease at early time points following ischemia, possibly due to cell death. In fact, on histologic examination only neurons in the infarcted contralateral hemispheres displayed

any positive staining for CB1R or CB2R. CB1R and CB2R were not found on astroglia, microglia, and CD45 positive leukocytes until later time point between days 3 and 5, and treatment with the CB1R agonist ACEA, the CB2R agonist JWH-133, or the combined CB1R/CB2R agonist KN38-72717 all provided significant decreases in infarct size at 24 hours post ischemia. Thus, the authors propose that the cannabinoid mediated effects observed in this study must be acting on neurons.

Learning and Memory and the Endocannabinoid System:

There is a large body of literature exploring the role of the endocannabinoid system in learning and memory, specifically focusing on the CB1R. Primarily expressed on neurons, CB1R is expressed in high numbers in the hippocampus, a brain region theorized to play an important role in storage, categorization, and recollection of memory (Marsicano et al., 1999). Depolarization-induced Suppression of Inhibition (DSI) is thought to play a key mechanistic role in memory (Alger, 2002). DSI occurs when endocannabinoids are transiently released from depolarized pyramidal neurons post-synaptically across the synaptic cleft where they bind CB1R on presynaptic interneurons (Alger, 2002; Freund et al., 2003). Activation of CB1R inhibits suppression on pyramidal neurons by presynaptic GABAnergic neurons, allowing for continued pyramidal cell depolarization. DSI has been found to induce long-term synaptic potentiation (LTP), a mechanism of cellular storage of information (Carlson et al., 2002).

Initial studies of the potential role of the eCS in learning and memory utilized CB1 agonists such as THC, finding impairment of short term and working memory formation (Ranganathan et al., 2006). However, DSI and activation of CB1R in learning and memory is thought to be under tight temporal and spatial regulation, an effect that can be lost with systemic or cerebral administration of a CB1R agonist (Marsicano et al., 2009). Doses of CB1R administered in this way, by binding CB1R, may interfere with physiologic signaling of the eCS and interfere with its efficiency. Thus, later studies moved toward the use of CB1R antagonists. Indeed, mice treated with a CB1R antagonist showed improved short-term olfactory memory in a social recognition memory task (Terranova et al., 1996). The eCS has also been implicated in working

memory function, fear extinction, and appetitive extinction (Carter et al., 2007; Kamprath et al., 2006; Marsicano et al., 2006; Marsicano et al., 2002; Ward et al., 2009).

Animal Models of Stroke

There are several different models used in the field of ischemic stroke research today, each with their own strengths and weaknesses. This is advantageous as stroke is a highly heterogeneous disease both in terms of causes, which include local thrombus formation, cardiac thromboembolism, and trauma among others, as well as symptomatology and location. Perhaps the most significant classification of stroke models is transient vs. permanent occlusion. It is postulated that transient occlusion more closely mirrors what is considered an "ideal" stroke treatment scenario, where blood flow is restored and tissue potentially salvaged either following an infarct or recanalization therapy with tPA treatment. As mentioned in the introduction, less than 10% of patients are eligible for tPA treatment, necessitating the study of permanent models of stroke as well (Reeves et al., 2005). A disadvantage of the plethora of different animal models used in stroke research is different animal subjects and disease inducting techniques can give different results, presenting a challenge in comparing and integrating data.

The most commonly used animal model for induction of ischemic stroke is the endovascular filament method (Hata et al., 1998). In this technique a focal infarct is achieved by inserting a coated suture into the external carotid artery, through the carotid bifurcation and into the internal carotid artery where it is left in place to occlude the origin of the Middle Cerebral Artery (MCA). Regional cerebral blood flow can be measured using a laser Doppler to confirm suture location and the occurrence of the infarct. Furthermore, this technique can be used to study transient infarction of a specified length of time and thus injury severity by removing the suture after a specific interval or left in place until animal sacrifice to achieve a permanent occlusion. In

addition to the clinical relevance of the model in allowing for study of reperfusion injury, the endovascular nature of the surgery limits non-ischemic injury to the animal during the surgery which could potentially confound results.

There are a number of disadvantages to the endovascular filament method. While infarct size is relatively uniform after a 60 minute occlusion, the technique is inconsistent at earlier time points such as 30 minutes of occlusion (Belayev et al., 1999). The significance of this is two-fold. The inability to consistently produce the smaller infarcts achieved with shorter occlusion times results in only large infarcts consistenty resulting in very high mortality. This prevents the study of infarct progression over time as well as the pathophysiology of the infarct expansion and cognitive decline in the subacute phase. In addition, the infarcts produced in this technique are significantly larger than the average size ischemic infarct in patients as most patients as the average human infarct occupies 4.5-15% of the brain which is significantly smaller than that achieved with this model (Brott et al., 1989; Carmichael, 2005; Lyden et al., 1994; Nopoulos et al., 2000; Sowell et al., 2003). These large infarcts also result in significant motor deficits and morbidity, resulting in mice that are too sick to perform behavioral testing.

The second most common technique for focal ischemia induction is the Tamura method (Tamura et al., 1981; Welsh et al., 1987). This technique is more invasive than the previously listed method and involves a transtemporal craniotomy and then direct cauterization of the MCA on the exposed brain surface. Cauterization can be achieved at different levels of the MCA to control stroke severity and the corresponding symptoms and infarct size. The craniotomy itself can lead to unintended injury during the surgery and requires a high level of surgical skill. However, the craniotomy potentially alleviates

a lot of edema associated injury, leading to lower mortality among animals. The technique induces infarcts of low size variability, and modifications using clamps can be applied to achieve transient infarction (Panahian, 2001).

Cerebral Photothrombosis (PT) is another noninvasive technique in which a photoreactive dye such as Rose Bengal is injected ip (Lee et al., 2007; Watson et al., 1985). A skin incision is then performed over the skull in the desired location with removal of the periosteum. A cold light source is then used to illuminate the skull resulting in activation of the dye and formation of hydrogen peroxide. The resulting formation of hydrogen peroxide causes severe acute endothelial damage leading to vessel occlusion, platelet aggregation, and blood brain barrier (BBB) breakdown, all features of cerebral ischemia (Dietrich et al., 1987; Dietrich et al., 1986; Wilson et al., 1991). The chief advantages of this technique are its ease of application allowing for high throughput experimentation, its statistical rigidity, and the low mortality of animals. There is some controversy about the technique which has limited its use. While platelet aggregation has been observed, evidence of complete occlusion in the cerebral vasculature is lacking in the literature, although it has been seen when PT has been used in the vasculature of other vessel beds (Frederix et al., 2007; Furie et al., 2005; D. D. Wagner, 1993). Our lab has recently reported the observation of complete pial vessel occlusion in cerebral PT through intravital microscopy, suggesting that light intensity may be a factor. Questions have also arisen about the clinical relevancy of this model after cell adhesion molecules crucial for inflammation such as Vonwillebrand Factor, Glycoprotein-1bα, beta-3 integrin and P-selectin KO mice failed to show a reduction in infarct size compared to wild-type controls in this model (Frederix et al., 2007). In addition, PT does not appear to be

platelet-dependent as both platelet depletion via anti-GPIbα antibodies and receptor blockade with GPIIb/IIIa- $F(ab)_2$ fragments did not affect outcome (Kleinschnitz et al., 2007). Additionally, FXII (Factor XII) KO mice did not have different outcomes from wild type controls (Kleinschnitz et al., 2006). This result is different than that seen in the same experiment applied to mice that underwent the endovascular filament method suggesting a lack of dependency on platelet aggregation (Kleinschnitz et al., 2007). In addition, E-selectin may perform a similar functional role when P-selectin is absent. Indeed, our results show a significant increase in the expression of *E-selectin* after PT injury.

Inflammation and Learning and Memory

The immune system plays an important role in learning and memory under physiologic conditions in the absence of a triggered inflammatory response. These effects begin during embryogenesis with neuronal pruning and neurogenesis and persist at lower levels through adulthood. There is a large body of evidence that suggests a role for specific leukocytes, such as microglia and T-cells, and proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6 in modulating neurogenesis, hippocampal LTP, neuroplasticity, learning, and memory (Yirmiya et al., 2011).

In the setting of strong inflammatory responses, such as seen in infection or with chronic degenerating diseases, the delicate balance of the immune system becomes dysregulated, and damages many of these processes (Yirmiya et al., 2011). High levels of IL-6 are found in the CSF of Alzheimer's dementia patient. Serum levels of IL-6 corresponded with poorer performance on memory tests in patients with multiple sclerosis and diabetes mellitus type II (Akiyama et al., 2000; Marioni et al., 2010; Patanella et al., 2010; Shaftel et al., 2008). Furthermore, high levels of IL-6 have been associated with inhibition of neurogenesis, LTP, and neuronal differentiation (Bellinger et al., 1995; Nakanishi et al., 2007; Vallieres et al., 2002).

Inappropriately high levels of IL-1B have also been found to have a detrimental effect on learning and memory. Rodents administered IL-1B experienced worsened performance in a water maze test, suggesting a cytokine-induced decreases in spatial memory (Oitzl et al., 1993). Similarly to IL-6, IL-1B has also been found to decrease neurogenesis and LTP (Bellinger et al., 1993; Goshen et al., 2008). The inhibitory effects

of IL-1B were shown to be abolished in a study of long-term memory through administration of the anti-inflammatory agent a-melanocortin (Gonzalez et al., 2009).

CHAPTER 4

RESULTS: MANUSCRIPT 1: THE CANNABINOID-2 RECEPTOR AGONIST O-1966 ATTENUATES INFARCT EXPANSION IN THE SUBACUTE PHASE IN A PHOTOTHROMBOSIS MODEL OF STROKE

Introduction:

Tissue injury in stroke progresses over a period of days to weeks following an ischemic insult as a result of various processes, including inflammation. There are currently no agents that are effective clinically in this critical subacute phase. The development of anti-inflammatory agents that act to attenuate infarct expansion in the subacute phase requires recognition of systems that may be modulated at several steps of the inflammatory process and characterization of the inflammatory response in animal models of stroke.

Drugs that modulate the Endocannabinoid Systems (eCs) have been shown act on several steps in the ischemic cascade, including attenuation of release of excitatory neurotransmitters and increasing regional blood flow (Goshen et al., 2008; M. Zhang et al., 2008). Activation of the Cannabinoid-2 system specifically results in an anti-inflammatory response and these drugs have shown benefit in animal models of secondary inflammatory mediated injury including Alzheimer's disease, multiple sclerosis, spinal cord injury, traumatic brain injury and stroke (Croxford, 2003; Grundy et al., 2001; Molina-Holgado et al., 2002; Ni et al., 2004; Ramirez et al., 2005; M. Zhang et al., 2007). However, the effect of anti-inflammatory CB2R agonists has only been demonstrated in the early acute phase of cerebral ischemia. A better understanding of the

effects of this drug in the subacute phase is of great clinical importance. The acute phase in clinical stroke is the first 48 hours following the initial ischemic insult and the subacute phase consists of day 3 to day 10 post ischemia. It must be considered that the average lifespan of a mouse is 6 years and thus the subacute phase defined in the rodent is proportionately greater than in a human. We sought to examine the effects of the CB2R selective agonist O-1966 in the subacute phase and to elucidate its mechanism of action in a Photothrombosis (PT) model of permanent ischemia. Inflammation plays an important role in stroke progression in the subacute phase of cerebral ischemia, and we hypothesized that the anti-inflammatory properties of O-1966 would reverse this process. Additionally we sought to characterize the inflammatory response in PT, a less severe animal model of cerebral ischemia that allows for study of inflammatory dynamics in the subacute phase.

QT-PCR Results:

Microglial Activation:

The expression of the proinflammatory cytokines *Tnf-α* $p<0.05$ vs. sham) and *Il-1β* peaked early at 1 day following ischemia, before decreasing at day 3. (Fig 1A). *Il-1β* peaked again at day 7 while *Tnf-α* only moderately increased from day 3 values. This pattern of biphasic expression seen with *Il-1β* was also seen in *Tlr-4* and Inducible Nitrous Oxide Synthase (*inos*) expression (Fig 1B), although the magnitude of expression was much lower than the former cytokines (Fig 1C). The anti-inflammatory *Il-10* was significantly decreased at 1 day following ischemia.

Endothelial Activation and Blood Brainer Integrity

E-selectin expression increased at day 1 in comparison to sham controls, before undergoing a decrease at day 3, and then peaking at 7 days where the difference from sham reached significance ($p<0.05$). Sham control mice underwent all steps of the PT procedure with the exception of illumination, and were sacrificed on day 7. Both *Mmp-2* and *Mmp-9* increased in expression over time following photothrombosis and were significantly higher than sham values at day 7. *Mmp-2* reached slightly higher levels than *Mmp-9* (Fig 1A).

Cannabinoid Receptor Expression:

Expression of the *Cb1r* remained similar to baseline levels, undergoing a slight increase at 24 hours. Sham animals were used to approximate baseline expression levels and were labeled "To." Conversely, expression of *Cb2r* was greatly decreased following photothrombosis (Fig 1B). The reduction in *Cb2r* expression corresponded with a decrease in microglia cell counts as seen with FACS and suggests it may be due to death of *Cb2r*-expression microglia following PT. Expression increased from day 1 to day 7 following ischemia, but these values remained low, never reaching baseline controls. None of these differences reached statistical significance.

T-Cell Activation:

The regulatory T cell marker *Foxp3* decreased greatly 1 day following ischemia, recovered back above baseline at day 3, and then returned to very low levels of expression at day 7 (Fig 1C). *Foxp3* expression levels were calculated to estimate the presence of anti-inflammatory regulatory T-cells. Likewise, expression levels of *Inf-γ*, a powerful proinflammatory cytokine produed by T-cells, decreased sharply at day 1, and remained very low throughout the post ischemia period (Fig 1C).

FACS Results:

Total number of microglia and leukocytes remained constant 24 hour following photothrombosis and peaked at 3 days with a 5 fold increase over the earlier time points. CD45 is a cell marker common to microglia and leukocytes and was used to quantify the number of immune cells in the brain. By day 7 the total number of leukocytes had regressed, but was still larger than in animals sacrificed at 24 hours or the sham control animals.

The pattern of changes in cell number between CD45+ microglia and invading myeloid cells underwent similar decreases in cell number at 24 hours following photothrombosis, before undergoing a dramatic increase at day 3 and peaking at day 7 (Fig 2B).

Conversely, T-cell numbers began to increase as early as 24 hours post ischemia with a large peak at day 3 ($p<0.05$ vs. sham) before regressing at day 7 back toward baseline levels. Only very small changes in myeloid dendritic cells and neutrophils were observed. Microglia made up the highest percentage of cells, followed by myeloid cells, and lastly T-cells (2C).

Infarct Size Results:

1 hour O-1966 pretreatment significantly decreased stroke volume and infarct fraction 24 hours following PT (Fig. 3A, Fig. 3B). A repeated dose regiment of 1 hour pretreatment, 2 days post ischemia, and 5 days post ischemia significantly decreased both stroke volume and infarct size 7 days following ischemia. Stroke size was also decreased at day 3 but this value did not reach significance for stroke volume or infarct fraction (Fig. 3A). O-1966 treatment on day 2 and day 5 postischemia showed no difference in stroke size compared to vehicle controls (Fig. 3C)

Figure 1: Gene Expression over Time

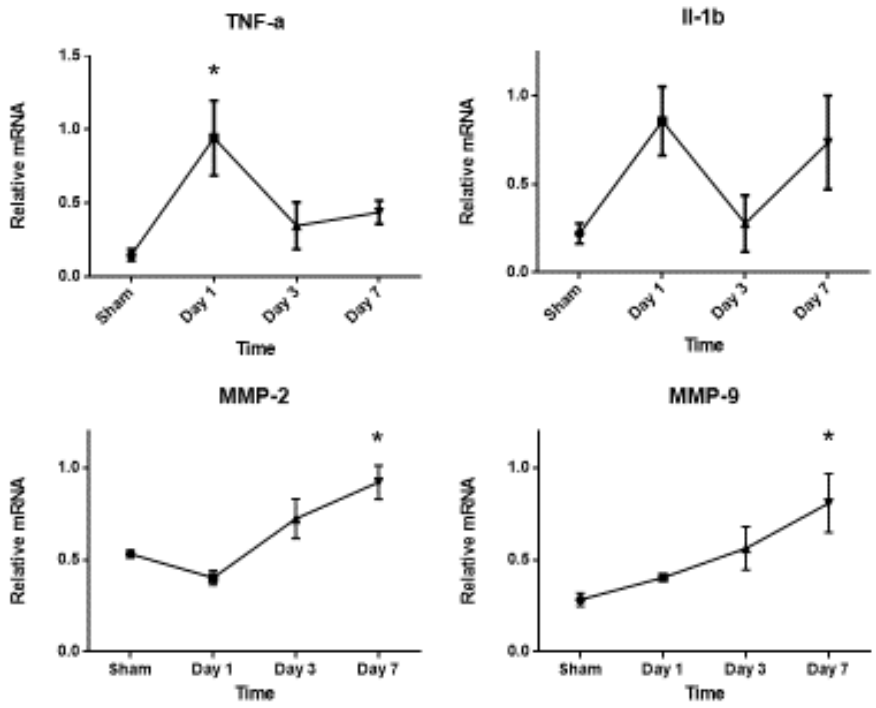

Figure 1: mRNA expression of TNF-α,, IL-1β, MMP-2, and MMP-9 over time in ischemic hemisphere after PT. Data expressed as Mean +/- SEM. * $p < 0.05$

Figure 2: Gene Expression over Time

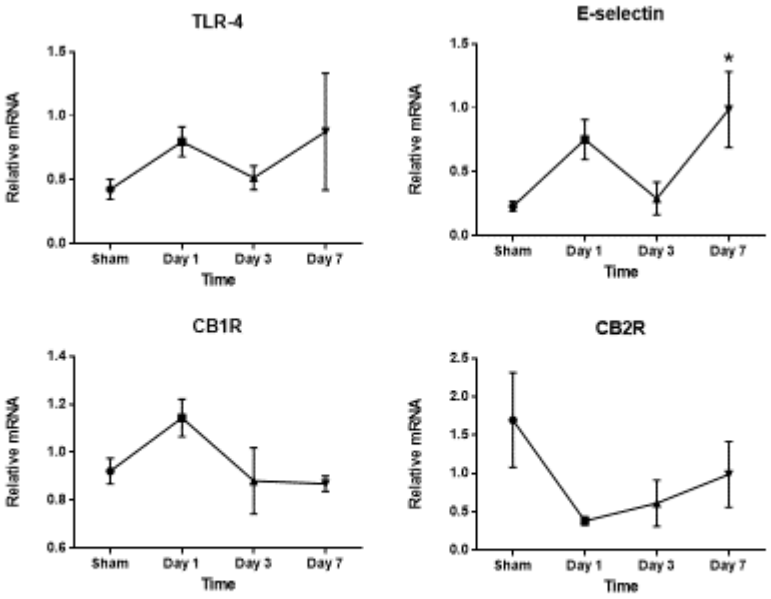

Figure 2: mRNA expression of TLR-4, E-Selectin, CB1R, and CB2R over time in ischemic hemisphere after PT. Data expressed as Mean +/- SEM. * $p < 0.05$

Figure 3: Gene Expression over Time

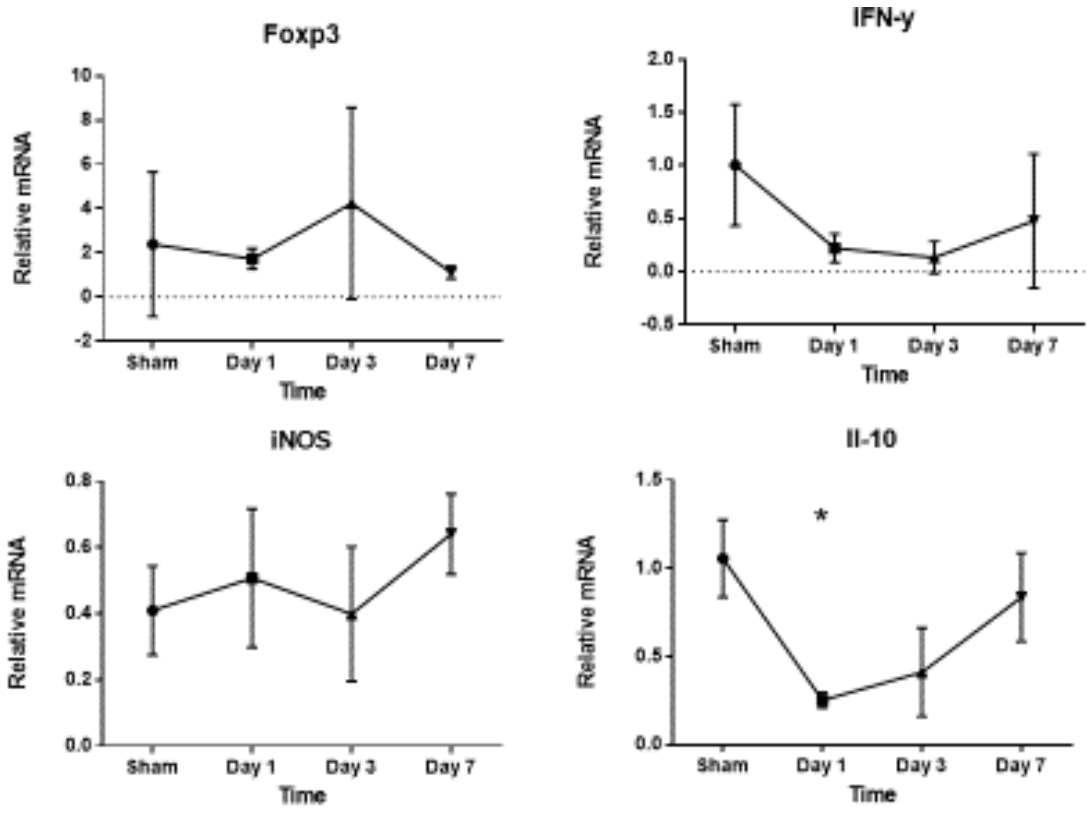

Figure 3: mRNA expression of FoxP3, IFN-γ, iNOS, and IL-10 over time in ischemic hemisphere n after PT. Data expressed as Mean +/- SEM. * $p < 0.05$

Figure 4: Total Number of Immune Cells over Time.

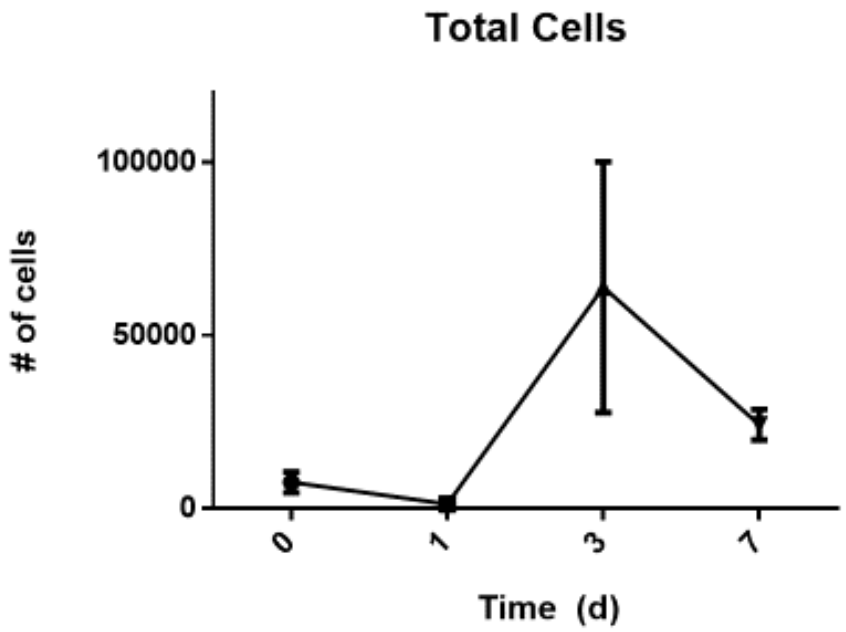

Figure 4: Total number of CD45+ cells in ischemic hemisphere over time following PT. Includes microglia and leukocytes. Data expressed as Mean +/- SEM. * $p < 0.05$

Figure 5: Number of Immune Cell Subpopulations Over Time

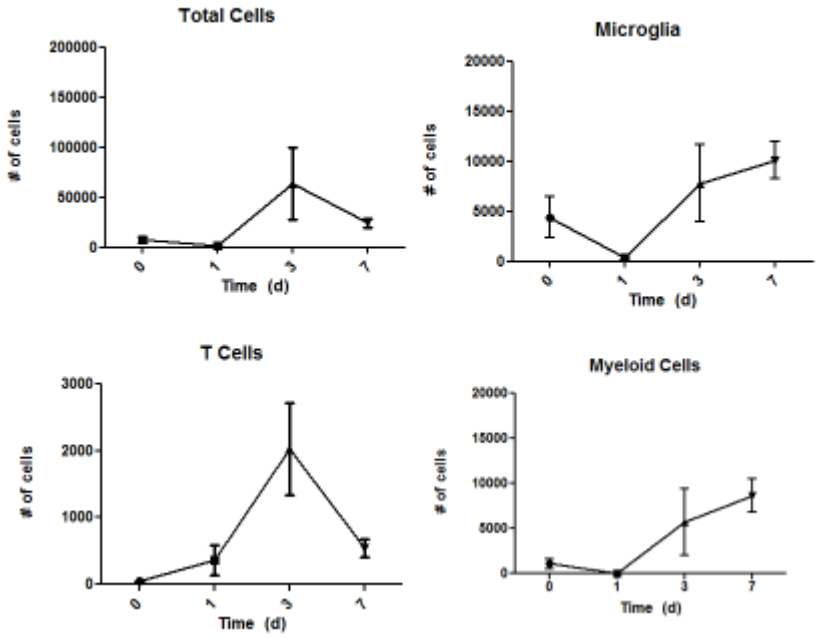

Figure 5: Total number of total cells, microglia, T cells, and myeloid cells in ischemic hemisphere over time following PT. Includes microglia and leukocytes. Data expressed as Mean +/- SEM. * $p < 0.05$

Figure 6: Proportion of Immune Cell Subpopulations

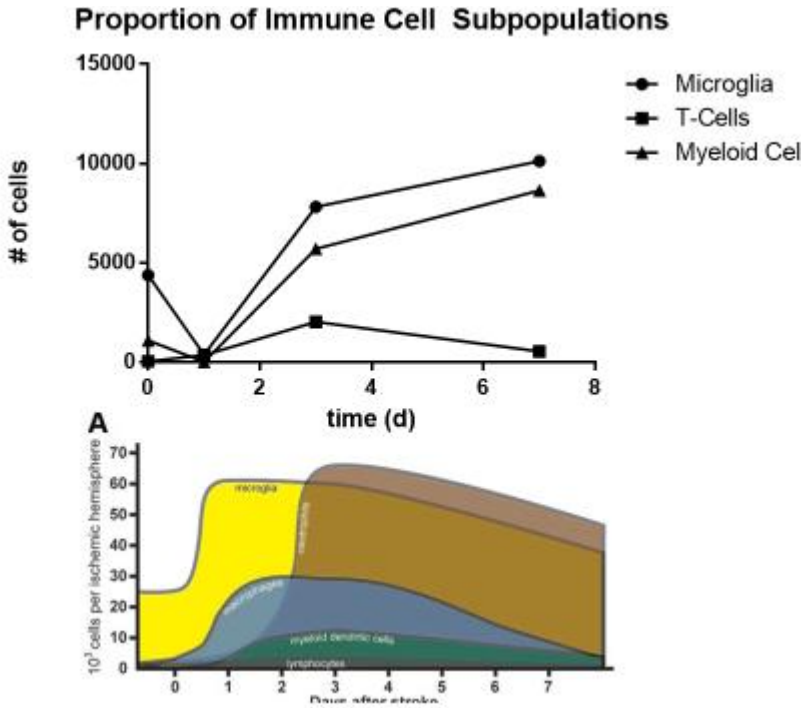

Figure 6: Relative proportion of different immune cell subpopulations over time. Number of microglia, T-cells, and myeloid cells in ischemic hemisphere after PT (top) and filament model of cerebral ischemia (bottom) (Gelderblom et al., 2009) * $p < 0.05$

Figure 7: Infarct Volume in O-1966 Treated vs. Vehicle Control Mice

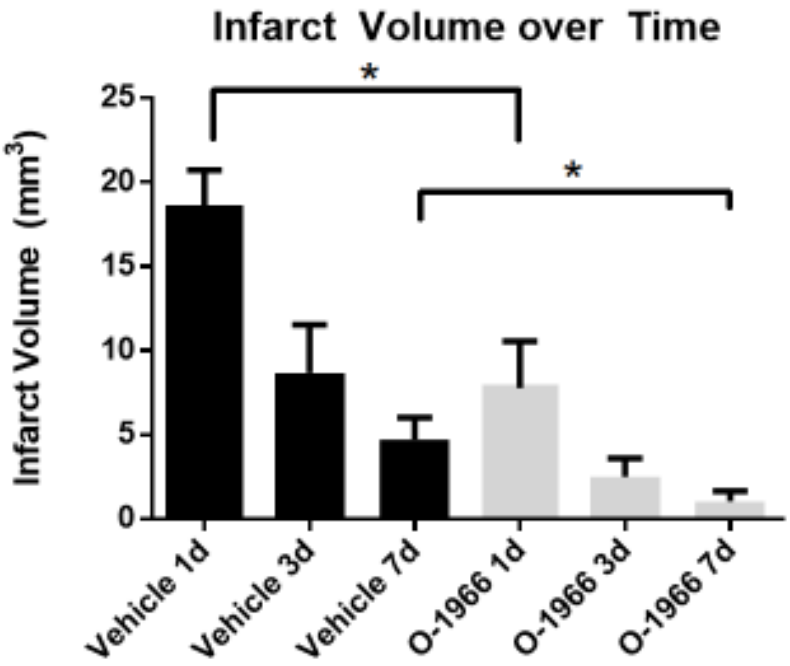

Figure 7: Infarct Volume in O-1966 treated mice vs. vehicle control mice at different time points. Mice sacrificed at 24 hours received one dose 5 mg/kg O-1966 one hour before PT (top). Those sacrificed on day 3 received an additional dose on day 2 (middle) and those sacrifice at day 7 received another dose on day 5 (bottom). Data expressed as Mean +/- SEM and was calculated using two way ANOVA for time and treatment. * $p < 0.05$. n=4-5 per group

Figure 8: Infarct Fraction in O-1966 Treated vs. Vehicle Control Mice

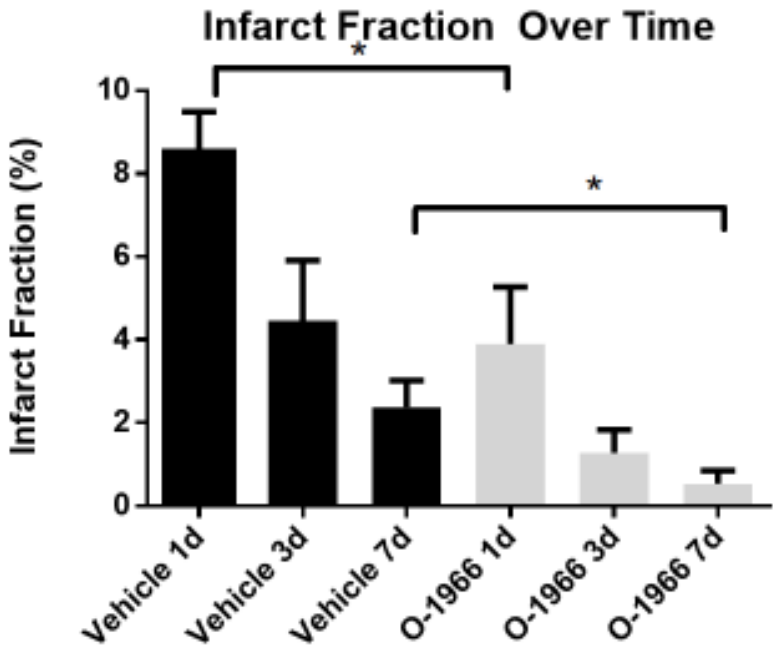

Figure 8: Infarct Fraction in O-1966 treated mice vs. vehicle control mice at different time points. Mice sacrificed at 24 hours received one dose 5 mg/kg O-1966 one hour before PT (top). Those sacrificed on day 3 received an additional dose on day 2 (middle) and those sacrifice at day 7 received another dose on day 5 (bottom). Data expressed as Mean +/- SEM and was calculated using two way ANOVA for time and treatment. * $p < 0.05$ n =4-5 per group.

Figure 9: Infarct Volume in O-1966 Treated vs. Vehicle Control Mice

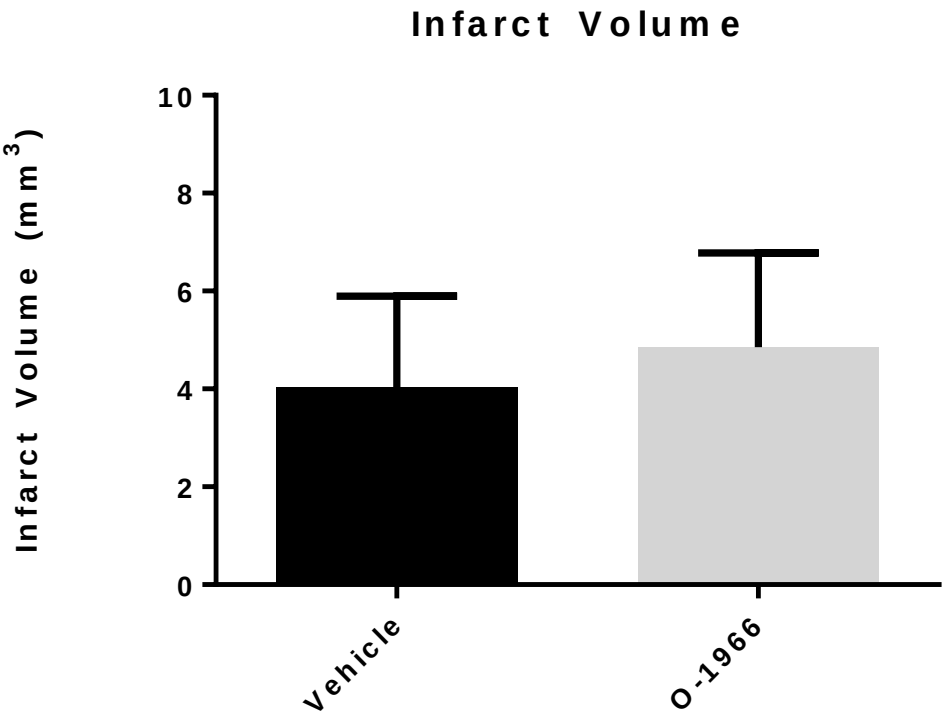

Figure 9: Infarct volume in O-1966 treated mice vs. vehicle control mice on day 7 after delayed treatment only. Delayed treatment included doses on day 2 and day 5 following PT. Data expressed as Mean +/- SEM. * $p < 0.05$. n=4 per group.

Discussion:

Several studies have demonstrated a neuroprotective effect of CB2R selective agonist administration in cerebral ischemia, both in transient and permanent models of the disease (Murikinati et al., 2010; Zarruk et al., 2012; M. Zhang et al., 2007). These compounds are thought to act on several different cell types, including microglia, neutrophils, endothelial cells, and even neurons (Schmidt et al., 2012). The potential of CB2R selective agonists was first examined by Zhang et al after CB1R targeting drugs have proved controversial (M. Zhang et al., 2007). Zhang found that treatment with two different O-1966 selective agonists, O-3853 and O-1966, resulted in significantly decreased infarct volumes when compared with vehicle controls in a transient filament model (tMCA) of focal cerebral ischemia. Furthermore, the mechanism was determined to be anti-inflammatory in nature, as intravital microscopy through a cranial window showed decreases in rolling and adhesion of rhodamine-labeled leukocytes along cerebral arterioles and postcapillary venules (M. Zhang et al., 2009). This evidence is consistent with the anti-inflammatory effect of CB2R in other disease models, including models of multiple sclerosis, spinal cord disease, traumatic brain injury, and Alzheimer's disease (Croxford, 2003; Grundy et al., 2001; Molina-Holgado et al., 2002; Ni et al., 2004; Ramirez et al., 2005). The dose response curve for O-1966 treatment demonstrated an inverted "U" shape, and was most effective at 5mg/kg. The specific molecular mechanisms by which rolling and adhesion were down regulated was not determined. Studies using P-selectin knockouts failed to show differences in infarct size in the permanent PT model of ischemia, in contrast to tMCAO studies (Frederix et al., 2007).

Thus, we hypothesized that E-selectin may perform a redundant role in the PT model. Indeed, E-selectin is increased following PT.

1 hour pretreatment with O-1966 significantly decreased stroke volume and infarct fraction. To our knowledge this is also the first study to demonstrate a neuroprotective effect of the CB2R selective agonist, O-1966, in a permanent model of cerebral ischemia, as well as the first to observe the effects of cannabinoid treatment in the PT model. O-1966 pretreatment had previously been shown to be effective in attenuating infarct expansion at 24 hours in a transient one hour occlusion filament model, and it was shown to decrease stroke volume by 33% (M. Zhang et al., 2007). The CB2R selective agonist JWH-133 was previously shown to be neuroprotective in a direct cauterization model of permanent cerebral ischemia (Zarruk et al., 2012).

Improvements in outcome upon repeated doses of O-1966 have been observed in other disease models in which secondary inflammatory plays an important role, including spinal cord injury and multiple sclerosis. In addition, studies of JWH-133 administration in a transient endothelin-1 model also showed significant decreases in infarct size at day 7 following repeated dose measurement (Schmidt et al., 2012). However, treatment with JWH-133 in this transient model did not require pretreatment but was successful with combined treatment during and following occlusion. Thus, "priming" of the immune response with pretreatment may only be necessary in the more severe permanent models of occlusion. This is important because it suggest that cell types native to the CNS, such as neurons and microglia, may be responsible. Alternatively, these two CB2R agonist may act through different mechanisms.

Inflammation has emerged as an important component of the ischemic cascade and a potential target for neuroprotective therapeutics in the treatment of ischemic stroke. Following cerebral ischemia, inflammation occurs over several weeks to months in the and there is a correlation between the magnitude of the inflammatory response and stroke outcomes (Dawson et al., 1996; Dutka et al., 1987; Dutka et al., 1988; Dutka et al., 1989; Hallenbeck et al., 1988; Hallenbeck et al., 1986; Kochanek et al., 1987; Kochanek et al., 1988; Kochanek & Hallenbeck, 1992). While some important clinical evidence exists to support this correlation, the majority of studies investigating inflammatory-mediated secondary damage in ischemic stroke comes from studies in animal models. However, there is great variability in the temporal dynamics of inflammation between the different models of stroke employed in animals, both in terms of cerebral proinflammatory cytokine expression and immune cell invasion. Each experimental approach models a different aspect of the disease, and the various advantages and disadvantages of each model as well as their pathologic mechanism must be tailored to the specific hypothesis being investigated. A more comprehensive, model-specific understanding of the cells and molecules involved in post-ischemic inflammation is essential in the utilization of these techniques to develop effective, translatable therapeutics

The endovascular filament method is the most commonly used and well characterized of the animal models of cerebral ischemia (Hata et al., 1998). Several studies employing FACS analysis and QT-PCR have been performed to elucidate the specific inflammatory mechanisms that occur in the filament model (Gelderblom et al., 2009). However, inflammation begins within minutes of cerebral infarction and progresses over several weeks to months, and the high mortality associated with tMCAO

prevents the study of the subacute phase of these processes. Additionally, outcome is dependent on differences in anatomy between different strains of mice and variability is high (Belayev et al., 1999) (Barone et al., 1993).

Less severe models of injury are required to study the later phases of inflammation in ischemic stroke. One, less severe alternative to the filament model is cerebral photothrombosis (PT) (Lee et al., 2007; Watson et al., 1985). In the PT model of cerebral ischemia, the photo-reactive dye rose Bengal is injected ip into the animal. The skin is removed over the target area of the skull, the periosteum scraped away, and a cold light source is placed in the desired location. The light passes through the skull where it activates rose Bengal perfusing through the cerebral vasculature, resulting in its decomposition and production superoxide, leading to severe, focal endothelial damage, blood-brain barrier breakdown, and impairment of blood flow (Dietrich et al., 1987; Dietrich et al., 1986; Wilson & Hatchell, 1991).

Unfortunately, a relatively small number of studies have examined the specific mechanism and timing of inflammation in PT. PT was first devised in a rat model before being implemented in a mouse (Lee et al., 2007; Watson et al., 1985). These studies demonstrated well circumscribed lesions on cresyl violet staining, and measurable differences in motor activity upon stair case and rotarod testing up to one month following PT. Mice are relatively less expensive to maintain, require shorter breeding times, and are more ideal for genetic and gene knockout studies. The first studies to examine changes in transcription levels of proinflammatory cytokines in a murine model of photothrombosis was performed by Jander et al (Schroeter et al., 2003). They examined the differences in transcription level over time of *Tnf-α*, *Il-1β*, and *inos*

between rat and mouse and discovered the expression level to of less magnitude and a longer time course in the latter. Levels of *Tnf-α* and *Il-1β* peaked at 24 hours before sharply decreasing but remaining elevated up to 28 days following PT. *Inos* levels were only slightly increased, and did not reach their peak until days 3 through 14 post ischemia. Our findings show a similar peak at 24 hours for *Tnf-α* and *1L-1β*, while the increase in *inos* expression is of similarly lower magnitude. Thus, the temporal dynamics of *Tnf-α*, *Il-1β*, and *inos* is consistent between different laboratories.

There has been some controversy around the mechanism of the PT model following a series of papers that showed marked differences between the more commonly utilized filament model, specifically with respect to the role of platelets. While platelet aggregation has been demonstrated in different murine PT models, questions exist about the necessity of platelets in the mechanism of injury. Studies using anti-GP1ba to deplete platelets, and anti-GPIIb/IIIa to inhibit platelet receptors resulted in no change in infarct size from controls (Frederix et al., 2007). In addition, neither mice lacking coagulation factor XII or with blocked factor X, factors essential for clot formation, showed differences in infarct size. This shifted the theory behind the mechanism from clot-based to more blood-brain barrier based. Indeed, studies looking at vascular leakage demonstrated significant leakage and edema into the cerebral parenchyma following PT (Clausen et al., 2008).

Molecules involved in postischemic vascular leakage include Matrix Metalloproteases, molecules activated during an inflammatory reaction that digest extracellular matrix molecules and lead to breakdown of the brain's tight blood-brain barrier, leading to increased leukocyte extravasation. Potential targeting of MMP

expression with anti-inflammatory agents such as O-1966 may be neuroprotective. MMP-9 KO studies have shown decreases in infarct size in a filament model (G. Wang et al., 2009). Both variants of this molecule, MMP-2 and MMP-9 were found to increase following rat PT (Piao et al., 2009). MMP-9 levels were found to increase early and peaked at 48 hours, before decreasing sharply toward baseline levels at 72 hours post ischemia. Conversely, MMP-2 levels underwent only slight increases in expression at earlier time points, before increasing significantly between 48 hours and 7 days. Indeed, *Mmp-9* is more commonly involved in the early pro-inflammatory stages of ischemia, while *Mmp-2* may be involved in tissue remodeling (Marsicano & Lafenetre, 2009). These increased levels of *Mmp* expression corresponded to vascular leakage as seen on Evans blue dye extravasation, which remained increased from 2 hours after PT until 72 hours, with a peak at 24 hours. MMP activity has been measured in a mouse PT model using gelatin gel zymography, showing increases in MMP-9 at 4 hours and 24 hours, consistent with early MMP-9 activity in the rat PT model. However, MMP-9 levels decreased back to baseline by day 7. In comparison, our results show increases in *Mmp-2* and *Mmp-9* from day 1 to 7, where they peak and are significantly larger than sham controls. Thus, our data shows that *Mmp*s continue to increase during the inflammatory response, consistent with the duration of edema formation as observed in other studies using Evans blue extravasation. Differences in results obtained may be due to the age of the mice used in the previously mentioned study, which were less than a month old, while our experiments used mice 8-12 weeks old.

Our study is the first to examine the temporal expression of the anti-inflammatory molecule *Il-10,* the marker of ant-inflammatory regulatory T cells *Foxp3*. Il-10 was

significantly decreased 24 hours post ischemia, and this taken together with the previously mentioned findings of increased *Tnf-α* and *Il-1β* expression at 24 hours may be evidence of a shift in microglial polarity toward a proinflammatory M1 phenotype rather than the M2 anti-inflammatory phenotype early after PT. *Foxp3* levels were not found to significantly increase following PT, suggesting that regulatory T cells may not be an important contributor in the pathophysiology of murine PT. Our study was also the first to quantify expression levels of *Ifn-γ* an important inflammatory mediator associated with T cell activity. *Ifn-γ* levels decreased up to day 3, before undergoing a slight but nonsignificant increase at day 7 back toward baseline values. The lack of significant changes in *Ifn-γ* suggest that though present, T cells may exert their effects through other means.

Cb2r is most predominantly expressed on microglia and peripheral leukocytes. Previous studies in our lab using a 60 minute tMCAO mouse model showed *Cb1r* and *Cb2r* expression increased and decreased respectively between 1 and 3 hours postischemia (M. Zhang et al., 2007). The present study shows similar patterns to the tMCAO study, with early increases in *Cb1r* expression and decreases in *Cb2r* expression, although of a protracted nature. Decreases in *Cb2r* expression 24 hours following PT does not exclude the CB2R receptor as target for O-1966 in providing neuroprotection at this time point, as inflammation-mediated changes in receptor sensitivity may play a role. Decreases in *Cb2r* expression may be due to the death of neurons and microglia in the ischemic core following PT. *Cb1r* expression decreased at day 3 and remained below baseline until day 7, while cb2r increased until day 7 but never reached preischemic control values. This is the first evidence of changes in *Cb1r* and *Cb2r* expression level

changes in a PT model, and first study of their expression at later time points in the ischemic mouse brain.

We also report for the first time the expression levels of Toll-Like Receptor 4 (*Tlr4*) and *E-selectin*. *Tlr*-4 levels were not significantly changed throughout ischemia, while *E-selectin* increased sharply at day 1 and peaked ($p<0.05$) at day 7. This is especially interesting given that much of the evidence suggesting a minor role for inflammation in PT comes from a study that showed no difference between *P-selectin* KO mice and wild type control mice (Frederix et al., 2007). This study, however did not examine *E-selectin*. It is possible that in PT, *E-selectin* plays more of an important role, and takes up the functions of the normally essential *P-selectin*.

The timing, magnitude, and relative contributions of different leukocyte subpopulations differs between human and animals, and between different animal models of cerebral ischemia. In humans, neutrophils invade infarcted tissue early, followed several days later by monocytes and macrophages. This differs greatly from reports on cell invasion observed in the most commonly used animal model of focal cerebral ischemia, the one hour tMCAO. In the tMCAO model, FACS analysis showed that microglial proliferation peaks at 24 hours (Gelderblom et al., 2009). However, monocytes, and not neutrophils as was commonly believed, begin to infiltrate first at 12 hours. Neutrophils do not begin to invade the parenchyma until day 2, when the magnitude of their invasion is several times larger than that of the monocytes. T-cells make up a very minor contribution of invading leukocytes and peak at day 3. The results of our study differ in a number of important ways. We show decreases in microglia and myeloid cells (mainly monocytes) at 24 hours possibly due to severe focal injury of a

faster time course than tMCAO. While the order of total cell number is maintained (microglia > myeloid cells > T cells), the relative levels of T cells and myeloid cells to microglia is much greater in Photothrombosis. In fact, T cells were significantly increased ($p<0.050$) on day 3 compared to sham animals. The shape of the T cell invasion curve is similar to tMCAO despite the greater magnitude in PT, as the number of T cells declined between day 3 and 7. In contrast to the tMCAO study, myeloid and microglial populations continued to increase between days 3 and 7.

The first study of temporal and spatial dynamics of PT in the mouse brain used immunohistochemical staining using different specific leukocyte markers (Schroeter et al., 2002). CD11b was used as a marker of microglial activation and a thin band of these cells surrounded the ischemic lesion. The number of CD11b+ microglial cells increased over time and had formed a very dense infiltrate in the infarct border by day 7. On day 28 CD11b+/F4/80+ microglia and macrophages had a strong presence in the ischemic core, although no time points between day 7 and 28 were examined so it is not possible to determine when they first entered this region. Interestingly, microglia activation was not confined to the peri-infarct region, as CD11b+ cells were seen in remote locations including the descending fiber tracts at the corpus collosum as well as the junction between the cortex and thalamus. CD 4+ or CD 8+ cell invasion was characterized as "low to absent" in the study. It is difficult to compare studies as this study did not used quantitative measures. However, the increase between the numbers of microglia from day 1 to day 7 was also observed in our study. The greater sensitivity of FACS in comparison to immunohistochemical staining could explain the differences in

observation. Even in low levels, T-cells are powerful cytokine factories, and the importance of their role is not to be discarded due to a lower number of cells.

Conclusion:

The development of a therapeutic agent that is effective in attenuating infarct progression in the subacute phase is a clinical imperative. Our results demonstrate the efficacy of a repeated dose regiment of the CB2R agonist O-1966 in the subacute phase in a photothrombosis model of permanent ischemic stroke. As inflammation is a major mediator of secondary damage and infarct progression in the subacute phase, the action of O-1966 in the subacute phase may be inflammation mediated.

Characterization and consideration of the inflammatory response in different animal models is necessary for the development of anti-inflammatory therapeutics. We report for the first time a quantitative assessment of immune cell invasion in a murine PT model of stroke. The total number of leukocytes peaked at day 3, and remained elevated compared to sham animals. Microglia made up the highest proportion of cells, followed by myeloid cells. This is consistent with immunohistochemical staining in a previous study of inflammation in PT, but differs greatly from what is seen in the tMCAO model where monocytes peaked at day 1 (Gelderblom et al., 2009; Schroeter et al., 2002). In fact, our studies show that the timing of monocyte invasion is more closely related to that seen in the clinic than in the tMCAO model, where monocyte invasion also peaks early in the process.

We verified the early postischemic increase in *Tnf-α* and *Il-1β* levels seen in the studies of Schroeter and present for the first time the temporal profile of expression of the cannabinoid receptors, *Cb1r* and *Cb2r*, *E-selectin*, *Tlr*-4 and the anti-inflammatory molecule *Il-10* in PT (Schroeter et al., 2003). *Il-10*, in contrast to the pro-inflammatory cytokines *Tnf-α and Il-1b*, underwent a significant decrease at 24 hours which may suggest a more M1 polarized phenotype following mouse PT. *Foxp3* and *Ifn-γ* levels did not significantly change, thus invading T cells, if important in the post ischemic immune response in PT, must act through different mechanisms.

CHAPTER 5

RESULTS: MANUSCRIPT 2: THE CANNABINOID-2 RECEPTOR AGONIST O-1966 REVERSES POSTISCHEMIC LEARNING AND MEMORY DEFICITS THROUGH ANTI-INFLAMMATORY PROCESSES

Introduction:

Cognitive impairments, including memory deficits, are an important source of morbidity in stroke patients. Of all patients that survive a stroke, 20-50% report memory difficulties (Nys et al., 2005; Rasquin et al., 2002; Sorensen et al., 1982; Wade et al., 1986). Unfortunately, treatment for stroke is limited to tissue-plasminogen activator (tPa), which has a narrow therapeutic window of 4 hours and is contraindicated in many patients. There are currently no effective treatments for stroke in the subacute phase and no treatments that act to specifically alleviate postischemic cognitive defects. Activation of the cannabinoid-2 receptor (CB2R) in the Endocannabinoid System (eCs) has been shown to modulate injury progression in the subacute phase. In addition, the eCs has been shown to play a role in memory and learning functions under both physiologic and pathologic conditions (Carlson et al., 2002; Marsicano & Lutz, 1999).

However, all studies have examined the Cannabinoid-1 Receptor in learning and memory, and not CB2R. We hypothesized that administration of the CB2R selective agonist O-1966 would result in attenuation of infarct expansion, resulting in the sparing of neuronal tissue important in learning and memory processes. PT results in injury to both the cerebral cortex and underlying hippocampus. As these structures are involved in several forms of memory, including spatial and operational learning, we predicted PT would result in deficits in these categories of learning. In addition, we demonstrate a

very strong correlation between Photothrombotic injury and performance on an Autoshaping test of recognition memory, a finding which demonstrates a role for these combined techniques in the study of postischemic cognitive defects and the development of drugs to preserve these functions.

Infarct Size Results:

Mice that received 5 mg/kg O-1966 one hour prior to ischemia had smaller infarct surface areas at 24 hours in comparison to vehicle control mice (Fig. 1). This difference approached statistical significance ($p=0.052$). Mice that received a regiment of 5mg/kg one hour prior to, 2 days, and 5 days following PT showed smaller infarct surface areas than vehicle controls at 7 days ($p<0.05$).

Learning and Memory Results:

A significant difference was observed in % time exploring the novel object. However, there was no statistical difference found between groups upon post hoc testing (Fig 2A). In addition, a significant difference was observed in adjusted latency, the time it took the mouse to learn the behavior, on day 6 testing in the O-1966 treated sham group compared to the vehicle treated sham group on day 6 as the latter performed better (Fig. 2B). Autoshaping was chosen because operational memory is the most common form of memory deficit in stroke patients. On day 7 testing there is a significant effect of drug and a significant interaction. However, there was no significance on post hoc testing.

Correlation between Cognitive Performance and Infarct Size

There was a moderate negative correlation between infarct surface area and percentage of time exploring the novel object (Fig 3.A). The correlation between latency of acquisition and infarct surface area was strongly positive and nearly reached statistical significance ($p=0.054$) on day 6. There was a significant ($p<0.05$) strongly positive relationship between latency of retention, the time it took to perform the behavior 10 times 24 hours later, and infarct surface area on day 7.

Q-PCR Results:

Il-6 expression was significantly decreased 24 hours following ischemia onset compared to vehicle controls ($p<0.05$) in O-1966 treated animals (Fig.4A). Expression of *E-selectin* and *Il-1β* was also decreased, but these values did not reach statistical significance. The expression of the anti-inflammatory cytokines *Il-10* and *Tgf-β* was increased in comparison to vehicle controls, but these values also did not reach statistical significance (Fig. 4B). These molecules were tested at 24 hours due to the role of early cytokine expression in the delayed phases of inflammation. *E-selectin* was attenuated at day 7 in animals that were given the O-1966 treatment regiment, and a nonsignificant decrease in *Mmp-9* expression was also seen at this time point compared to vehicle controls (Fig. 4C).

Figure 10: Infarct Size in Mice Treated with O-1966 vs. Vehicle Controls

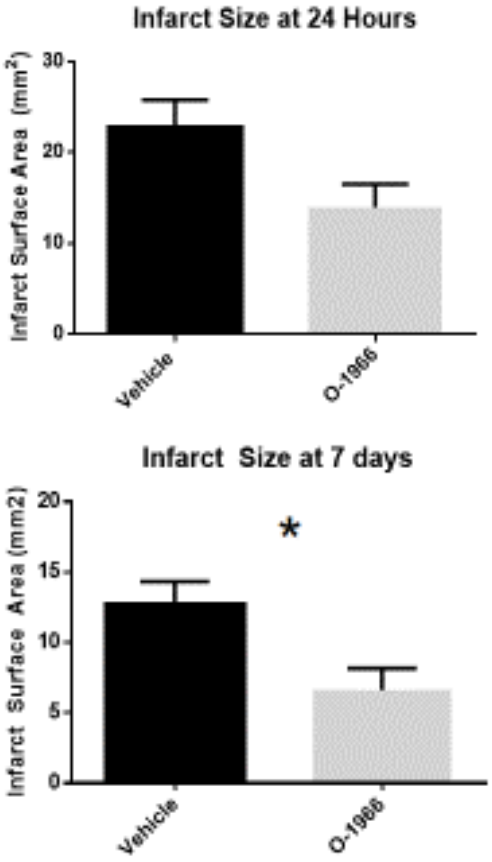

Figure 10: Infarct Surface Area in O-1966 treated mice vs. vehicle control mice at 24 hours (top) and 7 days (bottom) following PT. Mice sacrificed at 24 hours received one dose 5 mg/kg O-1966 one hour before PT. Those sacrificed on day 7 received additional doses on day 2 and day 5. Data expressed as Mean +/- SEM. * $p < 0.05$ n=5 per group

Figure 11: Novel Object Testing O-1966 Treated vs. Vehicle Control Mice.

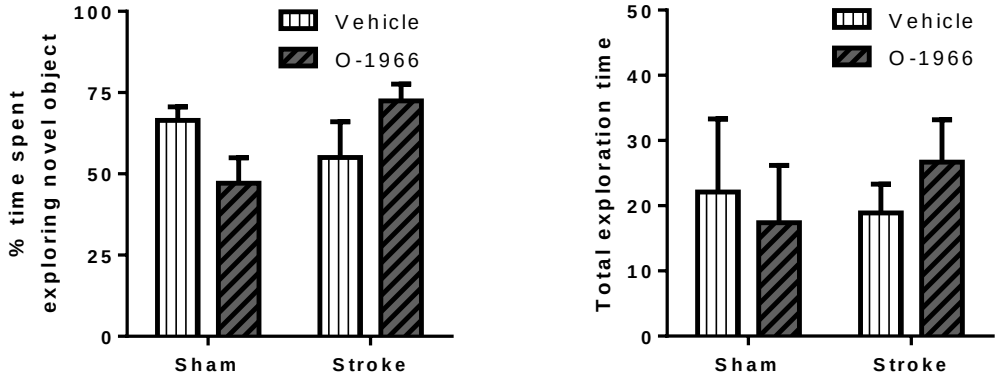

Figure 11: A significant difference was observed in % time exploring the novel object. However, there was no statistical difference found between groups upon Bonferonni's post hoc test.. Data expressed as Mean +/- SEM. * $p < 0.05$ n=5 per group

Figure 12: Autoshaping O-1966 Treated vs. Vehicle Control Mice.

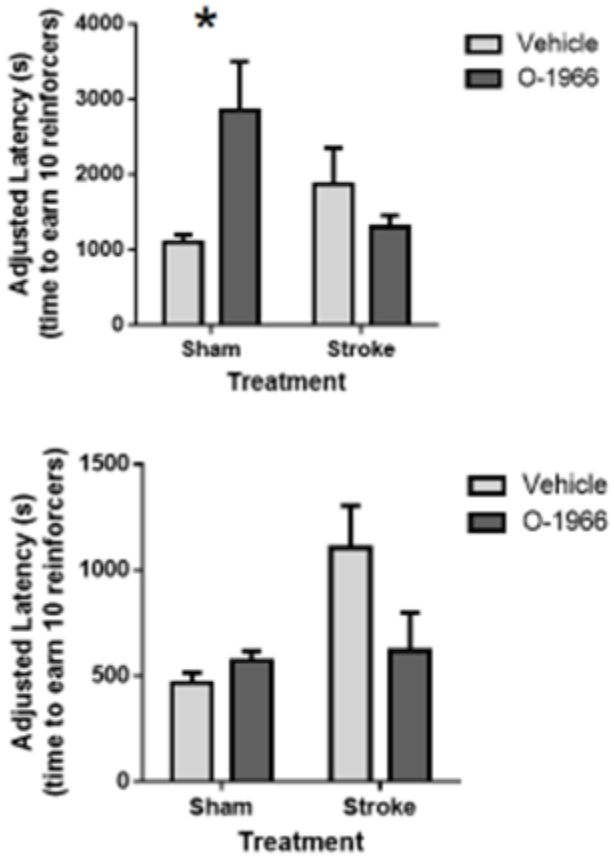

Figure 12: A significant difference was observed in adjusted latency on day 6 testing in the O-1966 treated sham group compared to the vehicle treated sham group on day 6 as the latter performed better (top). In addition, a significant effect of the drug and a significant interaction were seen on day 7 ($p<0.05$). Data expressed as Mean +/- SEM. * $p < 0.05$

Figure 13: Correlation Between Infarct Size and Cognitive Function

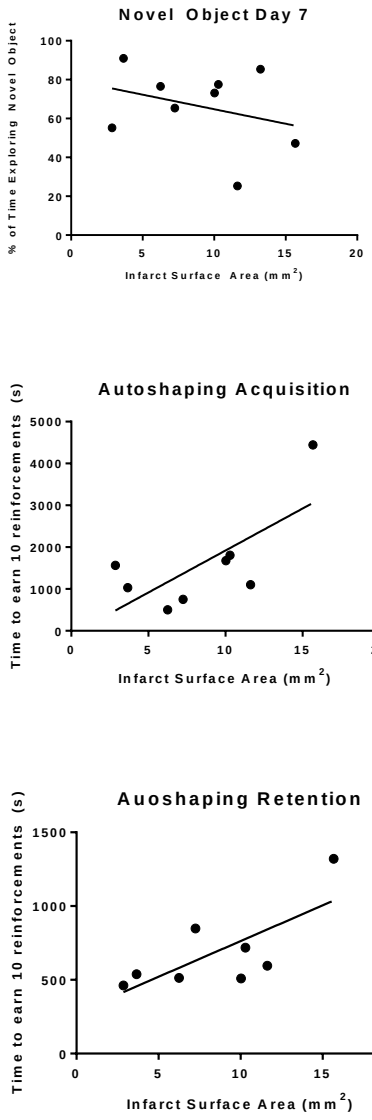

Figure 13: There was a moderate negative correlation between infarct surface area and percentage of time exploring the novel object (top figure). The correlation between latency of acquisition and infarct surface area was strongly positive and nearly reached statistical significance ($p=0.054$) on day 6 (middle figure). There was a significant ($p<0.05$) strongly positive relationship between latency of retention and infarct surface area on day 7 (bottom figure). Data expressed as Mean +/- SEM. * $p < 0.05$

Figure 14: Inflammatory Gene Expression in O-1966 Treated vs. Vehicle Control Mice on Day 1

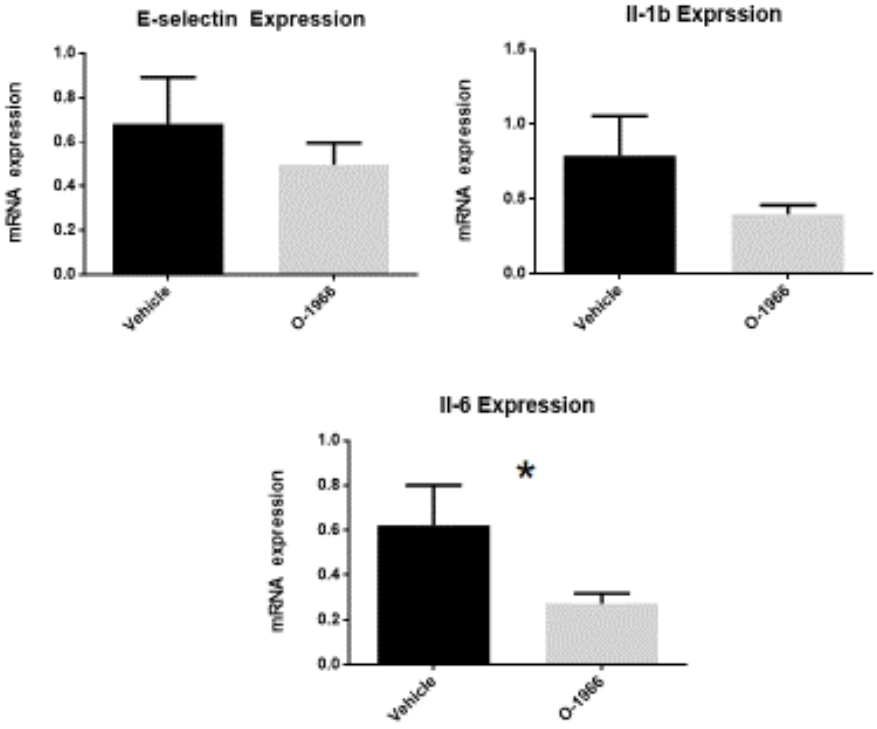

Figure 14: *Il-6* expression was significantly decreased 24 hours following ischemia onset in O-1966 treated mice compared to vehicle controls (bottom). Expression of *E-selectin* and *Il-1β* was also decreased, but these values did not reach statistical significance. Data expressed as Mean +/- SEM. * $p < 0.05$

Figure 15: Anti-inflammatory Gene Expression in O-1966 Treated vs. Vehicle Control Mice on Day 1

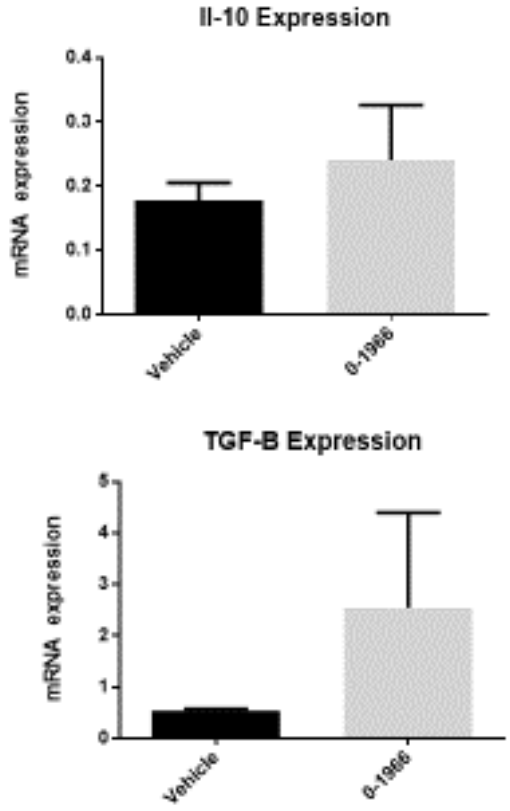

Figure 15: The expression of the anti-inflammatory cytokines *Il-10* and *Tgf-β* was increased in comparison to vehicle controls, but these values did not reach statistical significance. Data expressed as Mean +/- SEM. * $p < 0.05$

Figure 16: Inflammatory Gene Expression in O-1966 Treated vs. Vehicle Control Mice on Day 7

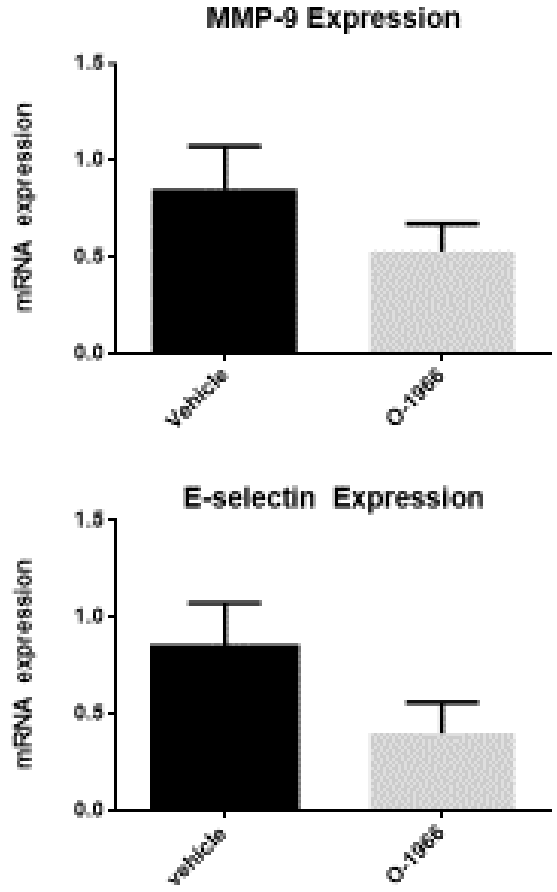

Figure 16: *E-selectin* was attenuated at day 7 in animals that were given the O-1966 treatment regiment, and a nonsignificant decrease in *Mmp-9* expression was also seen at this time point compared to vehicle controls. Data expressed as Mean +/- SEM. * p <0.05

Discussion:

Our results are the first to demonstrate beneficial effects on learning and memory of CB2R agonists in cerebral ischemia. Both the endocannabinoid and immune systems play important, direct roles in learning and memory processes (Carlson et al., 2002; Marsicano & Lutz, 1999; Yirmiya & Goshen, 2011). Low level, target specific release of cannabinoids act at presynaptic CB1R on inhibitory neurons. This phenomenon of attenuation of inhibitory interneuron firing alleviates suppression of postsynaptic excitatory neurons in a process termed depolarization-induced suppression of inhibition (DSI) (Alger, 2002; Freund et al., 2003). This DSI phenomenon can trigger long term potentiation (LTP), the electrical basis of neural plasticity (Carlson et al., 2002). However, effects of CB2R on learning and memory processes has not been previously studied.

Likewise, low basal levels of inflammatory cells and cytokines are also important in these processes (Yirmiya & Goshen, 2011). Interestingly, sham animals that received O-1966 performed more poorly than their vehicle counterparts. It is possible that the O-1966 is only beneficial in the setting of ischemic disease, and that under physiologic conditions it may be detrimental. O-1966 administration in the absence of a strong inflammatory response may deplete these necessary mediators and damage learning and memory functions. Correlation studies between the effects of O-1966 treatment on gene expression and memory testing performance in sham animals would offer further insight into this question.

Disruption of the cytokine balance can also be due to inappropriately high levels. of IL-6 and IL-1B. Patents with Alzheimer's Dementia, a disease characterized by severe, progressive cognitive impairment, have high CSF levels of IL-6 (Akiyama et al., 2000; Shaftel et al., 2008). In addition, several autoimmune disease, such as multiple sclerosis and diabetes type II are associated with memory loss. Studies in patients with these diseases revealed patients with higher levels of IL-1B and Il-6 performed poorer on memory based tasks (Marioni et al., 2010; Patanella et al., 2010). Mechanisms of the effects of these cytokines on learning and memory has been studied at the cellular level, and high levels of both IL-6 and IL-1B have been shown to inhibit neurogenesis and LTP (Bellinger et al., 1993; Bellinger et al., 1995; Goshen et al., 2008; Nakanishi et al., 2007; Vallieres et al., 2002).

We hypothesize that the effect of the CB2R agonist O-1966 on learning and memory is a result of the attenuation of inflammation mediated infarct expansion through CB2R, specifically as a result of the decrease in expression of the IL-1B and IL-6. We believe that this effect was a result of CB2R-mediated downregulation of the immune response resulting in sparing of tissue, as strong correlations were observed between infarct size and cognitive performance (Fig 3A). However, it is possible that learning and memory improvements may also be due to alleviation of the direct detrimental effects of high levels of IL-1B and IL-6 on these functions.

Of the proinflammatory cytokines examined, only *Il-6* was significantly decreased in response to O-1966 treatment, a finding also observed in a direct coagulation model of permanent cerebral ischemia. This suggests that CB2R agonists are active through direct or indirect effects on microglia or neurons, as *Il-6* may be secreted

by both of these cell types. Evidence using fluorescent labeling in an endothelin-1 model of transient cerebral ischemia in rats showed co-labeling with NeunN and CB2R 24 hours after injury, but no colocalization of CB2R and microglia was seen at this time point (Schmidt et al., 2012). Thus, it is possible that neurons, and not microglia, are the source of *Il-6.* Only a combined regiment of pretreatment and repeated doses post treatment showed an improvement in infarct size. The necessity of pretreatment implicates cell neurons and microglia as potential targets by which O-1966 is effective, as peripheral leukocytes are not present in large quantities in cortical tissue under physiologic conditions. *Il-1β* was found to be decreased, but similar to the results obtained in the literature, this difference was not significant (Zarruk et al., 2012).

Our study is the first to examine the effect of CB2R on proinflammatory molecules at 7 days, and differences in *E-selectin* and *Mmp-9* were decreased against vehicle controls. Expression of *E-selectin* and *Mmp-9* is not significantly increased until day 7, when post-ischemic blood brain barrier permeability is high. Thus, the effect on *E-selectin* expression at 7 days, but not 24 hours, may be due to *E-selectin* playing a more prominent role in disease progression at later time points, as suggested by its temporal expression profile in PT. MMP-9 is thought to play a more detrimental role in inflammatory damage, while MMP-2 is important in tissue repair (Marsicano & Lafenetre, 2009). The decrease in *Mmp-9* by O-1966 is consistent with studies that showed decreased transendothelial electrical resistance (TEER) with CB2R agonists (Chi et al., 2012).

Interestingly, the results obtained with the direct coagulation model showed downregulation of several other markers of microglial activation in addition to Il-6,

including IL-12/Il-23p40, MCP-1, MIP-1a, RANTES and iNOS (Zarruk et al., 2012). Interestingly, alternative, anti-inflammatory markers of microglia activation, namely IL-10 and TGF-B, were also attenuated, leading the authors to hypothesize that the CB2R agonist JWH-133 is effective via induction of an overall quiescent state of microglia as opposed of a cannabinoid-induced switch in polarity from the pro-inflammatory M1 microglia phenotype to the anti-inflammatory M2 phenotype. This contrasts with our own results, as although *Il-6* was decreased, decreases in the anti-inflammatory factors *Il-10* and *Tgf-β* was not observed. In fact, both of these cytokines were increased, although not significantly. Inflammation functions as a double edged sword in a number of pathologic disease states, and may be essential in the reparative phase of the disease. A drug that selectively attenuates production of powerful pro-inflammatory factors such as IL-6, while sparing the restorative anti-inflammatory factors such as IL-10 and TGF-B would be of potentially great therapeutic benefit.

There are several potential explanations that account for the difference in results obtained in the present study and the previously mentioned paper. Although both models utilized were models of permanent cerebral ischemia, they are very different models, with the former involving severe endothelial damage and blood brain barrier breakdown, while the latter involved direct cauterization of the middle cerebral artery with relative sparing of other vessels and surrounding tissue. Another possibility is that CB2R agonists of different binding affinities may interact with different pathways through the CB2R receptor. Finally, cannabinoids are considered to be very promiscuous agonists and can act on CB1, Vanilloid, or Serotonin receptors (Akerman et al., 2004; Baker & McDougall, 2004; Barann et al., 2002; Chemin et al., 2001; Dannert et al., 2007). Thus,

action by either or both of the agonists, JWH-133 and O-1966, on other receptors in addition to CB2R may account for the differences in the effect on the expression of anti-inflammatory factors.

Conclusion:

We show for the first time the beneficial effects of a CB2R agonist on postischemic learning and memory functions. The role of CB2R has not previously been studied in learning and memory. It is likely that these improvements are mediated by CB2R agonist-induced alleviation of the direct, damaging effects of IL-6 and IL-1B on these processes. Cognitive defects are a common symptom following ischemic stroke, and compounds that could potentially improve these symptoms would be valuable in preventing cognitive morbidity. The very strong correlation between PT induced infarct size and performance in Autoshaping behavioral testing suggest the combination of these techniques may be useful in the development of therapeutic agents that act to treat postischemic cognitive deficits.

Inflammation plays both a damaging role in exacerbating injury following cerebral ischemia as well as an essential role in repair. The development of drugs that selectively attenuate damaging inflammatory mechanisms while preserving anti-inflammatory function is essential for development of neuroprotective therapeutics in ischemic stroke. Our data shows downregulation of the expression of proinflammatory cytokine *Il-6* with O-1966 treatment, without altering levels of the anti-inflammatory mediators *Il-10* and Tgf-β. In addition, *Mmp-9* and *E-selectin* levels mRNA levels were decreased at 7 days.

CHAPTER 6

DISCUSSION

Stroke is the leading cause of morbidity in the United States, with many patients experiencing various forms of cognitive impairment. Memory deficits are a significant contributor of poststroke cognitive decline, and of all patients that survive a stroke, 20-50% report memory difficulties (Nys et al., 2005; Rasquin et al., 2002; Sorensen et al., 1982; Wade et al., 1986). This issue is compounded by indirect effects of the disease on memory including effects from medication and depression. Despite the high prevalence of the disease, there are currently no therapeutics available to treat cognitive decline in stroke patients. We report that a repeated dose regiment of the CB2R selective agonist O-1966 improved learning and memory 6 and 7 days postischemia, respectively. We propose that this phenomenon occurs from attenuation of inflammation mediated infarct expansion. CB2R agonist-induced reductions proinflammatory cytokine transcription, specifically *Il-1β* and *Il-6* may also play a direct role.

The direct effects of the CB2R on learning and memory processes may play a role in the mechanism behind our findings in this study since O-1966 sham and naïve mice performed differently from their vehicle counterparts on day 6. While there is a large volume of literature on the potential role of the endocannabinoid system in learning and memory, to our knowledge this is the first study to examine a potential role for CB2R.

Results obtained from learning and memory studies of exogenous cannabinoids has often conflicted with those obtained in studies of the endocannabinoid system under

physiologic conditions. For example, THC administration was found to damage short-term memory (Ranganathan & D'Souza, 2006). THC is a partial CB1 agonist. However, there is no evidence that CB1R activation under normal physiologic conditions can have a negative impact on these processes. This difference is believe to be due to the specific temporal and spatial regulation of the eCS in these processes. eCS activation in learning and memory probably occur over a very small amount of time, possibly milliseconds, and only in very specific regions (Marsicano & Lafenetre, 2009). Complete activation of all CB1R through systemic or cerebral administration of a potent exogenous cannabinoid agonist does not mimic the delicate balance and regulation required in complex brain functions such as learning and memory. Thus, our results suggest a potential direct role for CB2R activation in learning and memory processes.

The effects of O-1966 on memory and learning may also be a result of the attenuation of the direct effects of cytokines on learning and memory. Inflammation plays an important role in modulating the efficiency of many essential physiologic processes under quiescent conditions in the absence of tissue damage or pathogen invasion. An example of this is bone remodeling, by which marcrophage-like osteoclasts absorb bone and regulate bone density and efficiency in response to different physiologic inputs. Another example of this is the role of microglia in pruning of neurons in infancy and throughout adulthood to increase the efficiency of neuronal contacts (M. Zhang et al., 2008). Indeed, this is one way in which inflammation can affect learning and memory functions. Many proinflammatory cytokines also have direct neurotropic functions. In fact, it is has been shown in many different studies that low, baseline levels of cytokines

such as IL-6 and IL-1β play an important role in learning and memory under quiescent conditions (Yirmiya & Goshen, 2011).

Perturbance of the delicate cytokine balance as is the case with strong acute and chronic inflammatory reactions impairs learning and memory. There is ample evidence that inappropriately high IL-6 levels are detrimental in learning and memory processes. High levels of Il-6 were correlated with poorer performance on memory-based tasks in patients with MS and Type-II diabetes (Marioni et al., 2010; Patanella et al., 2010). In addition, Alzheimer's Dementia patients have elevated CSF levels of several cytokines, including IL-6 (Akiyama et al., 2000; Shaftel et al., 2008).

In vitro studies of the association between IL-6 and memory have found a similar correlation between increased levels of IL-6 and attenuation of neural plasticity and neurogenesis. Hippocampal slices with brain cells overexpressing IL-6 featured dampened LTP (Long Term Potentiation), a cellular mechanism of neural plasticity (Bellinger et al., 1995). Another study using transgenic astrocytes overexpression IL-6 decreased neurogenesis in the hippocampus (Vallieres et al., 2002). Studies utilizing neural progenitor cells in the presence of IL-6 showed non-specific decreases in NPC proliferation and differentiation (Nakanishi et al., 2007). Therefore, there are a number of potential mechanisms by which high levels of Il-6 could directly exacerbate postischemic memory loss, and therefore, ways in which O-1966-induced attenuation of IL-6 transcription could be beneficial in restoring these functions.

Other studies have implicated a negative effect of inappropriately high levels of IL-1B on learning and memory. Intraventricular and systemic injection of IL-1B

adversely affected recollection of spatial memory in a water maze test as well as fear conditioning (Oitzl et al., 1993). Long-term memory was also found to be impaired by IL-1B administration in rodents, and interestingly this response was extinguished by administration of the anti-inflammatory agent a-melanocortin (Gonzalez et al., 2009). It is possible that O-1966 acted in a similar manner in our study. Similarly to Il-6, high levels of Il-1B have been found to inhibit LTP and neurogenesis (Bellinger et al., 1993; Goshen et al., 2008). The mechanism by which Il-1B inhibits neurogenesis is two-fold, and is thought to include both as a result of Il-1B effects on glucocorticoid secretion as well as a direct effect on progenitor cells (Yirmiya & Goshen, 2011).

Transient and permanent cerebral ischemia represent two extremes of cerebrovascular disease. Permanent cerebral ischemia occurs when a vessel is permanently occluded, while transient ischemia involves reperfusion, either iatrogenic or otherwise. It is believed that blood flow plays a more significant role in permanent ischemia, and inflammation plays less of a role. This is the opposite for transient ischemia, where reperfusion of blood containing leukocytes, cytokines, and free radicals is hypothesized to be a relatively more important contributor to stroke outcome. Permanent ischemia is probably the more common clinical variant, as less than 10% of patients are eligible for tPa reperfusion therapy (Reeves et al., 2005). However, many forms of the disease exhibit aspects of both disease variants, and permanent ischemia can transition to transient ischemia as the disease progresses. While there are differences in the spatial and temporal dynamics, inflammation plays an important role in both transient and permanent ischemia. Thus, potential therapeutics for inflammation should be effective in animal models of both transient and permanent ischemia. The results

contained within provide further evidence for the effectiveness of CB2R agonists in attenuating infarct expansion in permanent cerebral ischemia.

While cell death occurs early via necrosis within hours of the initial ischemic insult, injury in cerebral ischemia progresses over several days to weeks. Several pathologic mechanisms account for this progression, particularly secondary damage associated with inflammation. In animal models of other disease of the CNS with strong inflammatory components such as Alzheimer's Disease, Traumatic Brain Injury, Multiple Sclerosis and Spinal Cord Injury, a repeated dose regiment of a CB2R agonist was necessary to attenuate inflammatory damage progression in the subacute phase (Croxford, 2003; Grundy et al., 2001; Molina-Holgado et al., 2002; Ni et al., 2004; Ramirez et al., 2005). Furthermore, studies using a repeated dose regiment of JWH-133 were effective in decreasing infarct size in a transient endothelin-1 administration model (Schmidt et al., 2012). Our results are the first to demonstrate the effectiveness of a CB2R agonist repeated dose regiment in improving infarct size in the subacute phase of permanent ischemia.

Although 1 hour single dose pretreatment with O-1966 resulted in decreased infarct size at 24 hours, this effect was lost at 7 days. Furthermore, post-ischemic treatment regiments only, including repeated dose regiments of 2 days and 5 days post ischemia did not result in a decrease in infarct size compared with vehicle controls. Additionally, the regiment of 6 hours, 2 days, and 5 days post ischemia treatment did not have an effect. Only the combined 1 hour pretreatment and 2 and 5 day posttreatment regiment was sufficient to significantly decrease infarct size. Indeed, the repeated dose regiment in the endothelin-1 study required an initial treatment during ischemia. This

taken together with the present results suggest that intervention is necessary at multiple time points, and that one of these time points must occur either before or during ischemia. Thus, some form of "priming" of neurons or microglia may be of mechanistic necessity to account for the means by which CB2R repeated dose agonists offer neuroprotection. The necessity of a repeated dose regiment may suggest attenuation of the innate immune response rather than the adaptive immune response.

At 24 hours following PT, pretreatment with O-1966 resulted in decreased expression of the potent pro-inflammatory cytokine *Il-6*. This is consistent with studies using a direct cauterization model of permanent ischemia and is of clinical and translational significance, as much of the evidence for a detrimental effect of inflammation in clinical stroke comes from studies of pro-inflammatory cytokine, specifically IL-6. Several studies have shown conflicting results in animal models of the effects of TNF-a, and while more conclusively linked with worsening outcomes in animal models, Il-1B levels do not correlate with stroke outcome in patients (Lambertsen et al., 2012). Indeed, only Il-6 levels, specifically in cerebrospinal fluid, correlate with stroke severity (Beridze et al., 2011). CB2R induced attenuation of Il-6 expression was also seen in a direct cauterization of cerebral ischemia (Zarruk et al., 2012).

Inflammation can be viewed as a double edged sword in pathologic states, and this remains true in the setting of cerebral ischemia. Many pro-inflammatory factors are considered to be detrimental to stroke resolution, while it is commonly accepted that anti-inflammatory factors are necessary not only for ending the inflammatory response, but for promotion of healing and repair. The development of a therapeutic agent with the ability to selectively attenuate pro-inflammatory responses, while sparing or promoting

anti-inflammatory factors and tissue repair would be invaluable. Interestingly, while our studies showed attenuation of the pro-inflammatory cytokine *Il-6*, there was an increase in the levels of the anti-inflammatory mediators *Il-10* and *Tgf-β*. O-1966 has been shown to increase IL-10 levels in in vitro studies utilizing a mixed lymphoid reaction system (Eisenstein, data not yet published). The authors of that study concluded that Regulatory T cells were the source of the increase in IL-10 (Reeves et al., 2005). The potential role of anti-inflammatory mediators and cells such as Regulatory T cells in improving stroke outcomes was seen in studies using anti-FoxP3 antibodies for Treg depletion in stroked mice (Liesz et al., 2009). Mice depleted of Tregs had higher infarct sized than the untreated mice, and this mechanism involved Treg expression of *Il-10*. Our studies also showed an increase in *Il-10* expression, and an interesting future direction would be to examine the potential effect of O-1966 on Treg invasion and *Foxp3* expression

Our studies are the first to demonstrate a CB2R agonist-mediated upregulation of these anti-inflammatory cytokines in the setting of cerebral ischemia. In contrast to our results, previous studies using a direct cauterization model showed significant decreases in *Il-10* and *Tgf-β* upon treatment with JWH-133 (Zarruk et al., 2012). It is interesting that while both CB2R agonist, JWH-133 and O-1966 attenuate *Il-6* expression, there would be opposite effects on anti-inflammatory mediator levels. There are several possible explanations for this. Exogenous cannabinoids are highly promiscuous ligands in terms of their binding affinity, and may interact with vanilloid, serotonin, and even cannabinoid-1 receptors. Thus, one or both of these CB2R agonist may be acting on other receptors. Additionally, differences in treatment doses and the pathophysiologic

mechanism of the model used may account for the difference in the effect on *Il-10* and *Tgf-β*.

E-selectin levels were decreased 7 days post ischemia in mice that received a repeated dose regiment of O-1966. E-selectins and P-selectins are important cell adhesion molecules in the early rolling stages of inflammation. These molecules are upregulated soon after inflammation and continue to increase up to 7 days post ischemia where they reach their peak. While P-selectin is stored in granules, E-selectin is transcribed in situ in response to injury. Both of these molecules interact with glycoproteins on leukocytes, specifically sialyl-Lewis^x groups, where they form weak interactions that allow time for initiation of later activation and adhesion steps in the inflammatory cascade (Bevilacqua, 1993; Butcher, 1991; Patarroyo et al., 1990; R. L. Zhang et al., 1996). Our results are consistent with studies of O-1966 in a transient model of ischemia that showed decreased leukocyte rolling and adhesion on arterioles and postcapillary venules in the cerebrovasculature 24 hours after ischemia (M. Zhang et al., 2009). However, no difference was seen in *E-selectin* at 24 hours, but rather at 7 days post ischemia. This is the time point when E-selectin expression is at its highest, and while O-1966 may act on attenuating expression at earlier time points, the lower levels of *E-selectin* may mask detection of a difference.

Levels of *Mmp-9*, a molecule involved in blood brain barrier breakdown, degradation of the extracellular matrix, and resulting increases in leukocyte invasion, was also decreased at 7 days with O-1966 treatment. There is evidence that tPa treatment may induce MMP-9, thus it would be interesting to examine the effect of O-1966 in downregulating its expression as a potential adjuvant to tPa treatment (Tsuji et al., 2005).

O-1966 treatment downregulates *Mmp-9* expression, a finding consistent with studies that showed decreased blood brain barrier permeability with transendothelial electrical resistance studies upon similar treatment (Chi et al., 2012).

The majority of studies of potential therapeutic agents in ischemic stroke are performed utilizing animal models. There are several different models of transient and permanent ischemia, necessary to study the various components and variants of the disease. Thus, the detailed elucidation of these different models is essential for the development of new treatment strategies. The most commonly used animal model of cerebral ischemia is the filament occlusion model. This model is believed to have considerable similarities to clinical disease and is the most widely studied. However, infarcts induced as a result of filament occlusion are large in size, far larger than what is commonly found in clinical disease, where the average infarct size is 4.5-15% of the brain (Brott et al., 1989; Carmichael, 2005; Lyden et al., 1994; Nopoulos et al., 2000; Sowell et al., 2003). Furthermore, stroke progresses over several days to weeks beyond the initial ischemic insult. The relatively more severe strokes of the filament model result in high early mortality, preventing the study of the effectiveness of therapeutics in the subacute phase (Kitagawa et al., 1998). In addition, the model has high statistical variability owing in part to its dependence on uniform vascular anatomy (Barone et al., 1993). Different strains of mice, and even mice within a given strain, can have high variability in terms of cerebrovasculature anatomy, which greatly affects the consistency of the filament model. As a result of the disadvantages, as well as the high level of surgical skill required, alternate models of cerebral ischemia, such as PT, have been developed. Post-ischemic secondary injury as a result of inflammatory damage is an

important aspect of the pathophysiology of ischemic stroke, and a potential target of treatment strategies. Unfortunately, the mechanism of injury in PT, especially with respect to inflammation, has not been as fully elucidated in comparison to the filament model.

We aimed to present a more comprehensive understanding of the temporal and spatial dynamics of PT with respect to the immune response. Immune cell invasion was studied using flow cytometry and proinflammatory cytokine expression using RT-PCR. Large increase in CD45+hi microglia and CD45+ low or intermediate peripheral leukocytes) remained at baseline 24 hours after injury, peaked at day 3, and then regressed to levels slightly higher than baseline at day 7. The invasion and proliferation of specific immune cell subpopulations were also examined. Microglia and myeloid cells (e.g monocytes) increased at day 3 and were further increased at day 7, a finding consistent with previous reports using immunohistochemical staining (Schroeter et al., 2002). Interestingly, the delayed increase in invaded monocytes at 3 days more closely resembles that seen in human stroke than in the filament occlusion model, where monocyte levels peak early at 24 hours post ischemia. There was a significant increase in T-cells at day 3, but these levels remained far below the other subpopulations and returned to baseline by day 7, mirroring what is reported in the literature for similar studies of the filament occlusion model. In contrast to human stroke and the filament occlusion model, neutrophil levels were so low as to make accurate quantification impossible. Thus, there are some important differences in the dynamics of immune cell invasion between PT, human stroke, and the filament occlusion model that should be considered when designing animal studies and translating data to human studies.

The present studies revealed increases in *Tnf-α* ($p<0.05$) and *Il-1β* at 24 hours, consistent with reports in the literature (Schroeter et al., 2003). Our studies were the first to examine these increases at later time points, where *Tnf-α* continued to increase and IL-1B reached a second peak at 7 days. The dynamics of *E-selectin*, *Tlr-4*, Ifn-γ, *Il-10*, and *Foxp*3 were also examined for the first time. *E-selectin* increased over time and peaked at 7 days ($p<0.05$) while *Tlr-4* levels did not change significantly. *Il-10* levels were significantly decreased 24 hours following ischemia, potentially representing a shift from an anti-inflammatory M2 microglia phenotype toward a proinflammatory M1 phenotype. *Ifn-γ* and *Foxp3* levels did not change significantly. This data suggests that T-cell function may not play a critical role in post ischemic inflammatory damage in PT.

CHAPTER 7

CONCLUSION

The present studies demonstrate the effectiveness of a CB2R selective agonist O-1966 in attenuating infarct expansion and preserving memory and learning functions in the subacute phase following permanent cerebral ischemia induced by photothrombosis. The very strong correlation between infarct size and performance on autoshaping behavioral testing suggests the mechanism involves prevention of infarct expansion into tissue involved in learning and memory function. Additionally, this correlation shows that these techniques may be used in tandem to assess the efficacy of potential therapeutics that target postischemic cognitive deficits.

O-1966 induced decreases in *Il-6* and *Il-1β* provides evidence that this attenuation of infarct expansion is anti-inflammatory in nature, but it is yet unknown whether this is a result of direct or indirect effects of these molecules on learning and memory functions. Correlation studies between cytokine expression and performance on cognitive tests may provide clues to test this theory. Interestingly, in contrast to a previous study, only the pro-inflammatory cytokines were affected, while the anti-inflammatory mediators *Tgf-β* and *Il-10* were unaffected. These anti-inflammatory mediators are essential for the beneficial functions of inflammation including tissue repair, and the lack of an effect of O-1966 may be valuable in selective targeting of only the harmful profinflammatory components of the postischemic inflammatory response.

Of equal importance in designing anti-inflammatory drugs for use in stroke treatment is characterization of the different animal models of ischemic stroke. We

sought to characterize the temporal dynamics of inflammation in PT in terms of immune cell invasion, microglia proliferation, and cytokine expression. In contrast to the commonly used filament model of cerebral ischemia, there was a more protracted invasion of monocytes, better resembling the time course of clinical stroke (160). However, neutrophils were not present in PT, a finding that conflicts with both clinical stroke and the filament method. The relative contributions of different immune cells, and the time course of their invasion and proliferation must be defined and taken into consideration when designing experiments examining anti-inflammatory neuroprotective agents in cerebral ischemia.

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