Alternative Targets for the Treatment of Stroke

by

Craig T. Ajmo, Jr.

A dissertation in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
Department of Molecular Pharmacology & Physiology
College of Medicine
University of South Florida

Major Professor: Keith R. Pennypacker, Ph.D.
Alison E. Willing, Ph.D.
Javier Cuevas, Ph.D.
Paul E. Gottschall, Ph.D.
Lynn Wecker, Ph.D.

Date of Approval:
June 15, 2007

Keywords: sigma receptor, spleen, neurodegeneration, inflammation, infarction

© Copyright 2007, Craig T. Ajmo, Jr.
Note to reader: The original of this document contains color that is necessary for understanding the data. The original dissertation is on file with the USF library in Tampa, Florida.
DEDICATION

This dissertation is dedicated to all my family, friends and wife. Their love and support helped see me through this adventure.
ACKNOWLEDGEMENTS

While this is an individual work, I could have never completed this venture without the guidance and support of many people. I would first like to thank my major professor, Dr. Keith R. Pennypacker for taking me into his laboratory. His tireless efforts, constant vigilance, our continual exchange of humor and common interests made the conclusion of this work possible. I would like to thank Lisa A Collier and Dionne Vernon for their help on all the projects. Without them I may still be sectioning brains. I would like to thank Shane N. Collier for his help with the image analysis, and Jennifer Newcomb for her help with the MCAO surgery. I would like to thank Aaron A. Hall for help and brain storming sessions regarding these projects. I extend my gratitude to Dr. Alison E. Willing, and Dr. Javier Cuevas for their mentorship and guidance with these projects. I could not have done this without the support of my family, both those who were present and those who have past during this time. Finally I would like to thank my wife Joanne, whose support, love, and devotion saw me through all the good and bad times.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of Figures</td>
<td>iii</td>
</tr>
<tr>
<td>Abstract</td>
<td>v</td>
</tr>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Stroke</td>
<td>1</td>
</tr>
<tr>
<td>Stroke Health Risks</td>
<td>2</td>
</tr>
<tr>
<td>Types of Stroke</td>
<td>3</td>
</tr>
<tr>
<td>The Infarction</td>
<td>4</td>
</tr>
<tr>
<td>Neurodegeneration</td>
<td>5</td>
</tr>
<tr>
<td>Inflammation: Peripheral Immune Cell Response</td>
<td>8</td>
</tr>
<tr>
<td>Inflammation: Resident Immune Cell Response and Astrogliosis</td>
<td>12</td>
</tr>
<tr>
<td>Inflammation: Biochemical Mediators of Stroke</td>
<td>14</td>
</tr>
<tr>
<td>Current Treatment</td>
<td>20</td>
</tr>
<tr>
<td>Tissue Plasminogen Activator</td>
<td>20</td>
</tr>
<tr>
<td>Failed Treatments</td>
<td>21</td>
</tr>
<tr>
<td>Novel Approaches for Treatment of Stroke</td>
<td>24</td>
</tr>
<tr>
<td>Human Umbilical Cord Blood</td>
<td>25</td>
</tr>
<tr>
<td>Sigma Receptors</td>
<td>26</td>
</tr>
<tr>
<td>1,3-Di-o-tolylguanidine</td>
<td>27</td>
</tr>
<tr>
<td>Spleen</td>
<td>27</td>
</tr>
<tr>
<td>The Current Study</td>
<td>29</td>
</tr>
<tr>
<td>References</td>
<td>30</td>
</tr>
</tbody>
</table>

## Chapter 1: Sigma Receptor Activation Reduces Infarct Size at 24 Hours after Permanent Middle Cerebral Artery Occlusion in Rats.

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>55</td>
</tr>
<tr>
<td>Introduction</td>
<td>57</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>60</td>
</tr>
<tr>
<td>Animals</td>
<td>60</td>
</tr>
<tr>
<td>Permanent Middle Cerebral Artery Occlusion</td>
<td>60</td>
</tr>
<tr>
<td>Treatments and Tissue Preparation</td>
<td>61</td>
</tr>
<tr>
<td>Fluoro-Jade Histochemistry</td>
<td>61</td>
</tr>
<tr>
<td>Immunohistochemistry and Histochemistry</td>
<td>62</td>
</tr>
<tr>
<td>Infarct Area and Immunohistochemistry Quantification</td>
<td>63</td>
</tr>
<tr>
<td>Statistical Analysis</td>
<td>64</td>
</tr>
<tr>
<td>Results</td>
<td>64</td>
</tr>
<tr>
<td>Discussion</td>
<td>68</td>
</tr>
<tr>
<td>References</td>
<td>88</td>
</tr>
</tbody>
</table>
Chapter 2: The Spleen Contributes to Stroke Induced Neurodegeneration

Abstract 98
Introduction 99
Materials and Methods 101
Animals 101
Splenectomy 101
Lazar Doppler Radar Blood Flow Measurement 101
Permanent Middle Cerebral Artery Occlusion 102
Brain Extraction and Sectioning 102
Fluoro-Jade Histochemistry 103
Nissl Staining with Thionin 103
Immunohistochemistry 104
Infarct Volume Quantification 104
Blood Smear Preparation and Giemsa Staining 105
Statistical Analysis 105
Results 106
Discussion 111
References 121

Chapter 3: Splenic Response to Stroke is not Dependent on Direct Autonomic Neurotransmission via the Splenic Nerve

Abstract 127
Introduction 129
Materials and Methods 131
Animals 131
Splenectomy 132
Lazar Doppler Radar Blood Flow Measurement 132
Permanent Middle Cerebral Artery Occlusion 133
Brain Extraction and Sectioning 133
Fluoro-Jade Histochemistry 133
Nissl Staining with Thionin 134
Immunohistochemistry 134
Infarct Volume Quantification 135
Flow Cytometry 135
Blood Smear Preparation and Giemsa Staining 137
Statistical Analysis 137
Results 138
Discussion 141
References 158

Conclusions 161
References 180
ABOUT THE AUTHOR END PAGE
LIST OF FIGURES

Chapter 1:

Figure 1  Effects of sigma receptor ligands on MCAO survival rates.  
Figure 2  Figure 2: DTG treatment reduced Fluoro-Jade staining when administered 24 hours post-MCAO.  
Figure 3  Quantification of DTG-elicited reduction in post-MCAO Fluoro-Jade staining.  
Figure 4  DTG treatment increases NeuN immunostaining when administered 24 hours post-MCAO.  
Figure 5  DTG treatment at delayed time points significantly increases the number of NeuN positive cells following MCAO.  
Figure 6  DTG decreases the intensity of GFAP immunostaining surrounding the infarct zone when administered 24 hours post-MCAO.  
Figure 7  DTG treatment decreases Isolectin IB4 binding when administered 24 hours post-MCAO.  

Chapter 2:

Figure 8  Histological pathology of infarct in brain tissue of splenectomized rats 96hr post MCAO.  
Figure 9  Immunohistochemical labeling of activated microglia and infiltrating macrophages at the level of infarction.  
Figure 10  MPO staining of neutrophils in the infarct zone.  
Figure 11  White blood cell profiles during treatments.
Chapter 3:

Figure 12  Denervation has no effect on spleen shrinkage resulting from stroke.  144

Figure 13  Fluoro-Jade labeled neurodegeneration of the infarct in brain tissue of denervated rats 48hr post MCAO.  146

Figure 14  Thionin labeling of Nissl bodies of surviving brain tissue of denervated rats 48hr post MCAO.  148

Figure 15  Analysis of infarction volumes labeled by histological staining.  150

Figure 16  Flow cytometry analysis of spleen homogenates.  152

Figure 17  Flow cytometry analysis of blood samples.  154

Figure 18  Analysis of blood smear white blood cell populations by Giemsa staining.  156

Conclusions:

Figure 19  Summary schematic of the effects of DTG on MCAO when administered 24 hours after MCAO.  174

Figure 20  Summary schematic of the effects of splenectomy prior to MCAO.  176

Figure 21  Summary schematic of the effects of denervation of the spleen prior to MCAO.  178
Alternative Targets for the Treatment of Stroke

Craig T. Ajmo, Jr.

ABSTRACT

Stroke is cerebrovascular injury that has been reported to be the third leading cause of death and the first leading cause of disability in the world (W. H. O. 2007). Currently, there is only one FDA approved treatment for stroke which is recombinant tissue plasminogen activator. This treatment has a narrow therapeutic window of three hours after ischemic stroke and can adversely cause the production of oxygen free radicals and intracranial hemorrhage. These limitations result in only 2-3% of all stroke victims as being candidates for this therapy as many patients do not arrive at the hospital in time to receive treatment, are not properly diagnosed, or do not know that they have had a stroke within this three hour time period. The purpose of these experiments was to elucidate alternative targets of stroke for the benefit of developing new treatments that stimulate neuroprotective and anti-inflammatory effects at the site of injury. It has been shown that transfusion of human umbilical cord blood cells up to 48 hours after stroke significantly reduces infarction and we have examined other targets that mimic these effects. We have shown that sigma receptor activation by DTG, a high affinity universal sigma agonist, reduces infarct volume when administered 24 hours after stroke. This suggests that modulation of
neurodegenerative and inflammatory responses can extend the therapeutic window of treatment. For the first time, evidence is provided that shows that the spleen enhances the neurodegeneration caused by stroke as splenectomy prior to stroke profoundly decreased infarction volume. Finally, we studied signaling mechanisms of the splenic reaction to stroke and determined that this response is not directly dependent on neurotransmission via the splenic nerve. Denervation of the spleen prior to stroke showed no changes in neurodegenerative load at the site of injury in rat brains when compared to those subjected to stroke only. Overall, these experiments provide evidence showing that targets mediating neuroprotective and anti-inflammatory effects can lead to novel therapeutic interventions of stroke.
INTRODUCTION

Stroke

Stroke is a cerebrovascular injury that is the result of the obstruction or hemorrhagic interruption of blood flow to the brain causing damage to the cerebral tissue. Annually, 15 million people worldwide suffer a stroke. Of these, 5 million die and another 5 million are left permanently disabled, placing a burden on family and community making this injury the third leading cause of death and first leading cause of disability in the world. The major risk factors for stroke are similar to those for coronary heart disease, with high blood pressure and tobacco use the most significant modifiable risks. Atrial fibrillation, heart failure and heart attack are other important risk factors (World Health Organization 2007).

Stroke carries a high risk of death and survivors can experience loss of vision and/or speech, paralysis, and confusion. Even with advances in technology and facilities, 60% of those who suffer a stroke die or become dependent (W.H.O. 2007). In 2004 The American Heart Association estimated that the direct and indirect costs of stroke are 53.6 million dollars (American Heart Association 2007). Based on these statistics and costs, stroke needs to be a main concern for researchers and health care providers for the purpose of developing novel treatments as well as reducing the risk factors that increase the chances of this cerebral injury.
Stroke Health Risks

There are several health risks that can increase the incidence of stroke in an individual. Some health risks can not be changed which are a function of heredity. Those threats that can not be changed revolve around age, heredity, sex, and prior stroke. However, the majority of the causes of stroke are based on personal lifestyle and can be altered with proper knowledge. The chance of stroke more than doubles for each decade of life after the age of 55 and people who have had parents or grandparents that have suffered a stroke are also at higher risk. Heredity is also an issue of stroke resulting from race. Blacks have a higher risk than Caucasians partly because of higher risks of high blood pressure, diabetes and high blood pressure in this group. Gender is a factor in that men are more likely to have a stroke than women. However, more than half of all total stroke deaths occur in women (A.H.A 2007).

Risk factors that can be monitored and altered are very similar to those for coronary heart disease. High blood pressure, smoking, diabetes, high cholesterol, poor diet, obesity and physical inactivity can all lead to stroke. Physical defects in the circulatory system are problematic but are also treatable. Atrial fibrillation, a heart rhythm disorder, can result in the pooling of blood and clot formation at the atria. Carotid and other artery diseases are caused by the narrowing of arteries by fatty deposits from atherosclerosis. These fatty deposits can eventually break free and cause vessel blockage. These risk factors all have the potential of instigating one of the types of stroke and need to be routinely monitored so as to inhibit such an injury.
Types of Stroke

There are two main types of stroke that occur, hemorrhagic which is blood vessel rupture and ischemic caused by blockage of a blood vessel. There are two subclasses of hemorrhagic stroke, subarachnoid and cerebral hemorrhage. Subarachnoid hemorrhage occurs when a blood vessel on the brain’s surface ruptures and bleeds into the space between the skull and brain. Cerebral hemorrhage is the rupturing of a blood vessel inside the brain. This results in the flooding of the surrounding cerebral tissue with blood.

Subarachnoid or cerebral hemorrhage results in the loss of a constant blood supply such that the cells of the brain are no longer obtaining the nutrients and oxygen they need to continue to function. Pooled blood from the hemorrhage can also put pressure on surrounding brain tissue and interfere with brain function (A.H.A 2007). The amount of bleeding establishes the harshness of the cerebral hemorrhages. Typically, people with cerebral hemorrhages die of increased pressure on their brains; however pressure from the blood compresses part of the brain. If the person survives, gradually the pressure goes away. Then the brain may regain some of its former function (A.H.A 2007).

Ischemic stroke is the most common type of stroke accounting for 88% of all reported stroke incidents and is the main focus of the following studies. This type of stroke occurs when a thrombus, a blood clot that forms in a blood vessel, blocks blood flow in an artery bringing blood to part of the brain. When the blood clot forms within an artery of the brain, it's called a cerebral thrombotic stroke.
An embolus, a plaque that is formed elsewhere in the body, may also cause an ischemic stroke. The embolus is carried by the bloodstream until it becomes lodged in an artery leading to or in the brain and is referred to as a cerebral embolism (A.H.A 2007).

**The Infarction**

Blockage of an artery leading to the brain by an embolus or thrombus inhibits blood flow bringing oxygen and nutrients to the cells of the tissue. This lack of sustenance results in two areas of expanding damage surrounding the artery or arteries that are no longer being perfused with blood. The first region directly adjacent to those arteries is the core ischemic region, which is widely considered to be an area of irreversible cell death. At the ischemic core, drastic changes in physiology, biochemistry, and energy metabolism occur (Lipton 1999). Almost immediately after blood perfusion inhibition, there is a rapid depolarization within brain cells leading to an increase of extracellular $K^+$ and a large influx of $Ca^{2+}$ into the cells. Levels of adenosine triphosphate (ATP) at the core drop markedly to approximately 25% showing arrested metabolism (Lipton 1999). Reactive oxygen species are also present at the central region and can be produced by a variety of mechanisms including glutamate excitation, intracellular calcium overload, mitochondrial cytochrome C release, nitric oxide synthetase, caspase activation, and xanthine oxidase (Dirnagl et al. 1999; Lewen et al. 2000). Damage is caused at the core ischemic region as a result of these reactive oxygen species, but because the ischemic core has little or no blood
The levels of oxygen free radicals are less than at the penumbra, the region surrounding the ischemic core (Dirnagl et al. 1999).

The penumbra is classified as a functionally impaired yet still viable tissue region surrounding the ischemic core as it receives minor amounts of blood perfusion from collateral arteries (Bandera et al. 2006). The penumbra is central to most stroke-related investigations as the penumbra contains areas that have the potential of recovering if appropriate treatment is administered. Physiology, biochemistry, and energy metabolism alterations occur at a slower rate than in the core but the progression of these alterations will eventually lead to the same cellular fate as the core. Within the penumbra there are no rapid depolarizations. However, sporadic transient depolarizations (Ginsberg and Pulsinelli 1994; Mies et al. 1991) are much more common in the penumbra and are dependent on glutamate release from the infarct core (Lipton 1999). ATP levels are maintained at approximately 50-70% of base levels and glucose utilization becomes elevated in the penumbra at the early stages of the ischemic insult but fall off to approximately 50% of base levels, equal to the core, by 3.5 hours after stroke (Yao et al. 1995). Cell death eventually follows in the penumbra due to the failure of energy (ATP) production, glutamate-mediated toxicity with influx of Na+ and Ca²⁺, and mitochondrial failure.

**Neurodegeneration**

Neurodegeneration is characterized by the loss of neuron function and eventually cell death. Degeneration and neuronal cell death occur through necrotic and apoptotic mechanisms (Weinberger 2006). Necrosis is
characterized by the disruption of cellular homeostasis with nuclear dissolution, membrane dysfunction, cell swelling, and severe mitochondrial damage (Bhardwaj et al. 2003; Choi 1992). Excessive release of excitatory neurotransmitters such as glutamate (Park et al. 1988; Wu and Fujikawa 2002) results in an increased activation of the glutamate receptors α-amino-3-hydroxy-5-methyl-4-isozazolone-propionic acid (AMPA), receptors which increase sodium uptake by the neurons (Endres and Dirnagl 2002) and N-methly-D-aspartate (NMDA), receptors that cause an influx of intracellular calcium (Ientile et al. 2002; Kahn et al. 1997). While sodium and calcium exchange pumps are able to remove some of the calcium that has entered the cells, three sodium ions enter for every two calcium ions that are removed. This results in edema or cell swelling as water is retained by the osmotic imbalance (Rosenberg 1999). Energy requiring calcium pumps fail as ATP levels fall, adding to the calcium levels in the cells (Rosenberg 1999). The increase of intracellular calcium resulting from glutamate excitotoxicity will eventually lead to the activation of proteases, such as caspases and matrix metalloproteinases, which cause irreversible damage to cellular membranes and DNA (White et al. 2000). Cells are unable to respond to proteolytic injury and die (White et al. 2000).

Besides calcium toxicity, NMDA activation by glutamate also leads to the production of the free radical nitric oxide (NO). After the activation of NMDA receptors, calcium is transiently increased in the cytosol and forms a complex with calmodulin (Moncada and Bolanos 2006). This complex binds to and activates constitutive neuronal nitric oxide synthase (nNOS) (Moncada and
The nNOS will then produce NO through enzymatic oxidation of the guanidino group of L-arginine in the presence of O$_2$, NADPH and tetrahydrobiopterin (Marletta et al. 1998). Peroxynitrite, a free oxygen metabolite, is eventually formed when NO and superoxide anion react (Beckman and Koppenol 1996). Superoxide anions are a byproduct of mitochondrial respiration and are constantly produced in conjunction with NADPH oxidases (Virag et al. 2003). Peroxynitrite initiates lipid peroxidation, causes DNA breakage and reacts with thiols (Radi et al. 1991; Salgo et al. 1995a; Salgo et al. 1995b). Cells are again incapable of repairing this damage and die.

Catecholamines are neurotransmitters that contribute to the necrosis of neurons by being excessively released at the time of stroke. Ischemic events inhibit the uptake of the neurotransmitters resulting in a build up of catecholamines outside the cells (Weinberger and Cohen 1982; Weinberger et al. 1985). Dopamine, a catecholamine that acts as a neurotransmitter in the central nervous system, is released at the time of initial ischemic event and is converted to dihydroxyphenylacetic acid (DOPAC) (Weinberger and Nieves-Rosa 1987). The formation of DOPAC results in the production of hydrogen peroxide which forms reactive oxygen species. These reactive oxygen species cause lipid peroxidation and denature proteins resulting in the necrosis of the neuropil, the unmyelinated neuronal processes of the neurons, in the infarction (Rabinovic et al. 2000).

Apoptotic cellular death occurs mainly in the penumbra of the infarction. It is a process defined as a self-destructive cell death that involves activation of
mechanisms encoded in the genomes of all higher eukaryotes and identified by chromatin condensation and increased mitochondria membrane permeability (Weinberger 2006). Compared to necrosis, there is no cell membrane and mitochondria disruption and there is no inflammation or injury to surrounding tissue (Johnson et al. 1995; Kerr et al. 1972). Although there is no inflammation in surrounding tissue, inflammatory mediators such as the cytokine tumor necrosis factor alpha (TNF-α) play an initial role in the events that lead to apoptosis (Hallenbeck 2002). Cytokines are increased within hours of the initial insult and result from immediate early gene products, such as c-fos and c-jun, activating transcription sites of the cytokine genes (Rosenberg 1999). TNF-α initiates apoptotic pathways by activating the cell membrane bound TNF receptors immediately starting caspase cascades. Activation of this receptor also results in mitochondria-mediated apoptosis by stimulating the production of Bid, a proapoptotic Bcl-2 family member. Bid then activates Bax, another Bcl-2 family member, which localizes to the mitochondrial membrane increasing its permeability. Translocation of these Bcl-2 members into the permeable membrane of the mitochondria induces cytochrome c release. Cytochrome c promotes the activation of procaspase-9 which allows caspase-9 to activate caspase-3. Caspase-3 causes irreparable DNA damage leading to apoptosis (Atlante et al. 2003; Zukin et al. 2004).

**Inflammation: Peripheral Immune Cell Response**

Inflammation is the complex biological response of the body to harmful stimuli and in the case of stroke is the response of the brain parenchyma to
insult. It is typically characterized by the infiltration of leukocytes, which in the case of cerebral ischemia are mainly polymorphonuclear leukocytes including neutrophils, monocytes/macrophages, lymphocytes and the activation of microglia, the resident immune cells of the brain (Kochanek and Hallenbeck 1992). Astrocytes, neuronal support cells, also contribute to inflammation during insult (Ridet et al. 1997). The collective action of these immune and neuronal support cells is to remove damaged tissue, but they have the potential to enhance infarction expansion ultimately resulting in more damage in the brain. In response to ischemia, transcription factors of brain cells produce inflammatory proteins that orchestrate necrosis and apoptosis (Feuerstein 2006). After interruption of cerebral blood flow, tissue injury begins with an inflammatory reaction. This reaction requires the infiltration of leukocytes, neutrophils and macrophages, which are the cellular mediators of subsequent microvessel obstruction, edema formation, cellular necrosis, and tissue infarction (Chou et al. 2004; Clark et al. 1993). Intervention of this reaction can reduce the deleterious effects that result from the inflammatory response. Studies have shown that post-ischemic administration of antibodies directed against adhesion molecules and infiltrating monocytes/macrophages, such as CD11b/CD18 and ED1 respectively, reduce ischemic cell damage and apoptosis in rats subjected to transient MCAO (Chopp et al. 1996; Chopp et al. 1994; Zhang et al. 1995a; Zhang et al. 1994b; Zhang et al. 1995b).

Leukocytes circulate in the bloodstream and enter the tissues only when they are recruited to sites of infection or inflammation. Under normal conditions
neutrophils do not interact closely with the cerebral endothelium. However, as a result of blood flow obstruction, these cells are the first inflammatory peripheral cell type present in the infarction (Kochanek and Hallenbeck 1992). Accumulation of these cells begins within three hours at the site of injury and peaks at about 24 hours after infarction. Afterwards the number of neutrophils declines (Pantoni et al. 1998). Neutrophil infiltration is preceded by increased expression of cytokine-induced neutrophil chemoattractant (CINC). Migration to the site of injury in response to CINC results in binding of neutrophils to the endothelial cells. This accumulation of neutrophils impedes blood vessels causing further obstruction of blood flow extending the infarction (Pantoni et al. 1998). Increased expression of CINC mRNA has been detected in the cortex and caudate putamen in rat brains injured by ischemia as early as 6 hours (Yamasaki et al. 1995). Evidence that shows that CINC attracts neutrophils to ischemic damage comes from transient stroke rodent experiments that show that CINC is detectable in brain tissue before neutrophil infiltration at three hours after reperfusion (Yamasaki et al. 1995). Once attracted to endothelial and brain cells, neutrophils incite tissue damage through the release of oxygen-free radicals, cytokines, and proteolytic enzymes (Akopov et al. 1996; Barone and Feuerstein 1999; Emerich et al. 2002; Zhang et al. 1994a). In addition, neutrophils have also been implicated in ischemic injury as a source of MMP-9, a protease that degrades the basal lamina and mediates breakdown of the blood brain barrier after injury (Justicia et al. 2003).
Monocytes/macrophages are the most abundant leukocyte that enters the brain after focal cerebral ischemia (Stoll et al. 1998). These cells contribute to inflammation and infarction by releasing vasoconstrictive mediators (superoxidase anions), cytokines, free oxygen radicals, NO, and proteolytic enzymes that damage cell membranes, alter the blood brain barrier, and contribute to post-ischemic edema (Feuerstein 2006; Geng 1997; Hendriks et al. 2005; Laffi et al. 1995). Permanent middle cerebral artery occlusion (MCAO) causes monocytes/macrophages to start infiltrating the parenchyma at 12 hours after injury (Clark et al. 1993). Once in the parenchyma monocytes/macrophages become indistinguishable from microglia, the resident macrophages of the brain, based on morphological grounds, the expression of the same immunocytochemical markers and performing the same inflammatory actions during stroke (Stoll et al. 1998). Monocyte/macrophage infiltration is preceded by the expression of monocyte chemoattractant protein-1 (MCP-1). This chemoattractant expression has been reported to be found early after MCAO, MCP-1 mRNA increases six hours after MCAO and remains elevated up to 5 days after injury (Yamasaki et al. 1995). Studies have localized MCP-1 expression at early stages of ischemic injury to endothelial cells, astrocytes, and microglia (Wang et al. 1995).

While the inflammatory response that occurs after stroke is antigen nonspecific and mediated by the innate immune system, there is an antigen nonspecific inflammatory response mediated by lymphocytes as well (Gee et al. 2007). Breaching of the blood brain barrier allows the adaptive immune system
to encounter novel central nervous antigens (Gee et al. 2007). In the brain neutrophils are the first leukocytes to accumulate and infiltrate the parenchyma, followed by monocytes and then T lymphocytes (Schroeter et al. 1994). Although data supporting the role of T cells is limited, there is evidence that T lymphocyte infiltration occurs at the early phase of ischemia and accompanies neutrophil and macrophage infiltration (Jander et al. 1995; Schroeter et al. 1994; Schwab et al. 2001; Vendrame et al. 2005). Studies suggest that T cells work in vascular beds to recruit other leukocyte subsets, release inflammatory cytokines and attack antigens found in myelin sheaths which result in demyelination of neurons (Gee et al. 2007; Yilmaz et al. 2006; Zipp and Aktas 2006). Relatively little information is known about the actions of B cells in ischemia (Yilmaz et al. 2006). Flow cytometry analysis of ipsilateral hemisphere brain homogenates show increased presence of the B cell membrane molecular marker CD45⁺/CD11b⁺ from rats subjected to MCAO (Vendrame et al. 2005). Recent studies have shown that B cells contribute to ischemia/reperfusion injury in the kidneys through release of soluble factors and are independent of neutrophil, macrophage, and T cell recruitment (Yilmaz et al. 2006). This suggests that B cells contribute to the overall damage occurring in the brain after stroke and must be investigated further.

**Inflammation: Resident Immune Cell Response and Astrogliosis**

In the resident immune response of the brain, microglia, which arise from peripheral mesoderm (Chan et al. 2007), are the first cells to respond to cerebral
ischemia (Lai and Todd 2006). Microglia are resident brain macrophages that adopt a typical ramified morphology in the central nervous system environment (Stoll et al. 1998). In response to pathogens, microglia exert a multiple of functions including transformation into phagocytes, presenting antigens to T cells, or producing neurotoxic factors for pathogen removal (Stoll et al. 1998). Under extreme brain injury conditions, such as ischemia, microglia become over activated inducing significant and high detrimental neurotoxic effects by the excess production of cytotoxic factors such as superoxide, NO, and TNF-α (Danton and Dietrich 2003; Dirnagl et al. 1999; Polazzi and Contestabile 2002). Activation of these cells by pathogens or injury is also accompanied by morphological transition with the loss of the ramified appearance and the microglia taking on a more amoeboid shape (Stoll et al. 1998). At the time of stroke, microglia have also been shown to be involved in neuroprotection by releasing neurotropins, and growth hormones, as well as aid in glutamate re-uptake which reduces necrotic and apoptotic cascades (Lai and Todd 2006). Recent evidence continues to support the neuroprotective role of microglia. Selective ablation of the proliferation of microglial cells during ischemic injury actually increases infarct damage in the brain of transgenic mice (Lalancette-Hebert et al. 2007).

Astrocytes, brain cells of neuroectodermal origin, also respond to ischemic injury (Mucke and Eddleston 1993). Under normal conditions, astrocytes perform several functions that are essential for normal neuronal activity, including glutamate uptake, glutamate release, $K^+$ and $H^+$ buffering, and metabolic and
In response to injury, astrocytes undergo morphological changes resulting in elongated processes and up-regulate intermediate filament proteins, particularly glial fibrillary acidic protein (GFAP) (Swanson et al. 2004). When these cells exhibit these changes the astrocytes are referred to as reactive and this is perhaps the best known hallmark of gliosis (Pekny and Nilsson 2005). Reactive gliosis is characterized by the hypertrophy of astrocytes and proliferation of both astrocytes and microglial cells around the injured region forming the glial scar (Ridet et al. 1997). Reactive astrocytes produce and release inflammatory mediators such as cytokines and chemokines. These mediators will increase the inflammatory effects at the site of injury by activating necrotic/apoptotic pathways and aiding in inflammatory cell recruitment (Ridet et al. 1997). Astrocytes also amplify inflammation and neurodegeneration by releasing glutamate, reducing glutamate uptake, and secreting nitric oxide (Swanson et al. 2004).

**Inflammation: Biochemical Mediators of Stroke**

In response to ischemia in the brain, immune and injury response cells transcribe inflammatory mediators that coordinate the biochemical pathways of stroke. Such mediators include cytokines, chemokines, adhesion molecules, lipid mediators and kinins. The uninjured brain has virtually no or extremely low levels of expression of the above mentioned mediators, but in response to ischemic injury, these molecules become highly expressed (Pantoni et al. 1998).
Pro-inflammatory cytokines, which include interleukins, are low molecular weight glycoproteins that act as intercellular messengers and are produced by macrophages, monocytes, lymphocytes, endothelial cells, fibroblasts, platelets, and many other cell types (Pantoni et al. 1998). These cytokines have various functions during cerebral ischemia and act at low concentrations on specific target cell receptors, whose expression is also mediated by the cytokines (Pantoni et al. 1998). Binding of cytokines to receptors activates protein kinases and phosphatases which initiate apoptosis through the activation of transcription factors and DNA damage (Pantoni et al. 1998). Cytokines attract leukocytes to the site of insult as well as stimulate the synthesis of adhesion molecules in the invading leukocytes, endothelial, glial, and other cells promoting the inflammatory response (Pantoni et al. 1998). Cytokines also contribute to ischemic tissue damage by altering levels of endogenous mediators of thrombogenesis, such as tissue plasminogen activator (Huang et al. 2006).

There is variation and overlap in the functions of the cytokines in ischemic injury. IL-1 is an extensively studied cytokine that exits in two forms IL-1α and IL-1β. These forms act mainly through two receptors types, type I and II. The type I receptor is found on a variety of cells and binds both forms of IL-1 with similar affinity. The type II receptor is found on the cell surface of neutrophils, B lymphocytes, and macrophages and binds IL-1β with a higher affinity (Buttini et al. 1994; Dinarello 1996; Huang et al. 2006; Mayadas et al. 1993; Polazzi and Contestabile 2002; Zhang et al. 1998). IL-1, particularly IL-1β, induces the expression of ICAM-1, an adhesion molecule on the surface endothelial cells
The increase of ICAM-1 is presumably a link between the up-regulation of IL-1 after ischemia and the following influx of neutrophils (Huang et al. 2006). IL-6 has similar biological activities as IL-1 in the development of the inflammatory response (Lindsberg et al. 1996) but has also been shown to induce phospholipase A$_2$ gene expression which stimulates the production of leukotrienes, prostaglandins, and platelet activating factor, all of which are involved in ischemic brain damage (Crowl et al. 1991). Like the interleukins, TNF-$\alpha$ induces the expression of adhesion molecules by glial and endothelial cells. This promotes neutrophil adherence and accumulation into the microvasculature (Liu et al. 1994). This cytokine also causes blood brain barrier alterations, pro-adhesive transformations of endothelial cell surfaces to take hold of circulating immune cells and glial cell activation to form scars as part of the repair and remodeling process in response to ischemia (Pantoni et al. 1998).

While TNF-$\alpha$ and IL-1 promote adhesion between endothelial cells and leukocytes, they are actually poor attractants for leukocytes (Pantoni et al. 1998). Other chemoattractant cytokines, CINC and MCP-1, are referred to as “chemokines” and are specifically involved in guiding leukocytes through brain parenchyma towards the site of ischemic injury (Pantoni et al. 1998). Chemokines are low molecular weight molecules that share a structural pattern of four cystine residues (Furie and Randolph 1995). They are divided into two main families (C-x-C and C-C) according to the presence or absence of an amino acid between the residues of the two most amino-proximal cystines (Furie and Randolph 1995). The structural distinction results in different attraction of cells.
The C-X-C chemokines tend to attract neutrophils, where the C-C chemokines preferentially act on monocytes/macrophages (Pantoni et al. 1998). Each cytokine family binds to specific receptors formed by seven transmembrane domains that activate G proteins. This activation leads to the production of intracellular protein kinases which activate cellular inflammatory responses (Gourmala et al. 1997).

Cell adhesion molecules mediate the adhesion of leukocytes to endothelial cells as well as the immune cell migration from the microvasculature to the site of injury by a variety of molecules found on the membranes of both leukocytes and endothelial cells (Pantoni et al. 1998). Adhesion molecules are divided into three structural classes: (1) selectins, (2) integrins, and (3) proteins of the immunoglobulin superfamily. Selectins are glycoproteins that mediate low-affinity endothelial-leukocyte interactions, thus promoting the margination and rolling of leukocytes along endothelial cells. Integrins are heterodimeric membrane glycoproteins that play a role in the extracellular matrix and cell-cell interaction. At the level of the basal lamina, integrins link endothelial cells to components of the extracellular matrix, such as laminin and collagen. In the brain, integrins join the endothelial cells, astrocytes, and basal lamina that comprise the blood brain barrier and are crucial in maintaining the integrity of the cerebral microvasculature (Haring et al. 1996; Huang et al. 2006). The immunoglobulin superfamily mediates cell adhesion of leukocytes to endothelia at low shear forces and creates a stronger attachment compared to the selectins (Pantoni et al. 1998).
Lipid mediators of arachidonic acid play a role in the inflammation that is occurring at the site of injury (Tzeng et al. 2005). Prostaglandins, one subset of these lipid mediators, are synthesized from arachidonic acid which is produced from the release of phospholipase A$_2$ (PLA$_2$) (Tzeng et al. 2005). Calcium accumulation in brain cells by energy failure resulting from loss of blood activates PLA$_2$ which hydrolyses the release of arachidonic acid from glycerophospholipids (Chen and Bazan 2005). Prostaglandins are then synthesized from arachidonic acid and cyclooxygenase (COX) enzymes (Tzeng et al. 2005). Prostaglandins contribute to inflammation by altering the integrity of membranes, disrupt the activity of ion channels, and inhibit glutamate uptake (Griffin et al. 1994). Prostaglandins are excessively produced in the brain at the time of injury by astrocytes and microglia. Astrocytes and microglia respond to pro-inflammatory cytokines by releasing COX which increases the production of the prostaglandins and then act as the pro-inflammatory mediators (Tzeng et al. 2005).

Another group of inflammatory lipid mediators synthesized from arachidonic acid are the leukotrienes (Funk 2005). Production of these mediators begins at the nuclear membranes of inflammatory cells (macrophages, neutrophils, and microglia) in response to inflammatory stimuli such as cytokines (Funk 2001; Henderson 1994; Lewis et al. 1990; Samuelsson 1983). Where prostaglandin synthesis was dependent on the cyclooxygenase enzyme, leukotriene production is dependent on 5-lipoxygenase (5-LO) which catalyses the conversion of arachidonic acid to 5-hydroperoxyeicosatetraenoic acid and the unstable leukotrieneA$_4$ (LTA$_4$) with the aid of the accessory protein 5-LO-
activating protein (FLAP) (Dixon et al. 1990; Miller et al. 1990). Here, the
pathway branches as LTA$_4$ hyrolase can metabolize LTA$_4$ to leukotriene B$_4$
(LTB$_4$). LTB$_4$ acts as a potent neutrophil chemotaxin, promotes the adhesion of
leukocytes to vascular endothelium and recruits T lymphocytes to sites of
inflammation (Ford-Hutchinson et al. 1980; Gimbrone et al. 1984; Goodarzi et al.
2003; Ott et al. 2003; Radmark et al. 1984). Alternatively, LTA$_4$ can be
conjugated with glutathione to yield a series of leukotrienes known as LTC$_4$,
LTD$_4$, and LTE$_4$ (Samuelsson 1983). Collectively these are referred to as
cysteiny1 leukotrienes (CysLT) (Samuelsson 1983). During anaphylactic
reactions, this group of leukotrienes causes airway smooth-muscle contraction
and vascular edema (Funk 2005). Leukotrienes are then secreted into the
extracellular space where they bind to G-protein couple receptors BLT$_1$, BLT$_2$,
CysLT$_1$ and CysLT$_2$. These receptors are expressed on different target cells,
including leukocytes, smooth-muscle cells and endothelial cells (Heise et al.

Kinins are most known as vasoconstrictors and vasodilators of smooth
muscle, but they have been implicated in inflammatory responses (Couture et al.
2001; Rodi et al. 2005). Kinins bond a specific receptor referred to as the
bradykinin receptor which has two subsets, B$_1$ and B$_2$ (Couture et al. 2001).
During ischemia, B$_2$ receptor activation promotes vascular permeability of
endothelial cells and edema (Dray and Perkins 1993). The pro-inflammatory
effects of B$_1$ receptors include promotion of blood leukocyte migration to the site
of injury and edema. Besides kinin binding, bradykinin receptors can also be stimulated by prostaglandins, cytokines, and nitric oxide (Dray 1997).

**Current Treatment**

Presently there is only one Food and Drug Administration approved drug for treating stroke which is recombinant tissue plasminogen activator (rtPA) (Marler and Goldstein 2003). This treatment, however, has several therapeutic limitations including a three hour therapeutic widow, use only with embolic stroke, no anti-inflammatory or neuroprotective effects, the risk of causing intracranial hemorrhage, oxygen free radical production and recurrent stroke (Marler and Goldstein 2003; NINDS 1995). These limitations result in approximately 2-3% of all stroke victims being candidates for this therapy as many patients do not arrive at the hospital to receive treatment, are not properly diagnosed, or even know that they are having a stroke in this three hour time period. For those individuals who are candidates for rtPA therapy, only 13% show improved outcomes (Albers et al. 2004; Albers et al. 1998).

**Recombinant Tissue Plasminogen Activator**

Recombinant tissue plasminogen activator is a secreted serine protease which converts the proenzyme plasminogen to plasmin, a fibrinolytic enzyme. Plasminogen is a glycoprotein produced by the liver and found in blood and most extravascular fluids. The generation of plasmin occurs preferentially on the fibrin surface, which offers binding sites for plasminogen and rtPA at the C-terminal
lysine residues (Chromogenix 1995). This binding stimulates plasminogen activation, but also localizes the action of plasmin to sites of fibrin formation which promotes efficient clot lysis. Plasmin can then dissolve a blood clot by degrading the fibrin polymers of the clot into soluble fragments. If rtPA causes increased enzymatic activity of the plasmin, hyperfibrinolysis can occur resulting in excessive bleeding, producing the potential of hemorrhagic stroke (Marx 2004).

**Failed Treatments**

Presently there are two main strategies in developing new treatments for embolic stroke; attacking the clot and protecting the neurons that are being subjected to ischemia. While rtPA had been deemed sufficient for clot busting, there have been many failed attempts to develop a treatment for stroke that promotes neuroprotection after ischemia insult. Failures in treatment usually arise from discrepancies between modeling of cerebral ischemia in animals compared to clinical trials in human test subjects (del Zoppo 1998). Animal models become unpredictable for reasons related to incomplete understanding of the pathophysiology of ischemic stroke, species differences, cellular reactivity, excessive toxicity, vascular disease processes, inflammation, and the use of other pharmacological modifiers such as anesthesia (del Zoppo 1998). These factors are all potential contributors to ischemic tissue injury and assessment of these may affect other species differently.
Treatments promoting neuroprotection work to block those pathways that stimulate necrosis and apoptosis during or after an ischemic insult. The most notable of these interventions are antagonists of glutamate receptors and calcium channels. One of the focuses of glutamate receptor antagonists is to inhibit the actions of the NMDA receptor. Blockage of this receptor results in reduction of calcium influx into neurons through post synaptic calcium channels (Hoyte et al. 2004). Clinical testing of this treatment resulted in several adverse affects. Blockage of the NMDA channels by competitive antagonism required high concentrations of the antagonist due to the high affinity of the NMDA receptor to glutamate. High concentrations were also needed to reach effective concentrations of the antagonist in the penumbra. This, however, led to hallucinations, delirium, and psychosis (Hoyte et al. 2004). Short neuroprotective time windows were a concern also because of the development of infarction. In animal models, there is a slow expansion of damage over the first few days of injury. Therefore, neuroprotective effects were a result of postponed injury development. Finally antagonism of glutamate receptor activation needed to occur very early after ischemia onset making this treatment potentially effective only within the first hour or less of injury (Hoyte et al. 2004).

Calcium channel blockers have been used in an attempt to promote neuroprotection in stroke victims by reducing calcium influx by voltage gated calcium channels. Nimodipine is a specific antagonist of calcium channels that has been evaluated in neuroprotective studies and works by blocking the L-type calcium channel (Langley and Sorkin 1989). Unfortunately, this treatment and
other calcium channel blockers have shown no efficacy in alleviating neurodegenerative load or behavioral improvements in clinical trials (Horn et al. 2001). These antagonists have also been shown to have adverse effects of severely lowering blood pressure of stroke victims (Horn et al. 2001).

Anti-inflammatory agents have been investigated as another avenue to reduce infarct volume. Common anti-inflammatory drugs such as aspirin and ibuprofen have been assessed in animal models of stroke and have found only minor success in reductions of behavioral deficits. Aspirin was used as an antithrombotic agent for its antiplatelet properties. Aspirin administration resulted in a 27% reduction of vascular death and infarction (Berger et al. 2004). Side effects resulted in resistance to the drug and between 15% and 45% of the patients in the studies were tolerant to the antiplatelet effects (Berger et al. 2004). Ibuprofen has been shown to inhibit cyclooxygenase expression and reduce the synthesis of prostaglandins from arachidonic acid in transient MCAO (Cole et al. 1993). Animal models using permanent MCAO showed an increase in infarction when treated with Ibuprofen suggesting that the beneficial effects of this drug only work during reperfusion of transient MCAO, which showed a decrease in infarction (Cole et al. 1993).

Cytokine inhibition has been examined as a potential treatment in an attempt to reduce activation of inflammatory pathways and migration of inflammatory cells during cerebral ischemia. Reduction of the expression of the pro-inflammatory cytokine TNF-α and molecular inhibition of the adhesion
molecule MCP-1 have shown promise in the decrease of neurological deficits (Hughes et al. 2002; Nawashiro et al. 1997a; Nawashiro et al. 1997b). Again, however, clinical and some animal trials of these agents have produced unfavorable results. TNF-α, while inflammatory, also provides some neuroprotection after stroke and inhibition of this cytokine can result in increased damage (Barone and Feuerstein 1999). Reduction of the chemoattractant MCP-1 results in accelerated atherogenesis, the increased formation of lipid deposits on the inner walls of arteries. These deposits can eventually break free causing embolic blockage of arteries leading to stroke (Fosslien 2005).

**Novel Approaches for Treatment of Stroke**

To develop a novel therapy for stroke, successes and failures must be taken into account. While rtPA, neuroprotective agents, and anti-inflammatory agents show some promise, individually these treatments do not result in a feasible therapy of stroke that promotes neuroprotection, reduces infarct volume, alleviates behavioral impairment and expands the therapeutic window of administration. Recent research from the Pennypacker and Willing laboratories, however, have shown that pharmacological treatments that combine both neuroprotection and anti-inflammation greatly reduce infarction in the rat MCAO stroke model which was first described by Longa et al. in 1989 (Longa et al. 1989). Human umbilical cord blood cell transfusion achieved these goals by reducing infarction volume, decreasing motor deficits and showing protection at delayed time points.
Human Umbilical Cord Blood Cells

Recent studies have shown that human umbilical cord blood cells (HUCBC) when used as a treatment for stroke, promote neuroprotection and anti-inflammation to expand the therapeutic window of treatment up to 48 hours after the initial insult (Newcomb et al. 2006; Vendrame et al. 2004). HUCBC are a mononuclear fraction of cells containing hematopoietic stem cells that are derived from the umbilical cord blood that is normally discarded after the neonate is born. The mononuclear fraction consists of a heterogeneous population of cells that can be distinguished from one another by cell surface antigens. Flow cytometry analysis shows that monocytes and lymphocytes make up the majority of the HUCBC mononuclear fraction, while the hematopoietic stem cells, bearing the CD34 antigen, make up only 1% of the total fraction (Pranke et al. 2001). The first beneficial effects of this treatment were shown when there was a reduction in deficits of behavioral motor response in rats that received a transfusion of HUCBC after MCAO (Chen et al. 2001). Further investigation of the transfusion of HUCBC after MCAO showed that there was a 50 and 70% reduction in infarction volume when administered 24 and 48 hours post MCAO, respectively (Newcomb et al. 2006; Vendrame et al. 2004). Alternative treatments that mimic the actions of HUCBC, such as sigma receptor activation, were investigated to confirm that promoting both neuroprotection and anti-inflammation result in the positive effects seen with cord blood cell therapy.
Sigma Receptors

Activation of sigma receptors at delayed time points is a novel treatment for stroke. Stimulation of these receptors promotes both neuroprotection and anti-inflammatory effects in the CNS (Goyagi et al. 2001; Harukuni et al. 2000; Harukuni et al. 1998; Su 1991). Sigma receptors were first discovered in 1976 by W. R. Martin but they were originally believed to be opioid receptors (Martin et al. 1976). They are now known to be their own class of receptors with unique structure and function. At this time an endogenous ligand for the sigma receptors has not yet been determined. However, it has been postulated that neurosteroids are these ligands. Sigma receptors can be divided into two distinct classes based of pharmacological profile, sigma-1 and sigma-2 (Bowen et al. 1989).

Of the two receptors, more is known about sigma-1 since it has been cloned (Hanner et al. 1996). While the three dimensional structure is still unknown, it has been postulated to be a several transmembrane molecule. From a protective perspective, sigma-1 receptor activation has been shown to be neuroprotective by reducing glutamate toxicity in cultured neurons (Kume et al. 2002; Takahashi et al. 1996) and decreasing neuronal nitric oxide production in rat transient MCAO (Goyagi et al. 2001; Lesage et al. 1995). Sigma-1 activation can also result in an anti-inflammatory response by inhibiting inflammatory cytokine production in macrophages and up-regulating the expression of IL-10, an anti-inflammatory cytokine that can inhibit the endotoxin-induced production of
proinflammatory cytokines (Bourrie et al. 1996; Bourrie et al. 1995; Bourrie et al. 2002; Derocq et al. 1995).

Much less is known regarding sigma-2. Although structure, cloning, function and endogenous ligand have yet to be determined, it has been discovered that sigma-2 has neuroprotective properties. Experiments have shown that activation of this receptor modulates voltage-gated calcium and sodium channels (Zhang and Cuevas 2002; Zhang and Cuevas 2005). Modulation of these two channels at the time of stroke may result in inhibition of excessive calcium influx into the neurons preventing necrotic and apoptotic pathway activation.

1, 3-Di-o-tolyguanidine

To confirm the protective actions of HUCBC affecting stroke, a sigma receptor ligand was selected based on the need to promote both neuroprotective and anti-inflammatory effects. Therefore the sigma receptor ligand 1, 3-di-o-tolyguanidine (DTG), was picked for examination for its high affinity for both the sigma-1 and sigma-2 binding sites (Quirion et al. 1992). We hypothesized that activation of both the sigma-1 and sigma-2 receptors by this ligand would result in increased neuroprotection and anti-inflammation at the site of injury.

Spleen

Another alternative target for the treatment of stroke is splenic reaction to ischemic injury. Identification of HUCBC by human nuclei immunoreactivity and
PCR analysis for human DNA showed that HUCBC localized to only the hemisphere of the brain subjected to MCAO and to the spleen (Vendrame et al. 2004). These findings suggest that the spleen is involved in the overall stroke mechanism that is affecting the body and HUCBC is potentially working through the spleen to modulate immune cell responses to the brain.

The spleen is a secondary lymphoid organ whose functions are centered on the peripheral circulation. It is located in the left hypochondriac region of the abdominal cavity between the fundus of the stomach and the diaphragm. This organ acts to remove old and damaged cells, filters the blood and initiates immune responses to blood born antigens (Cesta 2006). The spleen is comprised of two functionally and morphologically distinct compartments, the red pulp and the white pulp. The red pulp filters blood to remove foreign material and damaged erythrocytes. It is also a storage site for iron, erythrocytes, and platelets. The functional charge of the white pulp is to initiate immune responses, and store large amounts of the body’s lymphocytes and macrophages (Balogh et al. 2004; Nolte et al. 2002). The spleen itself is surrounded by a capsule composed of dense fibrous tissue, elastic fibers, and smooth muscle (Cesta 2006).

Recent studies have reported that in animal models the spleen shrinks in reaction to Parkinson’s and stroke injury to the brain (Benner et al. 2004; Offner et al. 2006a; Offner et al. 2006b; Vendrame et al. 2006). These studies have observed that shrinkage of the spleen is followed by a release of splenocytes into the circulation. In the specific case of stroke, the spleen shrinks by 50% of its
weight. Treatment with HUCBC, besides reducing infarct volume, caused the spleen to retain its size (Vendrame et al. 2006). This interaction with stroke suggests that cerebral infarction relays a signal to the spleen, by soluble chemicals or sympathetic innervation that results in a massive release of immune response cells causing the organ to decrease in size. These released immune cells can then infiltrate the brain via the disrupted blood brain barrier resulting in enhancement of the peripheral immune response and the up-regulation of inflammation at the site of insult. Determination of the exact interactions between stroke and the spleen could produce a launching point for the development of novel therapies for cerebral ischemia and is a focal point of the following experiments.

The Current Study

The overall hypothesis of this work is that treatments that promote neuroprotective and anti-inflammatory effects can modulate the neurodegenerative deficits of ischemic injury. Evidence from the literature and data from these experiments supports the theory that reduction of neurodegeneration, modulation of immune inflammatory responses and the contribution of peripheral inflammatory systems are effective targets for reducing infarction expansion after stroke. The data presented a) shows universal sigma receptor activation decreases neurodegenerative load of the infarction at a delayed administration after stroke, b) the spleen contributes to stroke-induced neurodegeneration, and c) autonomic innervation does not control splenic
response to stroke. Here, it is proposed that simultaneous activation of neuroprotective and anti-inflammatory effects by alternative targets of stroke promote neuron survival. These treatments will also lead to reduced inflammation by decreased activation or migration of peripheral immune cells resulting in reduced infarction expansion, morbidity and mortality.

References


Cesta MF. 2006. Normal structure, function, and histology of the spleen. 


piperidine (PPBP) is dependent on treatment duration in rats. Anesthesia and analgesia 87(6):1299-1305.


Rabinovic AD, Lewis DA, Hastings TG. 2000. Role of oxidative changes in the
degeneration of dopamine terminals after injection of neurotoxic levels of

membrane lipid peroxidation: the cytotoxic potential of superoxide and
nitric oxide. Archives of biochemistry and biophysics 288(2):481-487.

hydrolase in human leukocytes. Purification and properties. The Journal of
biological chemistry 259(20):12339-12345.

and molecular cues to biological function. Trends in neurosciences
20(12):570-577.

Rodi D, Couture R, Ongali B, Simonato M. 2005. Targeting kinin receptors for the
treatment of neurological diseases. Current pharmaceutical design
11(10):1313-1326.

diseases 42(3):209-216.

Salgo MG, Bermudez E, Squadrito GL, Pryor WA. 1995a. Peroxynitrite causes
DNA damage and oxidation of thiols in rat thymocytes [corrected].
Archives of biochemistry and biophysics 322(2):500-505.


CHAPTER 1
SIGMA RECEPTOR ACTIVATION REDUCES INFARCT SIZE AT 24 HOURS AFTER PERMANENT MIDDLE CEREBRAL ARTERY OCCLUSION IN RATS

Craig T. Ajmo Jr., MS, Dionne O. L. Vernon BS, Lisa A. Collier BS, Keith R. Pennypacker PhD*, Javier Cuevas PhD*

*contributed equally to this study and manuscript

Department of Molecular Pharmacology & Physiology, University of South Florida, Tampa, Florida 33612

Aknowledgments: We would like to thank Shane N. Collier for his help with the image analysis, and Jennifer Newcomb for her help with the MCAO surgery.

This study was supported by the NIH R01NS39141 (K.R.P.) and AHA Florida/Puerto Rico Affiliate Grant-In-Aid 0455210B (J.C.).

This work was published in Current Neurovascular Research, 2006 May; 3(2): 89-98
Abstract

The only available treatment for embolic stroke is recombinant tissue plasminogen activator, which must be administered within three hours of stroke onset. We examined the effects of 1,3-di-o-tolyguanidine (DTG), a high affinity sigma receptor agonist, as a potential treatment for decreasing infarct area at delayed time points. Rats were subjected to permanent embolic middle cerebral artery occlusion (MCAO) and allowed to recover before receiving subcutaneous injections of 15 mg/kg of DTG at 24, 48, and 72 hours. At 96 hours the rats were euthanized, and brains harvested and sectioned. Infarct areas were quantified at the level of the cortical/striatal and cortical/hippocampal regions in control (MCAO-only) and DTG treated animals using the marker for neurodegeneration, Fluoro–Jade. DTG treatment significantly reduced infarct area in both cortical/striatal and cortical/hippocampal regions by >80%, relative to control rats. These findings were confirmed by immunohistochemical experiments using a neuronal marker, mouse anti-neuronal nuclei monoclonal antibody (NeuN), which showed that application of DTG significantly increased the number of viable neurons in these regions. Furthermore, DTG blocked the inflammatory response evoked by MCAO, as indicated by decreases in the number of reactive astrocytes and activated microglia/macrophages detected by immunostaining for glial fibrillary acidic protein (GFAP) and binding of isolectin IB4, respectively. Thus, our results demonstrate that the sigma receptor-selective agonist, DTG, can enhance neuronal survival when administered 24 hr after an ischemic stroke. In addition, the efficacy of sigma receptors for stroke treatment at delayed time
points is likely the result of combined neuroprotective and anti-inflammatory properties of these receptors.

Key words:

Stroke ♦ Sigma receptors ♦ Neuroprotection ♦ Anti-inflammation ♦ Middle Cerebral Artery Occlusion ♦ Fluoro-Jade ♦ Infarction ♦ Ischemia
Introduction

Stroke is the leading cause of severe disability and the third leading cause of death in the United States of America (AHA, 2005). Intravenous application of recombinant tissue plasminogen activator (tPA), a thrombolytic agent, is the only FDA approved treatment for stroke and has a very limited therapeutic time window (1995). This “clot-buster” must be administered within three hours of stroke onset (Albers et al. 2004), and can produce possible adverse effects such as hemorrhage and reperfusion damage from oxygen free radicals (Hacke et al. 1999; Kumura et al. 1996; Peters et al. 1998). The limitations and adverse effects of tPA have stimulated the search for alternative treatments for stroke.

When a cerebral embolic stroke occurs, a thrombus blocks blood perfusion to the brain and triggers a series of events that ultimately result in neuronal death. The disruption in blood supply directly results in the cessation of oxygen and nutrient delivery, which metabolically compromises the neurons and produces an infarction. The infarct zone contains two regions associated with ischemic cell death. The center of the infarction or “core” is the area directly affected by the decrease in blood perfusion, and is where the greatest concentration of cell death can be found. Surrounding the core is the penumbra, a region with diminished blood flow but where collaterals provide some oxygen and nutrients. However, perfusion in the penumbra is sufficiently reduced that physiological function is arrested and some degeneration of neurons occurs (Ginsberg 2003).
Neuronal death is enhanced by secondary inflammation caused by the immune response in the penumbra. The inflammatory response is primarily from resident activated microglia and infiltrating macrophages, which enter the central nervous system through the degrading blood brain barrier (Stoll et al. 1998). Reactive astrocytes and microglia exacerbate cerebral inflammation via their production of pro-inflammatory cytokines and chemokines (Trendelenburg and Dirnagl 2005). These immune cells, which normally protect the brain via destruction of pathogens and promotion of tissue repair, become overactivated, and further promote the expansion of tissue damage by releasing high levels of nitric oxide (NO), glutamate, tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)), and interleukin-1 (IL-1) (Bal-Price and Brown 2001; Heales et al. 1999; Hertz et al. 2001).

It is our hypothesis that therapeutic interventions that provide neuroprotection and reduce inflammation will decrease damage to the penumbra, which will reduce the extent of the damage produced by stroke. Our hypothesis is based on recent studies which have shown that transfusion with human umbilical cord blood cells (HUCBC), which possess both of these properties, results in both infarct volume reduction and expansion of the therapeutic window (Newcomb et al. 2005; Vendrame M et al. 2005; Vendrame et al. 2004). Activation of sigma receptors has been shown to decrease neuronal death associated with hypoxia and to elicit an anti-inflammatory response (Bourrie et al. 2002; Goyagi et al. 2001). Thus, activation of sigma receptors may mimic the effects of HUCBC following stroke and provide for the expansion of the therapeutic time window for treatment after such injury.
Two distinct subtypes of sigma receptors have been identified on the basis of their pharmacological profile (Bowen et al. 1989; Quirion et al. 1992). Thus far, only the sigma-1 receptor has been cloned (Hanner et al. 1996), but the sigma-2 receptor has been shown to be a separate molecular entity (Langa et al. 2003). Sigma-1 receptor activation has been shown to prevent neuronal death associated with glutamate toxicity in cultured neurons (Kume et al. 2002; Takahashi et al. 1996) and to diminish infarct damage by decreasing neuronal nitric oxide production in vivo (Goyagi et al. 2001; Lesage et al. 1995). Sigma-1 also modulates the immune response by inhibiting TNF-α production in endotoxin-activated macrophages and acts as an anti-inflammatory agent by stimulating the expression of interleukin-10 during in vivo and in vitro ischemic simulations (Bourrie et al. 1996; Bourrie et al. 1995; Bourrie et al. 2002; Derocq et al. 1995). In contrast, less is known about sigma-2 receptors. However these receptors have been shown to regulate voltage-activated calcium and sodium channels in neurons (Zhang and Cuevas 2002; Zhang and Cuevas 2005), which is likely to enhance neuronal survival following stroke (Tanaka et al. 2002).

The ability of sigma-1 and sigma-2 receptors to target different ion channels and different processes likely involved in neuronal demise following ischemia suggests that both should be targeted for stroke therapy. 1,3-di-o-tolyguanidine (DTG), the sigma ligand used here, has a high affinity for both sigma 1 and 2 receptors (Quirion et al. 1992). We believe that activation of both sigma receptors will result in additive or synergistic neuroprotective and anti-inflammatory effects. In this study, we administered DTG subcutaneously to rats
starting at 24 hours after permanent embolic middle cerebral artery occlusion (MCAO) and found that this treatment results in a significant decrease in infarction area.

**Materials and Methods**

**Animals** - Fifty eight adult male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 300 to 350 g were housed in a climate controlled room with water and laboratory chow available *ad libitum*. Animals were cared for according to the guidelines of the IACUC of the University of South Florida’s College of Medicine.

**Permanent Middle Cerebral Artery Occlusion** - MCAO surgery was performed as previously reported by Vendrame *et al* (Vendrame *et al*. 2004) and originally described by Longa *et al* (Longa *et al*. 1989). Laser Doppler Radar (LDR) was used to monitor decrease in blood perfusion which indicates successful occlusion (Moor Instruments Ltd, Devon, England). A 2 mm diameter hole was drilled into the right parietal bone (1 mm posterior and 4 mm lateral from bregma), and a guide screw was set. The LDR probe (MP10M200ST; Moor) was inserted into the guide screw, and the tip of the probe was placed against the pial surface of the brain. Rats that did not show >55% reduction in perfusion during MCAO were excluded from the study because they generally failed to exhibit infarct damage. For MCAO, the embolus (4cm long, 6lb test monofilament) was advanced up the internal carotid artery into the middle cerebral artery and tied off at the internal/external carotid junction to produce permanent occlusion. The rat
was then sutured, given a 1 ml subcutaneous injection of saline, and allowed to wake in a fresh cage.

**Treatments and Tissue Preparation** - Rats were randomly assigned to 1 of 7 groups: (1) MCAO (n=7); (2) MCAO and 15 mg/kg DTG in a 3% lactic acid vehicle (n=11); (3) MCAO and 30 mg/kg DTG (n=16); (4) MCAO and bi-daily injections of 30 mg/kg DTG (n=12); (5) MCAO and 10 mg/kg \(N\text{-}[2-(3,4-\text{dichlorophenyl}) \text{ethyl}]\text{-N-methyl-2-(dimethylamino) ethylamine (BD 1047)}\) (n=4); (6) MCAO and 10 mg/kg BD1047 + 30 mg/kg DTG (n=4); (7) sham/MCAO and 15 mg/kg DTG (n= 4). DTG and BD1047 were obtained from Sigma Chemical Co (St. Louis, MO) and Tocris (Ellisville, Missouri), respectively. Injections were administered 24, 48, and 72 hours post MCAO. All rats received daily injections of 0.04 ml of ketophen and 1 ml of saline. The animals were sacrificed at 96 hours, and perfused with saline and 4% paraformaldehyde. The brains were harvested, fixed in paraformaldehyde, immersed in serial solutions of 20% and 30% sucrose, and sliced into 30µm sections. Sections were either cold mounted on slides or placed in Walter’s Anti-freeze cryopreservative.

**Fluoro-Jade Histochemistry** - Coronal brain sections from 1.7 to -3.3 mm from bregma containing cortical, striatal and hippocampal regions were stained with Fluoro-Jade. Fluoro-Jade labels degenerating neurons, and is more sensitive than triphenyltetrazolium chloride (TTC) for identifying neurodegeneration (Duckworth et al. 2005). This method was adapted from that originally described
by Schmued et al (Schmued et al. 1997) and has been detailed previously by Duckworth et al (Duckworth et al. 2005). Tissue was cold mounted, thawed, and dried onto glass slides. Slides were sequentially placed in 100% ethanol for 3 min, and 70% ethanol and deionized water for 1 min each. Sections were then oxidized using 0.06% KMnO$_4$ solution for 15 min followed by three rinses for 1 minute each in PBS. Sections were stained in a 0.001% solution of Fluoro-Jade (Histochem, Jefferson, AR) in 0.1% acetic acid for 30 min. Slides were rinsed with PBS, allowed to dry at 45 °C for 20 min, cleared with xylene, and cover slipped were affixed to slides with DPX medium (Electron Microscopy Sciences, Ft. Washington, PA).

**Immunohistochemistry and Histochemistry** - Immunohistochemistry was performed as previously detailed by Butler et al (Butler et al. 2002). Free floating brain sections were pre-incubated in permeabilization buffer, 0.3% lysine, 0.3% TritonX-100, and 2% goat serum in phosphate buffered saline (PBS) for 1 hour. Sections were washed three times with PBS between each incubation step. The tissue was then incubated in primary antibody solution overnight either with mouse anti-glial fibrillary acidic protein monoclonal antibody (GFAP) at a 1:10,000 dilution (MAB3402, Chemicon, Temecula, CA) or mouse anti-neuronal nuclei monoclonal antibody (NeuN) at a 1:30,000 dilution (Chemicon, Temecula, CA). Sections exposed to antibodies directed against these proteins were subsequently incubated for 1 hour in biotinylated horse anti-mouse secondary antibody solution (Vector Laboratories, Burlingame, CA) followed by avidin /
biotin / horseradish peroxidase complex (VectastainElite ABC kit; Vector) for 1 h. Sections were then washed 3x in PBS, and metal-enhanced 3, 3’-diaminobenzidine (Pierce, Rockford, IL) was used for color development. For experiments involving *Griffonia simplicifolia* Isolectin (IB4/Alexa Fluor 488; Molecular Probes Eugene, Oregon), sections were incubated overnight in isolectin IB4 (5µg/ml). All sections were mounted on slides, dried, cleared sequentially with 100%, 95%, and 70% ethanol and xylene, and coverslips affixed with DPX.

**Infarct Area and Immunohistochemistry Quantification** - Images of FluoroJade stained brain sections, 5 per cortical/striatal and 4 per cortical/hippocampal regions for each rat from 1.7 to -3.3 mm from bregma, were acquired with the Olympus IX71 microscope controlled by DP manager software (Olympus America Inc, Melville, NY) at a magnification of 12.5x. All other images were taken with a Zeiss Axicam Color (model 412-312) camera and Zeiss Axioscope 2 (model 801572) microscope controlled by Openlab software (Improvision Ltd, Lexington MA). Images were edited with Jasc Paintshop Pro to sharpen and enhance contrast of the images to the same specifications. Image analysis was performed utilizing NIH Image J software to determine the area of neurodegeneration by particle analysis in the regions of interest. The area of the contralateral side of the brain tissue was measured and used to compensate for possible edema in ipsilateral hemispheres. NeuN immunostaining was analyzed using the NIH Image J software to count NeuN positive cells by particle analysis.
in the ipsilateral hemispheres of the cortical/striatal and cortical/hippocampal regions of the rat brains at a magnification of 10X.

**Statistical Analysis** - Data were analyzed using SigmaPlot 2000 (SPSS Science, Chicago, IL). Data points represent means ± standard error of the mean (SEM). Multiple group comparisons were conducted using a One-Way or a Two-Way ANOVA, as appropriate, followed by post-hoc analysis with a Tukey or Dunn's Test to identify differences between individual groups. Differences were considered significant if $p < 0.05$. Survival rates were analyzed using a Kaplan-Meier Survival Analysis and post-hoc with a Holm-Sidak test for pairwise multiple comparison.

**Results**

**DTG Dose Quantification**

Reports in the literature indicate that doses of DTG as high as 30 mg/kg are well tolerated by rats (Rawls et al. 2002). Thus, this high dose of DTG was used to determine if stimulation of sigma receptors was neuroprotective at delayed time points. Furthermore, to confirm that the effects of DTG were mediated by activation of sigma receptors, the sigma receptor selective antagonist, BD1047, was used at concentrations (10 mg/kg) previously shown to abolish systemic effects of DTG (Rawls et al. 2002). Rats received subcutaneous injections of DTG at 30 mg/kg daily, 15 mg/kg daily, 30 mg/kg b.i.d., 10 mg/kg daily BD1047, or BD1047 (10 mg/kg) + DTG (30 mg/kg)
administered at 24, 48 and 72 hours post surgery with animals being sacrificed at 96 hours. The percent survival was defined as the number of rats that survived 96 hours post MCAO divided by the total number of rats that awoke from anesthesia. The MCAO and 15 mg/kg DTG daily groups produced survival rates of over 70%, while 30 mg/kg DTG daily had a survival rate under 40% Fig. (1). In contrast, the survival rates of BD1047 alone, BD 1047 + DTG, and 30 mg/kg bi-daily were ≤25%, indicating that high concentrations of DTG and inhibition of sigma receptors worsen survival outcomes. Given that none of the sham animals died following DTG application, high concentrations of DTG are not lethal in the absence of MCAO Fig. (1). The dose of 15 mg/kg per day did not enhance mortality relative to the MCAO only group, and therefore was the dose chosen for the subsequent study.

**DTG Treatment 24-hr post-MCAO Decreases Infarct Size**

The marker for neurodegeneration, Fluoro-Jade, was used to determine infarct area after MCAO. Figure 2 shows representative photomicrographs of coronal sections from cortical/striatal (Figure 2a and 2c) and cortical/hippocampal (Figure 2b and 2d) regions obtained from rats in the absence and presence of DTG treatment post-MCAO. Fluoro-Jade staining was observed in brain sections from all animals that underwent MCAO, but absent from sections collected from sham controls. While Fluoro-Jade staining was observed in DTG treated (15 mg/kg) animals (Fig 2c and 2d), this was less pronounced than in MCAO only rats (Figure 2a and 2b). Analysis of similar sections obtained from the individual
groups showed that Fluoro-Jade labeled 44 ± 5% and 29 ± 3% of the
cortical/striatal and cortical/hippocampal regions, respectively, in MCAO-only
rats. However, in DTG-treated animals (n=7) infarct areas were 6±4% and 2±2%
in the cortical/striatal and cortical/hippocampal regions, respectively Fig. (3).
This decrease in infarct area was statistically significant (p < 0.001), and
demonstrates that DTG application at delayed time points can effectively
decrease stroke-induced neurodegeneration.

**DTG-induced Enhancement of Neurosurvival**

To confirm that DTG increase the number of neurons surviving MCAO in rats, we
immunostained for the neuron-specific protein, NeuN, to selectively label the
nuclei of viable cells. Figure 4 shows representative brain sections from DTG
treated and untreated rats immunostained for NeuN. Whereas NeuN expression
was absent from the infarct zone of MCAO-only rats ( Figure 4a-c), NeuN
staining was readily visible in the infarct zone of animals injected with DTG
(Figure 4d-f). In identical experiments, the total number of NeuN-positive
neurons were counted by image analysis, and average number per visual field
determined. These values are shown in Figure 5, and demonstrate that MCAO
significantly decreases the number of viable neurons in the ipsilateral
cortical/striatal and cortical/hippocampal infarct zones, relative to the respective
regions in the contralateral hemisphere. Injection with DTG significantly
increased the number of viable neurons in both ipsilateral cortical/striatal and
cortical/hippocampal infarct zones (p < 0.001). Furthermore, the number of
neurons detected in these areas of the ipsilateral hemisphere were comparable to those observed in equivalent areas of the contralateral side (Figure 5). Thus, DTG increases neuronal survival to the extent that the number of surviving cells was not statistically different from that observed in sham controls (Figure 5).

**DTG Treatment Decreases Expression of Inflammatory Markers**

Experiments in our laboratory have shown that reduced brain inflammation is a key component to treating stroke at delayed time points (Newcomb et al. 2005; Vendrame M et al. 2005). Thus, to ascertain if stimulation of sigma receptors by DTG exerts an anti-inflammatory response, we examined inflammatory markers for both astrocytes and microglia in brain sections from the experiments discussed above. Reactive astrocytes, which participate in the inflammatory response in the brain following injury (O'Callaghan 1994), exhibit high levels of the distinguishing marker, GFAP. Thus, GFAP immunoreactivity was used to label reactive astrocytes responding to the ischemic injury produced by our model. Representative images showing GFAP labeling in tissue sections collected from MCAO-only and DTG treated animals are shown in Figure 6. Astrocytes containing high levels of GFAP were always observed in MCAO-only animals, and these astrocytes were primarily located in the areas surrounding the infarction Figure 6a-c). However, the area inside of the infarction was noticeably devoid of astrocytes expressing GFAP in these sections. In animals receiving DTG, brain sections exhibited a marked decrease in the level of GFAP
expression. Moreover, astrocytes with this low level GFAP-labeling were detected throughout the infarct zone Figure 6d-f).

Inflammatory response of the central nervous system also involves activation of microglia and infiltration of systemic macrophages. Both of these cells express the surface protein IB4 when activated in response to injury, and can be selectively labeled in this state using isolectin IB4 (Goldstein and Winter 1999). Figure 7 shows representative photomicrographs of tissue sections of cortical/striatal and cortical/hippocampal regions labeled with isolectin IB4 from animals subjected to MCAO with and without DTG treatment. MCAO evokes a pronounced increase in isolectin IB4 labeled cells in the infarct zone of untreated animals (Figure 7a and 7b). However, MCAO fails to elicit these elevations in isolectin IB4-positive cells in the infarct zone of sections taken from animals treated with DTG (Figure 7c and 7d). Isolectin IB4 labeling was also absent from sections taken from sham control animals (Figure 7e and 7f). Taken together, our data suggest that application of DTG blunts the inflammatory response of the brain following MCAO. This depression of neuroinflammation is likely to contribute to the enhanced neuronal survival reported here.

Discussion

The major finding reported here is that the application of DTG 24 hr after stroke injury significantly decreases neurodegeneration in rats subjected to MCAO. Moreover, it was observed that the administration of this sigma ligand depresses the inflammatory response evoked by the ischemic insult. The role of sigma receptors in the effects of DTG were supported by the observation that the
sigma-selective antagonist, BD-1047, worsened stroke outcomes. Thus, our data support the hypothesis that the window for treatment of stroke extends beyond the limitations of the currently available therapy, and that sigma receptors are a viable target for stroke therapy at delayed time points.

Two lines of evidence presented here confirm that DTG is neuroprotective when administered 24 hr post stroke. First, DTG significantly decreased Fluoro-Jade staining, which is consistent with reduced neurodegeneration, by >85% relative to untreated MCAO animals. Second, the number of surviving cells detected in the infarct zone with the neuron specific marker, NeuN, was increased by >83% in DTG treated animals relative to MCAO-only animals. Moreover, the number of viable cells observed in the ischemic region of DTG treated animals was similar to the number of cells present in equivalent regions of sham controls. Previous studies on the neuroprotective properties of sigma receptor activation following MCAO have focused on a transient ischemia model (1-2 hr occlusion) and have commenced intravenous application of sigma-1 selective ligands, such as 4-phenyl-1-(4-phenylbutyl) piperidine (PPBP), 1-2 hr following reperfusion (Takahashi et al. 1996). Brain sections taken from animals treated with PPBP showed ~40% decrease in neurodegeneration (Takahashi et al. 1996). Unlike observations reported here, some studies suggest that acute application of PPBP following MCAO fails to decrease infarct injury in the striatum of rats (Harukuni et al. 2000). The sigma-1 ligand, JO 1784, has also been shown to be effective for decreasing brain injury following global ischemia when applied ≥ 1 hr following the ischemic insult (O'Neill et al. 1995). Our
studies, however, indicate that DTG can exert its neuroprotective properties even when treatment begins 24 hr post-stroke.

The dose of DTG used here to diminish stroke injury without compromising survival rates (15 mg/kg) is comparable to doses previously used of this sigma ligand to elicit sigma receptor-mediated effects in vivo, such as hypothermia (1-30 mg/kg) and antinociception (10-20 mg/kg) (Kest et al. 1995; Rawls et al. 2002). The mechanism(s) by which higher concentrations of DTG (30 mg/kg) increase mortality following MCAO remains to be determined. However, sigma receptors have been shown to modulate cardiac function directly by acting on cardiac muscle and to regulate neurons that mediate autonomic control of the heart (Novakova et al. 1998; Zhang and Cuevas 2002; Zhang and Cuevas 2005). Given the fact that cardiovascular abnormalities, including cardiac arrhythmias, are associated with stroke (Klingelhofer and Sander 1997), high concentrations of DTG may exert their deleterious effects via enhancing cardiac dysfunction following stroke injury.

The inflammatory response that occurs in the central nervous system following injury, such as that produced by ischemic stroke, plays a significant role in enhancing neurodegeneration. The sequence of events leading to cerebral inflammation after acute focal ischemia begins with the rapid activation of microglia within 24 hours of the insult (Schroeter et al. 1997). This event is followed by systemic macrophage infiltration through the compromised blood brain barrier at 48 hours post-stroke (Schroeter et al. 1997). Subsequently, reactive astrocytes begin to release glutamate, nitric oxide, TNF-α, and other
factors, which result in increased inflammation contributing to delayed neuronal death (Swanson et al. 2004). Based on data presented here, DTG blunts the glia-mediated inflammatory response evoked by MCAO. Three mechanisms may account for the observed sigma receptor-mediated anti-inflammatory response. First, direct neuroprotection by DTG administration may result in reduced production and release of signaling molecules which trigger the pro-inflammatory response. Various in vitro studies have shown that stimulation of sigma receptors decreases neuronal death in response to hypoxia and glutamate excitotoxicity (DeCoster et al. 1995; Lockhart et al. 1995). Second, stimulation of sigma receptors on cells involved in inflammation in the CNS may dampen this response. Sigma receptor agonists like SSR125329A and SR 31747 decrease inflammation by inducing the release of anti-inflammatory cytokines such as interleukin-10 and by decreasing the release of pro-inflammatory cytokines such as TNF-α (Bourrie et al. 2002) (Derocq et al. 1995). Results from our laboratory have show that DTG treatment inhibits the production of nitric oxide and TNF-α from cultured microglia in response to lipopolysaccharide (Hall et al. 2005), suggesting that DTG could directly inhibit the inflammation associated with ischemic injury in our in vivo model of stroke. Finally, the anti-inflammatory effects of DTG may involve a combination of both decreased signaling due to neuroprotection and an arrest of the endogenous inflammatory response. Regardless of the mechanisms involved, the anti-inflammatory effects of sigma receptor stimulation is in part responsible for the DTG-evoked depression of delayed neuronal death induced by ischemia.
Few treatments have shown success in expanding the therapeutic window for stroke. One approach that has exhibited promise in treating stroke at delayed time points is intravenous infusion of HUCBC. HUCBC have been shown to effectively decrease infarct volume by 50-80% when injected 24-48 hr in rats following MCAO (Newcomb et al. 2005; Vendrame M et al. 2005; Vendrame et al. 2004). Like DTG, HUCBC treatment shows both neuroprotective and anti-inflammatory properties. This observation is consistent with our hypothesis that both of these properties are essential for effective treatment of stroke at delayed time points. Moreover, HUCBC are potentially activating sigma receptors upon transfusion through release of neurosteroids. Umbilical plasma has been shown to contain more than double the concentration of neuroactive steroids when compared to adult plasma (Hill et al. 2000). Neurosteroids have been shown to have high affinity for sigma receptors and have been proposed as the endogenous ligand for these receptors (Maurice 2004).

The specific sigma receptor subtype mediating the neuroprotective effects of DTG remains to be identified. However, data collected in our laboratory and by other investigators suggests that both sigma-1 and sigma-2 receptors are likely to be involved in the enhanced neurosurvival reported here. Sigma-1 receptors have been shown to block both voltage-gated $K^+$ channels and NMDA receptors (Aydar et al. 2002; Zhang and Cuevas 2005);(Nuwayhid and Werling 2003). Both of these ion channel types have been linked to the neuronal damage which is produced by ischemic stoke (Bonde et al. 2005; Gido et al. 1997). Our laboratory has now shown that sigma-1 receptors can decrease
calcium elevations elicited by ischemia in neurons in vitro (Zhang and Cuevas 2005), which would also provide neuroprotection (Mattson et al. 2000). Sigma-2 receptors have been shown to inhibit voltage-gated Ca\textsuperscript{2+} channels (Zhang and Cuevas 2002), and the inhibition of these channels is neuroprotective (Kristian and Siesjo 1997). In addition to regulating ion channels, sigma receptors are likely to modulate other processes that contribute to neuronal injury following stroke. Sigma-1 and sigma-2 receptors have been detected in lipid rafts in various cell types, and are likely to modulate cytokine signaling involving these microdomains (Hayashi and Su 2005). Both sigma receptor subtypes have also been implicated in the regulation of apoptosis in tumor cell lines, with sigma-1 receptors inhibiting apoptosis and sigma-2 receptor promoting apoptosis in these cells (Crawford and Bowen 2002; Spruce et al. 2004). However, the role of sigma receptors in regulation of cell survival in native non-tumor cells remains to be established.

In conclusion, our results clearly demonstrate that the sigma receptor-selective agonist, DTG, can enhance neuronal survival when administered 24 hr after an ischemic stroke. Conversely, application of the sigma receptor antagonist, BD-1047, decreases survival rates following MCAO. Thus, our studies identify sigma receptors as one of the first potential targets for expanding the therapeutic window beyond that provided by the currently available pharmacological treatments. In addition, the efficacy of sigma receptors for stroke treatment at delayed time points is likely the result of combined neuroprotective and anti-inflammatory properties of these receptors.
Chapter 1, Figure 1

![Bar chart showing survival rates for different treatments.](image-url)
Figure 1: Effects of sigma receptor ligands on MCAO survival rates.

Survival rate of sham control injected with 15mg/kg of DTG (Sham + DTG 15), and MCAO rats under the indicated conditions. Rats subjected to MCAO received vehicle alone (No Drug), DTG at 15 mg/kg or 30 mg/kg (DTG 15 QD, DTG 30 QD, DTG 30 BID), 10 mg/kg BD 1047 (BD 1047 10 QD), or 10 mg/kg BD 1047 and 30 mg/kg DTG (BD 1047 + DTG 30 QD). QD and BID denote once daily or twice daily administration of the compounds, respectively. Asterisks indicate significant difference from DTG 30 BID. All injections commenced at 24 hr and continued to 72 hr post surgery.
Chapter 1, Figure 2

Cortical/Striatal Region

MCAO

Cortical/Hippocampal Region

MCAO + DTG (15 mg/kg)
Figure 2: DTG treatment reduced Fluoro-Jade staining when administered 24 hours post-MCAO. Photomicrographs of brain sections were taken from MCAO rats in the absence (MCAO; panels A and B) and presence of DTG treatment (MCAO + DTG 15 mg/kg; panels C and D). Coronal sections were collected at the level of the cortical/striatal (Striatum; panels A and C) or cortical/hippocampal (Hippocampus; panels B and D) regions. Fluoro-Jade staining appears as bright green coloration and is indicative of area damaged by the ischemic insult. Scale bars represent 5mm at a magnification of 12.5X.
Chapter 1, Figure 3

- Relative infarct area – to compensate for edema – ipsilateral FJ area/contralateral hemisphere area
  - * p<0.001

![Diagram showing relative infarct area with comparison between SHAM, MCAO, and MCAO+DTG groups, with asterisks indicating statistical significance.](image)
**Figure 3: Quantification of DTG-elicited reduction in post-MCAO Fluoro-Jade staining.** Fluoro-Jade staining was analyzed in the cortical/striatal (Striatum, black bars) and cortical/hippocampal (Hippocampus, gray bars) regions of sham controls (Sham), rats subjected to MCAO receiving only vehicle (MCAO) and rats subjected to MCAO receiving 15 mg/kg DTG 24 hours post-surgery (DTG). Bars represent mean ± standard error. Asterisks denote significant difference from respective areas of sham control and DTG treated rats, and was determined using a two way ANOVA followed by post hoc analysis with a Dunn’s Test for multiple group comparison (p<0.01).
**Figure 4: DTG treatment increases NeuN immunostaining when administered 24 hours post-MCAO.** Photomicrographs of brain sections were taken from MCAO rats in the absence (MCAO; panels A-C) and presence of DTG treatment (MCAO + DTG 15 mg/kg; panels D-F). Coronal sections were collected at the level of the ipsilateral cortical/striatal (Striatum; panels A, C, D and F) or cortical/hippocampal (Hippocampus; panels B and D) regions. Boxes in panels A and D indicate field of view shown at higher magnification in panels C and F, respectively. Individually stained nuclei from neurons of the cortical/striatal regions were visualized using higher magnification (200X; panels C and F). NeuN staining appears as dark brown coloration and is indicative of viable neurons. Scale bars (A, B, D, and E) represent 30µm at a magnification of 40X. Scale bars for C and F represent 50µm at 200X.
Chapter 1, Figure 5

* $p<0.01$ significant difference from equivalent contralateral hemisphere

# $p<0.01$ significant difference from ipsilateral hemisphere of SHAM and DTG groups
Figure 5: DTG treatment at delayed time points significantly increases the number of NeuN positive cells following MCAO. The number of NeuN positive neurons detected in ipsilateral (IPSI) and contralateral (CONTRA) hemispheres of cortical/hippocampal (Hippocampus) and cortical/striatal (Striatum) regions of sham control rats (SHAM, white bars), MCAO rats injected with vehicle alone (MCAO, black bars) and MCAO rats treated with 15 mg/kg of DTG (DTG, gray bars). Bars represent mean count ± standard error. Statistical significance from equivalent region in the contralateral hemisphere is indicated by the asterisks, and from the ipsilateral hemisphere of SHAM and DTG groups by the pound symbol (p<0.01 for both). Statistical significance was determined using a two way ANOVA followed by post hoc analysis with a Tukey Test for multiple group comparison.
Chapter 1, Figure 6

(A) Striatum MCAO
(B) Hippocampus
(C) 200X

(D) Striatum MCAO + DTG (15 mg/kg)
(E)
(F)
Figure 6: DTG decreases the intensity of GFAP immunostaining surrounding the infarct zone when administered 24 hours post-MCAO.

Photomicrographs of brain sections were taken from MCAO rats in the absence (MCAO; panels A-C) and presence of DTG treatment (MCAO + DTG 15 mg/kg; panels D-F). Coronal sections were collected at the level of the ipsilateral cortical/striatal (Striatum; panels A, C, D and F) or cortical/hippocampal (Hippocampus; panels B and D) regions. Boxes in panels A and D indicate field of view shown at higher magnification in panels C and F, respectively. Reactive astrocytes showing high levels of GFAP were observed in the cortical/striatal region using higher magnification (200X; panels C and F). GFAP staining appears as dark brown coloration. Scale bars in panels A, B, D and E represent 30\(\mu\)m at a magnification of 40X, and in panels C and F represent 50\(\mu\)m at 200X.
Chapter 1, Figure 7

A B
MCAO

C D
MCAO+DTG

E F
SHAM
Figure 7: DTG treatment decreases Isolectin IB4 binding when administered 24 hours post-MCAO. Photomicrographs of coronal brain sections were taken from the ipsilateral cortical/striatal region of MCAO rats in the absence (MCAO; panels A and B) and presence of DTG (15 mg/kg) treatment (MCAO + DTG; panels C and D) as well as sham controls treated with DTG (SHAM; panels E and F). Boxes in panels A, C and E indicate field of view shown at higher magnification in panels B, D and F, respectively. Individual labeled activated microglia and/or macrophages from cortical/striatal regions were visualized using higher magnification (200X; panels B, D and F). Isolectin IB4 labeling appears as bright green coloration. Scale bars (A, C, E) represent 20\(\mu\)m at 40X, and scale bars (B, D, F) represent 50\(\mu\)m at 200X.
References


O'Callaghan, J. P.: Biochemical analysis of glial fibrillary acidic protein as a quantitative approach to neurotoxicity assessment: advantages, disadvantages and application to the assessment of NMDA receptor


CHAPTER 2

THE SPLEEN CONTRIBUTES TO STROKE-INDUCED NEURODEGENERATION

Craig T. Ajmo Jr., MS*, Dionne O. L. Vernon BS*, Lisa A. Collier BS*, Svitlana Garbuzova-Davis Ph.D. †, Alison E. Willing Ph.D. †, Keith R. Pennypacker Ph.D.*

*Department of Molecular Pharmacology & Physiology, University of South Florida, Tampa, Florida 33612

†Center of Excellence in Aging and Brain Repair, University of South Florida, Tampa, Florida 33612
Abstract

Stroke, a cerebrovascular injury, is the first cause of disability and third cause of death in the world (W.H.O. 2007). Recent reports implicate that anti-inflammation enhances neurosurvival to limit expansion of the infarction. The immune response that initiates from the spleen has been linked to the systemic inflammatory response to stroke, contributing to neurodegeneration. Here we show that removal of the spleen significantly reduces neurodegeneration after ischemic insult. Rats splenectomized two weeks prior to permanent middle cerebral artery occlusion had a >80% decrease in infarction volume in the brain compared to those rats that were subjected to the stroke surgery alone. Splenectomy also resulted in decreased activated microglia, macrophages, and neutrophils present in the brain tissue. Our results demonstrate that the peripheral immune response as mediated by the spleen is a major contributor to the inflammation that enhances neurodegeneration occurring in the brain after stroke.
Introduction

Protecting neurons and blunting the inflammatory response are key components for developing new treatments to alleviate the cerebral damage, disability and ultimately death caused by stroke. Currently the only FDA approved treatment for stroke is tissue plasminogen activator (tPA), a clot buster that has a limited three hour therapeutic window of administration after stroke leaving only a 33% success rate (Marler and Goldstein 2003). This treatment has no neuroprotective or anti-inflammatory properties making it unsuitable for treating those key components of stroke that result in expanding ischemic damage. Therefore it is necessary to develop new therapies for this injury with a complete understanding of the mechanism of interaction between the body’s immune response to the ischemic injury as well as the neurodegeneration that is occurring in the brain.

After the initial ischemic injury, a compromised blood brain barrier and expression of adhesion molecules by the vascular endothelial cells permit an influx of peripheral immune cells including macrophages, neutrophils, leukocytes, T cells and B cells (Emsley et al. 2003). This peripheral immune reaction to the brain ischemia exacerbates the local brain inflammatory response leading to enhanced neurodegeneration. Previous studies have demonstrated altered splenic function after a stroke and increased circulating proinflammatory cytokines (Offner et al. 2006b; Vendrame et al. 2006). These infiltrating cells and increased pro-inflammatory cytokines may negatively impact stroke outcome
(Lucas et al. 2006). Therefore a potential target for new stroke therapies to decrease stroke-induced inflammation may be the peripheral immune system.

Animal models of brain injury like 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Benner et al. 2004), and transient (Offner et al. 2006b) or permanent middle cerebral artery occlusion (MCAO) (Vendrame et al. 2006) report that there is a reduction in the spleen weight, which may result from the release of splenocytes. Transfusion of human umbilical cord blood cells (HUCBC) 24 hours after permanent MCAO in rats results in retention of the spleen’s mass as well as a reduction of infarct size by 60% (Vendrame et al. 2006). Further, there was an increase in the anti-inflammatory cytokine, interleukin-10, with this treatment. These results are consistent with the hypothesis that the spleen responds to the injury in the brain by releasing stored immune cells into the bloodstream which infiltrate the brain and promote a secondary inflammatory response to enhance neurodegeneration. By examining the effects of splenectomy in the in vivo MCAO stroke model, we now show that the spleen greatly contributes to the peripheral immune cells that invade the CNS at the time of ischemic injury. Furthermore, our findings imply that the spleen will be a starting point in development of novel therapies that target the splenic immune response after stroke, and ultimately produce a treatment that can target stroke at an extended therapeutic window.
Materials and Methods

Animals - Male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 300 to 350 g were housed in a climate controlled room with water and laboratory chow available ad libidum. Animals were cared for according to the guidelines of the IACUC of the University of South Florida’s College of Medicine.

Splenectomy – Male Sprague-Dawley rats weighing 300 to 350 g were anesthetized and maintained with 3 to 4% isofluorane in 100% oxygen. Splenectomy was performed by making a 2 cm dorsal midline skin incision in the rat at the caudal terminus at the level of the 13th rib. Opening the abdominal wall on the anatomical left, 1.5 to 2.5 cm from midline exposed the spleen. With blunt forceps, the organ, (with accompanying blood vessels and pancreatic tissue), was exteriorized through the incision. The blood vessels were ligated; the spleen was removed and stored at -80°C. The skin incision was closed with 3-0 sutures.

Laser Doppler Radar Blood Flow Measurement – Rats were anesthetized and maintained with 3 to 4% isofluorane in 100% oxygen. The rat’s head and neck were shaved. Using a scalpel, an incision was made to expose the plates of the skull on the side that was ipsilateral to the MCAO. Once the incision was made, the skin was spread open and the membrane covering the skull pushed aside by rubbing with a cotton tipped applicator. Using a microdrill a small hole was drilled through the skull at 1 mm posterior to bregma and 4mm lateral to bregma. A stainless steel hollow screw guide was screwed into the skull. A fiber optic cable
(500 \mu m) was inserted into the screw guide and the incision was sealed back together with vetbond. Blood pressure in the brain was then detected using the Moor Instruments LTD laser Doppler with MoorLAB propriety Windows based software on a standard laptop. Once surgery was complete, the screw guide was removed and the scalp incision was closed with surgical sutures. Rats that did not show \geq 60\% reduction in perfusion during MCAO were excluded from the study.

**Permanent Middle Cerebral Occlusion Model** - Permanent focal ischemia was achieved during MCAO by using the intraluminal suture technique. A 4cm long monofilament was advanced up the internal carotid artery into the middle cerebral artery and was then tied off at the internal/external carotid junction to produce permanent occlusion. The incision was then sutured closed, given a 1ml subcutaneous injection of saline and allowed to wake in a fresh cage.

**Brain Extraction and Sectioning** - The animals were euthanatized with 0.5 ml of pentobarbital at 96 hours, and perfused with saline and 4\% paraformaldehyde. The brains was harvested, post fixed in paraformaldehyde, and cryoprotected in 20\% and 30\% sucrose in PBS. Brains were frozen and sliced into 30\mu m sections with a cryostat. Sections were either thaw mounted on glass slides or placed in Walter’s Anti-freeze cryopreservative.
**Fluoro-Jade Histochemistry** – Six coronal brain sections at 1 mm intervals were cut from 1.7 to -3.3 mm from bregma were stained with Fluoro-Jade (Histochem, Jefferson, AR), which labels degenerating neurons and is more sensitive than triphenyltetrazolium chloride (TTC) in identifying neurodegeneration. This method was adapted from that originally described by Schmued et al (1997) and has been detailed previously by Duckworth et al (2005). Tissue was thaw mounted and dried to glass slides. Slides were then placed in absolute ethanol for 3 min followed by 70% ethanol and deionized water for 1 min each. Sections were then oxidized using 0.06% KMnO₄ solution for 15 min followed by three rinses in ddH₂O for 1 minute each. Sections were then placed in a 0.001% solution of Fluoro-Jade in 0.1% acetic acid for 30 min. Slides were again rinsed, and then allowed to dry at 45 °C for 20 min, cleared with xylene and coverslipped with DPX mounting medium (Electron Microscopy Sciences, Ft. Washington, PA).

**Nissl Staining with Thionin** - Six coronal brain sections at 1 mm intervals were cut from 1.7 to -3.3 mm from bregma were stained with Thionin (Sigma-Aldrich, St. Louis, MO) which labels Nissl bodies in cells. Tissue was thaw mounted and dried on glass slides. Slides were then dehydrated in alcohol series, stained in the Thionin stock solution for 1.5 minutes, dehydrated, cleared with xylene and coverslipped with Permount (Fisher Scientific, Fair Lawn, NY).
**Immunohistochemistry** - The tissue slides were thawed, rinsed with PBS (pH 7.2), and then placed in permeabilization buffer containing 10% goat serum, 3% 1M lysine, and 0.3% Triton X-100 in PBS for 1 hr at room temperature. Next, the sections were stained with either Isolectin IB4 (*Griffonia simplicifolia*) conjugated to Alexa-Fluor® 488 (5 µl/ml, Molecular Probes, Eugene, OR) or myeloperoxidase (MPO) rabbit polyclonal antibody (1:400, Affinity Bioreagents, Golden, CO) overnight at 4°C in a PBS solution with 2% goat serum and 0.3% Triton X-100 in a foil covered humidified chamber. The next morning the slides were washed with PBS (3 x 5 min). The MPO slides were then incubated with Alexa-Fluor® 488 goat anti-rabbit secondary (1:300, Molecular Probes) antibody solution (PBS, 2% goat serum, 0.3% Triton X-100) in a foil covered humidified chamber for 1 hour. The isoelectin slides required no additional incubation step. After final washing the slides were cover slipped with Vectashield hard set mounting media with DAPI (Vector Laboratories, Burlingame, CA).

**Infarct Volume Quantification** - Images of six 30 µm brain sections were taken at millimeter intervals from 1.7 to -3.3 mm from bregma to determine the volume of infarction. The Fluoro-Jade stained tissue was digitally photographed with an Olympus IX71 microscope controlled by DP manager software (Olympus America Inc, Melville, NY) at a magnification of 1.25X. Images were edited with Jasc Paintshop Pro. Area of neurodegeneration was measured using the NIH Image J software. The area of the contralateral side of the brain tissue was also measured and used to compensate for possible edema in the ipsilateral
hemispheres. Infarct volumes were then calculated by the summation of the infarct areas.

**Blood Smear Preparation and Giemsa Staining** – During harvest, a small drop of blood was placed near the frosted end of a clean glass slide. A second slide was used as a spreader. Holding the spreader slide at a 30 degree angle, the spreader was drawn back against the drop of blood. The blood was then pulled to the opposite edge of the slide producing a thin film on the slide. After air drying and the smears were fixed in Methanol for 5 minutes and again allowed to air dry. For Giemsa (Sigma-Aldrich) staining, slides were first washed three times for 2 minutes each with distilled water. Slides laying flat were then flooded with Giemsa stain (1 to 2 ml) for 4 minutes. Equal volumes of distilled water were added to the slides to stop staining. Slides were then dunked in a large container of water to remove excess stain. Stained slides were rinsed thoroughly three times for three minutes in distilled water, air dried vertically overnight and coverslipped with Permount.

**Statistical Analysis** - Infarct size was analyzed using ANOVA followed by Bonferroni’s or Tukey’s post hoc tests. A value of p<0.05 was considered significant.
Results

Splenectomy Prior to MCAO Reduces Neurodegeneration

A hallmark of certain brain injury models is the infiltration of peripheral immune cells that work to remove damaged tissue from the CNS (Stoll et al. 1998). A high concentration of these cells, however, will often times result in enhanced damage instead of aid through the release of free radicals, inflammatory cytokines, chemokines and cytotoxic substances (Raivich et al. 1999; Stoll et al. 2000) as these immune cells are responding to the ischemic injury as if it were invading pathogens. In conjunction with the increased peripheral immune cells infiltrating the brain, it has been observed that the spleen, an organ related to the immune system, shrinks in size. To determine whether the physiological change of the spleen is contributing to increased peripheral immune cell activity in the brain, as well as the neurodegeneration occurring at the site of ischemic injury, we compared rats that underwent splenectomy followed two weeks later by MCAO or sham MCAO with naïve rats that had MCAO only or sham MCAO. At 96 hours post-MCAO the animals were perfused, brains were sectioned at 30 μm, and infarct volume determined either with Fluoro-Jade, a histological stain that labels degenerating neurons and Thionin, a histological stain that labels the Nissl bodies of intact neurons.

In rats subjected to stroke-only, the Fluoro-Jade stain labeled extensive degeneration in the cortex, striatum and hippocampus of the hemisphere ipsilateral to the MCAO surgery (Figure 8a, center panel). Brain sections from rats that underwent splenectomy prior to MCAO displayed significantly less
Fluoro-Jade staining in the stroked side relative to brain sections of MCAO-only rats (Figure 8a, center and right panels) and more closely resembled Fluoro-Jade stained brain tissue from the sham MCAO animals (Figure 8a, left panel). Quantification of the Fluoro-Jade stained infarct volumes demonstrated that the splenectomy prior to MCAO significantly decreased infarct volume by 82.3% compared to volumes from the brains of rats subjected to MCAO-only (*p<0.001; significance determined by ANOVA followed by Dunn’s post hoc test) (Figure 8c).

To confirm these results we employed another method to measure infarct size by labeling Nissl bodies of intact neurons with Thionin. In contrast to Fluoro-Jade, absence of staining denotes neurodegeneration and cell death in the brain section. Again we observed the same effects of splenectomy on stroke; the brain sections of the MCAO-only treated rat showed absence of staining across the majority of the ipsilateral hemisphere including the cortical, striatal and hippocampal regions (Figure 8b, center panel). The brain sections from animals subjected to splenectomy and stroke showed a uniform labeling of Nissl bodies across the tissue, similar to brain sections from sham-operated rats, with only minute areas deficient in staining (Figure 8b, left and right panels). Infarct volume analysis again showed a significant decrease of 80.8% in those animals whose spleens were removed before stroke (*p<0.001; significance determined by ANOVA followed by Bonferroni’s post hoc test) (Figure 8d). Taken together, the data gathered from these neuronal stains shows that the presence of the spleen at the time of stroke contributes to the neurodegeneration occurring at the site of ischemic injury.
Microglia, infiltrating macrophages, and neutrophils all enhance neuroinflammation at the site of ischemia (Allan and Rothwell 2003; Barone and Feuerstein 1999; Barone et al. 1995; Lucas et al. 2006). These cells may be released from the spleen into the circulation during size reduction in response to stroke injury. We, therefore, examined this inflammatory response in the brain with immunohistochemical stains for activated microglia and peripheral immune cells.

We first investigated microglia, the resident macrophages of the brain that lie dormant in a ramified state until activated by some insult (Allan and Rothwell 2003; Lyons et al. 2000) and systemic macrophages, which are chemically called to the ischemic injury and invade through a compromised blood brain barrier (Kim et al. 2006; Stoll et al. 2002). To this date these cell types are not readily distinguishable due to lack of differential cellular markers and act in similar fashion in their expression of chemokines, inflammatory cytokines and free radical production (Raivich et al. 1999). To observe the actions of these cells in response to splenectomy and stroke, we labeled the cells with Isolectin IB4 which binds sugar residues found on activated microglia, infiltrating macrophages, and endothelial cells. The morphology of the immune cells, however, is easily discerned from that of the endothelial cells (Abbott 2000; Kim et al. 2006). Brain sections of MCAO-only rats showed cell labeling in the ipsilateral hemisphere (Figure 9b). Brain sections from splenectomized rats show Isolectin labeling of activated microglia and macrophages in small localized regions within the cortex.
and striatum (Figure 9c). In sham rat tissue only endothelial cells of the blood vessels and intact fiber tracts were labeled for background staining (Figure 9a).

Neutrophil granulocytes, part of the innate immune system, are reported to be one of the first damaging cell types present in the infarction and are involved in the exacerbating neurodegeneration resulting from stroke (Akopov et al. 1996; Barone and Feuerstein 1999; Emerich et al. 2002; Zhang et al. 1994a). Inhibition of neutrophils at the time of stroke has also shown a promising reduction of infarction (Chen et al. 1994; Chopp et al. 1994). These cells adhere to endothelium (Barone and Feuerstein 1999), release inflammatory cytokines, and chemoattractants (Barone and Feuerstein 1999) and produce radicals resulting in tissue damage (Dirnagl et al. 1999; Iadecola and Alexander 2001; Matsuo et al. 1995; Matsuo et al. 1996). Since these innate immune cells are found in the brain during ischemic injury and have been reported to reside in the spleen (Shi et al. 2001) neutrophils, as affected by splenectomy and MCAO, were discerned by immunohistochemically labeling brain sections for myeloperoxidase (MPO), a peroxidase enzyme found in lysosomes of these cells (Feuerstein et al. 1998; Nauseef 1998; Weston et al. 2006). Brain tissue sections of rats that were subjected to MCAO-only showed an increase of MPO labeling and a disappearance of fiber tracks in the infarct at the level of the striatum (Figure 10b). Tissue sections from splenectomized rats that underwent MCAO showed few MPO stained cells comparable to tissue sections from sham rats (Figure 10a and 10c). Labeled neutrophil analysis showed a significant decrease of 90.1% in MPO labeled cells in the brain tissue of splenectomized rats that underwent...
stroke (*p<0.001, significance determined by ANOVA followed by Dunn’s post hoc test) (Figure 10d). These results indicate that the spleen modulates the systemic immune response occurring at the site of ischemic injury and may be a reservoir for peripheral immune cells which are released at the time of cerebral injury and migrate to the brain exacerbating damage.

**Splenectomy has no Effect on Peripheral White Blood Cell Populations**

Since infiltrating peripheral immune cells travel through the vasculature migrating to the damaged tissue in the brain (Lucas et al. 2006), we examined whether spleen removal altered the profile of leukocytes in the blood. Smears were obtained from all rats at 0, 48, and 96 hours after the stroke surgery. These blood smears were stained with Giemsa and white blood cell populations tabulated. In the stroke-only animals blood leukocytes did not significantly change in the blood at 48 and 96 hours (Figure 11a).

In those animals that had their spleens removed prior to the stroke surgery, there was no significant change in the white blood cell populations in the blood post-MCAO. There was a tendency for the lymphocyte population to decrease and the neutrophil population to increase at 48 hours but both of these cell types returned to baseline levels at 96 hours but these changes were not significant (Figure 11b). Sham MCAO (Figure 11c) and splenectomy sham MCAO (Figure 11d) blood smears demonstrated no change in blood cell populations. Blood smears taken during the two week recovery period and
stained with Giemsa showed no significant change in leukocyte populations as a result of splenectomy (Figure 11e).

**Discussion**

Our results show that splenectomy prior to stroke significantly reduces infarction size in the brain. A proposed mechanism of action is activation of the sympathetic nervous system when a stroke occurs resulting in spleen reduction through a potential release of red and white blood cells (Stewart and McKenzie 2002). This reduction causes a release of pro-inflammatory immune cells, which are attracted to the brain by chemokines where they increase neuroinflammation and neurodegeneration (Abraham et al. 2002; Hausmann et al. 1998). Removal of the spleen prior to stroke decreases the peripheral immune cells entering the brain through a compromised blood brain barrier resulting in decreased neuroinflammation and neurodegeneration. This is consistent with the data reported from the transfusion of HUCBC into stroked rats (Offner et al. 2006b; Vendrame et al. 2006).

Alternatively, these data also yield insight into the systemic nature of the body’s response to a brain injury. Activated microglia have been thought to be the main cause of the second wave of neurodegeneration in the infarct area as these cells have been shown to outnumber the macrophages at the infarction (Liu and Hong 2003; Schilling et al. 2003). However, systemic inhibition of macrophages by anti-CD11b, anti-CD18, and anti-Mac-1 monoclonal antibodies 1 hour after transient middle cerebral artery occlusion show significant reduction
of infarction volume in the ischemic brain (Chen et al. 1994; Chopp et al. 1994; Zhang et al. 1995b). This suggests that the synergistic actions of both the activated microglia and infiltrating macrophages result in the secondary enhanced neuroinflammation and neurodegeneration. Splenectomy dramatically reduces infarction demonstrating that inhibition of systemic immune response cells by removing a population of infiltrating macrophages, neutrophils, B cells and T cells reduces cellular interaction with the activated microglia at the site of ischemic injury resulting in decreased damage.

We believe that the removal of the spleen does not result in a preconditioning response that ultimately reduces infarction size in the rat. If this were the case, removal of the spleen would result in an immune response in the rat and yet white blood cell populations are stable during the two week recovery period prior to stroke. In light of our findings of decreased infarct volume and modulation of immune cells we strongly recommend further testing of the splenic interaction with ischemic brain injury and propose that this organ is an excellent target for developing novel treatments that will minimize stroke damage.

Acknowledgements This work was supported by grants from the National Institute for Health NS052839-01A1 (to A.W.)
Chapter 2, Figure 8

Sham MCAO Splenectomy-MCAO

Infarct Volume (% of ipsilateral hemisphere)

SHAM MCAO Splenectomy-MCAO

Infarct Volume (% of ipsilateral hemisphere)

SHAM MCAO Splenectomy-MCAO

* denotes significant difference.
Figure 1: Histological pathology of infarct in brain tissue of splenectomized rats 96hr post MCAO.  

a, Fluoro-Jade staining shows a large area damage in the ipsilateral hemisphere (a, center panel) whereas splenectomy reduces Fluoro-Jade labeled degenerating neurons (a, right panel) and is similar in staining to sham tissue (a, left panel).  

b, The area lacking Thionin staining in the brain section from the MCAO-only treated rat is representative of the infarction (b, center panel) while tissue sections from sham (b, left panel) and splenectomized rats (b, right panel) that were subjected to stroke show reduced loss of staining (a and b scale bar = 5 mm).  

c, ANOVA followed by the post hoc Dunn’s test showed that splenectomy significantly reduced Fluoro-Jade stained degenerating neurons when performed prior to stroke (*p<0.001).  

d, ANOVA followed by the post hoc Bonferroni’s test showed that splenectomy significantly reduces absence of Thionin staining in brain sections from rats that underwent stroke (*p<0.001).
Chapter 2, Figure 9
Figure 2: Immunohistochemical labeling of activated microglia and infiltrating macrophages at the level of infarction 96 hours post MCAO.  

a, Isolectin IB4 labeling shows no activated microglia or macrophages in brain sections of sham operated rats. Arrows point to intact fasiculae of the striatum that pick up background staining.  
b, Box denotes area labeled with Isolectin IB4 and shows activated microglia and macrophages. The arrow points to endothelial cells of a blood vessel labeled by Isolectin IB4.  
c, Isolectin IB4 labeling of brain sections from splenectomized MCAO rats show reduced microglia and macrophage activity and is localized to a smaller area seen inside box. Scale bar = 400 \( \mu \text{m} \)
Chapter 2, Figure 10

(a) and (b) show images of tissue sections stained for MPO labeling. Arrows indicate areas of interest. (c) is a control image. (d) presents a bar graph showing the average MPO labeled cell count for different groups: SHAM, MCAO, and Splenectomy-MCAO. The graph indicates a significant difference (*).
Figure 3: MPO staining of neutrophils in the infarct zone.  

Immunohistochemical labeling of brain sections for MPO shows no neutrophils in the striatum of the sham rats; the arrow indicates fasiculae in the striatum were intact.  

b, MPO staining in brain sections from rats treated with MCAO-only show many neutrophils in the striatum and a disappearance of fasiculae. Arrows denote labeled neutrophils.  

c, Brain sections from rats treated with splenectomy prior to stroke show similar pathology to the sham brain section (panel a) with intact fasiculae designated by arrow. Scale bars = 200 µm.  

d, ANOVA followed by the post hoc Bonferroni’s test showed that splenectomy significantly reduces average number of cells labeled with MPO that were counted in representative areas of the infarction (*p<0.001).
Chapter 2, Figure 11

(a) [Graph showing WBC percentage over time for different cell types.]

(b) [Graph showing WBC percentage over time for different cell types.]

(c) [Graph showing WBC percentage over time for different cell types.]

(d) [Graph showing WBC percentage over time for different cell types.]

(e) [Graph showing WBC percentage over time for normal and spleen conditions.]

(normal)
Figure 4: White blood cell profiles during treatments.  

a, White blood cell profiles at 0, 48, and 96 hours after stroke for rats that underwent MCAO-only.  
b, White blood cell profiles of splenectomized rats that were subjected to stroke taken at 0, 48, and 96 hours after stroke.  
c, White blood cell profiles of sham MCAO operated rats at 0, 48, and 96 hours after sham stroke.  
d, White blood cell profiles of splenectomy sham MCAO operated rats at 0, 48 and 96 hours after sham stroke.  
e, White blood cell profiles of the two week recovery period between splenectomy and stroke.  

ANOVA followed by the post hoc Tukey’s test showed no significant differences between the groups (*p<0.05).
Reference


CHAPTER 3

SPLENIC RESPONSE TO STROKE IS NOT DEPENDENT ON DIRECT AUTONOMIC NEUROTRANSMISSION VIA THE SPLENIC NERVE


*Department of Molecular Pharmacology & Physiology, University of South Florida, Tampa, Florida 33612

†Center of Excellence in Aging and Brain Repair, University of South Florida, Tampa, Florida 33612
Abstract

Splenectomy two weeks prior to stroke results in a significant decrease in infarct volume, and immune cell modulation in the rat brain parenchyma. Also stroke itself causes splenic shrinkage suggesting that the spleen contributes the mechanisms of damage occurring at the site of ischemic injury. To determine if autonomic neurotransmission produces in the splenic response to stroke, spleens of male Sprague-Dawley rats were isolated and denervated two weeks prior to permanent middle cerebral artery occlusion (MCAO). At 48 hours after the stroke surgery, blood and spleen tissue were harvested for flow cytometry analysis and labeled with various leukocyte specific antibodies. At this time, the rats were also perfused with 4% paraformaldehyde, brains were harvested, sectioned at 30 µm, and labeled with markers of neurodegeneration and inflammation. Flow cytometry analysis showed a significant increase in the percentage of monocytes present in spleen samples from denervated rat spleens compared to the spleens of rats subjected to MCAO-only. Flow cytometry analysis showed no significant difference in all other cellular percentages when comparing the blood and spleen tissue from the denervated rats to rats that underwent MCAO-only. Infarct volume analysis by Fluoro-Jade, which labels degenerating neurons, showed no significant changes in the volume of ischemic damage in the brains of those rats whose spleens were denervated prior to stroke when compared to infarct volumes in the brains of MCAO-only rat. This previous observation was supported by thionin Nissl staining showing that infarction from the denervated spleen sample group equaled those from MCAO-
only. Denervation of the spleen prior to stroke also failed to inhibit splenic shrinkage. Weights of these spleens showed no significant difference from the spleen weights of MCAO-only animals. These results indicate that splenic shrinkage at the time of stroke is not contingent on autonomic neurotransmission. This suggests that spleen shrinkage is not a result of direct signal transmission from the splenic nerve.
Introduction

Stroke causes several dysfunctions in autonomic control that can lead to hypertension, arrhythmias, myocardial necrosis and death (Meyer et al. 2004). These alterations become most prevalent during the acute or early phase of stroke where sympathetic neurotransmission is up-regulated (Tokgozoglu et al. 1999; Wang et al. 1997). Shortly after ischemic insult, there is an increase of both norepinephrine and epinephrine in the circulation which are sympathetic neurotransmitters (Meyer et al. 2004). Activation of $\alpha$ adrenergic receptors by these agonists results in elevated heart rate by cardiac muscle contraction and hypertension by vascular smooth muscle contraction (Butcher et al. 1993; Cechetto et al. 1989; Klingelhofer and Sander 1997). The spleen may be affected by this increase in sympathetic tone as it has been shown that the splenic nerve is composed of approximately 98% sympathetic nerve fibers (Klein et al. 1982) and most studies involving the autonomic nervous system and the spleen suggest that innervation is predominantly sympathetic (Elenkov et al. 2000). Splenic smooth muscle has also been shown to contract, which is facilitated by sympathetic neurotransmission and to autotransfuse large amounts of red blood cells into the circulation during times of physiological stress (Stewart and McKenzie 2002).

Recent studies transfusing human umbilical cord blood cells (HUCBC) have showed that therapeutic window of treatment can be extended up to 48 hours after permanent middle cerebral artery occlusion (MCAO) (Newcomb et al. 2006; Vendrame et al. 2004). This treatment elevates neuroprotective and anti-
inflammatory effects resulting in significant decreases in infarction volume in the rat brain (Vendrame et al. 2004). PCR analysis for human DNA of the cord blood cells showed that they migrated only to the injured hemisphere of the brain and the spleen when transfused 24 hours after MCAO (Vendrame et al. 2004). Identification of HUCBC by immunoreactivity for human nuclei confirmed the presence of the HUCBC in the spleen (Vendrame et al. 2004). Further investigation of the spleen/stroke interaction showed that the spleen shrinks in response to ischemic injury; this is accompanied by an increase of regulatory T cells and macrophages in the circulation (Offner et al. 2006b) suggesting that the spleen contributes to the peripheral immune response of stroke by releasing stored inflammatory cells which then infiltrate the brain and enhance inflammation and neurodegeneration at the site of injury.

Our laboratory has shown that removal of the spleen two weeks prior to MCAO in rats results in a >80% decrease in infarction. This effect was accompanied by a significant reduction in the number of infiltrating neutrophils and decreased activated microglia/macrophages. These results confirm that the spleen contributes to stroke-induced neurodegeneration and supports the hypothesis that the spleen is a target for developing alternative treatments for stroke that can modulate neurodegeneration and inflammation. Determination of the signaling mechanism that initiates splenic reaction to stroke is vital to inhibiting the splenic response to ischemia. We hypothesize that sympathetic neurotransmission is the signaling mechanism that causes the spleen to shrink,
releasing inflammatory factors that contribute to the neurodegeneration occurring at the site of injury.

By examining the effects of denervation of the spleen prior to stroke on histological and immunohistochemical markers of neurodegeneration and flow cytometry analysis of leukocyte profile, we show that splenic reaction to stroke is not dependent on sympathetic innervation. These findings imply that another mechanism of action creates the link between ischemic injury and splenic shrinkage.

**Materials and Methods**

**Animals** - Male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 300 to 350 g were housed in a climate controlled room with water and laboratory chow available *ad libidum*. Animals were cared for according to the guidelines of the IACUC of the University of South Florida’s College of Medicine.

**Splenic Denervation** – Rats were anesthetized with 3 to 4% isofluorane and maintained with 100% oxygen with. Denervation was performed by making a 4 cm dorsal midline skin incision at the caudal terminus at the level of the 13th rib. Opening the abdominal wall on the anatomical left, 1.5 to 2.5 cm from midline exposed the spleen. With blunt forceps, the organ, (with accompanying blood vessels and adipose tissue), was exteriorized through the incision. Under a surgical microscope, splenic nerves were located at the anterior and posterior underside of the spleen and ligated taking care not to damage the splenic blood
vessels. The spleen and adipose tissue were then reinserted into the animal through the incision which was then closed with suture.

**Splenectomy** – Male Sprague-Dawley rats weighing 300 to 350 g were anesthetized and maintained with 3 to 4% isofluorane in 100% oxygen. Splenectomy was performed by making a 2 cm dorsal midline skin incision in the rat at the caudal terminus at the level of the 13th rib. Opening the abdominal wall on the anatomical left, 1.5 to 2.5 cm from midline exposed the spleen. With blunt forceps, the organ, (with accompanying blood vessels and pancreatic tissue), was exteriorized through the incision. The blood vessels were ligated; the spleen was removed and stored at -80°C. The skin incision was closed with 3-0 sutures.

**Laser Doppler Radar Blood Flow Measurement** – Rats were anesthetized and maintained with 3 to 4% isofluorane in 100% oxygen, The rat’s head and neck were shaved. Using a scalpel, an incision was made to expose the plates of the skull on the side that was ipsilateral to the MCAO. Once the incision was made, the skin was spread open and the membrane covering the skull pushed aside by rubbing with a cotton tipped applicator. Using a microdrill a small hole was drilled through the skull at 1 mm posterior to bregma and 4mm lateral to bregma. A stainless steel hollow screw guide was screwed into the skull. A fiber optic cable (500 µm) was inserted into the screw guide and the incision was sealed back together with vetbond. Blood pressure in the brain was then detected using the Moor Instruments LTD laser Doppler with MoorLAB propriety Windows based
software on a standard laptop. Once surgery was complete, the screw guide was removed and the scalp incision was closed with surgical sutures. Rats that did not show ≥60% reduction in perfusion during MCAO were excluded from the study.

**Permanent Middle Cerebral Occlusion Model** - Permanent focal ischemia was achieved during MCAO by using the intraluminal suture technique. A 4 cm long monofilament was advanced up the internal carotid artery into the middle cerebral artery and was then tied off at the internal/external carotid junction to produce permanent occlusion. The incision was then sutured closed, given a 1 ml subcutaneous injection of saline and allowed to wake in a fresh cage.

**Brain Extraction and Sectioning** - The animals were euthanatized with 0.5 ml of pentobarbital at 96 hours, and perfused with saline and 4% paraformaldehyde. The brains were harvested, post fixed in paraformaldehyde, and cryoprotected in 20% and 30% sucrose in PBS. Brains were frozen and sliced into 30 µm sections with a cryostat. Sections were either thaw mounted on glass slides or placed in Walter’s Anti-freeze cryopreservative.

**Fluoro-Jade Histochemistry** – Six coronal brain sections at 1 mm intervals were cut from 1.7 to -3.3 mm from bregma were stained with Fluoro-Jade (Histochem, Jefferson, AR), which labels degenerating neurons and is more sensitive than triphenyltetrazolium chloride (TTC) in identifying
neurodegeneration. This method was adapted from that originally described by Schmued et al (1997) and has been detailed previously by Duckworth et al (2005). Tissue was thaw mounted and dried to glass slides. Slides were then placed in absolute ethanol for 3 min followed by 70% ethanol and deionized water for 1 min each. Sections were then oxidized using 0.06% KMnO₄ solution for 15 min followed by three rinses in ddH₂O for 1 minute each. Sections were then placed in a 0.001% solution of Fluoro-Jade in 0.1% acetic acid for 30 min. Slides were again rinsed, and then allowed to dry at 45 °C for 20 min, cleared with xylene and coverslipped with DPX mounting medium (Electron Microscopy Sciences, Ft. Washington, PA).

**Nissl Staining with Thionin** - Six coronal brain sections at 1 mm intervals were cut from 1.7 to -3.3 mm from bregma were stained with Thionin (Sigma-Aldrich, St. Louis, MO) which labels Nissl bodies in cells. Tissue was thaw mounted and dried on glass slides. Slides were then dehydrated in alcohol series, stained in the Thionin stock solution for 1.5 minutes, dehydrated, cleared with xylene and coverslipped with Permount (Fisher Scientific, Fair Lawn, NY).

**Immunohistochemistry** - The tissue slides were thawed, rinsed with PBS (pH 7.2), and then placed in permeabilization buffer containing 10% goat serum, 3% 1M lysine, and 0.3% Triton X-100 in PBS for 1 hr at room temperature. Next, the sections were stained with either Isolectin IB4 (*Griffonia simplicifolia*) conjugated to Alexa-Fluor® 488 (5 μl/ml, Molecular Probes, Eugene, OR) or
myeloperoxidase (MPO) rabbit polyclonal antibody (1:400, Affinity Bioreagents, Golden, CO) overnight at 4°C in a PBS solution with 2% goat serum and 0.3% Triton X-100 in a foil covered humidified chamber. The next morning the slides were washed with PBS (3 x 5 min). The MPO slides were then incubated with Alexa-Fluor® 488 goat anti-rabbit secondary (1:300, Molecular Probes) antibody solution (PBS, 2% goat serum, 0.3% Triton X-100) in a foil covered humidified chamber for 1 hour. The isolectin slides required no additional incubation step. After final washing the slides were cover slipped with Vectashield hard set mounting media with DAPI (Vector Laboratories, Burlingame, CA).

Infarct Volume Quantification - Images of six 30 µm brain sections were taken at millimeter intervals from 1.7 to -3.3 mm from bregma to determine the volume of infarction. The Fluoro-Jade stained tissue was digitally photographed with an Olympus IX71 microscope controlled by DP manager software (Olympus America Inc, Melville, NY) at a magnification of 1.25X. Images were edited with Jasc Paintshop Pro. Area of neurodegeneration was measured using the NIH Image J software. The area of the contralateral side of the brain tissue was also measured and used to compensate for possible edema in the ipsilateral hemispheres. Infarct volumes were then calculated by the summation of the infarct areas.

Flow Cytometry – Spleen and blood samples were both processed for flow cytometry analysis. The spleen was removed from the rat prior to perfusion. It
was then placed in a stomacher bag containing 15 ml of ice cold PBS pH 7.4 with 0.2% BSA (PBS/BSA). The spleen was then mashed with a stomacher and the suspension was drained through a 40µm cell strainer into a 50ml conical tube. Volume was brought up to 15ml with PBS/BSA and then centrifuged at 1500 rpm for 10 minutes at room temperature. Supernatant was removed and the pellet was resuspended in 0.5ml ACK lysis buffer (4.01g NH₄Cl + 0.5g KHCO₃ + 0.01g EDTA in 500ml ddH₂O) for 5 minutes. The solution was then diluted in 1ml PBS/BSA and spun at 1500 rpm for 10 minutes. Supernatant was removed and the pellet was resuspended in 2ml PBS/BSA.

For blood preparation, 1 ml of blood was removed from the rat just prior to perfusion. The blood was then added to a tube containing 1ml of Dextran solution (10 mM EDTA + 1% Dextran (Mw 400,000 – 500,000) in 500ml of PBS pH 7.4) and was allowed to incubate at 37 °C for 1 hour. Supernatant was removed and diluted with 1ml PBS/BSA and spun at 15 rpm for 10 minutes. Supernatant was removed and the pellet was resuspended in 0.5 ml ACK lysis buffer for 5 minutes. The solution was then diluted with 1ml PBS/BSA and spun at 1500 rpm for 10 minutes. Supernatant was removed and the pellet was resuspended in 2ml PBS/BSA. Cells were counted using a hemocytometer and Trypan Blue exclusion method. At least 10⁶ cells were aliquoted into 100 µl of PBS/BSA. Aliquots then received 20 µl of Fc Block per 100 µl and were incubated in solution for 10 minutes in ice. Antibodies were then added and allowed to incubate for 15 – 30 minutes. Aliquots were then centrifuged, washed with PBS/BSA and the process repeated to remove excess antibody.
Analysis of staining was performed at the Moffitt Cancer Center flow
cytometry core center in Tampa, Florida. At this core facility 5000 events were
counted from the aliquots, cellular populations were identified based on staining
profile, and percentages of these populations were determined.

**Blood Smear Preparation and Giemsa Staining** – During harvest, a small drop
of blood was placed near the frosted end of a clean glass slide. A second slide
was used as a spreader. Holding the spreader slide at a 30 degree angle, the
spreader was drawn back against the drop of blood. The blood was then pulled
to the opposite edge of the slide producing a thin film on the slide. After air
drying and the smears were fixed in Methanol for 5 minutes and again allowed to
air dry. For Giemsa (Sigma-Aldrich) staining, slides were first washed three
times for 2 minutes each with distilled water. Slides laying flat were then flooded
with Giemsa stain (1 to 2 ml) for 4 minutes. Equal volumes of distilled water
were added to the slides to stop staining. Slides were then dunked in a large
container of water to remove excess stain. Stained slides were rinsed thoroughly
three times for three minutes in distilled water, air dried vertically overnight and
coverslipped with Permount.

**Statistical Analysis** - Infarct size, spleen weight, and % of blood cell populations
were analyzed using ANOVA followed by Bonferroni’s or Tukey’s post hoc tests.
A value of p<0.05 was considered significant.
Results

Denervation Prior to MCAO does not Inhibit Splenic Response

To determine whether sympathetic neurotransmission was modulating the physical size reduction of the spleen at the time of stroke, we looked at spleen weight changes at the time of stroke. We compared spleens of rats that were subjected to splenic denervation prior to MCAO to those rats that underwent MCAO only, sham-MCAO, and denervated spleen/sham-MCAO (Figure 12). Analysis of the spleen weights of these groups showed that denervated spleens of rats subjected to stroke had a significantly smaller weight when compared to sham-MCAO and denervated spleen/sham-MCAO (p<0.05) (Figure 12). There was no significant difference in the size of the spleens from the denervated spleen-MCAO and MCAO-only treatment groups. These results indicate that direct sympathetic innervation via the splenic nerve does not result in splenic shrinkage that is observed after stroke.

Splenic Denervation Prior to MCAO does not affect Infarct Volume

We have previously determined that splenectomy prior to stroke results in a significant reduction in neurodegeneration at the site of ischemic injury. To determine if lack of neuronal signaling was the reason why splenectomy resulted in a decrease in infarction volume, we compared denervated spleen/MCAO with spleens of rats that received splenectomies two weeks prior to MCAO as another form of control.
Fluoro-Jade extensively labeled degenerating neurons in the cortex, striatum and hippocampus of the hemisphere ipsilateral to the stroke in the brains of rats subjected to MCAO-only (Figure 13). Brain sections of rats that underwent splenic denervation two weeks prior to stroke showed a similar pattern of Fluoro-Jade labeling as observed in the rats subjected to MCAO-only. Splenectomy prior to stroke again showed reduced Fluoro-Jade staining of the brain parenchyma. Quantification of the infarction volumes revealed no significant difference in volumes between the denervated spleen-MCAO and MCAO-only groups. Splenectomy prior to stroke resulted in led to a significant decrease in infarct volume when compared to the volumes in denervated spleen-MCAO and MCAO-only rats (p<0.05) (Figure 15a).

To confirm these results we employed another method to measure infarct size by labeling Nissl bodies of intact neurons with Thionin. In contrast to Fluoro-Jade, absence of staining denotes neurodegeneration and cell death in the brain section; even so the results were similar. MCAO only-treated rats and denervated spleen-MCAO rats showed an absence of Nissl staining across the majority of the ipsilateral hemisphere including the cortical, striatal and hippocampal regions (Figure 14). Splenectomy prior to stroke decreased neurodegeneration in the brain sections as demonstrated by increased of labeling of the Nissl bodies of neurons. When we quantified and analyzed the infarct volumes there was no significant differences between MCAO-only and denervated spleen-MCAO group infarct volumes while the infarct volumes of rats whose spleens were removed prior to stroke again showed a significantly lower
average infarct volume when compared to the afore mentioned experimental
groups (Figure 15b). These results indicate that while the spleen contributes to
stroke-induced neurodegeneration, elimination of sympathetic neurotransmission
in the spleen does not mimic the neuroprotective effects seen with splenectomy
prior to stroke.

Flow Cytometry Analysis Shows Increased Splenic Monocytes
To determine if denervation affected cell populations blood and spleen, tissue
samples were analyzed by cytometry. Monocytes, neutrophils, B cell and T cell
surface antibodies were used to label the individual cell types for analysis.
Cytometry analysis showed that there was a significant increase in the
percentage of CD4+ labeled monocytes in those spleens subjected to
denervation prior to stroke when compared to the percentage of CD4+ cells
found in the spleens of rats subjected to MCAO-only (Figure 16). Flow cytometry
analysis showed no other significant differences in all other cellular
compartments of blood and spleen tissue from the denervated rats compared to
rats that underwent MCAO-only (Figures 16 and 17).

Denervation has no Effect on Giemsa Stained Leukocyte Populations after
MCAO
Peripheral immune cells have been shown to travel through the vasculature,
infiltrate the brain and migrate to the site of damage after ischemic insult. While
our laboratory has previously shown that splenectomy has no affect on leukocyte
populations after stroke, we examined if spleen presence and inhibition of sympathetic neurotransmission would result in cell population alterations. Blood smears were obtained at 0 and 48 hours after stroke surgery, stained with Giemsa and white blood cell profiles were tabulated. While cell populations changed from 0 to 48 hours in each group we were interested in how treatment affected the populations at 48 hours after MCAO. We found that there were no significant differences between experimental groups as lymphocytes, monocytes and neutrophils all showed the same cellular population alterations between the sham-MCAO, denervated spleen/sham-MCAO, denervated spleen MCAO, splenectomy MCAO, and MCAO alone (Figure 18).

**Discussion**

In response to stroke, the periphery reacts by increasing cardiac contraction and vasoconstriction which can potentially lead to cardiac arrhythmia and hypertension (Klingelhofer and Sander 1997). These responses have been shown to be a result of increased sympathetic neurotransmission as a response to the injury that has occurred in the brain (Butcher et al. 1993; Cechetto et al. 1989; Klingelhofer and Sander 1997). While sympathetic neurotransmission is driving many of the peripheral processes after stroke, our results show that denervation of sympathetic neurotransmission to the spleen has no affect on splenic contribution to stroke-induced neurodegeneration. We hypothesized that removal of the splenic innervation would inhibit sympathetic neurotransmission to the spleen resulting in organ size retention, and decreased neurodegeneration
and inflammation at the time of ischemic injury. During stroke there is a substantial increase of epinephrine and norepinephrine that is released from the adrenal gland into the circulation that activates sympathetic tone (Rosol et al. 2001; Wong 2006). While the sympathetic nerves to the spleen have been removed, it is quite possible that these neurotransmitters are still reaching the spleen through the circulation where they can bind to the adrenergic receptors located on the smooth muscle capsule and splenocytes of the spleen, causing contraction and spleen shrinkage.

Flow cytometry analysis of spleen tissue for different molecular markers of leukocytes showed a significant increase only in the percentage of macrophages in denervated spleens. Membranes of blood monocytes are shown to highly express the $\beta_2$ adrenergic receptor. In most studies activation of the $\beta_2$ receptor by an agonist, such as epinephrine, has been shown to be immunosuppressive by inhibiting the release of proinflammatory cytokines and down regulating the expression of intracellular monocyte adhesion molecules on monocyte cell surfaces (Kuroki et al. 2004; Mizuno et al. 2005). This suggests that removal of sympathetic innervation to the spleen decreases anti-inflammatory effects of $\beta_2$ agonist receptor activation allowing for the potential increased macrophage activity observed in the spleen. Denervation of the spleen may also systemically signal an injury to the organ resulting in the increased proliferation of blood monocytes. In light of our findings of the lack of sympathetic neurotransmission contribution to splenic response to stroke, we strongly recommend further testing of the splenic interaction with ischemic brain injury for the purpose of determining
the mechanism of action for the development of alternative that will decrease neurodegenerative and inflammatory effects of stroke.
Chapter 3, Figure 12

Spleen Weights of Experimental Groups

*\[p<0.05\]
Figure 1: Denervation has no effect on spleen shrinkage resulting from stroke. Spleens of rats subjected to denervation prior to stroke showed reduced weight when removed at 48 hours after stroke. ANOVA followed by the post hoc Dunnett’s test showed that there was no significant difference between denervated spleen weight and the weights of spleens taken from rats subjected to MCAO-only.
Chapter 3, Figure 13
Figure 2: Fluoro-Jade labeled neurodegeneration in brain tissue of denervated rats 48hr post MCAO. There was a large area of damage as indicated by Fluoro-Jade labeled cells in the ipsilateral hemisphere of rats whose spleens were denervated prior to stroke (panel d), which was similar to the Fluoro-Jade labeled neurodegeneration in the ipsilateral hemisphere in rats subjected to MCAO-only (panel c). Sham-MCAO (panel a) and denervated spleen/sham-MCAO (panel b) had little Fluoro-Jade labeled neurodegeneration. (a and c scale bar = 5 mm) (b and d scale bar = 2 mm)
Chapter 3, Figure 14
Figure 3: Thionin labeling of Nissl bodies of surviving brain tissue of denervated rats 48hr post MCAO. Tissue lacking staining is indicative of the infarction. Similar lack of tissue staining shows large infarct areas in both denervated spleen MCAO brain tissue (panel d) and MCAO only brain tissue (panel c). Sham-MCAO (panel a) and denervated spleen/sham-MCAO (panel b) showed no Fluoro-Jade labeled neurodegeneration. Tissue sections from Sham-MCAO (panel a) and denervated spleen/Sham-MCAO (panel b) showed no loss of Thionin staining (b scale bar = 5 mm) (a, c and d scale bar = 2 mm)
Average Infarct Volume Labeled by Fluoro-Jade

Average Infarct Volume Designated by Absence of Thionin Labeling

*\(p<0.05\)
Figure 4: Analysis of infarction volumes labeled by histological staining.

a, ANOVA followed by the post hoc Bonferroni’s test showed that denervation of the spleen prior to stroke produced no significant difference in the average infarction volume of the brain tissue determined with Fluoro-Jade labeling compared to average infarction volume of rats subjected to MCAO only. Analysis did show that splenectomy prior to stroke significantly reduced average Fluoro-Jade stained infarction volume compared to the average infarct volumes from the brain of rats that underwent denervation of the spleen prior to MCAO and MCAO only. (*p<0.05)

b, ANOVA followed by the post hoc Bonferroni’s test of average infarct volumes determined from Thionin staining shows that there was no significant difference between average volumes from denervated spleen/MCAO compared to MCAO only. Splenectomy prior to MCAO significantly reduced infarct volume compared to denervated spleen/MCAO and MCAO only infarct volumes. (*p<0.05)
Chapter 3, Figure 16

A. CD3 T cells

B. CD4 Helper T Cells

C. CD8a Cytotoxic T Cells

D. CD45R B Cells

E. H13416 Neutrophils

F. CD1 Monocyte/Macrophage

*p<0.001
Figure 5: Flow cytometry analysis of spleen homogenates.

a, Flow cytometry analysis of T cells positive for the CD3 antibody showed no significant difference in percentage of cell populations across experimental groups. b, There was no significant difference in the percentage of helper T cell populations stained with the CD4 antibody. c, There was no significant difference in the percentage of cytotoxic T cells labeled by CD8a. d, Flow cytometry analysis of B cells stained positive for the CD45R showed no significant changes in the percentage of cells in the populations. e, Neutrophils labeled with the HIS48 antibody showed no changes in the percentage of cells in the population. f, Flow cytometry analysis of monocytes labeled with the CD4 antibody showed a significant increase in the percentage of positively stained cells in the population in the spleen homogenates from the denervated spleen/MCAO group when compared to those of the MCAO only group (*p<0.01). Significance was determined by ANOVA followed by the post hoc Bonferroni’s test.
Chapter 3, Figure 17

A. CD3 T Cells

B. CD4 Helper T Cells

C. CD8 Cytotoxic T Cells

D. CD45RA Cells

E. H940 Neutrophils

F. CD4 Monocytes/Macrophages
Figure 6: Flow cytometry analysis of blood samples.

a, Flow cytometry analysis of T cells stained positive for the CD3 antibody showed no significant difference in percentage of cell populations across experimental groups. b, There was no significant difference in the percentage of helper T cell populations stained with the CD4 antibody. c, There was no significant difference in the percentage of cytotoxic T cells labeled by CD8a. d, Flow cytometry analysis of B cells stained positive for the CD45R showed no significant changes in the percentage of cells in the populations. e, Neutrophils labeled with the HIS48 antibody showed no changes in the percentage of cells in the population. f, Flow cytometry analysis of monocytes labeled with the CD4 antibody showed no significant changes in the percentage of labeled cells within the population. Significance was determined by ANOVA followed by the post hoc Bonferroni’s test. Absence of data in b and f suggest malfunction in staining process prior to flow cytometry analysis.
**Figure 7: Analysis of blood smear white blood cell populations by Giemsa staining.** Analysis of white blood cell populations at 48 hours after MCAO showed no significant changes in lymphocyte (a), monocyte (b), and neutrophil (c), populations. Significance was determined by ANOVA followed by the post hoc Bonferroni’s test.
References


Vendrame M, Cassady J, Newcomb J, Butler T, Pennypacker KR, Zigova T,
Sanberg CD, Sanberg PR, Willing AE. 2004. Infusion of human umbilical
cord blood cells in a rat model of stroke dose-dependently rescues


Wong DL. 2006. Epinephrine biosynthesis: hormonal and neural control during
CONCLUSION

During embolic stroke, an obstruction of blood flow, reduced nutrients and failure of energy production results in progressive damage to the central nervous system. Depending on the severity of these homeostatic alterations, stroke can lead to death, or severe physical and mental disabilities that require specialized care (W.H.O. 2007). The sole treatment for ischemic stroke is recombinant tissue plasminogen activator (rtPA). This treatment can restore blood flow to the injured brain by dissolving the embolus Only 13% of the time (Albers et al. 2004). The major caveat to this treatment is that it must be administered within 3 hours of stroke onset or 6 hours after stroke when intra-arterially dispensed (Albers et al. 2004; Marler and Goldstein 2003). This treatment may also produce adverse effects related to its mechanism of action such as intracranial bleeding and the production of oxygen free radicals. Thus, rtPA can only be administered to a select group of stroke victims that are diagnosed during this time period that meet specific criteria for this therapy (Albers et al. 2004). The rtPA must be used within such a short time period because it can cause intracranial bleeding. Therefore, rtPA must be administered prior to the induction of neurodegenerative cascades and the inflammatory response. There has been increasing evidence that any treatment of stroke at delayed timepoints must decrease the inflammatory response in addition to treating neurodegeneration (Newcomb et al. 2004).
Understanding the mechanisms by which these treatments counteract stroke and the therapeutic targets that they act on will shed new light on possible treatments for stroke.

Transfusion of human umbilical cord blood cells (HUCBC) promotes neuroprotection and reduces inflammation at the site of injury in the permanent middle cerebral artery occlusion (MCAO) model of stroke. Injections of cord blood cells were first used in Parkinson's, Alzheimer's and Huntington's disease animal models of brain injury and showed increased longevity (Ende and Chen 2001; Ende et al. 2001a; Ende et al. 2001b). Further experimentation of the intravenous injection of these cells in the stroke animal model resulted in the improvement of both neurological impairment and motor function deficits (Chen et al. 2001). Examination of animals subjected MCAO surgery and treated with HUCBC revealed that these cells migrated to the ipsilateral hemisphere of the brain and to the spleen (Vendrame et al. 2004). Immunohistochemical processing of brain sections from HUCBC treated animals revealed less neurodegeneration and reduced microglial activation when compared to sections from vehicle treated animals. Biochemical analysis of the brain tissue and the blood sera revealed reduced NFκB binding activity and a shift to an anti-inflammatory IL-10 cytokine profile in HUCBC treated animals when compared to vehicle controls (Vendrame et al. 2004; Vendrame et al. 2005; Vendrame et al. 2006). Treatment of animals at various timepoints after MCAO demonstrated that cord blood cells could be transfused up to 48 hours after MCAO and still continue to cause significant reductions in infarction volume (Newcomb et al. 2006; Vendrame et al. 2004).
This shows that a therapeutic window of treatment can be extended past the three hour limitation of rtPA and have beneficial effects.

Standard dogma of infarct formation contends that almost immediately after blockage of blood flow, dysregulation of neuronal homeostasis leads to necrosis and apoptosis of brain parenchyma. Interestingly many of the cellular markers for neuronal death such as TUNEL, activated caspase 3, and absence of Nissl staining are evident at 48 hours post stroke (Newcomb et al. 2006). Reliance on the aforementioned markers as evidence of apoptotic cell death has led investigators to begin therapeutic intervention either prior to or rapidly following stroke onset. Treatment with HUCBC at delayed time points after MCAO, however, causes reductions in the neurodegenerative labeling of TUNEL and absence of Nissl staining (Newcomb et al. 2006). This suggests that these stains are not indicators of cell death, but are instead labeling compromised neurons. The observation that many of the markers for neuronal loss used by the scientific community are reversible is extremely controversial. However, treatment with HUCBC provides evidence that the infarction does not consist of entirely dead cells at extended time points after stroke but is made up of tissue that still has the potential of being “rescued” when a neuroprotective and anti-inflammatory treatment is given (Newcomb et al. 2006).

To find other agents that work at delayed time points, it is necessary to choose treatments that work in a similar fashion to HUCBC’s promotion of neuroprotective and anti-inflammatory effects. Therefore, we chose a sigma receptor agonist as this drug class has well known neuroprotective and anti-
inflammatory properties (Bourrie et al. 2002; Goyagi et al. 2001; Katnik et al. 2006; Takahashi et al. 1996; Wagnerova et al. 2006; Zhang and Cuevas 2005).

Sigma receptors are highly dispersed throughout the brain and periphery, where they bind a vast assortment of substances including neurosteroids, antipsychotics, antidepressants, phencyclidine (PCP)-related compounds, and opiates (Bowen 2000; Katnik et al. 2006; Walker et al. 1990). Sigma receptors are classified into two subtypes based on their pharmacological binding profiles. Sigma-1 receptors preferentially bind (+) benzomorphans (Walker et al. 1990) and have been shown to modulate voltage gated potassium channels and IP3 receptor function while sigma-2 receptors preferentially bind ibogaine and alter voltage gated sodium and calcium channels (Zhang and Cuevas 2002; Zhang and Cuevas 2005). Sigma receptors function in several physiological and pathophysiological processes in the brain including learning and memory, movement disorders, and drug addiction (Matsumoto et al. 1990; McCracken et al. 1999; Senda et al. 1996). Importantly, sigma receptor activation has been shown to be neuroprotective in various stroke models. Previous studies regarding sigma receptor activation and focal ischemia have focused on the activation of the sigma-1 receptor (Goyagi et al. 2001; Takahashi et al. 1996). Voltage gated sodium and calcium channels, which are modulated by sigma-2 receptors, are involved in neuronal excitability and contribute to spreading depression during stroke (Gribkoff and Winquist 2005). Sigma receptor activation also leads to potent anti-inflammatory properties. Activation in vivo enhances lipopolysaccharide-induced systemic release of the anti-inflammatory
cytokine IL-10 while simultaneously suppressing TNF-α synthesis (Bourrie et al. 2002). We postulated that activation of both the sigma 1 and sigma 2 receptors would result in better protection of the brain parenchyma than activating only one of the receptor types. Additionally administration of the sigma ligands in these studies occurred either prior to stroke or shortly after the insult providing no insight on the efficacy of these compounds when administered at delayed time points (Goyagi et al. 2001; Takahashi et al. 1996). Examination of these agents at delayed time points is important to make treatment more available to individuals who are not candidates for rtPA.

We designed a series of experiments in which the sigma receptor ligand DTG was subcutaneously administered 24 hours post MCAO. As seen with HUCBC treatment, DTG significantly reduced infarct volume, enhanced neuronal survival, reduce astrogliosis and modulated immune cell responses (Figure 1) (Ajmo et al. 2006). This observation reinforces the hypothesis that treatment regimens which are both neuroprotective and anti-inflammatory can reverse or prevent neurodegenerative processes at delayed time points.

From these experiments, however, it is impossible to discern what proportion of the observed neuroprotection is due to the anti-inflammatory or the direct neuroprotective properties of these compounds. Sigma receptors have been shown to be neuroprotective in vitro by preventing intracellular calcium dysregulation in cortical neurons during ischemia and up-regulating anti-apoptotic Bcl family proteins (Katnik et al. 2006). Subsequent studies on transgenic mice subjected to MCAO however, suggest that the protective effects of sigma
receptor activation *in vivo* are mainly a product of its neuroprotective properties (Vagnerova et al. 2006). In those studies, administration of the sigma-1 receptor agonist (+) pentazocine to iNOS knockout mice failed to provide significant decreases in infarct volume when compared to iNOS knockout mice that received MCAO alone (Vagnerova et al. 2006). Inducible nitric oxide synthase (iNOS) is an enzyme which is upregulated primarily by microglia and macrophages during the inflammatory response and produces nitric oxide, a reactive oxygen species implicated in neuronal death (Virag et al. 2003).

Sigma receptor activation has also been shown to modulate inflammation in the periphery. These receptors have been identified in organs including endocrine-related structures, gastrointestinal tract, liver, kidney, and have been found to be distributed on rat splenocytes. The sigma agonist, SR 31747 is an immunomodulator that elicits high affinity for sigma receptors expressed on lymphocytes (Paul et al. 1994). Activation of the these receptors by this agonist suggests that sigma receptors may be considered as targets for immunomodulation in that they markedly suppress lipopolysaccharide (LPS)-induced production of the inflammatory cytokines interleukin (IL)-1, IL-6, and tumor necrosis factor-α (TNF-α) *in vivo*. Conversely, activation with SR 31747 stimulates the production of the anti-inflammatory cytokine IL-10. These reports, in conjunction with our sigma receptor activation data, suggest that up-regulation of anti-inflammatory effects coming from the periphery are causing a reduction in inflammation thereby decreasing neurodegeneration.
Previous studies with HUCBC treatment for stroke revealed that cord blood cells migrate to the spleen after ischemic insult (Vendrame et al. 2004). PCR analysis for human DNA showed that cord blood cell DNA was found only in the damaged hemisphere of the brain and in the spleen. Identification by human nuclei immunoreactivity confirmed the presence of HUCBC in this organ (Vendrame et al. 2004). Further investigation of the effects of HUCBC on MCAO demonstrated that the spleen shrinks by 50% of its size in response to stroke alone and transfusion of cord blood cells 24 hours after stroke resulted in spleen size retention paired with reduced infarct volume at the site of injury (Vendrame et al. 2006). To substantiate the spleen-related findings from the HUCBC studies, it was essential to establish splenic reaction to stroke. Therefore, we chose to remove the spleen from our animal model prior to stroke eliminating this variable from the overall mechanism that contributes to ischemic injury.

The spleen is secondary lymphoid organ whose function is to filter blood, store erythrocytes and leukocytes, remove damaged blood cells, and initiate immune responses (Cesta 2006). The spleen modulates the functions through its unique internal structure that is composed of red pulp, which filters and stores the red blood cells, and white pulp, which stores the leukocytes (Brendolan et al. 2007). The spleen’s internal structures are housed by a capsule composed of dense fibrous tissue, elastic fibers and smooth muscle (Cesta 2006). In times of physiological stress, smooth muscle of the spleen contracts giving it the ability to autotransfuse a large quantity of blood cells into the circulation for the purpose of increasing oxygen transport to afflicted areas (Stewart and McKenzie 2002).
This is consistent with studies that have shown that the spleen shrinks and releases splenocytes into the circulation in response to neurodegenerative diseases (Benner et al. 2004; Offner et al. 2006b).

As seen with HUCBC and DTG treatment, splenectomy prior to stroke significantly reduced infarct volume, decreased activated microglia/macrophages, and significantly reduced peripheral neutrophil presence (Figure 2). These findings support the hypothesis that the spleen contributes to stroke-induced neurodegeneration and is a part of the overall mechanism of stroke. These data suggest that activated microglia are not the major contributors of the second wave of neurodegeneration within the infarction as these cells have been shown to outnumber infiltrating macrophages (Liu and Hong 2003; Schilling et al. 2003). Our results are supported by the systemic inhibition of macrophages by anti-CD11b, anti-CD18, anti-ED1 and anti-Mac-1 monoclonal antibodies 1 hour after transient MCAO, which show significant reduction of infarction volume in the ischemic brain (Chen et al. 1994; Chopp et al. 1994; Zhang et al. 1995b). We now suggest that there is a strong synergistic effect between activated microglia and the infiltrating macrophages and neutrophils. Diminishing of the peripheral immune response by deletion of a population of macrophages, neutrophils, T cells and B cells through splenectomy dramatically reduces infarction by decreasing cellular interaction with activated microglia at the site of injury. Splenectomy prior to stroke also indicates that the majority neutrophils infiltrating the infarction are of splenic origin. Staining for myeoperoxidase (MPO), a peroxidase enzyme found in lysosomes of these cells, showed little or no cellular
presence in brain sections of animals whose spleens were removed prior to stroke.

While removal of the spleen validated the organ’s involvement in stroke, these experiments gave no insight to the signaling mechanism connecting the brain and the spleen at the time of injury. As stated previously, the spleen is encased in a layer of smooth muscle which can contract to autotransfuse the circulation with a large amount of blood cells in response to stress (Stewart and McKenzie 2002). Intuitively, smooth muscle contraction is a function of adrenergic receptor activation. Therefore, we hypothesized that spleen shrinkage and inflammatory cell release in response to the stress of stroke is a function of autonomic neurotransmission. Clinical and experimental studies have indicated that autonomic nervous system dysfunction is a complication of stroke (Meyer et al. 2004). Early mortality in stroke patients is associated with cardiac arrhythmias and myocardial damage (Tokgozoglu et al. 1999; Wang et al. 1997). It has also been documented that norepinephrine, epinephrine, and blood pressure all significantly rise after stroke, indicating that sympathetic tone becomes elevated (Meyer et al. 2004). Interestingly the splenic nerve contains approximately 98% sympathetic nerve fibers (Klein et al. 1982), and most studies suggest that the innervation of the spleen is predominantly sympathetic (Elenkov et al. 2000).

To confirm sympathetic neurotransmission as the signaling mechanism to the spleen during stroke, we removed neuronal innervation to the organ prior to MCAO (Figure 3). We speculated that denervation would mimic the effects of
spleenectomy. What we found, however, was that splenic denervation had no
effects on stroke pathology. Sections from the brains of rats whose spleens had
been denervated prior to stroke showed no decrease in infarct volume and
continued to show spleen shrinkage in contrast to sections from the brains of rats
treated with MCAO only. Comparing tissue from denervated rats to MCAO-only
rats showed an up-regulation of activated microglia/macrophages and infiltration
of neutrophils. Flow cytometry analysis of spleen tissue for different molecular
markers of leukocytes resulted only in a significant increase of macrophages in
denervated spleens. The $\beta_2$ adrenergic receptors are highly expressed in the
membranes of blood monocytes and receptor activation by an agonist has been
shown to be anti-inflammatory by inhibition of proinflammatory cytokine release
and down regulation of intracellular monocyte adhesion molecules (Kuroki et al.
2004; Mizuno et al. 2005). This suggests that removal of sympathetic innervation
to the spleen suppresses anti-inflammatory effects of $\beta_2$ agonist receptor
activation allowing for the potential increased proliferation of macrophages
observed in the spleen. Denervation of the spleen may also systemically signal
an injury to the organ resulting in the increased proliferation of blood monocytes.

Based on these observations it would appear that splenic reaction to
stroke is not dependent on direct innervation. The bulk release of epinephrine
and norepinephrine are from the adrenal gland and are released into the
circulation during times of intense physiological stress (Rosol et al. 2001; Wong
2006). These adrenergic agonists could still activate the $\alpha$ adrenergic receptors
of the smooth muscle capsule surrounding the spleen causing contraction
resulting in the observed organ shrinkage. Investigation of denervation of spleen prior to stroke will need to be carried out to 96 hours after injury to truly determine if direct sympathetic neurotransmission by the splenic nerve causes enhanced neurodegeneration. Time course experiments of stroke and transfusion of HUCBC showed that infarct damage can be reversed at 96 hours after MCAO when treated at 48 hours with cord blood cells (Newcomb et al. 2006). This showed that neurons in this animal model are severely compromised at this time but still have the potential of recovery. While we see increased neurodegeneration at 48 hours after stroke, denervation may dampen signal transmission enough to allow compromised nerves to be rescued.

The conclusion of these experiments has led to the deliberation of several avenues for the further investigation of the alternative targets examined in this study. While it has been shown that the spleen contributes to stroke-induced neurodegeneration, investigation into which specific cell types leave the spleen, migrate to the site of injury, and induce the most damage would aid in the development of a treatment that could be administered which blocks those cells. This would be markedly less invasive compared to splenectomy. Cellular migration could be examined by the labeling of cells directly in the spleen and then subjecting the animal model to stroke. While not shown in the above experiments we have gathered data showing that monocyte labeling in the spleen with fluorescent carboxyfluorescein diacetate succinimidyl ester (CFSE) can be detected in brain tissue at delayed time points after stroke. Double immunohistochemical staining of the brain tissue of animals whose spleens were
removed prior to stroke compared to animals subjected to stroke only could identify the different splenic cell types that are infiltrating the brain. Another possible experiment is to remove the spleen prior to stroke, culture splenocytes, separate the cell types and reinsert these individual cell populations back into the animal after stroke. Separation of cell population could be done by magnetic absorbents. Magnetic absorbents attach to specific surface molecular markers found on the cells of interested and then are isolated from the stock solution of cells by application of a magnetic source (Franzreb et al. 2006). A potential pitfall of this experiment could arise from the culture of the cells. Cells removed from the system and cultured could potentially activate in response to environment changes. Reinsertion of these cells could lead to increased damage skewing results.

Based on the experimental results of denervation of the spleen prior to stroke, we need to consider the actions of chemical mediators as modulators of splenic contribution to neurodegeneration resulting from stroke. Monocyte chemoattractant protein-1 (MCP-1) has been shown to be a strong starting point for the mechanisms responsible for the in vivo migration of HUCBC to the brain after the induction of stroke (Newman et al. 2005). MCP-1 released into the circulation from the brain may also be the cause of the observed migration of HUCBC to the spleen (Newman et al. 2005). MCP-1 expression has been detected in astrocytes and microglia at six hours after ischemic insult and remains elevated from twelve to forty-eight hours (Huang et al. 2006; Wang et al.
This chemokine is also detectable in macrophages within ischemic tissue four days after MCAO (Gourmala et al. 1997). Overexpression of MCP-1 in MBP-JE transgenic mice lead to increased migration of monocytes and increased brain infarction size in stroke-induced by MCAO (Chen et al. 2003). MCP-1 deficient mice also show decreases in infarct volume after MCAO (Hughes et al. 2002). This suggests that high levels of chemoattractants are circulating in the blood stream, potentially being filtered by the spleen and drawing splenic inflammatory cells to the site of injury. Suppression of this chemoattractant during stroke may show spleen size retention along with infarct reduction suggesting that splenic reaction to stroke is dependent on chemical mediators.

Overall we have provided evidence of novel treatments and targets for stroke by promoting neuroprotection and anti-inflammation, expanding the therapeutic window of treatment and promoting further understanding of a total mechanism of stroke. These components must be taken into account in the development of a universal treatment of stroke for use in a clinical setting.
Discussion, Figure 19

Ischemic stroke

Blood flow obstruction decreased oxygen/nutrients to cells

DTG

Sigma receptors

Neurodegeneration

Cell Death

Infarct Expansion

Death

Immune response

Resident response

Peripheral response

Inflammatory factors

Neutrophils

Macrophages

T cells/B cells

Microglia activation

Reactive astrocytes

DTG

Sigma receptors

Neurodegeneration

Cell Death

Infarct Expansion

Death
Figure 1: Summary schematic of the effects of DTG on MCAO when administered 24 hours after MCAO. Administration of DTG 24 hours after MCAO results in decreased infarction area by reducing neurodegeneration. It modulated the immune cell response to stroke by showing reductions in activated microglia and infiltrating macrophages at the site of injury. DTG mediates astrocyte activation causing a reducing in astrogliosis and glial scar formation. These alterations lead to an overall reduction of cell death.
Discussion, Figure 20

Ischemic stroke

Blood flow obstruction decreased oxygen/nutrients to cells

Blood born signal

Immune response

Neurodegeneration

Cell Death

Infarct Expansion

Death

Resident response

Microglia activation

Reactive astrocytes

Peripheral response

Inflammatory factors

Neutrophils

Macrophages

T cells/B cells

Innervation

Spleen

Innervation
**Figure 2: Summary schematic of the effects of splenectomy prior to MCAO.**

Removing the spleen prior to stroke results in modulation of the peripheral immune response to stroke by decreasing infiltrating neutrophils at the site of injury. It mediates peripheral and resident inflammatory responses by showing reductions in activated microglia and infiltrating macrophages. These effects cause a decrease in neurodegeneration at the site of injury and reduce the potential cellular death caused by ischemia.
Discussion, Figure 21

Ischemic stroke

Blood flow obstruction decreased oxygen/nutrients to cells

Blood born signal

Spleen

Neurodegeneration

Cell Death

Infarct Expansion

Death

Immune response

Resident response

Peripheral response

Microglia activation

Reactive astrocytes

Inflammatory factors

Neutrophils
Macrophages
T cells/B cells

Denervation

Inflammatory factors
Figure 3: Summary schematic of the effects of denervation of the spleen prior to MCAO. Denervation of the sympathetic innervation to the spleen prior to stroke produced no anti-inflammatory or neuroprotective effects in the brain. Denervation resulted in considerable infarction volume which was analogous with the infarct volumes observed with MCAO only. Flow cytometry analysis showed a significant increase of monocytes in homogenates of spleens that had been denervated by stroke. Removal of sympathetic innervation to the spleen may cause increased proliferation or inhibition of release of monocytes from the spleen during MCAO. Chemical signaling mechanisms may be affecting the spleen at the time of stroke.
References


ABOUT THE AUTHOR

Craig T. Ajmo Jr. received his Bachelor of Science degree in Biochemistry at the University of Florida in 2003. Upon completion of his undergraduate degree, Craig entered the graduate program at the University of South Florida’s College of Medicine in 2003. He received his Master of Science degree in Molecular Pharmacology and Physiology in 2005 under the tutelage of Dr. Keith R. Pennypacker investigating alternative targets for the treatment of stroke. Craig successfully defended his doctoral dissertation in June of 2007 at the University of South Florida.