Involvement of Central Endothelin B Receptors in Focal Cerebral Ischemia

BY

MARY G. LEONARD
B.A., University of Missouri – Columbia, 2003
B.S., University of Missouri – Columbia, 2003
M.S., Midwestern University, 2008

THESIS

Submitted as partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Bioengineering
in the Graduate College of the
University of Illinois at Chicago, 2013

Chicago, Illinois

Defense Committee:

Anil Gulati, Advisor
Norman Katz, Chair, Biopharmaceutical Sciences
Michael Cho
John Hetling
Shridhar Andurkar, Midwestern University
Shaifali Bhalla, Midwestern University

ACKNOWLEDGEMENTS

I would like to thank my thesis committee – Dr. Anil Gulati, Dr. Norman Katz, Dr. Shridhar Andurkar, Dr. Shaifali Bhalla, Dr. Michael Cho and Dr. John Hetling – without whom this would not have been possible. Your support and guidance throughout the entire process have been invaluable. I would also like to thank the members of my lab group throughout the years – George Matwyshyn, Manish Lavhale, Seema Briyal and Gwen Pais. They saw me through my day-to-day trials, listened to me as I complained, advised me when I was uncertain of my direction, and generally provided a sane and knowledgeable base for me to complete my research.

I am certain that I could not have gotten to this point without the constant support and patience of my family and friends. It has been a long and difficult journey that I could never have made on my own, without knowing that they were there behind me. I would like to especially thank my parents – Bill and Jill Leonard, my sister and brother – Tracy and John Leonard, and my grandmother – Mary Ellen Brown for pushing me to see my goals achieved, no matter how long it might take. Finally, I would like to thank Jensen Hornick, who has made these last few years not only bearable, but also enjoyable.

Thank you all for being there when I needed someone, for alleviating my worries when they were unfounded, for giving me assistance to overcome the obstacles that were presented, and, most of all, for believing in me.

MGL

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BBB	Blood Brain Barrier
BrdU	5-Bromo-2’-deoxyuridine
CNS	Central Nervous System
ET	Endothelin
GFAP	Glial Fibrillary Acidic Protein
GSH	Reduced glutathione
MCAO	Middle Cerebral Artery Occlusion
MDA	Malondialdehyde
NeuN	Neuronal Nuclei
NGF	Nerve Growth Factor
rtPA	Recombinant Tissue Plasminogen Activator
SOD	Superoxide dismutase

SUMMARY

Stroke is one of the leading causes of mortality and long-term disability worldwide. Over 80% of strokes are classified as ischemic in nature, a condition generally referred to as cerebral ischemia. Despite the devastating personal, social and economic burdens of this disease, there remains a dearth of treatment options for ischemic stroke. Moreover, many aspects of the pathophysiology of cerebral ischemia are still incompletely understood.

While it has long been known that part of the pathology of cerebral ischemia involves a disruption of the endogenous endothelin (ET) system, very little is known about the involvement of endothelin B (ET_B) receptors specifically. The purpose of this study, therefore, was to determine the involvement of central ET_B receptors in an experimental model of cerebral ischemia. Male Sprague-Dawley rats were subject to permanent middle cerebral artery occlusion and treated with either vehicle (isotonic saline) at a dose of 1 ml/kg or ET_B receptor agonist IRL-1620 at a dose of 5 µg/kg intravenously (i.v.) at 2, 4 and 6 hours post infarct. Endothelin B antagonist BQ788 was also administered at a dose of 1 mg/kg, i.v., 15 minutes prior to treatment with either vehicle or IRL-1620 in order to further classify the role of ET_B receptors in cerebral ischemia. Two distinct endpoints were used in this study to reflect the various stages of cerebral ischemia – 24 hours (24 h), reflecting the acute phase, and 1 week (1 w), reflecting the sub-acute phase. Animals were assessed for neurological and motor deficit and infarct volume. In order to determine the mechanism of action of any effects resulting from the stimulation and/or blockade of ET_B receptors, the brains of animals subject to ischemia were then evaluated for oxidative stress parameters and ET receptor estimation. Additionally, as the ET system may play a role in the repair and remodeling of neurovascular tissue which can occur following cerebral ischemia,

the effects of ET_B receptor agonist and antagonist treatment was determined on astrocytes, neurons and vascular endothelial cells.

Animals during both the acute and sub-acute phases of cerebral ischemia demonstrated significant neurological and motor function impairment. When treated with IRL-1620, however, animals showed a significant reduction in functional impairment. This reduction in motor deficit coincided with a significantly smaller infarct volume, with animals treated with IRL-1620 presenting with infarcts of 24.47 ± 4.37 mm^3 and 54.06 ± 14.12 mm^3 at 24 h and 1 w, respectively, as compared to vehicle-treated animals who presented with infarcts of 153.23 ± 32.18 mm^3 and 177.06 ± 13.21 mm^3 at 24 h and 1 w, respectively. Pretreatment with ET_B antagonist BQ788, on the other hand, blocked the effects of IRL-1620, confirming the role of ET_B receptor stimulation in the reduction of motor impairment and infarct volume.

In order to determine the mechanism of action responsible for improvement observed following ET_B receptor stimulation in cerebral ischemia, oxidative stress parameters were determined. Whereas cerebral ischemia increased levels of lipid peroxidation and decreased antioxidants, treatment with IRL-1620 resulted in lower levels of the lipid peroxidation parameter, malodialdehyde, and higher levels of antioxidants, reduced glutathione and superoxide dismutase, during both the acute and sub-acute phases of cerebral ischemia. These results indicate that the stimulation of ET_B receptors following cerebral ischemia provides significant neuroprotection. Additionally, while no alteration in the expression of ET_B receptors was noted in the first 24 hours, these receptor levels were significantly increased in the infarcted hemisphere of IRL-1620-treated animals at 1 w following induction of cerebral ischemia, indicating that an up-regulation of these receptors may play a role in neuroprotection.

In confirmation of the observed neuroprotection, we found that neuronal numbers within the cortex, striatum and subventricular zones of animals treated with IRL-1620 were preserved at 24 h post infarct. Moreover, at this time point, there was a reduction in astrocytic conversion and an increase in the number of vessels staining positive for vascular endothelial growth factor (VEGF), indicating that vascular remodeling was occurring. By 1 w following middle cerebral artery occlusion, VEGF+ vessels in the IRL-1620 treatment group numbered 11.33 ± 2.13 versus 4.19 ± 0.79 per 30 µm-thick brain slice in the vehicle-treated group. Furthermore, animals treated with IRL-1620 displayed increased numbers of proliferating cells as well as cells staining positively for nerve growth factor in the cortex, striatum, and subventricular zones of the infarcted brains. Pretreatment with BQ788 blocked these effects. The results indicate that treatment with IRL-1620, administered on the day of infarct, enhances angiogenic and neurogenic remodeling as well as neuroprotection for up to 1 w following experimental cerebral ischemia.

The results of this study clearly demonstrate that ET_B receptors are involved in cerebral ischemia. This is the first report indicating that stimulation of ET_B receptors with selective ET_B agonist IRL-1620 following cerebral ischemia results in neuroprotection. We have also shown, for the first time, that the mechanism behind ET_B receptor-induced neuroprotection involves a reduction in oxidative stress parameters and an enhancement of vascular and neuronal growth factors. We therefore speculate that selective stimulation of ET_B receptors may be a novel therapeutic target for the treatment of focal ischemic stroke.

I. INTRODUCTION

1. Ischemic Stroke – Definition and Significance

When the supply of blood to the brain is disturbed, it is called a cerebrovascular accident, otherwise known as a stroke. The two main ways in which this can occur are through a blockage or a rupture of the blood vessels. The former, known as an ischemic stroke, accounts for more than 80% of all cerebrovascular accidents. Typically, a clot forms either at the site of injury (thrombotic) or forms elsewhere and travels to the brain (embolic), occluding the blood vessel and cutting off the blood supply, oxygen and nutrients to the nervous tissue (Donnan, Fisher et al. 2008). The results of this type of incident can be devastating and long-lasting.

Stroke is a leading cause of death and serious, long-term disability throughout the world. In the United States alone, stroke is responsible for the death of 1 in every 18 people – nearly 130,000 deaths per year (Miniño, Murphy et al. 2011). Each year, approximately 795,000 people suffer a stroke, with one occurring on average every 40 sec (Roger, Go et al. 2012). Financially, the cost of health care, medication and missed days of work that can be attributed to stroke is estimated at approximately $54 billion each year (Heidenreich, Trogdon et al. 2011). When one considers that the risk factors for stroke include everything from high blood pressure to obesity, diabetes and age, it is not hard to see that the burden of this disease will only continue to grow. A thorough understanding of the pathological changes that occur as a result of an ischemic cerebrovascular accident may help to illuminate potential new therapeutic strategies for dealing with this disease and easing this current and future burden.

1.1. Pathophysiology of Cerebral Ischemia

The phrase "Time is Brain" was coined to emphasize the need for rapid treatment immediately following the onset of cerebral ischemia. This is due to the fact that, within minutes of infarct, a host of complex mechanisms are set in play that greatly impact the survival of the brain tissue surrounding the ischemic core. The ischemic core consists of tissue which is almost immediately unsalvageable, representing the terminal effects of the ischemic cascade, a series of neurochemical events that occur when the neuronal tissue surrounding the core is deprived of oxygen and nutrients. This surrounding area is termed the penumbra, and salvaging this tissue is the main goal of treatments for ischemic stroke. In order to do this successfully, however, it is of the utmost importance to understand the pathophysiological changes that develop over time once the infarct has occurred. A detailed picture of the mechanisms involved in the pathological conditions brought about by the ischemic event may lead to new and improved treatments, ultimately resulting in a more complete recovery.

1.1.1. Hyper-acute and Acute Stages of Cerebral Ischemia

Ischemic stroke may be broken down into four stages based on pathological changes within the brain – hyper-acute, acute, sub-acute, and chronic (Kanekar, Zacharia et al. 2012). The first 12 hours following the onset of ischemia are termed the "hyper-acute" phase and are generally considered the target window of time in which to administer therapy for a neuroprotective effect. The "acute" stage, 12-24 hours after infarct, is characterized by increasing levels of oxidative stress, cellular death, and irreversible damage to the penumbra.

The penumbra is the area around the ischemic core, which, although deprived of oxygen and nutrients due to a reduction in blood supply, is still described as salvageable. Whereas the

core is incapable of de- and repolarization, the penumbral cells are capable of repolarizing at the expense of energy and depolarizing in response to elevated glutamate and potassium. These repetitive depolarizations lead to an increased release of glutamate, resulting in glutamate excitotoxicity and subsequent cellular damage as part of the ischemic cascade (Hossmann 1996). The series of events that make up the ischemic cascade occur within the penumbra within minutes of the infarct. These events include everything from sodium-potassium pump failure to an increase in intracellular calcium, depolarization, generation of free radicals, blood brain barrier disruption, inflammation and apoptosis (Durukan and Tatlisumak 2007).

Penumbral tissue that is deprived of oxygen and glucose due to a decrease in cerebral blood flow suffers from a depletion of adenosine triphosphate (ATP), a critical component in the transport of sodium and potassium ions across the cellular membrane. This decrease in ATP results in the breakdown of this transport system, causing a passive diffusion of sodium, and subsequently fluid, into the cell, leading to cytotoxic edema (Huang and McNamara 2004). Cellular depolarization and the resulting release of glutamate cause an influx of calcium into the cells. The increase in intracellular calcium activates a variety of enzymes, leading to mitochondrial damage, inflammation and cell death (Dirnagl, Iadecola et al. 1999). At the same time, a disruption occurs in the balance of oxidants and antioxidants within the ischemic brain, resulting in oxidative stress. Under oxidative stress conditions, superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (-OH) are produced as antioxidants such as superoxide dismutase (SOD) and reduced glutathione (GSH) are depleted. This shift leads to damage to the membranes of both the cells and the mitochondria by lipid peroxidation, as well as inflammation and apoptosis (Lakhan, Kirchgessner et al. 2009). All of these events contribute to eventual

breakdown of the blood brain barrier, which in itself may lead to complications such as cerebral edema and hemorrhagic transformation.

1.1.2. Sub-acute and Chronic Stages of Cerebral Ischemia

The blood brain barrier (BBB) begins to break down around 18-24 hours after the onset of cerebral ischemia, resulting in an increase in extracellular fluid. This rise in fluid, known as vasogenic edema, continues to increase with time, becoming maximized by 48-72 hours post infarct. Cerebral edema is the hallmark of the "sub-acute" stage of ischemic stroke, which lasts from 2 days to 2 weeks post infarct (Kanekar, Zacharia et al. 2012). The cerebral swelling remains at its maximal level for 1 week as noted by contrast-enhanced T1-weighted imaging and diffusion weighted imaging (Schaefer, Grant et al. 2000).

In addition to edema, the sub-acute stage of stroke is also characterized by a chance for hemorrhagic transformation, or an extravasation of blood in the infarcted area (Slivka and Pulsinelli 1987, Kanekar, Zacharia et al. 2012). Hemorrhagic transformation is believed to be a result of the breakdown of the BBB, a disrupted endothelium and reperfusion. The concept that reperfusion may lead to hemorrhage 1-2 weeks following an ischemic stroke is of particular importance as reperfusion via thrombolysis is the mainline therapy for this condition. In fact, the chances of a hemorrhage have been shown to increase 2-3 times in patients receiving thrombolytic agents (Slivka and Pulsinelli 1987, Hacke, Donnan et al. 2004). As hemorrhagic transformation is only one of the many drawbacks in the current line of therapy for ischemic stroke, it is important to not only understand the mechanisms for these ischemia-related pathological changes but to also look into how they may be prevented.

The “chronic” stage of cerebral ischemia generally lasts from 2 weeks to 2 months after infarct, although the effects may be seen for years. Imaging during this stage has shown brain atrophy, gliosis, cavitations and calcifications with an increase in Wallerian degeneration and necrosis (Siskas, Lefkopoulos et al. 2003, Kanekar, Zacharia et al. 2012). Not everything that occurs in these stages, however, is negative.

Beginning in the sub-acute and continuing through the chronic stage of ischemic stroke, the body and brain attempt to repair themselves. The BBB is restored, leading to the resolution of edema and allowing the immune system to begin to clear away the necrotic tissue (Kanekar, Zacharia et al. 2012). The brain does not simply clean up the damage, however, but it makes an attempt to replace its lost materials and functions. As a result of the hypoxia, vascular endothelial growth factor (VEGF) is elevated. This increase in VEGF not only leads to angiogenesis, but also provides neuroprotection (Góra-Kupilas and Jośko 2005). In fact, endothelial cell proliferation may occur within 24 hours of infarct, shortly leading to a new vascular network. This new network is then capable of providing the energy needed to promote both tissue survival and remodeling (Yanev and Dijkhuizen 2012). With the repair of the BBB and an endogenous increase in mitogens and neuroprotective factors, new neurons also begin to appear, migrating out from the adult neuronal stem cell niche in the subventricular and subgranular zones to the peri-infarct areas (Minger, Ekonomou et al. 2007, Li, Yu et al. 2010). Understanding the pathways and mechanisms involved in this repair process may provide researchers with future targets to help improve the ultimate physiological and functional outcomes for patients suffering from ischemic stroke.

1.2. Current Treatments for Ischemic Stroke

Currently, there are only three acute treatments for ischemic stroke that are approved by the Food and Drug Administration (FDA). All of the approved treatments involve some form of thrombolysis, restoring blood flow to the ischemic brain by removing the blocking clot.

The first treatment to be approved by the FDA for emergency treatment of acute stroke was a recombinant form of tissue plasminogen activator (rtPA). Approved in June of 1996, rtPA is a recombinant form of a naturally occurring enzyme which activates plasminogen to dissolve a blood clot (1995). The results of the definitive clinical trial conducted by the National Institute for Neurological Disorders and Stroke led to FDA approval but gave relatively specific guidelines regarding the use of rtPA. In accordance with the statement "Time is Brain", the earlier that a patient receives intravenous rtPA, the better his or her outcome is likely to be. Specifically, the approved time window for efficacy between infarct and treatment with intravenous rtPA is only 3-4 hours. Partially due to this small therapeutic time window, only a very small number of people are actually able to benefit from this treatment. In fact, although numbers vary, only approximately 1-5% of stroke patients receive rtPA treatment (Donnan, Fisher et al. 2008, Kidd 2009). This miniscule percentage is due not only to the short time window, but also to the unpreparedness of the treatment center, the restrictive patient selection criteria, and the associated risks with the use of rtPA. Although rtPA is very effective in reducing disability following ischemic stroke, it does not improve overall mortality (Hacke, Donnan et al. 2004). In addition, treatment with rtPA comes with a significant risk of intracerebral hemorrhage, occurring in 6-7% of cases. The risks of symptomatic hemorrhage are higher in older patients presenting with high blood pressure, severe hyperglycemia and very severe neurological deficits, possibly indicative of malignant middle cerebral artery infarction

(Donnan, Fisher et al. 2008). Careful patient selection processes and accurate imaging and diagnostic techniques may help to lower the risks associated with rtPA administration, but these solutions often require time – time during which the brain is undergoing more and more damage.

Despite the obvious limitations to rtPA therapy for ischemic stroke, it remained the only approved treatment for eight years, and today still maintains its status as the "go to" treatment for an acute cerebral ischemic infarct. In 2004, however, another method for removal of the clot and restoration of blood flow to the brain was approved by the FDA – mechanical thrombectomy by the MERCI retrieval system®. The MERCI, standing for Mechanical Embolus Removal in Cerebral Ischemia, system consists of a tiny corkscrew-like catheter that can be wrapped around the clot and pulled back, removing the obstruction and restoring blood flow (Donnan, Fisher et al. 2008, Kidd 2009). The mechanical retrieval system is recommended for patients who are either ineligible for or have failed to respond to rtPA therapy. While the mechanical thrombectomy can be performed well outside the 3 hour time window, its efficacy in the clinical setting remains unclear.

More recently, a system partially based on the MERCI retrieval system® was developed, pushed through clinical trials and approved by the FDA in 2008. The new system, known as the Penumbra system™, uses suction or aspiration to grab and remove the clot from the larger blood vessels of the brain (Tarr, Hsu et al. 2010). Thus far, the Penumbra system™ has been shown to be efficacious when used within 8 hours of the infarct. Mechanical thrombectomy has the advantage of a larger, purportedly efficacious, therapeutic window. It is, however, still a surgical intervention, requiring catheterization and opening the possibility for mechanical damage to blood vessels other than those within the brain. Given our increased comprehension of the pathophysiologic changes that occur following cerebral ischemia, it is not surprising that many

researchers have therefore sought to find or develop a new, improved treatment for acute ischemic stroke.

1.3. Trends in Current Research for the Treatment of Cerebral Ischemia

Given the small number of patients who actually qualify for and receive the frontline therapy for acute ischemic stroke, it is unsurprising that research into new therapies is constantly occurring. A quick search of the current clinical trials in the United States with the keywords "cerebral ischemia" brings up over 1,500 trials, more than 650 of which are currently open (Health 2012). Limiting the search to open trials for "acute" cerebral ischemia brings the number down to near 150, which demonstrates that ischemic stroke treatment is still a substantial area of research. In general, it is possible to break down the investigational therapies into three categories – pharmaceutical, surgical and rehabilitative.

A vast majority of pharmaceutical interventions for the treatment of cerebral ischemia are being designed to prevent or reverse certain points in the ischemic cascade, thereby salvaging the tissue in the penumbra. Others are aimed at restoring perfusion, falling into the category of thrombolytics such as rtPA. The former are often referred to as neuroprotectancts, although it may be argued that both the restoration of blood flow and the prevention or slowing of the ischemic cascade are neuroprotective. In any case, investigational pharmaceutical interventions aimed at reperfusion include direct thrombolytics, such as desmoteplase – an engineered protein based off of an enzyme in the saliva of vampire bats, and anti-aggregation or "blood-thinning" compounds, such as monoclonal antibodies or heparin (Schleuning 2001, Camerlingo, Salvi et al. 2005, Green 2008). The so-called neuroprotectant drugs run the gambit from glutamate receptor antagonists to metal chelators to free radical scavengers and trapping agents (Green 2008).

Unfortunately, to this point, with the notable exception of rtPA, no pharmaceutical interventions have proven efficacious in human clinical trials. The reasons for these failures are manifold in nature. While a drug may show benefit in animal trials, the differences between species with regards to grey versus white matter, typical recovery rate, and dosage variations combine with the fact that animal studies are conducted under very rigid time and patient health controls – something which, while it would be ideal, is rarely as possible in human clinical trials (Pérez de la Ossa and Dávalos 2007, Green 2008, Krafft, Bailey et al. 2012). In hopes of ensuring that the drugs reaching clinical trial status had a relatively reasonable chance of displaying efficacy, strict guidelines for new drug development for the treatment of ischemic stroke were published in 1999 as a result of the Stroke Therapy Academic Industry Roundtable (STAIR) (1999). Nevertheless, as the first drug developed with these guidelines in mind, NXY-059, famously failed to demonstrate efficacy in clinical trials, updates on these STAIR guidelines are continuously being made (Fisher and Roundtable 2003, Fisher, Feuerstein et al. 2009, Saver, Albers et al. 2009).

While mechanical thrombectomy using either the MERCI retrieval system® or the Penumbra system™ are currently the only FDA-approved surgical interventions for the treatment of acute ischemic stroke, other treatments are being investigated in both animal and human cases of cerebral ischemia. One of the better studied surgical interventions is the use of hypothermia during the first 48 hours of stroke. An increase in body temperature occurring within the first few hours following an ischemic attack is associated with a poorer outcome (den Hertog, van der Worp et al. 2007). Both physical and pharmacological cooling methods have been put to the test in clinical trials, and, while more trials are warranted to prove or disprove efficacy, the ease of use of certain methods have led to their adoption in a majority of cases. Notably, the

administration of antipyretics such as acetaminophen or aspirin is common following a stroke, although their effectiveness in this situation is still under debate (den Hertog, van der Worp et al. 2007, Donnan, Fisher et al. 2008). In cases of malignant middle cerebral artery infarction, more drastic surgical interventions may be called upon. Specifically, although its use is still somewhat controversial, decompressive hemicraniectomy has been shown to benefit a small number of select patients. Decompressive hemicraniectomy is a neurosurgical procedure in which part of the skull is removed to allow space for the edematic pressure resulting from the cerebral ischemia to dissipate without further compression and blood flow reduction in the brain. Three large clinical trials, termed DECIMAL (decompressive craniectomy in malignant middle cerebral artery infarcts), DESTINY (decompressive surgery for the treatment of malignant infarction of the middle cerebral artery) and HAMLET (hemicraniectomy after middle cerebral artery infarction with life-threatening edema trial), demonstrated that certain patients, based on age, time from infarct to treatment, and with certain clear CT signs do show a significant benefit from this surgery (Donnan, Fisher et al. 2008, Treadwell and Thanvi 2010, Wartenberg 2012). Nonetheless, despite these advances in surgical treatments, only a small number of patients will qualify to receive them. Additionally, as with current treatments, each of these interventions comes with its own risks, from cardiovascular complications to infections, without the guarantee of a complete or even a good recovery.

In a nod to the difficulty and uncertainty of treating a cerebral ischemic attack both properly and within the relatively short time frame for efficacy, much current research is now focusing on rehabilitation. Even if patients are lucky enough to receive early acute treatment for their ischemic strokes, they will likely have side effects and symptoms of the brain damage for years to come. Physical and speech therapy may help patients to regain certain abilities long

after the initial ischemic attack. The question is, how is this recovery possible? The answer lies with the brain's own regenerative mechanisms. Studies in experimental stroke models and functional MRI imaging of the adult brain following injury have shown that the brain is capable of reorganizing and remodeling in order to compensate for damage (Calautti and Baron 2003, Eliassen, Boespflug et al. 2008). In fact, endogenous stem cells with the adult brain are capable of proliferating and migrating from the neuronal stem cells niches in the subventricular and subgranular zones outward toward the area of injured tissue (Yamashita, Ninomiya et al. 2006, Minger, Ekonomou et al. 2007). While there is currently insufficient evidence for the treatment of ischemic stroke with the implantation of exogenous stem cells, it is possible that the administration or stimulation of growth factors may encourage the proliferation and migration of the endogenous neuronal stem cells, leading to a better overall functional recovery from cerebral ischemia (Ninomiya, Yamashita et al. 2006, Kidd 2009, Boncoraglio, Bersano et al. 2010).

The ideal treatment for cerebral ischemia, barring our ability to completely prevent these attacks, would restore blood flow to the brain, protect the penumbra from oxidative stress and the brain from secondary damage due to the ischemic cascade and/or cerebral edema, and, ultimately, assist the brain in its attempts at reorganization and remodeling. In the meantime, researchers must continue to investigate these conditions and the pathways and mechanisms which lead to them in an attempt to fully understand the etiology of the disease and where it may be curtailed by human intervention.

2. <u>The Endothelin System</u>

Endothelin (ET) was discovered more than a quarter of a century ago as an endogenous endothelium-derived contracting factor (Hickey, Rubanyi et al. 1985, Yanagisawa, Kurihara et

al. 1988). Today, the ET system consists of three endogenous isopeptides termed ET-1, ET-2 and ET-3 which exert their effects upon two main classes of G-protein-coupled receptors, ET_A and ET_B. This system of peptides plays an extremely important role in vasoregulation under both normal and pathological conditions. The regulatory functions of the ET system with regards to vasculature as well as neuronal tissue are thought to play an important role in many of the pathophysiologic events following cerebral ischemia (Kaundal, Deshpande et al. 2012).

2.1. Endothelins and Endothelin Receptors

Endothelin peptides, ET-1, ET-2 and ET-3, consist of 21 amino acids forming an open loop tertiary structure. The peptides are encoded by preproendothelin genes and translated into respective 212 amino acid long preproendothelin (preproET) peptides. The biologically inert precursor to the ET peptide isoforms, known as “big ET”, is then formed by cytoplasmic cleavage from preproET by neuronal endopeptidase furin. Endothelin converting enzymes next work on big ET to form the 21 amino acid peptides, all of which share a similar structure with two disulfide bonds and a hydrophobic C terminal. Endothelin-2 is produced by the small intestine and kidney, ET-3 by the intestine and nervous tissue, and ET-1 mainly by the endothelium (Khimji and Rockey 2010, Kaundal, Deshpande et al. 2012). All three endogenous ET isopeptides have been implicated in a number of physiological and pathological roles including regulation of blood flow and pressure, apoptosis and immune modulation.

The biological responses to ET are mediated by the activation of two distinct G-protein-coupled receptors – ET_A and ET_B. These receptors consist of seven hydrophobic transmembrane domains, with an intracytoplasmic C terminus and an extracellular N terminus (Arai, Hori et al. 1990). Variations in the C terminus account for differences in the binding affinities of the three

isopeptides to each receptor. While all three ETs have an equal affinity for ET_B receptors, ET-3 has a lower affinity for ET_A receptors than either ET-1 or ET-2 (Sakurai, Yanagisawa et al. 1992). Within the vasculature, ET_A receptors are located on the vascular smooth muscle where they mediate vasoconstriction via a biphasic increase in intracellular calcium. Endothelin A activation by ET-1 leads to an initial transient rise in intracellular calcium as a result of the inositol triphosphate (IP3) pathway, followed by a sustained increase in intracellular calcium as depolarization triggers an influx of calcium via voltage-dependent L-type Ca^{2+} channels or non-selective ion channels (Goto, Kasuya et al. 1989, Iwamuro, Miwa et al. 1998). Endothelin B receptors, on the other hand, are mainly located on the vascular endothelial cells where they are coupled to the activation of endothelial nitric oxide synthase (eNOS) by Ca^{2+}-calmodulin and protein tyrosine kinase and/or Akt phosphorylation (Tsukahara, Ende et al. 1994, Liu, Premont et al. 2003, Tang, Luo et al. 2007). Figure 1 highlights the main pathways triggered by ET transmembrane signaling.

2.2. Endothelin in the Central Nervous System

Although first identified through and perhaps best known for its vasoregulatory properties, the ET system is by no means limited to the vasculature. It is well spread throughout the body, including within the central nervous system (CNS). Histological studies of both human and animal brains have demonstrated a wide distribution of ET peptides and their receptors (Matsumoto, Suzuki et al. 1989, Takahashi, Ghatei et al. 1991, Takahashi, Ghatei et al. 1991). Within the brain, ETs are not localized only to the vasculature, but may also be found on neurons, astrocytes and occasionally microglial cells (Schinelli 2006).

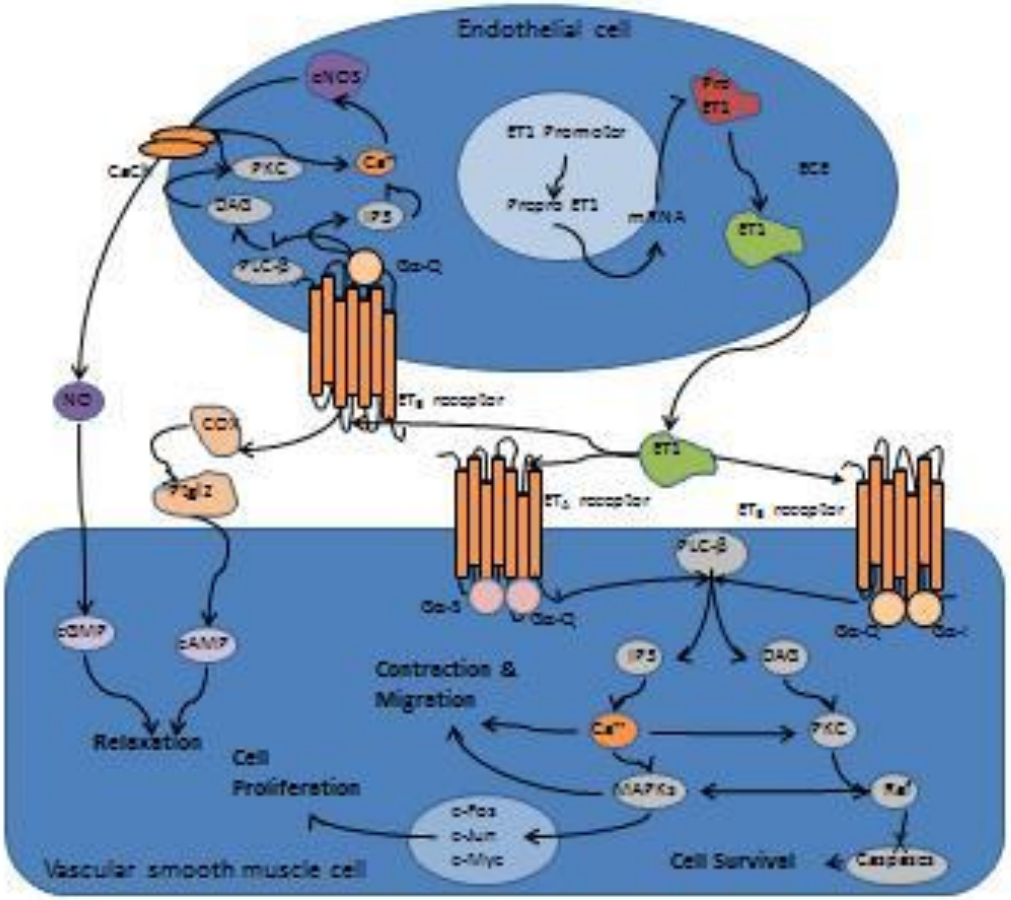


Figure 1: Transmembrane signaling of ET receptors. CaCh: calcium channel; cAMP: cyclic adenosine monophosphate; cGMP: cyclic guanosine monophosphate; COX: cyclooxygenase; DAG: diacylglycerol; eNOS: endothelial nitric oxide synthase; ET: endothelin; IP3: inositol-1,4,5-triphosphate; MAPK: mitogen-activated protein kinases; NO: nitric oxide; PLC: phospholipase C; PKC: protein kinase C; Ptgl2: prostaglandin 2.

Endothelins are known to play a particularly important role in the developing brain. Endothelin B receptors, specifically, concurrently enhance proliferation of neurons while preventing apoptosis during development. Endothelin B-deficient rats demonstrate a decrease in neuronal progenitors and an increase in apoptosis within the dentate gyrus and cerebellum postnatally (Ehrenreich, Nau et al. 2000, Vidovic, Chen et al. 2008). The ET_B knockout model leads to a congenital aganglionosis within the gut and associated CNS disturbances, and often serves as a rodent model for human Hirschsprung disease (Dembowski, Hofmann et al. 2000, Riechers, Knabe et al. 2004). A recent study performed in our laboratory showed that, while levels of ET_B receptors are high immediately following birth in rat brains, levels drop off by postnatal day 21, indicating that ET_B receptors are important during the very early postnatal development phase (Briyal, Lavhale et al. 2012). The role of ETs and their receptors in adult neurovascular development and repair is currently unknown.

Endothelins are thought to be important in the regulation of the blood brain barrier (BBB). The BBB consists of tight junctions around the capillaries of the CNS which do not exist in normal circulation. It serves as a barrier between the circulating blood and the cerebrospinal fluid within the CNS, restricting the type of molecules and metabolites that have access to the brain. Administration of ET-1 disrupts the BBB by impairing Cx43, an astrocytic specific connexin, while an increase in ET-1acting on ET_A receptors impairs the BBB, elevating levels of aquaporin 4, a type of water channel protein important in regulating water homeostasis in the CNS (Blomstrand, Venance et al. 2004, Lo, Chen et al. 2005). This results in an exacerbation of edema and poor outcome following transient cerebral edema. Conversely, stimulation of ET_B receptors by administering an ET_B agonist intracerebroventricularly (i.c.v.) causes a decrease in aquaporin 4 (Koyama and Tanaka 2010). This type of dual regulation is common with the ET

system and is important to consider when stimulating, blocking or knocking out either or both types of ET receptors.

2.3. Endothelin in Cerebral Ischemia

The vasoregulatory properties and the overwhelming presence of ETs throughout the brain, has led to numerous studies of ET and its possible roles in the pathologies of cerebrovascular accidents such as ischemic stroke. Indeed, it has been found that the ET system is highly disrupted as a result of cerebral ischemia. Both plasma and tissue levels of ET-1 are elevated following ischemic stroke. Specifically, it was found that plasma ET-1 levels begin to rise starting at 6 h post stroke and remain elevated until 48 h in both the core and the penumbral region of ischemic rat brains (Matsuo, Mihara Si et al. 2001). Similarly, plasma ET levels have been shown to be elevated in humans at the first 24 h following the cerebral ischemic attack, and these levels correlate with the size of the lesion (Ziv, Fleminger et al. 1992, Franceschini, Gandolfo et al. 2001). Higher levels of ET-1 seem to indicate increased damage and precede a poorer outcome. Interestingly, it is not only ET peptide levels which change following cerebral ischemia, but also ET receptor levels within the brain. Both ET_A and ET_B receptors have been shown to be up-regulated in the middle cerebral artery following ischemic stroke (Stenman, Malmsjo et al. 2002, Henriksson, Stenman et al. 2003). Endothelin A receptors at mossy fiber terminals, however, are down-regulated following ischemia, leading to an increase secretion of glutamate and subsequent excitotoxicity . Conversely, expression of ET_B receptors is increased in neurons, glia and macrophages, and has been hypothesized to activate various survival mechanisms following cerebral ischemia (Kreipke, Reynolds et al. 2011). Other reports, however, have found no change in ET_B receptor expression, while ET_B knockout models have displayed a concurrent down-regulation of ET_A receptors, all of which suggests that the

expression of both receptors may be dependent on each other, and therefore potentially quite variable (Chuquet, Benchenane et al. 2002, Davenport and Kuc 2004).

The high levels of ET and its receptors are thought to exacerbate, directly and indirectly, brain damage following cerebral ischemia. Endothelin has been implicated in everything from delayed hypoperfusion to excitotoxicity, BBB disruption and edema to inflammation (Kaundal, Deshpande et al. 2012). Because the main effects of ET are a result of activation of ET receptors, targeting these receptors by using agonists and/or antagonists is one of the best methods for further elucidating the individual roles of ET_A versus ET_B receptors. This method has the added benefit of illuminating potential future therapeutic targets for the ET system in ischemic stroke. As a majority of the known deleterious effects of ET in stroke occur as a result of ET_A receptor activation, research has thus far focused on antagonism of ET_A receptors or dual antagonism of both ET_A and ET_B receptors.

To date, a number of ET antagonists have been tested as pre- or post-treatments for ischemic stroke in animal models. One of the first experiments to test the theory that ET antagonists may be beneficial in the treatment of cerebral ischemia involved $ET_{A/B}$ antagonist bosentan in a permanent middle cerebral artery occlusion (MCAO) model of stroke in rats. No improvement with regards to cerebral blood flow (CBF) or neurological deficit was observed (McAuley, Breu et al. 1996). On the other hand, pretreatment with $ET_{A/B}$ antagonist SB217242 produced a 30% reduction in infarct volume, and pretreatment with dual antagonist TAK-044 led to a significant reversal of oxidative stress and prevented motor impairment in rat MCAO models (Barone, White et al. 1995, Gupta, Briyal et al. 2005). In an effort to separate out the effects of ET_A selective antagonism versus dual antagonism, Barone, et al, examined the effects of ET_A antagonist SB234551 and $ET_{A/B}$ antagonist SB209670 treatment following permanent MCAO.

While the ET_A–specific antagonist reduced neurological deficits, cerebral edema and degree of infarction, the $ET_{A/B}$ non-specific antagonist did not appear to provide any neuroprotection (Barone, Ohlstein et al. 2000). Endothelin A antagonist SB234551 has also been shown to improve CBF following permanent MCAO in rats (Zhang, Belayev et al. 2005). Endothelin A antagonist S-0139, used in conjunction with rtPA, provided neuroprotection by suppressing ischemia- and rtPA-triggered molecules that would normally evoke thrombosis and BBB disruption in an embolic stroke model in rodents (Zhang, Zhang et al. 2008).

Although a majority of the research on ET and stroke thus far has focused on antagonizing ET_A receptors selectively or non-selectively in order to prevent excessive vasoconstriction, only a few studies have examined the effects of selectively antagonizing ET_B receptors. Additionally, no one has examined the effects of selectively stimulating ET_B receptors in a focal stroke model. Complete deficiency or blockade of ET_B receptors shifts the ET vasomotor balance to constriction and exacerbates ischemic brain damage (Ehrenreich, Oldenburg et al. 1999, Chuquet, Benchenane et al. 2002). In murine astrocytes, there is a close co-localization between bundles of intermediate filament proteins and ET_B receptors. A deletion of these intermediate filament proteins in a mouse model of ischemic stroke was associated with an altered distribution of ET_B receptors, less efficient ET_B-mediated gap junction communication, a reduced ability to take up glutamate, and, ultimately, an increase in infarct volume (Li, Lundkvist et al. 2008). Our lab has recently demonstrated that intravenous administration of ET_B selective agonist IRL-1620 was capable of significantly elevating CBF in normal rats (Figure 2) (Leonard and Gulati 2009). In addition to studies showing relaxation of normal animal and human carotid and temporal arteries as a result of ET_B stimulation, topical application of IRL-1620 has been shown to cause vasodilatation of the basilar artery in stroke-

prone spontaneously hypertensive rats (Kitazono, Heistad et al. 1995, Lucas, White et al. 1996, Tirapelli, Casolari et al. 2005). Beyond the direct vasodilatory effect, ET_B receptors have been implicated in direct and indirect neuroprotection via their actions on astrocytes, gap junctions, and the release of endothelial nitric oxide synthase (eNOS). Nevertheless, despite these promising indications, no one has yet attempted to selectively stimulate ET_B receptors in a model of cerebral ischemia.

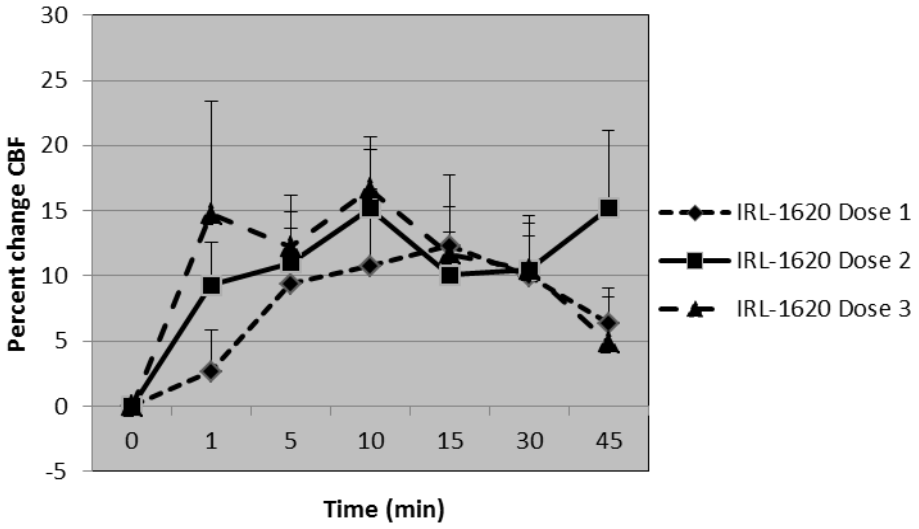


Figure 2: Effect of ET_B agonist IRL-1620 on cerebral blood flow. IRL-1620 was administer in 3 doses of 5µg/kg, i.v., at 1 h intervals and CBF was measured using a laser Doppler probe.

2.4. Endothelin B Receptor Agonist IRL-1620

IRL-1620 [N-Succinyl-[Glu9, Ala11,15] endothelin 1] is a synthetic analog of the endogenous vasoactive peptide ET-1. IRL-1620 was synthesized and its binding affinity was characterized by Takai, et al, in 1992 (Takai, Umemura et al. 1992). IRL-1620 was synthesized by replacing four amino acids in the ET-1 peptide sequence – Ala^7 was replaced with Suc^7, Lys^9 with Glu^9, and $Cys^{11,15}$ with $Ala^{11,15}$ (Figure 3). This synthetic peptide was found to be a potent and specific ligand for ET_B receptors, with Ki values for ET_A and ET_B of 1.9 µM and 16 pM, respectively ($Ki_{ETA}/Ki_{ETB} \approx 120{,}000$). IRL-1620 was also shown to be 60 times more selective than ET-3 for ET_B receptors ($Ki_{ETA}/Ki_{ETB} \approx 1900$ for ET-3) (Takai, Umemura et al. 1992). Studies conducted on rat aorta demonstrated that IRL-1620 (10^{-9} – 10^{-7} M) increased cytosolic Ca^{2+} in the vascular endothelium with little effect on resting muscle tone but with a relaxant effect on norepinephrine-stimulated tone. The relaxant effect was abolished in the endothelium-denuded aorta, while L-Arg(Me), an inhibitor of nitric oxide synthesis, inhibited relaxation but not the increase in Ca^{2+} (Karaki, Sudjarwo et al. 1993). Studies of radio-iodinated IRL-1620, [^{125}I]IRL-1620 determined that this agonist displays species-specific binding characteristics, with rapid and reversible binding to dog cerebellum and human lung tissues but rapid and irreversible binding to rat cerebellum and lung tissues (Nambi, Pullen et al. 1994).

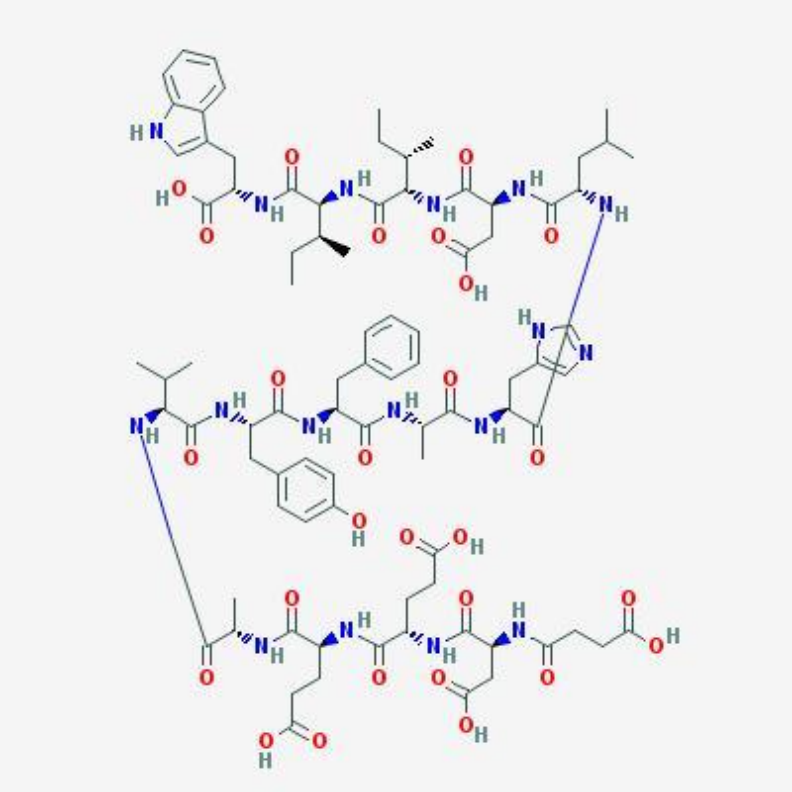


Figure 3: Molecular structure of IRL-1620 [N-Succinyl-[Glu9, Ala11,15] endothelin 1], a selective ET_B receptor agonist (PubChem).

Since its synthesis, IRL-1620 has been used in a wide variety of studies to determine the effects of selective stimulation of ET_B receptors. Studies utilizing IRL-1620 have been performed in the pulmonary, hepatic, renal, gastrointestinal, dermatological, and endocrine systems, where a number of ET_B mediated effects have been described. In addition to these studies, IRL-1620 has notably been used as a means of studying the effects of ET_B stimulation in

the cardiovascular and central nervous systems. In the cardiovascular system, stimulation of ET_B receptors by IRL-1620 elicits positive inotropic and chronotropic effects on the vascular smooth muscle, and negative inotropic effects and regulation of blood pressure via endothelial cell nitric oxide release (Beyer, Slesak et al. 1996, Leite-Moreira and Bras-Silva 2004, Bras-Silva, Fontes-Sousa et al. 2006). Cardiovascular studies performed in our laboratory determined that multiple intravenous injections of IRL-1620 at 1 h intervals led to tachyphylaxis of the hypotensive phase of IRL-1620's effects on mean arterial pressure (Figure 4) (Leonard and Gulati 2009). This same study demonstrated that i.v. administration of IRL-1620 was capable of increasing cerebral blood flow in normal rats (Figure 2). The CBF results confirmed the findings of Kitazono, et al, and Kobari, et al, who demonstrated that IRL-1620 stimulation of ET_B receptors caused vasodilatation and increased blood flow via NO release in the basilar artery of rats and cerebral microvessels of cats, respectively (Kobari, Fukuuchi et al. 1994, Kitazono, Heistad et al. 1995). In addition to its effects on CBF, IRL-1620-stimulation of central ET_B receptors has been shown to activate phospholipase D in astrocytes, modulating inflammatory activation (Servitja, Masgrau et al. 1998, Morga, Faber et al. 2000). Overall, given the high selectivity of IRL-1620 for ET_B receptors and the previous findings demonstrating its beneficial effects on cerebral blood flow and astrocytic inflammation, the use of this agonist would seem to be a good method for studying the effects of central ET_B receptors in a model of cerebral ischemia.

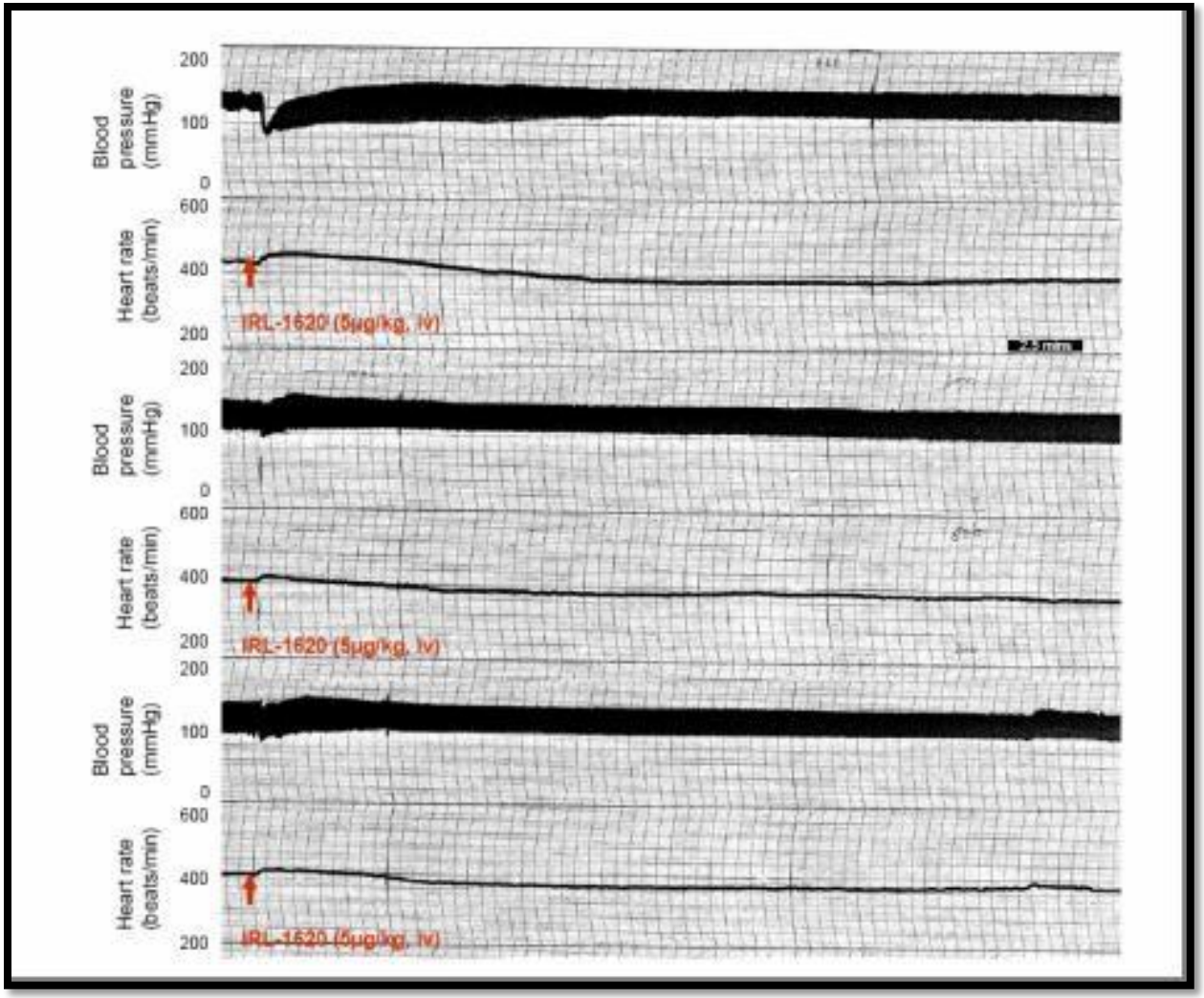


Figure 4: Tachyphylaxis of multiple doses of ET_B agonist IRL-1620 on hypotensive effect. IRL-1620 was administered in 3 doses of 5 µg/kg, i.v. at 1 h intervals. Blood pressure and heart rate were recorded using a Grass Polygraph.

II. PROBLEM STATEMENT

Despite ischemic stroke being a leading cause of death and long-term disability worldwide, there is a dearth of options for the treatment of this condition. Additionally, several biochemical pathways involved in the pathophysiology of cerebral ischemia are still poorly understood. While it is known that the endothelin system plays a role in cerebral ischemia, little is known regarding the involvement of ET_B receptors specifically. The goal of this study, therefore, is to determine and characterize the role of central ET_B receptors in a model of focal cerebral ischemia. Because recent findings have indicated cerebral vasodilator and neuroprotective effects following stimulation of ET_B receptors, it is of interest to determine whether such ET_B-specific stimulation results in a reduction of neurological damage following permanent middle cerebral artery occlusion in rats. The mechanism of action for any such neuroprotective changes ought also to be examined. Specifically, knowledge of the effects of ET_B receptor stimulation over time following cerebral ischemia on biochemical parameters such as oxidative stress, as well as on anatomical changes, such as neurovascular remodeling, could lead to new therapeutic targets for this condition. Overall, in addition to potentially revealing a novel therapeutic target for the treatment of ischemic stroke, this study will enhance our understanding of the pathways involved in the pathophysiology of cerebral ischemia, thus improving our ability to model, treat and/or prevent this condition.

In order to determine the involvement of ET_B receptors and their effects on neurological damage following cerebral ischemia, male Sprague-Dawley rats were subjected to permanent middle cerebral artery occlusion (MCAO). Animals were then treated with vehicle (isotonic saline) alone, ET_B agonist IRL-1620 alone, ET_B antagonist BQ788 followed by either vehicle or IRL-1620, or ET_A antagonist BQ123 followed by either vehicle or IRL-1620. Two

endpoints were utilized – 24 hours (24 h), representing the results of the acute phase of cerebral ischemia, and 1 week (1 w), representing the sub-acute phase of cerebral ischemia. Animals were assessed for neurological and motor function deficit before and at various time points post occlusion using neurological deficit score, grip test for muscular strength, foot fault error test and rota rod for muscular coordination, and locomotor activity for spontaneous movement. At the end of each experiment, animals were decapitated and their brains were stained with 2% TTC to determine infarct volume. Normal and ischemic brains were evaluated for ET_A and ET_B receptor expression using immunoblotting procedures.

Secondary damage to the penumbra following ischemic stroke occurs as part of the ischemic cascade, resulting in increased oxidative stress and subsequent deleterious changes to the surrounding tissue. It was therefore of interest to establish the effects ET_B receptors might have on oxidative stress parameters such as lipid peroxidation and antioxidant levels in the ischemic rat brain. Rats underwent MCAO as described above. At either 24 h or 1 w following occlusion, the brains were evaluated for levels of malondialdehyde, superoxide dismutase, and reduced glutathione to determine whether there was any alteration in oxidative stress following treatment with the ET_B agonist or antagonist.

An important consideration in the research of novel treatments for pathological conditions is the determination not only of which pathways are affected by the treatment, but also whether the treatment results in significant structural changes within the tissue. This is of particular importance in the treatment of ischemic stroke, where it is known that endogenous mechanisms for the repair of ischemic brain tissue are occasionally activated. In determining the effects of ET_B receptors following ischemic stroke, it was of interest to observe whether the neurovascular tissue was not only “rescued” but remodeled. Immunohistochemical techniques

were employed to stain the brain tissue following MCAO in rats subject to treatment with vehicle, ET_B agonist IRL-1620 or ET_B antagonist BQ788. The location and distribution of ET_B receptors as well as markers for normal, proliferating, angiogenic and neurogenic tissue were observed.

The goals of this study fall under three specific aims – 1. Determine the involvement of ET_B receptors in neurological damage following cerebral ischemia; 2. Determine whether ET_B receptors are mediated in oxidative stress mechanisms following cerebral ischemia; and 3. Determine whether ET_B receptors are mediated in neurovascular repair and remodeling following cerebral ischemia. Overall, these objectives were designed to determine the involvement of central ET_B receptors in cerebral ischemia and the mechanisms responsible for any effects resulting from stimulation and/or blockade of these receptors.

III. METHODS AND MATERIALS

1. Animals

Male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 300-350 g were housed in a room with controlled temperature (23 ± 1˚C), humidity (50 ± 10%), and light (6:00 A.M. to 6:00 P.M.). Food and water were available *ad libitum*. Animals were allowed to acclimate for at least 4 days prior to experimentation. All animal care and use for experimental procedures were in compliance with and approved by the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Midwestern University (MWU File #2103) and the Animal Care Committee (ACC) of the University of Illinois at Chicago (ACC #12-109).

2. Drugs

Ketamine (Butler Animal Health Supply, Dublin, OH) and xylazine (Lloyd Laboratories, Shenandoah, IA) were administered intraperitoneally (i.p.) at a dose of 100 mg/kg and 10 mg/kg, respectively. IRL-1620 [N-Succinyl-[Glu9, Ala11,15] endothelin 1] (American Peptide Co, Inc., Sunnyvale, CA) was dissolved in isotonic saline and administered at a dose of 5 µg/kg, intravenously (i.v.) at 2, 4 and 6 hours post middle cerebral artery occlusion. While ET_B receptor agonist, IRL-1620, is known to have transitory hypotensive effects, a previous study performed in our laboratory demonstrated that tachyphylaxis of the hypotensive response occurs when IRL-1620 is delivered in multiple doses of 5 µg/kg, i.v., over time (Leonard and Gulati 2009). As the first 12-24 h are critical in regards to protection of the penumbra, and in order to ameliorate any potential side effects due to transient hypotension, it was determined to administer IRL-1620 in 3 doses of 5 µg/kg, i.v., at 2 h intervals on day 1. 2 h post occlusion was chosen as the ideal time to begin treatment as it allows sufficient time for the animal to recover from surgery and to begin

displaying neurological and/or motor impairment, confirming that cerebral ischemia is occurring. ET_B receptor antagonist, BQ788 (American Peptide Co, Inc., Sunnyvale, CA), and ET_A receptor antagonist, BQ123 (American Peptide Co, Inc., Sunnyvale, CA), were dissolved in isotonic saline and administered at a dose of 1 mg/kg, i.v., 15 min prior to administration of either vehicle or IRL-1620. 5-Bromo-2'-deoxyuridine (BrdU, Millipore Corp., Temecula, CA), a thymidine analog that is incorporated into the DNA of dividing cells during S-phase, was injected at a dose of 10 mg/kg, i.p., on day 5 for subsequent immunofluorescent labeling of proliferating cells within the ischemic brain. In preparation for immunofluorescent labeling, animals were anesthetized with urethane (0.15 g/kg, i.p.; Acros Organics, New Jersey) and transcardially perfused with isotonic saline followed by a 4% paraformaldehyde (PFA; Sigma-Aldrich, St. Louis, MO) phosphate buffered saline solution.

3. <u>Experimental Protocols</u>

To determine the involvement of ET_B receptors and their effects on cerebral ischemia, three distinct studies were performed. The first 24 hours, known as the acute phase of ischemic stroke, is hallmarked by a strong oxidative stress reaction, whereas the period from 2 to 14 days after ischemic onset, the sub-acute phase, is characterized by microvascular damage, inflammation and a breakdown of the blood brain barrier leading to cerebral edema (Lakhan, Kirchgessner et al. 2009, Kanekar, Zacharia et al. 2012). Throughout these phases, the brain begins to take steps toward recovery via the initiation of angiogenesis, neurogenesis and neuroblast migration toward the ischemic boundary (Minger, Ekonomou et al. 2007). With these distinct pathologic changes in mind, we therefore examined the involvement of ET_B receptors in both acute and sub-acute cerebral ischemia, as well as post-ischemic neurovascular remodeling.

3.1. Involvement of Endothelin B Receptors in Acute Cerebral Ischemia

The involvement of ET_B receptor agonist, IRL-1620, and antagonist, BQ788, on the acute phase of ischemic stroke was assessed by their effects on neurologic and motor deficit, infarct volume, oxidative stress parameters and $ET_{A/B}$ receptor levels 24 hours (24 h) following permanent middle cerebral artery occlusion. The effects of ET_A antagonist, BQ123, both alone and in combination with IRL-1620, on neurological and motor deficit and infarct volume were also evaluated to see whether a combination treatment involving both sides of the ET system might be synergistic. Oxidative stress and ET receptor estimation was not performed on BQ123-treated animals. Rats were randomly divided into seven groups of 6 animals each. Group 1 animals were subjected to a sham operation. Rats in groups 2-7 underwent middle cerebral artery occlusion (MCAO) and were treated as follows – Group 2: MCAO + vehicle; Group 3: MCAO + IRL-1620; Group 4: MCAO + BQ788 + vehicle; Group 5: MCAO + BQ788 + IRL-1620; Group 6: MCAO + BQ123 + vehicle; Group 7: MCAO + BQ123 + IRL-1620. All drugs were administered via intravenous tail vein injection. A total of three injections of vehicle (isotonic saline, 1ml/kg) and IRL-1620 (5µg/kg) were given at 2, 4, and 6 h post MCAO. BQ788 or BQ123 (1mg/kg) was given 15 min prior to administration of the first dose of either vehicle or IRL-1620. A total of 72 rats were used for this study, with n=6 per group for infarct volume and edema estimation, and n=6 for endothelin receptor and oxidative stress analysis.

3.2. Involvement of Endothelin B Receptors in Sub-acute Cerebral Ischemia

To determine the involvement of ET_B receptors on the sub-acute phase of ischemic stroke, the effects of IRL-1620 and BQ788 on neurologic and motor deficit, infarct volume, edema, oxidative stress, and ET receptor levels were determined 1 week (1 w) following

permanent MCAO. Rats were randomly divided into five groups. Animals in group 1 were subject to a sham operation. Animals in groups 2-5 were subject to middle cerebral artery occlusion and were treated as follows – Group 2: MCAO + vehicle; Group 3: MCAO + IRL-1620; Group 4: MCAO + BQ788 + vehicle; Group 5: MCAO + BQ788 + IRL-1620. All drugs were administered via intravenous tail vein injection as described above. A total of 70 rats were used for this study, with n=8 per group for neurological and motor deficit and infarct volume analysis, and n=6 per group for endothelin receptor estimation and oxidative stress analysis.

3.3. Involvement of Endothelin B Receptors in Post-Ischemic Neurovascular Remodeling

In order to determine the progression of tissue changes, two endpoints were chosen – 24 h (acute) and 1 w (sub-acute) following induction of cerebral ischemia. Immunofluorescent labeling was performed on ischemic rat brains to determine the effects of the ET_B receptor agonist and antagonist treatment on reactive astrocytes and neurons, as well as angiogenic and neurogenic markers. Rats for each endpoint were randomly divided into five groups. Animals in group 1 were subject to a sham operation. Animals in groups 2-5 were subject to middle cerebral artery occlusion and were treated as follows – Group 2: MCAO + vehicle; Group 3: MCAO + IRL-1620; Group 4: MCAO + BQ788 + vehicle; Group 5: MCAO + BQ788 + IRL-1620. All drugs were administered via intravenous tail vein injection as described above. A total of 50 rats were used for this study, with n=5 per group.

4. Middle Cerebral Artery Occlusion

Permanent middle cerebral artery occlusion was performed according to the method of Koizumi, et al (Koizumi 1986). Rats were anesthetized with ketamine and xylazine. Rectal core temperature was measured with a Cole Palmer Animal Monitoring Thermometer colonic probe

(Vernon Hills, IL) and maintained throughout surgery at 37±1˚C using the thermo-controlled base of the operating table. With the anesthetized rat in a secure supine position, a midline incision was made and the right common, internal, and external carotid arteries were exposed. A 4.0 monofilament nylon filament (CP Medical, Portland, OR) with a flame-rounded tip was advanced from the external carotid artery into the lumen of the internal carotid artery until resistance was felt (~20-22 mm), indicating occlusion of the middle cerebral artery. In order to create a permanent model of cerebral ischemia, the filament was securely tied and allowed to remain in place until the end of the experiment. The incision was closed with 3.0 silk surgical sutures (Ethicon, Inc.). In sham-operated animals, the common and external right carotid arteries were exposed and the incision was sutured without touching the internal carotid artery. Rats were monitored twice daily to assess appearance, activity, and behavior. Animals were sacrificed if there was a gradual but continuous decline in body weight or if they were moribund with an unhealthy appearance such as a rough coat, hunched posture and/or distended abdomen. Survival of the animals was documented. Proper and intact placement of the filament was verified in all animals at the time of sacrifice.

5. <u>Motor Performance Tests</u>

Five assessments were used to determine neurological and motor deficit following permanent middle cerebral artery occlusion – neurological evaluation, grip test, foot fault error test, rota rod, and spontaneous locomotor activity. Animals were subject to blinded assessments 15 min prior to occlusion to establish a baseline and at 1, 4 and 7 days post occlusion to determine the effects of ischemia with and without treatment.

5.1. Neurological Evaluation

The neurological evaluation was based on a 6 point scale as described by Tatlisumak, et al (Tatlisumak, Carano et al. 1998). The scoring was as follows: 0 = no deficits, 1 = failure to fully extend left forepaw, 2 = circling to the left, 3 = paresis to the left, 4 = no spontaneous walking, 5 = death.

5.2. Grip Test

The grip test for muscular strength consisted of a string elevated 40 cm above a flat surface pulled taut between two vertical supports spaced 50 cm apart. The animal was placed on the string midway between the supports and evaluated according to a 6 point scale (Moran, Higgins et al. 1995). The scoring was as follows: 0 = falls off, 1 = hangs on by two forepaws, 2 = hangs on by two forepaws and attempts to climb on, 3 = hangs on by 3+ paws, 4 = hangs on by all paws plus tail, and 5 = escapes.

5.3. Foot Fault Error Test

Animals were placed on an elevated grid floor with a mesh size of 30 mm^2 for one minute to acclimate. They were then observed for one minute and evaluated for foot fault errors (i.e. a misplaced limb falling through the grid) compared with paired steps as follows (Markgraf, Green et al. 1992):

% foot fault error = (number of faults/number of paired steps) x 100

5.4. Rota Rod

Animals were acclimated to the rotating spindle of the rota rod (Rota-Rod 47700, Ugo Basile, Italy) prior to occlusion. They were placed on the rotating spindle, set to a constant 8

rotations per minute (RPM), until they demonstrated the ability to remain on the spindle for at least 60 sec. Animals were then subject to a baseline test trial on the accelerating spindle (4-40 RPM) over 5 min. The acceleration trial was repeated at 1, 4 and 7 d post occlusion, and the time (in sec) at which the animals fell off was recorded (Rogers, Campbell et al. 1997).

5.5. Spontaneous Locomotor Activity

Spontaneous locomotor activity was assessed using an animal activity meter (Opto-Varimex-4 Auto-Track System, Columbus Instruments, Columbus, OH) prior to and at 1, 4, and 7 d following occlusion. Each animal was observed for a period of 10 min in a square enclosed area equipped with infrared photocells along the X, Y, and Z axes to quantitatively measure spontaneous horizontal and vertical motion (Shen and Wang 2010).

6. Assessment of Cerebral Infarct Volume

Animals were euthanized by decapitation at 24 h or 1 w following middle cerebral artery occlusion, and the brains were removed for assessment of infarct volume. The brains were washed in chilled saline at 4˚C for 5 min and then cut into 2 mm thick slices using a Brain Matrix (Harvard Apparatus, Holliston, MA). The sections were incubated at 37˚C for 15 min in 2% 2,3,5-triphenyltetrazolium (TTC, Sigma, St. Louis, MO) dissolved in saline. The stained sections were then stored in 10% formalin at 4˚C for further analysis (Li, Irie et al. 1997). TTC is reduced to red formazan by succinate dehydrogenase, a mitochondrial enzyme. Normal tissue therefore stains red whereas infarcted tissue which lacks the enzyme does not stain, thus leaving a clearly demarcated border between the ischemic and normal tissue (Bederson, Pitts et al. 1986). Infarct volume was calculated by sampling each side of the coronal sections with a digital camera (Nikon). The infarct area, outlined in white, was measured by image analysis software

(Adobe Photoshop CS4). Edema was determined by taking the percent increase in size of the ischemic over the contralateral hemisphere (Barone, White et al. 1995). Total infarct size is expressed as infarct volume in mm^3 as the sum of infarct areas in each slice, corrected for edema.

7. Estimation of Oxidative Stress Parameters

Brain levels of malondialdehyde, reduced glutathione, and superoxide dismutase were estimated at 24 h and 1 w following middle cerebral artery occlusion. Animals were decapitated, and the brains removed and washed in chilled saline and stored at -80˚C. The biochemical analyses were performed within 48 h.

7.1. Measurement of Lipid Peroxidation

An indicator of lipid peroxidation, malondialdehyde (MDA), was estimated according to the method of Okhawa, et al (Ohkawa, Ohishi et al. 1979). Brains were homogenized in 10 times (w/v) 0.1 M sodium phosphate buffer (pH 7.4). To 0.1 ml of the processed tissue sample, 1.5 ml acetic acid (20%, pH 3.5), 1.5 ml thiobarbituric acid (0.8%), and 0.2 ml sodium dodecyl sulfate (8.1%) were added, and the mixture was incubated at 100˚C for 60 min. After cooling, 5 ml n-butanol:pyridine (15:1% v/v) and 1 ml distilled water were added. The mixture was then shaken vigorously and centrifuged at 4000 RPM for 10 min. The absorbance of the organic layer was measured at 532 nm using a spectrophotometer (Spectronic Instruments, Rochester, NY).

7.2. Measurement of Reduced Glutathione

Brain levels of the antioxidant, reduced glutathione (GSH), were measured according to the method of Ellman with minor modifications (Ellman 1959). Brains were homogenized with 10 times (w/v) sodium phosphate buffer (pH 7.4). The homogenate was then centrifuged with

5% trichloroacetic acid to separate out the proteins. 0.1 ml of the supernatant was then added to 2 ml phosphate buffer (pH 8.4), 0.5 ml 5'5-dithio-bis-2-nitrobenzoic acid, and 0.4 ml distilled water. The mixture was then vortexed and the absorbance was read within 15 min at 412 nm using a spectrophotometer.

7.3. Measurement of Superoxide Dismutase

Antioxidant superoxide dismutase (SOD) was measured in accordance with the method of Kakkar, et al (Kakkar, Das et al. 1984). 1.2 ml sodium pyrophosphate buffer (0.052 M, pH8.3), 0.1 ml phenzanine methosulfate (186 µM), 0.3 ml nitro blue tetrazolium (300 µM), and 0.2 ml NADH (780 µM) were added to 0.1 ml homogenate, and the mixture was incubated at 30˚C for 90 min. Following the addition of 4 ml n-butanol and 1 ml acetic acid, the mixture was shaken vigorously and then centrifuged at 4000 RPM for 10 min. The absorbance of the organic layer was measured at 560 nm using a spectrophotometer.

8. Endothelin Receptor Estimation

Endothelin receptors in the infarcted brain were measured via Western blotting. Animals were decapitated at 24 hours and one week post occlusion, and the brains, sectioned into right and left hemispheres, were flash frozen and stored at -80˚C. The tissue was homogenized in 10 times (w/v) RIPA lysis buffer. Protein concentration was measured according to the Lowry method, using a spectrophotometer (Lowry, Rosebrough et al. 1951). 20 µg of protein, denatured in Laemmli sample buffer, was resolved in 10% SDS-PAGE and transferred onto nitrocellulose membrane. After blocking, the membranes were incubated with rabbit polyclonal anti-ETA (1:1000) or anti-ETB (1:500) antibodies, followed by HRP-conjugated secondary antibodies (1:1000). The labeled proteins were then visualized using an ECL Plus western

blotting detection system (GE Healthcare, Buckinghamshire, UK). Stripped membranes were reprobed with β-actin primary antibody (1:1000) for a protein loading control (Lavhale, Briyal et al. 2010).

9. <u>Immunofluorescence</u>

Immunofluorescent labeling was used to determine the location of ET_B receptors as well as any potential angiogenesis and/or neurogenesis following MCAO in rats. At either 24 h or 1 w following middle cerebral artery occlusion, animals underwent transcardial perfusion to fix the brains. Briefly, the animals were anesthetized with urethane, and the chest opened to expose the heart. A feeding needle, connected to the infusion pump by a tube, was inserted into the left ventricle up to the aorta. Two ventricles were blocked with hemostatic forceps, and the right atrium was cut open to allow the perfusate to drain. A perfusion of normal saline (200-300 ml) was infused to displace the blood in the vessels, until the draining perfusate was clear. An infusion of 4% paraformaldehyde (~300 ml) then displaced the saline to fix the tissue. The animals were then decapitated and the brains removed. The brains were post-fixed in 50 ml of 4% PFA in $NaPO_4$ buffer solution for 2 h, then placed in 20% sucrose/4% PFA, pH 7.4, 50 ml/brain at 4ºC for 48 h. The brains were then sliced into 30 µm thick slices for immunofluorescent analyses at - 30˚C using a cryostat (Microtome cryostat HM 505 E; Walldorf, Germany). Sections were processed for immunofluorescent staining as described by Loo, et al (Loo, Ng et al. 2002). The primary antibody for the ET receptors was an anti-ET_B receptor antibody raised in sheep against the carboxy-terminal peptide of the rat ET_B receptor (1:200; Calbiochem, EMD Biosciences, Inc., La Jolla, CA). The phenotypic markers for localization included anti-NeuN (1:100; Millipore Corporation, Temecula, CA) for neurons and anti-GFAP (1:500; Millipore Corporation, Temecula, CA) for astrocytes. Determination of

angiogenic and neurogenic markers was performed using primary antibodies against vascular endothelial growth factor (anti-VEGF;1:500; Abcam, Cambridge, MA), BrdU – a proliferating cell marker (anti-BrdU; 1:100; Abcam, Cambridge, MA), and nerve growth factor (anti-NGF; 1:500; Abcam, Cambridge, MA). The sections were washed in phosphate-buffered saline (PBS) four times, and then blocked with 10% v/v serum in PBS containing 0.3% Triton X-100 for 1 h. The sections were incubated with the primary antibody at 4˚C overnight. The sections were then washed again with PBS and incubated with the appropriate secondary fluorescent-labeled antibody for 2 h at room temperature. Double labeling for co-localization studies on the same tissue section were performed sequentially. The sections were rinsed with PBS and mounted on glass slides with Vectashield (Vector Laboratories, Inc., Burlingame, CA). Fluorescence was detected using an inverted fluorescent microscope (Nikon Eclipse, Melville, NY).

10. <u>Immunofluorescent Analysis</u>

Analyses were performed specifically on the cortex, striatum, corpus collosum and subventricular zones of the right (infarcted) and left (non-infarcted) hemispheres. The number of cells staining positively for GFAP, NeuN, NGF, or BrdU was determined in six randomly selected, nonconsecutive 100 μm^2 sections per brain slice in each area. For the evaluation of angiogenesis, the total number of VEGF+ blood vessels in the infarcted hemisphere was determined per brain slice. In order to determine co-localization with the ET_B receptor, binary layers were created to label the positively stained areas for each antibody and the total area fraction of overlap was calculated using NIS-Elements 3.01 imaging software from Nikon Instruments, Inc. (Melville, NY).

11. <u>Statistical Analysis</u>

A power analysis was conducted using GraphPad Instat-2.00. The power was set to 80% (beta = 0.8) and the level of significance (alpha) used was 0.05. Power analysis indicated that a sample size of 5 per group was sufficient to achieve a power of 80%, when level of significance alpha=0.05. The data is represented as mean ± S.E.M. Behavioral data was analyzed via a two-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test with treatment as the factor and time as the repeated measure. One-way analysis of variance (ANOVA) followed by Bonferroni's post hoc comparison test was used for intergroup comparison in evaluating infarct volume, edema, oxidative stress parameters, and immunofluorescent data. Two-tailed unpaired t-test was used for endothelin receptor comparison. A *P* value of less than 0.05 was considered to be significant. The statistical analysis was processed with GraphPad Prism 5.00 (GraphPad, San Diego, CA, USA).

IV. RESULTS

1. Effect on Motor Function and Coordination Following Cerebral Ischemia

Neurological and motor function deficit are often the first sign that a stroke has occurred. The signs that the public is told to look for to indicate the onset of a stroke fall under the acronym FAST for Face, Arms, Speech and Time. A droop on one side of the face, a weakness in one or both arms and/or legs and slurred speech are all signs pointing to a stroke. The Time component refers to the "Time is Brain" scenario and the fact that the sooner these signs are recognized, the better the chances are that the patient will be able to receive proper treatment leading to recovery. The fact that one side of the body is often more affected than the other reflects the physiology of the brain and the location of the stroke. For instance, a stroke localized on the right side of the brain will mainly affect the left side of the body.

For this study, we focused mainly on symptoms affecting the overall movement and muscular strength capabilities of the animals following the induction of right-hemisphere localized middle cerebral artery occlusion. The neurological scores focused on the observable motor changes occurring on the left side of the animal's body, such as a weakness or paralysis of the left limbs (Tatlisumak, Carano et al. 1998, Gupta, Briyal et al. 2005). The grip test evaluated the strength of the animals, particularly that of the forepaws, while the foot fault error and rota rod duration tests evaluated their overall muscular coordination (Markgraf, Green et al. 1992, Moran, Higgins et al. 1995, Rogers, Campbell et al. 1997). Spontaneous movement following any surgery tends to decrease, but a decrease in vertical rearing for the rats is associated with a poorer outcome following cerebral ischemia (Shen and Wang 2010). It was important for us to observe not only the baseline and endpoint motor capabilities of the animals during acute and sub-acute ischemia, but also to observe when any potential improvement or worsening of the

condition might occur. For this reason, we observed the animals at multiple time points throughout the experiments.

Prior to occlusion, there were no significant differences between the groups with regards to motor function and coordination. On the other hand, permanent middle cerebral artery occlusion of the right hemisphere in rats resulted in significant neurological deficit and decreased muscular strength as measured by the grip test, as well as impaired coordination as measured by foot fault and rota rod tests at 1, 4, and 7 days post infarction (Tables I and II).

1.1. Effect on Neurological Deficit Following Acute Cerebral Ischemia

No neurological deficits were observed in any animals prior to occlusion. Twenty-four hours after middle cerebral artery occlusion of the right side, however, paresis of the left hind paw was observed. Compared to sham-operated rats, the mean neurological score of vehicle-treated middle cerebral artery occluded rats was significantly higher (3.13 ± 0.24; $P<0.001$), indicative of neurological impairment following induction of cerebral ischemia. In contrast, middle cerebral artery occluded rats treated with IRL-1620 demonstrated significant improvement in neurological function when compared with the vehicle group (0.85 ± 0.32; $P<0.001$) (Leonard, Briyal et al. 2011). Blockade of ET_B receptors with BQ788 prior to treatment with either vehicle or IRL-1620 resulted in significant neurological impairment when compared with both sham-operated ($P<0.001$) and IRL-1620 ($P<0.001$) alone. Interestingly, while animals treated with ET_A antagonist BQ123 followed by vehicle presented with less neurological deficit than those receiving vehicle alone, there did not appear to be a synergistic effect with the combination of BQ123 and IRL-1620 treatment. Conversely, animals receiving the combination faired significantly worse than those receiving IRL-1620 alone (Table I).

Table I

EFFECT ON NEUROLOGICAL AND MOTOR DEFICIT FOLLOWING ACUTE CEREBRAL ISCHEMIA[a]

Treatment Groups		Neurological Evaluation (6 point scale)	Grip Test (6 point scale)	Foot Fault Error (%)	Rota Rod Duration (sec)	Distance Traveled (cm)	Vertical Breaks
Sham	Baseline	0 ± 0	3.75 ± 0.24	7.25 ± 0.53	123.63 ± 15.28	4833 ± 227	55.67 ± 10.75
	24 hr	0 ± 0	3.25 ± 0.37	7.14 ± 1.05	161.14 ± 25.24	3025 ± 229	28.17 ± 5.17
MCAO + Vehicle	Baseline	0 ± 0	3.73 ± 0.23	5.93 ± 0.66	126.00 ± 7.05	4997 ± 354	48.43 ± 8.67
	24 hr	3.13 ± 0.24*	0.93 ± 0.28*	61.13 ± 8.88*	51.20 ± 10.07*	703 ± 188*	1.00 ± 0.46
MCAO + IRL-1620	Baseline	0 ± 0	4.07 ± 0.21	4.31 ± 0.58	124.23 ± 6.05	5154 ± 391	46.86 ± 12.99
	24 hr	0.85 ± 0.32#	2.85 ± 0.27#	15.62 ± 4.03#	122.00 ± 16.45#	1754 ± 340	18.40 ± 5.41
MCAO + BQ788 + Vehicle	Baseline	0 ± 0	4.17 ± 0.31	5.00 ± 0.86	143.67 ± 16.00	5352 ± 326	51.00 ± 4.25
	24 hr	2.67 ± 0.42*@	0.67 ± 0.21*@	60.17 ± 13.74*@	33.67 ± 9.46*@	1592 ± 399	8.00 ± 2.64
MCAO + BQ788 + IRL-1620	Baseline	0 ± 0	4.33 ± 0.33	6.83 ± 0.60	133.00 ± 8.55	5774 ± 18	49.00 ± 13.52
	24 hr	3.33 ± 0.21*@	0.33 ± 0.21*@	73.33 ± 11.44*@	32.50 ± 10.51*@	775 ± 45*	9.50 ± 7.17
MCAO + BQ123 + Vehicle	Baseline	0 ± 0	3.80 ± 0.37	4.60 ± 1.08	116.20 ± 10.13	NA	NA
	24 hr	2.00 ± 0.63*	1.20 ± 0.58*	46.20 ± 14.51	95.40 ± 7.16	NA	NA
MCAO + BQ123 + IRL-1620	Baseline	0 ± 0	4.00 ± 0.32	5.80 ± 1.16	125.40 ± 12.50	NA	NA
	24 hr	1.80 ± 0.58*@	1.00 ± 0.63*	53.60 ± 16.68*	79.80 ± 21..98*	NA	NA

[a] IRL-1620 (5 µg/kg, i.v.) or isotonic saline (1 mg/kg, i.v.) was injected at 2, 4, and 6 h post middle cerebral artery occlusion. BQ788 or BQ123 (1 mg/kg, i.v.) was administered 15 min prior to the first injection of IRL-1620 or vehicle. Values are expressed as mean ± SEM. *P<0.05 vs. sham. #P<0.01 vs. MCAO + vehicle. @P<0.01 vs. MCAO + IRL-1620.

1.2. Effect on Neurological Deficit Following Sub-acute Cerebral Ischemia

Animals prior to middle cerebral artery occlusion had no neurological deficit. For the week following middle cerebral artery occlusion, however, vehicle-treated occluded animals showed significantly more deficit than sham-operated animals, as evidenced mainly by paresis of the left side ($P<0.001$). Whereas vehicle-treated occluded rats performed worse with each assessment, animals treated with ET_B receptor agonist, IRL-1620, showed minimal deficit at day one following occlusion, and actually improved over the course of 7 days (Leonard, Briyal et al. 2012). Pretreatment with ET_B receptor antagonist, BQ788, followed by either vehicle or IRL-1620 resulted in significantly more deficits than both sham-operated ($P<0.001$) or IRL-1620 ($P<0.05$) treated, indicating that the improvement observed with IRL-1620 is specific to the stimulation of ET_B receptors (Table II).

1.3. Effect on Muscular Strength Following Acute Cerebral Ischemia

All animals prior to occlusion demonstrated similar muscular strength in their ability to grip/climb onto the string. Vehicle-treated rats demonstrated a significant decrease in muscle strength as measured by the grip test following middle cerebral artery occlusion as compared to sham-operated rats ($P<0.001$). In contrast, occluded rats treated with IRL-1620 showed significant improvement ($P<0.001$) when compared with vehicle-treated rats, with mean scores of 2.85 ± 0.27 vs. 0.93 ± 0.28, respectively (Leonard, Briyal et al. 2011). Pretreatment with BQ788 followed by treatment with either vehicle or IRL-1620 resulted in significant impairment of muscle strength as compared with sham ($P<0.001$) and IRL-1620 ($P<0.001$) alone groups. Interestingly, blockade of ET_B receptors by BQ788 caused an even greater deficit in motor function following cerebral ischemia than that seen in animals treated with vehicle alone.

Animals pretreated with BQ123, demonstrated a slightly improved ability to hold onto the string as compared to vehicle-treated animals, yet still presented with significantly less muscular strength than those in the sham-operated group ($P<0.05$). Again, no synergism was noted when BQ123 was combined with IRL-1620 treatment following MCAO (Table I).

1.4. Effect on Muscular Strength Following Sub-acute Cerebral Ischemia

Muscular strength was evaluated both prior to and at 1, 4, and 7 days post middle cerebral artery occlusion using the grip test. Prior to occlusion, all animals were approximately equal in their ability to cling onto/climb the string. Muscular strength significantly diminished following occlusion, with animals in the vehicle-treated group barely able to grasp the string with their forepaws and often falling off. Animals in the sham-operated group, on the other hand, maintained their strength. Animals treated with IRL-1620 following occlusion remained significantly ($P<0.01$) stronger than the vehicle-treated rats (Leonard, Briyal et al. 2012). Animals pretreated with BQ788 fared far worse in muscle strength following occlusion than either sham-operated ($P<0.001$) or IRL-1620 ($P<0.05$) treatment (Table II).

1.5. Effect on Motor Coordination using Foot Fault Error Following Acute Cerebral Ischemia

Prior to occlusion, animals in all groups averaged less than 10% foot fault error. Following occlusion, however, the percentage of foot fault errors was significantly higher in vehicle-treated occluded rats as compared to sham-operated rats ($P<0.001$), indicating lack of motor coordination. Treatment with IRL-1620 improved coordination, resulting in a significantly lower percentage of error as compared with vehicle-treated rats ($15.62 \pm 4.03\%$ vs. $61.13 \pm 8.88\%$; $P<0.001$) (Leonard, Briyal et al. 2011). In contrast, animals treated with BQ788

followed by either vehicle or IRL-1620 showed levels of impairment similar to those of the vehicle group. Once again, while BQ123 alone demonstrated some improvement in coordination, the combination of BQ123 with IRL1620 resulted in a worse outcome (Table I).

1.6. Effect on Motor Coordination using Foot Fault Error Following Sub-acute Cerebral Ischemia

Foot fault errors for all animals at baseline (prior to middle cerebral artery occlusion) were less than 10% (Table II). As can be seen in the vehicle-treated group, occlusion resulted in significant motor impairment with errors increasing to 82% at 7 days after the infarct. IRL-1620 treatment resulted in animals making far fewer errors (less than 20%) on the foot fault grid as compared to the vehicle group ($P<0.01$) (Leonard, Briyal et al. 2012). BQ788 blocked the positive effect of IRL-1620, resulting in a 60-80% error ($P<0.05$).

1.7. Effect on Motor Coordination using Rota Rod Following Acute Cerebral Ischemia

There were no significant differences between groups prior to occlusion with animals remaining on the rotating spindle for approximately 2 min. Twenty-four hours after occlusion, however, vehicle-treated middle cerebral artery occluded rats displayed a significant lack of coordination when compared with sham-operated animals ($P<0.001$). Whereas sham rats were able to remain on the accelerating spindle for a mean of 160 sec, vehicle-treated MCAO rats fell off after only a mean of 50 sec. IRL-1620-treated middle cerebral artery occluded rats demonstrated significant improvement in motor coordination as compared with vehicle-treated rats ($P<0.01$), remaining on the spindle for an average of 120 sec (Leonard, Briyal et al. 2011). Rats in both BQ788-treated groups, however, demonstrated significant motor coordination impairment when compared to sham ($P<0.001$) and IRL-1620 ($P<0.01$) alone groups. As with

the grip test, blockade of ET_B receptors led to an even greater deficit in motor function following cerebral ischemia compared to that seen in the vehicle-treated group. Treatment with ET_A antagonist BQ123alone led to an improvement in motor coordination, albeit less than that of the IRL-1620 treated group, with animals remaining on the spindle for approximately 95 sec. The combination of BQ123 and IRL-1620 treatment, however, again led to a poorer result, with animals falling off at 80 sec (Table I). Overall, while treatment with BQ123 does appear to have some efficacy on its own, it pales in comparison to that seen with IRL-1620. The combination the two, as opposed to creating a synergistic beneficial effect, instead appears to worsen the outcome. A combined therapy of ET_A antagonism with ET_B agonism is therefore not recommended in the treatment of ischemic stroke.

1.8. Effect on Motor Coordination using Rota Rod Following Sub-acute Cerebral Ischemia

There were no significant differences between groups prior to middle cerebral artery occlusion regarding their ability to remain on the rotating, accelerating spindle of the rota rod. All animals displayed sufficient motor coordination to remain on the spindle for approximately 2 min. Following middle cerebral artery occlusion, however, vehicle-treated rats displayed significantly ($P<0.05$) greater motor coordination deficit that sham-operated animals, remaining on the spindle for only a short duration. This condition became especially marked as the week progressed. IRL-1620 treated animals, on the other hand, retained coordination, remaining on the spindle for almost as long as the sham-operated group (Table II) (Leonard, Briyal et al. 2012). Animals receiving BQ788 demonstrated a lack of coordination following occlusion, retaining coordination for less than half of the time as compared with the sham and IRL-1620 treated groups.

1.9. Effect on Spontaneous Locomotor Activity Following Cerebral Ischemia

Whilst all animals undergoing middle cerebral artery occlusion showed a decrease in spontaneous locomotor activity as measured by distance traveled and vertical rearing, animals in the IRL-1620 group were the most active after 24 h and demonstrated the most improvement as the week progressed (Tables I and II). The effects of IRL-1620 on neurological and motor function were blocked in animals pretreated with ET_B antagonist BQ788. It is interesting to note that the surgery itself, as seen with the sham-operated group, resulted in an initial lack of activity on the first day. This may be attributed to general soreness at the site of incision which abates after a few days as the wound heals.

Table II

EFFECT ON NEUROLOGICAL AND MOTOR DEFICIT FOLLOWING SUB-ACUTE CEREBRAL ISCHEMIA[a]

Treatment Groups		Neurological Evaluation (6 point scale)	Grip Test (6 point scale)	Foot Fault Error (%)	Rota Rod Duration (sec)	Distance Traveled (cm)	Vertical Breaks
Sham	Baseline	0 ± 0	4.25±0.06	5.00±0.91	115.00±7.79	4968 ± 242	65.62 ± 3.18
	Day 1	0 ± 0	4.25 ± 0.24	3.75 ± 1.24	129.50 ± 6.34	3325 ± 324	37.63 ± 3.33
	Day 4	0 ± 0	4.50 ± 0.24	2.50 ± 0.66	165.25 ± 10.63	5323 ± 474	58.67 ± 1.53
	Day 7	0 ± 0	4.50 ± 0.47	1.00 ± 0.95	155.50 ± 6.00	4306 ± 314	53.67 ± 12.31
MCAO + Vehicle	Baseline	0 ± 0	4.00±0.22	6.67±0.94	115.00±10.11	5069 ± 329	54.56 ± 7.65
	Day 1	3.00 ± 0.45*	0.83 ± 0.35*	68.00 ± 10.11*	12.40 ± 8.28*	764 ± 216*	1.33 ± 0.53*
	Day 4	3.50 ± 0.43*	0.67 ± 0.29*	71.17 ± 11.35*	34.40 ± 17.67*	2353 ± 787*	15.00 ± 7.53*
	Day 7	3.83 ± 0.57*	0.33 ± 0.29*	81.67 ± 10.23*	0.80 ± 0.46*	2366 ± 660	20.25 ± 7.80*
MCAO + IRL-1620	Baseline	0 ± 0	4.57±0.19	3.71±0.81	132.13±9.48	5073 ± 334	61.50 ± 4.94
	Day 1	1.14 ± 0.38	2.86 ± 0.43#	17.00 ± 4.77#	99.50 ± 29.15	1611 ± 325*	12.75 ± 4.76*
	Day 4	1.00 ± 0.41#	3.00 ± 0.35#	12.43 ± 1.96#	137.67 ± 26.72#	2898 ± 451	26.33 ± 2.08
	Day 7	0.86 ± 0.43#	3.00 ± 0.35#	12.14 ± 2.92#	137.67 ± 28.52#	3472 ± 732	34.33 ± 3.78
MCAO + BQ788	Baseline	0 ± 0	4.67±0.29	3.67±0.29	129.00±7.44	5141 ± 285	55.17 ± 3.74
	Day 1	3.50 ± 0.54*@	0.50 ± 0.30*@	67.17 ± 9.36*@	26.80 ± 11.05	1168 ± 417*	5.33 ± 2.47*
	Day 4	3.17 ± 0.57*@	1.17 ± 0.35*@	52.50 ± 14.21*	31.00 ± 22.93*@	1246 ± 410*	4.33 ± 1.52*
	Day 7	3.33 ± 0.48*@	0.17 ± 0.14*@	59.50 ± 11.64*@	60.20 ± 23.32	2280 ± 836	11.00 ± 3.97*
MCAO + BQ788 + IRL-1620	Baseline	0 ± 0	4.50±0.30	4.50±1.13	114.00±5.85	5642 ± 358	48.00 ± 9.68
	Day 1	3.00 ± 0.39*	0.83 ± 0.41*@	64.00 ± 14.42*@	32.00 ± 14.44	742 ± 85*	2.00 ± 1.48*
	Day 4	3.67 ± 0.36*@	1.33 ± 0.43*@	65.00 ± 10.80*@	55.80 ± 19.75*	1223 ± 414	6.67 ± 3.42*
	Day 7	3.83 ± 0.35*@	0.83 ± 0.35*@	78.00 ± 12.21*@	47.60 ± 19.00*	2185 ± 818	17.33 ± 8.56*

[a] IRL-1620 (5 µg/kg, i.v.) or isotonic saline (1 ml/kg, i.v.) was injected at 2, 4, and 6 h post MCAO. BQ788 (1 mg/kg, i.v.) was administered 15 min prior to the first injection of IRL-1620 or vehicle. Values are expressed as mean ± SEM. *P<0.05 vs. sham. #P<0.05 vs. MCAO + vehicle. @P<0.05 vs. MCAO + IRL-1620.

2. Effect on Survival Following Cerebral Ischemia

During the acute study, no animals in either the sham-operated or IRL-1620-treated groups expired. Middle cerebral artery occlusion followed by vehicle treatment resulted in a 33% mortality rate, while the BQ788 + vehicle and BQ788 + IRL-1620 treated groups each demonstrated a 17% mortality rate in the 24 h period of acute ischemia. By the 7th day of the sub-acute study, the mortality rates of the vehicle-, BQ788-, and BQ788+IRL-1620-treated groups was 38%, 25% and 25%, respectively (Leonard, Briyal et al. 2012). There was no mortality in either the sham-operated or IRL-1620-treated groups throughout the 7 day period following surgery or induction of cerebral ischemia (Figure 5).

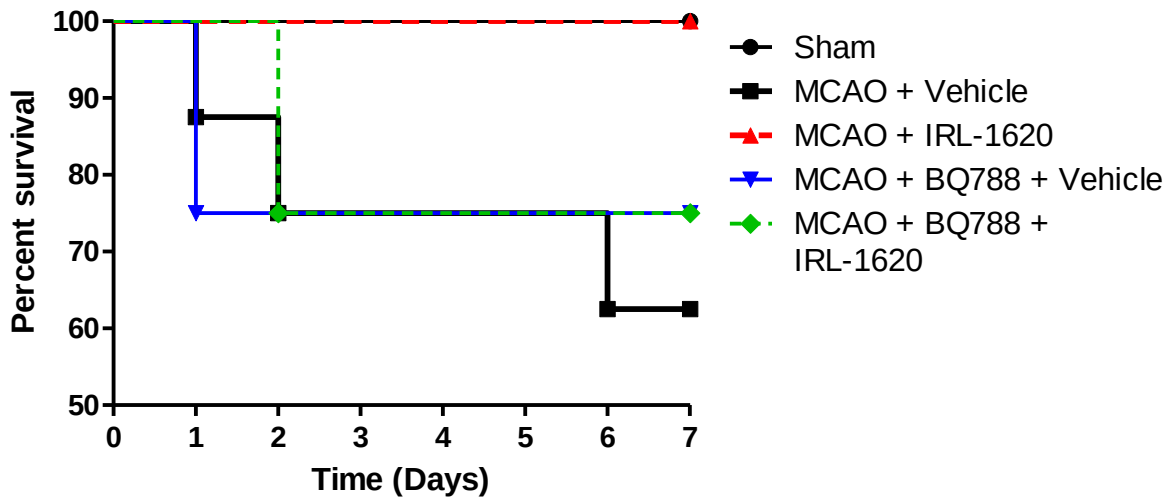


Figure 5: Effect on survival following sub-acute cerebral ischemia.

3. <u>Effect on Infarct Volume Following Cerebral Ischemia</u>

Overall infarct volume is an indicator of the extent of ischemic damage with the brain. A small infarct volume may indicate that the ischemia remained at or near the core of the attack, and that the normal secondary damage to the penumbra as a result of the ischemic cascade did not occur. This scenario could indicate a transient ischemic attack, for which there is simultaneously a better chance for recovery and an increased risk of future infarcts, or, in the case of treatment being given, may indicate that the selected treatment is providing neuroprotection. Conversely, a significantly large infarct volume is associated with a poor outcome, and, in the case of treatment, may mean that the treatment is exacerbating one or more pathways involved in the ischemic cascade (Durukan and Tatlisumak 2007, Kanekar, Zacharia et al. 2012).

The delineation of infarct volume by 2,3,5-triphenyltetrazolium (TTC) is well established (Bederson, Pitts et al. 1986, Li, Irie et al. 1997). TTC is reduced to red formazan by succinate dehydrogenase, a mitochondrial enzyme. Normal tissue therefore stains red whereas infarcted tissue which lacks the enzyme does not stain, thus leaving a clearly demarcated border between the ischemic and normal tissue. Additionally, the staining and sectioning of the brain in this fashion creates an easy method for determining the extent of edematic formation, by calculating the differing sizes of the infarcted and non-infarcted hemispheres (Barone, White et al. 1995). The formation of edema due to the breakdown of the BBB during the sub-acute phase of ischemic stroke is associated with a poor outcome. The increased intracranial pressure as fluids fill the brain cavity presses the neuronal tissue against the skull and can lead to intracerebral hemorrhage, post-stroke epilepsy and seizure activity as the neurons fire in response to the mechanical stimulation (Myint, Staufenberg et al. 2006).

For this study, animals subject to permanent middle cerebral artery occlusion were sacrificed at either 24 h (acute) or 1 w (sub-acute) following induction of cerebral ischemia. Their brains were then removed, sliced into 2 mm thick sections and stained with 2% TTC to determine the volume of the infarcted tissue.

3.1. Effect on Infarct Volume Following Acute Cerebral Ischemia

Middle cerebral artery occlusion resulted in an infarct volume of 153.23 ± 32.18 mm^3 at 24 h in rats treated with vehicle alone (Figure 6). Administration of IRL-1620 significantly reduced infarct volume (24.47 ± 4.37 mm^3; $P<0.01$) as compared with vehicle (Leonard, Briyal et al. 2011). In contrast, administration of BQ788 prior to either vehicle or IRL-1620 resulted in significantly large infarct volumes (163.51 ± 25.41 and 139.21 ± 15.20 mm^3; $P<0.01$) when compared with IRL-1620 alone. The BQ788-treated groups did not vary significantly from the vehicle-treated group. BQ123 treatment alone did result in an infarct volume reduction (77.08 ± 22.45 mm^3) as compared to the vehicle-treated group, but it was not significant. The combination of BQ123 and IRL-1620, on the other hand, resulted in a significantly large infarct volume of 156.98 ± 47.16 mm^3, comparable with the vehicle-treated group. These results, along with the neurological and motor function data, suggest that combination therapy involving BQ123 and IRL-1620 is not advisable.

3.2. Effect on Infarct Volume Following Sub-acute Cerebral Ischemia

Middle cerebral artery occlusion for 7 days resulted in an infarct volume of 177.06 ± 13.21 mm^3 in vehicle-treated rats (Figure 7). Administration of IRL-1620 significantly reduced infarct volume (54.06 ± 14.12 mm^3; $P<0.05$) as compared with vehicle (Leonard, Briyal et al. 2012). Infarct volumes did not reduce when ET_B receptor antagonist, BQ788, was given with

either vehicle or IRL-1620. A substantial edema was noted in the vehicle-treated animals, with the infarcted hemisphere 9.73 ± 1.26% larger than the contralateral hemisphere, whereas IRL-1620-treated animals showed no significant edema, with the infarcted hemisphere only 1.51 ± 1.81% larger than non-infarcted hemisphere. Conversely, blockade of ET_B receptors with BQ788 followed by either vehicle or IRL-1620 treatment significantly increased edema (17.02 ± 3.17 and 17.97 ± 5.17%, respectively, $P<0.01$).

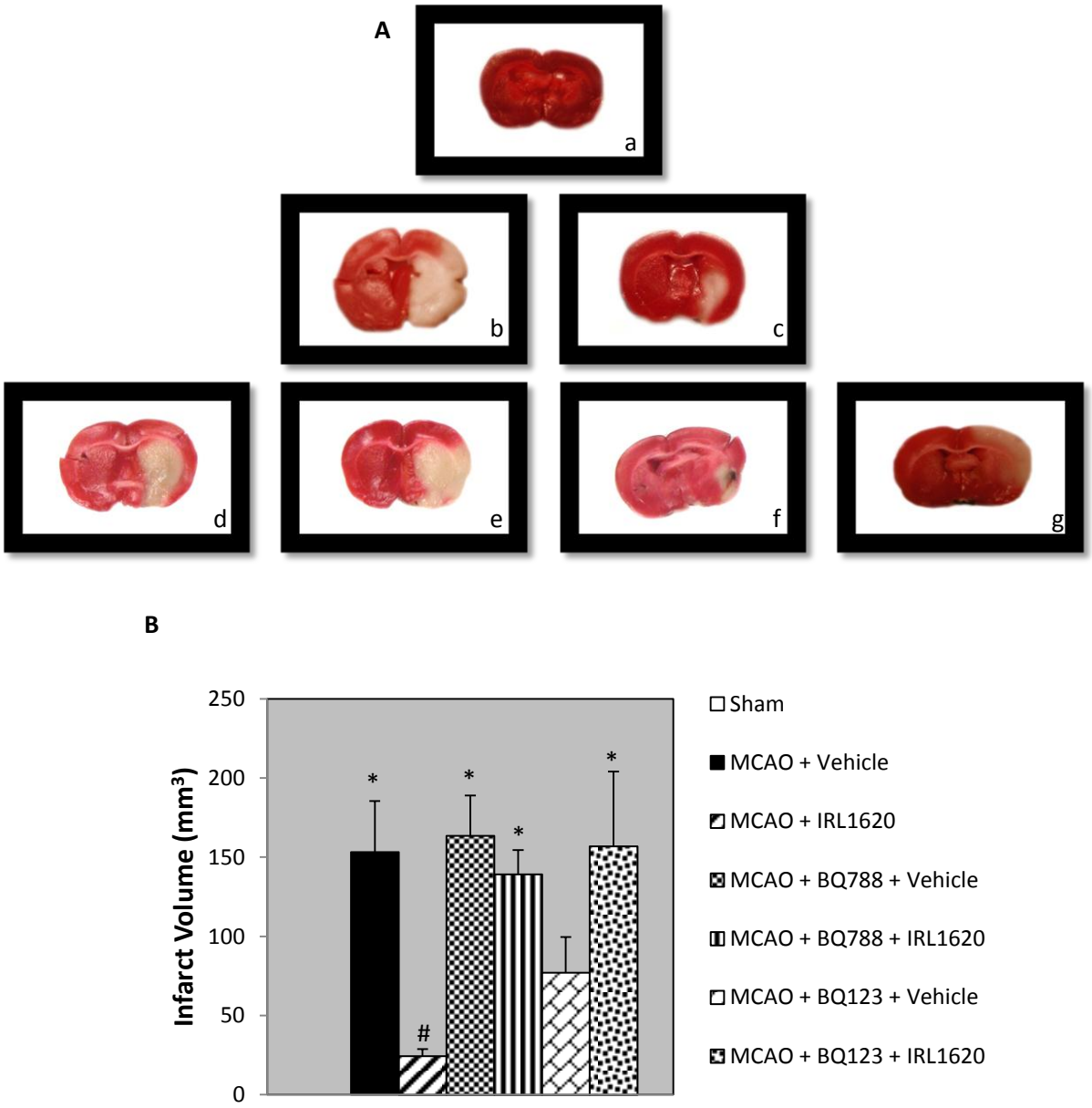


Figure 6: Effect on infarct volume following acute cerebral ischemia. **A.** 2mm coronal sections of brains stained with TTC to visualize the infarct area (red indicates normal tissue and white indicates infarct tissue). Representative slices from groups are as follows: a. sham, b. MCAO + Vehicle, c. MCAO + IRL-1620, d. MCAO + BQ788 + vehicle, e. MCAO + BQ788 + IRL-1620, f. MCAO + BQ123 + vehicle, g. MCAO + BQ123 + IRL-1620. IRL-1620 (5 µg/kg, i.v.) or isotonic saline (1 ml/kg, i.v.) was injected at 2, 4, and 6 h post MCAO. BQ788 or BQ123 (1 mg/kg, i.v.) was administered once 15 min prior to the first injection of IRL-1620 or vehicle. **B.** Effect of IRL-1620, BQ788, and BQ123 on infarct volume in middle cerebral artery occluded rats. Values are expressed as mean ± SEM. *P<0.001 vs. sham. #P<0.01 vs. MCAO + Vehicle. @P<0.01 vs. MCAO + IRL-1620.

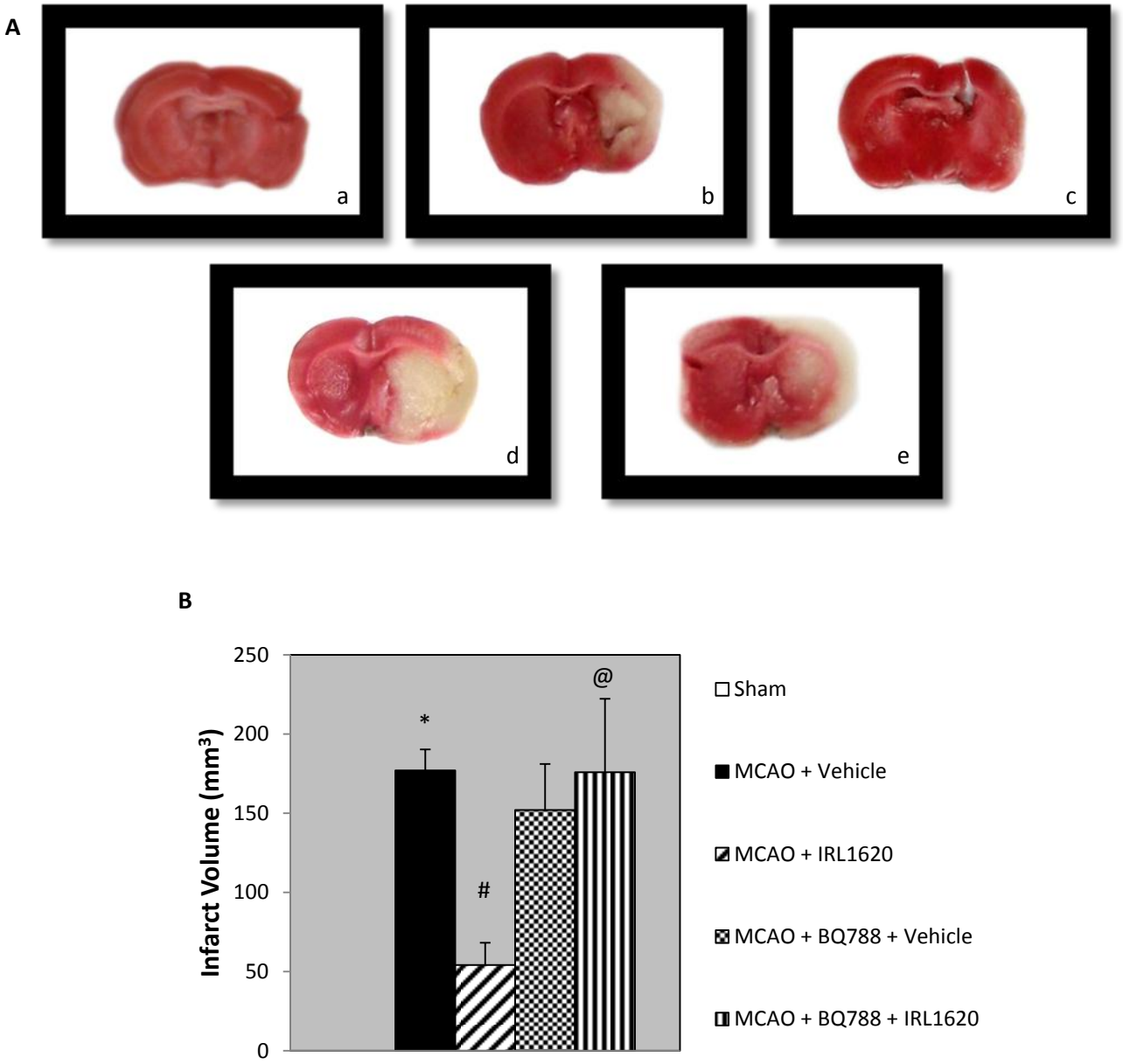


Figure 7: Effect on infarct volume following sub-acute cerebral ischemia. **A.** 2 mm coronal sections of brains stained with TTC to visualize the infarct area 7 days post middle cerebral artery occlusion (red indicates normal tissue and white indicates infarct tissue). Representative slices from groups are as follows: a. sham, b. MCAO + Vehicle, c. MCAO + IRL-1620, d. MCAO + BQ788 + Vehicle, e. MCAO + BQ788 + IRL-1620. IRL-1620 (5 µg/kg, i.v.) or isotonic saline (1 ml/kg, i.v.) was injected at 2, 4, and 6 h post MCAO. BQ788 (1 mg/kg, i.v.) was administered once 15 min prior to the first injection of IRL-1620 or vehicle. **B.** Effect of IRL-1620 and BQ788 on infarct volume in middle cerebral artery occluded rats. Values are expressed as mean ± SEM. *P<0.001 vs. sham. #P<0.05 vs. MCAO + Vehicle. @P<0.05 vs. MCAO + IRL-1620.

4. <u>Effect on Oxidative Stress Parameters Following Cerebral Ischemia</u>

Oxidative stress occurs as a part of the ischemic cascade and contributes to the secondary damage to the penumbral tissue surrounding the ischemic core. Within hours of the infarct, a disruption occurs in the balance of oxidants and antioxidants within the ischemic brain, resulting in oxidative stress (Hossmann 1996). Under oxidative stress conditions, superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (-OH) are produced as antioxidants such as superoxide dismutase (SOD) and reduced glutathione (GSH) are depleted. This shift leads to damage to the membranes of both the cells and the mitochondria by lipid peroxidation, as well as inflammation and apoptosis (Lakhan, Kirchgessner et al. 2009). All of these events contribute to eventual breakdown of the BBB, which in itself may lead to complications such as cerebral edema and hemorrhagic transformation. Endothelin A selective antagonist BQ123 and $ET_{A/B}$ antagonist TAK-044 have both been shown to reduce certain oxidative stress parameters following experimental ischemia (Gupta, Briyal et al. 2005, Briyal and Gulati 2010). Given that ET_B receptors enhance the release of nitric oxide which could potentially lead to increased reactive nitrogen species and thereby exacerbate oxidative stress conditions, it was of interest to determine how selective ET_B stimulation would affect oxidative stress parameters following MCAO in rats. Additionally, while most studies have shown that oxidative stress is a particular hallmark of the acute phase of ischemic stroke, they do not address the length of duration of this particular pathology. Therefore, it was important to determine the effects of week-long permanent occlusion, treated and untreated, on oxidative stress levels.

To determine the effect of ET_B receptors during middle cerebral artery occlusion on oxidative stress parameters, malondialdehyde, reduced glutathione and superoxide dismutase

levels in the brains of sham-operated and occluded rats treated with vehicle, IRL-1620 and/or BQ788 were determined 24 h and 1 w after cerebral infarction (Tables III and IV).

4.1. Effect on Malondialdehyde Levels Following Acute Cerebral Ischemia

Brain levels of malondialdehyde (MDA) were measured to determine the effect of ET_B receptor stimulation on lipid peroxidation following acute cerebral ischemia (Table III). As expected, the levels of MDA in vehicle-treated middle cerebral artery occluded rats were significantly high (574.30 ± 34.83 nmol/g wet tissue) when compared with sham-operated animals (113.16 ± 1.87 nmol/g wet tissue; $P<0.001$). Malondialdehyde levels were significantly reduced in the IRL-1620-treated animals when compared to the vehicle-treated group (179.12 ± 26.59; $P<0.001$), indicating a possible antioxidant effect of ET_B receptor stimulation following cerebral ischemia (Leonard, Briyal et al. 2011). Administration of BQ788 prior to either vehicle or IRL-1620 resulted in MDA levels comparable to those seen with vehicle alone (568.94 ± 18.94 and 601.89 ± 17.28 nmol/g wet tissue, respectively).

4.2. Effect on Malondialdehyde Levels Following Sub-acute Cerebral Ischemia

Week-long occlusion of the middle cerebral artery in vehicle-treated rats produced a significant increase in lipid peroxidation, with malondialdehyde levels of 698.91 ± 24.06 nmol/g wet tissue as compared to 128.40 ± 23.37 nmol/g wet tissue in the sham-operated group ($P<0.001$). Occluded animals treated with IRL-1620, on the other hand, had a significant ($P<0.001$) reduction in MDA as compared with the vehicle group (Leonard, Briyal et al. 2012). Blockade of the ET_B receptors with BQ788 followed by either vehicle or IRL-1620 resulted in high levels of MDA (Table IV).

4.3. Effect on Reduced Glutathione Levels Following Acute Cerebral Ischemia

Twenty-four hours after induction of ischemia, reduced glutathione (GSH) levels in vehicle-treated middle cerebral artery occluded rats were significantly lower (97.37 ± 7.16 µg/g wet tissue) than those of sham-operated animals (250.20 ± 15.01 µg/g wet tissue; $P<0.05$). Treatment with IRL-1620, on the other hand, significantly increased the levels of GSH in the brains of occluded rats (187.72 ± 13.31 µg/g wet tissue) as compared with vehicle alone (Table III) (Leonard, Briyal et al. 2011). Pretreatment with BQ788 blocked the positive effects of IRL-1620 treatment on GSH levels (85.71 ± 6.96 µg/g wet tissue; $P<0.001$).

4.4. Effect on Reduced Glutathione Levels Following Sub-acute Cerebral Ischemia

Antioxidant reduced glutathione levels remain decreased at 1 w following middle cerebral artery occlusion, as can be seen in the vehicle (52.63 ± 17.67 µg/g wet tissue) versus the sham-operated (293.23 ± 38.67 µg/g wet tissue) groups ($P<0.001$; Table IV). Stimulation of ET_B receptors with IRL-1620 resulted in a far lesser ($P<0.05$) reduction in GSH than seen in the group treated with vehicle only (Leonard, Briyal et al. 2012). Blockade of these receptors by BQ788 resulted in GSH levels which were close to those of the vehicle group and significantly lower than those of the IRL-1620 ($P<0.05$) alone group.

4.5. Effect on Superoxide Dismutase Levels Following Acute Cerebral Ischemia

The levels of the antioxidant marker, superoxide dismutase (SOD), in the brains of vehicle-treated middle cerebral artery occluded rats were significantly lower (8.26 ± 0.82 units/mg protein) than those of the sham-operated group (29.87 ± 1.66 units/mg protein; $P<0.001$) at 24 h after induction of cerebral ischemia. Administration of IRL-1620 significantly improved SOD levels (13.17 ± 0.69 units/mg protein; $P<0.05$) as compared with the vehicle-

treated group, again indicating a possible antioxidant effect of ET_B receptor stimulation following cerebral infarction (Table III) (Leonard, Briyal et al. 2011). As with GSH, SOD levels were significantly lower when occluded animals were pretreated with BQ788 prior to administration of either vehicle or IRL-1620 (5.11 ± 0.23 and 4.66 ± 0.17 units/mg protein, respectively; $P<0.001$).

4.6. Effect on Superoxide Dismutase Levels Following Sub-acute Cerebral Ischemia

Levels of superoxide dismutase in the brains of vehicle-treated middle cerebral artery occluded animals were significantly lower (8.49 ± 2.40 units/mg protein) than those of the sham-operated group (20.32 ± 1.29 units/mg protein; $P<0.01$) at 1 w post infarction. IRL-1620 treatment significantly ($P<0.001$) improved levels of SOD in the occluded rat brain as compared to vehicle-treated rats (Leonard, Briyal et al. 2012). On the other hand, SOD levels in animals pretreated with BQ788 followed by either vehicle or IRL-1620 were significantly ($P<0.001$) lower than those of the IRL-1620 alone group (Table IV). Overall, it was interesting to note that the markers for oxidative stress remained disturbed even as late as 1 w post induction of cerebral ischemia, and particularly significant that treatment on only the first day with IRL-1620 was sufficient to keep the levels of antioxidants high and lipid peroxidation low. This data indicates that an early, one-time treatment stimulating ET_B receptors after ischemic stroke may have long-term beneficial effects with regards to oxidative stress and, potentially, inflammatory damage.

Table III

EFFECT ON OXIDATIVE STRESS PARAMETERS FOLLOWING ACUTE CEREBRAL ISCHEMIA[a]

Treatment Group	Malondialdehyde (nmol/g wet tissue)	Reduced glutathione (µg/g wet tissue)	Superoxide dismutase (units/mg protein)
Sham	113.16±1.87	250.20±15.01	29.87±1.66
MCAO + Vehicle	574.30±34.83*	97.37±7.16*	8.26±0.82*
MCAO + IRL1620	179.12±26.59#	187.72±13.31#	13.17±0.69#
MCAO + BQ788 + Vehicle	568.94±18.94*@	90.23±6.00*@	5.11±0.23*@
MCAO + BQ788 + IRL1620	601.89±17.28*@	85.71±6.96*@	4.66±0.17*@

[a] IRL-1620 (5 µg/kg, i.v.) or isotonic saline (1 ml/kg, i.v.) was injected at 2, 4, and 6 h post MCAO. BQ788 (1 mg/kg, i.v.) was administered 15 min prior to the first injection of IRL-1620 or vehicle. Values are expressed as mean ± SEM. *P<0.001 vs. sham. #P<0.05 vs. MCAO + vehicle. @P<0.001 vs. MCAO + IRL-1620.

Table IV

EFFECT ON OXIDATIVE STRESS PARAMETERS FOLLOWING SUB-ACUTE CEREBRAL ISCHEMIA[a]

Treatment Group	Malondialdehyde (nmol/g wet tissue)	Reduced glutathione (µg/g wet tissue)	Superoxide dismutase (units/mg protein)
Sham	128.41±23.37	293.23±38.66	20.32±1.29
MCAO + Vehicle	698.61±24.06*	52.63±17.67*	8.49±2.40*
MCAO + IRL1620	227.18±45.49#	172.93±14.52#	23.14±1.32#
MCAO + BQ788 + Vehicle	691.67±15.58*@	58.65±23.42*@	8.99±3.16*@
MCAO + BQ788 + IRL1620	638.26±24.84*@	60.52±23.15*@	7.60±1.85*@

[a] IRL-1620 (5 µg/kg, i.v.) or isotonic saline (1 ml/kg, i.v.) was injected at 2, 4, and 6 h post MCAO. BQ788 (1 mg/kg, i.v.) was administered 15 min prior to the first injection of IRL-1620 or vehicle. Values are expressed as mean ± SEM. *P<0.001 vs. sham. #P<0.05 vs. MCAO + vehicle. @P<0.05 vs. MCAO + IRL-1620.

5. Effect on Brain Endothelin Receptor Expression Following Cerebral Ischemia

Numerous studies have already shown that various portions of the ischemic brain show either increased or decreased levels of ET receptors following infarct (Loo, Ng et al. 2002, Stenman, Malmsjo et al. 2002, Henriksson, Stenman et al. 2003, Kreipke, Reynolds et al. 2011). While the increase in ET_A receptor expression is thought to increase the risks of vasospasm following cerebral ischemia, the effects of alterations in ET_B receptor expression are less clear.

In an attempt to elucidate the role of ET_B receptors in cerebral ischemia and to determine whether stimulation of these receptors altered their expression, Western blot analysis was performed for ET_A and ET_B receptors at both 24 h and 1 w post middle cerebral artery occlusion.

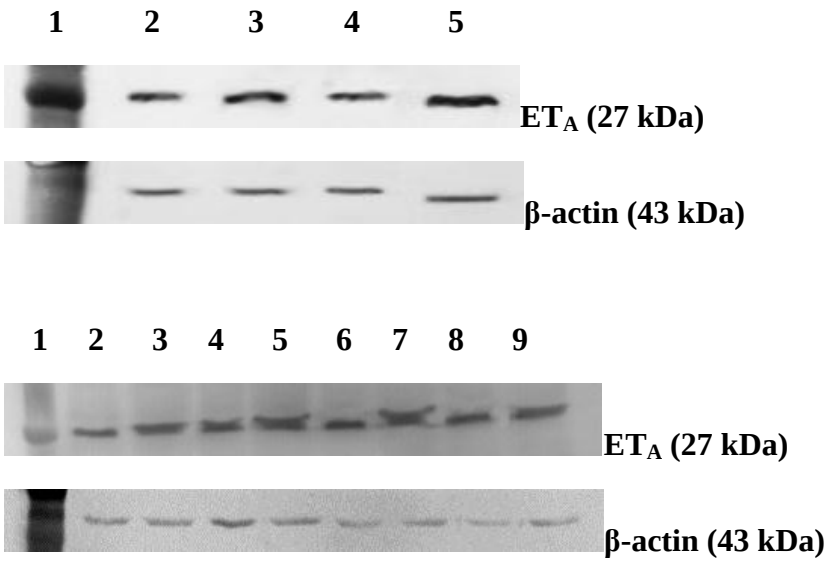
5.1. Effect on Brain Endothelin Receptor Expression Following Acute Cerebral Ischemia

Following the acute phase of cerebral ischemia, no significant differences were observed between vehicle-, IRL-1620-, and BQ788-treated middle cerebral artery occluded rats in either ET_A or ET_B receptor levels (Leonard, Briyal et al. 2011). All groups receiving the occlusion showed significantly higher levels of ET_A receptors in the right infarcted hemisphere as compared with the left non-infarcted hemisphere (Figure 8A). Endothelin B receptor levels were similar in all groups and in both infarcted and non-infarcted hemispheres, indicating that the protective effects of IRL-1620 during the acute phase of stroke are not due to a change in the number of ET_B receptors (Figure 8B).

5. 2. Effect on Brain Endothelin Receptor Expression Following Sub-acute Cerebral Ischemia

Both ET_A and ET_B receptor levels in the infarcted and non-infarcted hemispheres of sham-operated and middle cerebral artery occluded rats were examined on day 7 following

ischemia. Endothelin A receptor levels were found to be similar in vehicle-, IRL-1620-, and BQ788-treated middle cerebral artery occluded rats (Figure 9A). Endothelin B receptor levels were similar in vehicle- and BQ788-treated rats but were significantly raised in the infarcted hemisphere of IRL-1620-treated animals ($P<0.05$; Figure 9B) (Leonard, Briyal et al. 2012). Early, one-time treatment with IRL-1620 produced a notable up-regulation of ET_B receptors in the infarcted brain hemisphere, occurring at some point after the acute phase of the ischemic attack, potentially contributing to the continued neuroprotective and possible neuroregenerative effects of ET_B receptor agonist treatment.



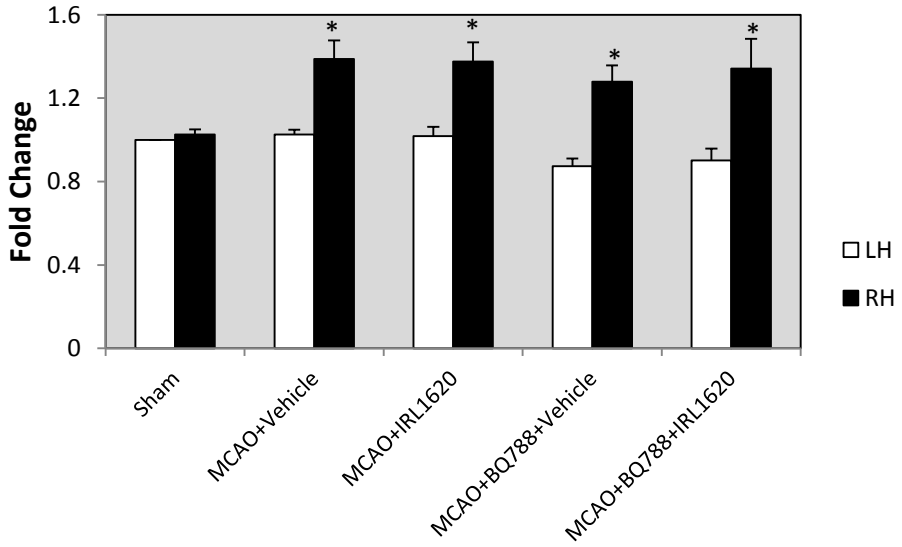


Figure 8A: Expression of ET_A receptor protein levels following acute cerebral ischemia. Top: Lane 1- Protein marker; Lane 2 – Sham (LH); Lane 3 – Sham (RH); Lane 4 – Vehicle + MCAO (LH); Lane 5 – Vehicle + MCAO (RH). Bottom: Lane 1- Protein marker; Lane 2 – Vehicle + MCAO (LH); Lane 3 – Vehicle + MCAO (RH); Lane 4 – MCAO + IRL1620 (LH); Lane 5 – MCAO + IRL1620 (RH); Lane 6 – MCAO + BQ788 + Vehicle (LH); Lane 7 – MCAO + BQ788 + Vehicle (RH); Lane 8 – MCAO + BQ788 + IRL1620 (LH); Lane 8 – MCAO + BQ788 + IRL1620 (RH). The blot is representative of four different experiments with similar results and bar graph showing fold change in the expression of ET_A receptor in brain 24 h post middle cerebral artery occlusion. IRL-1620 (5 µg/kg, i.v.) or isotonic saline (1 ml/kg, i.v.) was injected at 2, 4, and 6 h post MCAO. BQ788 (1 mg/kg, i.v.) was administered 15 min prior to the first injection of IRL-1620 or vehicle. LH=Left hemisphere; RH= Right hemisphere. Values are expressed as mean ± SEM. *P<0.05 compared to LH.

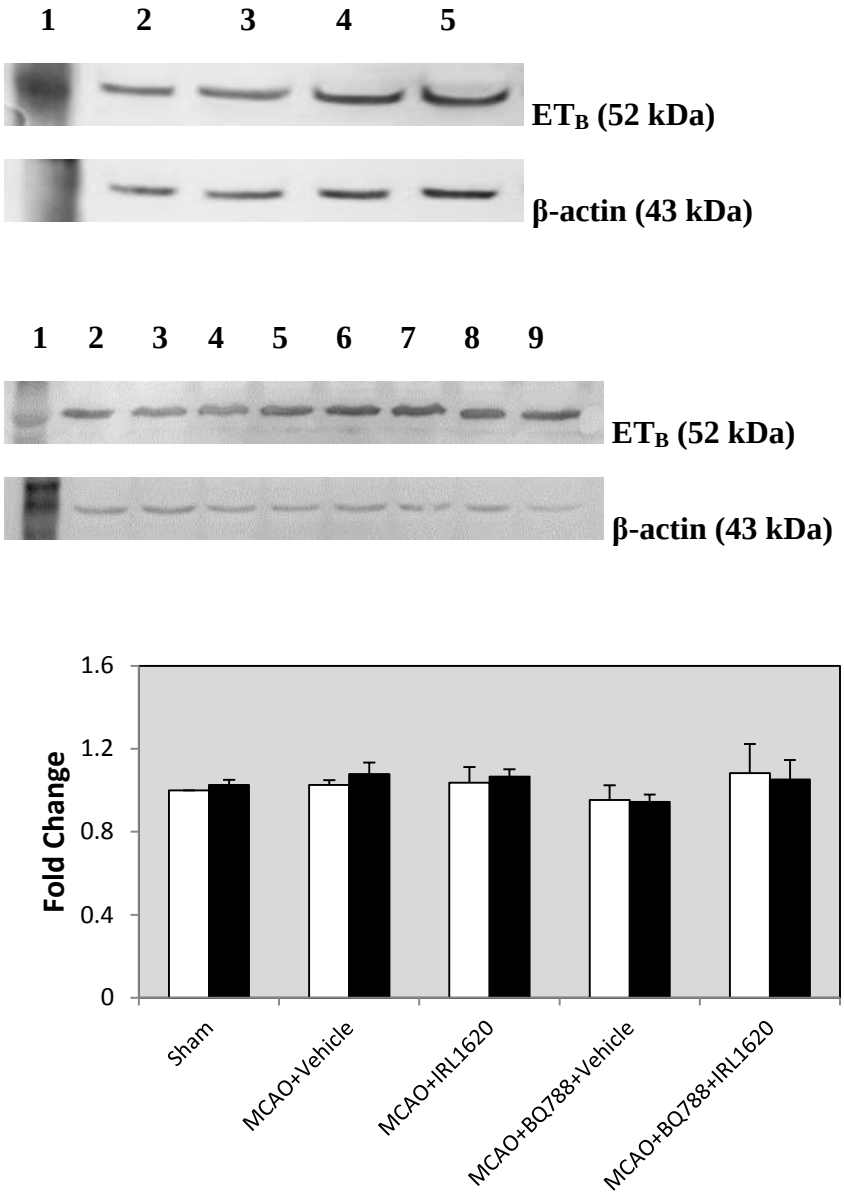
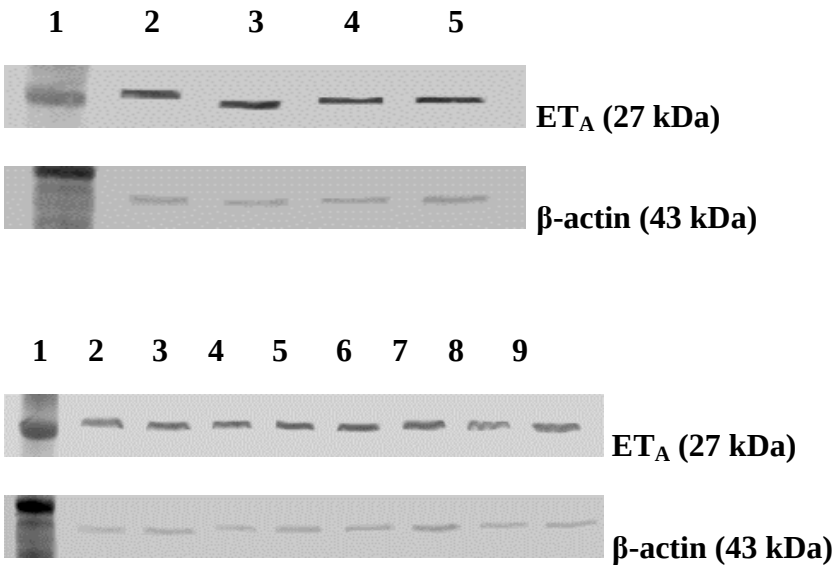


Figure 8B: Expression of ET_B receptor protein levels following acute cerebral ischemia. Top: Lane 1- Protein marker; Lane 2 – Sham (LH); Lane 3 – Sham (RH); Lane 4 – Vehicle + MCAO (LH); Lane 5 – Vehicle + MCAO (RH). Bottom: Lane 1- Protein marker; Lane 2 – Vehicle + MCAO (LH); Lane 3 – Vehicle + MCAO (RH); Lane 4 – MCAO + IRL1620 (LH); Lane 5 – MCAO + IRL1620 (RH); Lane 6 – MCAO + BQ788 + Vehicle (LH); Lane 7 – MCAO + BQ788 + Vehicle (RH); Lane 8 – MCAO + BQ788 + IRL1620 (LH); Lane 8 – MCAO + BQ788 + IRL1620 (RH). The blot is representative of four different experiments with similar results and bar graph showing fold change in the expression of ET_B receptor in brain 24 h post middle cerebral artery occlusion. IRL-1620 (5 µg/kg, i.v.) or isotonic saline (1 ml/kg, i.v.) was injected at 2, 4, and 6 h post MCAO. BQ788 (1 mg/kg, i.v.) was administered 15 min prior to the first injection of IRL-1620 or vehicle. LH=Left hemisphere; RH= Right hemisphere. Values are expressed as mean ± SEM.



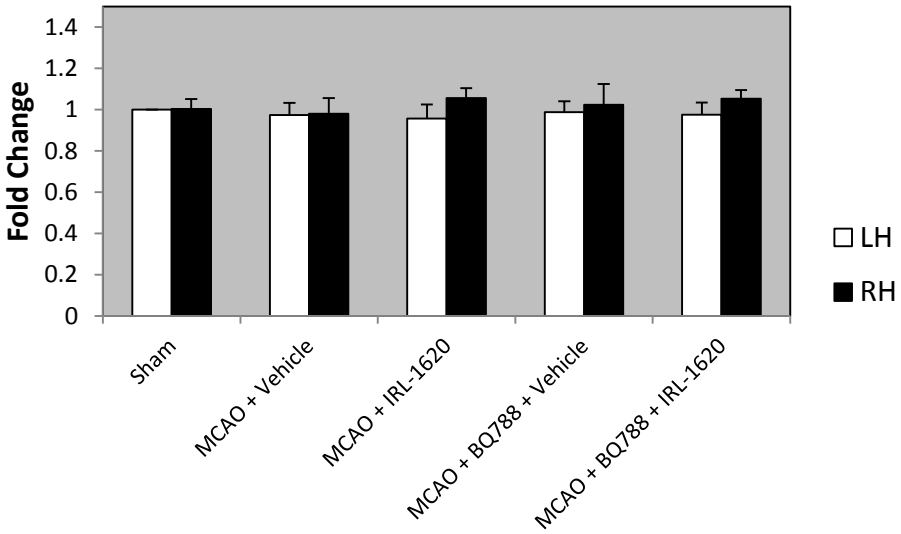
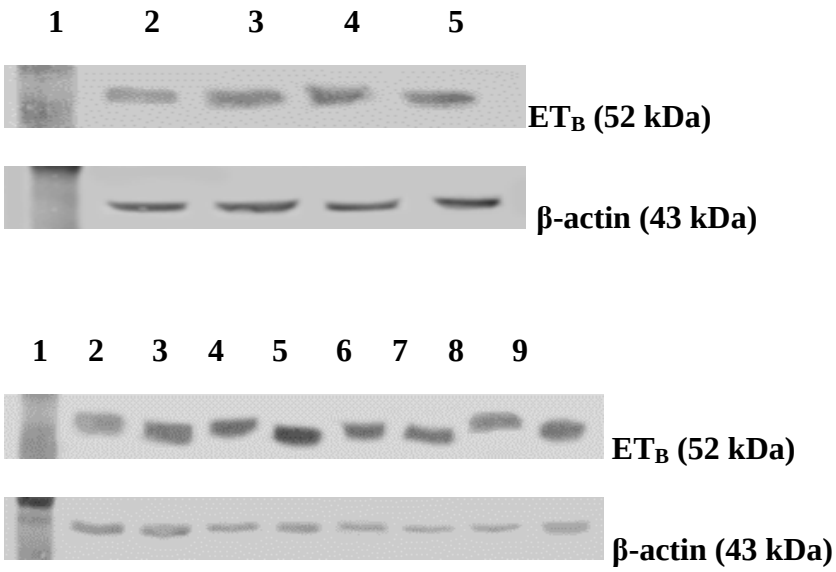


Figure 9A: Expression of ET_A receptor protein levels following sub-acute cerebral ischemia. Top: Lane 1- Protein marker; Lane 2 – Sham (LH); Lane 3 – Sham (RH); Lane 4 – Vehicle + MCAO (LH); Lane 5 – Vehicle + MCAO (RH). Bottom: Lane 1- Protein marker; Lane 2 – Vehicle + MCAO (LH); Lane 3 – Vehicle + MCAO (RH); Lane 4 – MCAO + IRL1620 (LH); Lane 5 – MCAO + IRL1620 (RH); Lane 6 – MCAO + BQ788 + Vehicle (LH); Lane 7 – MCAO + BQ788 + Vehicle (RH); Lane 8 – MCAO + BQ788 + IRL1620 (LH); Lane 9 – MCAO + BQ788 + IRL1620 (RH). The blot is representative of six different experiments with similar results and bar graph showing fold change in the expression of ET_A receptor in brain 7 days following middle cerebral artery occlusion. IRL-1620 (5 µg/kg, i.v.) or isotonic saline (1 ml/kg, i.v.) was injected at 2, 4, and 6 h post MCAO. BQ788 (1 mg/kg, i.v.) was administered 15 min prior to the first injection of IRL-1620 or vehicle. LH=Left hemisphere; RH= Right hemisphere. Values are expressed as mean ± SEM.



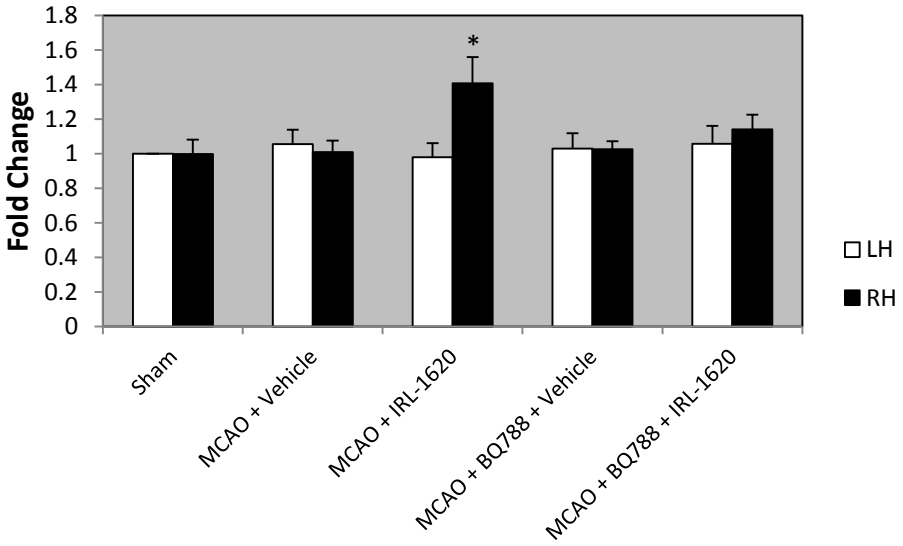


Figure 9B: Expression of ET_B receptor protein levels following sub-acute cerebral ischemia. Top: Lane 1- Protein marker; Lane 2 – Sham (LH); Lane 3 – Sham (RH); Lane 4 – Vehicle + MCAO (LH); Lane 5 – Vehicle + MCAO (RH). Bottom: Lane 1- Protein marker; Lane 2 – Vehicle + MCAO (LH); Lane 3 – Vehicle + MCAO (RH); Lane 4 – MCAO + IRL1620 (LH); Lane 5 – MCAO + IRL1620 (RH); Lane 6 – MCAO + BQ788 + Vehicle (LH); Lane 7 – MCAO + BQ788 + Vehicle (RH); Lane 8 – MCAO + BQ788 + IRL1620 (LH); Lane 9 – MCAO + BQ788 + IRL1620 (RH). The blot is representative of six different experiments with similar results and bar graph showing fold change in the expression of ET_B receptor in brain 7 days following middle cerebral artery occlusion. IRL-1620 (5 µg/kg, i.v) or isotonic saline (1 ml/kg, i.v.) was injected at 2, 4, and 6 h post MCAO. BQ788 (1 mg/kg, i.v.) was administered 15 min prior to the first injection of IRL-1620 or vehicle. LH=Left hemisphere; RH= Right hemisphere. Values are expressed as mean ± SEM. *P<0.05 compared to LH.

6. Effect on Reactive Astrocytes Following Cerebral Ischemia

In response to injuries to the central nervous system, astrocytes undergo morphological and functional changes, initiating both pro- and anti-inflammatory pathways (Li, Lundkvist et al. 2008, Lakhan, Kirchgessner et al. 2009). These 'reactive' astrocytes stain immuno-positive for glial fibrillary acidic protein (GFAP). As ET_B receptors are known to be present on astrocytes throughout the brain and our previous results demonstrated that ET_B receptors may play a role in reducing certain inflammatory reactions following MCAO, it was of interest to determine the effect of treatment with ET_B agonist, IRL-1620, and ET_B antagonist, BQ788, on reactive astrocytes during the acute and sub-acute phases of cerebral ischemia. The number of GFAP+ cells were determined in the cortex, striatum, corpus collosum (CC) and subventricular zones (SVZ) of the both the 'normal', left hemisphere and the infarcted, right hemisphere following permanent middle cerebral artery occlusion.

6.1. Effect on Reactive Astrocytes Following Acute Cerebral Ischemia

Cerebral ischemia caused an increase in the number of reactive astrocytes as marked by cells positively stained for glial fibrillary acidic protein. With 4.00 ± 0.29, 5.95 ± 0.46,8.83 ± 0.75 and 6.76 ± 0.32 GFAP+ cells per 100 μm^2 in the cortex, striatum, CC and SVZ, respectively, vehicle-treated animals displayed an increase in reactive astrocytes as early as 24 h post infarct as compared with sham-operated animals ($P<0.0001$; Table V). While the numbers of reactive astrocytes were often significantly higher in the infarcted hemisphere, GFAP+ cells also appeared to be elevated in the contralateral hemisphere, most notably in the CC and SVZ (Figures 11-14). Interestingly, when compared to sham, animals receiving IRL-1620 treatment only displayed an increase in GFAP+ cells in the CC and SVZ at 24 h (7.22 ± 0.42 and 5.17 ±

0.57, respectively; P<0.001), the areas closest to the core of the infarct. No significant differences in the area fraction of ET_B receptor staining was observed between groups, with 6-8% of the total area positive for ET_B and approximately one-third of the reactive astrocytes showing co-localization with ET_B (Figure 10A).

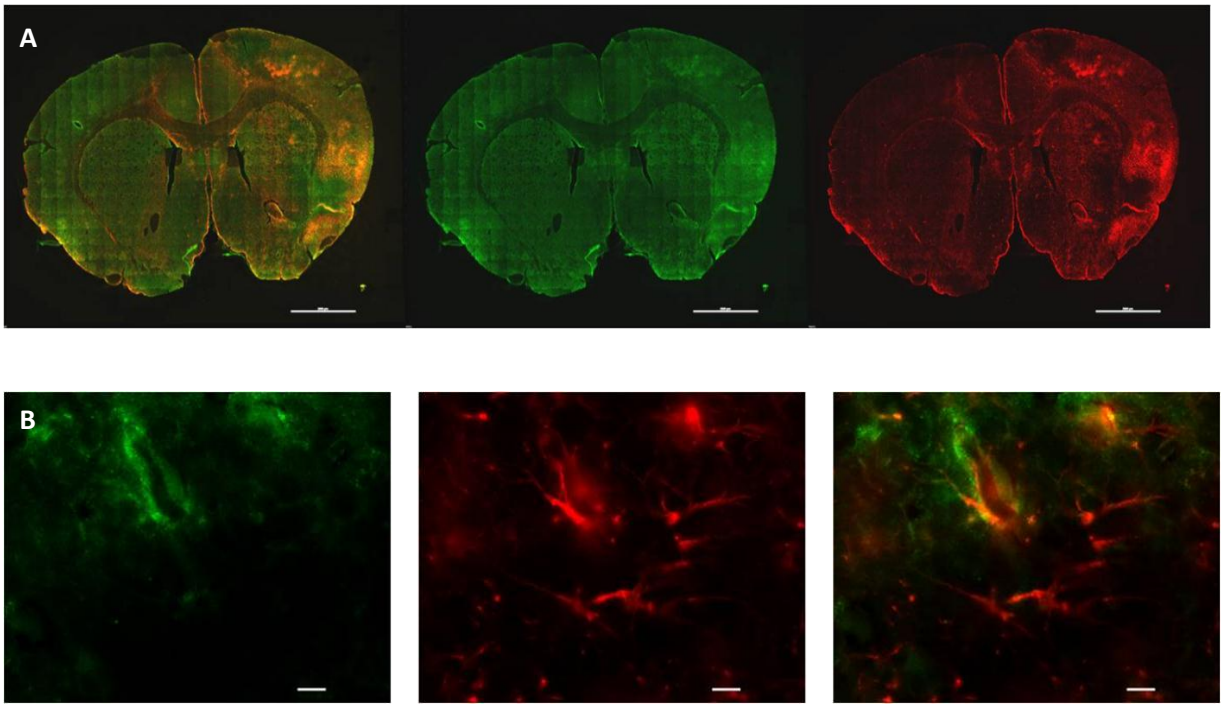


Figure 10: Effect on glial fibrillary acidic protein following cerebral ischemia. **A.** Representative 30 µm-thick ischemic brain slice stained for the ET_B receptor (green) and GFAP (red) 24 h post MCAO. Scale bar = 2000 µm. **B.** Representative image of the striatum of a BQ788-treated animal 1 week following MCAO, stained for the ET_B receptor (green) and GFAP (red). Scale bar = 10 µm.

6.2. Effect on Reactive Astrocytes Following Sub-acute Cerebral Ischemia

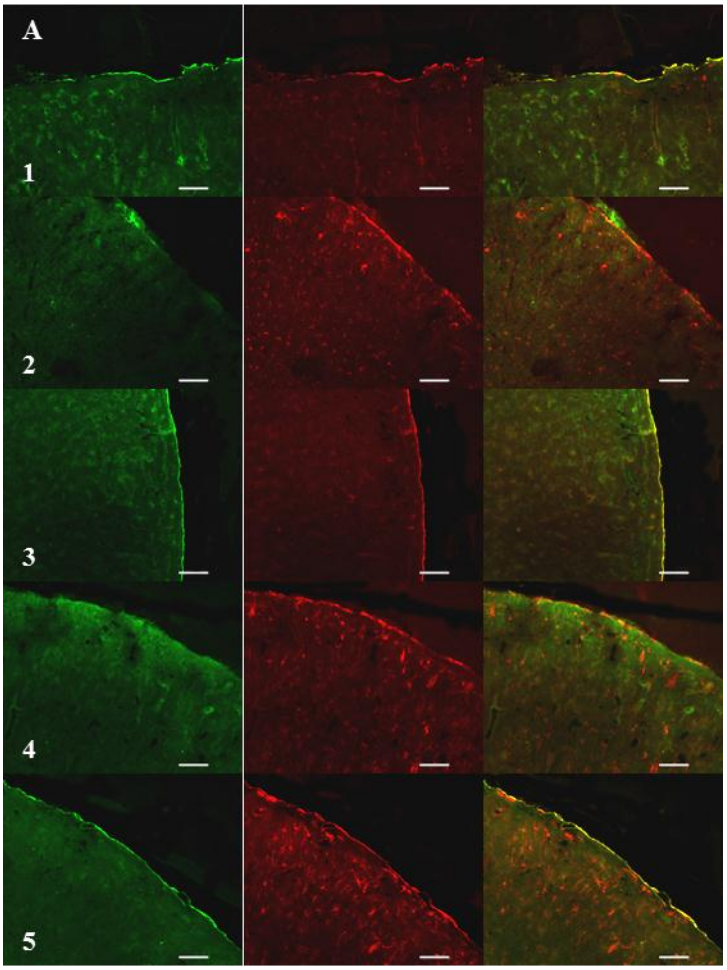
By the end of the week, animals in all treatment groups presented with increased reactive astrocytes in all observed zones of the infarcted hemisphere (Table V). Astrocytes in the left, non-infarcted hemisphere were relatively sparse in all zones except for the corpus collosum (Figures 15-18). Endothelin B receptor staining accounted for 8-14% of the total area with no significant differences between the groups, indicating a slight elevation in ET_B receptors at 1 week post induction of cerebral ischemia. Endothelin B receptors co-localized with the reactive astrocytes, with double staining occurring in just over one-third of astrocytes in all groups (Figure 10B).

Table V

EFFECT ON GLIAL FIBRILLARY ACIDIC PROTEIN (GFAP) FOLLOWING ACUTE AND SUB-ACUTE CEREBRAL ISCHEMIA[a]

Treatment Group	Infarct Duration	Cortex	Striatum	Corpus Collosum	Subventricular Zone
Sham	**24 hours**	1.73±0.27	1.27±0.28	2.44±0.38	1.40±0.27
	1 week	1.96±0.21	1.79±0.19	4.83±0.45	2.90±0.21
MCAO + Vehicle	**24 hours**	4.00±0.29*	5.95±0.46*	8.83±0.75*	6.76±0.32*
	1 week	7.38±0.44*	7.88±0.51*	10.52±0.52*	6.95±0.47*
MCAO + IRL-1620	**24 hours**	2.89±0.43	2.44±0.27#	7.22±0.42*	5.17±0.57*
	1 week	8.04±0.33*	8.25±0.43*	9.57±0.52*	7.58±0.55*
MCAO + BQ788 + Vehicle	**24 hours**	3.89±0.31*	5.61±0.46*@	6.72±0.47*	6.48±0.33*
	1 week	8.28±0.46*	8.31±0.31*	8.29±0.58*	6.61±0.61*
MCAO + BQ788 + IRL-1620	**24 hours**	6.22±0.26*@	7.43±0.39*@	8.87±0.57*	6.27±0.47*
	1 week	7.70±0.43*	6.89±0.48*	7.89±0.60*	6.67±0.62*

[a] IRL-1620 (5 µg/kg, i.v.) or isotonic saline (1 ml/kg, i.v.) was injected at 2, 4, and 6 h post MCAO. BQ788 (1 mg/kg, i.v.) was administered 15 min prior to the first injection of IRL-1620 or vehicle. Number of reactive astrocytes (GFAP+ cells) per 100 μm^2 in the cortex, striatum, corpus colossum and subventricular zone of middle cerebral artery occluded rats at 24 h and 1 week after infarct. Values are presented as mean ± SEM. *P<0.001 vs. sham. #P<0.0001 vs. MCAO + vehicle. @P<0.01 vs. MCAO + IRL-1620.



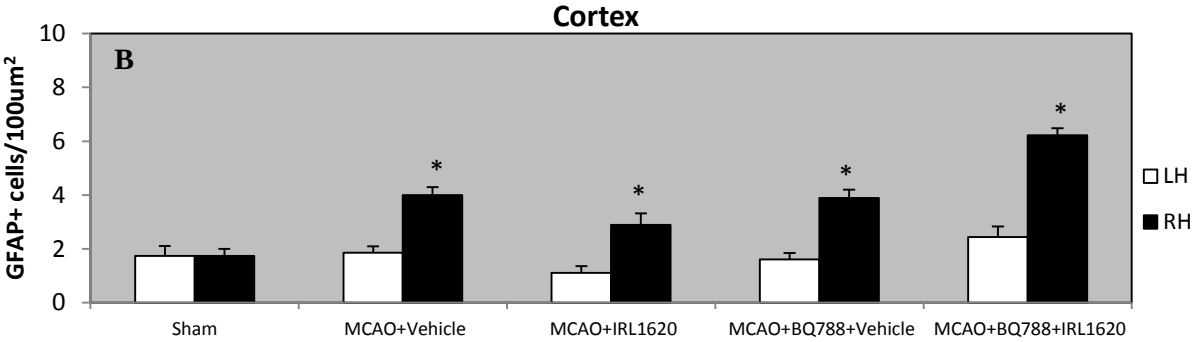
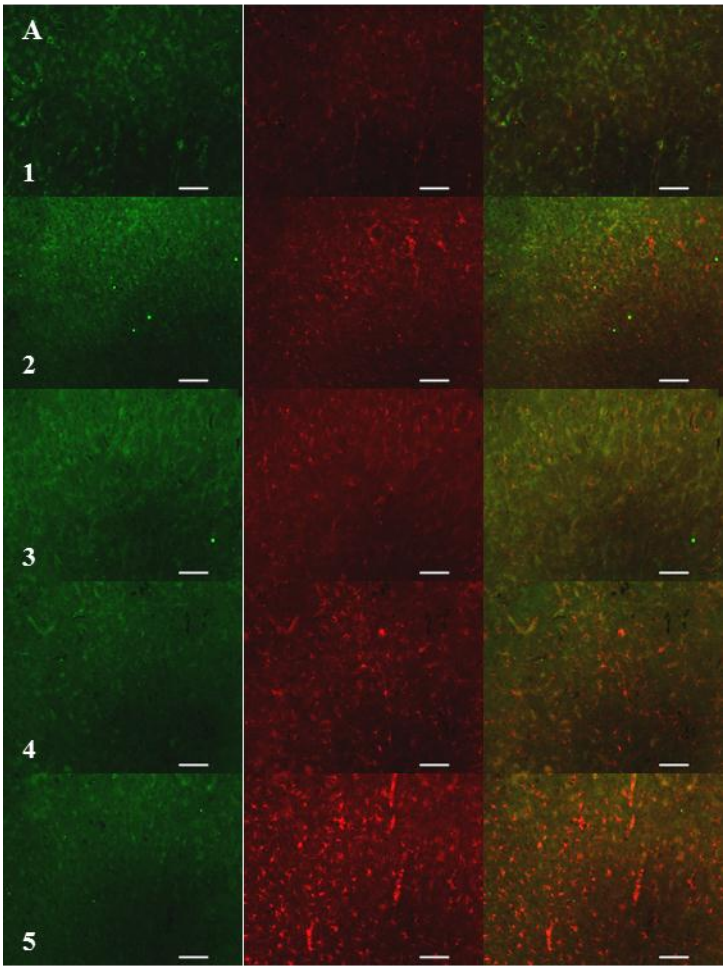


Figure 11: Effect on cortical GFAP following acute cerebral ischemia. **A.** Representative 30 µm-thick sections of the right, infarcted cortex of rats 24 h post MCAO stained for ET_B receptor (green) and GFAP (red). Scale bar = 100 µm. Rows: 1 - Sham; 2 - MCAO + Vehicle; 3 - MCAO + IRL-1620; 4 – MCAO + BQ788 + Vehicle; 5 – MCAO + BQ788 + IRL-1620. **B.** Number of reactive astrocytes (GFAP+ cells) per 100 μm^2 in the cortex of middle cerebral artery occluded rats at 24 h after infarct. LH = Left (normal) hemisphere; RH = Right (infarcted) hemisphere. Values are presented as mean ± SEM. *P<0.05 vs. LH.



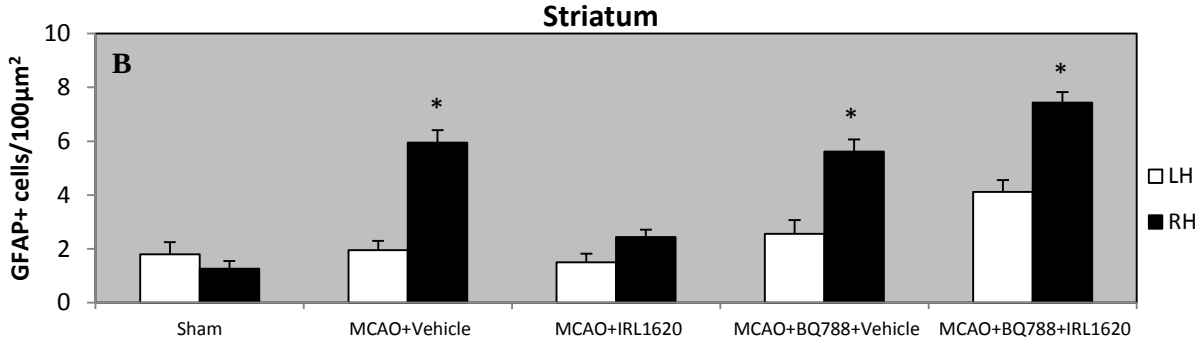
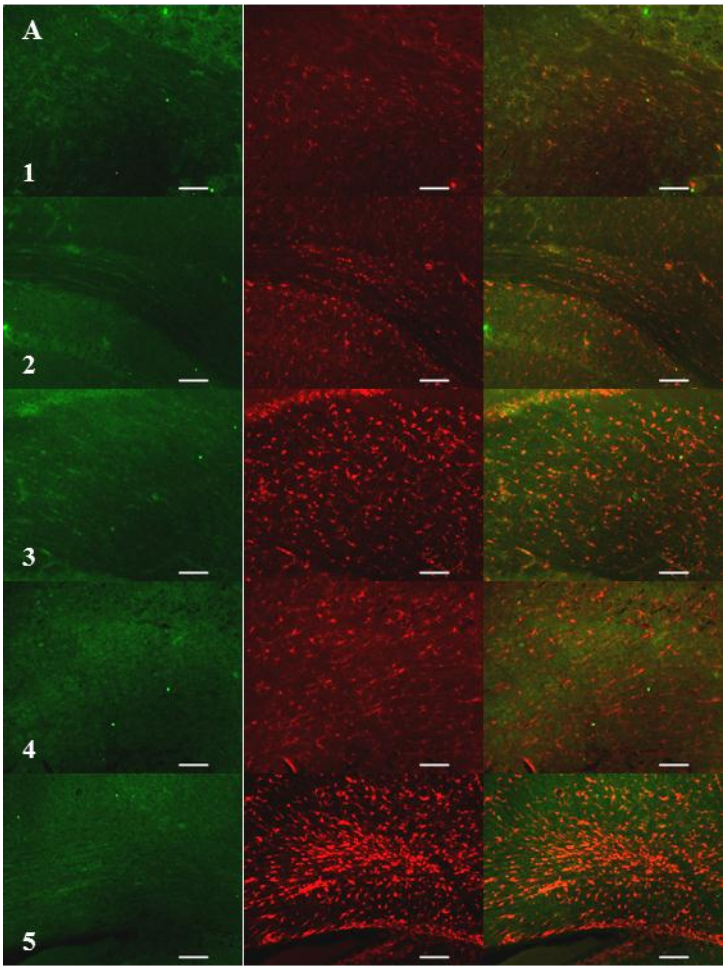


Figure 12: Effect on striatal GFAP following acute cerebral ischemia. **A.** Representative 30 µm-thick sections of the right, infarcted striatum of rats 24 hr post MCAO stained for ET_B receptor (green) and GFAP (red). Scale bar = 100 µm. Rows: 1 - Sham; 2 - MCAO + Vehicle; 3 - MCAO + IRL-1620; 4 – MCAO + BQ788 + Vehicle; 5 – MCAO + BQ788 + IRL-1620. **B.** Number of reactive astrocytes (GFAP+ cells) per 100 μm^2 in the striatum of middle cerebral artery occluded rats at 24 h after infarct. LH = Left (normal) hemisphere; RH = Right (infarcted) hemisphere. Values are presented as mean ± SEM. *P<0.001 vs. LH.



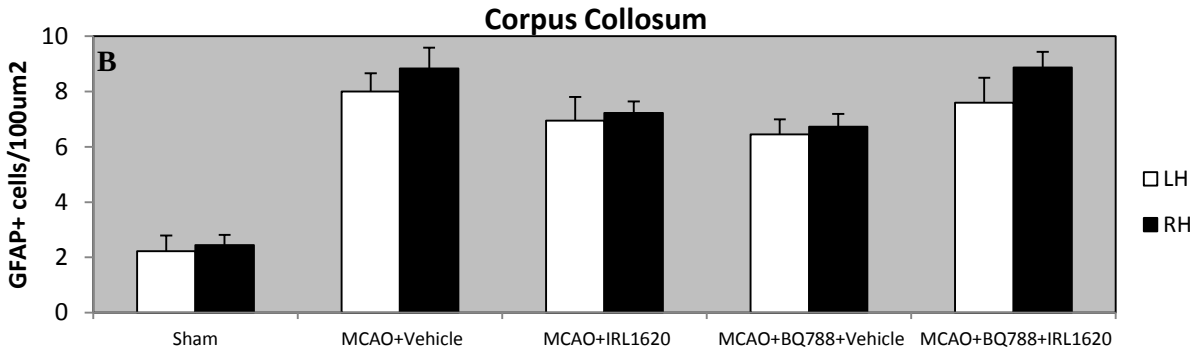
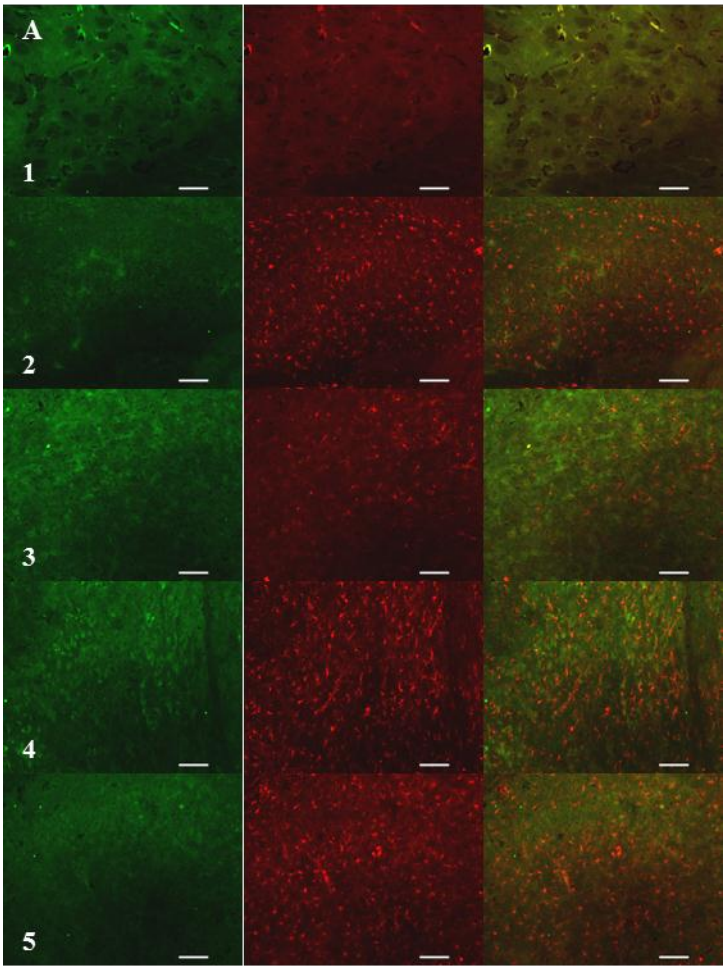


Figure 13: Effect on corpus collosum GFAP following acute cerebral ischemia. **A.** Representative 30 µm-thick sections of the right, infarcted corpus collosum of rats 24 h post MCAO stained for ET_B receptor (green) and GFAP (red). Scale bar = 100 µm. Rows: 1 - Sham; 2 - MCAO + Vehicle; 3 - MCAO + IRL-1620; 4 – MCAO + BQ788 + Vehicle; 5 – MCAO + BQ788 + IRL-1620. **B.** Number of reactive astrocytes (GFAP+ cells) per 100 μm^2 in the corpus collosum of middle cerebral artery occluded rats at 24 h after infarct. LH = Left (normal) hemisphere; RH = Right (infarcted) hemisphere. Values are presented as mean ± SEM.



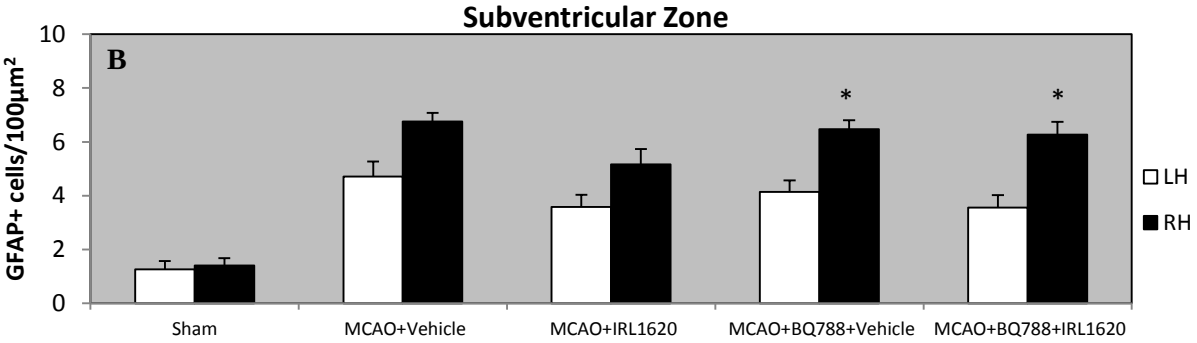
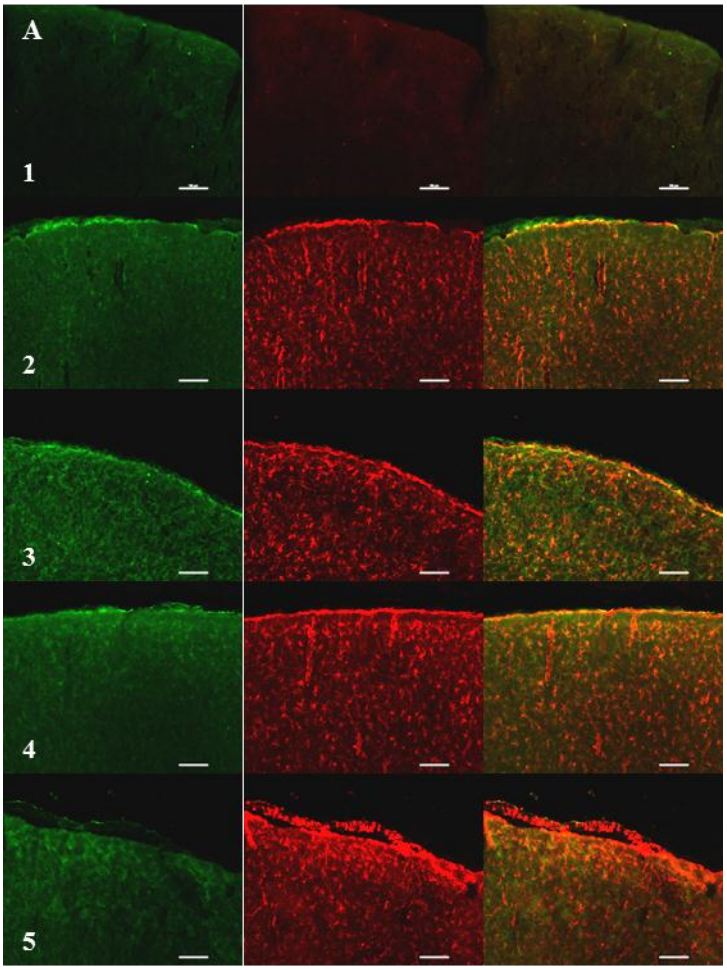


Figure 14: Effect on subventricular GFAP following acute cerebral ischemia. **A.** Representative 30 µm-thick sections of the right, infarcted subventricular zone of rats 24 h post MCAO stained for ET_B receptor (green) and GFAP (red). Scale bar = 100 µm. Rows: 1 - Sham; 2 - MCAO + Vehicle; 3 - MCAO + IRL-1620; 4 – MCAO + BQ788 + Vehicle; 5 – MCAO + BQ788 + IRL-1620. **B.** Number of reactive astrocytes (GFAP+ cells) per 100 μm^2 in the SVZ of middle cerebral artery occluded rats at 24 h after infarct. LH = Left (normal) hemisphere; RH = Right (infarcted) hemisphere. Values are presented as mean ± SEM. *P<0.05 vs. LH.



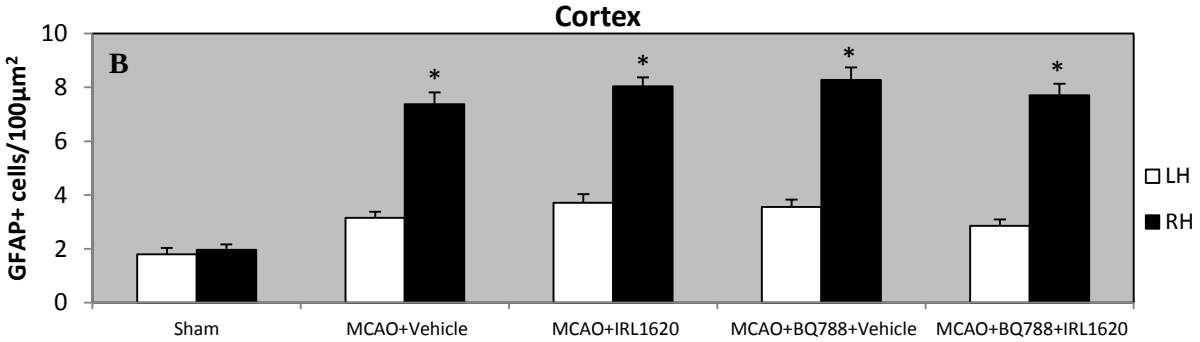
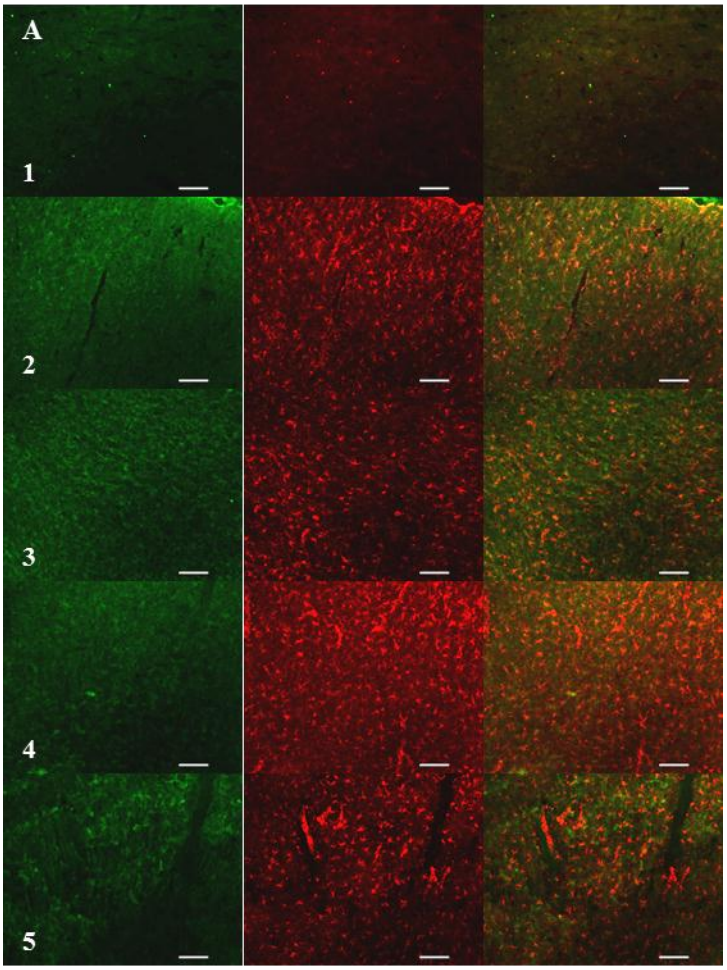


Figure 15: Effect on cortical GFAP following sub-acute cerebral ischemia. **A.** Representative 30 µm-thick sections of the right, infarcted cortex of rats 1w post MCAO stained for ET_B receptor (green) and GFAP (red). Scale bar = 100 µm. Rows: 1 - Sham; 2 - MCAO + Vehicle; 3 - MCAO + IRL-1620; 4 – MCAO + BQ788 + Vehicle; 5 – MCAO + BQ788 + IRL-1620. **B.** Number of reactive astrocytes (GFAP+ cells) per 100 μm^2 in the cortex of middle cerebral artery occluded rats at 1 w after infarct. LH = Left (normal) hemisphere; RH = Right (infarcted) hemisphere. Values are presented as mean ± SEM. *P<0.001 vs. LH.



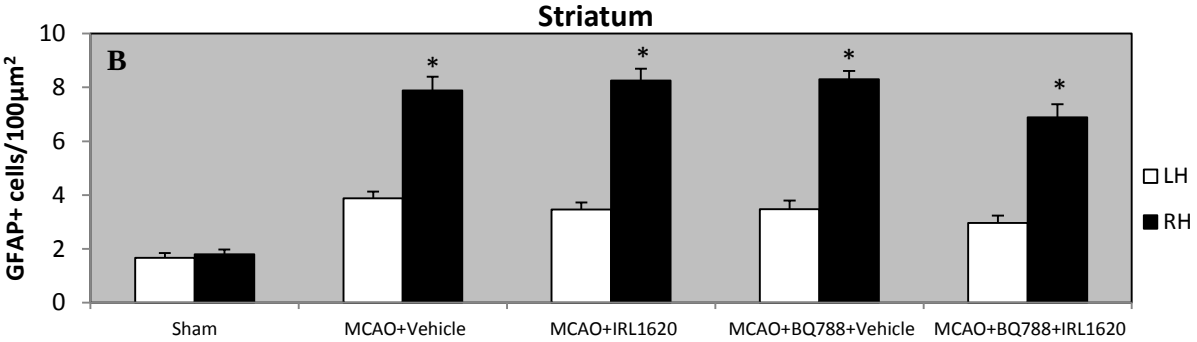
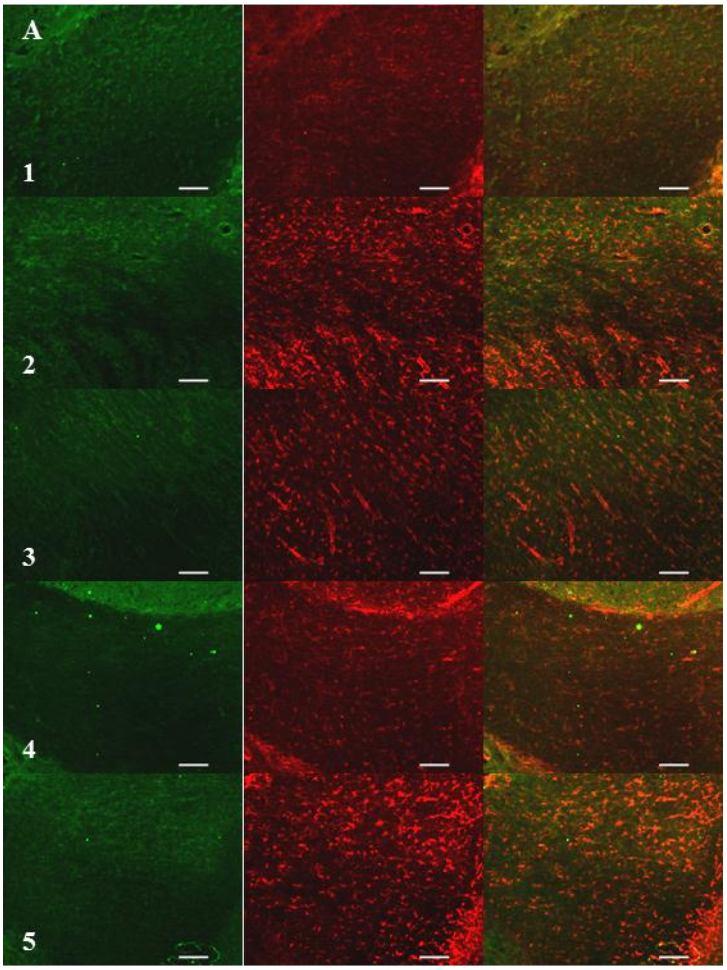


Figure 16: Effect on striatal GFAP following sub-acute cerebral ischemia. **A.** Representative 30 µm-thick sections of the right, infarcted striatum of rats 1 w post MCAO stained for ET_B receptor (green) and GFAP (red). Scale bar = 100 µm. Rows: 1 - Sham; 2 - MCAO + Vehicle; 3 - MCAO + IRL-1620; 4 – MCAO + BQ788 + Vehicle; 5 – MCAO + BQ788 + IRL-1620. **B.** Number of reactive astrocytes (GFAP+ cells) per 100 μm^2 in the striatum of middle cerebral artery occluded rats at 1 w after infarct. LH = Left (normal) hemisphere; RH = Right (infarcted) hemisphere. Values are presented as mean ± SEM. *P<0.001 vs. LH.



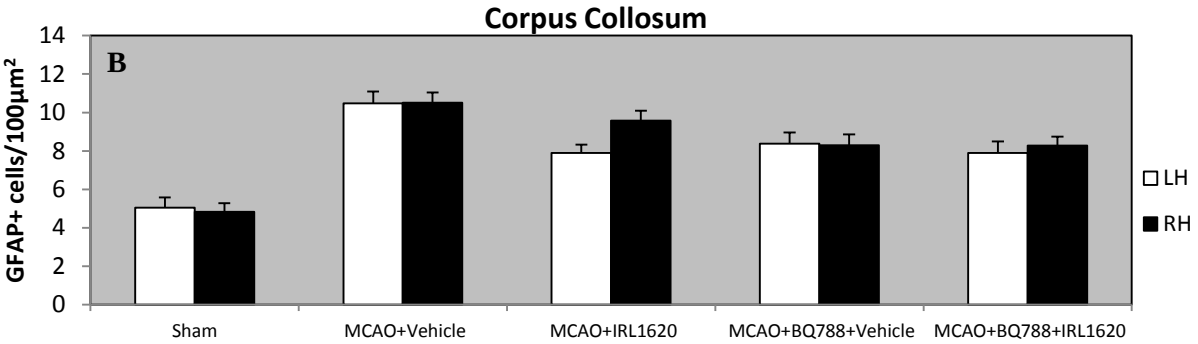
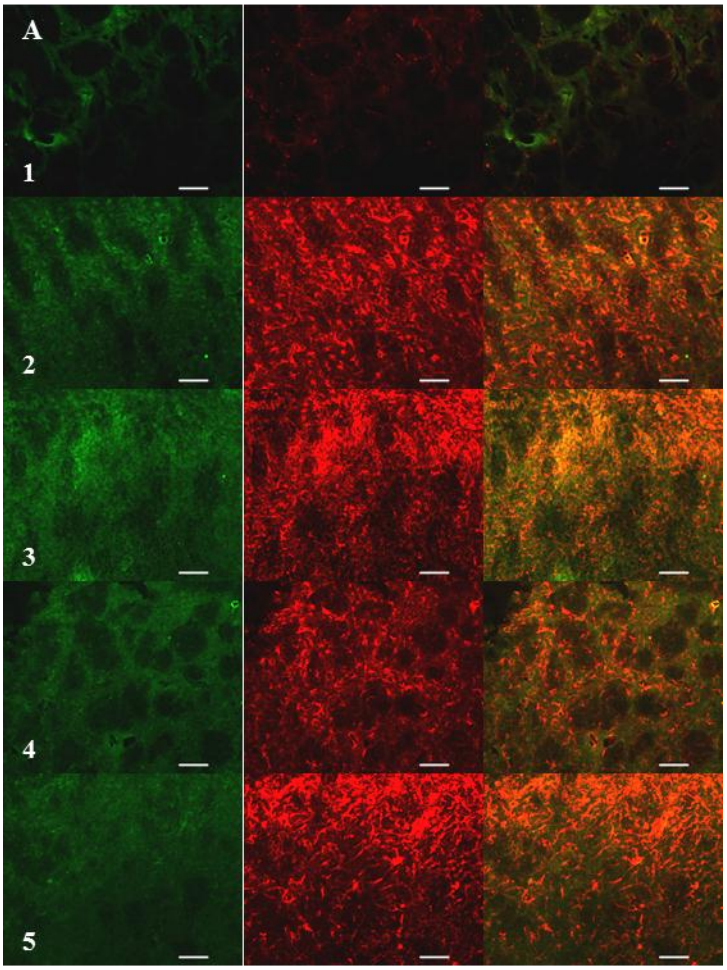


Figure 17: Effect on corpus collosum GFAP following sub-acute cerebral ischemia. **A.** Representative 30 µm-thick sections of the right, infarcted corpus collosum of rats 1 w post MCAO stained for ET_B receptor (green) and GFAP (red). Scale bar = 100 µm. Rows: 1 - Sham; 2 - MCAO + Vehicle; 3 - MCAO + IRL-1620; 4 – MCAO + BQ788 + Vehicle; 5 – MCAO + BQ788 + IRL-1620. **B.** Number of reactive astrocytes (GFAP+ cells) per 100 μm^2 in the corpus collosum of middle cerebral artery occluded rats at 1 w after infarct. LH = Left (normal) hemisphere; RH = Right (infarcted) hemisphere. Values are presented as mean ± SEM.



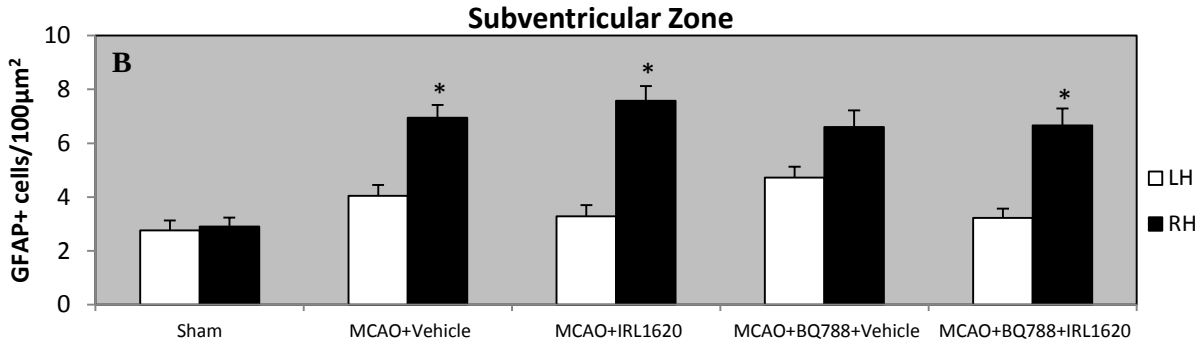


Figure 18: Effect on subventricular GFAP following sub-acute cerebral ischemia. **A.** Representative 30 µm-thick sections of the right, infarcted subventricular zone of rats 1 w post MCAO stained for ET_B receptor (green) and GFAP (red). Scale bar = 100 µm. Rows: 1 - Sham; 2 - MCAO + Vehicle; 3 - MCAO + IRL-1620; 4 – MCAO + BQ788 + Vehicle; 5 – MCAO + BQ788 + IRL-1620. **B.** Number of reactive astrocytes (GFAP+ cells) per 100 μm^2 in the SVZ of middle cerebral artery occluded rats at 1 w after infarct. LH = Left (normal) hemisphere; RH = Right (infarcted) hemisphere. Values are presented as mean ± SEM. *P<0.01 vs. LH.

7. Effect on Mature Neurons Following Cerebral Ischemia

While our initial studies clearly demonstrated a neuroprotective effect of ET_B receptor stimulation at both 24 h and 1 w following cerebral ischemia in terms of decreases in oxidative stress, infarct volume and neurological and motor function deficit, we had thus far not focused specifically on the survival of individual neurons (Leonard, Briyal et al. 2011, Leonard, Briyal et al. 2012). In order to confirm that the number of mature neurons was indeed remaining stable, we therefore directly examined the neuronal nuclei in the treated and untreated ischemic rat brain.

The nuclei of mature neurons along with the ET_B receptors were fluorescently labeled in the cortex, striatum and subventricular zones of rat brains at 24 h and 1 w following middle cerebral artery occlusion. The number of NeuN+ cells per 100 μm^2 in each zone was compared between the infarcted and non-infarcted hemispheres and between treatment groups to determine the effect of ET_B agonist, IRL-1620, and antagonist, BQ788, on mature neurons during the acute and sub-acute phases of cerebral ischemia.

7.1. Effect on Mature Neurons Following Acute Cerebral Ischemia

Cerebral ischemia resulted in a decrease in the number of neurons with 6.50 ± 0.90, 5.67 ± 0.77, and 4.08 ± 0.61 NeuN+ cells per 100 μm^2 in the cortex, striatum, and SVZ, respectively, of vehicle-treated animals 24 h post infarct ($P<0.05$; Table VI). In contrast, IRL-1620 treatment preserved neurons in the same period, with 10.44 ± 0.49, 10.52 ± 0.51, and 10.38 ± 0.56 NeuN+ cells/100 μm^2 in the cortex, striatum and SVZ. These counts are similar to those seen in the sham-operated group at 24 h. Animals pretreated with ET_B antagonist BQ788 blocked the neuroprotective effects of IRL-1620, with neurons at levels similar to those of the vehicle-treated

group. Interestingly, little difference was seen in NeuN+ cell numbers between the left non-infarcted and right infarcted hemispheres, indicating that the whole brain is affected despite localization of the infarct core to the right middle cerebral artery (Figures 20-22). The area stained for ET_B receptors ranged from 5.51 ± 0.61% in the BQ788 + IRL-1620 group to 14.44 ± 0.86% in the IRL-1620 alone group. Both BQ788 and vehicle treatment groups had significantly less ET_B receptor labeling than either the IRL-1620 or sham groups ($P<0.05$). Co-localization between the neuronal nuclei and ET_B receptors was also higher in the IRL-1620 group, at 49.73 ± 3.19% vs. 27.47 ± 2.14% for the vehicle-treated group ($P<0.001$).

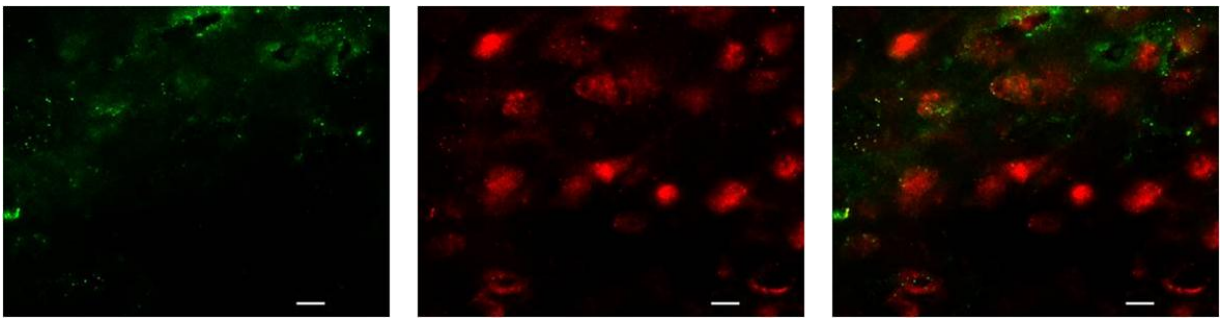


Figure 19: Effect on neuronal nuclei following cerebral ischemia. Representative image of the striatum of an IRL-1620-treated animal 1 week following MCAO, stained for the ET_B receptor (green) and NeuN (red). Scale bar = 10 µm.

7.2. Effect on Mature Neurons Following Sub-acute Cerebral Ischemia

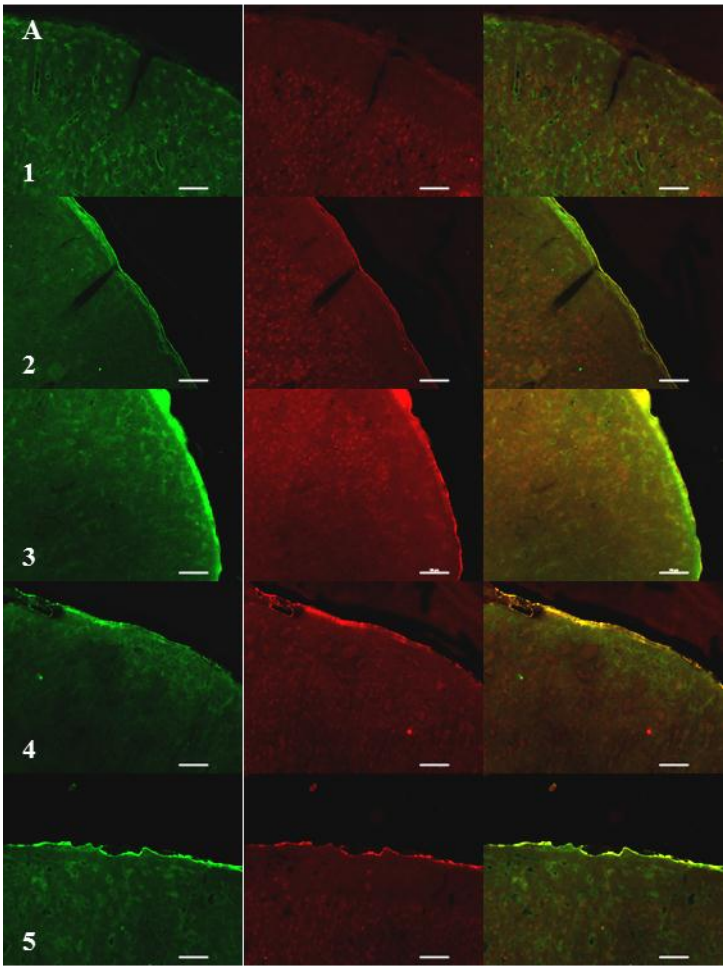
Intriguingly, while the numbers of NeuN+ cells in the sham group did not alter after 1 w, those in all groups undergoing MCAO were increased at 1 w as compared to the 24 h endpoint. Most notably, animals treated with IRL-1620 presented with 15.04 ± 0.74, 17.67 ± 0.53, and 15.58 ± 0.72 NeuN+ cells per 100 μm^2 in the cortex, striatum, and SVZ, respectively, a significant increase over the level of neurons in the sham-operated group ($P<0.05$; Table VI). Conversely, while the number of neurons in the vehicle- and BQ788-treated groups increased from 24 h to 1 w, they still remained significantly lower than those in the sham group in both the infarcted and non-infarcted hemispheres ($P<0.05$; Figures 23-25). These results indicate that some amount of neuronal remodeling occurs over the course of the week following induction of cerebral ischemia, and that this remodeling may be enhanced by the selective stimulation of ET_B receptors by IRL-1620. While approximately one-third of the NeuN+ cells co-localized with ET_B receptors in all groups, the IRL-1620-treated group again displayed an increase in ET_B receptor area fraction following the sub-acute phase of cerebral ischemia as compared with the vehicle-treated group (12.34 ± 0.35% vs. 8.44 ± 0.61%; $P<0.05$; Figure 19).

Table VI

EFFECT ON NEURONAL NUCLEI (NEUN) FOLLOWING ACUTE AND SUB-ACUTE CEREBRAL ISCHEMIA[a]

Treatment Group	Infarct Duration	Cortex	Striatum	Subventricular Zone
Sham	**24 hours**	10.00±0.77	10.58±0.56	11.33±0.64
	1 week	11.41±0.71	11.79±0.73	11.71±1.03
MCAO + Vehicle	**24 hours**	6.50±0.90*	5.67±0.77*	4.08±0.61*
	1 week	8.63±0.58	10.53±0.51	5.58±0.64*
MCAO + IRL-1620	**24 hours**	10.44±0.49#	10.52±0.38#	10.38±0.56#
	1 week	15.04±0.74#	17.67±0.53*#	15.58±0.72*#
MCAO + BQ788 + Vehicle	**24 hours**	4.44±0.52*@	4.06±0.43*@	2.09±0.32*@
	1 week	8.27±0.84@	8.23±0.57*@	5.80±0.52*@
MCAO + BQ788 + IRL-1620	**24 hours**	4.44±0.69*@	4.33±0.52*@	1.38±0.23*@
	1 week	5.19±0.52*@	5.28±0.41*@	2.73±0.29*@

[a] IRL-1620 (5 µg/kg, i.v.) or isotonic saline (1 ml/kg, i.v.) was injected at 2, 4, and 6 h post MCAO. BQ788 (1 mg/kg, i.v.) was administered 15 min prior to the first injection of IRL-1620 or vehicle. Number of mature neurons (NeuN+ cells) per 100 μm^2 in the cortex, striatum, and subventricular zone of middle cerebral artery occluded rats at 24 h and 1 week after infarct. Values are presented as mean ± SEM. *P<0.05 vs. sham. #P<0.01 vs. MCAO + vehicle. @P<0.0001 vs. MCAO + IRL-1620.



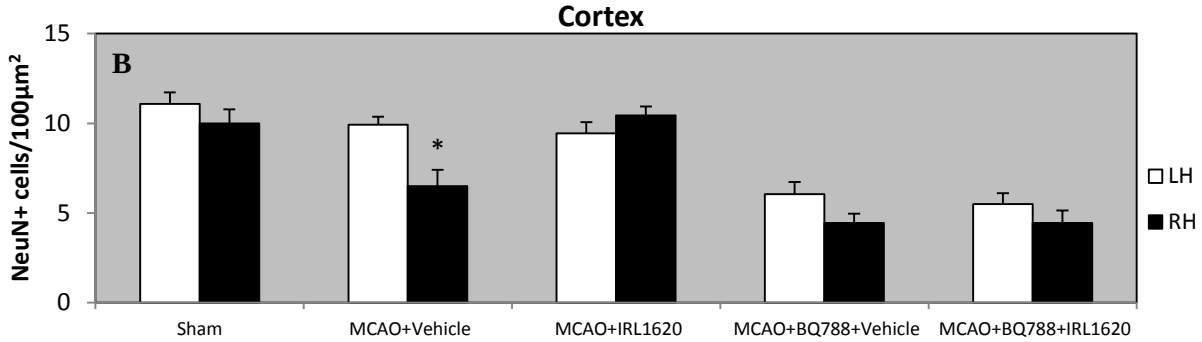
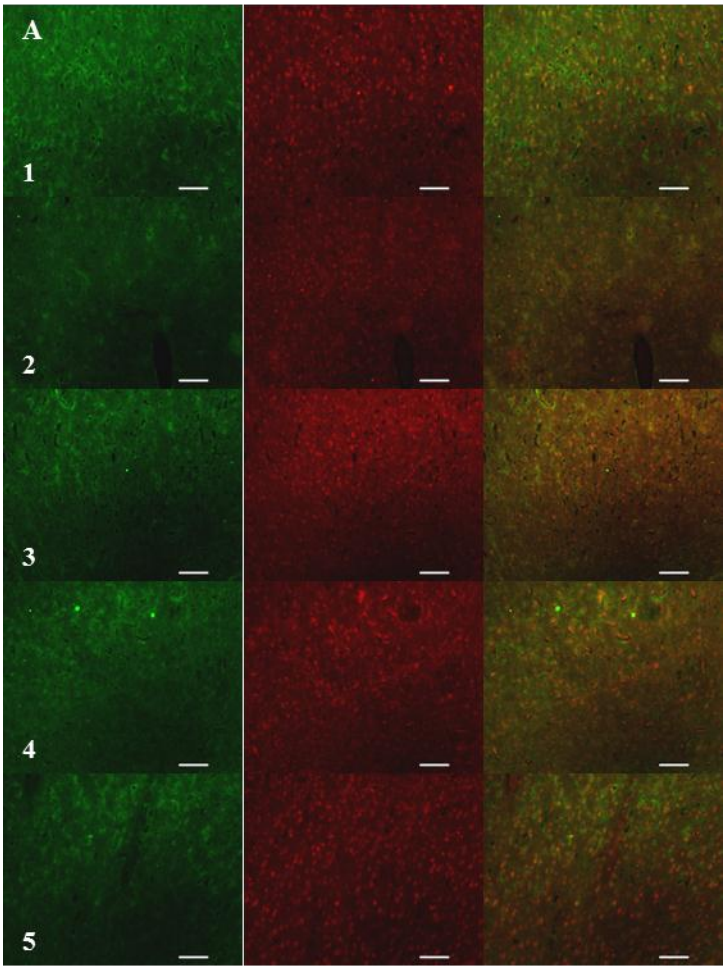


Figure 20: Effect on cortical NeuN following acute cerebral ischemia. **A.** Representative 30 µm-thick sections of the right, infarcted cortex of rats 24 h post MCAO stained for ET_B receptor (green) and NeuN (red). Scale bar = 100 µm. Rows: 1 - Sham; 2 - MCAO + Vehicle; 3 - MCAO + IRL-1620; 4 – MCAO + BQ788 + Vehicle; 5 – MCAO + BQ788 + IRL-1620. **B.** Number of mature neurons (NeuN+ cells) per 100 μm^2 in the cortex of middle cerebral artery occluded rats at 24 h after infarct. LH = Left (normal) hemisphere; RH = Right (infarcted) hemisphere. Values are presented as mean ± SEM. *P<0.05 vs. LH.



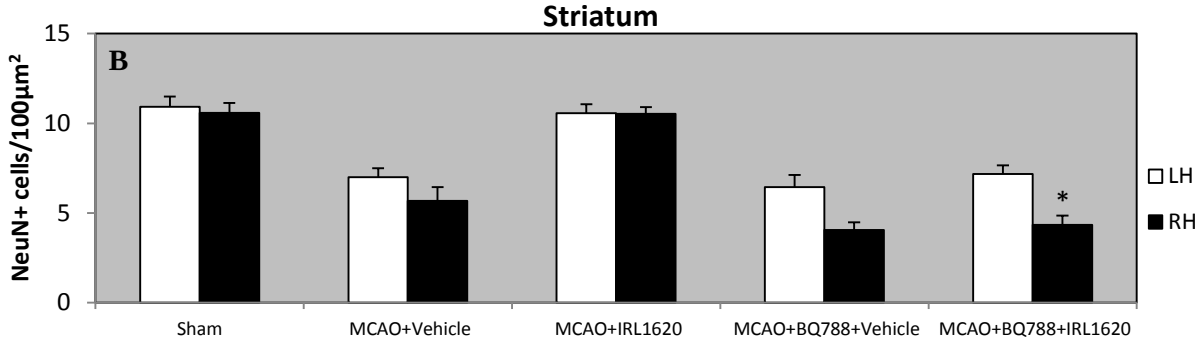
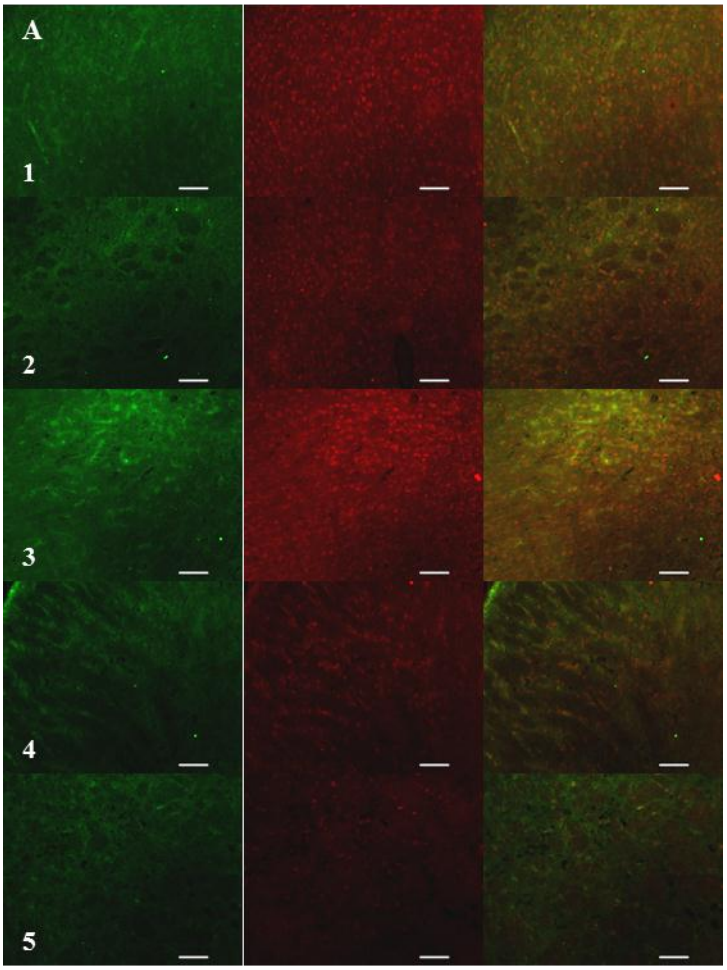


Figure 21: Effect on striatal NeuN following acute cerebral ischemia. **A.** Representative 30 µm-thick sections of the right, infarcted striatum of rats 24 h post MCAO stained for ET_B receptor (green) and NeuN (red). Scale bar = 100 µm. Rows: 1 - Sham; 2 - MCAO + Vehicle; 3 - MCAO + IRL-1620; 4 – MCAO + BQ788 + Vehicle; 5 – MCAO + BQ788 + IRL-1620. **B.** Number of mature neurons (NeuN+ cells) per 100 μm^2 in the striatum of middle cerebral artery occluded rats at 24 h after infarct. LH = Left (normal) hemisphere; RH = Right (infarcted) hemisphere. Values are presented as mean ± SEM. *P<0.05 vs. LH.



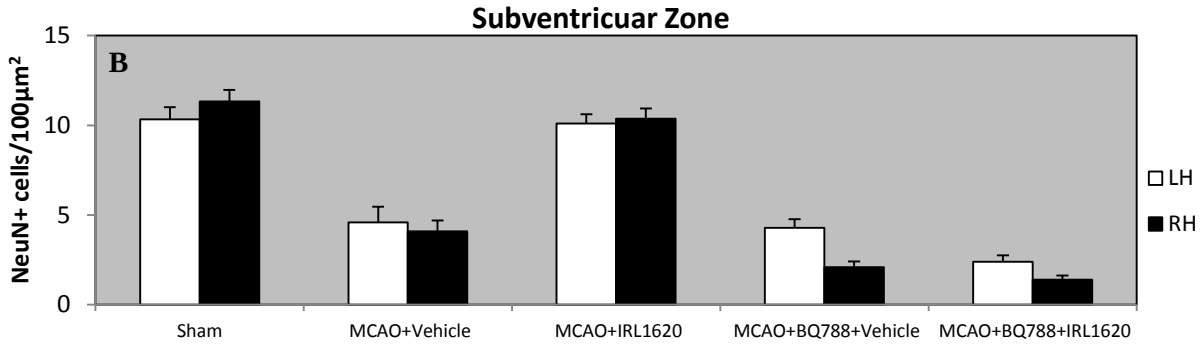
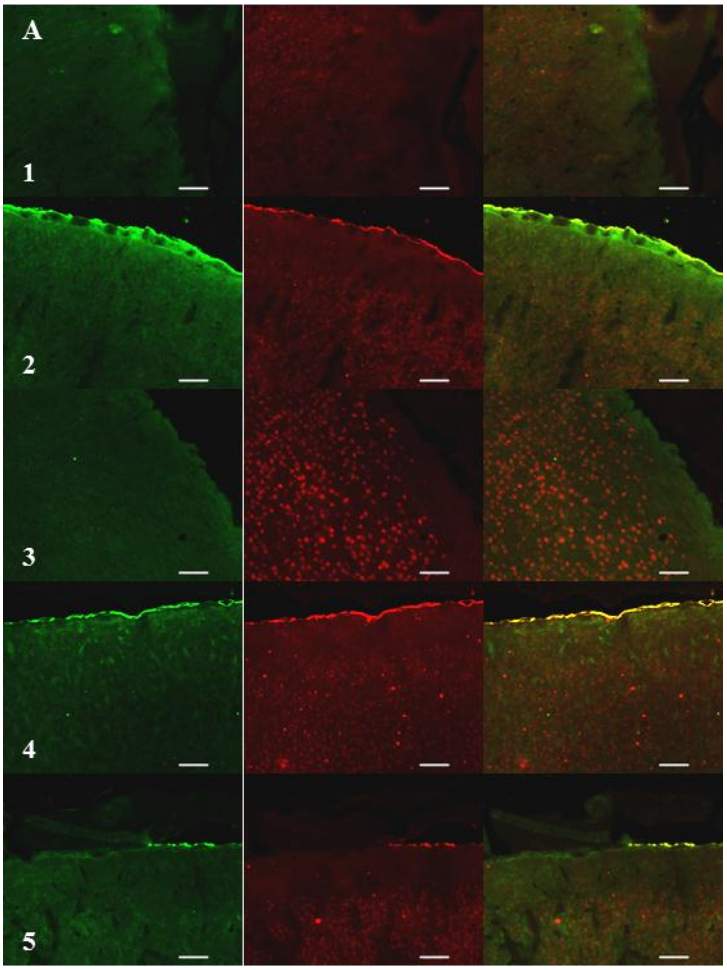


Figure 22: Effect on subventricular NeuN following acute cerebral ischemia. **A.** Representative 30 µm-thick sections of the right, infarcted subventricular zone of rats 24 h post MCAO stained for ET_B receptor (green) and NeuN (red). Scale bar = 100 µm. Rows: 1 - Sham; 2 - MCAO + Vehicle; 3 - MCAO + IRL-1620; 4 – MCAO + BQ788 + Vehicle; 5 – MCAO + BQ788 + IRL-1620. **B.** Number of mature neurons (NeuN+ cells) per 100 μm^2 in the SVZ of middle cerebral artery occluded rats at 24 h after infarct. LH = Left (normal) hemisphere; RH = Right (infarcted) hemisphere. Values are presented as mean ± SEM.



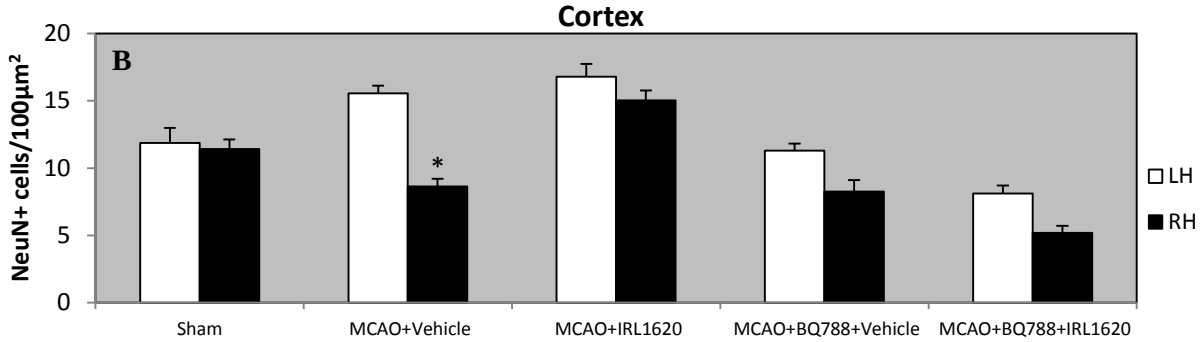
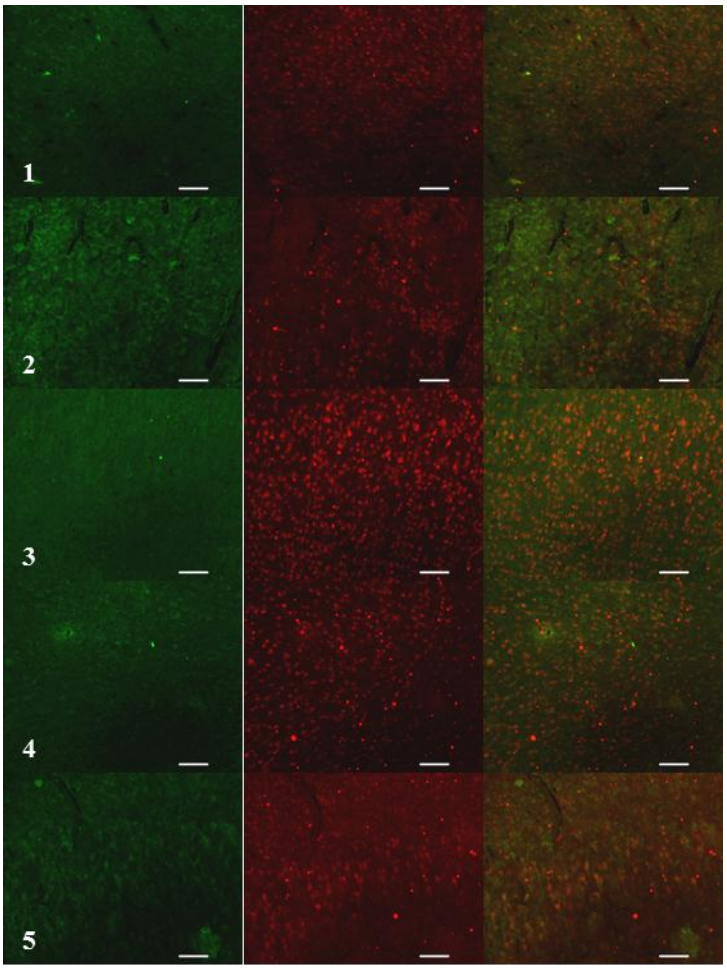


Figure 23: Effect on cortical NeuN following sub-acute cerebral ischemia. **A.** Representative 30 µm-thick sections of the right, infarcted cortex of rats 1 w post MCAO stained for ET_B receptor (green) and NeuN (red). Scale bar = 100 µm. Rows: 1 - Sham; 2 - MCAO + Vehicle; 3 - MCAO + IRL-1620; 4 – MCAO + BQ788 + Vehicle; 5 – MCAO + BQ788 + IRL-1620. **B.** Number of mature neurons (NeuN+ cells) per 100 μm^2 in the cortex of middle cerebral artery occluded rats at 1 w after infarct. LH = Left (normal) hemisphere; RH = Right (infarcted) hemisphere. Values are presented as mean ± SEM. *P<0.0001 vs. LH.



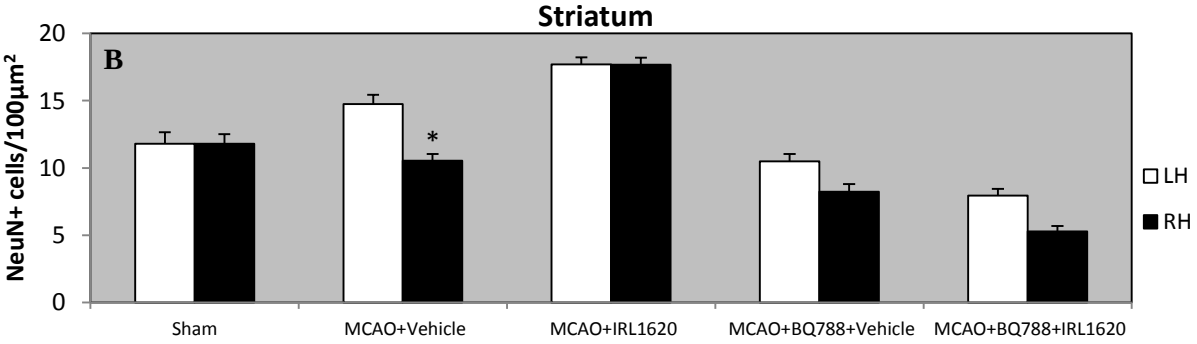
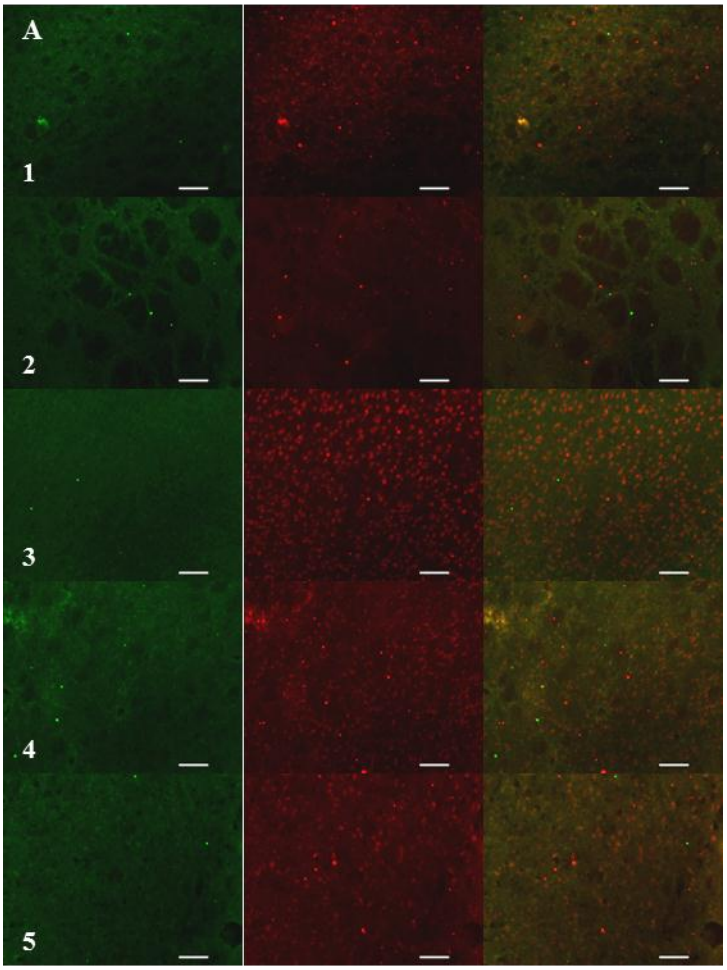


Figure 24: Effect on striatal NeuN following sub-acute cerebral ischemia. **A.** Representative 30 µm-thick sections of the right, infarcted striatum of rats 1 w post MCAO stained for ET_B receptor (green) and NeuN (red). Scale bar = 100 µm. Rows: 1 - Sham; 2 - MCAO + Vehicle; 3 - MCAO + IRL-1620; 4 – MCAO + BQ788 + Vehicle; 5 – MCAO + BQ788 + IRL-1620. **B.** Number of mature neurons (NeuN+ cells) per 100 μm^2 in the striatum of middle cerebral artery occluded rats at 1 w after infarct. LH = Left (normal) hemisphere; RH = Right (infarcted) hemisphere. Values are presented as mean ± SEM. *P<0.01 vs. LH.



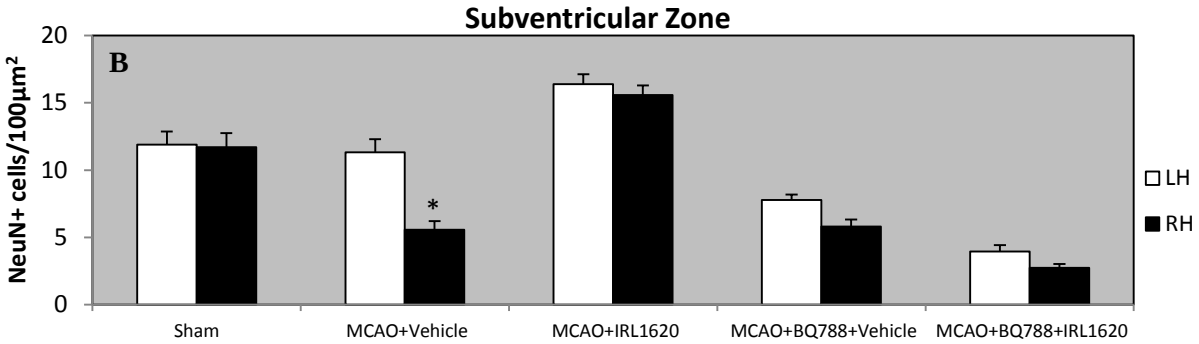
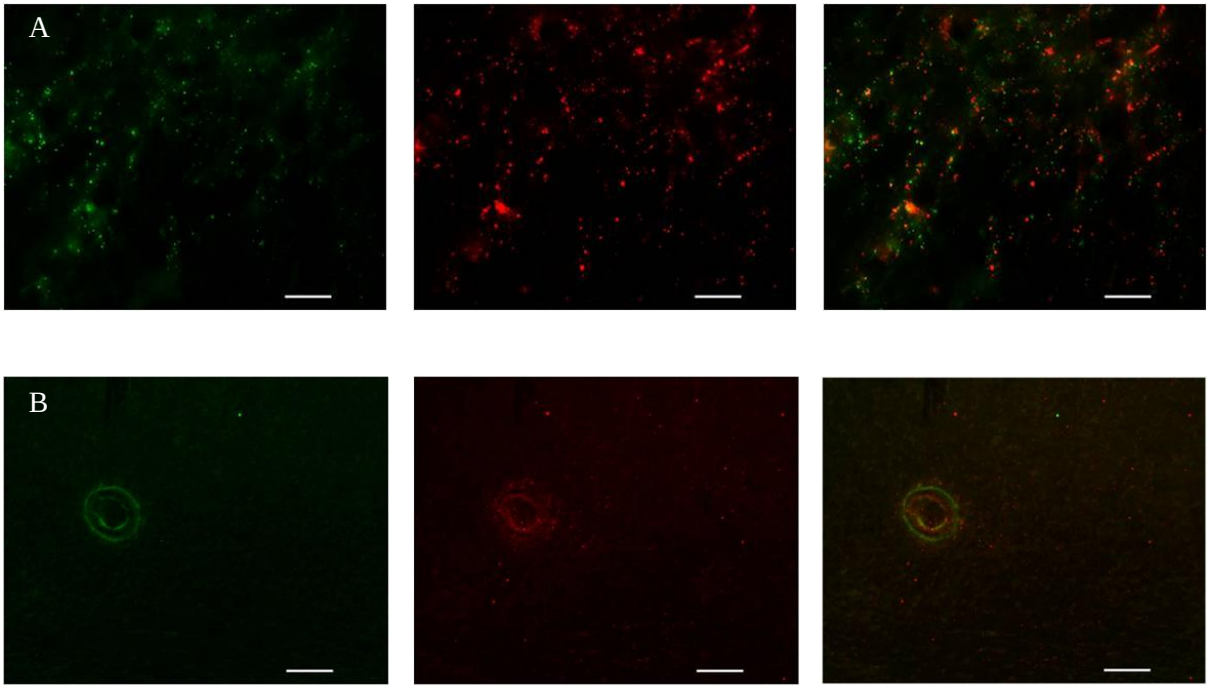


Figure 25: Effect on subventricular NeuN following sub-acute cerebral ischemia. **A.** Representative 30 µm-thick sections of the right, infarcted subventricular zone of rats 1 w post MCAO stained for ET_B receptor (green) and NeuN (red). Scale bar = 100 µm. Rows: 1 - Sham; 2 - MCAO + Vehicle; 3 - MCAO + IRL-1620; 4 – MCAO + BQ788 + Vehicle; 5 – MCAO + BQ788 + IRL-1620. **B.** Number of mature neurons (NeuN+ cells) per 100 μm^2 in the SVZ of middle cerebral artery occluded rats at 1 w after infarct. LH = Left (normal) hemisphere; RH = Right (infarcted) hemisphere. Values are presented as mean ± SEM. *P<0.001 vs. LH.

8. Effect on Proliferating Cells Following Cerebral Ischemia

In order to determine whether or not neuronal remodeling occurred as indicated by the increased NeuN+ cells in the IRL-1620-treated sub-acute MCAO group, animals in the week-long survival group were injected with bromodeoxyuridine (BrdU) 48 h prior to sacrifice. Brain slices were then stained for evidence of BrdU incorporation into the DNA of proliferating cells. Bromodeoxyuridine is a synthetic nucleoside, an analogue of thymidine, which is used to detect proliferating cells in live tissue. Because of its similarity to thymidine, BrdU can replace this nucleoside during DNA replication. Its presence can then be detected using a specific antibody and used to determine which cells were replicating during a given period of time (Kee, Sivalingam et al. 2002).

While some proliferating cells were observed in the vehicle-treated animals following MCAO, animals treated with IRL-1620 presented with significantly higher levels of BrdU incorporation ($P<0.001$; Figure 26C). IRL-1620 treated MCAO rats showed 4.63 ± 0.27, 4.24 ± 0.31, and 5.83 ± 0.36 BrdU-positive cells per 100 μm^2 in the cortex, striatum, and SVZ, respectively. Both hemispheres displayed evidence of proliferation, although proliferating cells were significantly more numerous in the infarcted hemisphere of the IRL-1620-treated animals than on the contralateral side (Figures 27-29). BQ788 blocked the effects of IRL-1620 on proliferating cells. Proliferating cells were highly co-localized with the ET_B receptor, with 45-50% of BrdU+ cells co-labeled with ET_B. Interestingly, neurons, astrocytes and blood vessels all showed proliferation, indicating that neurovascular remodeling was occurring post ischemia (Figure 26).



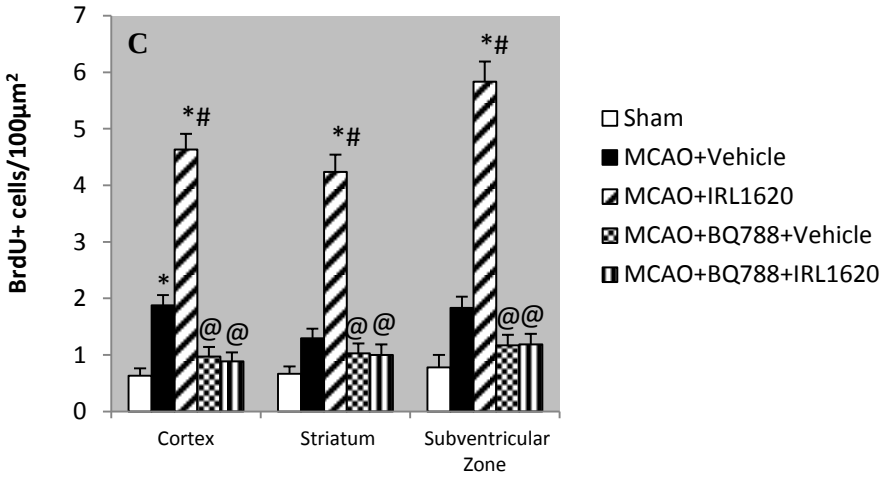
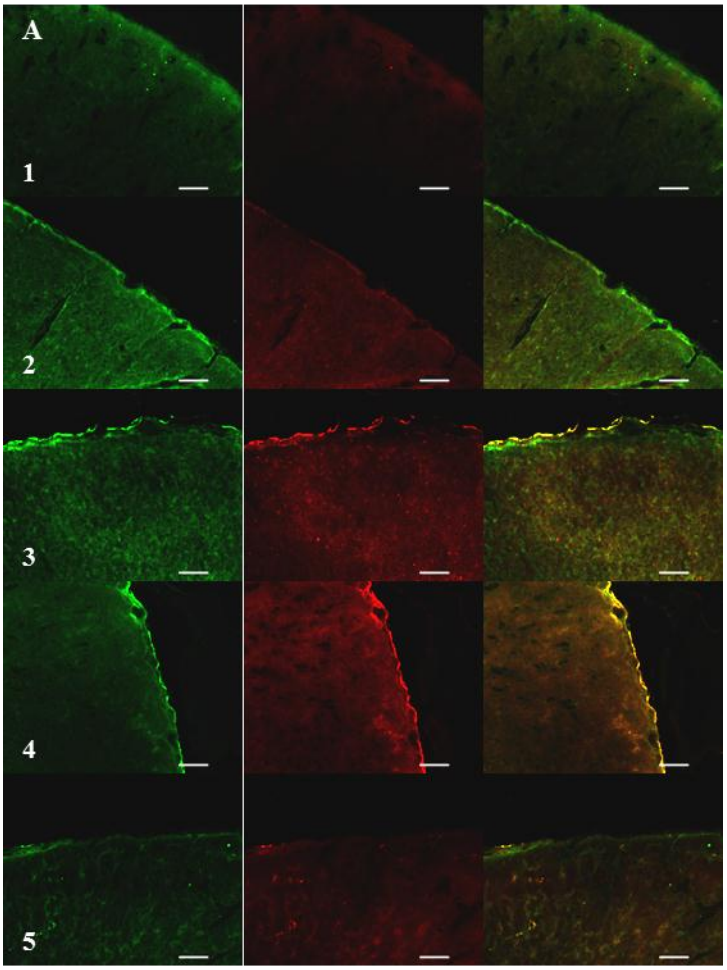


Figure 26: Effect on proliferating cells following sub-acute cerebral ischemia. **A.** Representative image of the striatum of an IRL-1620-treated animal 1 week following MCAO, stained for the ET_B receptor (green) and BrdU (red). Scale bar = 10 µm. **B.** Representative image of the cortex of an IRL-1620-treated animal 1 week following MCAO depicting a cerebral blood vessel, stained for the ET_B receptor (green) and BrdU (red). Scale bar = 100 µm. **C.** Number of proliferating cells (BrdU+) per 100µm^2 in the cortex, striatum, and subventricular zone of middle cerebral artery occluded rats at 1 week after infarct. *P<0.01 vs. sham. #P<0.0001 vs. MCAO + vehicle. @P<0.0001 vs. MCAO + IRL-1620.



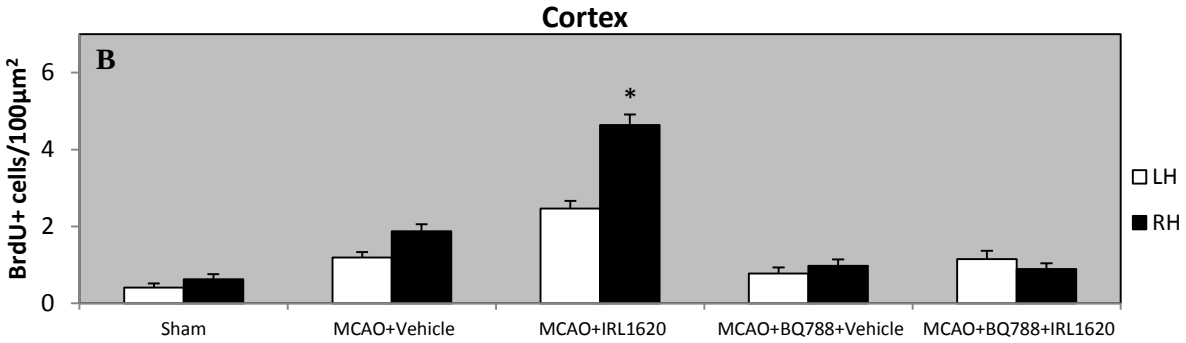
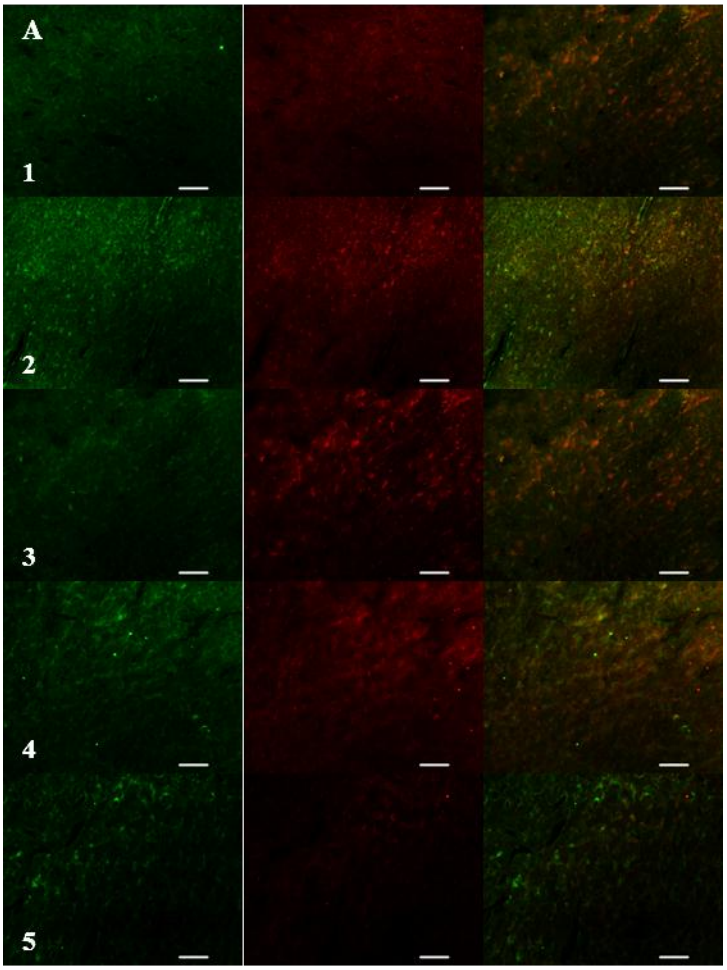


Figure 27: Effect on cortical proliferating cells following sub-acute cerebral ischemia. **A.** Representative 30 µm-thick sections of the right, infarcted cortex of rats 1 w post MCAO stained for ET_B receptor (green) and BrdU (red). Scale bar = 100 µm. Rows: 1 - Sham; 2 - MCAO + Vehicle; 3 - MCAO + IRL-1620; 4 – MCAO + BQ788 + Vehicle; 5 – MCAO + BQ788 + IRL-1620. **B.** Number of proliferating cells (BrdU+ cells) per 100 μm^2 in the cortex of middle cerebral artery occluded rats at 1 w after infarct. LH = Left (normal) hemisphere; RH = Right (infarcted) hemisphere. Values are presented as mean ± SEM. *P<0.0001 vs. LH.



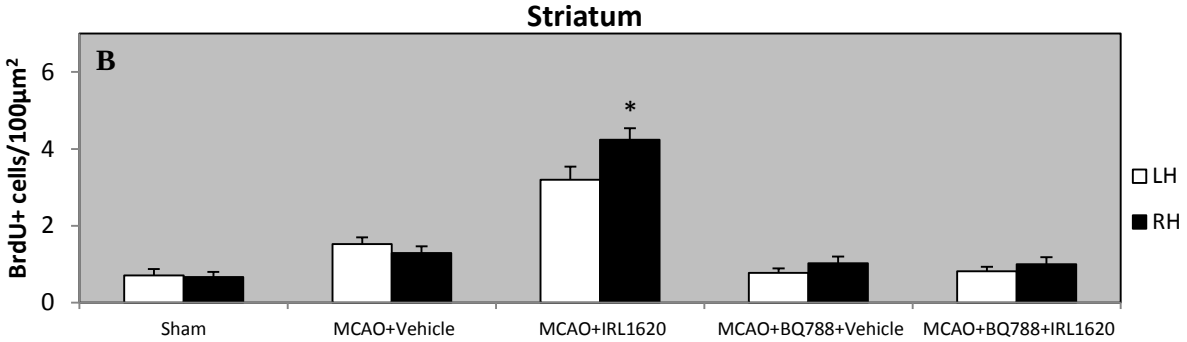
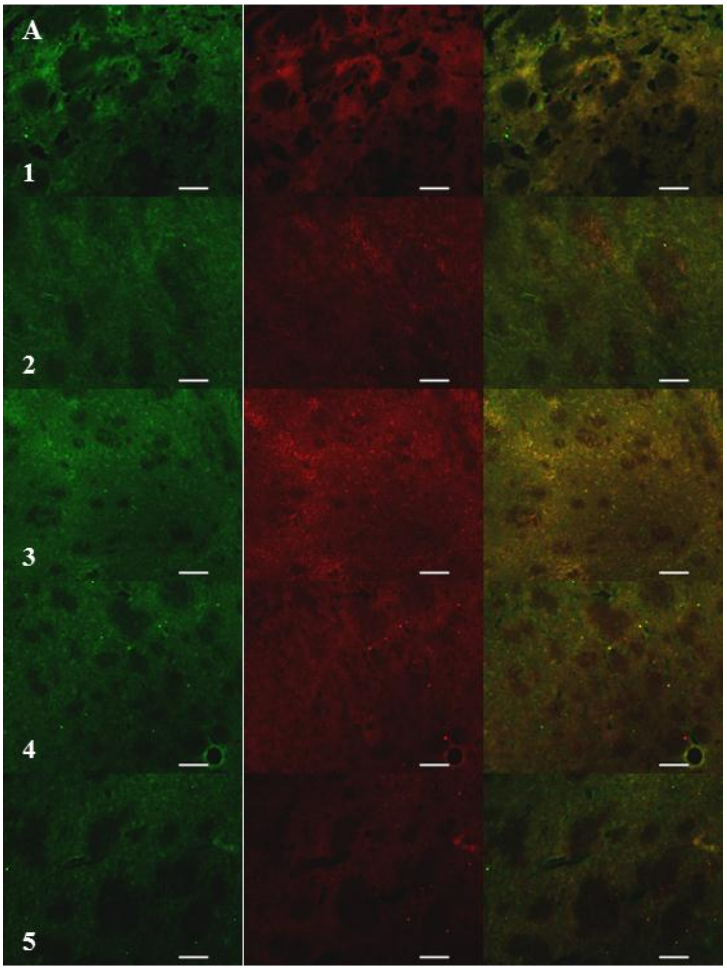


Figure 28: Effect on striatal proliferating cells following sub-acute cerebral ischemia. **A.** Representative 30 µm-thick sections of the right, infarcted striatum of rats 1 w post MCAO stained for ET_B receptor (green) and BrdU (red). Scale bar = 100 µm. Rows: 1 - Sham; 2 - MCAO + Vehicle; 3 - MCAO + IRL-1620; 4 – MCAO + BQ788 + Vehicle; 5 – MCAO + BQ788 + IRL-1620. **B.** Number of proliferating cells (BrdU+ cells) per 100 μm^2 in the striatum of middle cerebral artery occluded rats at 1 w after infarct. LH = Left (normal) hemisphere; RH = Right (infarcted) hemisphere. Values are presented as mean ± SEM. *P<0.05 vs. LH.



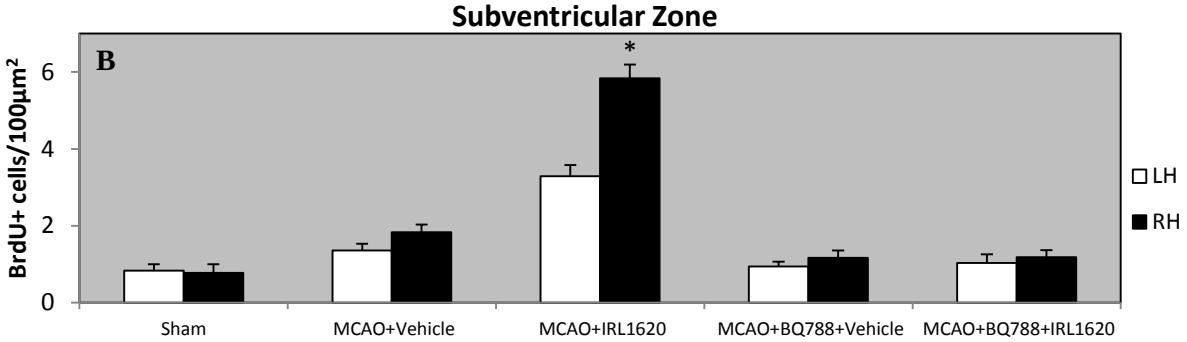
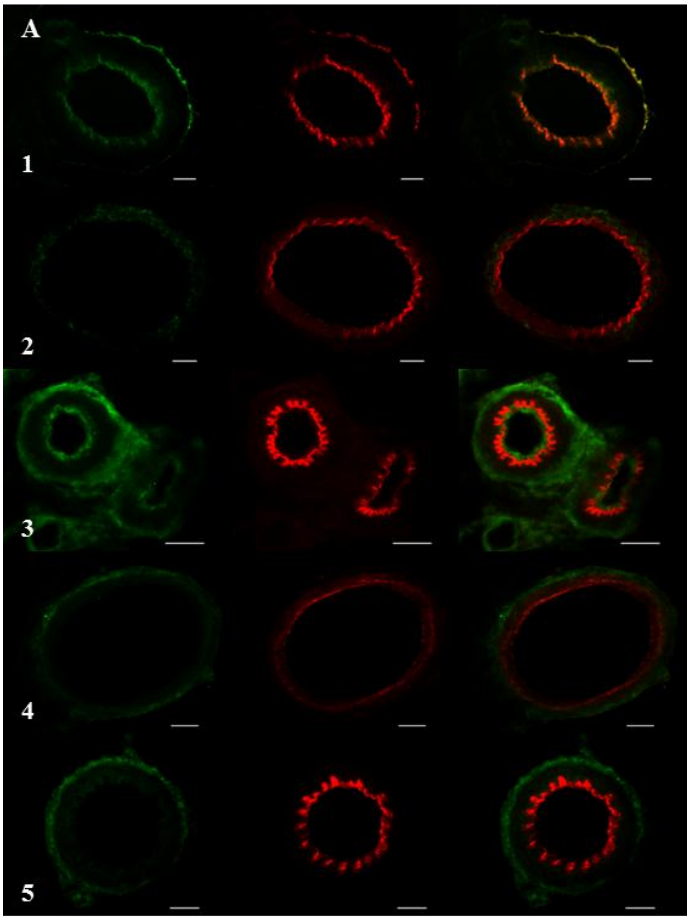


Figure 29: Effect on subventricular proliferating cells following sub-acute cerebral ischemia. **A.** Representative 30 µm-thick sections of the right, infarcted subventricular zone of rats 1 w post MCAO stained for ET_B receptor (green) and BrdU (red). Scale bar = 100 µm. Rows: 1 - Sham; 2 - MCAO + Vehicle; 3 - MCAO + IRL-1620; 4 – MCAO + BQ788 + Vehicle; 5 – MCAO + BQ788 + IRL-1620. **B.** Number of proliferating cells (BrdU+ cells) per 100 μm^2 in the SVZ of middle cerebral artery occluded rats at 1 w after infarct. LH = Left (normal) hemisphere; RH = Right (infarcted) hemisphere. Values are presented as mean ± SEM. *P<0.0001 vs. LH.

9. Effect on Vascular Endothelial Growth Factor Following Cerebral Ischemia

Following cerebral ischemia, angiogenesis, the formation of new blood vessels, may occur as a part of the innate repair and remodeling process (Góra-Kupilas and Jośko 2005). In order to determine whether or not ET_B receptor agonist IRL-1620 might affect this process and as our BrdU results indicated that blood vessels were demonstrating signs of proliferation, we examined the brain slices of ischemic rats for vascular endothelial growth factor (VEGF), both at 24 h and 1 w following MCAO.

Whereas occluded animals treated with vehicle presented with an average of 1.17 ± 0.46 VEGF+ vessels per 30 µm slice, IRL-1620 treated animals presented with an average of 4.17 ± 0.52 VEGF+ vessels/30 µm slice ($P<0.01$) as early as 24 h post MCAO (Figure 30B). This increase in the angiogenic marker remained evident up to 1 w following MCAO, with significantly more VEGF+ blood vessels in the group treated with IRL-1620 versus vehicle (11.33 ± 2.13 and 4.19 ± 0.79 VEGF+ vessels/30 µm slice, respectively; $P<0.0001$). Pretreatment with BQ788 resulted in levels of VEGF+ vessels similar to those in the vehicle-treated group, effectively blocking the actions of IRL-1620. As can be seen in Figure 30A, ET_B receptors are located both on the vascular smooth muscle surrounding the vessels and on the endothelial cells lining the interior vessel walls. At 24 h, the IRL-1620 group demonstrated a co-localization of ET_B receptors with VEGF+ endothelial cells of 35.18 ± 2.73%, while the vehicle group showed a co-localization of only 23.93 ± 4.03% (Figure 30A). At week's end, however, all groups presented with a co-localization between ET_B receptors and VEGF of approximately 40% (Figure 31A). Overall, these results indicate that ET_B receptor agonist IRL-1620 enhances the marker for angiogenesis for up to 1 w following cerebral ischemia.



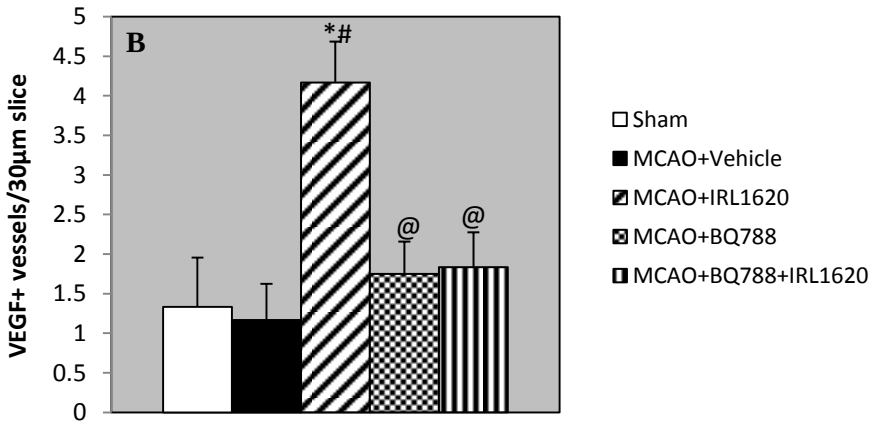
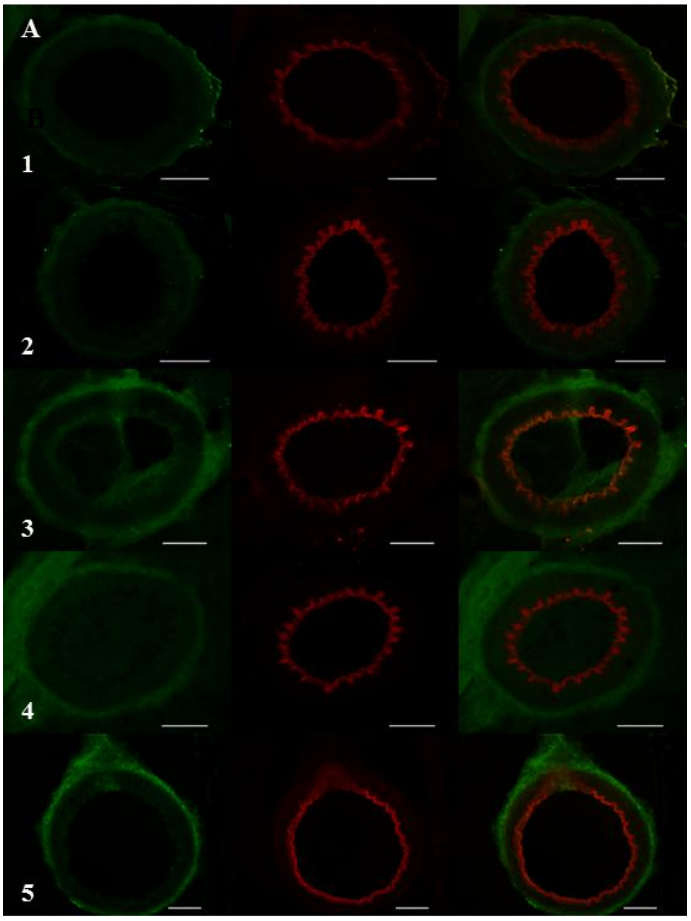


Figure 30: Effect on VEGF following acute cerebral ischemia. **A.** Representative images of blood vessels in the rat cortex 24 h following MCAO, stained for the ET_B receptor (green) and VEGF (red). Rows: 1. Sham; 2. MCAO + vehicle; 3. MCAO + IRL-1620; 4. MCAO + BQ788 + vehicle; 5. MCAO + BQ788 + IRL-1620. Scale bar = 10 µm. **B**. Number of VEGF+ vessels per 30 µm brain slice middle cerebral artery occluded rats at 24 h after infarct. *P<0.05 vs. sham. #P<0.01 vs. MCAO + vehicle. @P<0.05 vs. MCAO + IRL-1620.



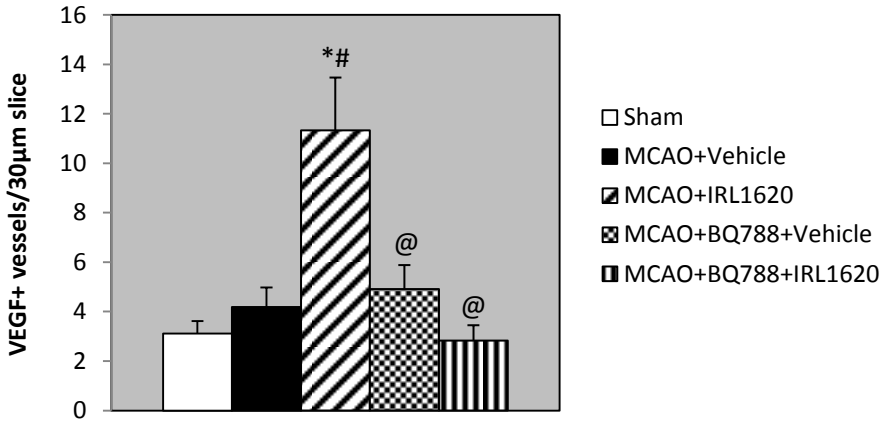
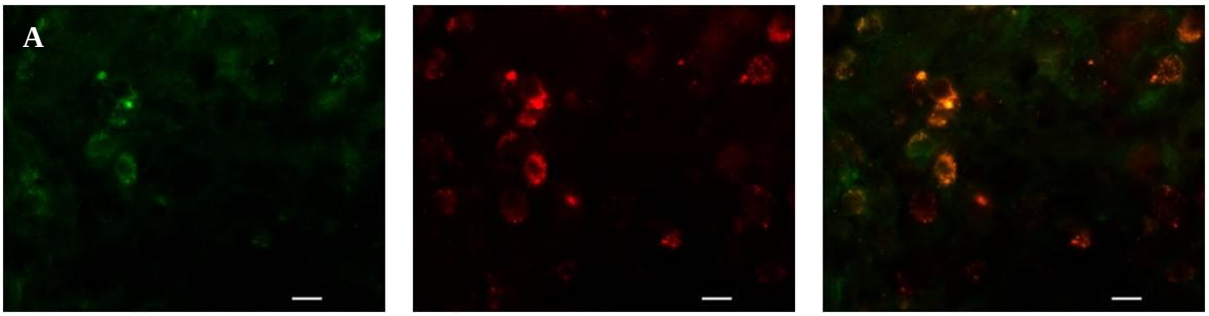


Figure 31: Effect on VEGF following sub-acute cerebral ischemia. **A.** Representative images of blood vessels in the rat cortex 1 w following MCAO, stained for the ET_B receptor (green) and VEGF (red). Rows: 1. Sham; 2. MCAO + vehicle; 3. MCAO + IRL-1620; 4. MCAO + BQ788 + vehicle; 5. MCAO + BQ788 + IRL-1620. Scale bar = 10 µm. **B.** Number of VEGF+ vessels per 30 µm brain slice middle cerebral artery occluded rats at 1 w after infarct. *P<0.01 vs. sham. #P<0.01 vs. MCAO + vehicle. @P<0.05 vs. MCAO + IRL-1620.

10. <u>Effect on Nerve Growth Factor Following Cerebral Ischemia</u>

In addition to angiogenesis, neurogenesis may occur in the recovering ischemic brain (Minger, Ekonomou et al. 2007). Our BrdU immunofluorescent results indicated that not only were blood vessels demonstrating new growth, but that neurons were also proliferating. In order to investigate this potential neurogenesis and its possible relation to ET_B receptors, we stained the ischemic brain slices for nerve growth factor (NGF), co-labeled with an ET_B receptor antibody.

Consistent with the proliferating cell data, the group treated with IRL-1620 presented with 2.29 ± 0.31, 2.08 ± 0.26, and 3.05 ± 0.38 NGF+ cells per 100 μm^2 in the cortex, striatum, and SVZ 1 w following induction of permanent cerebral ischemia (Figure 32B). These numbers were significantly greater than those in the sham-operated and vehicle- and BQ788-treated groups ($P<0.0001$). When comparing the hemispheres, significantly more NGF+ cells were present in cortex, striatum and SVZ of the right, infarcted hemisphere ($P<0.001$; Figures 33-35). As with the BrdU staining, co-localization between NGF and ET_B receptors occurred approximately 45-50% of the time (Figure 32A). These results confirm that treatment with ET_B receptor agonist IRL-1620 post cerebral ischemia enhances neurogenic remodeling of the damaged brain tissue.



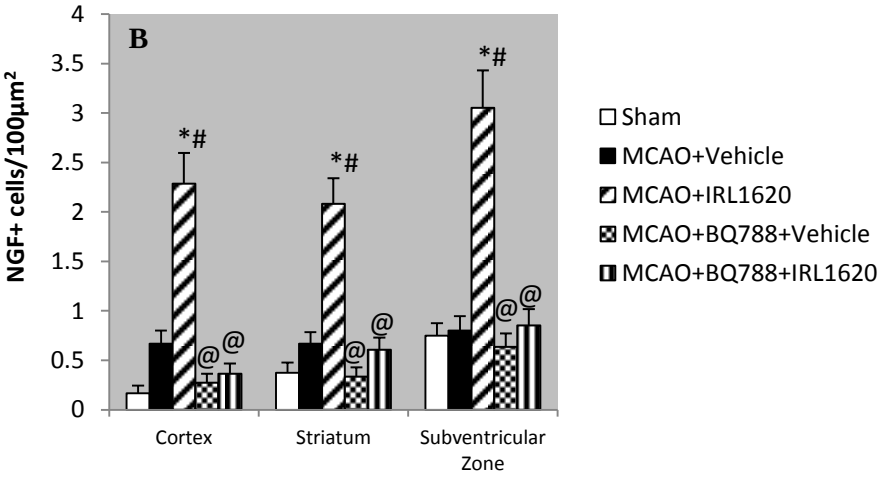
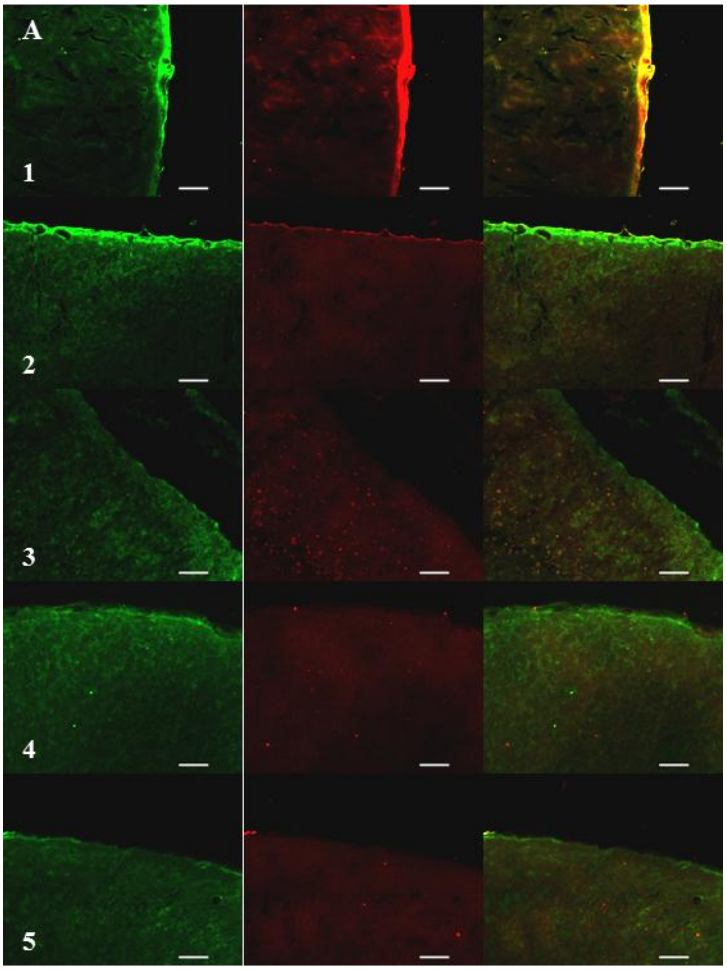


Figure 32: Effect on nerve growth factor following sub-acute cerebral ischemia. **A.** Representative image of the cortex of an IRL-1620-treated animal 1 week following MCAO, stained for the ET_B receptor (green) and NGF (red). Scale bar = 10 µm. **B.** Number of NGF+ cells per 100µm^2 in the cortex, striatum, and subventricular zone of middle cerebral artery occluded rats at 1 week after infarct. Values are presented as mean ± SEM. *P<0.0001 vs. sham. #P<0.0001 vs. MCAO + vehicle. @P<0.0001 vs. MCAO + IRL-1620.



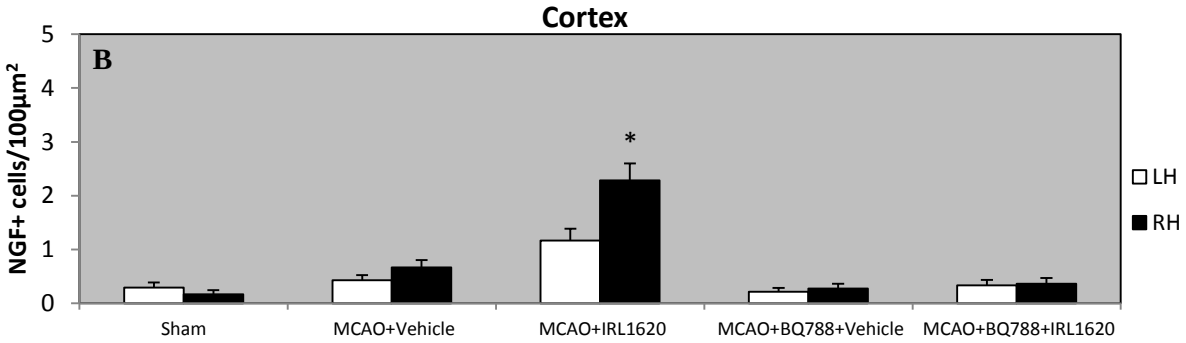
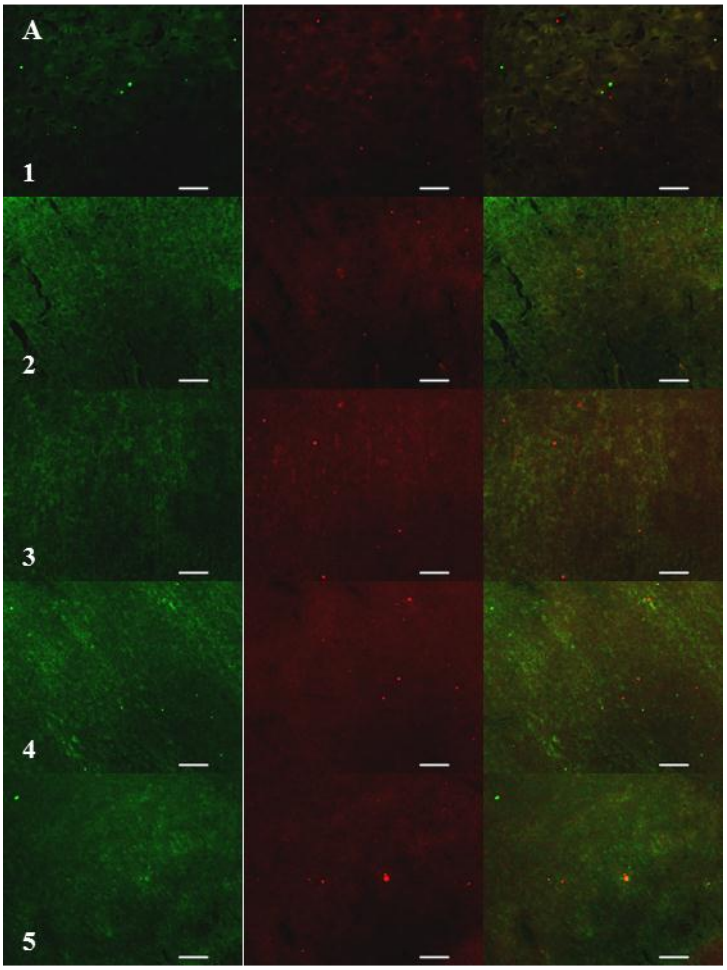


Figure 33: Effect on cortical NGF following sub-acute cerebral ischemia. **A.** Representative 30 µm-thick sections of the right, infarcted cortex of rats 1 w post MCAO stained for ET_B receptor (green) and NGF (red). Scale bar = 100 µm. Rows: 1 - Sham; 2 - MCAO + Vehicle; 3 - MCAO + IRL-1620; 4 – MCAO + BQ788 + Vehicle; 5 – MCAO + BQ788 + IRL-1620. **B.** Number of new neurons (NGF+ cells) per 100 μm^2 in the cortex of middle cerebral artery occluded rats at 1 w after infarct. LH = Left (normal) hemisphere; RH = Right (infarcted) hemisphere. Values are presented as mean ± SEM. *P<0.001 vs. LH.



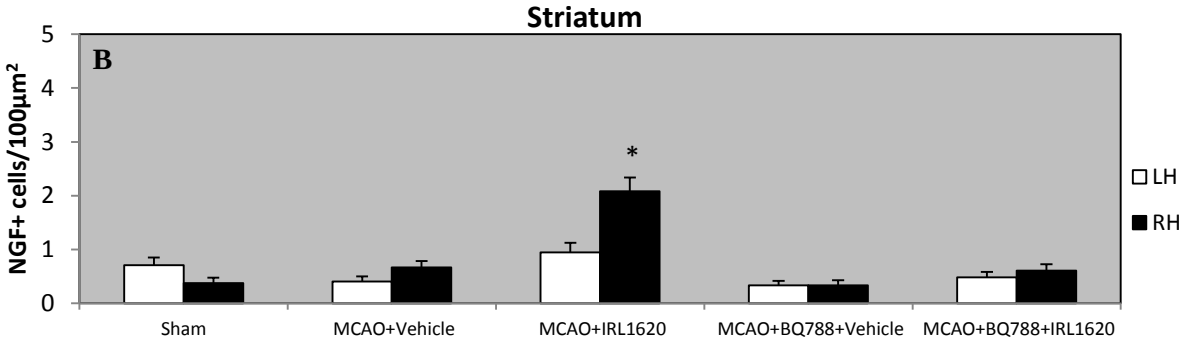
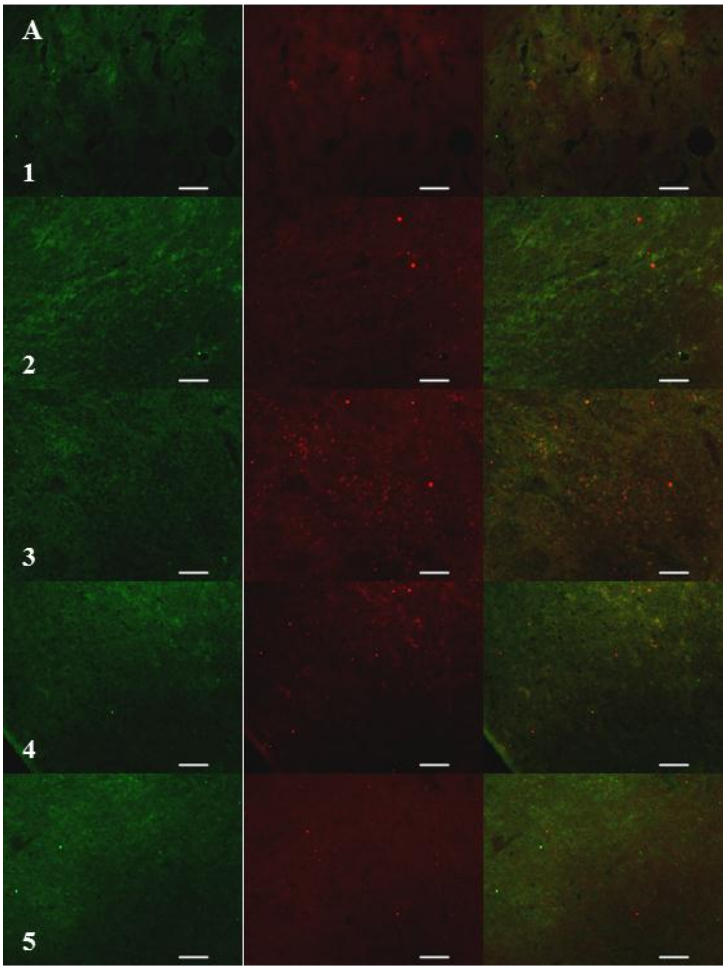


Figure 34: Effect on striatal NGF following sub-acute cerebral ischemia. **A.** Representative 30 µm-thick sections of the right, infarcted striatum of rats 1 w post MCAO stained for ET_B receptor (green) and NGF (red). Scale bar = 100 µm. Rows: 1 - Sham; 2 - MCAO + Vehicle; 3 - MCAO + IRL-1620; 4 – MCAO + BQ788 + Vehicle; 5 – MCAO + BQ788 + IRL-1620. **B.** Number of new neurons (NGF+ cells) per 100 μm^2 in the striatum of middle cerebral artery occluded rats at 1 w after infarct. LH = Left (normal) hemisphere; RH = Right (infarcted) hemisphere. Values are presented as mean ± SEM. *P<0.001 vs. LH.



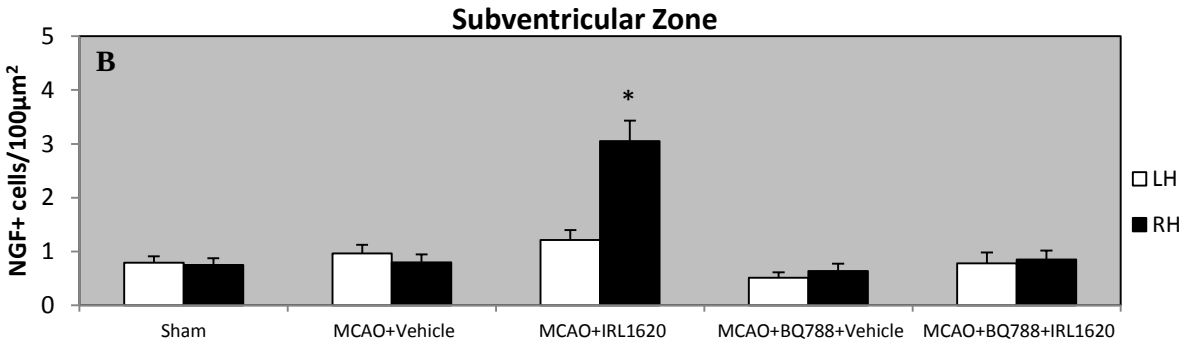


Figure 35: Effect on subventricular NGF following sub-acute cerebral ischemia. **A.** Representative 30 µm-thick sections of the right, infarcted subventricular zone of rats 1 w post MCAO stained for ET_B receptor (green) and NGF (red). Scale bar = 100 µm. Rows: 1 - Sham; 2 - MCAO + Vehicle; 3 - MCAO + IRL-1620; 4 – MCAO + BQ788 + Vehicle; 5 – MCAO + BQ788 + IRL-1620. **B.** Number of new neurons (NGF+ cells) per 100 μm^2 in the SVZ of middle cerebral artery occluded rats at 1 w after infarct. LH = Left (normal) hemisphere; RH = Right (infarcted) hemisphere. Values are presented as mean ± SEM. *P<0.0001 vs. LH.

V. DISCUSSION

The overarching purpose of this study was to determine the involvement of central endothelin B receptors in focal cerebral ischemia, in hopes of both further characterizing the elements involved in the pathophysiology and potentially identifying novel therapeutic targets for the treatment of this devastating disease. In line with these goals, we examined the effects of stimulation and blockade of ET_B receptors in a permanent model of middle cerebral artery occlusion in rats. We observed the influence of ET_B receptor agonist, IRL-1620, and ET_B receptor antagonist, BQ788, on neurological damage, oxidative stress, and neurovascular remodeling following both the acute and sub-acute phases of cerebral ischemia.

Amongst the first signs and symptoms of an ischemic stroke in humans are a weakness in the limbs and face, along with slurred speech. Similarly, experimental cerebral ischemia leads to a marked deficit in neurological and motor function, characterized by a weakness or paralysis of the limbs and a lack of coordinated movement. The progression of these symptoms is an indicator not only of the extent of the ischemic damage, but also of the potential for recovery. Our results clearly show that stimulation of ET_B receptors by administration of selective agonist IRL-1620 following the induction of cerebral ischemia leads to a dramatic improvement in neurological and motor function. This improvement is evident as early as 24 h post infarct (Leonard, Briyal et al. 2011). Animals receiving IRL-1620 treatment not only showed fewer deficits following the acute phase of cerebral ischemia, but also demonstrated a gradual improvement in muscular strength and coordination during the sub-acute phase (Leonard, Briyal et al. 2012). In fact, the motor function of animals that had undergone MCAO followed by only a single day of treatment with the ET_B agonist, were, by the end of the week, on par with the motor function of the sham-operated animals that had not received a stroke. Conversely, animals

that had been treated with ET_B antagonist BQ788 demonstrated no improvement in motor skills, thus confirming that the observed beneficial effects of IRL-1620 were due to the stimulation of ET_B receptors.

While a reduction in neurological and motor deficit is a good indicator of neuroprotection, the volume of the lesion is also important. Using TTC staining, we were able to clearly demarcate the extent of ischemic damage within the brains of rats undergoing middle cerebral artery occlusion. Whereas animals receiving only vehicle treatment presented with large hemispheric infarcts, animals that were treated with IRL-1620 presented with an approximately 85% reduction in infarct volume following the acute phase of cerebral ischemia (Leonard, Briyal et al. 2011). The infarct volumes of IRL-1620-treated rats remained significantly smaller than those of vehicle-treated rats throughout the sub-acute phase of ischemic stroke (Leonard, Briyal et al. 2012). Additionally, while the vehicle- and BQ788-treated groups presented with significant edematic formation, no edema was noted in the IRL-1620 group, indicating that the blood brain barrier remained intact. This preservation of the BBB may be explained at least in part by the fact that stimulation of central ET_B receptors decreases expression of aquaporin-4, a type of water channel protein important in regulating water homeostasis in the CNS (Koyama and Tanaka 2010). The formation of edema in the groups not receiving the ET_B receptor agonist in the present study led to several animals developing post-infarct seizures, a condition which is also noted in humans following ischemic stroke, often resulting in a poor recovery or even death (Ferro and Pinto 2004). Blockade of ET_B receptors with BQ788 also resulted in infarct volumes similar to those in the vehicle-treated group, further confirming that the beneficial effects seen with IRL-1620 were due to its actions on ET_B receptors. In an attempt to determine whether IRL-1620 could also be working through

ET_A receptors and/or whether a combination of ET_A receptor antagonism with ET_B receptor agonism would be synergistic, we pretreated rats with ET_A antagonist BQ123 followed by either vehicle or IRL-1620. Rats treated with BQ123 followed by vehicle showed some improvement in neurobehavioural parameters as well as a reduction in infarct volume, agreeing with previous reports (Briyal and Gulati 2010). Nevertheless, the efficacy of the ET_A antagonist appeared to be less than that of the ET_B agonist. Additionally, no synergism of beneficial effect was detected with the combination of BQ123 and IRL-1620. These results seem to indicate that IRL-1620 does not act directly through ET_A receptors, although the possibility of allosteric modulation cannot be excluded. The reduction in infarct volume taken together with the improvement in neurological and motor function seen in the IRL-1620-treated groups indicate, for the first time, that selective stimulation of ET_B receptors leads to physical and functional recovery following cerebral ischemia.

In an attempt to elicit the mechanism of action for IRL-1620's apparent neuroprotection, we investigated its effects on ET receptor levels as well as oxidative stress parameters in the rat brain following the acute and sub-acute phases of experimental cerebral ischemia. Following the acute phase, 24 h post occlusion, all ischemic brains demonstrated an increase in ET_A receptors in the infarcted hemisphere regardless of treatment. Conversely, ET_B receptor expression remained unaltered (Leonard, Briyal et al. 2011). The lack of ET_B receptor up-regulation seen in our study conflicts with previous reports which indicated an increase in ET_B receptors in the cerebrovasculature of rats subject to ischemia (Stenman, Malmsjo et al. 2002, Henriksson, Stenman et al. 2003). This discrepancy may be explained by the fact that different models of ischemia were used, permanent versus transient MCAO, and that the previously observed up-regulation occurred within the vasculature specifically. We observed no change in ET_B receptor

expression within the infarcted hemisphere as a whole, but there may have been a localized up-regulation that may not have been seen with our method. Interestingly, we discovered that the increase in ET_A receptors following acute cerebral ischemia was a transient phenomenon, with levels of these receptors returning to normal by the end of one week of permanent ischemia (Leonard, Briyal et al. 2012). Thus, while it would appear that ET_A receptors may be involved in the pathophysiology of the acute phase of ischemic stroke, this particular disruption in the ET system is resolved by the seventh day post infarction. This concept is somewhat supported by previous reports indicating that the elevation in ET-1 levels that occurs acutely following cerebral ischemia also appears to resolve over time, with ET-1 levels being within the normal range at 1 week, 1 month and 3 months post infarct (Haapaniemi, Tatlisumak et al. 2000). Following the sub-acute phase, however, we found that ET_B receptors were up-regulated, but only in the infarcted hemisphere of the IRL-1620-treated group (Leonard, Briyal et al. 2012). It is possible that, as has been previously suggested, ET_B receptors play a role in survival mechanisms following cerebral ischemia (Kreipke, Reynolds et al. 2011). Our limitation at this point in the study was that we were uncertain of the location of these up-regulated ET_B receptors. Studies were therefore planned to determine the location of ET_B receptors at both the acute and sub-acute stages of ischemic stroke and to attempt to further elucidate the effects that these receptors had on various cell types (i.e. astrocytes, neurons and vascular endothelial cells).

Prior to our attempt to locate central ET_B receptors via immunochemical methods, however, we examined one of the possible mechanisms for IRL-1620's apparent neuroprotective effects – namely, changes in oxidative stress parameters. Oxidative stress occurs as part of the ischemic cascade and contributes greatly to secondary ischemic damage to the penumbra. Oxidative stress is defined as an imbalance of oxidants and antioxidants within the ischemic

brain. Oxidants such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (-OH) are produced as antioxidants such as superoxide dismutase (SOD) and reduced glutathione (GSH) are depleted. This, in turn, causes damage to the membranes of both the cells and the mitochondria by lipid peroxidation, contributing to inflammation and apoptosis (Lakhan, Kirchgessner et al. 2009). The ischemic cascade and oxidative stress are hallmarks of the acute phase of cerebral ischemia, and, as such, are generally considered to be prime targets for therapeutic, neuroprotective treatment. In this study, we found that treatment with IRL-1620 effectively reduced oxidative stress during the acute phase of ischemic stroke, lowering levels of lipid peroxidation while simultaneously increasing levels of SOD and GSH (Leonard, Briyal et al. 2011). Oxidative stress is generally considered to last throughout the acute phase, with a gradual return to homeostasis. Instead, we found that the levels of MDA remained high and antioxidants SOD and GSH remained low in the vehicle- and BQ788-treated groups for up to one week following the onset of cerebral ischemia, i.e. during the sub-acute phase. Treatment with ET_B agonist IRL-1620 on the first day of ischemia, however, was able to restore the balance between oxidants and antioxidants within the ischemic rat brain for up to one week after infarct (Leonard, Briyal et al. 2012). This reduction in oxidative stress, combined with the reduction in infarct volume and the improvement in neurological and motor function all point to the idea that selective ET_B receptor stimulation is neuroprotective in cerebral ischemia. The mechanism of action for this neuroprotection, however, still remains to be elucidated.

Endothelin B receptors are known to be located on the cerebrovasculature as well as on neurons and astrocytes. Our results indicated an up-regulation of these receptors within the sub-acute ischemic brain, while others have shown an up-regulation as early as the acute phase (Stenman, Malmsjo et al. 2002, Henriksson, Stenman et al. 2003, Leonard, Briyal et al. 2012). It

was thought that determining where these receptors show increased expression in the various phases of cerebral ischemia might help clarify the mechanisms by which the ET_B receptors enhance neuroprotection.

Indeed, in confirmation of the neuroprotective effect of ET_B receptor stimulation, neuronal numbers in the cortex, striatum, and SVZ were preserved after 24 h of infarct in the IRL-1620 group. Additionally, there were fewer reactive astrocytes in this group. The conversion of astrocytes into their reactive form occurs in response to excitotoxicity and oxidative stress following cerebral ischemia (Lakhan, Kirchgessner et al. 2009). Intriguingly, the numbers of reactive astrocytes in the infarcted hemisphere of the IRL-1620 group increased to the levels seen in the vehicle- and BQ788-treated groups by the end of the sub-acute phase of cerebral ischemia. It would therefore appear that stimulation of ET_B receptors following cerebral ischemia results in a delayed conversion of astrocytes to their reactive form. This delay may be a result of the lower levels of oxidative stress which we previously demonstrated following treatment of cerebral ischemia with IRL-1620 (Leonard, Briyal et al. 2011, Leonard, Briyal et al. 2012). While lower oxidative stress is indicative of less inflammation, this does not explain the late rise in astrocytes within the IRL-1620 treatment group. In fact, our results indicate that oxidative stress remains low throughout the sub-acute phase and that inflammation is minimal in this group due to a lack of edematic formation. The question, therefore, is why there is a delay in the conversion of astrocytes to their reactive form when ET_B receptors are stimulated following cerebral ischemia. It may be that, while the initial rise in reactive astrocytes seen in the vehicle and BQ788-treated groups is due to the early oxidative stress reaction, the later conversion of these cells in the IRL-1620-treated group occurs as part of the repair process, regulating

inflammatory actions and potentially encouraging neurovascular genesis (Li, Lundkvist et al. 2008, Ekdahl, Kokaia et al. 2009).

Additionally, it should be noted that increased levels of ET-1 seen after infarct are known to promote inflammation by recruiting inflammatory cells and enhancing the release of inflammatory mediators, leading to further breakdown of the BBB (McCarron, Wang et al. 1993, Zidovetzki, Chen et al. 1999, Trevisi, Bova et al. 2002). One of the main physiological roles of ET_B receptors is to clear excess ET-1 from the system (Ozaki, Ohwaki et al. 1995, Hasselblatt, Lewczuk et al. 2001). At the same time, intracerebroventricular (i.c.v.) administration of an ET_B receptor agonist has been shown to increase production of tissue inhibitors of matrix metalloproteinases in normal rats (Koyama, Baba et al. 2007). These inhibitors are capable of counteracting the rise in matrix metalloproteinases which is seen following nerve damage and leads to breakdown of the extracellular matrix, edema and increased inflammation (Crocker, Pagenstecher et al. 2004). Stimulation of ET_B receptors on astrocytes may result in numerous beneficial actions, from clearance of excess ET-1 to the release of anti-inflammatory molecules to direct regulation of gap junctions, all resulting in a neuroprotective effect and decrease in inflammation and oxidative stress following cerebral ischemia.

While there were no significant differences in the percentage of reactive astrocytes co-localizing with ET_B receptors between groups, with approximately one-third of all GFAP+ cells co-labeled with ET_B receptors, the overall area covered by ET_B receptors did increase from the acute to the sub-acute phase of cerebral ischemia. This increase in ET_B receptor immunoreactivity was concurrent with the rise in reactive astrocytes and consistent with previous findings. Intriguingly, co-localization with neuronal cells following acute ischemia was higher in the IRL-1620-treated group than in the vehicle- or BQ788-treated groups, although the

expression of ET_B receptors in the IRL-1620 group was no higher than that of sham. During the later phase of stroke, however, all groups demonstrated similar levels of co-localization between mature neurons and ET_B receptors, although overall levels of ET_B receptors were higher in the IRL-1620-treated group. This increased immunoreactivity of ET_B receptors in the sub-acute phase of cerebral ischemia within the IRL-1620 group agrees with our earlier immunoblotting data. The combination of an increase in reactive astrocytes as well as a change in neuronal numbers may account for the observed increased expression of ET_B receptors.

Indeed, along with the rise in astrocytic conversion, we found that the number of neuronal cells increased during the sub-acute phase of cerebral ischemia in animals treated with IRL-1620. In order to confirm that this increase in neurons was due to proliferation and not simply a natural variation in neuronal numbers between animals, we examined the number of proliferating cells in the brains of animals subject to weeklong MCAO. Immunostaining for BrdU incorporation indicated that significant proliferation was indeed occurring, not only amongst neuronal cells and astrocytes, but also within the cerebrovasculature of animals treated with ET_B receptor agonist IRL-1620. These results led us to investigate whether or not IRL-1620 treatment following cerebral ischemia might be enhancing neuroprotection and the innate repair processes by increasing vascular and/or neuronal growth factors.

Vascular endothelial growth factor (VEGF) is an endogenous protein known for its ability to promote angiogenesis and enhance vascular permeability. It exerts its effects by binding to tyrosine-kinase receptors VEGFR-1 and VEGFR-2, which are located mainly on vascular endothelial cells. Expression of VEGF is induced under hypoxic condition such as cerebral ischemia in neurons, astrocytes and endothelial cells via hypoxia-inducible factor-1 (HIF-1) (Breier and Risau 1996). While recent research has shown that i.c.v. administration of

an ET_B receptor agonist in normal rats stimulates production of VEGF and activates VEGF receptors in the brain, nothing was known of the effect ET_B receptor stimulation would have on cerebral VEGF expression under ischemic conditions until now (Koyama, Nagae et al. 2011). In the present study, we show for the first time that intravenous administration of ET_B receptor agonist IRL-1620 following cerebral ischemia leads to an increase in VEGF+ cerebral blood vessels for up to one week following infarct.

Vascular endothelial growth factor initiates direct and indirect neuroprotective actions, inhibiting apoptosis, stimulating neurogenesis and angiogenesis, increasing glucose uptake and activating antioxidants (Góra-Kupilas and Jośko 2005). Triggering of the extracellular signal-regulated kinase (ERK) and mitogen-activated protein kinase (MAPK) signaling pathways occurs when VEGF binds to its receptor VEGFR-2. These pathways are, in turn, responsible for the neuroprotective and proliferative effects of VEGF (Nowacka and Obuchowicz 2012). Antagonism of ET_B receptors, on the other hand, blocks proliferation and increases apoptosis while down-regulating the ERK/MAPK pathways. These effects occur without altering VEGF mRNA levels (Paolillo, Russo et al. 2010). It has, however, previously been shown that blockade of ET_B receptors is capable of preventing the HIF-1-mediated VEGF up-regulation (Spinella, Rosanò et al. 2007). Conversely, in this study, we demonstrate that stimulation of ET_B receptors enhances the up-regulation of VEGF following cerebral ischemia, while simultaneously increasing proliferation and promoting neuroprotection.

Not only do both VEGF and ET_B receptors appear to promote survival and neuroprotection, they are also implicated in the enhancement of proliferation and migration of neuronal cells and astrocytes. During development, VEGF serves as a paracrine activator of endothelial cell survival and angiogenesis and as an autocrine and paracrine activator of neuronal

survival (Nowacka and Obuchowicz 2012). By forming new blood vessels, VEGF promotes the delivery of nutrients and oxygen to the developing nervous system. This enriched environment, along with direct activation of certain signaling pathways, helps to both encourage and regulate neuronal and astrocytic proliferation and migration (Autiero, De Smet et al. 2005, Ruiz de Almodovar, Lambrechts et al. 2009). Similarly, ET_B receptors are known to be a necessary component of the developing nervous system. Endothelin B receptors play a role in the differentiation, proliferation and migration of neural cells during pre- and post-natal development, helping to assure the proper formation of melanocytes, neurons and glia of the enteric as well as the central nervous system (Tsaur, Wan et al. 1997, Riechers, Knabe et al. 2004, Druckenbrod, Powers et al. 2008). Indeed, complete absence of these receptors leads to CNS pathologies along with aganglionosis of the gut in animals, and is used as a model for human Hirschsprung's disease (Dembowski, Hofmann et al. 2000, Riechers, Knabe et al. 2004). A recent ontogeny study performed in our lab showed that rats display a high level of ET_B receptors within the CNS until the 21st day after birth, suggesting that these receptors play a significant role in the early post-natal development of the CNS (Briyal, Lavhale et al. 2012). For the purposes of this study, however, we were interested in determining whether the central ET_B receptors could be involved in the proliferation and/or migration of adult neural cells.

Our early results which showed an increase in neuronal nuclei and proliferating cells in the ischemic rat brain following treatment with ET_B receptor agonist IRL-1620 led us to investigate the effects of this treatment on the expression of nerve growth factor (NGF). As suggested by our previous data, we did indeed demonstrate an increase in the number of cells staining positive for NGF in the cortex, striatum and SVZ of rats 1 week following the induction of permanent cerebral ischemia treated with IRL-1620. Nearly one-half of both BrdU+

proliferating cells and NGF+ cells demonstrated co-localization with ET_B receptors, a higher concentration than that seen in any other cell type investigated. These new, proliferating cells may therefore be the source of the increase expression in ET_B receptors within the infarcted brain treated with IRL-1620 which was seen with immunoblotting. Previous reports have shown the presence of ET_B receptors in the neuronal stem cell niches and indicated that i.c.v. administration of an ET_B receptor agonist was capable of increasing growth factors such as brain-derived neurotrophic factor, glial-derived neurotrophic factor, and neurotrophin-3 in normal rats (Koyama, Tsujikawa et al. 2003, Koyama, Baba et al. 2005, Castaneda, Cubilla et al. 2010). This, however, is the first report indicating that selective stimulation of ET_B receptors is capable of enhancing neurogenic remodeling in a neurodegenerative pathology such as ischemic stroke.

Migration of neuronal stem cells and neuroblasts from the SVZ outward toward the peri-infarct areas of the striatum and cortex are thought to occur along glial pathways, which coincides with the delayed conversion of reactive astrocytes as seen in the IRL-1620-treated animals following cerebral ischemia (Thored, Heldmann et al. 2009). Additionally, recruitment of existing pathways from the contralateral, non-infarcted hemisphere may have played a role in the observed neurovascular remodeling (Kidd 2009). This could explain the fact that little difference was noted between hemispheres, with fewer neurons overall in the vehicle- and BQ788-treated groups, and more in the IRL-1620 treated group. The cross-talk between hemispheres may occur along the corpus collosum, a tract of white matter which spans both hemispheres and is thought to allow communication between both sides of the brain. The general elevated level of GFAP+ astrocytes within this region in all animals receiving MCAO would explain the relatively small differences observed between hemispheres despite the localization of the infarct to the right side.

Overall, the decrease in oxidative stress along with the apparent increase in neurovascular remodeling observed in animals treated with ET_B agonist IRL-1620 on the first day of cerebral ischemia, led to a reduction in infarct volume and a substantial improvement in neurological and motor function. These effects were reversed when the animals were treated with ET_B antagonist BQ788. These results indicate that not only are ET_B receptors involved in both the acute and sub-acute phases of cerebral ischemia, but that selective stimulation of these receptors may be a viable and novel therapeutic target for the treatment of this disease.

VI. CONCLUSIONS AND FUTURE IMPLICATIONS

The present study has demonstrated that central endothelin B receptors are indeed involved in focal cerebral ischemia. While previous reports have indicated that a depletion of ET_B receptors results in a poorer outcome following experimental cerebral ischemia, this is the first report showing that selective stimulation of these receptors is beneficial. Stimulation of ET_B receptors via agonist IRL-1620, administered in three doses on the first day of ischemia, in a permanent model of middle cerebral artery occlusion in rats, resulted in significant neuroprotection and enhanced neurovascular remodeling during the acute and sub-acute phases of cerebral ischemia.

Stimulation of ET_B receptors resulted in functional and physiological recovery, improving muscular strength and motor coordination while reducing infarct volume. These results implied that ET_B receptors may play a role in both the early and late stages of the ischemic cascade and subsequent breakdown of the blood brain barrier. In fact, we found that treatment with ET_B agonist IRL-1620 led to a reduction in early and late stage oxidative stress and blocked the formation of cerebral edema. Blockade of ET_B receptors using a specific antagonist, BQ788, led to a reversal of these effects, confirming that the neuroprotective actions of IRL-1620 were due to their stimulation of endogenous ET_B receptors. These findings, along with an observed increase in central ET_B receptor expression within the ischemic brain one week following infarct and treatment with IRL-1620, led us to investigate the location of these receptors and whether or not they were involved in post-ischemic neurovascular repair and remodeling. We discovered that ET_B receptor stimulation is involved in and enhances the survival, proliferation and migration of neurons following MCAO. This enhancement most likely occurs through both direct and indirect pathways. The observed reduction in inflammation

and oxidative stress, combined with an increased production of vascular endothelial growth factor, would provide an enriched environment, thereby encouraging neuronal growth and repair. In addition, ET_B receptors on the neurons and astrocytes themselves may initiate survival and proliferation pathways. Further investigations into the pathways involved in the process of neurovascular genesis following ET_B receptor stimulation are warranted.

Additionally, while we have clearly demonstrated the involvement and beneficial effects of ET_B receptor stimulation following cerebral ischemia, much work remains before this novel therapeutic target can be said to have clinical relevancy. According to the STAIR requirements for novel therapeutic strategies in the treatment of ischemic stroke, the proposed treatment must show significant efficacy not only in a permanent model but also in a transient model of cerebral ischemia. It would also be of interest to observe the effects of ET_B receptor stimulation in an embolic, as opposed to a filament, model to determine any possible effects upon the clot itself. While the logistics of this study prohibited the use of a laser Doppler probe to determine any alterations in cerebral blood flow, future studies ought to incorporate a non-invasive method of determining this parameter, especially as we have previously demonstrated that IRL-1620 increases CBF in normal rats. In addition, as one of the main limitations to current treatment for ischemic stroke is the short therapeutic time window, further studies utilizing a later administration of the ET_B agonist are needed to determine the proper therapeutic window for this treatment. Finally, dosing strategies need to be optimized in order to determine whether an infusion of the ET_B agonist or single or multiple bolus administrations are required for efficacy.

In addition to being a potential novel therapeutic target for the treatment of cerebral ischemia, stimulation of ET_B receptors may prove efficacious in other neurodegenerative

diseases. Our findings demonstrating the reduction in oxidative stress and the enhancement of neurovascular remodeling following experimental cerebral ischemia treated with a selective ET_B agonist open the possibility that other cerebral and cerebrovascular pathologies may benefit from this treatment. Recent studies performed in our laboratory have indicated that i.c.v. administration of IRL-1620 improves learning and memory and reduces oxidative stress in a rat model of Alzheimer's disease (unpublished data). Alzheimer's disease is a neurodegenerative disease characterized by the formation of beta amyloid plaques and tau tangles within the brain. It is the most prevalent form of dementia worldwide and has no cure and very few questionably effective treatments. Given our recent findings, it would be of interest to study whether stimulation of ET_B receptors in an Alzheimer's model results in neurovascular alterations within the brain.

In conclusion, we have clearly demonstrated that central ET_B receptors are involved in focal cerebral ischemia. Stimulation of these receptors causes a reduction in oxidative stress and an enhancement of neurovascular remodeling within the ischemic brain, leading to a better physiological and functional recovery. These results indicate that stimulation of ET_B receptors may present a novel therapy for the treatment of ischemic stroke along with other neurodegenerative diseases.

CITED LITERATURE

(1995). "Tissue plasminogen activator for acute ischemic stroke. The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group." N Engl J Med **333**(24): 1581-1587.

(1999). "Recommendations for standards regarding preclinical neuroprotective and restorative drug development." Stroke **30**(12): 2752-2758.

Arai, H., S. Hori, I. Aramori, H. Ohkubo and S. Nakanishi (1990). "Cloning and expression of a cDNA encoding an endothelin receptor." Nature **348**(6303): 730-732.

Autiero, M., F. De Smet, F. Claes and P. Carmeliet (2005). "Role of neural guidance signals in blood vessel navigation." Cardiovasc Res **65**(3): 629-638.

Barone, F. C., E. H. Ohlstein, A. J. Hunter, C. A. Campbell, S. H. Hadingham, A. A. Parsons, Y. Yang and E. Shohami (2000). "Selective antagonism of endothelin-A-receptors improves outcome in both head trauma and focal stroke in rat." J Cardiovasc Pharmacol **36**(5 Suppl 1): S357-361.

Barone, F. C., R. F. White, J. D. Elliott, G. Z. Feuerstein and E. H. Ohlstein (1995). "The endothelin receptor antagonist SB 217242 reduces cerebral focal ischemic brain injury." J Cardiovasc Pharmacol **26 Suppl 3**: S404-407.

Bederson, J. B., L. H. Pitts, S. M. Germano, M. C. Nishimura, R. L. Davis and H. M. Bartkowski (1986). "Evaluation of 2,3,5-triphenyltetrazolium chloride as a stain for detection and quantification of experimental cerebral infarction in rats." Stroke **17**(6): 1304-1308.

Beyer, M. E., G. Slesak and H. M. Hoffmeister (1996). "Significance of endothelinB receptors for myocardial contractility and myocardial energy metabolism." J Pharmacol Exp Ther **278**(3): 1228-1234.

Blomstrand, F., L. Venance, A. L. Siren, P. Ezan, E. Hanse, J. Glowinski, H. Ehrenreich and C. Giaume (2004). "Endothelins regulate astrocyte gap junctions in rat hippocampal slices." Eur J Neurosci **19**(4): 1005-1015.

Boncoraglio, G. B., A. Bersano, L. Candelise, B. A. Reynolds and E. A. Parati (2010). "Stem cell transplantation for ischemic stroke." Cochrane Database Syst Rev(9): CD007231.

Bras-Silva, C., A. P. Fontes-Sousa, C. Moura, J. C. Areias and A. F. Leite-Moreira (2006). "Impaired response to ET(B) receptor stimulation in heart failure: functional evidence of endocardial endothelial dysfunction?" Exp Biol Med (Maywood) **231**(6): 893-898.

Breier, G. and W. Risau (1996). "The role of vascular endothelial growth factor in blood vessel formation." Trends Cell Biol **6**(12): 454-456.

Briyal, S. and A. Gulati (2010). "Endothelin-A receptor antagonist BQ123 potentiates acetaminophen induced hypothermia and reduces infarction following focal cerebral ischemia in rats." Eur J Pharmacol **644**(1-3): 73-79.

Briyal, S., M. S. Lavhale and A. Gulati (2012). "Repeated administration of centhaquin to pregnant rats did not affect postnatal development and expression of endothelin receptors in the brain, heart or kidney of pups." Arzneimittelforschung **62**(12): 670-676.

Calautti, C. and J. C. Baron (2003). "Functional neuroimaging studies of motor recovery after stroke in adults: a review." Stroke **34**(6): 1553-1566.

Camerlingo, M., P. Salvi, G. Belloni, T. Gamba, B. M. Cesana and A. Mamoli (2005). "Intravenous heparin started within the first 3 hours after onset of symptoms as a treatment for acute nonlacunar hemispheric cerebral infarctions." Stroke **36**(11): 2415-2420.

Castaneda, M. M., M. A. Cubilla, M. M. Lopez-Vicchi and A. M. Suburo (2010). "Endothelinergic cells in the subependymal region of mice." Brain Res **1321**: 20-30.

Chuquet, J., K. Benchenane, J. Toutain, E. T. MacKenzie, S. Roussel and O. Touzani (2002). "Selective blockade of endothelin-B receptors exacerbates ischemic brain damage in the rat." Stroke **33**(12): 3019-3025.

Crocker, S. J., A. Pagenstecher and I. L. Campbell (2004). "The TIMPs tango with MMPs and more in the central nervous system." J Neurosci Res **75**(1): 1-11.

Davenport, A. P. and R. E. Kuc (2004). "Down-regulation of ETA receptors in ETB receptor-deficient mice." J Cardiovasc Pharmacol **44 Suppl 1**: S276-278.

Dembowski, C., P. Hofmann, T. Koch, H. Kamrowski-Kruck, H. Riedesel, H. J. Krammer, F. J. Kaup and H. Ehrenreich (2000). "Phenotype, intestinal morphology, and survival of homozygous and heterozygous endothelin B receptor--deficient (spotting lethal) rats." J Pediatr Surg **35**(3): 480-488.

den Hertog, H., B. van der Worp, M. van Gemert and D. Dippel (2007). "Therapeutic hypothermia in acute ischemic stroke." Expert Rev Neurother **7**(2): 155-164.

Dirnagl, U., C. Iadecola and M. A. Moskowitz (1999). "Pathobiology of ischaemic stroke: an integrated view." Trends Neurosci **22**(9): 391-397.

Donnan, G. A., M. Fisher, M. Macleod and S. M. Davis (2008). "Stroke." Lancet **371**(9624): 1612-1623.

Druckenbrod, N. R., P. A. Powers, C. R. Bartley, J. W. Walker and M. L. Epstein (2008). "Targeting of endothelin receptor-B to the neural crest." Genesis **46**(8): 396-400.

Durukan, A. and T. Tatlisumak (2007). "Acute ischemic stroke: overview of major experimental rodent models, pathophysiology, and therapy of focal cerebral ischemia." Pharmacol Biochem Behav **87**(1): 179-197.

Ehrenreich, H., T. R. Nau, C. Dembowski, M. Hasselblatt, M. Barth, A. Hahn, L. Schilling, A. L. Siren and W. Bruck (2000). "Endothelin b receptor deficiency is associated with an increased rate of neuronal apoptosis in the dentate gyrus." Neuroscience **95**(4): 993-1001.

Ehrenreich, H., J. Oldenburg, M. Hasselblatt, J. Herms, C. Dembowski, B. M. Loffler, W. Bruck, H. Kamrowski-Kruck, S. Gall, A. L. Siren and L. Schilling (1999). "Endothelin B receptor-deficient rats as a subtraction model to study the cerebral endothelin system." Neuroscience **91**(3): 1067-1075.

Ekdahl, C. T., Z. Kokaia and O. Lindvall (2009). "Brain inflammation and adult neurogenesis: the dual role of microglia." Neuroscience **158**(3): 1021-1029.

Eliassen, J. C., E. L. Boespflug, M. Lamy, J. Allendorfer, W. J. Chu and J. P. Szaflarski (2008). "Brain-mapping techniques for evaluating poststroke recovery and rehabilitation: a review." Top Stroke Rehabil **15**(5): 427-450.

Ellman, G. L. (1959). "Tissue sulfhydryl groups." Arch Biochem Biophys **82**(1): 70-77.

Ferro, J. M. and F. Pinto (2004). "Poststroke epilepsy: epidemiology, pathophysiology and management." Drugs Aging **21**(10): 639-653.

Fisher, M., G. Feuerstein, D. W. Howells, P. D. Hurn, T. A. Kent, S. I. Savitz, E. H. Lo and S. Group (2009). "Update of the stroke therapy academic industry roundtable preclinical recommendations." Stroke **40**(6): 2244-2250.

Fisher, M. and S. T. A. I. Roundtable (2003). "Recommendations for advancing development of acute stroke therapies: Stroke Therapy Academic Industry Roundtable 3." Stroke **34**(6): 1539-1546.

Franceschini, R., C. Gandolfo, A. Cataldi, M. Del Sette, A. Rolandi, G. Corsini, E. Rolandi and T. Barreca (2001). "Twenty-four-hour endothelin-1 secretory pattern in stroke patients." Biomed Pharmacother **55**(5): 272-276.

Goto, K., Y. Kasuya, N. Matsuki, Y. Takuwa, H. Kurihara, T. Ishikawa, S. Kimura, M. Yanagisawa and T. Masaki (1989). "Endothelin activates the dihydropyridine-sensitive, voltage-dependent Ca2+ channel in vascular smooth muscle." Proc Natl Acad Sci U S A **86**(10): 3915-3918.

Green, A. R. (2008). "Pharmacological approaches to acute ischaemic stroke: reperfusion certainly, neuroprotection possibly." Br J Pharmacol **153 Suppl 1**: S325-338.

Gupta, Y. K., S. Briyal, U. Sharma, N. R. Jagannathan and A. Gulati (2005). "Effect of endothelin antagonist (TAK-044) on cerebral ischemic volume, oxidative stress markers and neurobehavioral parameters in the middle cerebral artery occlusion model of stroke in rats." Life Sci **77**(1): 15-27.

Góra-Kupilas, K. and J. Jośko (2005). "The neuroprotective function of vascular endothelial growth factor (VEGF)." Folia Neuropathol **43**(1): 31-39.

Haapaniemi, E., T. Tatlisumak, K. Hamel, L. Soinne, C. Lanni, T. J. Opgenorth and M. Kaste (2000). "Plasma endothelin-1 levels neither increase nor correlate with neurological scores, stroke risk factors, or outcome in patients with ischemic stroke." Stroke **31**(3): 720-725.

Hacke, W., G. Donnan, C. Fieschi, M. Kaste, R. von Kummer, J. P. Broderick, T. Brott, M. Frankel, J. C. Grotta, E. C. Haley, T. Kwiatkowski, S. R. Levine, C. Lewandowski, M. Lu, P. Lyden, J. R. Marler, S. Patel, B.

C. Tilley, G. Albers, E. Bluhmki, M. Wilhelm, S. Hamilton, A. T. Investigators, E. T. Investigators and N. r.-P. S. G. Investigators (2004). "Association of outcome with early stroke treatment: pooled analysis of ATLANTIS, ECASS, and NINDS rt-PA stroke trials." Lancet **363**(9411): 768-774.

Hasselblatt, M., P. Lewczuk, B. M. Löffler, H. Kamrowski-Kruck, N. von Ahsen, A. L. Sirén and H. Ehrenreich (2001). "Role of the astrocytic ET(B) receptor in the regulation of extracellular endothelin-1 during hypoxia." Glia **34**(1): 18-26.

Health, U. S. N. I. o. (2012). ClinicalTrials.gov.

Heidenreich, P. A., J. G. Trogdon, O. A. Khavjou, J. Butler, K. Dracup, M. D. Ezekowitz, E. A. Finkelstein, Y. Hong, S. C. Johnston, A. Khera, D. M. Lloyd-Jones, S. A. Nelson, G. Nichol, D. Orenstein, P. W. Wilson, Y. J. Woo, A. H. A. A. C. Committee, S. Council, C. o. C. R. a. Intervention, C. o. C. Cardiology, C. o. E. a. Prevention, C. o. Arteriosclerosis, T. a. V. Biology, C. o. Cardiopulmonary, C. Care, P. a. Resuscitation, C. o. C. Nursing, C. o. t. K. i. C. Disease and a. I. C. o. Q. o. C. a. O. R. Council on Cardiovascular Surgery and Anesthesia (2011). "Forecasting the future of cardiovascular disease in the United States: a policy statement from the American Heart Association." Circulation **123**(8): 933-944.

Henriksson, M., E. Stenman and L. Edvinsson (2003). "Intracellular pathways involved in upregulation of vascular endothelin type B receptors in cerebral arteries of the rat." Stroke **34**(6): 1479-1483.

Hickey, K. A., G. Rubanyi, R. J. Paul and R. F. Highsmith (1985). "Characterization of a coronary vasoconstrictor produced by cultured endothelial cells." Am J Physiol **248**(5 Pt 1): C550-556.

Hossmann, K. A. (1996). "Periinfarct depolarizations." Cerebrovasc Brain Metab Rev **8**(3): 195-208.

Huang, Y. and J. O. McNamara (2004). "Ischemic stroke: "acidotoxicity" is a perpetrator." Cell **118**(6): 665-666.

Iwamuro, Y., S. Miwa, T. Minowa, T. Enoki, X. F. Zhang, M. Ishikawa, N. Hashimoto and T. Masaki (1998). "Activation of two types of Ca2+-permeable nonselective cation channel by endothelin-1 in A7r5 cells." Br J Pharmacol **124**(7): 1541-1549.

Kakkar, P., B. Das and P. N. Viswanathan (1984). "A modified spectrophotometric assay of superoxide dismutase." Indian J Biochem Biophys **21**(2): 130-132.

Kanekar, S. G., T. Zacharia and R. Roller (2012). "Imaging of stroke: Part 2, Pathophysiology at the molecular and cellular levels and corresponding imaging changes." AJR Am J Roentgenol **198**(1): 63-74.

Karaki, H., S. A. Sudjarwo, M. Hori, M. Takai, Y. Urade and T. Okada (1993). "Induction of endothelium-dependent relaxation in the rat aorta by IRL 1620, a novel and selective agonist at the endothelin ETB receptor." Br J Pharmacol **109**(2): 486-490.

Kaundal, R. K., T. A. Deshpande, A. Gulati and S. S. Sharma (2012). "Targeting endothelin receptors for pharmacotherapy of ischemic stroke: current scenario and future perspectives." Drug Discov Today **17**(13-14): 793-804.

Kee, N., S. Sivalingam, R. Boonstra and J. M. Wojtowicz (2002). "The utility of Ki-67 and BrdU as proliferative markers of adult neurogenesis." J Neurosci Methods **115**(1): 97-105.

Khimji, A. K. and D. C. Rockey (2010). "Endothelin--biology and disease." Cell Signal **22**(11): 1615-1625.

Kidd, P. M. (2009). "Integrated brain restoration after ischemic stroke--medical management, risk factors, nutrients, and other interventions for managing inflammation and enhancing brain plasticity." Altern Med Rev **14**(1): 14-35.

Kitazono, T., D. D. Heistad and F. M. Faraci (1995). "Enhanced responses of the basilar artery to activation of endothelin-B receptors in stroke-prone spontaneously hypertensive rats." Hypertension **25**(4 Pt 1): 490-494.

Kobari, M., Y. Fukuuchi, M. Tomita, N. Tanahashi, S. Konno and H. Takeda (1994). "Dilatation of cerebral microvessels mediated by endothelin ETB receptor and nitric oxide in cats." Neurosci Lett **176**(2): 157-160.

Koizumi, J., Yoshida, Y., Nakazawa, T., Ooneda, G. (1986). "Experimental studies of ischemic brain edema. I. A new experimental model of cerebral embolism in rats in which recirculation can be introduced in the ischemic area." Jpn J Stroke **8**: 1-8.

Koyama, Y., A. Baba and T. Matsuda (2005). "Endothelins stimulate the expression of neurotrophin-3 in rat brain and rat cultured astrocytes." Neuroscience **136**(2): 425-433.

Koyama, Y., A. Baba and T. Matsuda (2007). "Intracerebroventricular administration of an endothelin ETB receptor agonist increases expression of tissue inhibitor of matrix metalloproteinase-1 and -3 in rat brain." Neuroscience **147**(3): 620-630.

Koyama, Y., R. Nagae, S. Tokuyama and K. Tanaka (2011). "I.c.v administration of an endothelin ET(B) receptor agonist stimulates vascular endothelial growth factor-A production and activates vascular endothelial growth factor receptors in rat brain." Neuroscience **192**: 689-698.

Koyama, Y. and K. Tanaka (2010). "Decreases in rat brain aquaporin-4 expression following intracerebroventricular administration of an endothelin ET B receptor agonist." Neurosci Lett **469**(3): 343-347.

Koyama, Y., K. Tsujikawa, T. Matsuda and A. Baba (2003). "Intracerebroventricular administration of an endothelin ETB receptor agonist increases expressions of GDNF and BDNF in rat brain." Eur J Neurosci **18**(4): 887-894.

Krafft, P. R., E. L. Bailey, T. Lekic, W. B. Rolland, O. Altay, J. Tang, J. M. Wardlaw, J. H. Zhang and C. L. Sudlow (2012). "Etiology of stroke and choice of models." Int J Stroke **7**(5): 398-406.

Kreipke, C. W., C. A. Reynolds, P. C. Schafer, S. Schafer, R. Pirooz and J. A. Rafols (2011). "Endothelin receptors A and B are expressed in distinct cellular compartments of rat hippocampus following global ischemia: an immunocytochemical study." Neurol Res **33**(2): 162-168.

Lakhan, S. E., A. Kirchgessner and M. Hofer (2009). "Inflammatory mechanisms in ischemic stroke: therapeutic approaches." J Transl Med **7**: 97.

Lavhale, M. S., S. Briyal, N. Parikh and A. Gulati (2010). "Endothelin modulates the cardiovascular effects of clonidine in the rat." Pharmacol Res **62**(6): 489-499.

Leite-Moreira, A. F. and C. Bras-Silva (2004). "Inotropic effects of ETB receptor stimulation and their modulation by endocardial endothelium, NO, and prostaglandins." Am J Physiol Heart Circ Physiol **287**(3): H1194-1199.

Leonard, M. G., S. Briyal and A. Gulati (2011). "Endothelin B receptor agonist, IRL-1620, reduces neurological damage following permanent middle cerebral artery occlusion in rats." Brain Res.

Leonard, M. G., S. Briyal and A. Gulati (2012). "Endothelin B receptor agonist, IRL-1620, provides long-term neuroprotection in cerebral ischemia in rats." Brain Res **1464**: 14-23.

Leonard, M. G. and A. Gulati (2009). "Repeated administration of ET(B) receptor agonist, IRL-1620, produces tachyphylaxis only to its hypotensive effect." Pharmacol Res **60**(5): 402-410.

Li, F., K. Irie, M. S. Anwer and M. Fisher (1997). "Delayed triphenyltetrazolium chloride staining remains useful for evaluating cerebral infarct volume in a rat stroke model." J Cereb Blood Flow Metab **17**(10): 1132-1135.

Li, L., A. Lundkvist, D. Andersson, U. Wilhelmsson, N. Nagai, A. C. Pardo, C. Nodin, A. Stahlberg, K. Aprico, K. Larsson, T. Yabe, L. Moons, A. Fotheringham, I. Davies, P. Carmeliet, J. P. Schwartz, M. Pekna, M. Kubista, F. Blomstrand, N. Maragakis, M. Nilsson and M. Pekny (2008). "Protective role of reactive astrocytes in brain ischemia." J Cereb Blood Flow Metab **28**(3): 468-481.

Li, Y., S. P. Yu, O. Mohamad, T. Genetta and L. Wei (2010). "Sublethal Transient Global Ischemia Stimulates Migration of Neuroblasts and Neurogenesis in Mice." Transl Stroke Res **1**(3): 184-196.

Liu, S., R. T. Premont, C. D. Kontos, J. Huang and D. C. Rockey (2003). "Endothelin-1 activates endothelial cell nitric-oxide synthase via heterotrimeric G-protein betagamma subunit signaling to protein jinase B/Akt." J Biol Chem **278**(50): 49929-49935.

Lo, A. C., A. Y. Chen, V. K. Hung, L. P. Yaw, M. K. Fung, M. C. Ho, M. C. Tsang, S. S. Chung and S. K. Chung (2005). "Endothelin-1 overexpression leads to further water accumulation and brain edema after middle cerebral artery occlusion via aquaporin 4 expression in astrocytic end-feet." J Cereb Blood Flow Metab **25**(8): 998-1011.

Loo, L. S., Y. K. Ng, Y. Z. Zhu, H. S. Lee and P. T. Wong (2002). "Cortical expression of endothelin receptor subtypes A and B following middle cerebral artery occlusion in rats." Neuroscience **112**(4): 993-1000.

Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall (1951). "Protein measurement with the Folin phenol reagent." J Biol Chem **193**(1): 265-275.

Lucas, G. A., L. R. White, R. Juul, J. Cappelen, J. Aasly and L. Edvinsson (1996). "Relaxation of human temporal artery by endothelin ETB receptors." Peptides **17**(7): 1139-1144.

Markgraf, C. G., E. J. Green, B. E. Hurwitz, E. Morikawa, W. D. Dietrich, P. M. McCabe, M. D. Ginsberg and N. Schneiderman (1992). "Sensorimotor and cognitive consequences of middle cerebral artery occlusion in rats." Brain Res **575**(2): 238-246.

Matsumoto, H., N. Suzuki, H. Onda and M. Fujino (1989). "Abundance of endothelin-3 in rat intestine, pituitary gland and brain." Biochem Biophys Res Commun **164**(1): 74-80.

Matsuo, Y., Mihara Si, M. Ninomiya and M. Fujimoto (2001). "Protective effect of endothelin type A receptor antagonist on brain edema and injury after transient middle cerebral artery occlusion in rats." Stroke **32**(9): 2143-2148.

McAuley, M. A., V. Breu, D. I. Graham and J. McCulloch (1996). "The effects of bosentan on cerebral blood flow and histopathology following middle cerebral artery occlusion in the rat." Eur J Pharmacol **307**(2): 171-181.

McCarron, R. M., L. Wang, D. B. Stanimirovic and M. Spatz (1993). "Endothelin induction of adhesion molecule expression on human brain microvascular endothelial cells." Neurosci Lett **156**(1-2): 31-34.

Minger, S. L., A. Ekonomou, E. M. Carta, A. Chinoy, R. H. Perry and C. G. Ballard (2007). "Endogenous neurogenesis in the human brain following cerebral infarction." Regen Med **2**(1): 69-74.

Miniño, A. M., S. L. Murphy, J. Xu and K. D. Kochanek (2011). "Deaths: final data for 2008." Natl Vital Stat Rep **59**(10): 1-126.

Moran, P. M., L. S. Higgins, B. Cordell and P. C. Moser (1995). "Age-related learning deficits in transgenic mice expressing the 751-amino acid isoform of human beta-amyloid precursor protein." Proc Natl Acad Sci U S A **92**(12): 5341-5345.

Morga, E., C. Faber and P. Heuschling (2000). "Stimulation of endothelin B receptor modulates the inflammatory activation of rat astrocytes." J Neurochem **74**(2): 603-612.

Myint, P. K., E. F. Staufenberg and K. Sabanathan (2006). "Post-stroke seizure and post-stroke epilepsy." Postgrad Med J **82**(971): 568-572.

Nambi, P., M. Pullen and W. Spielman (1994). "Species differences in the binding characteristics of [125I]IRL-1620, a potent agonist specific for endothelin-B receptors." J Pharmacol Exp Ther **268**(1): 202-207.

Ninomiya, M., T. Yamashita, N. Araki, H. Okano and K. Sawamoto (2006). "Enhanced neurogenesis in the ischemic striatum following EGF-induced expansion of transit-amplifying cells in the subventricular zone." Neurosci Lett **403**(1-2): 63-67.

Nowacka, M. M. and E. Obuchowicz (2012). "Vascular endothelial growth factor (VEGF) and its role in the central nervous system: a new element in the neurotrophic hypothesis of antidepressant drug action." Neuropeptides **46**(1): 1-10.

Ohkawa, H., N. Ohishi and K. Yagi (1979). "Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction." Anal Biochem **95**(2): 351-358.

Ozaki, S., K. Ohwaki, M. Ihara, T. Fukuroda, K. Ishikawa and M. Yano (1995). "ETB-mediated regulation of extracellular levels of endothelin-1 in cultured human endothelial cells." Biochem Biophys Res Commun **209**(2): 483-489.

Paolillo, M., M. A. Russo, D. Curti, C. Lanni and S. Schinelli (2010). "Endothelin B receptor antagonists block proliferation and induce apoptosis in glioma cells." Pharmacol Res **61**(4): 306-315.

PubChem IRL 1620 Compound Summary (CID 16130933), National Center for Biotechnology Information, U.S. National Library of Medicine.

Pérez de la Ossa, N. and A. Dávalos (2007). "Neuroprotection in cerebral infarction: the opportunity of new studies." Cerebrovasc Dis **24 Suppl 1**: 153-156.

Riechers, C. C., W. Knabe, A. L. Siren, C. E. Gariepy, M. Yanagisawa and H. Ehrenreich (2004). "Endothelin B receptor deficient transgenic rescue rats: a rescue phenomenon in the brain." Neuroscience **124**(4): 719-723.

Roger, V. L., A. S. Go, D. M. Lloyd-Jones, E. J. Benjamin, J. D. Berry, W. B. Borden, D. M. Bravata, S. Dai, E. S. Ford, C. S. Fox, H. J. Fullerton, C. Gillespie, S. M. Hailpern, J. A. Heit, V. J. Howard, B. M. Kissela, S. J. Kittner, D. T. Lackland, J. H. Lichtman, L. D. Lisabeth, D. M. Makuc, G. M. Marcus, A. Marelli, D. B. Matchar, C. S. Moy, D. Mozaffarian, M. E. Mussolino, G. Nichol, N. P. Paynter, E. Z. Soliman, P. D. Sorlie, N. Sotoodehnia, T. N. Turan, S. S. Virani, N. D. Wong, D. Woo, M. B. Turner and A. H. A. S. C. a. S. S. Subcommittee (2012). "Heart disease and stroke statistics--2012 update: a report from the American Heart Association." Circulation **125**(1): e2-e220.

Rogers, D. C., C. A. Campbell, J. L. Stretton and K. B. Mackay (1997). "Correlation between motor impairment and infarct volume after permanent and transient middle cerebral artery occlusion in the rat." Stroke **28**(10): 2060-2065; discussion 2066.

Ruiz de Almodovar, C., D. Lambrechts, M. Mazzone and P. Carmeliet (2009). "Role and therapeutic potential of VEGF in the nervous system." Physiol Rev **89**(2): 607-648.

Sakurai, T., M. Yanagisawa and T. Masaki (1992). "Molecular characterization of endothelin receptors." Trends Pharmacol Sci **13**(3): 103-108.

Saver, J. L., G. W. Albers, B. Dunn, K. C. Johnston, M. Fisher and S. V. Consortium (2009). "Stroke Therapy Academic Industry Roundtable (STAIR) recommendations for extended window acute stroke therapy trials." Stroke **40**(7): 2594-2600.

Schaefer, P. W., P. E. Grant and R. G. Gonzalez (2000). "Diffusion-weighted MR imaging of the brain." Radiology **217**(2): 331-345.

Schinelli, S. (2006). "Pharmacology and physiopathology of the brain endothelin system: an overview." Curr Med Chem **13**(6): 627-638.

Schleuning, W. D. (2001). "Vampire bat plasminogen activator DSPA-alpha-1 (desmoteplase): a thrombolytic drug optimized by natural selection." Haemostasis **31**(3-6): 118-122.

Servitja, J. M., R. Masgrau, E. Sarri and F. Picatoste (1998). "Involvement of ET(A) and ET(B) receptors in the activation of phospholipase D by endothelins in cultured rat cortical astrocytes." Br J Pharmacol **124**(8): 1728-1734.

Shen, H. and Y. Wang (2010). "Correlation of locomotor activity and brain infarction in rats with transient focal ischemia." J Neurosci Methods **186**(2): 150-154.

Siskas, N., A. Lefkopoulos, I. Ioannidis, A. Charitandi and A. S. Dimitriadis (2003). "Cortical laminar necrosis in brain infarcts: serial MRI." Neuroradiology **45**(5): 283-288.

Slivka, A. and W. Pulsinelli (1987). "Hemorrhagic complications of thrombolytic therapy in experimental stroke." Stroke **18**(6): 1148-1156.

Spinella, F., L. Rosanò, V. Di Castro, S. Decandia, M. R. Nicotra, P. G. Natali and A. Bagnato (2007). "Endothelin-1 and endothelin-3 promote invasive behavior via hypoxia-inducible factor-1alpha in human melanoma cells." Cancer Res **67**(4): 1725-1734.

Stenman, E., M. Malmsjo, E. Uddman, G. Gido, T. Wieloch and L. Edvinsson (2002). "Cerebral ischemia upregulates vascular endothelin ET(B) receptors in rat." Stroke **33**(9): 2311-2316.

Takahashi, K., M. A. Ghatei, P. M. Jones, J. K. Murphy, H. C. Lam, D. J. O'Halloran and S. R. Bloom (1991). "Endothelin in human brain and pituitary gland: comparison with rat." J Cardiovasc Pharmacol **17 Suppl 7**: S101-103.

Takahashi, K., M. A. Ghatei, P. M. Jones, J. K. Murphy, H. C. Lam, D. J. O'Halloran and S. R. Bloom (1991). "Endothelin in human brain and pituitary gland: presence of immunoreactive endothelin, endothelin messenger ribonucleic acid, and endothelin receptors." J Clin Endocrinol Metab **72**(3): 693-699.

Takai, M., I. Umemura, K. Yamasaki, T. Watakabe, Y. Fujitani, K. Oda, Y. Urade, T. Inui, T. Yamamura and T. Okada (1992). "A potent and specific agonist, Suc-[Glu9,Ala11,15]-endothelin-1(8-21), IRL 1620, for the ETB receptor." Biochem Biophys Res Commun **184**(2): 953-959.

Tang, L., B. Luo, R. P. Patel, Y. Ling, J. Zhang and M. B. Fallon (2007). "Modulation of pulmonary endothelial endothelin B receptor expression and signaling: implications for experimental hepatopulmonary syndrome." Am J Physiol Lung Cell Mol Physiol **292**(6): L1467-1472.

Tarr, R., D. Hsu, Z. Kulcsar, C. Bonvin, D. Rufenacht, K. Alfke, R. Stingele, O. Jansen, D. Frei, R. Bellon, M. Madison, T. Struffert, A. Dorfler, I. Q. Grunwald, W. Reith and A. Haass (2010). "The POST trial: initial

post-market experience of the Penumbra system: revascularization of large vessel occlusion in acute ischemic stroke in the United States and Europe." J Neurointerv Surg **2**(4): 341-344.

Tatlisumak, T., R. A. Carano, K. Takano, T. J. Opgenorth, C. H. Sotak and M. Fisher (1998). "A novel endothelin antagonist, A-127722, attenuates ischemic lesion size in rats with temporary middle cerebral artery occlusion: a diffusion and perfusion MRI study." Stroke **29**(4): 850-857; discussion 857-858.

Thored, P., U. Heldmann, W. Gomes-Leal, R. Gisler, V. Darsalia, J. Taneera, J. M. Nygren, S. E. Jacobsen, C. T. Ekdahl, Z. Kokaia and O. Lindvall (2009). "Long-term accumulation of microglia with proneurogenic phenotype concomitant with persistent neurogenesis in adult subventricular zone after stroke." Glia **57**(8): 835-849.

Tirapelli, C. R., D. A. Casolari, A. Yogi, A. C. Montezano, R. C. Tostes, E. Legros, P. D'Orleans-Juste and A. M. de Oliveira (2005). "Functional characterization and expression of endothelin receptors in rat carotid artery: involvement of nitric oxide, a vasodilator prostanoid and the opening of K+ channels in ETB-induced relaxation." Br J Pharmacol **146**(6): 903-912.

Treadwell, S. D. and B. Thanvi (2010). "Malignant middle cerebral artery (MCA) infarction: pathophysiology, diagnosis and management." Postgrad Med J **86**(1014): 235-242.

Trevisi, L., S. Bova, G. Cargnelli, G. Ceolotto and S. Luciani (2002). "Endothelin-1-induced arachidonic acid release by cytosolic phospholipase A2 activation in rat vascular smooth muscle via extracellular signal-regulated kinases pathway." Biochem Pharmacol **64**(3): 425-431.

Tsaur, M. L., Y. C. Wan, F. P. Lai and H. F. Cheng (1997). "Expression of B-type endothelin receptor gene during neural development." FEBS Lett **417**(2): 208-212.

Tsukahara, H., H. Ende, H. I. Magazine, W. F. Bahou and M. S. Goligorsky (1994). "Molecular and functional characterization of the non-isopeptide-selective ETB receptor in endothelial cells. Receptor coupling to nitric oxide synthase." J Biol Chem **269**(34): 21778-21785.

Vidovic, M., M. M. Chen, Q. Y. Lu, K. F. Kalloniatis, B. M. Martin, A. H. Tan, C. Lynch, G. D. Croaker, D. T. Cass and Z. M. Song (2008). "Deficiency in endothelin receptor B reduces proliferation of neuronal progenitors and increases apoptosis in postnatal rat cerebellum." Cell Mol Neurobiol **28**(8): 1129-1138.

Wartenberg, K. E. (2012). "Malignant middle cerebral artery infarction." Curr Opin Crit Care **18**(2): 152-163.

Yamashita, T., M. Ninomiya, P. Hernández Acosta, J. M. García-Verdugo, T. Sunabori, M. Sakaguchi, K. Adachi, T. Kojima, Y. Hirota, T. Kawase, N. Araki, K. Abe, H. Okano and K. Sawamoto (2006). "Subventricular zone-derived neuroblasts migrate and differentiate into mature neurons in the post-stroke adult striatum." J Neurosci **26**(24): 6627-6636.

Yanagisawa, M., H. Kurihara, S. Kimura, Y. Tomobe, M. Kobayashi, Y. Mitsui, Y. Yazaki, K. Goto and T. Masaki (1988). "A novel potent vasoconstrictor peptide produced by vascular endothelial cells." Nature **332**(6163): 411-415.

Yanev, P. and R. M. Dijkhuizen (2012). "In Vivo Imaging of Neurovascular Remodeling After Stroke." Stroke.

Zhang, R. L., C. Zhang, L. Zhang, C. Roberts, M. Lu, A. Kapke, Y. Cui, M. Ninomiya, T. Nagafuji, B. Albala, Z. G. Zhang and M. Chopp (2008). "Synergistic effect of an endothelin type A receptor antagonist, S-0139, with rtPA on the neuroprotection after embolic stroke." Stroke **39**(10): 2830-2836.

Zhang, Y., L. Belayev, W. Zhao, E. A. Irving, R. Busto and M. D. Ginsberg (2005). "A selective endothelin ET(A) receptor antagonist, SB 234551, improves cerebral perfusion following permanent focal cerebral ischemia in rats." Brain Res **1045**(1-2): 150-156.

Zidovetzki, R., P. Chen, M. Chen and F. M. Hofman (1999). "Endothelin-1-induced interleukin-8 production in human brain-derived endothelial cells is mediated by the protein kinase C and protein tyrosine kinase pathways." Blood **94**(4): 1291-1299.

Ziv, I., G. Fleminger, R. Djaldetti, A. Achiron, E. Melamed and M. Sokolovsky (1992). "Increased plasma endothelin-1 in acute ischemic stroke." Stroke **23**(7): 1014-1016.

APPENDICES

UIC UNIVERSITY OF ILLINOIS AT CHICAGO

Office of Animal Care and
Institutional Biosafety Committees (MC 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612-7227

June 5, 2012

Anil Gulati
Biopharmaceutical Sciences
M/C 865

Dear Dr. Gulati:

The protocol indicated below was reviewed in accordance with the Animal Care Policies of the University of Illinois at Chicago on **5/29/2012**. *The protocol was not initiated until final clarifications were reviewed and approved on* **6/1/2012**. *The protocol is approved for a period of* ***3*** years with annual continuation.

Title of Application: The Effect of ETB Receptor Agonist IRL-1620 on a Permanent Model of MCAO in Rats (Form G)

ACC Number: 12-109

Initial Approval Period: 6/1/2012 to 5/29/2013

Current Funding: *Not at UIC*

Performance Site: *Midwestern University*.

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare (OLAW), NIH. **This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the funding proposal are matched to this ACC protocol.**

In addition, all investigators are responsible for ensuring compliance with all federal and institutional policies and regulations related to use of animals under this protocol and the funding sources listed on this protocol. Please use OLAW's "*What Investigators Need to Know about the Use of Animals*" (http://grants.nih.gov/grants/olaw/InvestigatorsNeed2Know.pdf) as a reference guide. Thank you for complying with the Animal Care Policies and Procedures of UIC.

Sincerely yours,

Bradley Merrill, PhD
Chair, Animal Care Committee

BM/*mbb*
cc: BRL, ACC File, Mary Leonard

Phone (312) 996-1972 • Fax (312) 996-9088 • www.research.uic.edu

Pharmacological Research 60 (2009) 402–410

Contents lists available at ScienceDirect

Pharmacological Research

journal homepage: www.elsevier.com/locate/yphrs

Repeated administration of ET_B receptor agonist, IRL-1620, produces tachyphylaxis only to its hypotensive effect

Mary G. Leonard, Anil Gulati *

College of Health Sciences and Chicago College of Pharmacy, Midwestern University, 555 31st St., Downers Grove, IL 60515, USA

ARTICLE INFO

Article history:
Received 24 March 2009
Received in revised form 19 July 2009
Accepted 31 July 2009

Keywords:
Endothelin (ET)
IRL-1620
Tachyphylaxis
Blood flow
Mean arterial pressure

ABSTRACT

IRL-1620, a highly selective ET_B receptor agonist, is presently in a phase I clinical trial (NCT00613691) in the United States for patients with recurrent or progressive carcinoma. The effect of acute repeated administration of IRL-1620 on the development of tachyphylaxis to changes in blood pressure, heart rate and blood flow (renal and cerebral) has not been studied. The present studies were conducted in urethane anesthetized rats to determine the cardiovascular effects of acute repeated intravenous administration of IRL-1620. In order to determine the tachyphylactic effect, each dose of IRL-1620 was administered at 0, 60, and 120 min. It was found that IRL-1620 did not significantly affect heart rate. IRL-1620 produced a transient fall in blood pressure. A fall in mean arterial pressure (MAP) of 35.47% with 1.6 μg/kg, 38.87% with 5.0 μg/kg and 28.04% with 15.0 μg/kg dose of IRL-1620 was observed. Repeated administration of a low dose (1.6 μg/kg, i.v.) of IRL-1620 produced a fall in MAP but no tachyphylaxis was observed. However, repeated administration of IRL-1620 (5.0 μg/kg, i.v.) produced a fall in MAP of 40.12%, 29.15%, and 21.61% with the first, second and third injections, respectively. IRL-1620 produced a consistent decrease in renal blood flow and increase in cerebral blood flow without any evidence of tachyphylaxis. Pretreatment with ET_A antagonist BMS187824 (5 mg/kg, i.v.), followed by three doses of 5 μg/kg IRL-1620 at 60 min intervals eliminated the development of tachyphylaxis to the transient hypotension, confirming the involvement of the ET_A receptor in tachyphylactic development. The findings indicate development of tachyphylaxis to IRL-1620 only to the fall in blood pressure when given repeatedly at mid-high doses, while the decrease in renal and increase in cerebral blood flow were not affected with regards to tachyphylactic development.

1. Introduction

Endothelin (ET) is an endogenous 21-residue peptide secreted from the endothelial cells of blood vessels, where it displays potent vasoactive properties [1]. ETs are widely distributed throughout the body and are involved in a variety of physiological functions, most of which are cardiovascular in nature [2,3]. Three distinct isopeptides belonging to the human endothelin family have been identified. These isopeptides, delineated ET-1, ET-2, and ET-3, have multiple structural and pharmacologic differences despite their close genetic relationship. Of the three isopeptides, ET-1 appears to be the most potent vasoconstrictor. Interestingly, both ET-1 and ET-2 show a transient vasodilator action preceding the constriction [4].

ETs exert their effects by binding to two distinct types of cell surface receptors, ET_A and ET_B [5,6]. ET_A receptors have equal affinity for ET-1 and ET-2, and low affinity for ET-3 whereas ET_B receptors have equal affinity for ET-1, ET-2, and ET-3 [6]. Both ET_A and ET_B receptors belong to the G protein-coupled receptor (GPCR) family [3]. ET_A receptors in blood vessels mainly mediate the constrictor responses by acting on the intracellular Ca^{2+} concentration of vascular smooth muscle cells. ET_B receptors, on the other hand, induce vasodilatation via the release of nitric oxide (NO) from the endothelial cells [7,8]. In addition, ET_B receptors appear to buffer the vasoconstriction induced by the ET_A receptors by clearing ET from the plasma and reducing the concentration available to the ET_A receptors [9].

IRL-1620 (N-Succinyl-[Glu^9, Ala^{11,15}] endothelin-1, which is a fragment of ET-1 having amino acids 8–21 of ET-1) is a synthetic analogue of ET-1. IRL-1620 is a highly selective ET_B receptor agonist, being 120,000 times more selective to ET_B receptors than to ET_A receptors [10]. IRL-1620 has a molecular formula of $C_{86}H_{117}N_{17}O_{27}$ and a molecular weight of 1820.95. The molecular structure of IRL-1620 has an amino acid sequence of Suc-Asp-Glu-Glu-Ala-Val-Tyr-Phe-Ala-His-Leu-Asp-Ile-Ile-Trp.

It was recently found that IRL-1620 selectively enhanced breast tumor perfusion in rats [11–15]. Administration of BQ788, a highly selective ET_B receptor antagonist, blocked the tumor perfusion induced by IRL-1620 and confirmed the involvement of ET_B receptors in tumor vasodilatation [14,15]. It is thought that the increased tumor perfusion is due to the unique structure of the tumor vascu-

* Corresponding author. Tel.: +1 630 515 7379; fax: +1 630 515 6958.
E-mail address: AGULAT@midwestern.edu (A. Gulati).

1043-6618/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phrs.2009.07.015

lature, which has a higher ratio of endothelial (and therefore more ET_B receptors) to vascular smooth muscle cells than that of normal vascular beds. The selective enhancement of tumor blood flow resulted in a greater percentage of infused paclitaxel reaching the tumor as compared to the normal tissues [14,15]. As a result of these studies, IRL-1620 is presently in a phase I clinical trial in the United States for patients with recurrent or progressive carcinoma (ClinicalTrials.gov number NCT00613691).

Despite the increasing interest and significance of this drug, the effect of acute repeated administration of IRL-1620 on various cardiovascular parameters has not been determined. While a few studies have been performed to determine the effects of acute repeated administration of ET-1, which activates both ET_A and ET_B receptors, none have focused on selectively and repeatedly stimulating the ET_B receptor alone. As part of the carcinoma treatment regimen, it may be necessary to repeatedly administer IRL-1620. It is therefore important to determine if there are alterations to the hemodynamic effects of this agent as a result of such repeated administration. Early studies on the various properties of ET quickly brought to light the fact that ET-1 produces tachyphylaxis (rapidly decreasing response to a drug following multiple doses) and cross-tachyphylaxis between ET isopeptides to the hypotensive portion of its biphasic blood pressure response [16,17].

The purpose of this study was to investigate whether acute repeated administration of ET_B receptor agonist IRL-1620 leads to the development of tachyphylaxis on its blood pressure, heart rate, and cerebral and renal blood flow responses in rats.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats weighing 321 ± 11 g were obtained from Harlan, Indianapolis, IN. The animals were housed and allowed free access to standard dry rat food (Harlan Tech Lab, Madison, WI) and RO (reverse osmosis) water. They were kept 2 animals to a cage and allowed to acclimate for at least 3 days prior to any experimentation as per the Institutional Animal Care and Use Committee (IACUC) protocol. A total of 34 animals were used for this study.

2.2. Drugs

IRL-1620 (Suc-[Glu 9, Ala 11,15], American Peptide Co, Inc., Sunnyville, CA) was dissolved in isotonic saline (0.9% NaCl, Hospira, Inc., Lake Forest, IL). ET-1 (American Peptide Co, Inc., Sunnyvale, CA) was dissolved in isotonic saline. ET_A antagonist, BMS187824 hydrochloride (Tocris, Ellisville, MO) was dissolved in DMSO (Sigma Aldrich, St. Louis, MO). L-Arginine and nitric oxide donor, sodium nitroprusside (SNP) (Sigma Aldrich, St. Louis, MO) were dissolved in isotonic saline. Nitric oxide synthase inhibitor, N_ω-nitro-L-arginine methyl ester hydrochloride (L-NAME) (Sigma Aldrich, St. Louis, MO) was dissolved in isotonic saline.

2.3. Anesthesia

The animals were anesthetized with urethane (ethyl carbamate, Sigma Aldrich, St. Louis, MO) dissolved in isotonic saline. Urethane was administered in a dose of 0.15 g per 100 g body weight via intraperitoneal (i.p.) injection. Anesthetic efficacy was determined via toe pinch and corneal reflex reactions.

2.4. Cannulation of blood vessels

The anesthetized animals were shaved and immobilized on a surgical board. A 2–3 cm incision was made above the femoral vein and artery. The vessels were cleaned and isolated. The vein was clamped, a nick made in the vessel, and the cannula (PE 50 tubing) inserted and tied off using 3.0 silk thread (Deknatel, Research Triangle Park, NC) for the administration of drugs. The artery was similarly clamped, nicked, cannulated and tied off. The arterial cannula was connected to a Gould P23 ID pressure transducer for recording the blood pressure (BP) on a Grass P7D polygraph through a 7PI preamplifier. The heart rate (HR) was recorded through a 7P4B Grass tachograph, triggered from BP signals.

2.5. Cerebral perfusion

A burr hole was drilled into the rat skull approximately 2 mm to the left of midline, being careful not to disturb the brain tissue itself. Cerebrovascular perfusion was measured via a fiber optic probe (PF407) applied to the surface of the rat brain. The probe was connected to a Periflux PF2b 4000 Laser Doppler Flowmetry unit (Perimed, Stockholm, Sweden). The perfusion was determined by measuring the passage of red blood cells through the capillaries.

2.6. Renal perfusion

The right kidney was dissected retroperitoneally. Renal perfusion was measured via a fiber optic probe (PF408) applied to the surface of the rat kidney. The probe was connected to a Periflux PF2b 4000 Laser Doppler Flowmetry unit (Perimed, Stockholm, Sweden). The perfusion was determined in the same manner as described above per cerebral blood flow.

2.7. Study design

The animals were randomly separated for three studies. Animals in Study I were randomly divided into two groups. Group 1 (n=8) was administered IRL-1620 in cumulative doses of 1.6, 5.0 and 15.0 μg/kg at 0, 60, and 120 min, respectively. Group 2 (n=4) was administered ET-1 in cumulative doses of 0.25, 0.75 and 2.25 μg/kg ET-1 at 0, 60, and 120 min, respectively. In Study II, animals were randomly divided into three groups. Group 1 (n=7) was repeatedly administered IRL-1620 (1.6 μg/kg, i.v.) at 0, 60, and 120 min, respectively. Group 2 (n=5) was repeatedly administered IRL-1620 (5.0 μg/kg, i.v.) at 0, 60, and 120 min, respectively. Group 3 was repeatedly administered IRL-1620 (15.0 μg/kg, i.v.) at 0, 60, and 120 min, respectively, but animals were unable to survive acute repeated injections at this dose and were therefore not included in the study. Animals in Study III were randomly divided into three groups to clarify the effects of nitric oxide and the ET_A receptor on the development of tachyphylaxis. Group 1 (n=4) was pretreated with an infusion of nitric oxide synthase inhibitor L-NAME (3.3 mg/kg/h), followed by three injections of IRL-1620 (5.0 μg/kg, i.v.) at 60 min intervals. Group 2 (n=3) was pretreated with L-arginine (100 mg/kg/h) and nitric oxide donor sodium nitroprusside (10 μg/kg), followed by three injections of IRL-1620 (5.0 μg/kg, i.v.) at 60 min intervals. Group 3 (n=3) was pretreated with ET_A receptor antagonist BMS187824 (5 mg/kg), followed by three injections of IRL-1620 (5.0 μg/kg, i.v.) at 60 min intervals.

Animals were allowed to stabilize for 30 min following surgery, and then a 15 min baseline recording of all parameters was obtained prior to drug administration. Blood pressure, heart rate, cerebral blood perfusion and renal blood perfusion were recorded for 60 min following each injection. At the end of each experiment, the animals were euthanized with an intravenous overdose of urethane.

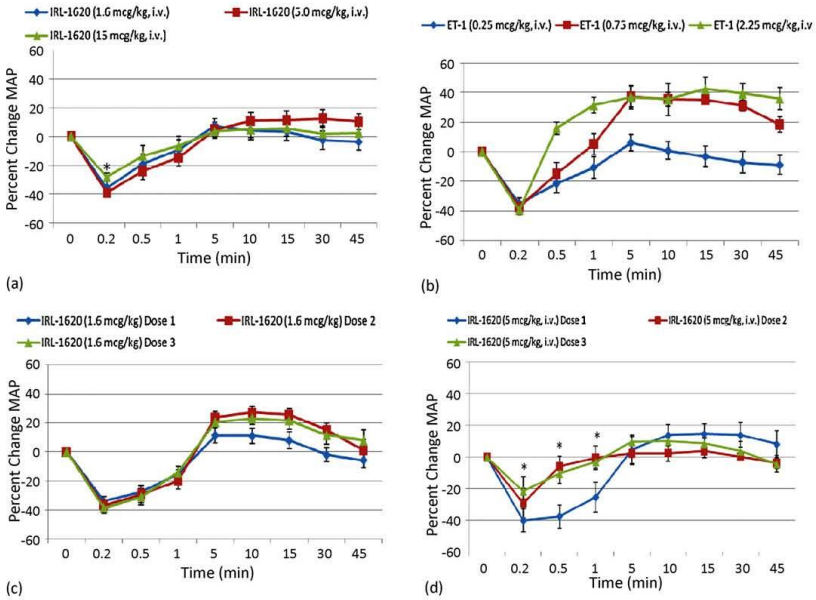


Fig. 1. Effect of repeated intravenous administration of IRL-1620 and ET-1 on mean arterial pressure. The values are expressed as mean ± SEM of the percent change in MAP. (a) IRL-1620 was administered in the doses of 1.6, 5.0 and 15.0 μg/kg at 60 min interval; $n = 8$; (b) ET-1 was administered in the doses of 0.25, 0.75, and 2.25 μg/kg at 60 min interval; $n = 4$; (c) IRL-1620 was administered thrice in the dose of 1.6 μg/kg at 60 min interval; $n = 7$; (d) IRL-1620 was administered thrice in the dose of 5.0 μg/kg at 60 min interval; $n = 5$. Tachyphylaxis to the fall in MAP was observed when IRL-1620 was administered at higher doses. *$P < 0.05$.

2.8. Statistical analysis

All data values are presented as mean ± SEM. Percent change from baseline was determined from the zero time point for each animal, after which the mean and standard error were calculated for each dose. One-way ANOVAs followed by post hoc tests (Bonferroni's test) were used to test the differences within and between the groups. A P-value of $P < 0.05$ was considered significant.

3. Results

3.1. Blood pressure

3.1.1. Effect of repeated administration of IRL-1620 (1.6, 5.0, and 15.0 μg/kg, i.v) on mean arterial pressure

Acute repeated administration of IRL-1620 (1.6, 5.0, and 15.0 μg/kg, i.v.) at 60 min intervals produced a transient fall in mean arterial pressure (MAP). IRL-1620 caused a maximal decrease in MAP of 35.47%, 38.87% and 28.04% at 1.6, 5.0, and 15.0 μg/kg, respectively. There was a significant ($P < 0.01$) tachyphylaxis to the hypotensive effects of IRL-1620 as noted between the second (5 μg/kg) and third (15 μg/kg) doses (Fig. 1a). No tachyphylaxis was observed between the low to medium doses, indicating that there may be a minimum concentration of the drug that must be achieved before tachyphylaxis is evident.

3.1.2. Effect of repeated administration of ET-1 (0.25, 0.75, and 2.25 μg/kg, i.v.) on mean arterial pressure

Acute repeated administration of ET-1 (0.25, 0.75, and 2.25 μg/kg, i.v.) at 60 min intervals produced a biphasic response in MAP, with a transient hypotension followed by a sustained hypertension. Administration of ET-1 in the doses of 0.25, 0.75, and 2.25 μg/kg produced a maximal fall of 34.67%, 36.98%, and 39.21%, respectively (Fig. 1b). No tachyphylaxis was observed to the hypotensive effects of ET-1 for this dosing regimen.

3.1.3. Effect of repeated administration of IRL-1620 (1.6 μg/kg, i.v.) on mean arterial pressure

Acute repeated intravenous administration of IRL-1620 (1.6 μg/kg) at 60 min intervals revealed a transient decrease in MAP followed by a return to basal levels. The initial dose caused a maximal fall in MAP of 33.82% while the second and third injections caused falls in MAP of 36.75% and 38.69%, respectively (Fig. 1c). No tachyphylaxis was observed at this dose, again indicating that there may be a minimum amount of IRL-1620 needed for the development of tachyphylaxis.

3.1.4. Effect of repeated administration of IRL-1620 (5.0 μg/kg, i.v.) on mean arterial pressure

Acute repeated intravenous administration of IRL-1620 (5.0 μg/kg, i.v) at 60 min intervals demonstrated a transient hypotension followed by a return to normal. The hypotensive response was recorded as a maximal decrease of 40.12%, 29.15%, and 21.61% for the first, second and third administrations, respectively (Fig. 1d and 4). Significant ($P < 0.01$) tachyphylaxis was observed to the hypotensive effect of IRL-1620 with the second and third doses.

3.1.5. Effect of repeated administration of IRL-1620 (5 μg/kg, i.v.) following pretreatment with L-NAME, L-arginine + SNP, or BMS187824 on mean arterial pressure

Pretreatment with nitric oxide synthase inhibitor L-NAME (3.3 mg/kg/h) significantly blocked the hypotensive effect of repeated intravenous administration of IRL-1620 (5.0 μg/kg). Animals pretreated with L-NAME followed by three doses of IRL-1620 at 60 min intervals displayed a maximal fall in MAP of 7.28%, 11.43%,

Table 1
Effect of acute repeated intravenous administration of IRL-1620 and ET-1 on heart rate (bpm). The values are expressed as mean ± SEM of heart rate immediately before and at 1 min after injection of IRL-1620 or ET-1. While heart rate increased with both ET-1 and IRL-1620, no significant alteration and no tachyphylaxis were observed.

Treatment	Baseline	Dose 1	Baseline	Dose 2	Baseline	Dose 3
IRL-1620 (1.6, 5.0 and 15 μg/kg)	361 ± 21	361 ± 14	372 ± 17	373 ± 19	354 ± 11	356 ± 17
ET-1 (0.25, 0.75, 2.25 μg/kg)	357 ± 11	371 ± 13	345 ± 9	358 ± 13	336 ± 5	337 ± 5
IRL-1620 (1.6, 1.6 and 1.6 μg/kg)	351 ± 11	349 ± 12	343 ± 22	356 ± 22	347 ± 17	353 ± 17
IRL-1620 (5.0, 5.0 and 5.0 μg/kg)	364 ± 19	377 ± 24	361 ± 21	364 ± 16	355 ± 11	367 ± 15

and 8.19% compared with a decrease of 40.12%, 29.15%, and 21.61% for animals receiving no pretreatment (Fig. 5a). These results support previous findings that the transient hypotensive effect is due, at least in part, to nitric oxide.

Pretreatment with L-arginine (100 mg/kg/h) and nitric oxide donor sodium nitroprusside (10 μg/kg) followed by repeated intravenous administration of IRL-1620 (5.0 μg/kg) produced significant tachyphylaxis to the transient hypotensive effect. A maximal decrease in MAP of 30.22%, 18.61%, and 11.11% after IRL-1620 administration in L-arginine + SNP pretreated rats for doses 1, 2, and 3, respectively, was observed (Fig. 5a).

On the other hand, pretreatment with ET_A receptor antagonist BMS187824 (5 mg/kg) followed by repeated intravenous administration of IRL-1620 (5.0 μg/kg) eliminated the development of tachyphylaxis to the transient hypotensive effects of IRL. The maximal decrease in MAP for the first, second, and third doses of IRL-1620 following pretreatment of BMS was found to be 31.73%, 28.21%, and 32.61%, respectively (Fig. 5a). These results suggest that the development of tachyphylaxis to the hypotensive effect of acute repeated administration of IRL-1620 is due to an interaction between the ET_A and ET_B receptors.

3.2. Heart rate

3.2.1. Effect of repeated administration of IRL-1620 and ET-1 on heart rate

Acute repeated intravenous administration of IRL-1620 and ET-1, while both slightly elevating heart rate did not cause any significant changes at any of the dosing regimens (Table 1). No development of tachyphylaxis was observed in any of the groups.

3.3. Cerebral blood flow

3.3.1. Effect of repeated administration of IRL-1620 (1.6, 5.0, and 15.0 μg/kg, i.v.) on cerebral blood flow

Administration of IRL-1620 produced an increase in cerebral blood flow (CBF). The CBF increase was 15.03%, 7.18% and 10.01% from baseline values 1 min following the 1.6, 5.0, and 15.0 μg/kg dose IRL-1620, respectively (Fig. 2a). No tachyphylaxis was observed with repeated administration. The significance of the increase in CBF itself was not determined, as the purpose of this experiment was solely to determine the development of tachyphylaxis.

3.3.2. Effect of repeated administration of ET-1 (0.25, 0.75, and 2.25 μg/kg, i.v.) on cerebral blood flow

Acute repeated administration of ET-1 caused a transient increase in CBF followed by a return to basal levels within 5 min. The maximal increase in CBF was 17.71%, 15.07% and 3.61% with 0.25, 0.75, and 2.25 μg/kg dose of ET-1, respectively (Fig. 2b). It is possible that the initial doses of ET-1 were interacting with the ET_B receptors causing a slight increase in CBF which quickly dissipated as the ET_A receptors became activated in response to the elevated levels of ET-1 in the circulation. No tachyphylaxis was observed to ET-1's effects on CBF.

3.3.3. Effect of repeated administration of IRL-1620 (1.6 μg/kg, i.v.) on cerebral blood flow

As shown in Fig. 2c, acute repeated administration of IRL-1620 (1.6 μg/kg, i.v.) at 60 min intervals produced an increase in CBF of 14.87%, 15.81%, and 9.42% 1 min after the first, second and third injections, respectively. No tachyphylaxis was observed with this dosing regimen. Again, the significance of the increase in CBF was not determined.

3.3.4. Effect of repeated administration of IRL-1620 (5.0 μg/kg, i.v.) on cerebral blood flow

Acute repeated administration of IRL-1620 (5.0 μg/kg, i.v.) produced an increase in CBF of 2.66%, 9.28%, and 14.74% 1 min after the first, second and third doses, respectively (Fig. 2d). It is interesting to note that the increase in CBF at this dose is lower than the increase with the low dose (1.6 μg/kg) of IRL-1620. No tachyphylaxis was observed with this dosing regimen.

3.3.5. Effect of repeated administration of IRL-1620 (5 μg/kg, i.v.) following pretreatment with L-NAME, L-arginine + SNP, or BMS187824 on cerebral blood flow

Acute repeated administration of IRL-1620 (5.0 μg/kg) following pretreatment with L-NAME, L-arginine + SNP, or BMS187824 produced an increase in cerebral blood flow (Fig. 5b). No tachyphylaxis was observed to the change in CBF with any of the pretreatment regimes.

3.4. Renal blood flow

3.4.1. Effect of repeated administration of IRL-1620 (1.6, 5.0, and 15.0 μg/kg, i.v.) on renal blood flow

Administration of IRL-1620 intravenously caused a decrease in renal blood flow (RBF) of 6.05%, 16.16%, and 9.16% with 1.6, 5.0 and 15.0 μg/kg dose of IRL-1620, respectively, after 1 min (Fig. 3a). No tachyphylaxis was observed. As noted with CBF data, the decrease in RBF was evaluated only for development of tachyphylaxis (Fig. 3).

3.4.2. Effect of repeated administration of ET-1 (0.25, 0.75, and 2.25 μg/kg, i.v.) on renal blood flow

ET-1 produced a decrease in renal blood flow of 3.21%, 6.37% and 42.76% 1 min after dosing with 0.25, 0.75, and 2.25 μg/kg of ET-1, respectively (Fig. 3b). No tachyphylaxis was observed with repeated administration of increasing doses of ET-1 to RBF.

3.4.3. Effect of repeated administration of IRL-1620 (1.6 μg/kg, i.v.) on renal blood flow

Acute repeated administration of IRL-1620 (1.6 μg/kg, i.v.) as shown in Fig. 3c only produced a slight decrease in renal blood flow. RBF dropped 11.60%, 6.07%, and 3.14% from baseline 1 min after the first, second and third doses, respectively. No tachyphylaxis was observed.

3.4.4. Effect of repeated administration of IRL-1620 (5.0 μg/kg, i.v.) on renal blood flow

Acute repeated administration of IRL-1620 (5.0 μg/kg, i.v.) produced a decrease after 1 min in RBF of 16.06%, 6.38% and 17.28%

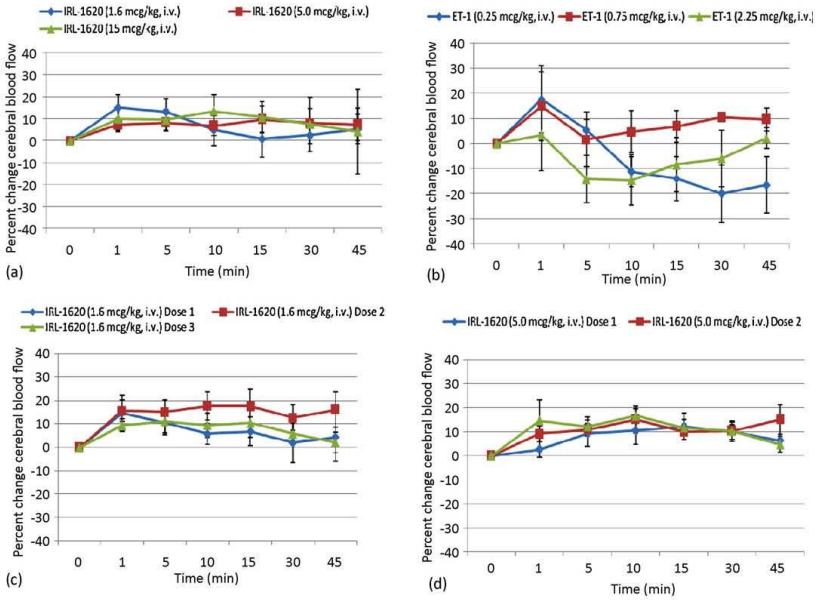


Fig. 2. Effect of repeated intravenous administration of IRL-1620 and ET-1 on cerebral blood flow. The values are expressed as mean ± SEM of the percent change in CBF. (a) IRL-1620 was administered in the doses of 1.6, 5.0 and 15.0 μg/kg at 60 min interval; $n = 6$; (b) ET-1 was administered in the doses of 0.25, 0.75, and 2.25 μg/kg at 60 min interval; $n = 4$; (c) IRL-1620 was administered thrice in the dose of 1.6 μg/kg at 60 min interval; $n = 6$; (d) IRL-1620 was administered thrice in the dose of 5.0 μg/kg at 60 min interval; $n = 5$. IRL-1620 produced an increase in cerebral blood flow at all doses. There was no evidence of any tachyphylaxis.

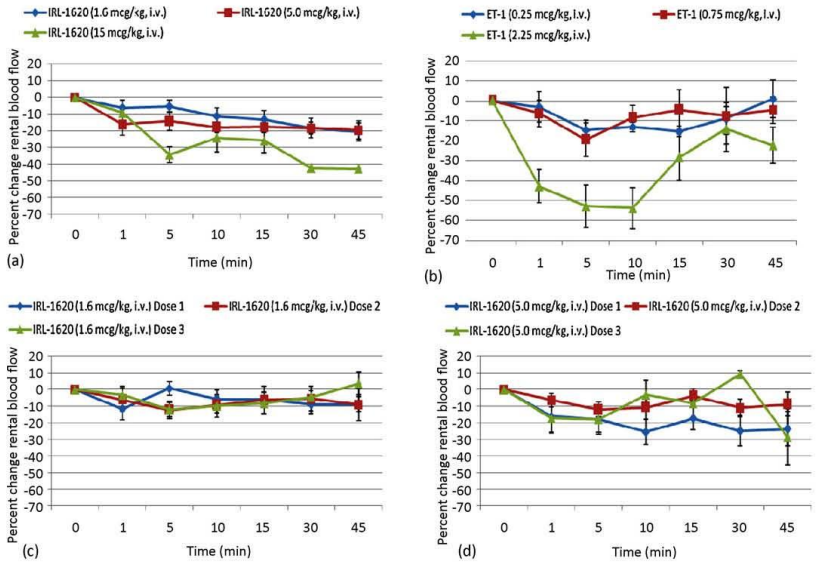


Fig. 3. Effect of repeated intravenous administration of IRL-1620 and ET-1 on renal blood flow. The values are expressed as mean ± SEM of the percent change in RBF. (a) IRL-1620 was administered in the doses of 1.6, 5.0 and 15.0 μg/kg at 60 min interval; $n = 5$; (b) ET-1 was administered in the doses of 0.25, 0.75, and 2.25 μg/kg at 60 min interval; $n = 4$; (c) IRL-1620 was administered thrice in the dose of 1.6 μg/kg at 60 min interval; $n = 6$; (d) IRL-1620 was administered thrice in the dose of 5.0 μg/kg at 60 min interval; $n = 5$. IRL-1620 produced a decrease in renal blood flow at all doses. There was no evidence of any tachyphylaxis.

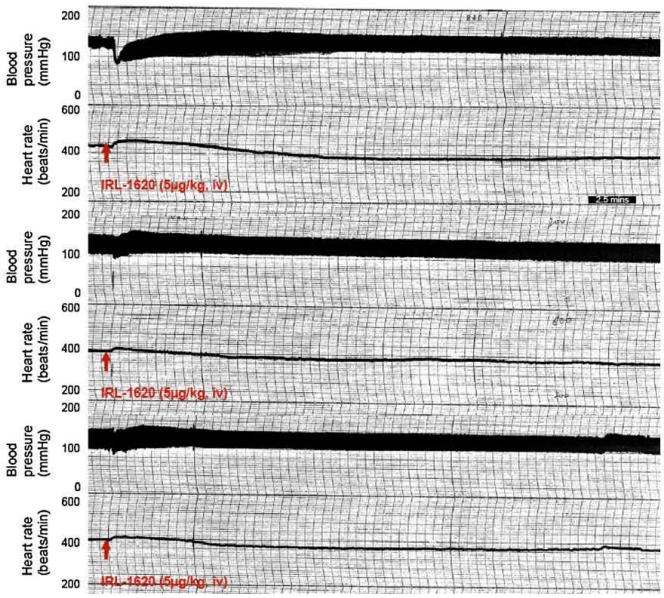


Fig. 4. Blood pressure and heart rate tracing of an experiment to determine the effect of repeated (thrice) administration of IRL-1620 (5.0 μg/kg, i.v.). IRL-1620 produced significant tachyphylaxis to its hypotensive effect.

with the first, second and third doses, respectively, as seen in Fig. 3d. No tachyphylaxis was observed.

3.4.5. Effect of repeated administration of IRL-1620 (5 μg/kg, i.v.) following pretreatment with L-*NAME,* L-*arginine + SNP, or BMS187824 on renal blood flow*

Acute repeated administration of IRL-1620 (5.0 μg/kg) following pretreatment with L-NAME, L-arginine + SNP, or BMS187824 produced a decrease in renal blood flow (Fig. 5c). No tachyphylaxis was observed to the change in RBF with any of the pretreatment regimes.

4. Discussion

The purpose of this study was to determine the hemodynamic effects of acute repeated administration of ET_B receptor agonist IRL-1620. The findings of this study indicate that IRL-1620 exhibits rapidly developing tachyphylaxis to its hypotensive effects but not to its effects on renal or cerebral blood flow. The development of tachyphylaxis to the hypotensive effects of IRL-1620 appears to be due, at least in part, to an interaction with the ET_A receptor.

4.1. Effect on mean arterial pressure

Intravenous injection of ET-1 produces a biphasic response on blood pressure. This response is characterized by a transient hypotension followed by a sustained hypertension. It has been shown that this transient hypotensive effect of ET-1 is due to the release of NO from endothelial cells via stimulation of the ET_B receptors [18,19]. This was confirmed in the present study, where pretreatment with nitric oxide synthase inhibitor L-NAME blocked the hypotensive effect of ET_B agonist IRL-1620.

Tachyphylaxis to ET-1's hypotensive effect has been indicated in previous studies [16,20]. In this study, however, we did not observe any development of tachyphylaxis to the hypotensive effect of ET-1. The difference in results between the previous and present studies may be attributed to the observation that tachyphylaxis to ET-1 is reversible and was not observed after a 2 h interval [16,17]. The previous studies administered ET-1 at 10-12 min intervals, while the current study administered ET-1 at 60 min intervals. It is possible that the recovery time of 60 min was enough to prevent the development of tachyphylaxis to the hypotensive effect of ET-1. It should also be noted that, in contrast to our findings, a study by Hollenberg et al. showed tachyphylactic development to the hypertensive effect of ET-1 in aortic ring segments [21]. This discrepancy could be due to the variations in the dosing regimens, as we were using a much lower dose of ET-1 for repeated injections than the Hollenberg study. Moreover, our study was performed *in vivo* compared to the *in vitro* studies by Hollenberg et al. with differing concentrations of the drug. Our results showing the lack of tachyphylactic development to the pressor effects of ET-1 are consistent with previous findings in rats given successive injections of ET-1 [17]. It may be possible, however, that tachyphylaxis could be observed if ET-1 were administered in high concentrations and with shorter time intervals. In our study, where the most commonly used doses of ET-1 were administered *in vivo*, there was no tachyphylaxis.

ET-1 acts on both the ET_A and ET_B receptors. It produces vasoconstriction by acting directly on the ET_A receptors on the vascular smooth muscle. On the other hand, ET-1 produces vasodilatation by acting on the ET_B receptors on the vascular endothelium. This dilatory action is mediated via the release of NO [22] or prostaglandins [16]. In the present study it was observed that tachyphylaxis develops to the transient hypotension following repeated administration of IRL-1620, a highly selective ET_B receptor agonist. The mechanism involved in development of tachyphylaxis to the hypotensive effects of ET-1 was thought to be due to the depletion of NO, although cross-tachyphylaxis between NO and ET-1 did not support this hypothesis [20]. Another possibility is that acute repeated administration of ET-1 causes the vasoconstrictor effects of the ET_A receptor to overshadow the vasodilator effects of the ET_B receptor.

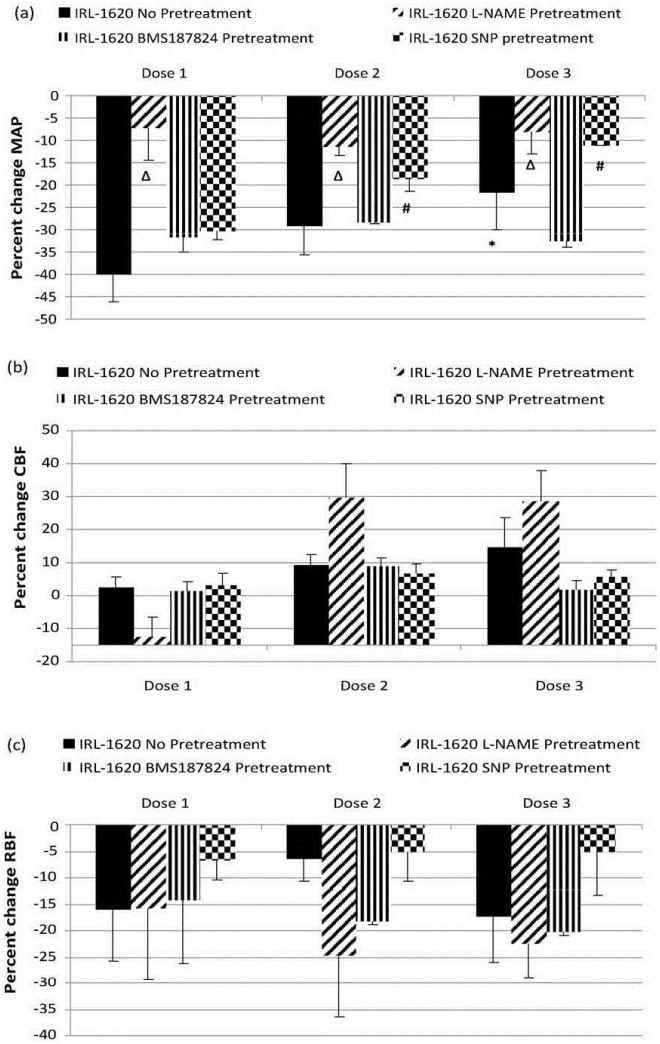


Fig. 5. Effect of repeated administration of IRL-1620 (5.0 μg/kg, i.v.) with or without pretreatment on mean arterial pressure, cerebral blood flow, and renal blood flow. Pretreatments include L-NAME (3.3 mg/kg/h) $n = 4$, L-arginine (100 mg/kg/h) + SNP (10 μg/kg) $n = 3$, and BMS187824 (5 mg/kg) $n = 3$. The values are expressed as mean ± SEM of the percent change in MAP, CBF, and RBF, respectively. (a) Effect of acute repeated administration of IRL-1620 on MAP. IRL-1620 without pretreatment produced tachyphylaxis to its hypotensive effects, $^{*}P < 0.01$. Pretreatment with L-NAME blocked the transient hypotension produced by IRL-1620, $^{\Delta}P < 0.05$. Pretreatment with BMS did not produce tachyphylaxis. Pretreatment with L-arginine and SNP produced tachyphylaxis, $^{\#}P < 0.05$. (b) Effect of acute repeated administration of IRL-1620 with and without pretreatment on CBF. No tachyphylaxis was observed. (c) Effect of repeated administration of IRL-1620 with and without pretreatment on RBF. No tachyphylaxis was observed.

There are several possible explanations for the development of tachyphylaxis to the hypotensive effect of ET_B selective agonist, IRL-1620:

1. As proposed previously with tachyphylactic development to the hypotensive effects of ET-1, a depletion of nitric oxide could lead to less of a fall in MAP with successive doses.
2. The vasodilatation caused by the activation of the ET_B receptors on the vascular endothelium could be overshadowed by the activation of the vasoconstrictor ET_A receptors on the vascular smooth muscle. Although IRL-1620 is highly selective for the ET_B receptors, high concentrations of this drug can act upon the ET_A receptors as well. It is possible that the multiple injections could have had a summing effect which could result in plasma concentrations high enough to activate the ET_A receptors.
3. There may be a change in the number, conformation or selective activation of the ET_B receptors, resulting in a decreased activation of the vasodilator action.

We tested the hypothesis that the tachyphylactic development to the hypotensive effects of IRL-1620 was due to a depletion of nitric oxide. Pretreating animals with L-arginine, a precursor for

nitric oxide synthesis, and sodium nitroprusside, a nitric oxide donor, however, did not eliminate the development of tachyphylaxis to the hypotensive effects of acute repeated administration of IRL-1620 (Fig. 5a). These results are consistent with the previous findings that tachyphylactic development to the hypotensive effects of ET-1 does not appear to be related to the depletion of NO [20].

In order to test the hypothesis that a significantly high concentration of IRL-1620 sufficiently activated the ET_A receptors to cause a constriction that overshadowed the ET_B receptor vasodilator effect, we pretreated animals with ET_A receptor antagonist BMS187824. If the tachyphylactic development to the hypotensive effects of IRL-1620 was due to the activation of ET_A receptors, one would expect that blocking these receptors would eliminate the tachyphylaxis. As shown in Fig. 5a, it is evident that antagonizing the ET_A receptor did in fact alter the development of tachyphylaxis to the transient hypotension elicited by acute repeated administration of ET_B receptor agonist IRL-1620. The maximal decrease in MAP in animals pretreated with BMS remained consistently around 30% with each successive dose of IRL-1620. These results are consistent with previous studies showing tachyphylaxis to the hypotensive effects of ET-1, which activates both ET_A and ET_B receptors. Although this study did not observe such tachyphylaxis with repeated doses of ET-1, it is thought that the intervals (60 min) between the doses of ET-1 were sufficient to mask this effect. Previous studies have shown that the tachyphylaxis to ET-1's hypotensive effects are reversible after 2 h, while our findings seem to indicate that it may be reversed in as little as 1 h [20]. While this study indicates that the tachyphylaxis to IRL-1620's hypotensive effects is evident at 60 min intervals *in vivo*, it is unknown when or if this effect is reversible.

It remains to be seen whether or not there is an alteration in the conformation, number, or activation of ET_B receptors with acute repeated administration of either ET-1 or IRL-1620. There has been some pharmacological evidence suggesting that ET_B receptors may be divided into two subtypes, ET_{B1} receptors present on endothelial cells mediating vasodilatation, and ET_{B2} receptors present on smooth muscle cells mediating vasoconstriction [23,24]. A complex interaction between all three ET receptors may occur when simultaneously stimulated. In 2006, Kusmic et al. proposed a theory that, under normotensive conditions, co-stimulation of the ET_A and ET_{B1} receptors may cause a negative feedback inactivating the ET_{B2} receptor. Under hypotensive conditions, this negative feedback is turned off, resulting in a net pressor effect as the ET_A and ET_{B2} receptors are both activated [19]. Further studies involving immunohistochemistry of the ET_B receptors in various tissues may help determine the possibility of such an interaction among receptors, as well as the possibility of a change in conformation or number of these receptors when subject to acute repeated administration of IRL-1620.

4.2. Effect on regional blood flow

An important observation made during the course of this study is that IRL-1620 is capable of producing an increase in cerebral blood flow. The stimulation of vasodilator ET_B receptors and the subsequent synthesis and release of NO could account for this phenomenon.

Alterations in cerebral blood flow following acute repeated administration of either IRL-1620 or ET-1 remained unaffected in regards to tachyphylaxis development. The tight junctions of the cerebral vasculature may prevent the activation of ET_A receptors on the vascular smooth muscle, thus limiting the ET-mediated constriction of those vessels, and prolonging the vasodilatation and subsequent increase in CBF.

As with the alterations in CBF, changes in renal blood flow did not reflect the development of tachyphylaxis. Our findings concur with previous studies that have indicated that stimulation of ET_B receptors in the kidney causes a decrease in RBF via constriction of the afferent arteriole (in conjunction with ET_A receptor activation) and dilation of the efferent arteriole [25,26]. The fact that the decrease in RBF displayed no tachyphylaxis to acute repeated activation of ET receptors via either IRL-1620 or ET-1 is not surprising. Even if the dilation of the efferent arteriole was somewhat abated via the previously mentioned mechanisms, the net effect of ET on constricting the afferent arteriole would be a decrease in RBF and no tolerance would therefore have been observed.

Although tachyphylactic development was observed to the hypotensive effects of IRL-1620, we did not observe tachyphylaxis to the effects on renal or cerebral blood flow. It is possible that other vascular beds may be developing tachyphylaxis to acute repeated administration of IRL-1620. This could be determined via the use of radioactive microspheres where the blood flow to all of the organs of the body can be studied at one time. It is also possible that pathological changes like hypoxia can produce an imbalance in the ET and nitric oxide physiology and hence development of tachyphylaxis to IRL-1620 may alter in disease states including hypoxia of the tumors.

5. Conclusion

The results of the present study demonstrate that acute repeated administration of ET_B receptor agonist, IRL-1620 produces tachyphylaxis only to its hypotensive effects. No tachyphylaxis was observed to the hypertensive effects or the alterations in cerebral and renal blood flow. It is therefore concluded that acute repeated administration of IRL-1620 may be carried out without developing tolerance to the alteration of renal or cerebral blood flow. This study did not examine whether IRL-1620 produces tachyphylaxis to an increase in tumor blood flow.

Acknowledgement

The present study was supported by funds from Midwestern University, Downers Grove, IL.

References

[1] Nunez DJ, Brown MJ, Davenport AP, Neylon CB, Schofield JP, Wyse RK. Endothelin-1 mrna is widely expressed in porcine and human tissues. J Clin Invest 1990;85:1537–41.
[2] Gulati A, Rebello S. Characteristics of endothelin receptors in the central nervous system of spontaneously hypertensive rats. Neuropharmacology 1992;31:243–50.
[3] Nelson J, Bagnato A, Battistini B, Nisen P. The endothelin axis: emerging role in cancer. Nat Rev Cancer 2003;3:110–6.
[4] Inoue A, Yanagisawa M, Kimura S, Kasuya Y, Miyauchi T, Goto K, et al. The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes. Proc Natl Acad Sci USA 1989;86:2863–7.
[5] Arai H, Hori S, Aramori I, Ohkubo H, Nakanishi S. Cloning and expression of a cdna encoding an endothelin receptor. Nature 1990;348:730–2.
[6] Sakurai T, Yanagisawa M, Takuwa Y, Miyazaki H, Kimura S, Goto K, et al. Cloning of a cdna encoding a non-isopeptide-selective subtype of the endothelin receptor. Nature 1990;348:732–5.
[7] Remuzzi G, Perico N, Benigni A. New therapeutics that antagonize endothelin: promises and frustrations. Nat Rev Drug Discov 2002;1:986–1001.
[8] Sakamoto A, Yanagisawa M, Sawamura T, Enoki T, Ohtani T, Sakurai T, et al. Distinct subdomains of human endothelin receptors determine their selectivity to endothelina-selective antagonist and endothelinb-selective agonists. J Biol Chem 1993;268:8547–53.
[9] Just A, Olson AJ, Arendshorst WJ. Dual constrictor and dilator actions of et(b) receptors in the rat renal microcirculation: interactions with et(a) receptors. Am J Physiol Renal Physiol 2004;286:F660–8.
[10] Takai M, Umemura I, Yamasaki K, Watakabe T, Fujitani Y, Oda K, et al. A potent and specific agonist, suc-[glu9,ala11,15]-endothelin-1(8–21), irl 1620, for the etb receptor. Biochem Biophys Res Commun 1992;184:953–9.

[11] Gulati A, Rai A. Endothelin-1-induced vasodilatation in rat breast tumor is mediated through endothelin-b receptors. J Cardiovasc Pharmacol 2004;44:S483–6.
[12] Rai A, Gulati A. Evidence for the involvement of et(b) receptors in et-1-induced changes in blood flow to the rat breast tumor. Cancer Chemother Pharmacol 2003;51:21–8.
[13] Rai A, Rajeshkumar NV, Gulati A. Effect of the et(b) receptor agonist, irl-1620, on paclitaxel plasma pharmacokinetics of breast tumor rats. Exp Biol Med (Maywood) 2006;231:1120–2.
[14] Rai A, Rajeshkumar NV, Shord S, Gulati A. Et(b) receptor agonist, irl 1620, does not affect paclitaxel plasma pharmacokinetics in breast tumour bearing rats. J Pharm Pharmacol 2005;57:869–76.
[15] Rajeshkumar NV, Rai A, Gulati A. Endothelin b receptor agonist, irl 1620, enhances the anti-tumor efficacy of paclitaxel in breast tumor rats. Breast Cancer Res Treat 2005;94:237–47.
[16] Le Monnier de Gouville AC, Lippton H, Cohen G, Cavero I, Hyman A. Vasodilator activity of endothelin-1 and endothelin-3: rapid development of cross-tachyphylaxis and dependence on the rate of endothelin administration. J Pharmacol Exp Ther 1990;254:1024–8.
[17] Le Monnier de Gouville AC, Mondot S, Lippton H, Hyman A, Cavero I. Hemodynamic and pharmacological evaluation of the vasodilator and vasoconstrictor effects of endothelin-1 in rats. J Pharmacol Exp Ther 1990;252:300–11.
[18] Cardillo C, Kilcoyne CM, Cannon 3rd RO, Panza JA. Interactions between nitric oxide and endothelin in the regulation of vascular tone of human resistance vessels in vivo. Hypertension 2000;35:1237–41.
[19] Kusmic C, Lazzerini G, Coceani F, Barsacchi R, L'Abbate A, Sambuceti G. Paradoxical coronary microcirculatory constriction during ischemia: a synergic function for nitric oxide and endothelin. Am J Physiol Heart Circ Physiol 2006;291:H1814–21.
[20] Le Monnier de Gouville AC, Cavero I. Cross tachyphylaxis to endothelin isopeptide-induced hypotension: a phenomenon not seen with proendothelin. Br J Pharmacol 1991;104:77–84.
[21] Hollenberg SM, Shelhamer JH, Cunnion RE. Tachyphylaxis to the vasopressor effects of endothelin in rat aortic rings. Am J Physiol 1993;264:H352–6.
[22] Fozard JR, Part ML. The role of nitric oxide in the regional vasodilator effects of endothelin-1 in the rat. Br J Pharmacol 1992;105:744–50.
[23] Brooks DP, DePalma PD, Pullen M, Gellai M, Nambi P. Identification and function of putative etb receptor subtypes in the dog kidney. J Cardiovasc Pharmacol 1995;26(Suppl. 3):S322–5.
[24] Leite-Moreira AF, Bras-Silva C. Inotropic effects of etb receptor stimulation and their modulation by endocardial endothelium, no, and prostaglandins. Am J Physiol Heart Circ Physiol 2004;287:H1194–9.
[25] Fellner SK, Arendshorst W. Endothelin-a and -b receptors, superoxide, and Ca^{2+} signaling in afferent arterioles. Am J Physiol Renal Physiol 2007;292:F175–84.
[26] Inscho EW, Imig JD, Cook AK, Pollock DM. Eta and etb receptors differentially modulate afferent and efferent arteriolar responses to endothelin. Br J Pharmacol 2005;146:1019–26.

BRAIN RESEARCH 1420 (2011) 48–58

Available online at www.sciencedirect.com

SciVerse ScienceDirect

www.elsevier.com/locate/brainres

BRAIN RESEARCH

Research Report

Endothelin B receptor agonist, IRL-1620, reduces neurological damage following permanent middle cerebral artery occlusion in rats

Mary G. Leonard[a,b], *Seema Briyal*[a], *Anil Gulati*[a,b,*]

[a]*Midwestern University Chicago College of Pharmacy, Downers Grove, IL 60515, USA*
[b]*Department of Bioengineering, University of Illinois at Chicago, Chicago, IL 60607, USA*

ARTICLE INFO

Article history:
Accepted 31 August 2011
Available online 7 September 2011

Keywords:
Endothelin B receptor
Cerebral ischemia
Oxidative stress
IRL-1620
Neuroprotection

ABSTRACT

Endothelin and its receptors have long been considered therapeutic targets in the treatment of ischemic stroke. Recent studies indicate that ET_B receptors may provide both vasodilatation and neuroprotection. The purpose of this study was to determine the effect of selectively activating the ET_B receptors following permanent middle cerebral artery occlusion in rats. IRL-1620 [Suc-[Glu9,Ala11,15]-Endothelin-1(8-12)], a highly selective ET_B agonist, was used alone and in conjunction with BQ788, an ET_B antagonist, to determine the role of ET_B receptors in cerebral ischemia. Rats were assessed for neurological deficit and motor function, and their brains were evaluated to determine infarct area, oxidative stress parameters, and ET receptor protein levels. Animals treated with IRL-1620 showed significant improvement in all neurological and motor function tests when compared with both vehicle-treated and BQ788-treated middle cerebral artery occluded groups. In addition, there was a significant decrease in infarct volume 24 h after occlusion in animals treated with IRL-1620 (24.47 ± 4.37 mm^3) versus the vehicle-treated group (153.23 ± 32.18 mm^3). Blockade of ET_B receptors by BQ788 followed by either vehicle or IRL-1620 treatment resulted in infarct volumes similar to those of rats treated with vehicle alone (163.51 ± 25.41 and 139.21 ± 15.20 mm^3, respectively). Lipid peroxidation, as measured by malondialdehyde, increased and antioxidants (superoxide dismutase and reduced glutathione) decreased following infarct. Treatment with IRL-1620 reversed these effects, indicating that ET_B receptor activation reduces oxidative stress injury following ischemic stroke. Animals pretreated with BQ788 showed similar oxidative stress damage as those in the vehicle-treated group. No significant difference was observed in ET_B receptor levels in any of the groups. The present study demonstrates that ET_B receptor activation may be a novel neuroprotective therapy in the treatment of focal ischemic stroke.

* *Corresponding author at:* Chicago College of Pharmacy, Midwestern University, 555 31st St., Downers Grove, IL 60515-1235, USA. Fax: +1 630 971 6097.
E-mail address: AGULAT@midwestern.edu (A. Gulati).

Abbreviations: ET, endothelin; MCAO, middle cerebral artery occlusion; MDA, malondialdehyde; GSH, reduced glutathione; SOD, superoxide dismutase; NO, nitric oxide; NOS, nitric oxide synthase

0006-8993/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.brainres.2011.08.075

1. Introduction

According to the American Heart Association, stroke is the third leading cause of death and the leading cause of long-term disability. Ischemic stroke, which accounts for more than 80% of all strokes, occurs when a portion of the brain is deprived of blood flow and therefore oxygen, often due to a clot (Roger et al., 2011). Despite the severity of this condition, the only currently available FDA-approved pharmacological treatment for ischemic stroke is recombinant tissue plasminogen activator (rtPA), which dissolves the clot and restores blood flow to the brain. This treatment is complicated by the relatively short window of time between infarct and treatment (3–4 h) and the increased risk of subarachnoid hemorrhage (Micieli et al., 2009). A large number of other agents, broadly classified as neuroprotective and aiming to slow or stop the secondary damage associated with the ischemic cascade following stroke, have shown promise in the initial stages of research but have thus far failed to demonstrate efficacy in clinical trials (Ly et al., 2006). A new approach is therefore needed, one which has the potential to address both the restoration of blood flow and attenuate secondary damage to the penumbral area.

More than two decades ago, endothelin (ET) and its receptors were identified as potential therapeutic targets in the treatment and prevention of ischemic stroke. ET is an endogenous vasoregulatory peptide which targets two main receptors — ET_A and ET_B. ET_A receptors are mainly located on vascular smooth muscle cells and mediate vasoconstriction, whereas ET_B receptors are mainly located on vascular endothelial cells and mediate vasodilatation (Goto et al., 1989; Tsukahara et al., 1994). ET and its receptors are present throughout the brain, expressed in vascular tissues, neurons and astrocytes (Schinelli, 2006). Following both ischemic stroke and subarachnoid hemorrhage, ET levels in the blood and ET immunoreactivity in the tissues are elevated (Asano et al., 1990; Viossat et al., 1993). A demonstration that the increase in ET levels coincides with a decrease in regional blood flow in the ischemic areas of the brain following experimental stroke led to the investigation of several ET antagonists in the treatment of focal ischemic stroke (Patel et al., 1995). Although some ET_A specific and $ET_{A/B}$ non-specific antagonists have shown promise in experimental stroke models, others have not (Barone et al., 1995; Barone et al., 2000; Briyal and Gulati, 2010; Gupta et al., 2005; Zhang et al., 2005; Zhang et al., 2008).

While the majority of research in this area has focused on selectively antagonizing the ET_A receptors in order to prevent excess vasoconstriction, no one has yet examined the effect of selectively activating ET_B receptors in a focal stroke model. Complete deficiency or blockade of ET_B receptors leads to exacerbation of ischemic brain damage, possibly due to the shift in ET vasomotor balance (Chuquet et al., 2002; Ehrenreich et al., 1999). Conversely, our lab has previously demonstrated that activation of ET_B receptors with intravenous IRL-1620, a highly selective ET_B agonist, results in a significant elevation in cerebral blood flow in normal rats (Leonard and Gulati, 2009). Moreover, functional ET_B receptors have been shown to enhance proliferation of neuronal progenitors and to protect against apoptosis in the dentate gyrus, olfactory epithelium, and cortical neurons (Ehrenreich et al., 1999; Laziz et al., 2011; Lee et al., 2003; Yagami et al., 2005).

ET_B receptors mediate their vasodilator effects via the synthesis and release of nitric oxide (NO) (Tsukahara et al., 1994). The three isoforms of nitric oxide synthase (eNOS, iNOS, and nNOS) have all been shown to play a role in either the endogenous neuroprotection or neurodegradation which occurs after an ischemic stroke. Overstimulation of nNOS and iNOS following an ischemic attack leads to excessive NO which reacts with O_2^- to form the neurotoxic oxidant $ONOO^-$, resulting in excytotoxic damage and exacerbation of the ischemic insult (Bauser-Heaton and Bohlen, 2007; Samdani et al., 1997). As activation of ET_B receptors results in the release of NO, which may be either therapeutic or deleterious, it was of interest to determine the levels of oxidative stress parameters that are altered during the ischemic cascade. Oxidative stress following ischemic stroke results in increases in lipid peroxidation compounds like malondialdehyde and decreases in antioxidants such as reduced glutathione and superoxide dismutase (Briyal and Gulati, 2010; Chan, 1996; Gupta et al., 2005).

As previous data has demonstrated that activation of ET_B receptors increases cerebral blood flow and may be neuroprotective, we conducted this study to determine whether activation of the ET_B receptor reduces neurological damage following cerebral ischemia. The effects of ET_B receptor agonist IRL-1620 and ET_B receptor antagonist BQ788 on infarct area, neurological deficit, motor function, oxidative stress parameters, and ET_A and ET_B receptor levels were determined in a rat model of middle cerebral artery occlusion.

2. Results

2.1. *Effect on motor function and coordination in middle cerebral artery occluded rats*

Prior to occlusion, there were no significant differences between the groups with regards to motor function and coordination. On the other hand, permanent middle cerebral artery occlusion of the right hemisphere in rats resulted in significant neurological deficit and decreased muscular strength as measured by the grip test, as well as impaired coordination as measured by foot fault and rota rod tests 24 h after induction of cerebral ischemia (Table 1).

2.1.1. *Effect on neurological deficit in middle cerebral artery occluded rats*

No neurological deficits were observed in any animals prior to occlusion. Twenty-four hours after middle cerebral artery occlusion of the right side, however, paresis of the left hind paw was observed. Compared to sham-operated rats, the mean neurological score of vehicle-treated middle cerebral artery occluded rats was significantly higher (3.13 ± 0.24; $P<0.001$), indicative of neurological impairment following induction of cerebral ischemia. In contrast, middle cerebral artery occluded rats treated with IRL-1620 demonstrated significant improvement in neurological function when compared with the vehicle group (0.85 ± 0.32; $P<0.001$). Blockade of ET_B receptors with BQ788 prior to treatment with either vehicle or IRL-1620 resulted in significant neurological impairment when compared with both sham-operated ($P<0.001$) and IRL-1620 ($P<0.001$) alone (Table 1). The vehicle-treated group resulted in a 33% mortality rate at 24 h post occlusion,

Table 1 – Effect of ET_B receptor agonist, IRL-1620, and antagonist, BQ788, on neurological deficit and motor function at baseline and 24 h post middle cerebral artery occlusion in rats. IRL-1620 (5 μg/kg, IV) or isotonic saline (1 ml/kg, IV) was injected at 2, 4, and 6 h post MCAO. BQ788 (1 mg/kg, IV) was administered 15 min prior to the first injection of IRL-1620 or vehicle. Values are expressed as mean ± SEM.

Treatment groups		Neurological evaluation (6 point scale)	Grip test (6 point scale)	Foot fault error (%)	Rota rod duration (s)
Sham	Baseline	0±0	3.75±0.24	7.25±0.53	123.63±15.28
	24 h	0±0	3.25±0.37	7.14±1.05	161.14±25.24
MCAO+vehicle	Baseline	0±0	3.73±0.23	5.93±0.66	126.00±7.05
	24 h	3.13±0.24 *	0.93±0.28 *	61.13±8.88 *	51.20±10.07 *
MCAO+IRL-1620	Baseline	0±0	4.07±0.21	4.31±0.58	124.23±6.05
	24 h	0.85±0.32 #	2.85±0.27 #	15.62±4.03 #	122.00±16.45 #
MCAO+BQ788	Baseline	0±0	4.17±0.31	5.00±0.86	143.67±16.00
	24 h	2.67±0.42 *,@	0.67±0.21 *,@	60.17±13.74 *,@	33.67±9.46 *,@
MCAO+BQ788+IRL-1620	Baseline	0±0	4.33±0.33	6.83±0.60	133.00±8.55
	24 h	3.33±0.21 *,@	0.33±0.21 *,@	73.33±11.44 *,@	32.50±10.51 *,@

* P<0.01 vs. sham.
\# P<0.01 vs. MCAO+vehicle.
@ P<0.01 vs. MCAO+IRL-1620.

while the BQ788+vehicle and BQ788+IRL-1620 treated groups each demonstrated a 17% mortality rate. No mortality was observed in either the sham operated or IRL-1620 treated groups during the 24 h period.

2.1.2. *Effect on muscular strength using grip test*

All animals prior to occlusion demonstrated similar muscular strength in their ability to grip/climb onto the string. Vehicle-treated rats demonstrated a significant decrease in muscle strength as measured by the grip test following middle cerebral artery occlusion as compared to sham-operated rats (P<0.001). In contrast, occluded rats treated with IRL-1620 showed significant improvement (P<0.001) when compared with vehicle-treated rats, with mean scores of 2.85±0.27 vs. 0.93±0.28, respectively. Pretreatment with BQ788 followed by treatment with either vehicle or IRL-1620 resulted in significant impairment of muscle strength as compared with sham (P<0.001) and IRL-1620 (P<0.001) alone groups. Interestingly, blockade of ET_B receptors by BQ788 caused an even greater deficit in motor function following cerebral ischemia than that seen in animals treated with vehicle alone (Table 1).

2.1.3. *Effect on motor coordination using foot fault*

Prior to occlusion, animals in all groups averaged less than 10% foot fault error. Following occlusion, however, the percentage of foot fault errors was significantly higher in vehicle-treated occluded rats as compared to sham-operated rats (P<0.001), indicating lack of motor coordination. Treatment with IRL-1620 improved coordination, resulting in a significantly lower percentage of error as compared with vehicle-treated rats (15.62±4.03% vs. 61.13±8.88%; P<0.001). In contrast, animals treated with BQ788 followed by either vehicle or IRL-1620 showed levels of impairment similar to those of the vehicle alone group (Table 1).

2.1.4. *Effect on motor coordination using rota rod*

There were no significant differences between groups prior to occlusion with animals remaining on the rotating spindle for approximately 2 min. Twenty-four hours after occlusion, however, vehicle-treated middle cerebral artery occluded rats displayed a significant lack of coordination when compared with sham-operated animals (P<0.001). Whereas sham rats were able to remain on the accelerating spindle for 161.14±25.24 s, vehicle-treated MCAO rats fell off after 51.20±10.07 s. IRL-1620-treated middle cerebral artery occluded rats demonstrated significant improvement in motor coordination as compared with vehicle-treated rats (P<0.01), remaining on the spindle for 124.23±6.05 s. Rats in both BQ788 treated groups, however, demonstrated significant impairment in motor coordination when compared to sham (P<0.001) and IRL-1620 (P<0.01) alone groups. Blockade of the ET_B receptors led to an even greater deficit in motor function following cerebral ischemia compared to that seen in the vehicle-treated group (Table 1).

2.2. *Effect on infarct volume in middle cerebral artery occluded rats*

Middle cerebral artery occlusion resulted in an infarct volume of 153.23±32.18 mm^3 in rats treated with vehicle alone (Fig. 1). Administration of IRL-1620 significantly reduced infarct volume (24.47±4.37 mm^3; P<0.01) as compared with vehicle. In contrast, administration of BQ788 prior to either vehicle or IRL-1620 resulted in significantly large infarct volumes (163.51±25.41 and 139.21±15.20 mm^3, respectively; P<0.01) when compared with IRL-1620 alone. The infarct volume in BQ788 treated group was similar to the vehicle-treated group.

2.3. *Effect on oxidative stress parameters in middle cerebral artery occluded rats*

To determine the effect of ET_B receptors during middle cerebral artery occlusion on oxidative stress parameters, malondialdehyde, reduced glutathione and superoxide dismutase levels in the brains of sham-operated and occluded rats treated with vehicle, IRL-1620 and/or BQ788 were determined 24 h after cerebral infarction (Fig. 2).

2.3.1. *Effect on malondialdehyde levels*

Brain levels of malondialdehyde were measured to determine the effect of ET_B receptor activation on lipid peroxidation following middle cerebral artery occlusion (Fig. 2A). As expected, the levels

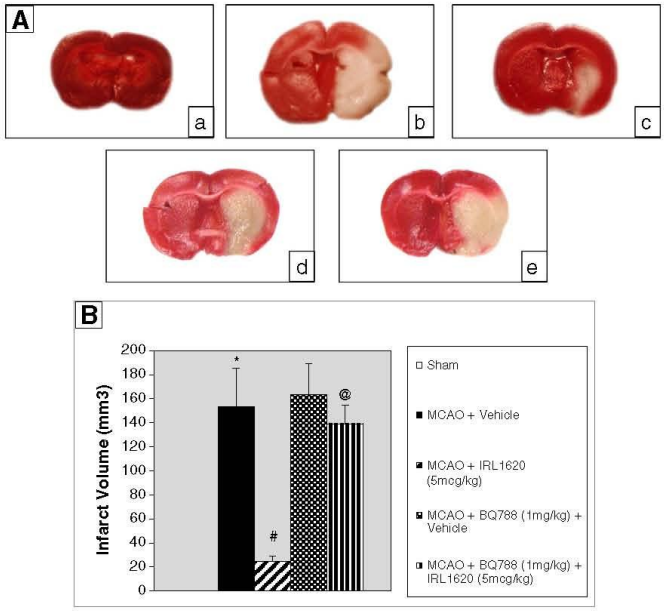


Fig. 1 – A. 2 mm coronal sections of brains stained with TTC to visualize the infarct area (red indicates normal tissue and white indicates infarct tissue). Representative slices from groups are as follows: a. sham, b. MCAO+Vehicle, c. MCAO+IRL-1620, d. MCAO+BQ788, e. MCAO+BQ788+IRL-1620. IRL-1620 (5 μg/kg, IV) or isotonic saline (1 ml/kg, IV) was injected at 2, 4, and 6 h post MCAO. BQ788 (1 mg/kg, IV) was administered once 15 min prior to the first injection of IRL-1620 or vehicle. B. Effect of IRL-1620, BQ788, and BQ788+IRL-1620 on infarct volume in middle cerebral artery occluded rats. Values are expressed as mean ± SEM. *P<0.001 vs. sham. #P<0.01 vs. MCAO+Vehicle. @P<0.01 vs. MCAO+IRL-1620.

of MDA in vehicle-treated middle cerebral artery occluded rats were significantly high (574.30±34.83 nmol/g wet tissue) when compared with sham-operated animals (113.16±1.87 nmol/g wet tissue; P<0.001). MDA levels were significantly reduced in the IRL-1620-treated animals when compared to the vehicle-treated group (179.12±26.59 nmol/g wet tissue; P<0.001), indicating a possible antioxidant effect of ET_B receptor activation following cerebral ischemia. Administration of BQ788 prior to either vehicle or IRL-1620 resulted in MDA levels (568.94±18.94 and 601.89±17.28 nmol/g wet tissue, respectively) comparable to those seen with vehicle alone.

2.3.2. *Effect on reduced glutathione levels*

Reduced GSH levels in vehicle-treated middle cerebral artery occluded rats were significantly lower (97.37±7.16 μg/g wet tissue) than those of sham-operated animals (250.20±15.01 μg/g wet tissue; P<0.05). Treatment with IRL-1620, on the other hand, significantly increased the levels of GSH in the brains of occluded rats (187.72±13.31 μg/g wet tissue) as compared with vehicle alone (Fig. 2B). Pretreatment with BQ788 blocked the positive effects of IRL-1620 treatment on GSH levels (85.71±6.96 μg/g wet tissue; P<0.001).

2.3.3. *Effect on superoxide dismutase levels*

The levels of the antioxidant marker, superoxide dismutase, in the brains of vehicle-treated middle cerebral artery occluded rats were significantly lower (8.26±0.82 units/mg protein) than those of the sham-operated group (29.87±1.66 units/mg protein; P<0.001). Administration of IRL-1620 significantly improved SOD levels (13.17±0.69 units/mg protein; P<0.05) as compared with the vehicle-treated group, again indicating a possible antioxidant effect of ET_B receptor activation following cerebral infarction (Fig. 2C). As with GSH, SOD levels were significantly lower when occluded animals were pretreated with BQ788 prior to administration of either vehicle or IRL-1620 (5.11±0.23 and 4.66±0.17 units/mg protein, respectively; P<0.001).

2.4. *Effect on levels of brain endothelin receptors in middle cerebral artery occluded rats*

To determine the effects of ischemia, with or without treatment, on the regulation of ET receptor levels within the brain, Western blot analysis was performed. No significant differences were observed between vehicle, IRL-1620, and BQ788 treated middle cerebral artery occluded rats in either ET_A or ET_B receptor levels as determined by Western blot analysis. All groups receiving the occlusion showed significantly higher levels of ET_A receptors in the right, infarcted hemisphere as compared with the left non-infarcted hemisphere (Fig. 3A). ET_B receptor levels were similar in all groups and in both infarcted and non-infarcted hemispheres (Fig. 3B), indicating that the protective effects of IRL-1620 are not due to a change in the number of ET receptors.

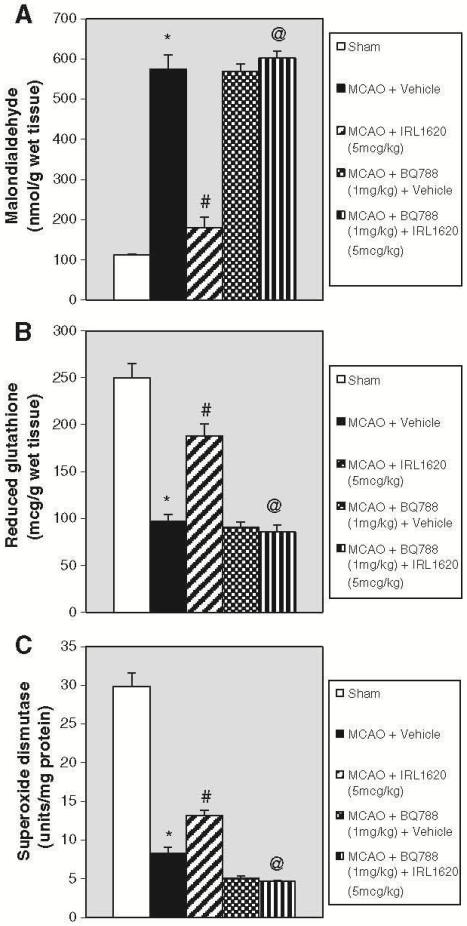


Fig. 2 – Effect of ET_B agonist, IRL-1620, on oxidative stress parameters 24 h post middle cerebral artery occlusion in rats. A. Malondialdehyde levels in the occluded rat brain. B. Reduced glutathione levels in the occluded rat brain. C. Superoxide dismutase levels in the occluded rat brain. IRL-1620 (5 μg/kg, IV) or isotonic saline (1 ml/kg, IV) was injected at 2, 4, and 6 h post MCAO. BQ788 (1 mg/kg, IV) was administered once 15 min prior to the first injection of IRL-1620 or vehicle. Values are expressed as mean ± SEM. *P < 0.001 vs. sham. #P < 0.05 vs. MCAO + Vehicle. @P < 0.001 vs. MCAO + IRL-1620.

3. Discussion

The purpose of this study was to determine the effect of selective ET_B receptor activation on physiological and functional recovery following a middle cerebral artery occlusion model of ischemic stroke in rats. In order to gain a complete picture of ET_B receptor involvement in cerebral ischemia, as well as to verify that the effects of IRL-1620 were specific to activation of ET_B receptors, a selective ET_B antagonist BQ788 was used alone and in combination with IRL-1620.

Experimental cerebral ischemia results in a marked deficit in neurological function and motor performance, as well as an increase in oxidative stress, within 24 h after MCAO. Our results indicate that treatment with selective ET_B receptor agonist, IRL-1620, significantly improves neurological and motor function while reducing oxidative stress and overall infarct area. Antagonizing the ET_B receptor, on the other hand, blocks the positive effects of IRL-1620 treatment, resulting in deficits similar to, and in some cases greater than, those seen in vehicle-treated middle cerebral artery occluded rats. To our knowledge, this is the first report indicating that stimulation of the ET_B receptors following cerebral ischemia is neuroprotective.

The endothelin system has long been known to play a role in the pathophysiology of stroke. ET-1 levels in the blood and ET immunoreactivity are elevated following cerebral ischemia (Viossat et al., 1993). Due to the highly potent vasoconstriction caused by ET-1 via the ET_A receptor, it was postulated that administration of ET antagonists would decrease the damage associated with ischemic stroke. ET_A receptor antagonists, SB234551 and S-1039, demonstrated a reduction in infarct area, edema, and neurological deficit following experimental cerebral ischemia (Zhang et al., 2005; Zhang et al., 2008). Mixed $ET_{A/B}$ receptor antagonists, however, have not been as consistently efficacious. While TAK-044 and SB17242 decreased oxidative stress and reduced ischemia, bosentan and SB209670 had no effect (Barone et al., 1995; Barone et al., 2000; Gupta et al., 2005; Patel and McCulloch, 1995). In fact, selective blockade of ET_B receptors has been shown to exacerbate ischemic brain damage, a finding consistent with our results in this study (Chuquet et al., 2002). Conversely, activation of endothelial ET_B receptors is known to elicit vasodilatation, and previous studies in our lab have indicated that this leads to an increase in cerebral blood flow in normal rats (Leonard and Gulati, 2009). We have not attempted to measure cerebral blood flow in the present study because placing the laser Doppler probe would have confounded our results, moreover in this study animals were allowed to recover for 24 h before measuring parameters to determine extent of cerebral ischemia. Since penumbra remains vulnerable to ischemia for a longer time, we carried out our observations at 24 h following middle cerebral artery occlusion in order to include any damage due to penumbra.

In the present study, rats undergoing middle cerebral artery occlusion received three intravenous doses of either vehicle (isotonic saline) or ET_B receptor agonist IRL-1620 (5 μg/kg) at 2, 4 and 6 h after MCAO. Twenty four hours after the occlusion, animals were evaluated for neurological and motor deficit, and their brains were removed for analysis of infarct area, oxidative stress parameters, and ET receptor estimation. Occlusion of the middle cerebral artery resulted in severe neurological and motor deficits, with animals demonstrating weakened or paralyzed limbs. Treatment with IRL-1620 resulted in a significant reduction in neurological deficit and motor impairment as measured by the grip test, foot fault and rota rod when compared with the vehicle-treated group. Muscle strength and coordination were significantly improved with treatment, demonstrated by an increased ability to hold onto the string and to remain on the rotating spindle.

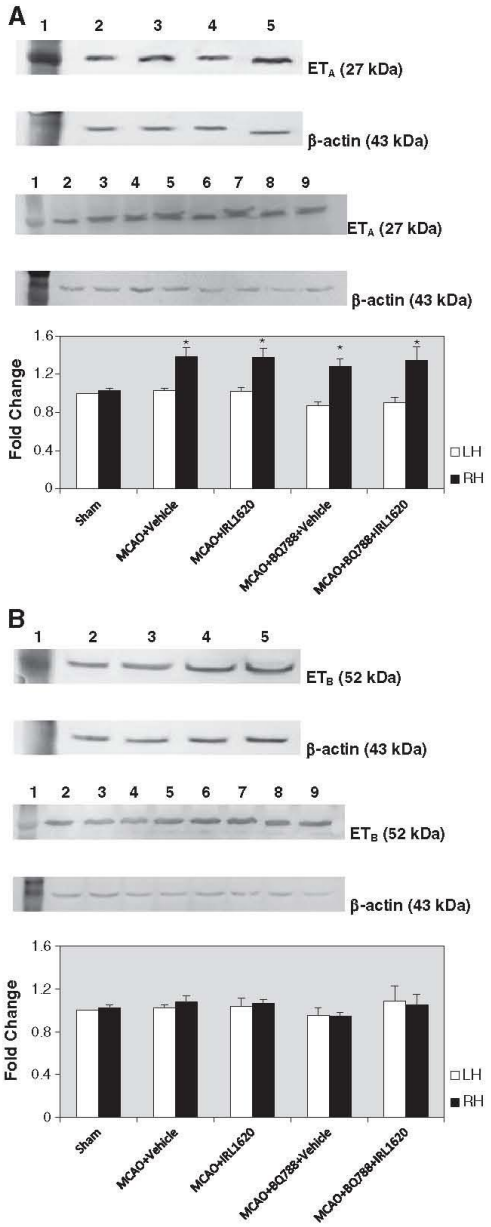


Fig. 3 – A. Expression of ET_A receptor protein levels with β-actin as a loading control. Top: Lane 1 — Protein marker; Lane 2 — Sham (LH); Lane 3 — Sham (RH); Lane 4 — Vehicle + MCAO (LH); Lane 5 — Vehicle + MCAO (RH). Bottom: Lane 1 — Protein marker; Lane 2 — Vehicle + MCAO (LH); Lane 3 — Vehicle + MCAO (RH); Lane 4 — MCAO + IRL1620 (LH); Lane 5 — MCAO + IRL1620 (RH); Lane 6 — MCAO + BQ788 + Vehicle (LH); Lane 7 — MCAO + BQ788 + Vehicle (RH); Lane 8 — MCAO + BQ788 + IRL1620 (LH); Lane 9 — MCAO + BQ788 + IRL1620 (RH). The blot is representative of four different experiments with similar results and bar graph showing fold change in the expression of ET_A receptor in brain 24 h post middle cerebral artery occlusion. B. Expression of ET_B receptor protein levels in the occluded rat brain with lane distribution same as in (A). IRL-1620 (5 μg/kg, IV) or isotonic saline (1 ml/kg, IV) was injected at 2, 4, and 6 h post MCAO. BQ788 (1 mg/kg, IV) was administered 15 min prior to the first injection of IRL-1620 or vehicle. LH = Left hemisphere; RH = Right hemisphere. Values are expressed as mean ± S.E.M. $^*P < 0.05$ compared to LH.

On the other hand, antagonism of the ET_B receptors with BQ788, given 15 min prior to either vehicle or IRL-1620, resulted in neurological and motor deficits similar to or greater than those seen in the vehicle group alone, reinforcing the hypothesis that the improvement seen with IRL-1620 alone is indeed due to selective activation of the ET_B receptors. The observed functional deficits coincided with the results of the TTC staining for infarct volume; IRL-1620 group presented with significantly smaller infarct volumes than those of the vehicle or BQ788 treated groups.

This is the first report indicating that selective stimulation of the ET_B receptors following ischemic stroke leads to physical and functional recovery. In an effort to elicit the mechanism of action for this apparent neuroprotection, we investigated the effects of middle cerebral artery occlusion, with and without treatment, on ET receptor levels as well as oxidative stress parameters in the rat brain. Treatment with IRL-1620 did not affect the levels of either ET_A or ET_B receptors in the occluded brain. All occluded animals, whether treated with vehicle, IRL-1620 or BQ788, showed higher levels of the ET_A receptor in the infarcted hemisphere as compared with the contralateral hemisphere, with no alteration in ET_B receptor levels. Our results for ET_B receptor levels conflict with a study conducted by Stenman et al. (2002), which demonstrated an upregulation of this receptor following cerebral ischemia. This discrepancy may be explained by the fact that they used a temporary as opposed to a permanent model of middle cerebral artery occlusion and that they observed the upregulation of the ET_B receptor in the middle cerebral artery whereas we tested the entire infarcted hemisphere. A localized upregulation of the receptor may have been present but not seen with our method. From the present results, neither activating nor blocking the ET_B receptors appears to cause an up- or down-regulation of these receptors in cerebral ischemia. Similarly, ET_A receptor levels, while elevated in ischemic pathology, did not increase or decrease in number as a result of the ET_B-selective treatment. It would thus appear that, while IRL-1620 is known to selectively activate ET_B receptors, its neuroprotective effects in cerebral ischemia are not due to a change in receptor number. It is, however, possible that 24 h is not a sufficient time to see a change in the ET_B receptor levels. Further studies with a longer survival time and immunohistochemical studies will investigate this possibility. We conducted a preliminary study to determine the effect of pretreatment with ET_A receptor antagonist, BQ123, on IRL-1620 in rats with cerebral ischemia. BQ123 treated rats showed improvement in neurobehavioral parameters and reduced infarct volume, however, the effect of BQ123 was less than that observed with IRL-1620. Furthermore, there was no synergistic effect when IRL-1620 was administered to rats pretreated with BQ123 (unpublished observation), indicating that IRL-1620 is not acting directly through ET_A receptors. However, the possibility of allosteric modulation of ET_A receptors by IRL-1620 cannot be excluded.

Treatment with IRL-1620 effectively reduced oxidative stress as measured by decreased levels of malondialdehyde (MDA) and increased levels of reduced glutathione (GSH) and superoxide dismutase (SOD) as compared with the vehicle-treated group. Blockade of the ET_B receptor with BQ788, on the other hand, resulted in an increase in oxidative stress. Increased levels of MDA along with decreased levels of antioxidants GSH and SOD are all hallmarks of oxygen free radical generation occurring as part of the ischemic cascade (Marnett, 1999). The ischemic cascade is a series of biochemical reactions that occur within seconds to minutes following the loss of adequate blood supply, where the lack of O_2 causes the neuron's normal process for making ATP to fail, leading to excess intracellular Ca^{2+}, glutamate excitotoxicity, production of reactive oxygen species, and eventual apoptosis. Agents that target these events in order to slow or prevent irreversible injury caused by cerebral ischemia are labeled neuroprotective. Previous and current targets for neuroprotection following cerebral ischemia include reactive oxygen species, NMDA, AMPA and GABA receptors, as well as neuronal Ca^{2+} and Na^+ channels (Tuttolomondo et al., 2009). The reduction in physiological deficit, infarct area, and oxidative stress following IRL-1620 administration in the current study indicates that the ET_B receptor may be a new therapeutic target for neuroprotection following ischemic stroke.

At present, we do not have a complete picture of the mechanism by which the ET_B receptor protects the brain against ischemia. Activation of ET_B receptors with IRL-1620 is known to cause cerebral vasodilatation and increased blood flow through the release of nitric oxide (NO) (Kitazono et al., 1995; Kobari et al., 1994; Leonard and Gulati, 2009; Tirapelli et al., 2005). Recent studies have demonstrated that endothelial NO stimulation and increased cerebral blood flow via pharmacological means enhance angiogenesis and improve recovery following stroke in rats (Chen et al., 2007; Ding et al., 2008). Along these lines, the ET_B receptor has been shown to enhance the formation of new blood vessels through eNOS (Goligorsky et al., 1999). Beyond the possible neuroprotection and/or angiogenesis induced by ET_B receptor activation of eNOS, these receptors, which are located on neurons and astrocytes as well as vascular endothelial cells, may work through other pathways to prevent the apoptosis associated with the ischemic cascade. Previous reports using an ET_B receptor deficient model have indicated that this receptor promotes neuronal survival and decreases apoptosis in the hippocampus, dentate gyrus, and olfactory epithelium (Ehrenreich et al., 1999; Laziz et al., 2011; Riechers et al., 2004). An in vitro study by Yagami et al. (2005) demonstrated that ET_B receptor agonist, ET-3, directly rescued cortical neurons from human group IIA secretory phospholipase A_2-induced apoptosis, a form of apoptosis associated with cerebral ischemia. Both the induction of eNOS and the direct anti-apoptotic neuronal effects of ET_B receptor activation may play a role in the reduction of oxidative stress and the improvement in functional and physiological recovery following cerebral ischemia.

The present study demonstrates that ET_B receptor activation may be a novel neuroprotective therapy in the treatment of focal ischemic stroke. Selective ET_B receptor agonist, IRL-1620, significantly decreases neurological deficit, motor impairment and infarct area following middle cerebral artery occlusion in rat. While this effect is to some degree explained by the observed reduction in oxidative stress, further investigations into the mechanism of neuroprotection by the ET_B receptor in cerebral ischemia are warranted.

4. Experimental procedures

4.1. Animals

Male Sprague–Dawley rats (Harlan, Indianapolis, IN) weighing 250–350 g were allowed to acclimate for at least 4 days before use. Animals were housed in a room with controlled temperature (23±1 °C), humidity (50±10%), and light (6:00 A.M. to 6:00 P.M.). Food and water were available continuously. Animal care and use for experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Midwestern University. All anesthetic and surgical procedures were in compliance with the guidelines established by the IACUC of Midwestern University.

4.2. Drugs

Ketamine (Butler Animal Health Supply, Dublin, OH, USA) was administered at a dose of 100 mg/kg, intraperitoneally (i.p.), and xylazine (Lloyd Laboratories, Shenandoah, IA, USA) was administered at a dose of 10 mg/kg, i.p. IRL-1620 [N-Succinyl-[Glu9, Ala11,15] endothelin 1] (American Peptide Co, Inc., Sunnyvale, CA, USA) was dissolved in isotonic saline and administered in a dose of 5 μg/kg, intravenously (i.v.) at 2, 4 and 6 h post middle cerebral artery occlusion. BQ788 (American Peptide Co, Inc., Sunnyvale, CA, USA) was dissolved in isotonic saline and administered at a dose of 1 mg/kg, i.v. prior to administration of IRL-1620 or vehicle. The doses of IRL-1620 and BQ788 were based on preliminary studies and previous work conducted in our laboratory (Briyal and Gulati, 2010; Lavhale et al., 2010; Leonard and Gulati, 2009).

4.3. Experimental protocol

Rats were randomly divided into five groups of 6 animals each. Group 1 animals were subjected to a sham operation. Rats in groups 2–5 underwent middle cerebral artery occlusion (MCAO) and were treated as follows — Group 2: MCAO+vehicle; Group 3: MCAO+IRL-1620; Group 4: MCAO+BQ788+vehicle; Group 5: MCAO+BQ788+IRL-1620. All drugs were administered via intravenous tail vein injection. A total of three injections of vehicle (isotonic saline, 1 ml/kg) and IRL-1620 (5 μg/kg) were given at 2, 4, and 6 h post MCAO. BQ788 (1 mg/kg) was given 15 min prior to administration of the first dose of either vehicle or IRL-1620.

4.4. Middle cerebral artery occlusion to induce focal cerebral ischemia

Induction of focal cerebral ischemia via middle cerebral artery occlusion (MCAO) was performed according to the method of Koizumi et al. (1986). Rats were anesthetized with ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). A rectal core temperature of 37±1 °C was maintained throughout surgery using the thermo-controlled base of the operating table, measured with a Cole Palmer Animal Monitoring Thermometer with colonic probe (Vernon Hills, Il, USA). With the animal in a secure supine position, a midline incision was made and the right common carotid artery, external carotid artery, and internal carotid artery were exposed. A 4.0 monofilament nylon thread (CP Medical, Portland, OR, USA) with its tip rounded by briefly heating the end was used to occlude the middle cerebral artery. The nylon filament was advanced from the external carotid artery into the lumen of the internal carotid artery until a resistance was felt (~20 mm), indicating occlusion of the middle cerebral artery. The nylon filament was allowed to remain in place to create a permanent model of focal cerebral ischemia. The incision was closed with 3.0 silk surgical sutures (Ehticon, Inc.). In sham-operated animals, the common carotid artery and external carotid artery were exposed and the incision was sutured without touching the internal carotid artery.

4.5. Motor performance tests

Neurological deficit and motor activity and coordination of the animals was blindly assessed 15 min prior to and 24 h post middle cerebral artery occlusion using a grip test, foot fault test, and rota rod.

4.5.1. Neurological evaluation

Animals were subjected to a neurological evaluation 15 min prior to and 24 h post middle cerebral artery occlusion. The neurological evaluation was based on a 6 point scale as described by Tatlisumak et al. (1998). The scoring was as follows: 0 = no deficits, 1 = failure to fully extend left forepaw, 2 = circling to the left, 3 = paresis to the left, 4 = no spontaneous walking, and 5 = death.

4.5.2. Grip test

The grip test consisted of a string 50 cm in length, pulled taut between two vertical supports and elevated 40 cm above a flat surface. The animal was placed on the string midway between the supports and evaluated according to a 6 point scale (Moran et al., 1995). The scoring was as follows: 0 = falls off, 1 = hangs on by two forepaws, 2 = hangs on by two forepaws and attempts to climb on, 3 = hangs on by 3+ paws, 4 = hangs on by all paws plus tail, and 5 = escapes.

4.5.3. Foot fault test

Animals were placed on an elevated grid floor with a mesh size of 30 mm for one minute to acclimate. They were then observed for one minute and evaluated for foot fault errors (i.e. a misplaced limb falling through the grid) compared with paired steps as follows (Markgraf et al., 1992):

% foot fault error = (number of faults/number of paired steps) × 100.

4.5.4. Rota rod

Prior to middle cerebral artery occlusion, animals were acclimated to the rotating spindle of the rota rod apparatus (Rota-Rod 47700, Ugo Basile, Italy). Animals were placed on the rotating spindle, set to a constant 8 rotations per minute (rpm), until they demonstrated the ability to remain on the spindle for 60 s. The animals were then subjected to a baseline test trial on the accelerating spindle (4–40 rpm) over 5 min. The acceleration trial was repeated at 24 h post middle cerebral artery occlusion, and the time at which they fell off was recorded in seconds (Rogers et al., 1997).

4.6. Assessment of cerebral infarct volume

Following neurological and motor function testing at 24 h post middle cerebral artery occlusion, animals were euthanized by decapitation, and the brains were removed to determine infarct volume. The brains were quickly removed and chilled in saline at 4 °C for 5 min. They were then cut into 2 mm thick coronal slices using a Brain Matrix (Harvard Apparatus). Sections were incubated in 2% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma, MO) dissolved in saline for 15 min at 37 °C. The stained sections were then stored in 10% formalin and refrigerated at 4 °C for further analysis (Li et al., 1997). Infarct volumes were calculated by sampling each side of the coronal sections with a digital camera (Nikon). The infarct area, outlined in white, was measured by image analysis software (Adobe Photoshop CS4). Edema was determined by taking the percent increase in size of the ischemic over the contralateral hemisphere (Barone et al., 1995). Infarct size is expressed as infarction volume in mm^3 as the sum of infarct areas in each slice, corrected for edema.

4.7. Estimation of oxidative stress markers

Brain levels of malondialdehyde (MDA), reduced glutathione (GSH), and superoxide dismutase (SOD) were estimated 24 h post middle cerebral artery occlusion. The animals were decapitated, and the brains were quickly removed and washed in chilled saline, then stored at −80 °C. The biochemical analyses were performed within 48 h.

4.7.1. Measurement of malondialdehyde

Malondialdehyde (MDA), the indicator of lipid peroxidation, was estimated according to the method of Ohkawa et al. (1979). Brains were homogenized with 10 times (w/v) in 0.1 M sodium phosphate buffer (pH 7.4). 1.5 ml acetic acid (20%, pH 3.5), 1.5 ml thiobarbituric acid (0.8%), and 0.2 ml sodium dodecyl sulfate (8.1%) were added to 0.1 ml of processed tissue sample. The mixture was heated at 100 °C for 60 min. Next, 5 ml of n-butanol:pyridine (15:1% v/v) and 1 ml of distilled water were added to the cooled mixture, shaking vigorously. After centrifugation at 4000 rpm for 10 min, the organic layer was withdrawn and absorbance was measured at 532 nm using a spectrophotometer.

4.7.2. Measurement of reduced glutathione

Reduced glutathione (GSH) was measured according to the method of Ellman (1959) with minor modifications. Brains were homogenized with 10 times (w/v) in 0.1 M sodium phosphate buffer (pH 7.4). This homogenate was then centrifuged with 5% trichloroacetic acid to separate out the proteins. Next, 2 ml of phosphate buffer (pH 8.4), 0.5 ml of 5′5 dithiobis (2-nitrobenzoic acid), and 0.4 ml of double distilled water were added to 0.1 ml of homogenate. The mixture was vortexed and the absorbance was read within 15 min at 412 nm using a spectrophotometer.

4.7.3. Measurement of superoxide dismutase

Superoxide dismutase (SOD) was measured according to the method of Kakkar et al. (1984). Brains were homogenized with 10 times (w/v) in 0.1 M sodium phosphate buffer (pH 7.4). 1.2 ml sodium pyrophosphate buffer (0.052 M, pH 8.3), 0.1 ml phenazanine methosulfate (186 μM), 0.3 ml nitro blue tetrazolium (300 μM), and 0.2 ml NADH (780 μM) were added. The mixture was incubated at 30 °C for 90 min. Next, 4 ml of n-butanol and 1 ml of acetic acid were added, and the mixture was shaken vigorously. After centrifugation at 4000 rpm for 10 min, the organic layer was removed and absorbance was measured at 560 nm using a spectrophotometer.

4.8. Estimation of endothelin receptors

Endothelin (ET) receptors were measured using the Western blotting technique. Animals were decapitated 24 h post middle cerebral artery occlusion, and brains were immediately sectioned into right and left hemispheres, flash frozen on dry ice, and stored at −80 °C for further analysis. The tissue was homogenized with 10 times (w/v) RIPA lysis buffer (20 mM Tris–HCl pH 7.5, 120 mM NaCl, 1.0% TritonX-100, 0.1% SDS, 1% sodium deoxycholate, 10% glycerol, 1 mM EDTA and 1X protease inhibitor, Roche). Proteins were isolated in solubilized form and concentrations were measured by Folin–Ciocalteu's phenol reagent (Lowry et al., 1951). Solubilized protein (20 μg) was denatured in Laemmli sample buffer (Bio-Rad), resolved in 10% SDS-PAGE and transferred on nitrocellulose membrane followed by blocking the membrane with 5% BSA (w/v) in TBST (10 mM Tris, 150 mM NaCl, 0.1% Tween20). The membranes were incubated with rabbit polyclonal anti-ETA (1:1000) or anti-ETB (1:500) antibodies, followed by HRP-conjugated secondary antibodies (1:1000) and visualized by ECL Plus western blotting detection system (GE Healthcare, Buckinghamshire, UK). Stripped membranes were reprobed with β-actin primary antibody (1:1000) for a protein loading control (Lavhale et al., 2010).

4.9. Statistical analysis

The data is represented as mean ± S.E.M. One-way analysis of variance (ANOVA) followed by Bonferroni's post hoc comparison test was used for intergroup comparison, while unpaired t-test was used for ET receptor comparison. $P<0.05$ was considered significant.

Acknowledgments

The present study was supported by funds from Midwestern University, Downers Grove, IL.

REFERENCES

Asano, T., Ikegaki, I., Satoh, S., Suzuki, Y., Shibuya, M., Sugita, K., Hidaka, H., 1990. Endothelin: a potential modulator of cerebral vasospasm. Eur. J. Pharmacol. 190, 365–372.

Barone, F.C., White, R.F., Elliott, J.D., Feuerstein, G.Z., Ohlstein, E.H., 1995. The endothelin receptor antagonist SB 217242 reduces cerebral focal ischemic brain injury. J. Cardiovasc. Pharmacol. 26 (Suppl 3), S404–S407.

Barone, F.C., Ohlstein, E.H., Hunter, A.J., Campbell, C.A., Hadingham, S.H., Parsons, A.A., Yang, Y., Shohami, E., 2000. Selective antagonism of endothelin-A-receptors improves outcome in both head trauma and focal stroke in rat. J. Cardiovasc. Pharmacol. 36, S357–S361.

Bauser-Heaton, H.D., Bohlen, H.G., 2007. Cerebral microvascular dilation during hypotension and decreased oxygen tension: a

role for nNOS. Am. J. Physiol. Heart Circ. Physiol. 293, H2193–H2201.
Briyal, S., Gulati, A., 2010. Endothelin-A receptor antagonist BQ123 potentiates acetaminophen induced hypothermia and reduces infarction following focal cerebral ischemia in rats. Eur. J. Pharmacol. 644, 73–79.
Chan, P.H., 1996. Role of oxidants in ischemic brain damage. Stroke 27, 1124–1129.
Chen, J., Cui, X., Zacharek, A., Jiang, H., Roberts, C., Zhang, C., Lu, M., Kapke, A., Feldkamp, C.S., Chopp, M., 2007. Niaspan increases angiogenesis and improves functional recovery after stroke. Ann. Neurol. 62, 49–58.
Chuquet, J., Benchenane, K., Toutain, J., MacKenzie, E.T., Roussel, S., Touzani, O., 2002. Selective blockade of endothelin-B receptors exacerbates ischemic brain damage in the rat. Stroke 33, 3019–3025.
Ding, G., Jiang, Q., Li, L., Zhang, L., Zhang, Z.G., Ledbetter, K.A., Panda, S., Davarani, S.P., Athiraman, H., Li, Q., Ewing, J.R., Chopp, M., 2008. Magnetic resonance imaging investigation of axonal remodeling and angiogenesis after embolic stroke in sildenafil-treated rats. J. Cereb. Blood Flow Metab. 28, 1440–1448.
Ehrenreich, H., Oldenburg, J., Hasselblatt, M., Herms, J., Dembowski, C., Loffler, B.M., Bruck, W., Kamrowski-Kruck, H., Gall, S., Siren, A.L., Schilling, L., 1999. Endothelin B receptor-deficient rats as a subtraction model to study the cerebral endothelin system. Neuroscience 91, 1067–1075.
Ellman, G.L., 1959. Tissue sulfhydryl groups. Arch. Biochem. Biophys. 82, 70–77.
Goligorsky, M.S., Budzikowski, A.S., Tsukahara, H., Noiri, E., 1999. Co-operation between endothelin and nitric oxide in promoting endothelial cell migration and angiogenesis. Clin. Exp. Pharmacol. Physiol. 26, 269–271.
Goto, K., Kasuya, Y., Matsuki, N., Takuwa, Y., Kurihara, H., Ishikawa, T., Kimura, S., Yanagisawa, M., Masaki, T., 1989. Endothelin activates the dihydropyridine-sensitive, voltage-dependent Ca2+ channel in vascular smooth muscle. Proc. Natl. Acad. Sci. U.S.A. 86, 3915–3918.
Gupta, Y.K., Briyal, S., Sharma, U., Jagannathan, N.R., Gulati, A., 2005. Effect of endothelin antagonist (TAK-044) on cerebral ischemic volume, oxidative stress markers and neurobehavioral parameters in the middle cerebral artery occlusion model of stroke in rats. Life Sci. 77, 15–27.
Kakkar, P., Das, B., Viswanathan, P.N., 1984. A modified spectrophotometric assay of superoxide dismutase. Indian J. Biochem. Biophys. 21, 130–132.
Kitazono, T., Heistad, D.D., Faraci, F.M., 1995. Enhanced responses of the basilar artery to activation of endothelin-B receptors in stroke-prone spontaneously hypertensive rats. Hypertension 25, 490–494.
Kobari, M., Fukuuchi, Y., Tomita, M., Tanahashi, N., Konno, S., Takeda, H., 1994. Dilatation of cerebral microvessels mediated by endothelin ETB receptor and nitric oxide in cats. Neurosci. Lett. 176, 157–160.
Koizumi, J., Yoshida, Y., Nakazawa, T., Ooneda, G., 1986. Experimental studies of ischemic brain edema. 1. A new experimental model of cerebral embolism in rats in which recirculation can be introduced in the ischemic area. Jpn. J. Stroke 8, 1–8.
Lavhale, M.S., Briyal, S., Parikh, N., Gulati, A., 2010. Endothelin modulates the cardiovascular effects of clonidine in the rat. Pharmacol. Res. 62, 489–499.
Laziz, I., Larbi, A., Grebert, D., Sautel, M., Congar, P., Lacroix, M.C., Salesse, R., Meunier, N., 2011. Endothelin as a neuroprotective factor in the olfactory epithelium. Neuroscience 172, 20–29.
Lee, H.O., Levorse, J.M., Shin, M.K., 2003. The endothelin receptor-B is required for the migration of neural crest-derived melanocyte and enteric neuron precursors. Dev. Biol. 259, 162–175.
Leonard, M.G., Gulati, A., 2009. Repeated administration of ET(B) receptor agonist, IRL-1620, produces tachyphylaxis only to its hypotensive effect. Pharmacol. Res. 60, 402–410.
Li, F., Irie, K., Anwer, M.S., Fisher, M., 1997. Delayed triphenyltetrazolium chloride staining remains useful for evaluating cerebral infarct volume in a rat stroke model. J. Cereb. Blood Flow Metab. 17, 1132–1135.
Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.
Ly, J.V., Zavala, J.A., Donnan, G.A., 2006. Neuroprotection and thrombolysis: combination therapy in acute ischaemic stroke. Expert Opin. Pharmacother. 7, 1571–1581.
Markgraf, C.G., Green, E.J., Hurwitz, B.E., Morikawa, E., Dietrich, W.D., McCabe, P.M., Ginsberg, M.D., Schneiderman, N., 1992. Sensorimotor and cognitive consequences of middle cerebral artery occlusion in rats. Brain Res. 575, 238–246.
Marnett, L.J., 1999. Lipid peroxidation-DNA damage by malondialdehyde. Mutat. Res. 424, 83–95.
Micieli, G., Marcheselli, S., Tosi, P.A., 2009. Safety and efficacy of alteplase in the treatment of acute ischemic stroke. Vasc. Health Risk Manag. 5, 397–409.
Moran, P.M., Higgins, L.S., Cordell, B., Moser, P.C., 1995. Age-related learning deficits in transgenic mice expressing the 751-amino acid isoform of human beta-amyloid precursor protein. Proc. Natl. Acad. Sci. U.S.A. 92, 5341–5345.
Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 95, 351–358.
Patel, T.R., Galbraith, S.L., McAuley, M.A., Doherty, A.M., Graham, D.I., McCulloch, J., 1995. Therapeutic potential of endothelin receptor antagonists in experimental stroke. J. Cardiovasc. Pharmacol. 26 (Suppl 3), S412–S415.
Patel, T.R., McCulloch, J., 1995. Postischemic hypoperfusion in transient global ischemia: a role for endothelins? J. Cardiovasc. Pharmacol. 26 (Suppl 3), S425–S428.
Riechers, C.C., Knabe, W., Siren, A.L., Gariepy, C.E., Yanagisawa, M., Ehrenreich, H., 2004. Endothelin B receptor deficient transgenic rescue rats: a rescue phenomenon in the brain. Neuroscience 124, 719–723.
Roger, V.L., Go, A.S., Lloyd-Jones, D.M., Adams, R.J., Berry, J.D., Brown, T.M., Carnethon, M.R., Dai, S., de Simone, G., Ford, E.S., Fox, C.S., Fullerton, H.J., Gillespie, C., Greenlund, K.J., Hailpern, S.M., Heit, J.A., Ho, P.M., Howard, V.J., Kissela, B.M., Kittner, S.J., Lackland, D.T., Lichtman, J.H., Lisabeth, L.D., Makuc, D.M., Marcus, G.M., Marelli, A., Matchar, D.B., McDermott, M.M., Meigs, J.B., Moy, C.S., Mozaffarian, D., Mussolino, M.E., Nichol, G., Paynter, N.P., Rosamond, W.D., Sorlie, P.D., Stafford, R.S., Turan, T.N., Turner, M.B., Wong, N.D., Wylie-Rosett, J., 2011. Heart disease and stroke statistics — 2011 update: a report from the American Heart Association. Circulation 123, e18–e209.
Rogers, D.C., Campbell, C.A., Stretton, J.L., Mackay, K.B., 1997. Correlation between motor impairment and infarct volume after permanent and transient middle cerebral artery occlusion in the rat. Stroke 28, 2060–2065 discussion 2066.
Samdani, A.F., Dawson, T.M., Dawson, V.L., 1997. Nitric oxide synthase in models of focal ischemia. Stroke 28, 1283–1288.
Schinelli, S., 2006. Pharmacology and physiopathology of the brain endothelin system: an overview. Curr. Med. Chem. 13, 627–638.
Stenman, E., Malmsjo, M., Uddman, E., Gido, G., Wieloch, T., Edvinsson, L., 2002. Cerebral ischemia upregulates vascular endothelin ET(B) receptors in rat. Stroke 33, 2311–2316.
Tatlisumak, T., Carano, R.A., Takano, K., Opgenorth, T.J., Sotak, C.H., Fisher, M., 1998. A novel endothelin antagonist, A-127722, attenuates ischemic lesion size in rats with temporary middle cerebral artery occlusion: a diffusion and perfusion MRI study. Stroke 29, 850–857 discussion 857–8.

Tirapelli, C.R., Casolari, D.A., Yogi, A., Montezano, A.C., Tostes, R.C., Legros, E., D'Orleans-Juste, P., de Oliveira, A.M., 2005. Functional characterization and expression of endothelin receptors in rat carotid artery: involvement of nitric oxide, a vasodilator prostanoid and the opening of K^+ channels in ETB-induced relaxation. Br. J. Pharmacol. 146, 903–912.

Tsukahara, H., Ende, H., Magazine, H.I., Bahou, W.F., Goligorsky, M.S., 1994. Molecular and functional characterization of the non-isopeptide-selective ETB receptor in endothelial cells. Receptor coupling to nitric oxide synthase. J. Biol. Chem. 269, 21778–21785.

Tuttolomondo, A., Di Sciacca, R., Di Raimondo, D., Arnao, V., Renda, C., Pinto, A., Licata, G., 2009. Neuron protection as a therapeutic target in acute ischemic stroke. Curr. Top. Med. Chem. 9, 1317–1334.

Viossat, I., Duverger, D., Chapelat, M., Pirotzky, E., Chabrier, P.E., Braquet, P., 1993. Elevated tissue endothelin content during focal cerebral ischemia in the rat. J. Cardiovasc. Pharmacol. 22 (Suppl 8), S306–S309.

Yagami, T., Ueda, K., Sakaeda, T., Okamura, N., Nakazato, H., Kuroda, T., Hata, S., Sakaguchi, G., Itoh, N., Hashimoto, Y., Fujimoto, M., 2005. Effects of an endothelin B receptor agonist on secretory phospholipase A2-IIA-induced apoptosis in cortical neurons. Neuropharmacology 48, 291–300.

Zhang, R.L., Zhang, C., Zhang, L., Roberts, C., Lu, M., Kapke, A., Cui, Y., Ninomiya, M., Nagafuji, T., Albala, B., Zhang, Z.G., Chopp, M., 2008. Synergistic effect of an endothelin type A receptor antagonist, S-0139, with rtPA on the neuroprotection after embolic stroke. Stroke 39, 2830–2836.

Zhang, Y., Belayev, L., Zhao, W., Irving, E.A., Busto, R., Ginsberg, M.D., 2005. A selective endothelin ET(A) receptor antagonist, SB 234551, improves cerebral perfusion following permanent focal cerebral ischemia in rats. Brain Res. 1045, 150–156.

BRAIN RESEARCH 1464 (2012) 14–23

Available online at www.sciencedirect.com

SciVerse ScienceDirect

www.elsevier.com/locate/brainres

BRAIN RESEARCH

Research Report

Endothelin B receptor agonist, IRL-1620, provides long-term neuroprotection in cerebral ischemia in rats

Mary G. Leonard[a,b], *Seema Briyal*[a], *Anil Gulati*[a,b,*]

[a]*Midwestern University Chicago College of Pharmacy, Downers Grove, IL 60515, USA*
[b]*Department of Bioengineering, University of Illinois at Chicago, Chicago, IL 60607, USA*

ARTICLE INFO

Article history:
Accepted 3 May 2012
Available online 9 May 2012

Keywords:
Endothelin B receptor
Cerebral ischemia
Oxidative stress
IRL-1620
Neuroprotection

ABSTRACT

We have earlier shown that stimulation of endothelin B receptors by IRL-1620 provides significant neuroprotection at 24 h following cerebral ischemia. However, the effect of IRL-1620 is not known in the subacute phase of cerebral ischemia, where development of cerebral edema further contributes towards brain damage. This study was designed to determine the effect of IRL-1620 on neurological functions, infarct volume, oxidative stress, and endothelin receptors following permanent middle cerebral artery occlusion for 7 days. Rats received three intravenous injections of either vehicle or IRL-1620 [Suc-[Glu9,Ala11,15]-Endothelin-1(8–12)] at 2, 4, and 6 h post occlusion. Treatment with IRL-1620 reduced infarct volume (54.06 ± 14.12 mm^3 vs. 177.06 ± 13.21 mm^3), prevented cerebral edema and significantly improved all neurological and motor function parameters when compared to the vehicle-treated group. Vehicle-treated middle cerebral artery occluded rats demonstrated high levels of malondialdehyde and low levels of reduced glutathione and superoxide dismutase; these effects were reversed in IRL-1620 treated rats. No change in expression of endothelin A receptor was observed 7 days after induction of cerebral ischemia in vehicle or IRL-1620 treated rats. Rats receiving IRL-1620 demonstrated an upregulation of endothelin B receptor only in the infarcted hemisphere 7 days following occlusion. All effects of IRL-1620 were blocked by endothelin B receptor antagonist, BQ788. Results of the present study demonstrate that IRL-1620, administered on day 1, provides significant neuroprotection till 7 days after the induction of cerebral ischemia in rats. Selective endothelin B receptor activation may prove to be a novel therapeutic target in the treatment of cerebral ischemia.

1. Introduction

Endothelin is an endogenous peptide which acts on endothelin A and B receptors. Endothelin A receptors are mainly located on the vascular smooth muscle where they mediate vasoconstriction, while endothelin B receptors are mainly located on the endothelium where they mediate vasodilatation (Goto et al., 1989; Tsukahara et al., 1994). Endothelin receptors are also found throughout the brain, on neurons and astrocytes as well as cerebrovasculature (Schinelli, 2006). These centrally located receptors have been found to play several roles from regulation of cerebral blood flow to cellular migration, proliferation, and apoptosis.

* *Corresponding author at:* Chicago College of Pharmacy, Midwestern University, 555 31st St., Downers Grove, IL 60515-1235, USA. Fax: +1 630 971 6097.
E-mail address: AGULAT@midwestern.edu (A. Gulati).

0006-8993/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.brainres.2012.05.005

A disruption of the endothelin system occurs under the condition of cerebral ischemia. Endothelin-1 plasma levels and endothelin immunoreactivity in the tissues are elevated in cases of ischemic stroke (Viossat et al., 1993). Early studies discovered that blockade or deficiency of endothelin B receptors leads to a possible shift in vasomotor balance, thereby exacerbating ischemic brain damage (Chuquet et al., 2002; Ehrenreich et al., 1999). These studies, combined with studies from our laboratory showing that endothelin B receptor stimulation increases cerebral blood flow, and by other researchers demonstrating the enhancement of neural progenitors and protection against neuronal apoptosis in the presence of functional endothelin B receptors, led us to investigate the activation of these receptors in an ischemic stroke model (Laziz et al., 2011; Lee et al., 2003; Leonard and Gulati, 2009; Yagami et al., 2005).

The pathology of ischemic stroke occurs in four distinct stages, each involving complex pathways and specific markers for neurodegeneration and/or neuroprotection and recovery — hyperacute (<12 h), acute (12–24 h), subacute (2–14 d), and chronic (>14 d). Whereas the acute phase of cerebral ischemia is hallmarked by a strong oxidative stress reaction, the subacute phase is characterized by microvascular damage, inflammation and a breakdown in the blood brain barrier, resulting in significant cerebral edema which is maximal at the end of the first week (Kanekar et al., 2012; Lakhan et al., 2009). During this stage, the brain also begins its long road to recovery, initiating angiogenesis, neurogenesis and neuroblast migration towards the ischemic boundary (Minger et al., 2007).

Initial studies in our laboratory demonstrated that endothelin B receptor activation via the selective agonist IRL-1620 significantly improved neurological and motor functions while drastically decreasing infarct volume twenty-four hours following permanent middle cerebral artery occlusion in rats (Leonard et al., 2011). This study also demonstrated that middle cerebral artery occluded rats treated with IRL-1620 presented with a reduced oxidative stress as compared with the vehicle-treated animals, indicating a further neuroprotective role for activating endothelin B receptors following cerebral ischemia. Given these initial promising results, it was of interest to determine the effects which activating the endothelin B receptors would have on later stages of cerebral ischemia.

The current study was conducted to determine the effects of endothelin B receptor agonist, IRL-1620, in the presence and absence of endothelin B receptor antagonist, BQ788, in a week-long permanent middle cerebral artery occlusion model of ischemic stroke in rats. Mortality was observed and surviving animals were assessed for neurological and motor functions over the course of 7 days, and their brains were removed and evaluated for brain edema, infarct volume, oxidative stress and endothelin receptor levels 7 days post occlusion.

2. Results

2.1. *Effect on motor function and coordination*

Prior to middle cerebral artery occlusion, there was no difference between the groups in motor function or coordination. Occluding the middle cerebral artery led to significant neurological deficits and decreased muscular strength. Coincident with these losses, cerebral ischemia resulted in a distinct loss of motor coordination as measured by the foot fault error and rota rod tests at 1, 4, and 7 days post infarction (Table 1).

2.1.1. *Effect on neurological deficit*

Animals prior to middle cerebral artery occlusion had no neurological deficit. For the week following middle cerebral artery occlusion, however, vehicle-treated occluded animals showed significantly more deficit than sham-operated animals, as evidenced mainly by paresis of the left side ($P<0.001$). Whereas vehicle-treated occluded rats performed worse with each assessment, animals treated with endothelin B receptor agonist, IRL-1620, showed minimal deficit at day one following occlusion, and actually improved over the course of 7 days. Pretreatment with endothelin B receptor antagonist, BQ788, followed by either vehicle or IRL-1620 resulted in significantly more deficits than both sham-operated ($P<0.001$) or IRL-1620 ($P<0.05$) treated, indicating that the improvement observed with IRL-1620 is specific to the activation of endothelin B receptors (Table 1).

2.1.2. *Effect on muscular strength*

Muscular strength was evaluated both prior to and at 1, 4, and 7 days post middle cerebral artery occlusion using the grip test. Prior to occlusion, all animals were approximately equal in their ability to cling onto or climb the string. Muscular strength significantly diminished following occlusion, with animals in the vehicle-treated group barely able to grasp the string with their forepaws and often falling off. Animals in the sham-operated group, on the other hand, maintained their strength. Animals treated with IRL-1620 following occlusion remained significantly ($P<0.01$) stronger than the vehicle-treated rats. Animals pretreated with BQ788 faired far worse in muscle strength following occlusion than either sham-operated ($P<0.001$) or IRL-1620 ($P<0.05$) treatment (Table 1).

2.1.3. *Effect on motor coordination using foot fault error*

Foot fault errors for all animals at baseline (prior to middle cerebral artery occlusion) were less than 10% (Table 1). As can be seen in the vehicle-treated group, occlusion resulted in significant motor impairment with errors increasing to 82% 7 days after the infarct. IRL-1620 treatment resulted in animals making far fewer errors (less than 20%) on the foot fault grid as compared to the vehicle group ($P<0.01$). BQ788 blocked the positive effect of IRL-1620, resulting in a 60–80% error ($P<0.05$).

2.1.4. *Effect on motor coordination using rota rod*

There were no significant differences between groups prior to middle cerebral artery occlusion regarding their ability to remain on the rotating, accelerating spindle of the rota rod. All animals displayed sufficient motor coordination to remain on the spindle for approximately 2 min. Following middle cerebral artery occlusion, however, vehicle-treated rats displayed significantly ($P<0.05$) greater deficit in motor coordination than sham-operated animals, remaining on the spindle for only a short duration. This deficit was more marked as days progressed. IRL-1620 treated animals, on the other hand, retained coordination, remaining on the spindle for almost as long as the sham-operated group (Table 1). Animals receiving BQ788

Table 1 – Effect of endothelin B receptor agonist, IRL-1620, and antagonist, BQ788, on neurological deficit and motor function at baseline and 1, 4 and 7 d post middle cerebral artery occlusion in rats. IRL-1620 (5 μg/kg, IV) or isotonic saline (1 ml/kg, IV) was injected at 2, 4, and 6 h post MCAO. BQ788 (1 mg/kg, IV) was administered 15 min prior to the first injection of IRL-1620 or vehicle. Values are expressed as mean ± SEM. *P < 0.05 vs. sham. #P < 0.05 vs. MCAO + vehicle. @P < 0.05 vs. MCAO + IRL-1620.

Treatment groups		Neurological evaluation (6 point scale)	Grip test (6 point scale)	Foot fault error (%)	Rota rod duration (s)
Sham	Baseline	0 ± 0	4.25 ± 0.06	5.00 ± 0.91	115.00 ± 7.79
	Day 1	0 ± 0	4.25 ± 0.24	3.75 ± 1.24	129.50 ± 6.34
	Day 4	0 ± 0	4.50 ± 0.24	2.50 ± 0.66	165.25 ± 10.63
	Day 7	0 ± 0	4.50 ± 0.47	1.00 ± 0.95	155.50 ± 6.00
MCAO + vehicle	Baseline	0 ± 0	4.00 ± 0.22	6.67 ± 0.94	115.00 ± 10.11
	Day 1	3.00 ± 0.45*	0.83 ± 0.35*	68.00 ± 10.11*	12.40 ± 8.28*
	Day 4	3.50 ± 0.43*	0.67 ± 0.29*	71.17 ± 11.35*	34.40 ± 17.67*
	Day 7	3.83 ± 0.57*	0.33 ± 0.29*	81.67 ± 10.23*	0.80 ± 0.46*
MCAO + IRL-1620	Baseline	0 ± 0	4.57 ± 0.19	3.71 ± 0.81	132.13 ± 9.48
	Day 1	1.14 ± 0.38	2.86 ± 0.43#	17.00 ± 4.77#	99.50 ± 29.15
	Day 4	1.00 ± 0.41#	3.00 ± 0.35#	12.43 ± 1.96#	137.67 ± 26.72#
	Day 7	0.86 ± 0.43#	3.00 ± 0.35#	12.14 ± 2.92#	137.67 ± 28.52#
MCAO + BQ788	Baseline	0 ± 0	4.67 ± 0.29	3.67 ± 0.29	129.00 ± 7.44
	Day 1	3.50 ± 0.54*@	0.50 ± 0.30*@	67.17 ± 9.36*@	26.80 ± 11.05
	Day 4	3.17 ± 0.57*@	1.17 ± 0.35*@	52.50 ± 14.21*	31.00 ± 22.93*@
	Day 7	3.33 ± 0.48*@	0.17 ± 0.14*@	59.50 ± 11.64*@	60.20 ± 23.32
MCAO + BQ788 + IRL-1620	Baseline	0 ± 0	4.50 ± 0.30	4.50 ± 1.13	114.00 ± 5.85
	Day 1	3.00 ± 0.39*	0.83 ± 0.41*@	64.00 ± 14.42*@	32.00 ± 14.44
	Day 4	3.67 ± 0.36*@	1.33 ± 0.43*@	65.00 ± 10.80*@	55.80 ± 19.75*
	Day 7	3.83 ± 0.35*@	0.83 ± 0.35*@	78.00 ± 12.21*@	47.60 ± 19.00*

demonstrated a lack of coordination following occlusion as compared with the sham and IRL-1620 treated groups (Table 1).

2.2. *Effect on survival*

There was no mortality in sham treated rats throughout the 7 day period. However, in middle cerebral artery occluded rats in the vehicle treated group, a 38% mortality by 7th day was observed. On the other hand, middle cerebral artery occluded rats treated with IRL-1620 showed no mortality throughout the 7 day period. There was 25% mortality in middle cerebral artery occluded rats treated with BQ788 + vehicle or BQ788 + IRL-1620 during the 7 day observation (Fig. 1).

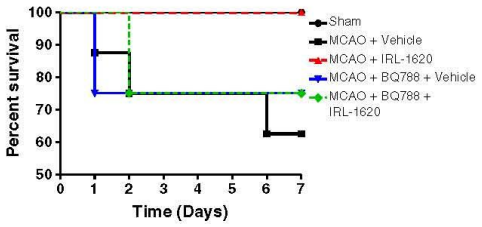


Fig. 1 – Effect of endothelin B receptor agonist, IRL-1620, in the presence and absence of BQ788 on 7 day survival of rats undergoing either sham surgery or middle cerebral artery occlusion.

2.3. *Effect on infarct volume*

Middle cerebral artery occlusion for 7 days resulted in an infarct volume of 177.06 ± 13.21 mm^3 in vehicle-treated rats (Fig. 2). Administration of IRL-1620 significantly reduced infarct volume (54.06 ± 14.12 mm^3; $P < 0.05$) as compared with vehicle. Infarct volumes did not reduce when endothelin B receptor antagonist, BQ788, was given with either vehicle or IRL-1620 (Fig. 2). A substantial edema was noted in the vehicle-treated animals, with the infarcted hemisphere 9.73 ± 1.26% larger than the contralateral hemisphere, whereas IRL-1620-treated animals showed no significant edema, with infarcted hemisphere only 1.51 ± 1.81% larger than non-infarcted hemisphere. Conversely, blockade of endothelin B receptor with BQ788 followed by either vehicle or IRL-1620 treatment significantly ($P < 0.01$) increased edema (17.02 ± 3.17 and 17.97 ± 5.17%, respectively).

2.4. *Effect on oxidative stress parameters*

To determine the effect of IRL-1620 on oxidative stress damage in the subacute phase of cerebral ischemia, malondialdehyde, reduced glutathione, and superoxide dismutase levels in the brains of middle cerebral artery occluded rats treated with vehicle, IRL-1620 and/or BQ788 were determined 7 days following infarction (Fig. 3).

2.4.1. *Effect on malondialdehyde levels*

Occlusion of the middle cerebral artery in vehicle treated rats produced a significant increase in lipid peroxidation, with malondialdehyde (MDA) levels of 698.91 ± 24.06 nmol/g wet tissue as compared to 128.40 ± 23.37 nmol/g wet tissue in the sham-operated group ($P < 0.001$). Occluded animals treated

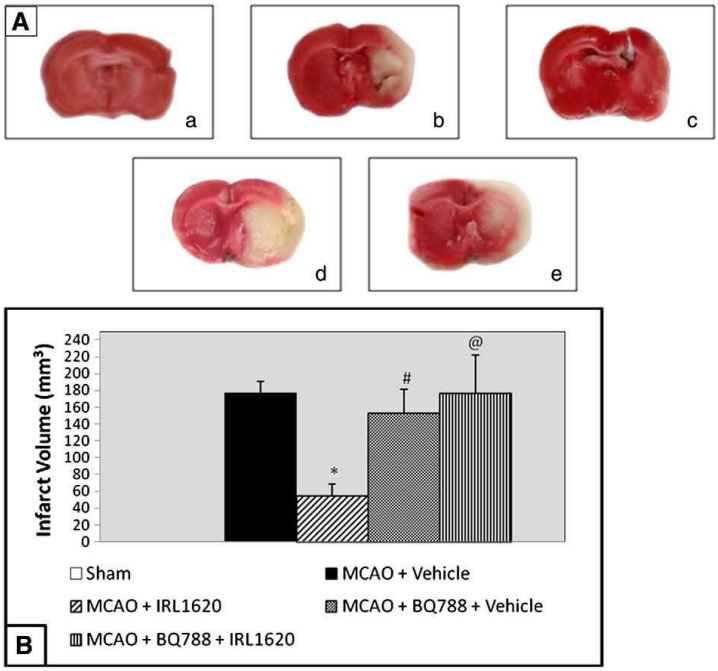


Fig. 2 – A. 2 mm coronal sections of brains stained with TTC to visualize the infarct area 7 days post middle cerebral artery occlusion (red indicates normal tissue and white indicates infarct tissue). Representative slices from groups are as follows: a. sham, b. MCAO + Vehicle, c. MCAO + IRL-1620, d. MCAO + BQ788, e. MCAO + BQ788 + IRL-1620. IRL-1620 (5 μg/kg, IV) or isotonic saline (1 ml/kg, IV) was injected at 2, 4, and 6 h post MCAO. BQ788 (1 mg/kg, IV) was administered once 15 min prior to the first injection of IRL-1620 or vehicle. B. Effect of IRL-1620, BQ788, and BQ788 + IRL-1620 on infarct volume in middle cerebral artery occluded rats. Values are expressed as mean ± SEM. *P < 0.001 vs. vehicle. #P < 0.05 vs. MCAO + IRL-1620. @P < 0.05 vs. MCAO + IRL-1620.

with IRL-1620, on the other hand, had a significant (P < 0.001) reduction in MDA as compared with the vehicle group. Blockade of the endothelin B receptors with BQ788 followed by either vehicle or IRL-1620 resulted in high levels of MDA (Fig. 3A).

2.4.2. *Effect on reduced glutathione levels*

Antioxidant reduced glutathione (GSH) levels decrease in middle cerebral artery occlusion, as can be seen in the vehicle (52.63 ± 17.67 μg/g wet tissue) versus the sham-operated (293.23 ± 38.67 μg/g wet tissue) groups (P < 0.001; Fig. 3B). Activation of endothelin B receptors with IRL-1620 resulted in a far lesser (P < 0.05) reduction in GSH than seen in the group treated with vehicle only. Blockade of these receptors by BQ788 resulted in GSH levels which were close to those of the vehicle group and significantly lower than those of the IRL-1620 (P < 0.05) alone group (Fig. 3B).

2.4.3. *Effect on superoxide dismutase levels*

Levels of antioxidant superoxide dismutase (SOD) in the brains of vehicle treated middle cerebral artery occluded animals were significantly lower (8.49 ± 2.40 units/mg protein) than those of the sham-operated group (20.32 ± 1.29 units/mg protein; P < 0.01; Fig. 3C). IRL-1620 treatment significantly (P < 0.001) improved levels of SOD in the occluded rat brain as compared to vehicle-treated rats. On the other hand, SOD levels in animals pretreated with BQ788 followed by either vehicle or IRL-1620 were significantly (P < 0.001) lower than those of the IRL-1620 alone group (Fig. 3C).

2.5. *Effect on endothelin receptor levels*

Both endothelin A and endothelin B receptor levels in the infarcted and non-infarcted hemispheres of sham-operated and middle cerebral artery occluded rats were examined on day 7 following ischemia. Endothelin A receptor levels were found to be similar in vehicle-, IRL-1620-, and BQ788-treated middle cerebral artery occluded rats (Fig. 4A). Endothelin B receptor levels were similar in vehicle and BQ788 treated rats but were significantly raised in the infarcted hemisphere of IRL-1620-treated animals (Fig. 4B; P < 0.05). Treatment with endothelin B receptor agonist, IRL-1620 after induction of

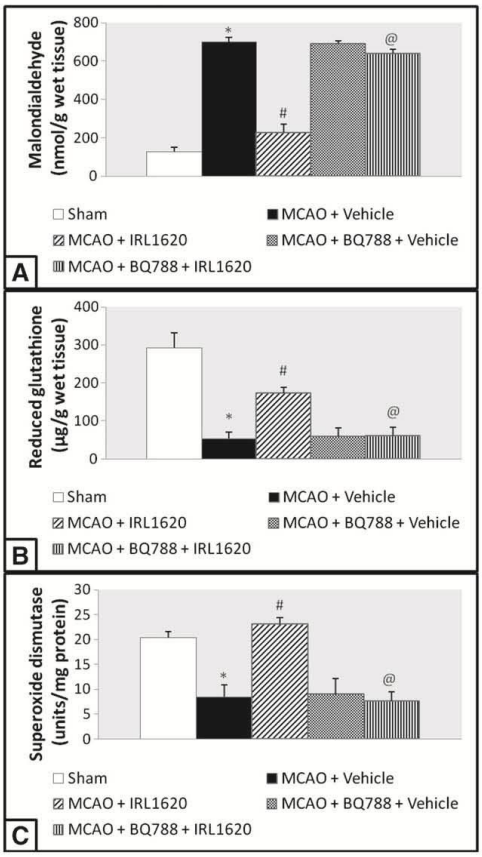


Fig. 3 – Effect of endothelin B receptor agonist, IRL-1620, on oxidative stress parameters 7 days following middle cerebral artery occlusion in rats. A. Malondialdehyde levels in the occluded rat brain. B. Reduced glutathione levels in the occluded rat brain. C. Superoxide dismutase levels in the occluded rat brain. IRL-1620 (5 µg/kg, IV) or isotonic saline (1 ml/kg, IV) was injected at 2, 4, and 6 h post MCAO. BQ788 (1 mg/kg, IV) was administered once 15 min prior to the first injection of IRL-1620 or vehicle. Values are expressed as mean ± SEM. *P < 0.001 vs. sham. #P < 0.05 vs. MCAO + Vehicle. @P < 0.05 vs. MCAO + IRL-1620.

cerebral ischemia produced a significant upregulation of endothelin B receptors in the infarcted brain hemisphere.

3. Discussion

The purpose of this study was to determine the effects of IRL-1620, a selective endothelin B receptor agonist, on the subacute phase of permanent cerebral ischemia in rats. IRL-1620 was used alone and in conjunction with a specific endothelin B receptor antagonist, BQ788, to confirm that any observed alterations in parameters were specific to endothelin B receptor. IRL-1620 administered only on day 1 significantly reduced the mortality of rats with cerebral ischemia when observed over a 7 day period. It was found to improve neurological and motor functions with a corresponding reduction in infarct volume, cerebral edema and oxidative stress for up to 7 days following middle cerebral artery occlusion. A unique finding was that treatment with IRL-1620 produced an upregulation of endothelin B receptors in the infarcted hemisphere, while the expression of these receptors was not altered in non-infarcted hemisphere of the same rat. In addition, an increase in the expression of endothelin A receptors observed at 24 h (Leonard et al., 2011) following cerebral ischemia was not observed on the 7th day. The effects of IRL-1620 are blocked by pretreatment with BQ788, confirming the role of endothelin B receptors in neuroprotection following cerebral ischemia.

Experimental cerebral ischemia leads to a marked neurological deficit accompanied by a weakness or paralysis and lack of motor coordination. In addition, there are increases in oxidative stress as determined by increases in lipid peroxidation and decreases in antioxidants (Briyal and Gulati, 2010; Chan, 1996; Gupta et al., 2005). All of these signs and symptoms were noted in the vehicle- and BQ788-treated rats undergoing middle cerebral artery occlusion. Middle cerebral artery occluded rats treated with IRL-1620, on the other hand, presented with significantly improved neurological and motor functions along with reduced MDA and increased GSH and SOD. These results concur with our previous study in which rats underwent a 24 h permanent occlusion (Leonard et al., 2011). Oxidative stress represents a fast cellular response and is an early feature of cerebral ischemia lasting for several hours from the onset of ischemia as was observed in our previous study of 24 hour occlusion (Leonard et al., 2011). Findings of the present study indicate that either a longer lasting oxidative stress occurs or a secondary delayed development may be taking place following cerebral ischemia. Selective endothelin B receptor activation was found to provide neuroprotection against oxidative stress even in cases of long-term permanent cerebral ischemia.

The endothelin B receptor, which is located on neurons and astrocytes as well as vascular endothelium, has been indicated in the promotion of neuronal survival and reduced apoptosis in the hippocampus, dentate gyrus, olfactory epithelium, and cortical neurons (Ehrenreich et al., 1999; Laziz et al., 2011; Riechers et al., 2004; Yagami et al., 2005). Conversely, deficiency of endothelin B receptors enhances neuronal susceptibility to hypoxia–ischemia in vivo, whereas endothelin B receptor deficient neuronal cultures demonstrate similar rates of hypoxia-induced cell death as compared with wildtype neurons (Siren et al., 2002). These results suggest that the endothelin B receptors located on the other cells, e.g. astrocytes, throughout the central nervous system may be exerting neuroprotective effects. Endothelin receptors on astrocytes mediate a reduction in gap junction permeability and influence the propagation of apoptotic signals (Blomstrand et al., 1999; Lin et al., 1998). Our results showing attenuated edema in the presence of IRL-1620 treatment and exacerbated edema in the presence of the endothelin B receptor antagonist seem to indicate that the endothelin B receptor is playing a role in gap

junction permeability and breakdown of the blood brain barrier which occurs during the subacute phase of cerebral ischemia. Heightened levels of endothelin as seen after an ischemic stroke are known to promote inflammation via recruitment of inflammatory cells and the release of key inflammatory mediators which lead to further breakdown of the blood brain barrier (McCarron et al., 1993; Trevisi et al., 2002; Zidovetzki et al., 1999). The endothelin B receptor acts as a clearance receptor for endothelin-1, which may account for some of its vasculo-protective effects (Ozaki et al., 1995). Attenuation of edema and inflammatory reactions via endothelin B receptors may also account for the sustained reduction in oxidative stress.

In a previous study conducted in rats with middle cerebral artery occlusion for 24 h, we did not observe any upregulation of endothelin B receptors in the non-infarcted or infarcted brain hemisphere. However, endothelin A receptor expression was increased in the infarcted brain hemisphere which was not affected by IRL-1620 treatment (Leonard et al., 2011). On the other hand, in the present study conducted in rats with middle cerebral artery occlusion for 7 days, it was found that endothelin A receptor expression was similar in the non-infarcted and infarcted brain hemispheres. Therefore, an increase in endothelin A receptor expression observed at 24 h of infarction resolves by 7th day indicating involvement of endothelin A receptors in acute phase of cerebral ischemia. In the present study, where measurements were made during the subacute phase of stroke, the unique finding is an increase in expression of endothelin B receptors in the infarcted hemisphere following IRL-1620 treatment, while the non-infarcted brain hemisphere was not affected. It is possible that endothelin B receptors play an important role in brain plasticity and that administration of IRL-1620 on the first day contributes towards long term plastic mechanisms in the brain leading to improvement in signs and symptoms of stroke. A limitation of our study is that we do not know the exact location of endothelin receptors, and that they could be located on astrocytes, neurons or blood vessels. However, because we studied brain tissue it is less likely that vascular receptors are reflected in our findings. Following cerebral ischemia astrocytes are converted from resting to reactive which then produce vascular endothelial growth factor in the damaged brains (Cobbs et al., 1998; Papavassiliou et al., 1997). Endothelin B receptors are extensively present on astrocytes and it has been shown that administration of a selective endothelin B receptor agonist converted resting astrocytes to reactive and increased the production of vascular endothelial growth factor (Koyama et al., 2011). It is possible that IRL-1620 acts through multiple mechanisms, including reduction in parameters of oxidative stress, direct anti-apoptotic effect, and increased production of vascular endothelial growth factors from astrocytes.

Finally, a recent study by Castaneda et al. demonstrated that the endothelin B receptor may be associated with the regulation and migration of adult neural stem cells through neurogenic and gliogenic pathways (Castaneda et al., 2010). An increased number of neuronal stem cells and new blood

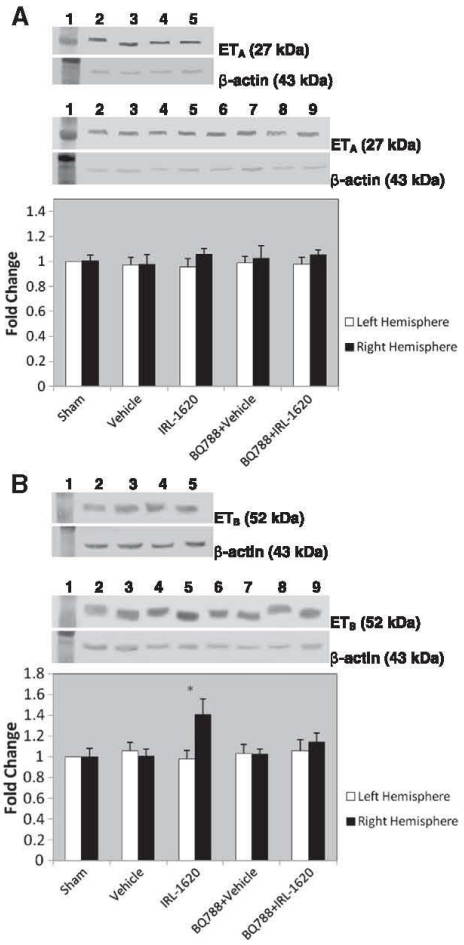


Fig. 4 – A. Expression of endothelin A receptor protein levels with β-actin as a loading control. Top: Lane 1 — protein marker; Lane 2 — sham (LH); Lane 3 — sham (RH); Lane 4 — vehicle + MCAO (LH); Lane 5 — vehicle + MCAO (RH). Bottom: Lane 1 — protein marker; Lane 2 — vehicle + MCAO (LH); Lane 3 — vehicle + MCAO (RH); Lane 4 — MCAO + IRL1620 (LH); Lane 5 — MCAO + IRL1620 (RH); Lane 6 — MCAO + BQ788 + vehicle (LH); Lane 7 — MCAO + BQ788 + vehicle (RH); Lane 8 — MCAO + BQ788 + IRL1620 (LH); Lane 9 — MCAO + BQ788 + IRL1620 (RH). The blot is representative of six different experiments with similar results and bar graph showing fold change in the expression of endothelin A receptor in brain 7 days following middle cerebral artery occlusion. B. Expression of endothelin B receptor protein levels in the occluded rat brain with lane distribution same as in (A). IRL-1620 (5 μg/kg, IV) or isotonic saline (1 ml/kg, IV) was injected at 2, 4, and 6 h post MCAO. BQ788 (1 mg/kg, IV) was administered 15 min prior to the first injection of IRL-1620 or vehicle. LH = left hemisphere; RH = right hemisphere. Values are expressed as mean ± S.E.M. *P < 0.05 compared to LH.

vessels have been noted in the human brain following cerebral ischemia (Minger et al., 2007). Whether stimulation of endothelin B receptors may enhance this process following cerebral ischemia is as yet unknown. Future studies, including double immunostaining for endothelin B receptors along with staining for astrocytes, neurons or endothelial cells, are planned which hope to address these questions in addition to gaining a more complete picture of the mechanism of action by which endothelin B receptors protect the brain following ischemia.

The present study indicates that selective endothelin B receptor activation is a promising new potential tool for neuroprotection in the treatment of ischemic stroke. Endothelin B receptor agonist, IRL-1620, significantly improves survival, reduces neurological and motor function deficit while effectively decreasing infarct volume, edema and oxidative stress for up to one week following permanent induction of cerebral ischemia in rats. These improvements are accompanied by an upregulation of endothelin B receptors in the infarcted hemisphere. Further investigations into the endothelin B receptor's mechanisms of neuroprotection and possible resultant tissue changes following cerebral ischemia are warranted.

4. Experimental procedures

4.1. Animals

Male Sprague–Dawley rats (Harlan, Indianapolis, IN) weighing 300–350 g were housed in a room with controlled temperature (23 ± 1 °C), humidity (50 ± 10%), and light (6:00 A.M. to 6:00 P.M.). Food and water were available ad libitum. Animals were allowed to acclimate for at least 4 days prior to experimentation. All animal care and use for experimental procedures were in compliance with and approved by the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Midwestern University.

4.2. Drugs

Ketamine (Butler Animal Health Supply, Dublin, OH) and xylazine (Lloyd Laboratories, Shenandoah, IA) were administered intraperitoneally (i.p.) at a dose of 100 mg/kg and 10 mg/kg, respectively. IRL-1620 [N-Succinyl-[Glu9, Ala11,15] endothelin 1] (American Peptide Co, Inc., Sunnyvale, CA) was dissolved in isotonic saline and administered at a dose of 5 μg/kg, intravenously (i.v.) at 2, 4 and 6 h post middle cerebral artery occlusion. 2 h post occlusion was chosen as the ideal time to begin treatment as it allows sufficient time for the animal to recover from surgery and to begin displaying neurological and/or motor impairment, confirming that cerebral ischemia is occurring. BQ788 (American Peptide Co, Inc., Sunnyvale, CA) was dissolved in isotonic saline and administered at a dose of 1 mg/kg, i.v., 15 min prior to administration of either vehicle or IRL-1620. While ETB receptor agonist, IRL-1620, is known to have transitory hypotensive effects, a previous study performed in our laboratory demonstrated that tachyphylaxis of the hypotensive response occurs when IRL-1620 is delivered in multiple doses of 5 μg/kg, i.v., over time (Leonard and Gulati, 2009). As the first 12–24 h are critical in regards to protection of the penumbra, and in order to ameliorate any potential side effects due to transient hypotension, it was determined to administer IRL-1620 in 3 doses of 5 μg/kg, i.v., at 2 h intervals on day 1. The doses of IRL-1620 and BQ788 were based on preliminary studies and previous work conducted in our laboratory (Briyal and Gulati, 2010; Lavhale et al., 2010; Leonard and Gulati, 2009).

4.3. Experimental protocol

Rats were randomly divided into five groups. Animals in group 1 were subject to a sham operation. Animals in groups 2–5 were subject to middle cerebral artery occlusion and were treated as follows — Group 2: occlusion + vehicle (isotonic saline, 1 ml/kg); Group 3: occlusion + IRL-1620; Group 4: occlusion + BQ788 + vehicle; and Group 5: occlusion + BQ788 + IRL-1620. All drugs were administered via intravenous tail vein injection as described above. A total of 70 rats were used for this study, with n = 8 per group for neurological and motor deficit and infarct volume analysis, and n = 6 per group for endothelin receptor estimation and oxidative stress analysis.

4.4. Middle cerebral artery occlusion

Permanent middle cerebral artery occlusion was performed according to the method of Koizumi et al. (1986). Rats were anesthetized with ketamine and xylazine. Rectal core temperature was measured with a Cole Palmer Animal Monitoring Thermometer colonic probe (Vernon Hills, IL) and maintained throughout surgery at 37 ± 1 °C using the thermocontrolled base of the operating table. With the anesthetized rat in a secure supine position, a midline incision was made and the right common, internal, and external carotid arteries were exposed. A 4.0 monofilament nylon filament (CP Medical, Portland, OR) with a flame-rounded tip was advanced from the external carotid artery into the lumen of the internal carotid artery until resistance was felt (~20–22 mm), indicating occlusion of the middle cerebral artery. In order to create a permanent model of cerebral ischemia, the filament was securely tied and allowed to remain in place until the end of the experiment. The incision was closed with 3.0 silk surgical sutures (Ethicon, Inc.). In sham-operated animals, the common and external right carotid arteries were exposed and the incision was sutured without touching the internal carotid artery. Rats were monitored twice daily to assess appearance, activity, and behavior. Animals were sacrificed if there was a gradual but continuous decline in body weight, or were moribund with unhealthy appearance such as rough coat, hunched posture and/or distended abdomen. Survival of the animals was documented. Proper and intact placement of the filament was verified in all animals at the time of sacrifice.

4.5. Motor performance tests

Four assessments were used to determine neurological and motor deficit following permanent middle cerebral artery occlusion — neurological evaluation, grip test, foot fault error test, and rota rod. Animals were subject to blinded assessments 15 min prior to occlusion to establish a baseline and at 1, 4 and 7 days post occlusion to determine the effects of ischemia with and without treatment.

4.5.1. *Neurological evaluation*

The neurological evaluation was based on a 6 point scale as described by Tatlisumak et al. (1998). The scoring was as follows: 0 = no deficits, 1 = failure to fully extend left forepaw, 2 = circling to the left, 3 = paresis to the left, 4 = no spontaneous walking, and 5 = death.

4.5.2. *Grip test*

The grip test for muscular strength consisted of a string elevated 40 cm above a flat surface pulled taut between two vertical supports spaced 50 cm apart. The animal was placed on the string midway between the supports and evaluated according to a 6 point scale (Moran et al., 1995). The scoring was as follows: 0 = falls off, 1 = hangs on by two forepaws, 2 = hangs on by two forepaws and attempts to climb on, 3 = hangs on by 3+ paws, 4 = hangs on by all paws plus tail, and 5 = escapes.

4.5.3. *Foot fault error test*

Animals were placed on an elevated grid floor with a mesh size of 30 mm^2 for one minute to acclimate. They were then observed for one minute and evaluated for foot fault errors (i.e. a misplaced limb falling through the grid) compared with paired steps as follows (Markgraf et al., 1992):

$$\%\text{foot fault error} = (\text{number of faults/number of paired steps}) \times 100.$$

4.5.4. *Rota rod*

Animals were acclimated to the rotating spindle of the rota rod (Rota-Rod 47700, Ugo Basile, Italy) prior to occlusion. They were placed on the rotating spindle, set to a constant 8 rotations per minute (RPM), until they demonstrated the ability to remain on the spindle for 60 s. Animals were then subject to a baseline test trial on the accelerating spindle (4–40 RPM) over 5 min. The acceleration trial was repeated at 1, 4 and 7 d post occlusion, and the time (in s) at which the animals fell off was recorded (Rogers et al., 1997).

4.6. *Assessment of cerebral infarct volume*

Animals were euthanized by decapitation one week following middle cerebral artery occlusion, and the brains were removed for assessment of infarct volume. The brains were washed in chilled saline at 4 °C for 5 min and then cut into 2 mm thick slices using a Brain Matrix (Harvard Apparatus, Holliston, MA). The sections were incubated at 37 °C for 15 min in 2% 2,3,5-triphenyltetrazolium (TTC, Sigma, St. Louis, MO) dissolved in saline. The stained sections were then stored in 10% formalin at 4 °C for further analysis (Li et al., 1997). Infarct volume was calculated by sampling each side of the coronal sections with a digital camera (Nikon). The infarct area, outlined in white, was measured by image analysis software (Adobe Photoshop CS4). Edema was determined by taking the percent increase in size of the ischemic over the contralateral hemisphere (Barone et al., 1995). Total infarct size is expressed as infarct volume in mm^3 as the sum of infarct areas in each slice, corrected for edema.

4.7. *Estimation of oxidative stress parameters*

Brain levels of MDA, GSH, and SOD were estimated one week following middle cerebral artery occlusion. Animals were decapitated, and the brains removed and washed in chilled saline and stored at −80 °C. The biochemical analyses were performed within 48 h.

4.7.1. *Measurement of lipid peroxidation*

An indicator of lipid peroxidation, MDA, was estimated according to the method of Ohkawa et al. (1979). Brains were homogenized in 10 times (w/v) 0.1 M sodium phosphate buffer (pH 7.4). To 0.1 ml of the processed tissue sample, 1.5 ml acetic acid (20%, pH 3.5), 1.5 ml thiobarbituric acid (0.8%), and 0.2 ml sodium dodecyl sulfate (8.1%) were added, and the mixture was incubated at 100 °C for 60 min. After cooling, 5 ml n-butanol:pyridine (15:1% v/v) and 1 ml distilled water were added. The mixture was then shaken vigorously and centrifuged at 4000 RPM for 10 min. The absorbance of the organic layer was measured at 532 nm using a spectrophotometer (Spectronic Instruments, Rochester, NY).

4.7.2. *Measurement of reduced glutathione*

Brain levels of the antioxidant, GSH, were measured according to the method of Ellman with minor modifications (Ellman, 1959). Brains were homogenized with 10 times (w/v) sodium phosphate buffer (pH 7.4). The homogenate was then centrifuged with 5% trichloroacetic acid to separate out the proteins. 0.1 ml of the supernatant was then added to 2 ml phosphate buffer (pH 8.4), 0.5 ml 5′5-dithio-bis-2-nitrobenzoic acid, and 0.4 ml distilled water. The mixture was then vortexed and the absorbance was read within 15 min at 412 nm using a spectrophotometer.

4.7.3. *Measurement of superoxide dismutase*

SOD was measured in accordance with the method of Kakkar et al. (1984). 1.2 ml sodium pyrophosphate buffer (0.052 M, pH 8.3), 0.1 ml phenzanine methosulfate (186 μM), 0.3 ml nitro blue tetrazolium (300 μM), and 0.2 ml NADH (780 μM) were added to 0.1 ml homogenate, and the mixture was incubated at 30 °C for 90 min. Following the addition of 4 ml n-butanol and 1 ml acetic acid, the mixture was shaken vigorously and then centrifuged at 4000 RPM for 10 min. The absorbance of the organic layer was measured at 560 nm using a spectrophotometer.

4.8. *Endothelin receptor estimation*

Endothelin receptors in the infarcted brain were measured via Western blotting. Animals were decapitated one week post occlusion, and the brains, sectioned into right and left hemispheres, were flash frozen and stored at −80 °C. The tissue was homogenized in 10 times (w/v) RIPA lysis buffer. Protein concentration was measured according to the Lowry method, using a spectrophotometer (Lowry et al., 1951). 20 μg of protein, denatured in Laemmli sample buffer, was resolved in 10% SDS-PAGE and transferred onto nitrocellulose membrane. After blocking, the membranes were incubated with rabbit polyclonal anti-ETA (1:1000) or anti-ETB (1:500) antibodies, followed by HRP-conjugated secondary antibodies (1:1000). The labeled proteins were then visualized using an ECL Plus western blotting detection system (GE Healthcare, Buckinghamshire, UK). Stripped membranes were reprobed with β-actin primary antibody (1:1000) for a protein loading control (Lavhale et al., 2010).

4.9. *Statistical analysis*

A Power Analysis was conducted using GraphPad Instat-2.00. The power was set to 80% (beta = 0.8) and the level of significance (alpha) used was 0.05. Power Analysis indicated that a sample size of 5 per group was sufficient to achieve a power of 80%, when level of significance alpha = 0.05. The data is represented as mean ± S.E.M. Behavioral data was analyzed via a two-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test with treatment as the factor and time as the repeated measure. One-way analysis of variance (ANOVA) followed by Bonferroni's post hoc comparison test was used for intergroup comparison in evaluating infarct volume and oxidative stress parameters. Two-tailed unpaired *t*-test was used for endothelin receptor comparison. A P value of less than 0.05 was considered to be significant. The statistical analysis was processed with GraphPad Prism 5.00 (GraphPad, San Diego, CA, USA).

REFERENCES

Barone, F.C., White, R.F., Elliott, J.D., Feuerstein, G.Z., Ohlstein, E.H., 1995. The endothelin receptor antagonist SB 217242 reduces cerebral focal ischemic brain injury. J. Cardiovasc. Pharmacol. 26 (Suppl. 3), S404–S407.

Blomstrand, F., Giaume, C., Hansson, E., Ronnback, L., 1999. Distinct pharmacological properties of ET-1 and ET-3 on astroglial gap junctions and Ca(2+) signaling. Am. J. Physiol. 277, C616–C627.

Briyal, S., Gulati, A., 2010. Endothelin-A receptor antagonist BQ123 potentiates acetaminophen induced hypothermia and reduces infarction following focal cerebral ischemia in rats. Eur. J. Pharmacol. 644, 73–79.

Castaneda, M.M., Cubilla, M.A., Lopez-Vicchi, M.M., Suburo, A.M., 2010. Endothelinergic cells in the subependymal region of mice. Brain Res. 1321, 20–30.

Chan, P.H., 1996. Role of oxidants in ischemic brain damage. Stroke 27, 1124–1129.

Chuquet, J., Benchenane, K., Toutain, J., MacKenzie, E.T., Roussel, S., Touzani, O., 2002. Selective blockade of endothelin-B receptors exacerbates ischemic brain damage in the rat. Stroke 33, 3019–3025.

Cobbs, C.S., Chen, J., Greenberg, D.A., Graham, S.H., 1998. Vascular endothelial growth factor expression in transient focal cerebral ischemia in the rat. Neurosci. Lett. 249, 79–82.

Ehrenreich, H., Oldenburg, J., Hasselblatt, M., Herms, J., Dembowski, C., Loffler, B.M., Bruck, W., Kamrowski-Kruck, H., Gall, S., Siren, A.L., Schilling, L., 1999. Endothelin B receptor-deficient rats as a subtraction model to study the cerebral endothelin system. Neuroscience 91, 1067–1075.

Ellman, G.L., 1959. Tissue sulfhydryl groups. Arch. Biochem. Biophys. 82, 70–77.

Goto, K., Kasuya, Y., Matsuki, N., Takuwa, Y., Kurihara, H., Ishikawa, T., Kimura, S., Yanagisawa, M., Masaki, T., 1989. Endothelin activates the dihydropyridine-sensitive, voltage-dependent Ca^{2+} channel in vascular smooth muscle. Proc. Natl. Acad. Sci. U. S. A. 86, 3915–3918.

Gupta, Y.K., Briyal, S., Sharma, U., Jagannathan, N.R., Gulati, A., 2005. Effect of endothelin antagonist (TAK-044) on cerebral ischemic volume, oxidative stress markers and neurobehavioral parameters in the middle cerebral artery occlusion model of stroke in rats. Life Sci. 77, 15–27.

Kakkar, P., Das, B., Viswanathan, P.N., 1984. A modified spectrophotometric assay of superoxide dismutase. Indian J. Biochem. Biophys. 21, 130–132.

Kanekar, S.G., Zacharia, T., Roller, R., 2012. Imaging of stroke: part 2, pathophysiology at the molecular and cellular levels and corresponding imaging changes. AJR Am. J. Roentgenol. 198, 63–74.

Koizumi, J., Yoshida, Y., Nakazawa, T., Ooneda, G., 1986. Experimental studies of ischemic brain edema. I. A new experimental model of cerebral embolism in rats in which recirculation can be introduced in the ischemic area. Jpn. J. Stroke 8, 1–8.

Koyama, Y., Nagae, R., Tokuyama, S., Tanaka, K., 2011. I.c.v administration of an endothelin ET(B) receptor agonist stimulates vascular endothelial growth factor-A production and activates vascular endothelial growth factor receptors in rat brain. Neuroscience 192, 689–698.

Lakhan, S.E., Kirchgessner, A., Hofer, M., 2009. Inflammatory mechanisms in ischemic stroke: therapeutic approaches. J. Transl. Med. 7, 97.

Lavhale, M.S., Briyal, S., Parikh, N., Gulati, A., 2010. Endothelin modulates the cardiovascular effects of clonidine in the rat. Pharmacol. Res. 62, 489–499.

Laziz, I., Larbi, A., Grebert, D., Sautel, M., Congar, P., Lacroix, M.C., Salesse, R., Meunier, N., 2011. Endothelin as a neuroprotective factor in the olfactory epithelium. Neuroscience 172, 20–29.

Lee, H.O., Levorse, J.M., Shin, M.K., 2003. The endothelin receptor-B is required for the migration of neural crest-derived melanocyte and enteric neuron precursors. Dev. Biol. 259, 162–175.

Leonard, M.G., Gulati, A., 2009. Repeated administration of ET(B) receptor agonist, IRL-1620, produces tachyphylaxis only to its hypotensive effect. Pharmacol. Res. 60, 402–410.

Leonard, M.G., Briyal, S., Gulati, A., 2011. Endothelin B receptor agonist, IRL-1620, reduces neurological damage following permanent middle cerebral artery occlusion in rats. Brain Res. 1420, 48–58.

Li, F., Irie, K., Anwer, M.S., Fisher, M., 1997. Delayed triphenyltetrazolium chloride staining remains useful for evaluating cerebral infarct volume in a rat stroke model. J. Cereb. Blood Flow Metab. 17, 1132–1135.

Lin, J.H., Weigel, H., Cotrina, M.L., Liu, S., Bueno, E., Hansen, A.J., Hansen, T.W., Goldman, S., Nedergaard, M., 1998. Gap-junction-mediated propagation and amplification of cell injury. Nat. Neurosci. 1, 494–500.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Markgraf, C.G., Green, E.J., Hurwitz, B.E., Morikawa, E., Dietrich, W.D., McCabe, P.M., Ginsberg, M.D., Schneiderman, N., 1992. Sensorimotor and cognitive consequences of middle cerebral artery occlusion in rats. Brain Res. 575, 238–246.

McCarron, R.M., Wang, L., Stanimirovic, D.B., Spatz, M., 1993. Endothelin induction of adhesion molecule expression on human brain microvascular endothelial cells. Neurosci. Lett. 156, 31–34.

Minger, S.L., Ekonomou, A., Carta, E.M., Chinoy, A., Perry, R.H., Ballard, C.G., 2007. Endogenous neurogenesis in the human brain following cerebral infarction. Regen. Med. 2, 69–74.

Moran, P.M., Higgins, L.S., Cordell, B., Moser, P.C., 1995. Age-related learning deficits in transgenic mice expressing the 751-amino acid isoform of human beta-amyloid precursor protein. Proc. Natl. Acad. Sci. U. S. A. 92, 5341–5345.

Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 95, 351–358.

Ozaki, S., Ohwaki, K., Ihara, M., Fukuroda, T., Ishikawa, K., Yano, M., 1995. ETB-mediated regulation of extracellular levels of endothelin-1 in cultured human endothelial cells. Biochem. Biophys. Res. Commun. 209, 483–489.

Papavassiliou, E., Gogate, N., Proescholdt, M., Heiss, J.D., Walbridge, S., Edwards, N.A., Oldfield, E.H., Merrill, M.J., 1997. Vascular endothelial growth factor (vascular permeability factor) expression in injured rat brain. J. Neurosci. Res. 49, 451–460.

Riechers, C.C., Knabe, W., Siren, A.L., Gariepy, C.E., Yanagisawa, M., Ehrenreich, H., 2004. Endothelin B receptor deficient transgenic rescue rats: a rescue phenomenon in the brain. Neuroscience 124, 719–723.

Rogers, D.C., Campbell, C.A., Stretton, J.L., Mackay, K.B., 1997. Correlation between motor impairment and infarct volume after permanent and transient middle cerebral artery occlusion in the rat. Stroke 28, 2060–2065 (discussion 2066).

Schinelli, S., 2006. Pharmacology and physiopathology of the brain endothelin system: an overview. Curr. Med. Chem. 13, 627–638.

Siren, A.L., Lewczuk, P., Hasselblatt, M., Dembowski, C., Schilling, L., Ehrenreich, H., 2002. Endothelin B receptor deficiency augments neuronal damage upon exposure to hypoxia-ischemia in vivo. Brain Res. 945, 144–149.

Tatlisumak, T., Garano, R.A., Takano, K., Opgenorth, T.J., Sotak, C.H., Fisher, M., 1998. A novel endothelin antagonist, A-127722, attenuates ischemic lesion size in rats with temporary middle cerebral artery occlusion: a diffusion and perfusion MRI study. Stroke 29, 850–857 (discussion 857–8).

Trevisi, L., Bova, S., Cargnelli, G., Geolotto, G., Luciani, S., 2002. Endothelin-1-induced arachidonic acid release by cytosolic phospholipase A2 activation in rat vascular smooth muscle via extracellular signal-regulated kinases pathway. Biochem. Pharmacol. 64, 425–431.

Tsukahara, H., Ende, H., Magazine, H.I., Bahou, W.F., Goligorsky, M.S., 1994. Molecular and functional characterization of the non-isopeptide-selective ETB receptor in endothelial cells. Receptor coupling to nitric oxide synthase. J. Biol. Chem. 269, 21778–21785.

Viossat, I., Duverger, D., Chapelat, M., Pirotzky, E., Chabrier, P.E., Braquet, P., 1993. Elevated tissue endothelin content during focal cerebral ischemia in the rat. J. Cardiovasc. Pharmacol. 22 (Suppl. 8), S306–S309.

Yagami, T., Ueda, K., Sakaeda, T., Okamura, N., Nakazato, H., Kuroda, T., Hata, S., Sakaguchi, G., Itoh, N., Hashimoto, Y., Fujimoto, M., 2005. Effects of an endothelin B receptor agonist on secretory phospholipase A2-IIA-induced apoptosis in cortical neurons. Neuropharmacology 48, 291–300.

Zidovetzki, R., Chen, P., Chen, M., Hofman, F.M., 1999. Endothelin-1-induced interleukin-8 production in human brain-derived endothelial cells is mediated by the protein kinase C and protein tyrosine kinase pathways. Blood 94, 1291–1299.

VITA

Name Mary G. Leonard

Education and Certification

Aug 2008 – May 2013 (projected)
Doctor of Philosophy – Bioengineering
University of Illinois at Chicago, Chicago, IL
Focus: Neural Engineering
Area of research: Endothelin B receptor involvement in permanent cerebral ischemia
Certification: Animal and Research at UIC – Sept 2009
Training: Laboratory and Radiation Safety

Aug 2006 – May 2008
Master of Science – Biomedical Sciences
Midwestern University, Downers Grove, IL
Thesis: Cardiovascular Effects of Endothelin B Receptor Agonist IRL-1620
Training: Good Laboratory Practices, Biosafety and Radiation Safety, Good Manufacturing Practices, IACUC Animal Care & Use

Aug 1999 – May 2003
Bachelor of Science – Biology
Bachelor of Arts – Russian Studies
University of Missouri, Columbia, MO

Professional Experience

June 2008 – Present
Research Associate – Chicago College of Pharmacy, Midwestern University, Downers Grove, IL

- Design and implement experiments using proper laboratory safety and methods and following proper IACUC protocol when handling animals.
- Perform statistical analysis of experimental data.
- Research, compile, and compose manuscripts.
- Train students and fellows in specific laboratory and experimental techniques.

Oct 2006 – July 2008
Pharmacy Technician – Licensed (IL), WellGroup Health Partners, Chicago Heights, IL

- Demonstrate familiarity with prescription and OTC medications.
- Greet patients/customers, check prescriptions and patient data, answer OTC questions, and assist pharmacist in varying tasks.

Research Expertise

In vivo techniques – rats and mice

- Cardiovascular pharmacology (blood pressure, heart rate, pressure-volume, cerebral and renal blood flow, blood gas analysis)
- Middle Cerebral Artery Occlusion
- Cannulation (femoral, carotid, jugular, ventricular)
- Analgesia (tail flick, hot/cold plate)
- Behavioral analysis (water maze learning and memory, locomotor activity, foot fault error, grip test, rota rod)
- Induction of various disease states (cerebral ischemia, hemorrhagic shock, diabetes mellitus, Alzheimer's disease)
- Drug administration (intravenous, subcutaneous, oral, intraperitoneal, intracerebroventricular)
- Optical Imaging (BioFLECT system)

In vitro techniques

- Western blotting
- Immunohistochemistry / Immunofluorescence
- Oxidative stress estimation (malondialdehyde, reduced glutathione, superoxide dismutase)
- Spectroscopy (UV-VIS)
- Microscopy (Fluorescent Inverted and Confocal)
- Thromboelastography
- HPLC

Software

- PowerLab, Varimex Tracking System, MatLab, GraphPad Prism, NIH ImageJ, EndNote, Nikon NIS Elements, MS-Excel, Word, Power Point, Photoshop

Areas of Research Interest

- Neural and tissue engineering
- Neural pharmacology
- CNS physiology and development – angiogenesis, neurogenesis, synaptic plasticity
- Learning and memory
- Cardiovascular pharmacology
- Endothelin pharmacology

Publications

1. **Leonard, M.G.**, S. Briyal and A. Gulati. *Endothelin B receptor agonist, IRL-1620, provides long-term neuroprotection in cerebral ischemia in rats.* Brain Res, 2012. 1464: 14–23.

PMID: 22580085; doi:10.1016/j.brainres.2012.05.005.

2. **Leonard, M.G.**, S. Briyal and A. Gulati. *Endothelin B Receptor Agonist, IRL-1620, Reduces Neurological Damage Following Permanent Middle Cerebral Artery Occlusion in Rats*. Brain Res, 2011. 1420: 48-58.
   PMID: 21959172; doi:10.1016/j.brainres.2011.08.075.
3. **Leonard, M.G.** *A Review of Deep Brain Stimulation*. UIC Bioengineering Student Journal. 2010; 2(1): 25-30.
4. **Leonard, M.G.** and A. Gulati. *Repeated administration of ET(B) receptor agonist, IRL-1620, produces tachyphylaxis only to its hypotensive effect.* Pharmacol Res, 2009. 60(5): 402-10.
   PMID: 19666119; doi:10.1016/j.phrs.2009.07.015.

Abstracts/Presentations

1. SCCM Annual Congress of the Society of Critical Care Medicine, San Juan, Puerto Rico – 19-23 January 2013; *IRL-1620, ETB receptor agonist provides neuroprotection and promotes angiogenesis following cerebral ischemia in rats*; Abstract 6160; **Leonard, M.**, Gulati, A.

2. ACCP American College of Clinical Pharmacology Annual Meeting, San Diego, CA - 23-25 September 2012; *IRL-1620, an Endothelin B Receptor Agonist, Provides Long-term Neuroprotection Following Cerebral Ischemia in Rats*; Abstract 1394590; **Leonard, M.**, Briyal, S., Gulati, A.
3. Kenneth A Suarez Annual Research Day – Midwestern University, Downers Grove, IL – 11 May 2012; *Endothelin B receptor agonist, IRL-1620, provides long-term neuroprotection in cerebral ischemia in rats*; **Leonard, M.**, Briyal, S., Gulati, A.
4. ET-12: Twelfth International Conference on Endothelin, Cambridge, England – 11-14 September 2011; *Endothelin B Agonist, IRL-1620, Reduces Neurological Damage Following Cerebral Ischemia in Rats*; **Leonard, M.**, Briyal, S., Gulati, A.
5. Kenneth A. Suarez Annual Research Day – Midwestern University, Downers Grove, IL – 6 May 2011; *Endothelin B Agonist, IRL-1620, Reduces Neurological Damage Following Cerebral Ischemia in Rats*; **Leonard, M.**, Briyal, S., Gulati, A.

6. AAPS Annual Meeting and Exposition, Los Angeles, CA – 8 November 2009; *Endothelin modulates cardiovascular effects of clonidine and centhaquin*; Lavhale, M., Parikh, N., **Leonard, M.**, Gulati, A.

7. ET-11: APS International Conference on Endothelin, Montreal, Canada – 9 September 2009; *Modulation of cardiovascular effects of clonidine and centhaquin by endothelin*; Lavhale, M., Parikh, N., **Leonard, M.**, Gulati, A.

8. Kenneth A. Suarez Annual Research Day – Midwestern University, Downers Grove, IL – 1 May 2009; *Modulation of cardiovascular effects of clonidine and centhaquin by endothelin*; Lavhale, M., Parikh, N., **Leonard, M.**, Gulati, A.

9. IXth World Conference on Clinical Pharmacology and Therapeutics, Quebec City, Canada – 29 July 2008; *ET_B receptor agonist, IRL-1620, increases cerebral blood perfusion in rats*; **Leonard, M.**, Gulati, A.

10. IXth World Conference on Clinical Pharmacology and Therapeutics, Quebec City, Canada – 29 July 2008; *Repeated administration of ET_B receptor agonist, IRL-1620, produces tachyphylaxis only to its hypotensive effect*; **Leonard, M.**, Gulati, A.
11. Kenneth A. Suarez Annual Research Day - Midwestern University, Downers Grove, IL – 2 May 2008; *Repeated administration of ET_B receptor agonist, IRL-1620, produces tachyphylaxis only to its hypotensive effect*; **Leonard, M.**, Gulati, A.
12. Midwestern University, Downers Grove, IL – 16 Nov 2007; *Alzheimer's and Parkinson's – A Devastating Combination*

Teaching Experience

- United States Peace Corps – Community Health Specialist; Yeghegnadzor Medical College – Aug 2003 – July 2005
    - Developed curriculum and taught in Eastern Armenian to approximately 60 students.
    - STDs and Contraception – 2 semesters
    - Nutrition – 2 semesters
    - Healthy Living - 2 semesters
    - English Medical Terminology – 4 semesters

Professional Affiliations

- National Association for Professional Women (NAPW) – Member 2013

Awards

- Young Investigator Award – Twelfth International Conference on Endothelin, Cambridge, England 09/11
- MBS Program Research Award – Midwestern University, Downers Grove, IL – 05/08; "For demonstrating the potential to make significant contributions to Biomedical Research."
- *Summa cum laude* – Midwestern University, Downers Grove, IL – 05/08
- *Magna cum laude* – Russian, University of Missouri, Columbia, MO – 05/03
- *Magna cum laude* – Biological Sciences, University of Missouri, Columbia, MO – 05/03