**HYPERTENSION AND THE DEVELOPMENT OF POST-STROKE COGNITIVE IMPAIRMENT: MECHANISMS AND THERAPEUTIC IMPLICATIONS**

by

MOHAMMED ADEL GOMAA SAYED

(Under the Direction of Susan C. Fagan)

ABSTRACT

Post-stroke cognitive impairment (PSCI) is a major source of disability, affecting up to two thirds of stroke survivors with no available therapeutic options. The condition remains understudied in preclinical models due to its delayed presentation. Although hypertension is a leading risk factor for dementia, how ischemic stroke contributes to this neurodegenerative condition is unknown. In this work, we used a model of hypertension to study the development of PSCI and its mechanisms, in addition to investigating the delayed activation of Angiotensin II type 2 receptor (AT2R) as a potential therapeutic mechanism. In the first set of investigations, spontaneously hypertensive rats (SHR) were compared to normotensive rats and were subjected to 1-hour middle cerebral artery occlusion (MCAO) or sham surgery. Several cognitive tests were used to assess cognition. Brain magnetic resonance images (MRI) were obtained 12-weeks post-stroke and tissue was collected for immunohistochemistry and protein quantification. Stroked animals developed memory impairment at 4-weeks post-stroke despite recovery from motor deficits. SHRs displayed grey matter atrophy and showed increased markers of inflammatory cell death and DNA

damage. This indicates that preexisting hypertension exacerbates neurodegeneration after stroke beyond its acute effects on neurovascular injury.

In the second set, we ran a randomized, controlled, blinded preclinical trial to determine the therapeutic potential of delayed administration of compound 21 (C21) on these animals. SHRs were subjected to 60-min MCAO or sham surgery. They received C21 or water (orally) for 8 weeks, starting 3 days post-MCAO. Several tests were utilized to assess sensorimotor and cognitive function. Markers of inflammation, cell-death and DNA damage were quantified in the brain lysates. Stroked animals suffered significant sensorimotor deficits that improved overtime and cognitive deficits compared to sham animals. However, delayed treatment with C21 was not effective in improving the rate of sensorimotor recovery or preventing cognitive deficits.

In conclusion, this dissertation provides a better understanding to the role hypertension plays in the development of post stroke cognitive impairment. It proves that there is still a long way to go to produce better treatments to reduce post-stroke disability, beyond treatments that target reducing the initial ischemic insult.

INDEX WORDS: Stroke, Hypertension, Cognitive Impairment, Neuroinflammation, Compound C21; middle cerebral artery occlusion; AT2R

# HYPERTENSION AND THE DEVELOPMENT OF POST-STROKE COGNITIVE IMPAIRMENT: MECHANISMS AND THERAPEUTIC IMPLICATIONS

by

MOHAMMED ADEL GOMAA SAYED

B.Pharm, Ain Shams University, Egypt, 2010

MS, University of Goettingen, Germany, 2013

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2020

# HYPERTENSION AND THE DEVELOPMENT OF POST-STROKE COGNITIVE IMPAIRMENT: MECHANISMS AND THERAPEUTIC IMPLICATIONS

by

MOHAMMED ADEL GOMAA SAYED

| | |
|---|---|
| Major Professor: | Susan C. Fagan |
| Committee: | Adviye Ergul |
| | Bradley G. Phillips |
| | Anilkumar Pillai |
| | Steven Stice |

Electronic Version Approved:

Ron Walcott
Interim Dean of the Graduate School
The University of Georgia
August 2020

# DEDICATION

I dedicate this work to my wife Sara Altantawi, who has been standing by my side since I embarked in this journey ten years ago, being the best companion one could ever dream of. I dedicate it to my daughter Sara Sayed who has been the light and joy of our lives since she was born. I dedicate it to my mother and father, Amany Ismail and Adel Sayed, without whom I could have achieved nothing in my life. I dedicate it to my brothers Ahmed and Omar, whose presence in my life made me stronger. I dedicate it to my grandfather, Abdelghany Ismail, who always believed in me and treated me like a scientist since I was 8.

ACKNOWLEDGEMENTS

I express my sincerest gratitude to Dr. Susan Fagan who taught me a lot in the past few years about how to be a great scientist, a great teacher and a great human being. In addition to providing me with the means and guidance to perform this work, Dr. Fagan, most importantly, provided me with a safe environment to learn, grow and explore with great independence. To that and to her patience with me, I am forever in debt.

I would like to express my thanks to all members of the stroke lab, past and present, Tauheed, Fouda, Heba, LaDonya, Waleed, Abdulkarim and Asmat. I learned a lot form you and each one of you left a mark on my journey. However, the two I am most indebted to are Wael and Bindu. Without these two, none of this work would have seen the light of day.

I would like to express my sincerest thanks to the members of my advisory committee. Dr. Ergul and her lab, specifically, were always by my side, helping and guiding me throughout this journey and her lab always felt like my second home. I would like to thank Dr. Stice, Dr. Phillips and Dr. Pillai for their time and effort, supporting me and attending my meetings, offering me all kinds of useful guidance.

I would like to thank my family in Augusta, the CET program. I had the best time of my life working with all of you guys and I would not trade you for the whole world. Finally, I would like to thank my friends from AU: Mahmoud Abdelbary, Mohamed Abdelsaid and Ahmet Alptekin. Each one of them supported me greatly on the professional, and most importantly, on the personal level. I could not imagine my life in Augusta without you.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

# CHAPTER 1

# INTRODUCTION AND LITERATURE REVIEW

## Development of cognitive impairment following ischemic stroke:

Stroke is the number 5 cause of death in the United States, behind diseases of the heart, cancer, chronic lower respiratory disease, and unintentional injuries/accidents. Globally, in 2013, it ranked as the second-leading cause of death behind ischemic heart disease.[1] Stroke mortality has been steadily decreasing over the last decade due to the continuous improvement in health care standards[2], however, the rate of stroke survivors with residual disability is steadily increasing.[3] Ischemic stroke is a condition characterized by an initial ischemic event that deprives brain tissue from blood supply and oxygenation, usually followed by a reperfusion event, leading to irreversible brain damage and subsequently motor and cognitive impairment.[4]

Post-stroke cognitive impairment (PSCI) is a condition that affects up to two-thirds of ischemic stroke patients, with up to one third eventually developing dementia.[5] Although post stroke cognitive impairment is highly prevalent among stroke survivors according to the data currently available, there is an accumulating body

of evidence showing that the criteria for diagnosis may underestimate the frequency of dementia and cognitive decline among stroke survivors.[6, 7]

From the pathophysiological point of view, it was previously common to describe cognitive impairment following stroke as a form of “vascular dementia”[8], however recent advances have shown that both halves of this definition are misleading. First, a substantial number of patients developing PSCI do not meet the diagnostic criteria of dementia, leading to the shift to a more appropriate term: “vascular cognitive impairment”. [3]. Second, while it has been thought traditionally that post-stroke cognitive impairment results from recurrence of “vascular” insults, recent evidence suggests that a very substantial portion of this impairment results from neuronal pathogenesis.[9] Further evidence from clinical studies suggests that up to one third of patients presenting with PSCI displayed pathology consistent with Alzheimer’s Disease.[10] Therefore, there is a substantial overlap between the vascular and neurodegenerative components of PSCI. According to a review of published autopsy studies on human brain, at least 50% of dementias can be attributed to a mixture of vascular and neurodegenerative causes, thus termed “mixed dementia”.[11]

As stated above, the classical view was that cognitive impairment following vascular injury develops in a stepwise fashion due to repeated ischemic insults. Recently published data from a large, NIH-funded, epidemiologic trial showed, however, that patients, in addition to acute changes, can suffer from a slowly progressive cognitive decline after a single-stroke lesion.[12, 13] This continuous

deterioration occurs even in the absence of any evidence of new ischemic injuries.[14]

The development of cognitive impairment in a progressive fashion after stroke follows a different trajectory compared to sensorimotor deficits. While sensorimotor deficits are usually maximal in the few days following the incidence of the first insult and resolve over time until it reaches a plateau, post-stroke cognitive impairment develops in a progressive manner over an extended period of time both in stroke patients[15] and in experimental animal models of stroke.[16]

Diagnosis and characterization of clinically apparent post-stroke cognitive impairment has proven to be a challenging task owing to the heterogeneity of the condition itself. The incidence of PSCI and its severity depends largely upon morphology of the vascular injury (focal or multifocal; large or small vessel), volume of brain tissue affected by ischemia and, most importantly, the location and number of lesions. [17] Histopathological studies provide crucial information regarding the link between the vascular aspects of brain injuries affecting cognition and the neurodegenerative aspects that resemble Alzheimer's disease pathology.[18] The main histopathological hallmarks of Alzheimer's disease are the deposition of extracellular neuritic plaques and the accumulation of intracellular neurofibrillary tangles. The plaques consist of aggregated amyloid-beta (Aβ) fibrils. These fibrils result from the sequential cleavage of the amyloid precursor protein (APP) by two enzymes, namely, beta and gamma secretase. The proteolytic action

of these secretases preferentially generate Aβ1–42 fibrils, a species of Aβ more hydrophobic and prone to aggregation.[19]

The importance of understanding both pathologies, the vascular and the neurodegenerative, can be put into perspective by the fact that a significant proportion of patients suffering from dementia/cognitive impairment present with both pathologies, ranging from approximately 25%[11] to 56%[20], according to different reviews. One of the most important clinical studies that highlights the importance of the overlap of these pathologies is the Nun Study. In this study, Snowdon and his colleagues demonstrated that the amount of Aβ plaques required to produce cognitive impairment in the patients was greatly reduced if concurrent infarct lesions were present in the thalamus, basal ganglia, or deep white matter.[21] These findings highlight the fact the location of the infarct injury in the brain plays a role of utmost importance in determining whether the patient will suffer from post-stroke cognitive impairment. Poor understanding of the mechanisms involved in the development of post-stroke cognitive impairment as well its time course of development, in addition to difficulty in understanding the roles played by comorbidities such as hypertension, led us to focus on characterizing the timeline and the mechanisms of development of post-stroke cognitive impairment in animal models of hypertension. Modeling the clinically described phenomenon of delayed PSCI will facilitate the development of effective therapeutics to reduce its impact.

## Hypertension as a risk factor for the development of PSCI:

Hypertension is the most commonly occurring modifiable risk-factor for stroke worldwide and is being increasingly recognized as a risk factor for the development of PSCI.[18, 22] Hypertension, a condition associated with vascular dysfunction, has been found to increase the risk of cognitive impairment both independently and by increasing the risk of stroke. In fact, the incidence of hypertension alone can be used to predict the development of dementia in nearly 60% of subjects with cognitive dysfunction.[23] Chronic hypertension, particularly midlife high blood pressure (BP), has been associated with an increased risk for cognitive decline, vascular dementia and even Alzheimer's disease[24]. One of the mechanisms by which hypertension is believed to contribute to the development of cognitive impairment is exposing the cerebral microvasculature to pulsatile pressure causing tearing of the brain vascular endothelium and smooth muscle cells leading to lipohyalinosis and fibrinoid necrosis.[25] Clinical studies on hypertensive patients have shown that acute disruption of blood perfusion can lead to the formation of lacunar infarcts, while chronic ischemia can lead to the development of white matter lesions which are associated with the development of cognitive impairment. [26, 27]

## Cognitive impairment and the brain renin-angiotensin system:

The Renin-Angiotensin system (RAS) is a complex physiological system that plays a pivotal role in regulation of water and electrolyte balance, systemic vascular

resistance, blood pressure and cardiovascular homeostasis. The chronic activation of RAS leads to the accumulation of oxidative stress, endothelial dysfunction and inflammation, and subsequently several pathological conditions ranging from hypertension to kidney disease and heart failure. [28, 29] Traditionally, RAS is thought of as a systemic (endocrine) system, however, recent research found that in addition to the systemic RAS, there is a local RAS that affects the brain, among several other body tissues. In the brain RAS, angiotensin II (Ang II) is the most studied vasoactive substance, as it has been found that Ang II exerts a direct effect at the brain cellular level impacting cell survival, differentiation and inflammation, in addition to its vascular and renal actions.[30, 31]

As part of an intrinsic protection system, the blood–brain barrier (BBB) restricts peripheral RAS components from reaching the majority of brain tissue, requiring the local synthesis of cerebral RAS.[28] Angiotensin is synthesized from a precursor called angiotensinogen. The vast majority of brain angiotensinogen is produced within astrocytes and constitutively secreted for conversion into various neuroactive peptides. Angiotensinogen is also produced by neurons, where it can either be secreted or remain as an intracellular component. These neuroactive peptides can bind to receptors in different cell types to produce intracellular signaling responses.[32, 33] The pathway for Ang II production begins with the cleavage of angiotensinogen into Ang I by renin, followed by further processing through the peptidase ACE. Ang II is the main angiotensin effector peptide

resulting from this synthesis pathway and it binds two major receptors, AT1R and AT2R.[28]

Ang II has the capability of binding to both the AT1R and AT2R. AT1Rs are G-protein coupled receptors (GPCRs) that can be found expressed in the brain on neurons, astrocytes, oligodendrocytes and microglia of the cortex, hippocampus, and basal ganglia. [34] ACE is the enzyme responsible for converting Ang I to Ang II. Although Ang II can bind to both AT1R and AT2R, ACE upregulation leads to a specific increase in AT1R activation. The upregulation of ACE and the resulting over-activation of AT1R are known to result in vasoconstriction systemically, and are linked to the development of cognitive impairment in the context of the brain, possibly through an increase in inflammation and cell death.[34-37] The AT2R is also a GPCR located on neurons, astrocytes and microglia of the cortex, hippocampus, and basal ganglia. [38] AT2R is known to play an important role in vasodilation, and within the brain, it is known to specifically enhance cognition, cell survival and has both antioxidant and anti-inflammatory properties.[34, 35, 39, 40] AT2R activation increases ACE2 expression, and when AT2Rs are knocked out, there is a decline in ACE2 mRNA, protein and activity. [41] AT2Rs can also heterodimerize with AT1Rs, forming AT2R/AT1R heterodimers to directly antagonize and inactivate AT1Rs, leading to a decrease in AT1R signaling pathways.[42] ACE2 significantly increases the formation of these AT2R/AT1R and ACE2 upregulation also results in the overall downregulation of AT1Rs.[43] Upregulation of AT1R activation also leads to downregulation of ACE2, suggesting

a synergistic interplay between the receptors and enzymes to maintain a delicate balance for cognition within a healthy brain. [28]

Hyperactivation of AT1R and ACE signaling in neurons plays an important role in exacerbating cognitive impairment, cell death, and inflammation.[34] This effect manifests through an increase in oxidative stress, resulting from an increase of the ROS produced by an upregulated NOX2.[41] Interestingly, AT1R activation can also lead to an increase in the synthesis of intracellular Ang II to bind AT1Rs, through a signaling pathway involving the nuclear translocation of the Ang II/AT1R complex and an increase in angiotensinogen, renin, and prorenin/renin receptor mRNA.[44] Nuclear AT1R activation increases AT2R expression leading to an increase in AT2R translocation to the mitochondria and the cell surface, in a compensatory fashion.[41] The activation of AT1R leads to an upregulation in Ang (1–7) levels and, subsequently, to an increase in AT2R expression. These effects are decreased in aging and in cognitive disorders.[34] Overactivation of AT1R signaling results in a subsequent activation of NOX4 which leads to increased ROS production. In neurons, this angiotensin-induced ROS production happens predominantly in the mitochondria.[41]

Through this cascade of oxidative stress, mediated by the activation of AT1R and the upregulation of ACE, cell death is induced in different areas of cortical and subcortical brain tissue resulting in the exacerbation of cognitive impairment.[28] These effects have been shown in many rodent studies, where, for example, upregulation of ACE expression reduced acetylcholine release from cholinergic

neurons, a hallmark for cognitive dysfunction.[45-47] Oxidative stress, mediated by AT1R upregulation, resulted in the release of pro-inflammatory cytokines and inflammation that induced cell death, compounded by the activation of pro-inflammatory microglia, ultimately leading to impaired cognition. [28]

Anti-hypertensive medications have been studied extensively as candidates for treating and preventing cognitive dysfunction, both of vascular and of neurodegenerative origins. Our lab has focused extensively on studying the effect of modulators of the renin-angiotensin system on the outcome of stroke in rodent models of experimental stroke, in general, and on the neuroprotective and neurorestorative effects of these agents, in particular. In stroke patients, although poor outcome was observed in association with acutely elevated blood pressure,[48] lowering blood pressure as a strategy for the early management of stroke was associated with an extremely poor stroke outcome, and a significantly worse extent of ischemic injury[49]. Two landmark studies in this regard are the Scandinavian Candesartan Acute Stroke Trial (SCAST) and the China Antihypertensive Trial in Acute Stroke (CATIS). Both of those studies demonstrated no benefit of blood pressure lowering as a strategy in the acute management of stroke.[50, 51] However, there is extensive experimental evidence from animal studies supporting neurovascular benefits for angiotensin receptor blockers (ARBs) independent from their blood-pressure lowering properties,[52, 53] which was not found in other classes of anti-hypertensive agents.[54] It

remains possible that low doses of the ARB, candesartan, may have beneficial effects on long-term recovery after ischemic stroke.

The beneficial effects of angiotensin receptor blockers (ARBs) can be attributed to their ability to activate endogenous restorative mechanisms (neuro-restoration), rather than simply reducing the extent of ischemic injury (neuroprotection). Indeed, most scientific efforts in the field, especially during the last few years, have started to focus on the former rather than the latter.[55-57] In our lab, Ishrat and colleagues have found that treating male Wistar rats (290-300 g), that underwent 90 min of middle cerebral artery occlusion (MCAO) with a subhypotensive dose of candesartan induced subsequent functional recovery through enhanced neurotrophic factor expression in rats subjected to ischemia reperfusion injury.[58] We found that the treatment increased the expression of vascular endothelial growth factor (VEGF) and brain-derived neurotrophic factor (BDNF) and its receptor (TrkB) significantly. Soliman and colleagues found that a single candesartan dose (1 mg/kg) induced a prolonged proangiogenic effect and a prolonged upregulation of VEGF-A and VEGF-B in male Wistar rats that were exposed to 90-minute middle cerebral artery occlusion and treated with candesartan (1 mg/kg) at reperfusion.[59] In addition, Alhusban and colleagues found that candesartan treatment significantly increased the expression of BDNF in the brains of spontaneously hypertensive rats (SHR). In addition, candesartan treatment reversed the antiangiogenic effect of Ang II. We also found that the

beneficial effects of candesartan were ablated by neutralizing the effects of BDNF.[60]

## Activation of AT2R as an alternative approach of targeting cognitive impairment

Opposite to the effects of AT1R, activation of cell surface AT2R can lead to the promotion of cell survival and can counteract the AT1R activation of pathways such as Src Homology Region 2 Domain-Containing Phosphatase-1 (SHP–1), serine-threonine phosphatase (PP2A) and Peroxisome Proliferator-activated Receptors (PPARγ).[28] In addition, activation of nuclear AT2R can have a neuroprotective effect after ischemic injury, through increased nitric oxide (NO) production that leads to decreased firing rates and hyperpolarization via decreased activity of T-type calcium channels and delayed rectifier potassium channels.[61] It is worth noting that, specifically in mitochondria, AT2Rs are much more numerous compared to AT1Rs. With aging, the expression of AT2R is often downregulated, while AT1R is upregulated.[28] The expression of mitochondrial AT2R was also found to be upregulated upon oxidative stress and to decrease mitochondrial respiration to relieve oxidative stress through NO production.[41]

These protective effects mediated by the activation of AT2R was found to play a role in alleviating cognitive impairment, affecting brain regions key to cognitive function such as the cortex, hippocampus and basal ganglia. For example, in rodents, AT2R activation was associated with an increased VEGF production and

enhanced survival of cortical neurons to improve neurological deficits after ischemic injury.[39] While on the other hand, reduced AT2R activation induced dendritic spine abnormalities in the hippocampus and lead to spatial memory deficits.[40] In the basal ganglia, AT2R downregulation was associated with dysfunctional signaling of dopaminergic neurons in animal models of Parkinson's disease.[62]

Among the best AT2R agonist candidates for use in humans is compound 21 (C21). This is a relatively novel non-peptide AT2R agonist, that was discovered in 2004, and has the benefits of both systemic and oral activity. [63] C21 was found to have an oral bioavailability of about 30% and a plasma half-life of almost 4–6 h in rats. [64] It was modelled on the C-terminal pentapeptide structure of Ang II, lacking AT1 receptor affinity and was demonstrated in human embryonic kidney cells to have 4000-fold selectivity to the AT2 receptor.[65, 66] C21 was approved by European Medicines Agency (EMA) and Food and Drug Administration (FDA) as an orphan drug for the treatment of pulmonary fibrosis.[67, 68] This specific compound was found to exhibit vast biological activities which includes anti-fibrotic, anti-inflammatory, anti-apoptotic, anti-oxidant and anti-hypertensive properties. As a result, it showed beneficial effects in the treatment of heart failure, myocardial infarction, chronic inflammatory diseases, and several neurological diseases. [69-71]

In the last few years, our lab has worked extensively on validating the therapeutic potential of C21 in the treatment of cerebral ischemia, including ischemic stroke.

Alhusban and colleagues found that after 3 h of MCAO, a single IP injected dose of C21 (0.03 mg/kg) reduced infarct size and improved behavioral outcome at 24 h without affecting blood pressure, an effect that was blocked by co-administration of the AT2R antagonist (PD123319). On the molecular level, we found that C21 decreased brain hemoglobin content, down-regulated apoptotic and oxidative markers, and increased pro-survival molecules in the brain. After 90 min of MCAO, we found that C21 treatment resulted in sustained functional improvement at 7 days, together with increased vascular density in the ischemic penumbra. In vitro, we showed that C21 showed a pro-angiogenic effect that was blocked with brain-derived neurotrophic factor neutralization.[72] Fouda and colleagues demonstrated that C21 treatment reduced infarct size, improved functional outcome and decreased the pro-inflammatory cytokine, tumor necrosis factor alpha (TNF-α) in the ischemic hemisphere compared to saline, after a single IP injection of C21 (0.03 mg/kg), following 3 hour MCAO in rats. We also demonstrated that anti-IL-10 co-treatment blocked the C21-induced reduction in infarct size and inflammation, and the improvement in behavioral outcome. In vitro, we showed that C21 treatment increased neuron survival and reduced cell apoptosis after oxygen glucose deprivation (OGD) and OGD/reoxygenation and that these effects were mediated through AT2R stimulation.[73] Eldahshan and colleagues found that the acute beneficial effects of C21 post-stroke was not limited to male rats. In females, a single IP dose of C21 resulted in an improvement in sensorimotor scores, one day after MCAO.[74] Despite disappointing effects on motor performance, Ishrat and colleagues found that C21 and performance in the

novel object recognition test in rats at 7 days following embolic MCAO.[75] The results of this study did not support further development of C21 for the acute neuroprotection against ischemic damage.

More recently, our lab garnered more evidence about the neurorestorative effects of C21 when administered in longer term studies. Ahmed and colleagues demonstrated the beneficial effects of C21 on the cognitive function of aged hypertensive rats with chronic cerebral hypoperfusion. We showed that C21 effectively preserved cognitive function and prevented progression of vascular cognitive impairment, using a series of blinded behavioral tests after 4 and 8 weeks of chronic cerebral hypoperfusion concurrent with oral administration of C21 in the drinking water.[35] Ahmed and colleagues also showed that oral C21 treatment for 28 days post MCAO preserved cognitive function, reduced cytotoxicity, and prevented chronic-reactive microgliosis in spontaneously hypertensive rats (SHRs) post-stroke. These protective effects were independent of blood pressure and β-amyloid accumulation.[76] We found similar results in aged animals, when subjected to permanent focal ischemia.[77]

We have now changed our focus to the effects of C21 on the longer-term complications of stroke, especially cognitive impairment. In our most recent study, by Jackson and colleagues examined the effect on C21 on the co-morbid condition of diabetes with control and diabetic rats were subjected to 1 h MCAO or sham surgery. Sensorimotor and cognitive function were tested after delayed administration of C21. Three days post-stroke, rats that met the inclusion criteria

were administered C21 or vehicle in drinking water at a dose of 0.12 mg/kg/day for 8 weeks. It was found that diabetes exacerbated the development of PSCI and increased inflammation and demyelination. Delayed administration of C21, starting 3 days post-stroke, reduced mortality and improved sensorimotor and cognitive deficits. It also reduced inflammation and demyelination through modulation of the pro-inflammatory to anti-inflammatory microglial ratio in the diabetic animals. [78] It is unclear whether hypertensive animals will react similarly to the delayed administration of C21 after stroke and whether similar mechanisms are at work.

**In Summary:**

Stroke is an extremely debilitating medical condition with effects that go beyond increased mortality. While the advances in healthcare have led to decreased stroke mortality, the higher rate of stroke survivors means that more patients are going to suffer from long term disability as a result. While the sensorimotor disabilities due to stroke tend to improve and stabilize with time, clinical observations suggest that cognitive disabilities have a delayed presentation and worsen with time. The mechanisms contributing to the delayed presentation of post-stroke cognitive impairment are still poorly understood, as well as the temporal and spatial aspects of this phenomenon. In addition, accumulating evidence from human and animal studies suggest that comorbid conditions, such as hypertension, play an important role in exacerbating this cognitive decline.

Meanwhile, in the past two decades there has been little success in providing stroke survivors with treatment options other than thrombolytic therapy and mechanical clot removal, two treatments which have an extremely small window of opportunity for intervention and strict eligibility requirements. Efforts have not been fruitful in developing treatments that rely on neuroprotection, by decreasing the damage of the initial insults. Therefore, scientific attention has shifted into neurorestoration, by harnessing and enhancing the natural regenerative capabilities of the human brain to stop or slow down cognitive decline. In this regard, activation of the AT2R has emerged as an extremely promising mechanism that has proven to be beneficial in different models of pulmonary, cardiovascular, neurovascular and ischemic conditions. In this regard, C21 is a prime candidate to target post-stroke cognitive impairment with its favorable pharmacokinetics, pharmacodynamics and toxicity profile. Therefore, we decided to test its potential as an experimental treatment for post-stroke cognitive impairment in hypertensive animals, with delayed administration.

Our **central hypothesis** is that cognitive impairment accumulates after stroke in a progressive manner due to ongoing inflammatory processes that lead to activation of cortical and hippocampal neuronal cell death and neurodegeneration. Delayed activation of AT2R in the brain following ischemic stroke can decrease the severity of PSCI by enhancing cell survival and decreasing inflammation and oxidative stress, beyond the acute effect on the initial ischemic insult. (Figure 1.1)

The **specific aims** for this project were as follows:

Aim 1: Determine the role of hypertension in the development of cognitive impairment following experimental stroke by comparing normotensive and hypertensive animals.

We hypothesized that hypertension plays a key role in exacerbating PSCI by inducing inflammatory processes, causing hypertensive animals to be more susceptible to PSCI compared to normotensive animals. We planned to determine the temporal aspects of the development of PSCI by performing a battery of cognitive and motor tests over the course of 3 months. In addition, we planned to examine the gross morphological changes by using magnetic resonance imaging and histologic changes using markers of inflammation, cell death, astrogliosis, apoptosis and neurodegeneration in the cortex of affected animals by means of immunostaining and molecular testing.

Aim 2: Determine the role of AT2R activation with C21, initiated 3 days after ischemic stroke, in preventing the development of PSCI.

C21 is a non-peptide compound that has been found to act as a potent and selective small-molecule agonist of AT2R. It has shown a beneficial effect in animal models of cerebral ischemic damage, including age-related cognitive impairment and various forms of brain injury. We planned to evaluate the impact of C21 as an experimental treatment for PSCI by studying its effect on various cognitive

domains, with delayed administration in hypertensive animals, to investigate its cell survival promoting abilities, beyond its pro-angiogenic effects that reduce the severity of the initial insult. We proposed the inhibition of inflammation and oxidative-stress induced DNA damage as candidate mechanisms for its action.

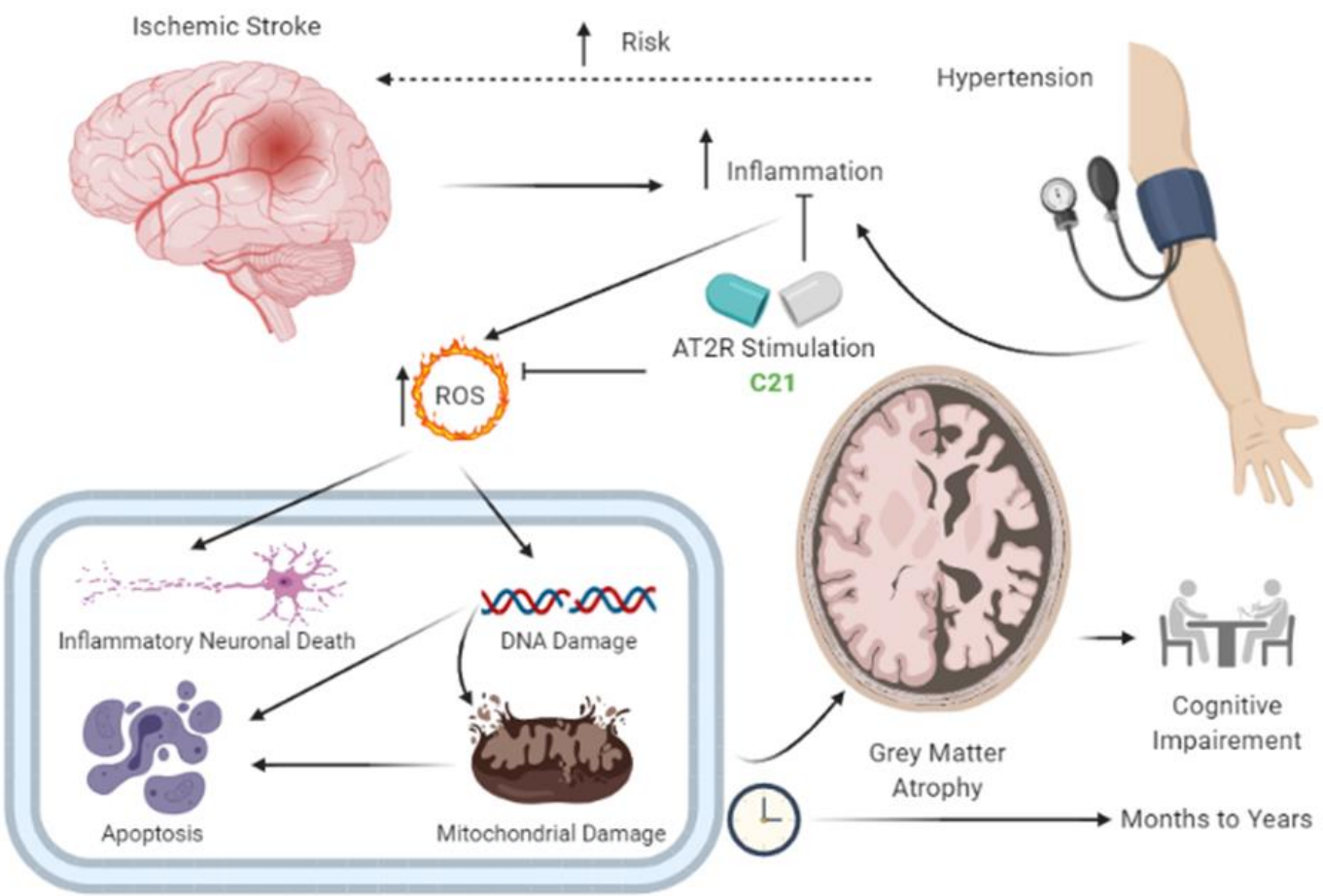
**Significance and Innovation:**

This project will have a significant positive impact on human health because it will provide a novel therapeutic strategy for addressing an under-studied aspect of stroke pathology. This will lead to decreased disability from brain ischemia and will benefit patients, their families and caregivers.

By the end of this project, we aim to have characterized the development of delayed post-stroke cognitive impairment in normotensive and hypertensive rats both temporally and mechanistically. This will allow future experiments to target the specific affected timepoints and cognitive functions in pharmacological evaluations. Furthermore, by building upon the previous work in our lab that proved that the beneficial effects of C21 in different models of cerebral ischemia were mediated through improved angiogenesis, we plan to establish C21 as a novel therapeutic candidate for the treatment of delayed post-stroke cognitive impairment in hypertensive animals as a stepping stone towards human studies.

**Figure (1.1): Hypothesis.** Ischemic stroke causes a marked inflammation in stroked animals. In hypertensive patients, who already suffer from an increase in ROS production, the ischemic insult and the oxidative stress result in chronic increase in DNA damage, mitochondrial damage and neuronal inflammatory cell death and apoptosis. The result of these processes is chronic neuronal loss in the form of grey matter atrophy and, subsequently, post stroke cognitive impairment. Activation of AT2R with C21 blocks these processes by reducing chronic inflammation and oxidative stress.

Fig. 1.1


Ischemic Stroke
Risk
Hypertension
Inflammation
AT2R Stimulation
C21
ROS
Inflammatory Neuronal Death
DNA Damage
Apoptosis
Mitochondrial Damage
Grey Matter Atrophy
Cognitive Impairement
Months to Years

## References


1. Benjamin, E.J., et al., *Heart Disease and Stroke Statistics-2017 Update: A Report From the American Heart Association.* Circulation, 2017. **135**(10): p. e146-e603.
2. Towfighi, A. and J.L. Saver, *Stroke declines from third to fourth leading cause of death in the United States: historical perspective and challenges ahead.* Stroke, 2011. **42**(8): p. 2351-5.
3. Sun, J.H., L. Tan, and J.T. Yu, *Post-stroke cognitive impairment: epidemiology, mechanisms and management.* Ann Transl Med, 2014. **2**(8): p. 80.
4. Woitzik, J., T. Back, and C. Thome, *Flow-dependent versus spreading-like impairment of brain tissue integrity during focal cerebral ischemia and its consequences for neuroprotective strategies.* Front Biosci, 2008. **13**: p. 1500-6.
5. Leys, D., et al., *Poststroke dementia.* Lancet Neurol, 2005. **4**(11): p. 752-9.
6. Pendlebury, S.T., et al., *Underestimation of cognitive impairment by Mini-Mental State Examination versus the Montreal Cognitive Assessment in patients with transient ischemic attack and stroke: a population-based study.* Stroke, 2010. **41**(6): p. 1290-3.
7. Bour, A., et al., *How predictive is the MMSE for cognitive performance after stroke?* J Neurol, 2010. **257**(4): p. 630-7.
8. Erkinjuntti, T., *Cerebrovascular Dementia.* CNS Drugs, 1999. **12**(1): p. 35-48.
9. Arba, F., et al., *Enlarged perivascular spaces and cognitive impairment after stroke and transient ischemic attack.* Int J Stroke, 2018. **13**(1): p. 47-56.

10. Desmond, D.W., et al., *Frequency and clinical determinants of dementia after ischemic stroke.* Neurology, 2000. **54**(5): p. 1124-31.

11. Jellinger, K.A., *The enigma of mixed dementia.* Alzheimers Dement, 2007. **3**(1): p. 40-53.

12. Delavaran, H., et al., *Cognitive function in stroke survivors: A 10-year follow-up study.* Acta Neurol Scand, 2017. **136**(3): p. 187-194.

13. Rouch, L., et al., *Antihypertensive drugs, prevention of cognitive decline and dementia: a systematic review of observational studies, randomized controlled trials and meta-analyses, with discussion of potential mechanisms.* CNS Drugs, 2015. **29**(2): p. 113-30.

14. Wiesmann, M., A.J. Kiliaan, and J.A. Claassen, *Vascular aspects of cognitive impairment and dementia.* J Cereb Blood Flow Metab, 2013. **33**(11): p. 1696-706.

15. Levine, D.A., et al., *Trajectory of Cognitive Decline After Incident Stroke.* Jama, 2015. **314**(1): p. 41-51.

16. Whitehead, S.N., et al., *Progressive increase in infarct size, neuroinflammation, and cognitive deficits in the presence of high levels of amyloid.* Stroke, 2007. **38**(12): p. 3245-50.

17. Jellinger, K.A., *The pathology of "vascular dementia": a critical update.* J Alzheimers Dis, 2008. **14**(1): p. 107-23.

18. Sahathevan, R., A. Brodtmann, and G.A. Donnan, *Dementia, stroke, and vascular risk factors; a review.* Int J Stroke, 2012. **7**(1): p. 61-73.

19. Hardy, J., *Amyloid, the presenilins and Alzheimer's disease.* Trends Neurosci, 1997. **20**(4): p. 154-9.

20. Riley, K.P., D.A. Snowdon, and W.R. Markesbery, *Alzheimer's neurofibrillary pathology and the spectrum of cognitive function: findings from the Nun Study.* Ann Neurol, 2002. **51**(5): p. 567-77.

21. Snowdon, D.A., et al., *Brain infarction and the clinical expression of Alzheimer disease. The Nun Study.* Jama, 1997. **277**(10): p. 813-7.

22. Tu, J.V., *Reducing the global burden of stroke: INTERSTROKE.* Lancet, 2010. **376**(9735): p. 74-5.

23. Lopez-Gil, X., et al., *DWI and complex brain network analysis predicts vascular cognitive impairment in spontaneous hypertensive rats undergoing executive function tests.* Front Aging Neurosci, 2014. **6**: p. 167.

24. Qiu, C., B. Winblad, and L. Fratiglioni, *The age-dependent relation of blood pressure to cognitive function and dementia.* Lancet Neurol, 2005. **4**(8): p. 487-99.

25. O'Rourke, M.F. and M.E. Safar, *Relationship between aortic stiffening and microvascular disease in brain and kidney: cause and logic of therapy.* Hypertension, 2005. **46**(1): p. 200-4.

26. Au, R., et al., *Association of white matter hyperintensity volume with decreased cognitive functioning: the Framingham Heart Study.* Arch Neurol, 2006. **63**(2): p. 246-50.

27. de Groot, J.C., et al., *Cerebral white matter lesions and subjective cognitive dysfunction: the Rotterdam Scan Study.* Neurology, 2001. **56**(11): p. 1539-45.

28. Jackson, L., et al., *Within the Brain: The Renin Angiotensin System.* International journal of molecular sciences, 2018. **19**(3): p. 876.

29. Mentz, R.J., et al., *The past, present and future of renin-angiotensin aldosterone system inhibition.* Int J Cardiol, 2013. **167**(5): p. 1677-87.

30. Fouda, A.Y., et al., *Renin-angiotensin system as a potential therapeutic target in stroke and retinopathy: experimental and clinical evidence.* Clin Sci (Lond), 2016. **130**(4): p. 221-38.

31. Paul, M., A. Poyan Mehr, and R. Kreutz, *Physiology of local renin-angiotensin systems.* Physiol Rev, 2006. **86**(3): p. 747-803.

32. Bodiga, V.L. and S. Bodiga, *Renin Angiotensin System in Cognitive Function and Dementia.* Asian Journal of Neuroscience, 2013. **2013**: p. 102602.

33. Grobe, J.L., D. Xu, and C.D. Sigmund, *An intracellular renin-angiotensin system in neurons: fact, hypothesis, or fantasy.* Physiology (Bethesda), 2008. **23**: p. 187-93.

34. Labandeira-Garcia, J.L., et al., *Brain Renin-Angiotensin System and Microglial Polarization: Implications for Aging and Neurodegeneration.* Front Aging Neurosci, 2017. **9**: p. 129.

35. Ahmed, H.A., et al., *Role of angiotensin system modulation on progression of cognitive impairment and brain MRI changes in aged hypertensive animals - A randomized double- blind pre-clinical study.* Behav Brain Res, 2018. **346**: p. 29-40.

36. Xia, H. and E. Lazartigues, *Angiotensin-converting enzyme 2 in the brain: properties and future directions.* J Neurochem, 2008. **107**(6): p. 1482-94.

37. Zhang, T.L., et al., *The neuroprotective effect of losartan through inhibiting AT1/ASK1/MKK4/JNK3 pathway following cerebral I/R in rat hippocampal CA1 region.* CNS Neurosci Ther, 2012. **18**(12): p. 981-7.

38. Lenkei, Z., et al., *Distribution of angiotensin II type-2 receptor (AT2) mRNA expression in the adult rat brain.* J Comp Neurol, 1996. **373**(3): p. 322-39.

39. Mateos, L., M.J. Perez-Alvarez, and F. Wandosell, *Angiotensin II type-2 receptor stimulation induces neuronal VEGF synthesis after cerebral ischemia.* Biochim Biophys Acta, 2016. **1862**(7): p. 1297-308.

40. Maul, B., et al., *Impaired spatial memory and altered dendritic spine morphology in angiotensin II type 2 receptor-deficient mice.* J Mol Med (Berl), 2008. **86**(5): p. 563-71.

41. Costa-Besada, M.A., et al., *Paracrine and Intracrine Angiotensin 1-7/Mas Receptor Axis in the Substantia Nigra of Rodents, Monkeys, and Humans.* Mol Neurobiol, 2018. **55**(7): p. 5847-5867.

42. AbdAlla, S., et al., *The angiotensin II AT2 receptor is an AT1 receptor antagonist.* J Biol Chem, 2001. **276**(43): p. 39721-6.

43. Jiang, T., et al., *ACE2-Ang-(1-7)-Mas Axis in Brain: A Potential Target for Prevention and Treatment of Ischemic Stroke.* Curr Neuropharmacol, 2013. **11**(2): p. 209-17.

44. Leonhardt, J., et al., *Evidence for Heterodimerization and Functional Interaction of the Angiotensin Type 2 Receptor and the Receptor MAS.* Hypertension, 2017. **69**(6): p. 1128-1135.

45. Barnes, J.M., et al., *Angiotensin-converting enzyme inhibition, angiotensin, and cognition.* J Cardiovasc Pharmacol, 1992. **19 Suppl 6**: p. S63-71.

46. Tota, S., et al., *Central angiotensin converting enzyme facilitates memory impairment in intracerebroventricular streptozotocin treated rats.* Behav Brain Res, 2012. **226**(1): p. 317-30.

47. Kumaran, D., et al., *Involvement of angiotensin converting enzyme in cerebral hypoperfusion induced anterograde memory impairment and cholinergic dysfunction in rats.* Neuroscience, 2008. **155**(3): p. 626-39.

48. Ishitsuka, K., et al., *High blood pressure after acute ischemic stroke is associated with poor clinical outcomes: Fukuoka Stroke Registry.* Hypertension, 2014. **63**(1): p. 54-60.

49. Jauch, E.C., et al., *Guidelines for the early management of patients with acute ischemic stroke: a guideline for healthcare professionals from the American Heart Association/American Stroke Association.* Stroke, 2013. **44**(3): p. 870-947.

50. Sandset, E.C., et al., *The angiotensin-receptor blocker candesartan for treatment of acute stroke (SCAST): a randomised, placebo-controlled, double-blind trial.* Lancet, 2011. **377**(9767): p. 741-50.

51. He, J., et al., *Effects of immediate blood pressure reduction on death and major disability in patients with acute ischemic stroke: the CATIS randomized clinical trial.* Jama, 2014. **311**(5): p. 479-89.

52. Nishimura, Y., T. Ito, and J.M. Saavedra, *Angiotensin II AT(1) blockade normalizes cerebrovascular autoregulation and reduces cerebral ischemia in spontaneously hypertensive rats.* Stroke, 2000. **31**(10): p. 2478-86.

53. Wilms, H., et al., *Neuroprotection with angiotensin receptor antagonists: a review of the evidence and potential mechanisms.* Am J Cardiovasc Drugs, 2005. **5**(4): p. 245-53.

54. Elewa, H.F., et al., *Blood pressure lowering after experimental cerebral ischemia provides neurovascular protection.* J Hypertens, 2007. **25**(4): p. 855-9.

55. Chen, J., et al., *Atorvastatin induction of VEGF and BDNF promotes brain plasticity after stroke in mice.* J Cereb Blood Flow Metab, 2005. **25**(2): p. 281-90.

56. Morris, D.C., et al., *Thymosin beta4: a candidate for treatment of stroke?* Ann N Y Acad Sci, 2010. **1194**: p. 112-7.

57. Cui, X., et al., *The neurorestorative benefit of GW3965 treatment of stroke in mice.* Stroke, 2013. **44**(1): p. 153-61.

58. Ishrat, T., et al., *Low-dose candesartan enhances molecular mediators of neuroplasticity and subsequent functional recovery after ischemic stroke in rats.* Mol Neurobiol, 2015. **51**(3): p. 1542-53.

59. Soliman, S., et al., *Candesartan induces a prolonged proangiogenic effect and augments endothelium-mediated neuroprotection after oxygen and glucose deprivation: role of vascular endothelial growth factors A and B.* J Pharmacol Exp Ther, 2014. **349**(3): p. 444-57.

60. Alhusban, A., et al., *AT1 receptor antagonism is proangiogenic in the brain: BDNF a novel mediator.* J Pharmacol Exp Ther, 2013. **344**(2): p. 348-59.

61. Guimond, M.O. and N. Gallo-Payet, *How does angiotensin AT(2) receptor activation help neuronal differentiation and improve neuronal pathological situations?* Front Endocrinol (Lausanne), 2012. **3**: p. 164.

62. Villar-Cheda, B., et al., *Nigral and striatal regulation of angiotensin receptor expression by dopamine and angiotensin in rodents: implications for progression of Parkinson's disease.* Eur J Neurosci, 2010. **32**(10): p. 1695-706.

63. Steckelings, U.M., et al., *AT2 receptor agonists: hypertension and beyond.* Current opinion in nephrology and hypertension, 2012. **21**(2): p. 142-146.

64. Pandey, A. and A.B. Gaikwad, *AT(2) receptor agonist Compound 21: A silver lining for diabetic nephropathy.* Eur J Pharmacol, 2017. **815**: p. 251-257.

65. Bosnyak, S., et al., *Relative affinity of angiotensin peptides and novel ligands at AT1 and AT2 receptors.* Clinical science, 2011. **121**(7): p. 297-303.

66. Wan, Y., et al., *Design, synthesis, and biological evaluation of the first selective nonpeptide AT2 receptor agonist.* Journal of medicinal chemistry, 2004. **47**(24): p. 5995-6008.

67. Bruce, E., et al., *Selective activation of angiotensin AT 2 receptors attenuates progression of pulmonary hypertension and inhibits cardiopulmonary fibrosis.* British Journal of Pharmacology, 2015. **172**(9): p. 2219-2231.

68. Rathinasabapathy, A., et al., *C21, a nonpeptide angiotensin II type 2 receptor agonist attenuates lung fibrosis and associated cardiac dysfunction*. 2015, Eur Respiratory Soc.

69. Chow, B.S. and T.J. Allen, *Angiotensin II type 2 receptor (AT2R) in renal and cardiovascular disease.* Clinical Science, 2016. **130**(15): p. 1307-1326.

70. McCarthy, C.A., et al., *Angiotensin II type 2 receptor stimulation initiated after stroke causes neuroprotection in conscious rats.* Hypertension, 2012. **60**(6): p. 1531-1537.

71. Rehman, A., et al., *Angiotensin type 2 receptor agonist compound 21 reduces vascular injury and myocardial fibrosis in stroke-prone spontaneously hypertensive rats.* Hypertension, 2012. **59**(2): p. 291-299.

72. Alhusban, A., et al., *Compound 21 is pro-angiogenic in the brain and results in sustained recovery after ischemic stroke.* J Hypertens, 2015. **33**(1): p. 170-80.

73. Fouda, A.Y., et al., *Role of interleukin-10 in the neuroprotective effect of the Angiotensin Type 2 Receptor agonist, compound 21, after ischemia/reperfusion injury.* Eur J Pharmacol, 2017. **799**: p. 128-134.

74. Eldahshan, W., et al., *Angiotensin II type 2 receptor stimulation with compound 21 improves neurological function after stroke in female rats: a pilot study.* Am J Physiol Heart Circ Physiol, 2019. **316**(5): p. H1192-h1201.

75. Ishrat, T., et al., *Dose-response, therapeutic time-window and tPA-combinatorial efficacy of compound 21: A randomized, blinded preclinical trial in a rat model of thromboembolic stroke.* J Cereb Blood Flow Metab, 2019. **39**(8): p. 1635-1647.

76. Ahmed, H.A., et al., *RAS modulation prevents progressive cognitive impairment after experimental stroke: a randomized, blinded preclinical trial.* Journal of neuroinflammation, 2018. **15**(1): p. 229-229.

77. Ahmed, H.A., et al., *Angiotensin receptor (AT2R) agonist C21 prevents cognitive decline after permanent stroke in aged animals-A randomized double- blind pre-clinical study.* Behav Brain Res, 2019. **359**: p. 560-569.

78. Jackson, L., et al., *Delayed Administration of Angiotensin II Type 2 Receptor (AT2R) Agonist Compound 21 Prevents the Development of Post-stroke Cognitive*

*Impairment in Diabetes Through the Modulation of Microglia Polarization.*

Transl Stroke Res, 2019.

# CHAPTER 2

# STROKE PROMOTES THE DEVELOPMENT OF BRAIN ATROPHY AND DELAYED CELL DEATH IN HYPERTENSIVE RATS: RELEVANCE TO COGNITIVE AND PSYCHOLOGICAL OUTCOMES[1]

[1] Sayed MA, Eldahshan W, Abdelbary A, Pillai B, Althomali W, Johnson MH, Arbab AS, Ergul A and Fagan SC. Submitted to [Scientific Reports], [April 30, 2020]

**Stroke promotes the development of brain atrophy and delayed cell death in hypertensive rats: relevance to cognitive and psychological outcomes**

Mohammed A. Sayed[1,2], Wael Eldahshan[1,2], Mahmoud Abdelbary[3], Bindu Pillai[1,2], Waleed Althomali[1,2], Maribeth H. Johnson[4], Ali S. Arbab[5], Adviye Ergul[6,7], and Susan C. Fagan* [1,2]

[1]Clinical and Experimental Therapeutics, College of Pharmacy, University of Georgia, Augusta, GA; [2]Charlie Norwood VA Medical Center, Augusta, GA; [3]Department of Physiology, Medical College of Georgia, Augusta, GA; [4]Department of Neuroscience and Regenerative Medicine, Augusta, GA; [5]Georgia Cancer Center, Augusta, GA; [6]Department of Pathology and Laboratory Medicine, Medical University of South Carolina, Charleston, SC; and [7]Ralph H. Johnson VA Medical Center, Charleston, SC

**Corresponding Author: Susan C. Fagan, Pharm.D., FAHA**

914 New Baillie Street, HM Building Room 116, Augusta, GA 30901

Tel.: 706-721-0130 Email: sfagan@uga.edu

**Abstract**

Post-stroke cognitive impairment (PSCI) is a major source of disability, affecting up to two thirds of stroke survivors with no available therapeutic options. The condition remains understudied in preclinical models due to its delayed presentation. Although hypertension is a leading risk factor for dementia, how ischemic stroke contributes to this neurodegenerative condition is unknown. In this study, we used a model of hypertension to study the development of PSCI and its mechanisms.

Spontaneously hypertensive rats (SHR) were compared to normotensive rats and were subjected to 1-hour middle cerebral artery occlusion or sham surgery. Novel object recognition, passive avoidance test and Morris water maze were used to assess cognition. In addition, brain magnetic resonance images were obtained 12-weeks post-stroke and tissue was collected for immunohistochemistry and protein quantification.

Stroked animals developed impairment in long-term memory at 4-weeks post-stroke despite recovery from motor deficits, with hypertensive animals showing some symptoms of anhedonia. Stroked SHRs displayed grey matter atrophy and had a two-fold increase in apoptosis in the ischemic borderzone and increased markers of inflammatory cell death and DNA damage at 12 weeks post-stroke. This indicates that preexisting hypertension exacerbates the development of secondary neurodegeneration after stroke beyond its acute effects on

neurovascular injury.

**Key Words:** Stroke, Hypertension, Cognitive Impairment, Neuroinflammation

## Introduction

Stroke has recently become the fifth leading cause of death in the United States.[1] While stroke mortality has been steadily declining over the last decade due to the continuous improvement in health care standards,[2] the number of stroke survivors with residual disability is steadily increasing.[3] Ischemic stroke is a condition characterized by an initial ischemic event that deprives brain tissue from blood supply and oxygenation, that is sometimes followed by reperfusion, leading to irreversible brain damage and subsequent motor and cognitive impairment.[4]

Post-stroke cognitive impairment (PSCI) is a condition that affects up to two-thirds of patients following ischemic stroke, with up to one third eventually developing dementia.[5, 6] Although PSCI is highly prevalent among stroke survivors, there is evidence suggesting that the criteria for diagnosis may underestimate the frequency of dementia and cognitive decline among stroke survivors.[7, 8] While it was traditionally thought that the cognitive impairment results from the recurrence of ischemic insults, newer evidence suggests that a very substantial portion of this impairment results from neuronal pathogenesis.[9] Published data from a large, NIH-funded, epidemiologic trial showed that patients, in addition to acute changes, suffer from a slowly progressive cognitive decline after a single-stroke lesion.[10, 11] This continuous deterioration occurs even in the absence of any evidence of new ischemic injuries.[12, 13]

Diagnosis and characterization of clinically apparent PSCI has proven to be a challenging task, owing to the heterogeneity of the condition itself. The incidence

of PSCI and its severity depends largely upon the morphology of the vascular injury (focal or multifocal; large or small vessel), volume of brain tissue affected by ischemia and, most importantly, the location and number of lesions. [14] Histopathological studies have shown a clear link between the vascular aspects of brain injuries affecting cognition and the neurodegenerative aspects that resemble Alzheimer's disease pathology.[15]

Hypertension is the most commonly occurring modifiable risk-factor for stroke worldwide and is being increasingly recognized as a risk factor for the development of PSCI.[15, 16] Chronic hypertension, particularly midlife high blood pressure (BP), has been associated with an increased risk for cognitive decline, vascular dementia and Alzheimer's disease.[17] One of the mechanisms by which hypertension is believed to contribute to the development of cognitive impairment is exposing the cerebral microvasculature to pulsatile pressure causing tearing of the brain vascular endothelium and smooth muscle cells leading to lipohyalinosis and fibrinoid necrosis.[18] Clinical studies on hypertensive patients have shown that acute disruption of blood perfusion can lead to the formation of lacunar infarcts, while chronic ischemia can lead to the development of white matter lesions which are associated with the development of cognitive impairment. [19, 20]

In this study, we aimed to determine the role of hypertension in the development of cognitive impairment following experimental stroke in rats by comparing normotensive (Wistar) rats to spontaneously hypertensive rats (SHR). We

hypothesized that hypertension plays a key role in exacerbating PSCI by inducing neurodegenerative processes, causing SHRs to be more susceptible to PSCI compared to normotensive animals.

## Methods

The data that support the findings of this study are available from the corresponding author on reasonable request. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Charlie Norwood VA Medical Center, Augusta, Georgia. Overall experimental design can be found in (Figure 2.1-A).

## Data Analysis

Statistical analyses were performed using SAS version 9.4 (SAS Institute, Inc, Cary, NC) by a biostatistician (Johnson MH). All data are presented as means ±SEM. Unless otherwise mentioned, all results presented were analyzed by 2-way ANOVA (2 Strain (Wistar vs. SHR) X 2 Surgery (sham vs. stroke)), with p-values for the tests presented as tables on the graphs. Letters in the graphs represent the results of Tukey post-hoc multiple comparisons tests for significant ANOVA results where pairs of means with different letters are significantly different. Otherwise, comparisons of two groups were analyzed by student t-test for parametric values,

and Wilcoxon's signed rank test for non-parametric values. Results are considered significant at a Type I error rate of 5%.

**Experimental Animals**

Both male Wistar and SHR animals were purchased from Charles River (Wilmington, MA) and were housed (1 rat per cage) in pathogen free, temperature-controlled facility (24 ±1 C; 12-12-hour light-dark cycle) with access to standard chow and water ad libitum.

**Transient Middle Cerebral Artery Occlusion (tMCAO) surgery**

Male Wistar and SHR rats (age: 10-14 weeks) (body weight: 320-400 g) were subjected to 60-min of tMCAO using 4–0 silicon coated nylon suture (Doccol 403756)) as previously described [22]. The original source of method description is our previously published work, Eldahshan et al. [21] Briefly, the animals were anesthetized using 2-5% isoflurane, a ventral mid-line neck incision was made, the right common carotid artery (CCA) was exposed, and the external carotid artery (ECA) was ligated and cut. The suture was advanced from a small nick at the ECA into the internal carotid artery (ICA) until a mild resistance was encountered, indicating the branching of the anterior and middle cerebral artery. The suture was tied in place for the duration of the occlusion and the animals were allowed to recover from anesthesia. Several minutes before the end of the occlusion time, the

animals were re-anesthetized, the suture was removed for reperfusion and the small nick at the ECA was permanently ligated. In sham surgeries, the CCA was isolated and manipulated without cutting or insertion of the suture and the skin was closed.

We defined a failed MCAO surgery as: “Insertion of the suture that did not succeed in producing an ischemic damage as evident from the lack of motor deficits after reperfusion, possibly due to an incomplete occlusion of the origin of the MCA”.

**Randomization and Blinding**

Animals were randomized to either the sham or stroke group using a stratified block randomization method, in which animals were arranged into strata based on body weight, with each stratum being divided equally between groups, to achieve an equal distribution of body weight (sham vs. stroke) within strain, before surgery. All behavioral, histochemical and molecular assessments and analyses were performed by blinded investigators. Animals were assigned numbers 1-50 after randomization, with their strain and surgery unknown to all investigators except the surgeon. Animals were assigned to groups A-D based on strain and surgery, and group assignments were revealed to the investigators after the conclusion of the tests and analyses.

### Inclusion and Exclusion

To ensure the success of the surgery, animals were tested for unilateral paresis immediately following reperfusion to exclude any animals that did not show a significant deficit. No animals were excluded for failed MCAO. Animals that showed spontaneous recovery at 24 hours post-surgery were excluded. Our inclusion criteria were: (1) At least 5% loss in body weight at 24 hours after stroke and (2) a score of 6 out of 8 or less on a 4-task neurological score based on the Bederson test. Animals that failed to satisfy one of these criteria at 24 hours post-stroke were deemed spontaneously recovered.

### Assessment of Functional Outcome

### Body Weight

As we described in our previously published work Ahmed et al. [22], weight monitoring is an extremely important tool that serves as an independent and unambiguous measure of an animal's overall health and welfare, specifically after stroke. For our studies, animals were weighed before surgery and then daily after stroke for the first 14 days, then once a week until the day of sacrifice. All animals selected for the study were in the range of 300-400 grams body weight at baseline.

## Neurobehavioral Testing

All neurobehavioral tests were conducted, recorded, and analyzed in a blinded manner.

## Sensorimotor Testing

To assess sensorimotor function, animals underwent an 8-point modified neurological assessment modified from the Bederson protocol [23] at days 1, 3, 5, 7 and 14 post surgery. Furthermore, motor recovery was assessed via measuring the locomotor activity during the open field, Y-maze and Morris water maze (MWM) tests.

## Modified Bederson Score

Animals were assessed neurologically on an 8-point scale measuring 4 basic functions (spontaneous rotation, resistance to lateral push and fore and back paw flexion) with a score of 2 points awarded in each category for an animal exhibiting a natural response, a score of 1 point for mildly impaired animals and a score of 0 for strongly impaired animals. Higher scores indicate better performance with a maximum possible score of 8/8 and a minimum possible score of 0/8.

## Cognitive Testing

Cognitive tests were performed according to the design in (Figure 2.1). Special consideration was taken for cognitive tests to allow sufficient period of time to prevent different tests from affecting one another. Open Field, Y-maze and Novel Object Recognition (NOR) were allowed to be performed on the same days as these tests were of minimal invasiveness, measuring the spontaneous activity of the animals. However, invasive tests such as passive avoidance (PAT) and exhausting tests such as the MWM were done individually. The NOR was performed to evaluate non-spatial working memory [24-27], while the passive avoidance test (PAT) assessed associative learning and reference memory. [28, 29]

## The Novel Object Recognition (NOR) test

The original source of method description is our previously published work, Ahmed et al.[22] The NOR test was performed to evaluate non-spatial working memory related to frontal-subcortical circuits. This test was based on the spontaneous tendency of animals to interact with a novel object more than a familiar one and consisted of 2 trials separated by a retention period. On the designated test day, animals were first subjected to an acquisition/sample trial, where the animal is presented with 2 identical (sample) objects and allowed to explore for 15 min. Following sample object exposure, the animal was returned to its home cage for a

1-h retention period. The 2nd preference trial/test session (5 min), which follows the retention period, was conducted in the same manner as the 1st trial, except that a new/novel object replaces one of the familiar/sample objects. The arena and objects were cleaned after each session with 70% ethanol. The time spent in exploring each object during the preference trial/test session was recorded and the discrimination index, which is the difference in exploration time for the objects divided by total time of exploration, was calculated. The discrimination index (DI) and the recognition index (RI), which is the time spent exploring the novel object relative to the total time of exploration, were taken as indicators of working memory.

Discrimination index (DI) = (TN − TF)/(TN + TF)

Recognition index (RI) = TN/(TN + TF)

- Time spent interacting with the familiar object (TF)
- Time spent interacting with the novel object (TN)

The required exploratory criteria was that animals should spend between 20-80% of the time exploring the objects out of the 5 min. Objects used were chosen as previously described.[30] Briefly, objects used were unified between animals and chosen according to recommendations of Heyser and Chemero in that they were symmetrical and transparent, and made of odorless, durable, and easy to clean plastic and glass.

**The Passive Avoidance Test (PAT)**

The passive avoidance test was used to assess aversive associative learning and related reference memory. The original source of method description is our previously published work, Ahmed et al.[22] For this test, one of the compartments of a Y-maze was equipped with a metal floor connected to an electric circuit box, adjusted to deliver brief, moderate intensity electric shocks (3 s duration, 0.75 mA). For the acquisition trial, the shock compartment/arm was blocked, and the animal placed in one of the “safe” arms and allowed 10 min to explore the 2 open arms. Upon completion of 10 min, the door blocking the shock arm was opened allowing the animal to enter. Once the animal had fully entered the shock arm, its initial latency was recorded, and it received a brief electric shock before being returned to its cage. After a 72-h retention period, the test trial was conducted. This was performed in a manner similar to that of the acquisition trial except that the foot shock was omitted, and all 3 arms were accessible to the animal from the start. The difference, between training and test sessions, the latency to enter the shock arm was used as a measure of retention. This latency was recorded for up to 300 s, as the index of long-term aversive associative memory consolidation.

**The Morris Water Maze (MWM)**

The MWM test was used to assess spatial learning and long-term memory. The original source of method description is our previously published work, Ahmed et

al.[23] All water maze tests were conducted in a large circular pool of water, 120cm in diameter, 55cm height, filled to a depth of 35 ± 1 cm with water at 25 ± 2 ◦C. This was separated into quadrants designated northeast (NE), northwest (NW), southeast (SE) and south-west (SW), based on the 4 equally spaced cardinal points N (North), S (South), E (East), and W (West) around the edge of the pool. One of these quadrants contained a transparent escape platform (10.5 cm diameter), submerged 1.5 cm below the water surface and obscured from view. Visual extra-maze cues were mounted to aid spatial navigation.

**MWM Training/Learning Sessions**

The initial training consisted of a single daily session of 8 trials (60 s each) for the first day, followed by a daily session of 4 trials per day for 3 consecutive days, for a total of 20 training trials. Each trial consisted of releasing the rat into the water from 1 of the 4 starting locations and allowing it to find the platform. If they did not reach the platform within 60 s, they were gently guided to it and kept there for 10 s, then removed. Trials were spaced at least 1 minute apart. All trials were recorded, and video tracked by the computerized tracking system Etho-Vision XT 7 (Noldus, Leesburg, VA, USA). This automated system monitored animals' swim patterns and calculated mean escape latency (s), total distance travelled to target (cm), and velocity to target (cm/s). Data from all training sessions were pooled for each individual animal, evaluated and compared between groups at the different time points.

**MWM Spatial Reference Memory Test**

Spatial reference memory was assessed with a probe test 24 h after the last daily session. For this test, all procedures were kept the same as during training, except that the platform was removed, and rats were allowed to swim for 60 s in an attempt to find it. Performance was evaluated by measuring time spent in the target quadrant/zones, proximity to the target location, and initial latency to the target zone. The target zone was centered on the platform location and was 3 times bigger.

**Magnetic Resonance Imaging (MRI)**

Method description is adapted, with changes, from our previously published work, Ahmed et al.[24] To determine the changes in the brain, ventricular volume, and the presence of white matter hyperintensities, animals underwent T2 -weighted and fluid attenuated inversion recovery (FLAIR), 8 weeks after MCAO. This was performed using a horizontal 7.0 T BioSpec MRI spectrometer (Bruker Instruments, Billerica, MA) equipped with an 8.9-cm micro imaging gradient insert (100 G/cm. All T2-weighted MRI and FLAIR images were obtained at Augusta University by the Core Imaging Facility for Small Animals (CIFSA). All MRI images were registered DICOM sequences, analyzed using FIJI. Total brain volume as well as hemispheric and ventricular volumes (regions of interest), were determined

on binarized sequences obtained by thresholding. For each region of interest, the volumes were calculated by adding the areas measured on each slice (11 slices total) and multiplying it by the slice thickness (1 mm in all cases).

**Animal Sacrifice and Tissue Collection**

The original source of method description is our previously published work, Ahmed et al.[22] At week 12, animals were anesthetized with IP ketamine/xylazine and transcardially perfused with 300 ml of PBS. Animals were decapitated, and their brains collected. Brains were sliced into 2 mm coronal sections, using a glass slicer matrix (Braintree Scientific, Braintree, MA, USA) and sections were labeled from A to F, anterior to posterior. Sections A and B, from brain matrix, were snap frozen and kept for molecular testing. The remaining brain tissue was immersed in 10% formalin (Fischer Scientific, Waltham, MA, USA) for 48 h and then transferred to a 30% sucrose solution until taken for frozen sectioning.

**Immunohistochemistry (IHC)**

Frozen brain sections (5 μm thick) were processed and stained following a standard technique. Method description is adapted from our previously published work, Jackson et al.[25] Briefly, sections were washed in PBS plus 0.03%/Triton X-100 with gentle agitation, blocked for 2 h in 10% normal goat serum/1% BSA, and incubated overnight at 4 °C with anti-IBA-1 (Ionized calcium-binding adaptor

molecule 1, 1:500, Wako, Japan) and anti-GFAP (Glial fibrillary acidic protein, 1:500, Sigma-Aldrich, Burlington, MA). After washing, slides were incubated with their appropriate fluorophore-conjugated (Texas Red, Alexa 488) secondary antibodies (1:1000; Abcam) for 1 h at room temperature and washed and cover-slipped with Fluoroshield mounting medium with DAPI (Millipore-Sigma, Burlington, MA). Imaging was performed using the Keyence Microscope (Itasca, IL). 20x magnification images were obtained from the ischemic border zone (penumbra) of the stroked animals and compared to the cortex of the sham animals. Images were analyzed by using the ImageJ software (NIH).

## Results

### Hypertension increases stroke mortality and worsens stroke outcome

As SHRs are known to develop hypertension over the course of the first few months of their life, it was important to make sure that our animals had already developed hypertension at the time of stroke (10-12 weeks of age). For this purpose, we assessed the mean arterial blood pressure of a cohort of 6 male SHR animals over the course of 2 weeks via telemetry. All of the animals developed elevated mean arterial blood pressure with an average ranging between 147-150 mm Hg compared to a normal expected value of 96.5±10.7 for Wistar rats under normothermia.[26] (Figure 2.1B).

Next, we evaluated the impact of ischemic stroke. Based on preset criteria (see Methods), 12 out of 19 animals from the Wistar group spontaneously recovered at 24 hours while there was no spontaneous recovery in the SHR group ($P<0.0001$, Fisher's exact test) (Figure 2.1-C).

Animals were weighed regularly over the course of the study to assess their recovery after the surgery. Animals from both strains started regaining weight 3 days after stroke, with Wistar animals continuing to gain weight until the end of the study, while SHR animals plateaued at an average of less than 400 grams. (Figure 2.1-D and 2.1-E). Three animals from the Stroke SHR group were sacrificed prior to the completion of the study due to excessive weight loss (>30%), in accordance with the study protocol. Survival curves are displayed in (Supp. Fig. 2.1). ($P=0.03$, Log-rank test for trend).

To assess motor recovery, animals were assessed neurologically, in a blinded manner, on a modified Bederson score, as described in the methods. Based on this assessment, both strains of animals recovered to near their baseline during the course of the first 14 days of follow-up. (Figure 2.2-A). All animals were able to ambulate freely. Animals were assessed at the end of week 1 post-stroke using the open field test, without significant differences from sham (Supp. Fig. 2.2).

**Hypertension does not affect working memory in the Novel Object Recognition test**

Short-term memory was assessed using the novel object recognition test (NOR). Animals that spent less than 20% of the total time exploring the objects, had their trials excluded from the results (0 trials). Neither Wistar nor SHR animals showed a significant impairment in recognizing the novel object at weeks 2 or 12 post-stroke. (Figure 2.2-B). Total exploration time was reduced in SHR animals (Figure 2.2-C) but it did not achieve significance.

**Hypertension induced depressive symptoms without affecting memory and learning in Morris Water Maze**

To assess the spatial learning and memory, animals were tested using the Morris Water Maze at week 4. All groups showed a significant learning behavior ($P<0.0001$) (Figure 2.3-A) and similar average swim speeds over the course of the experiment (Figure 2.3-B). There was no significant difference between the four groups during the probe-trial in the time spent in the platform zone or the time spent in the target quadrant, indicating an intact memory function. (Figure 2.3-C,D) Despite this, SHR animals exhibited a significant reduction in the total distance traveled during the probe test, regardless of the surgery, indicating a significantly impaired exploratory behavior, a sign of “behavioral despair” and depression [33] (Figure 2.3-E).

**Stroke induces long-term memory impairment in both hypertensive and normotensive animals**

Long-term memory was assessed using the passive avoidance test starting at week 4 post-stroke. Both strains of stroked animals showed a significant reduction in their latency to enter the shock arm, 3 days after receiving the shock, indicating an impairment in their avoidance-driven long-term memory ($p=0.0054$). Animals that entered the shock arm immediately after being introduced into the arena (< 30 sec latency) had their trials excluded from the results of this study (1 animal) (Figure 2.3-F). Since this test has a pronounced learning effect, it was not repeated.

**Stroke accelerates grey matter atrophy in hypertensive animals**

The animals were assessed via T2-weighted MRI on the brain at 12 weeks post-stroke to assess brain morphological changes (Figure 2.4-A). Due to small numbers in the Wistar group, the larger ischemic infarct size in the SHR animals did not achieve statistical significance. (Figure 2.4-B). The total volume of lateral ventricles, an indicator of cerebral atrophy, was significantly larger in SHR animals compared to Wistars, and this was more prominent in the stroked animals. (Figure

2.4-C) Interestingly, this difference was not apparent when comparing the size of the ipsilesional ventricles alone (Figure 2.4-D), where both strains showed a marked enlargement over their sham counterparts. This indicates that the loss of tissue in SHR animals is not confined to the stroked hemisphere, but also affects the contralesional hemisphere in a significant way. In both strains, as expected, the ipsilesional hemisphere was significantly atrophied compared to the contralesional one. (Figure 2.4-E).

We performed an MRI scan on all the excluded animals and found that only 1 out of the 12 excluded animals showed signs of a very minor ischemic damage in the brain, which added to our confidence in the validity of our exclusion criteria. (data not shown).

**Hypertension contributed to heightened apoptosis in the ischemic borderzone at 12 weeks following stroke.**

Quantification of TUNEL staining in the ischemic borderzone, showed increased apoptosis in SHR animals compared to Wistars (Figure 2.5-D), and both stroke groups exhibited a marked increase in apoptosis compared to their sham counterparts. Stroke exacerbated the effect of hypertension significantly when comparing the stroked SHRs to the stroked Wistars (Figure 2.5-B).

IBA1 is a marker of microglia/macrophage activation. To assess whether the increase in apoptosis was associated with increased inflammation, IBA1 positive

cells from the ischemic borderzone were quantified (Figure 2.5-E). Even 12 weeks after stroke, there was ongoing inflammation, IBA1 positive cells, in both hypertensive and normotensive animals to a similar extent (Figure 2.5-C). No significant difference was found in the levels of the pro-inflammatory cytokines IL1B and TNFa between groups using ELISA on the whole-brain homogenate of the ipsilesional brain hemisphere. (Supp. Fig. 3)

**Hypertension increases the late expression of markers of DNA damage and cell death after stroke, which are not increased in normotensive animals.**

Pharmacological poly(ADP-ribose)polymerase-1 (PARP1) is a marker of DNA damage that is associated with increased inflammation and the accumulation of reactive oxygen species. [27-29]. We found that stroke caused a significant increase in the expression of PARP1 in the brain homogenate of SHRs, an effect that was not present in normotensive animals (Figure 2.6-A). There was no difference in the expression level of cleaved (inactive) PARP1 (data not shown).

In SHRs, increased High mobility group box 1 protein (HMGB1) expression has been shown to be associated with neuronal damage, increased cell death and increased inflammation.[30] We found that stroke significantly increased the expression of HMGB1 in the brain homogenates of SHRs compared to shams, but not in normotensive animals (Figure 2.6-B).

Matrix metalloproteinase 9 (MMP9) is a member of the zinc-metalloproteinases family involved in the degradation of the extracellular matrix. In this study, hypertension was associated with increased expression of the cleaved active forms of MMP9 at 12 weeks after stroke, but this was not the case in normotensive animals. Furthermore, stroked SHRs displayed a significant increase in the expression of activated MMP9 compared to their sham counterparts, showing that this was not due to hypertension alone. (Figure 2.6-C and 6-D).

**Discussion**

Post-stroke cognitive and psychological problems are one of the most prevalent yet understudied aspects of stroke-related disability in human patients. In order to be able to develop treatments for PSCI, it is important to develop reliable animal models suitable for translational studies. Although numerous studies investigated the effects of stroke and MCAO on the development of post-stroke cognitive impairment,[31, 32] most studies used relatively healthy animals and concluded by 30 days post-stroke. In humans, most patients suffer from comorbid diseases like hypertension and diabetes and although maximum motor recovery is usually achieved within 6 months to one year after stroke, stroke survivors display increased incidence of cognitive impairment and dementia for decades after the initial event.[33] We now know that this is likely due to long term progressive

neurodegeneration, which is exacerbated by comorbidities.[12] The deleterious effect of hypertension on the development of cognitive impairment, even in the absence of stroke is well established.[18] However, it is usually studied in the context of assessing the effect of blood-pressure lowering medications on preventing these deleterious effects. Our study is one of the very few studies to compare normotensive and SHRs, with and without stroke, in order to determine the extent of contribution of hypertension and stroke to the development of cognitive impairment. Hypertension is known to worsen stroke outcome and increase mortality acutely following MCAO. [34, 35] In this study, we started by measuring the mean arterial blood pressure of a separate cohort of animals, of the same weight and age to our main SHR cohort, using telemetry, to prove that the animals had already developed hypertension before the initiation of the MCAO. This was done because the stress generated by telemetry was expected to impact the behavioral data. Although our lab has extensive experience working on both normotensive and hypertensive animals, comparing Wistar and SHR animals directly in a head-to-head fashion presented a unique challenge. Although a 90-min MCAO was found to reliably produce cognitive deficits in Wistar rats, it resulted in a high rate of mortality in SHRs, and while 60-min MCAO produced very little mortality in SHRs, Wistars had a high rate of spontaneous, 24-hour recovery. We elected to use the 60-min MCAO to reduce animal mortality, excluding spontaneously recovering Wistar animals from further analysis. Wistar rats were selected for this comparison instead of Wistar Kyoto, as Wistar Kyoto animals are known to have intrinsic problems in neurobehavior, regardless of hypertension.[36]

The choice of our timepoints for cognitive assessment was made taking into consideration the suitability of those behavioral tests for repetition, as well as the effect of the different tests on each other. For example, with more repetition we found that animals lost interest in exploring the objects in the NOR, which prompted us to retire the test from week 3 to week 12. Even with this long break, overall object exploration was decreased for all animals at week 12. Similarly, we found that the PAT and the MWM produced a great physical and psychological stress for the animals, thus potentially affecting other behavioral tests that could have been done in the same time period. Therefore, no other tests were administered concurrently with the PAT and MWM.

Our main hypothesis was that hypertension would exacerbate the deleterious effects of MCAO on memory and learning, based on our previously published findings in SHRs. [22, 24] Both SHRs and Wistars in this study developed impairments in learning and memory at 4 weeks after stroke, as demonstrated in the passive avoidance task. We were unable to demonstrate deficits due to stroke in NOR task in either strain, however, when tested at 3 and 12 weeks after stroke. Our previous investigation revealed profound deficits in NOR at 30 days in a similarly young SHR cohort.[22] Whether our failure to replicate that finding was due to the differences in timing of the testing (3 and 12 weeks vs. 30 days) or the experimental set-up remains unclear. It is also possible that depression-like behavior animals develop has a big impact on NOR and water maze tests which limits our ability to detect cognitive deficits. Additional tests designed to evaluate

the depression-like behavior need to be incorporated into future studies that monitor long term outcomes. While our previous study did not include a Wistar comparison group,[22] in the current study the PAT findings remained robust, assuring us that both strains developed PSCI. It is worth noting that our animals were housed individually following MCAO due to concerns that housing the animals in pairs would be dangerous due to the wounds present, and concerns regarding post-stroke feeding competition and recovery. We consider this to be one of the limitations of our study as social isolation is known to be an influencing factor on animal behavior in cognitive tests.[37] However, the differences in behavior we found between the experimental groups were all among animals of the same housing conditions, leading to our conclusion that the behavioral differences observed were a result of strain differences rather than the housing conditions.

This study is one of the first studies to establish the ongoing deleterious long-term effects of hypertension on recovery of the stroked brain. Although others have reported changes over time after stroke, they failed to include sham groups, so the effects of hypertension alone could not be teased out. [38, 39] It is known that SHR animals develop enlarged brain ventricles over time,[40] however in our study, the ventricular volume was not significantly different between the Wistar and SHR sham groups, which points to the fact that our SHR animals have not yet spontaneously developed larger ventricles at the time of the study. Similarly, the increase in the ventricular size in the ipsilateral hemisphere was similar in both

strains. We found that SHRs had a significant increase in the total ventricular size of both hemispheres, which did not disappear after standardizing on the ventricle size of the strain using the sham animals. We found that SHR animals had profound brain atrophy, in both hemispheres, at 12-weeks post-stroke, when compared to normotensive animals and even to sham-operated SHRs. This indicates that hypertension prolongs the ongoing neurogenic damage of stroke, and this continues to the late time-points. Our findings point to SHR animals being uniquely more vulnerable to the stroke induced ventricular enlargement, specifically in the contralateral hemisphere, which can possibly be attributed to the increased apoptosis post stroke. Our original hypothesis was that this damage was immunologic in nature due to increased inflammation in SHRs, however, our results obtained 12 weeks post-stroke indicate similar levels of post-stroke inflammation. Flow cytometry may have been able to better describe the nature of the activated microglia and macrophages in our samples, but we did not collect the tissue in a way that would allow that analysis. In our previous investigations, we showed that PSCI was associated with ongoing microglial activation and cell death at 30 days in SHRs, and it could be therapeutically targeted.[22] Here we see that a long-term increase in apoptotic cell death is more pronounced in SHRs. It was ideal to perform detailed histological analyses on the hippocampus to determine the extent of hippocampal damage. However, in most of our animals, the ischemic lesion was big enough to reach sub-cortical areas including destroying all or parts of the ipsilateral hippocampus, which made quantifiable analyses of hippocampal injury impossible for these animals.

The increase in PARP1, MMP9 and HMGB1 associated with stroke in SHRs points to the unique relationship between hypertension and the enhancement of post-stroke cell death (Figure 2.7). In addition, PARP1 plays a role in the development of a caspase-independent form of cell death, termed as parthanatos, in response to ischemia/reperfusion damage.[41, 42] Chronic inhibition of full-length (active) PARP1 in SHRs was found to reduce hypertension-related tissue damage in the brain and vascular tissue without affecting the blood pressure.[43] Both PARP1 knock-out and PARP1 inhibition are known to provide anti-inflammatory and neuroprotective effects in models of traumatic brain injury[44] and cerebral ischemia.[45]

In addition to its well-known deleterious effects on acute ischemic stroke, including disruption of the blood brain barrier (BBB), increased risk of hemorrhagic complications, and worsened stroke outcome,[46, 47] MMP9 expression has been found to be increased in SHR brains after MCAO, and associated with increased deficits in memory and learning.[48] In addition, it plays an important role in the cleavage of PARP1. [49] Although the increase in MMP-9 after acute ischemia has been well documented, the increased activity has been reported as transient, returning to baseline in the week following stroke. [50, 51] However, MMP9 expression is increased in several chronic inflammatory CNS pathologies such as multiple sclerosis and Devic's neuromyelitis optica.[41]. It has been reported that PARP1 inhibitors also inhibit MMPs, indicating that the beneficial effects of PARP1 inhibitors after stroke may be in part due to the inhibition of MMPs.[52] MMP9 is

secreted as an inactive pro form, which is cleaved and activated, appearing as two bands of cleaved MMP9.[53] MMP9 was found to play a critical role in the development of age-dependent post-operative cognitive decline, and MMP9 knockout mice were found to be protected from this phenomenon.[54] Lastly, MMP9 plays an important role in the secretion of pro-inflammatory cytokines and can act as one itself, particularly in response to tissue injury. [55]

HMGB1 is a protein involved in DNA organization and transcription regulation in the nucleus.[56] In the brain, HMGB1 acts on microglia to mediate chronic neuroinflammation that drives progressive neurodegeneration. [57] HMGB1 was also found to be elevated in models of traumatic brain injury, neuroinflammation, epilepsy, and cognitive dysfunction.[58, 59] Finally, HMGB1 was found to be released from necrotic cells in the ischemic core, activating an early inflammatory response and its concentrations were found to correlate with disease severity and outcome after brain injury. [60]

Our study demonstrated that comorbid hypertension not only worsens the initial injury due to stroke, it exacerbates ongoing tissue damage that occurs months after motor recovery. Although cognitive impairment was evident in both Wistars and SHRs at 30 days after stroke, molecular evidence of active neurodegeneration was more than 2-fold higher in the SHRs at 12 weeks. In fact, the statistically significant “interaction” we report suggests that the presence of hypertension actually reverses the normal response of these mediators to ischemia and reperfusion. It is likely that hypertension contributes to progressive post-stroke

cognitive impairment and differences may have been demonstrated with more sensitive tests or longer-term follow-up.

## Perspectives

Our study aimed to establish the contribution of hypertension to the clinically relevant phenomenon of delayed PSCI. We found that delayed cognitive impairment develops in both normotensive and SHRs. However, SHRs showed an increase in delayed DNA damage and cell death, resulting in overall tissue atrophy which was greater than that seen in hypertension alone. Neurodegeneration is a LATE target for intervention after stroke in hypertensive individuals.

## Author Contribution:

MAS was responsible for overall study design, data collection and analysis as well as performing behavioral testing, microscopy studies and writing the manuscript. WE performed the stroke surgeries. MA, BP and WA helped in data collection and analysis for molecular and behavioral experiments. MHJ was responsible for the statistical analysis. ASA supported the study with the MRI data. AE interpreted the data and reviewed the manuscript. SCF designed the study, managed the experiments, interpreted the data, and reviewed the manuscript. All authors read and approved the final manuscript.

**Acknowledgements:**

The authors would like to thank the core imaging facility for small animals (CIFSA) of the Georgia Cancer Center and Augusta University animal facility for performing the imaging and for help in performing analysis. We would also like to thank the Electron Microscope and Histology Core at Augusta University for sectioning the brain tissues. Special thanks to members of Dr. Adviye Ergul's lab for providing technical assistance during animal sacrifice. We would like to thank Mir Adil for his help in figure preparation.

**Sources of Funding:**

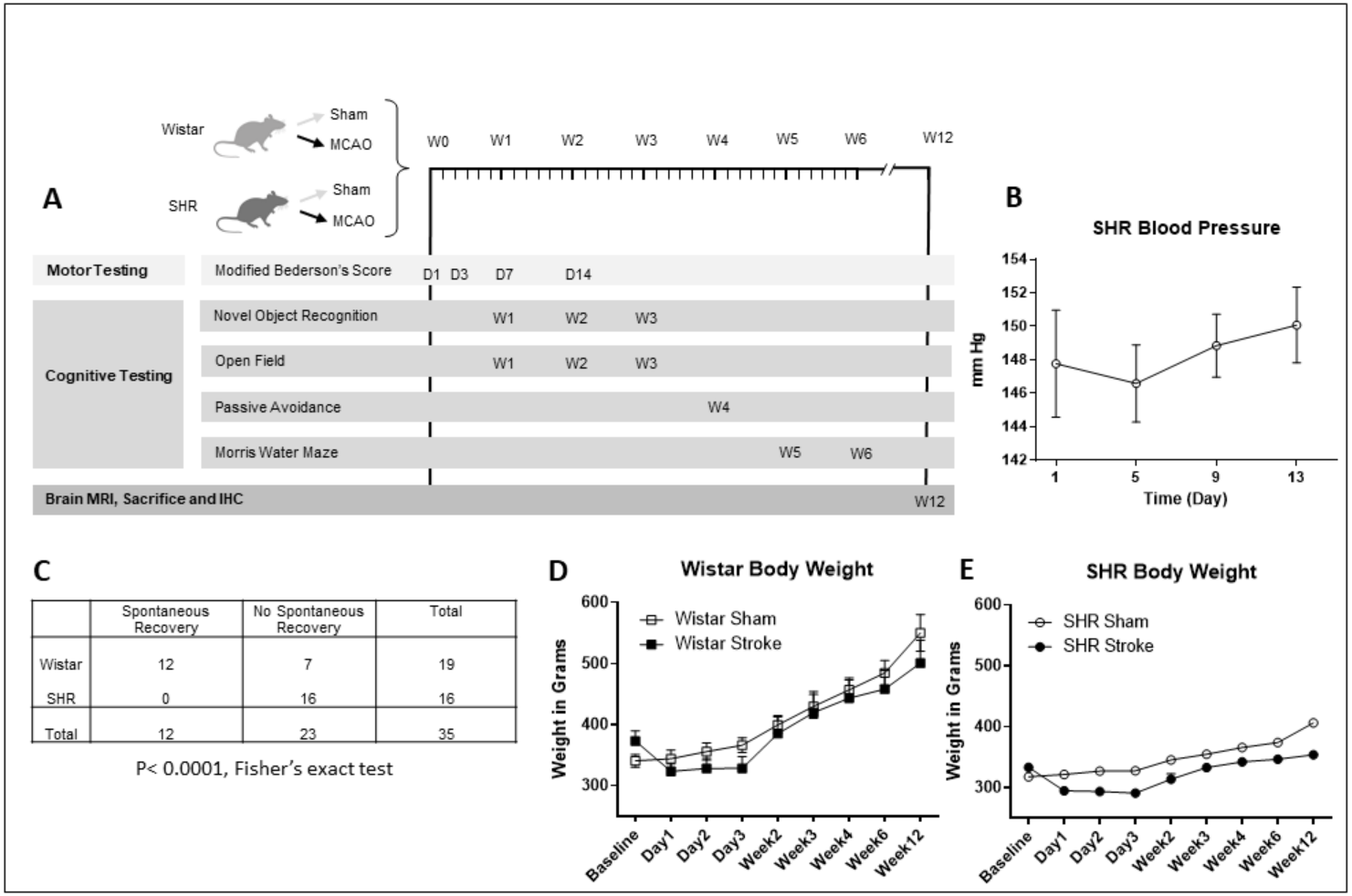
This study was supported by National Institute of Health (NIH) R01 NS104573 to Adviye Ergul and Susan C. Fagan; R21-NS088016 and Veterans Affairs (VA) Merit Review (BX000891) to Susan C. Fagan; R01 NS083559, VA Merit Review (BX000347), VA Senior Research Career Scientist Award (IK BX004471) to Adviye Ergul.

**Competing Interests:**

No conflicts of interest, financial or otherwise, are declared by the authors.

**Figure (2.1): Hypertension increases stroke mortality and reduces spontaneous recovery.** (A) Experimental design showing different time points for behavioral testing. (B) The mean arterial blood pressure of a cohort of SHR animals of the same age and weight of our stroke animals was followed for 2 weeks to establish their blood pressure (n=6). (C) 63% of the Wistar animals achieved complete spontaneous post-stroke recovery after 24 hours of MCAO, compared to 0% for SHR animals. ($P < 0.0001$, Fischer's exact test) (D) & (E) Body weight was recorded for 12 weeks following MCAO comparing sham animals from both strains to stroke animals (n=6-12 per group).

Fig. 2.1




| | Spontaneous Recovery | No Spontaneous Recovery | Total |
|---|---|---|---|
| Wistar | 12 | 7 | 19 |
| SHR | 0 | 16 | 16 |
| Total | 12 | 23 | 35 |

P< 0.0001, Fisher's exact test

**Figure (2.2): Hypertension causes slower sensorimotor recovery.** (A) Both strains had significant neurological impairment (Modified Bederson's Score) starting 24 hrs after stroke and up to day 14. (n=6-12 per group) (One sample t-test compared to the maximum score of 8). (B) There was no significant difference between the 4 groups in object discrimination in the novel object recognition test at 2- and 12-weeks post MCAO. (n=6-12 per group). (C) SHRs showed a trend for reduced total object exploration time at 2-weeks post stroke (p=0.058, 2-way ANOVA). The difference disappeared at 12-weeks post-stroke. (n=6-12 per group).

Fig. 2.2

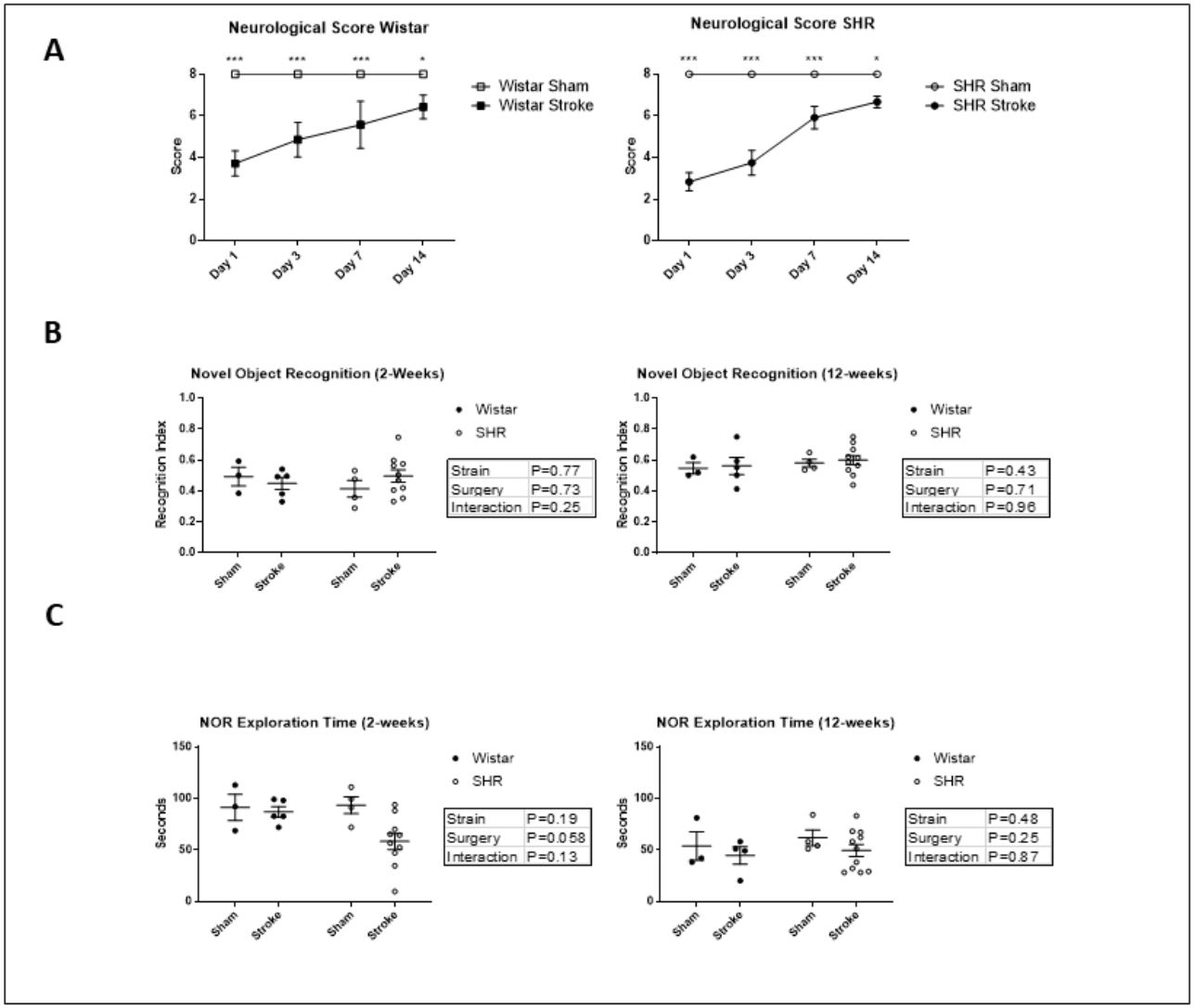

A
Neurological Score Wistar
Score
Day 1
Day 3
Day 7
Day 14
Wistar Sham
Wistar Stroke
Neurological Score SHR
SHR Sham
SHR Stroke
B
Novel Object Recognition (2-Weeks)
Recognition Index
Sham
Stroke
Wistar
SHR
Strain P=0.77
Surgery P=0.73
Interaction P=0.25
Novel Object Recognition (12-weeks)
Strain P=0.43
Surgery P=0.71
Interaction P=0.96
C
NOR Exploration Time (2-weeks)
Seconds
Strain P=0.19
Surgery P=0.058
Interaction P=0.13
NOR Exploration Time (12-weeks)
Strain P=0.48
Surgery P=0.25
Interaction P=0.87

**Figure (2.3): Stroke causes long-term fear associated memory dysfunction and hypertension induces behavioral despair.** (A) Time to reach the platform during the acquisition of the Morris Water Maze was recorded. All groups showed a consistent learning curve (P for time <0.001, 2-way ANOVA). (B) Average swim speed over the course of the experiment was measured for all groups. (P>0.05, 2-way ANOVA) (n=6-12 per group). (C) Time spent in the platform zone and (D) time spent in the target quadrant were measured in the probe trial to examine the memory function (P>0.05, 2-Way ANOVA). (E) Total distance travelled during the probe test was a measurement for behavioral despair and depression. (P for strain<0.001, 2-way ANOVA). (a,b: Tukey post-hoc multiple comparisons, pairs of means with different letters are significantly different) (F) Passive Avoidance showed a decrease in the average latency to enter the shock arm for stroked animals. (P<0.01, 2-way ANOVA) (n=6-12 per group).

Fig. 2.3

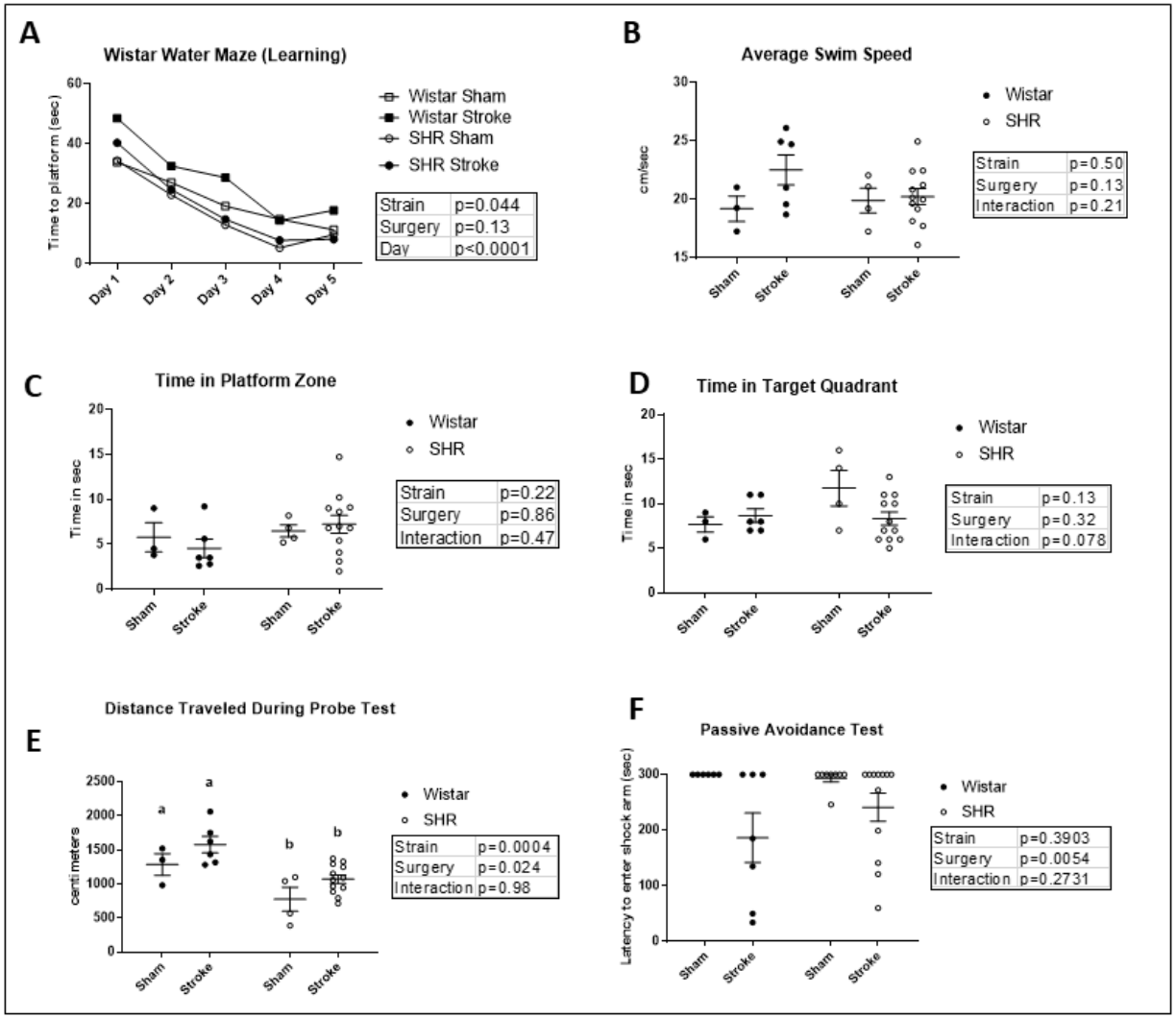

A
Wistar Water Maze (Learning)
Time to platform (sec)
Day 1
Day 2
Day 3
Day 4
Day 5
Wistar Sham
Wistar Stroke
SHR Sham
SHR Stroke
Strain p=0.044
Surgery p=0.13
Day p<0.0001
B
Average Swim Speed
cm/sec
Sham
Stroke
Wistar
SHR
Strain p=0.50
Surgery p=0.13
Interaction p=0.21
C
Time in Platform Zone
Time in sec
Wistar
SHR
Strain p=0.22
Surgery p=0.86
Interaction p=0.47
D
Time in Target Quadrant
Time in sec
Wistar
SHR
Strain p=0.13
Surgery p=0.32
Interaction p=0.078
E
Distance Traveled During Probe Test
centimeters
a
b
Wistar
SHR
Strain p=0.0004
Surgery p=0.024
Interaction p=0.98
F
Passive Avoidance Test
Latency to enter shock arm (sec)
Wistar
SHR
Strain p=0.3903
Surgery p=0.0054
Interaction p=0.2731

**Figure (2.4): Stroke induces enlarged ventricles and grey matter atrophy in SHRs, as measured by MRI at 12-weeks post-stroke.** (A) Representative images for diffusion weighted MRI, week 12 post-stroke. White indicates areas with high water content. (B) Ischemic tissue as % of the volume of the contralesional hemisphere ($P>0.05$, t-test) (n=6-12 per group). (C) Total volume of lateral ventricles as % of total brain volume, with SHR showing significant bilateral enlargement of lateral ventricles (P for strain <0.01, 2-way ANOVA). (D) Volume of the ipsilesional ventricle as % of the contralesional ventricle, with significant enlargement for stroked animals in both strains. (P for surgery <0.001, 2-way ANOVA). (F) Total volume of the ipsilesional hemisphere as % of total brain volume, decreased for stroked animals in both strains. (P for surgery <0.001, 2-way ANOVA). (a,b,c: Tukey post-hoc multiple comparisons, pairs of means with different letters are significantly different.)

Fig. 2.4

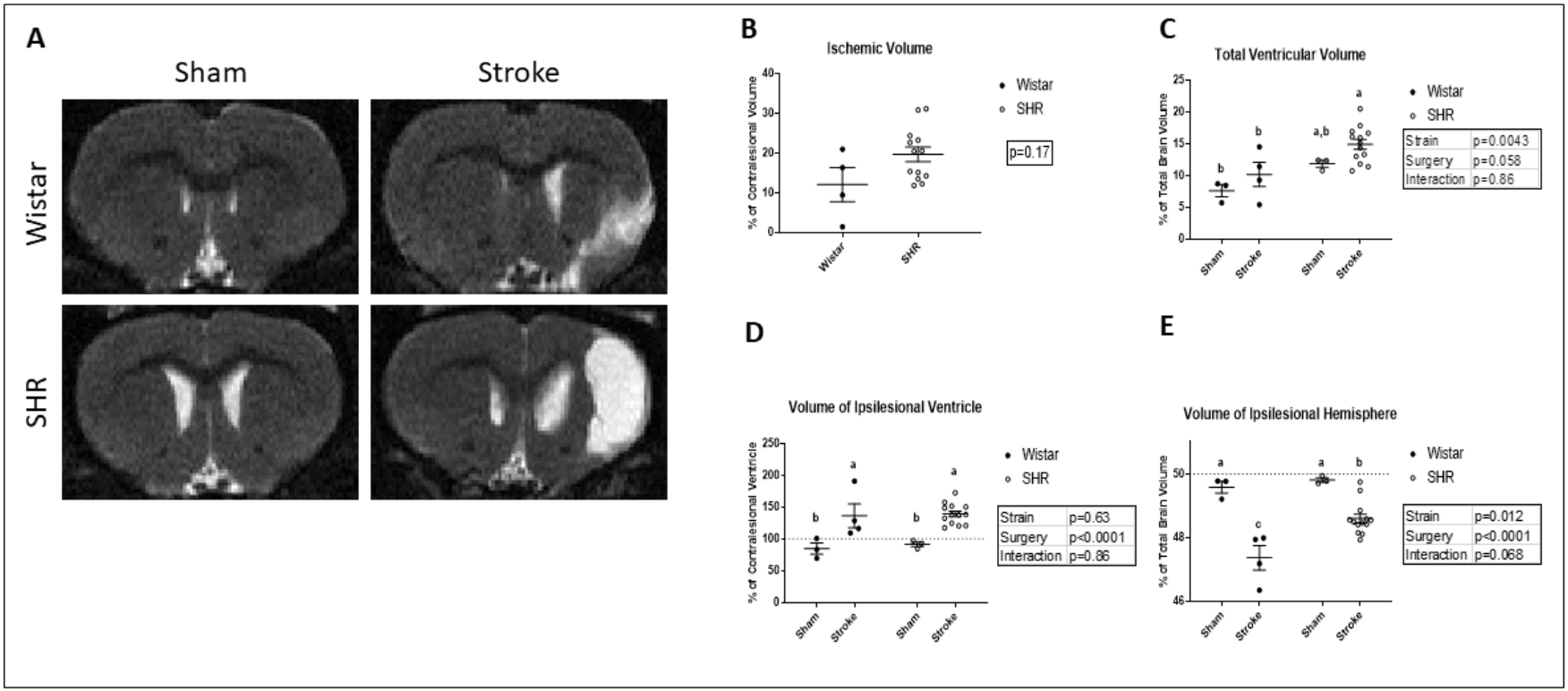

A
Sham
Stroke
Wistar
SHR
B
Ischemic Volume
% of Contralesional Volume
Wistar
SHR
p=0.17
C
Total Ventricular Volume
% of Total Brain Volume
Wistar
SHR
Strain p=0.0043
Surgery p=0.058
Interaction p=0.86
Sham
Stroke
D
Volume of Ipsilesional Ventricle
% of Contralesional Ventricle
Strain p=0.63
Surgery p<0.0001
Interaction p=0.86
E
Volume of Ipsilesional Hemisphere
% of Total Brain Volume
Strain p=0.012
Surgery p<0.0001
Interaction p=0.068

**Figure (2.5): Stroke induces neuronal apoptosis in the ischemic border zone region of brain tissue, which is exacerbated by hypertension.** (A) Representative image of the positions from which the TUNEL and IBA1 images were taken. The polygon outline represents the ischemic tissue, the squares represent fields for quantitation. (DAPI) (B) SHRs displayed a significant increase in apoptosis ($P<0.05$, 2-way ANOVA) compared to normotensive animals. Stroked animals displayed a significant increase in apoptosis ($P<0.01$, 2-way ANOVA) compared to Sham animals. (n=4-8 per group). (C) Stroked animals displayed a significant increase in IBA1 staining compared to shams ($P<0.001$, 2-way ANOVA) (n=4-8 per group). (a,b: Tukey post-hoc multiple comparisons, pairs of means with different letters are significantly different.) (D) Representative images from TUNEL staining. (E) Representative images from IBA1 staining (false color).

Fig. 2.5

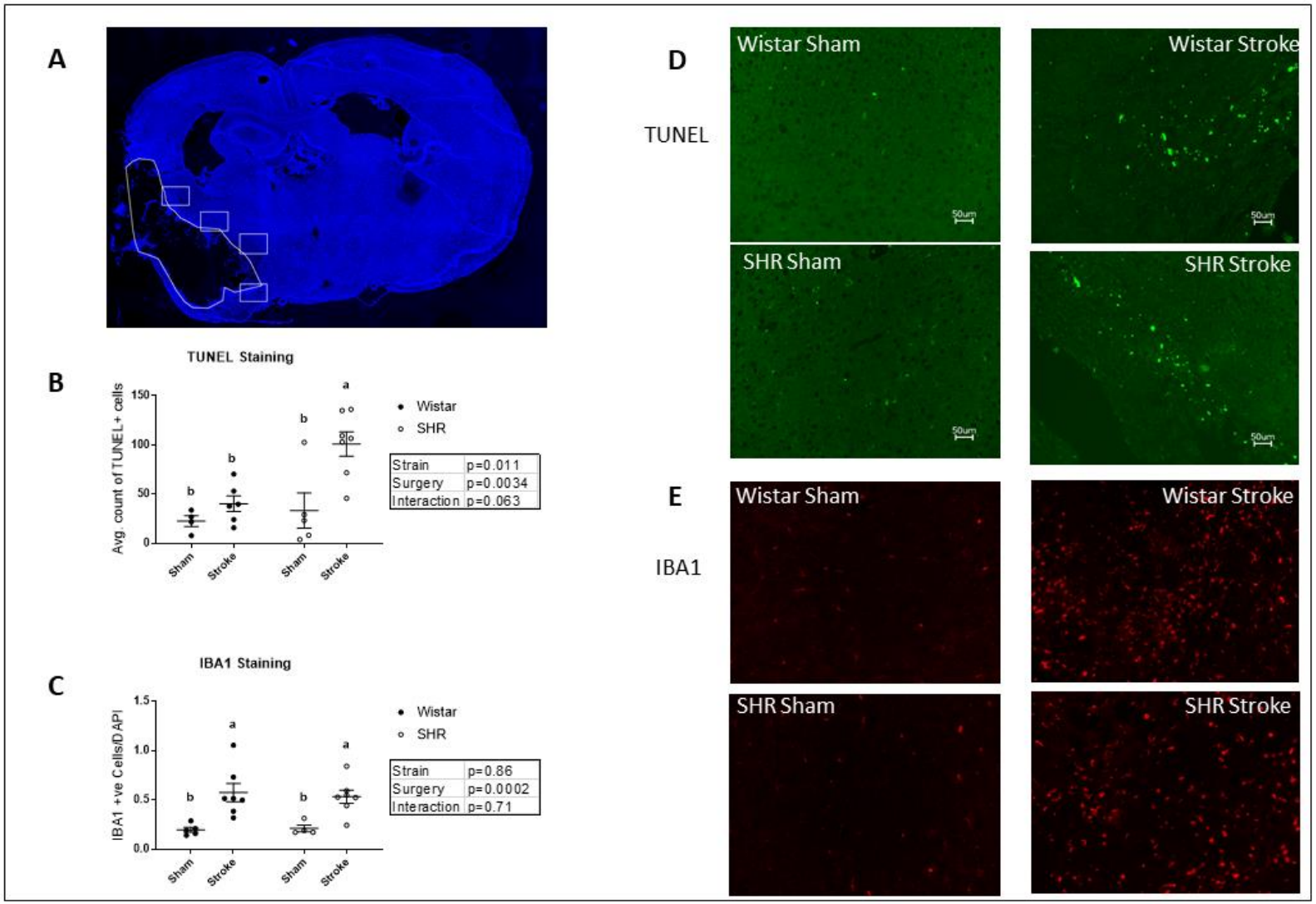

A
B
TUNEL Staining
Avg. count of TUNEL+ cells
150
100
50
0
Sham
Stroke
Sham
Stroke
Wistar
SHR
Strain p=0.011
Surgery p=0.0034
Interaction p=0.063
C
IBA1 Staining
IBA1 +ve Cells/0.API
1.5
1.0
0.5
0.0
Sham
Stroke
Sham
Stroke
Wistar
SHR
Strain p=0.86
Surgery p=0.0002
Interaction p=0.71
D
TUNEL
Wistar Sham
Wistar Stroke
SHR Sham
SHR Stroke
50um
E
IBA1
Wistar Sham
Wistar Stroke
SHR Sham
SHR Stroke

**Figure (2.6): SHRs display increases in markers of neuronal damage following stroke that are not increased in normotensive animals.** (A) Representative image for Western blot data. (B) Stroked SHR show increase in HMGB1 ($p$ (interaction)$<0.05$, 2-way ANOVA). (n=4-8, per group). (C) and (D) Stroked SHRs show increase in activated MMP9 ($p$ (interaction)$<0.01$ for upper band and $<0.001$ for lower band, 2-way ANOVA). Stroked SHRs show an increase of activated MMP9 ($p<0.05$ for upper band and $<0.01$ for lower band, Bonferroni post-hoc test) (n=4-8, per group). (E) SHR show increase in full-length PARP1 after stroke ($p$ (interaction)$<0.01$, 2-way ANOVA). Stroked SHR show an increase of PARP1 compared to stroked Wistars ($p<0.05$, Bonferroni post-hoc test) (n=4-8, per group). (a,b: Tukey post-hoc multiple comparisons, pairs of means with different letters are significantly different.)

Fig. 2.6

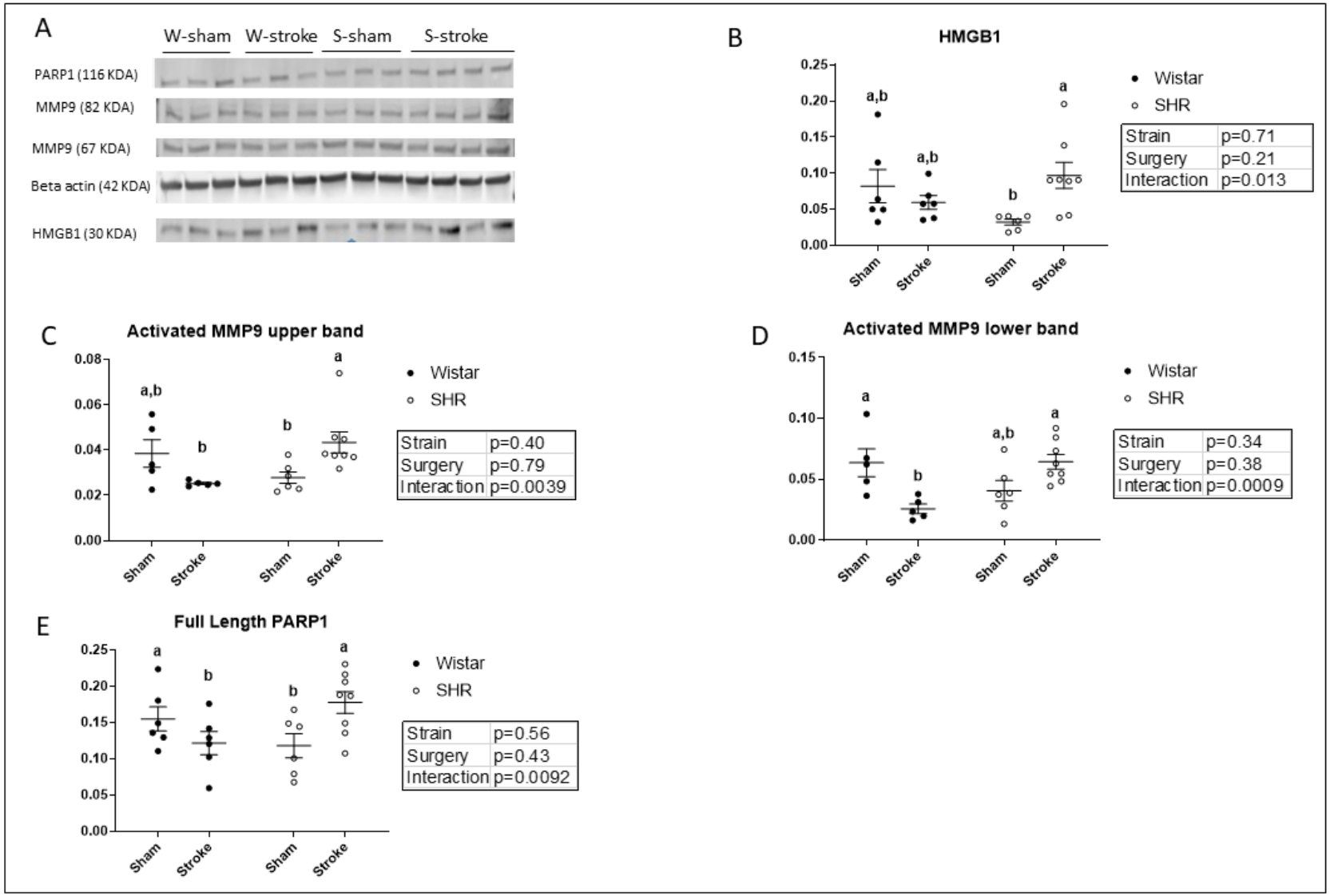
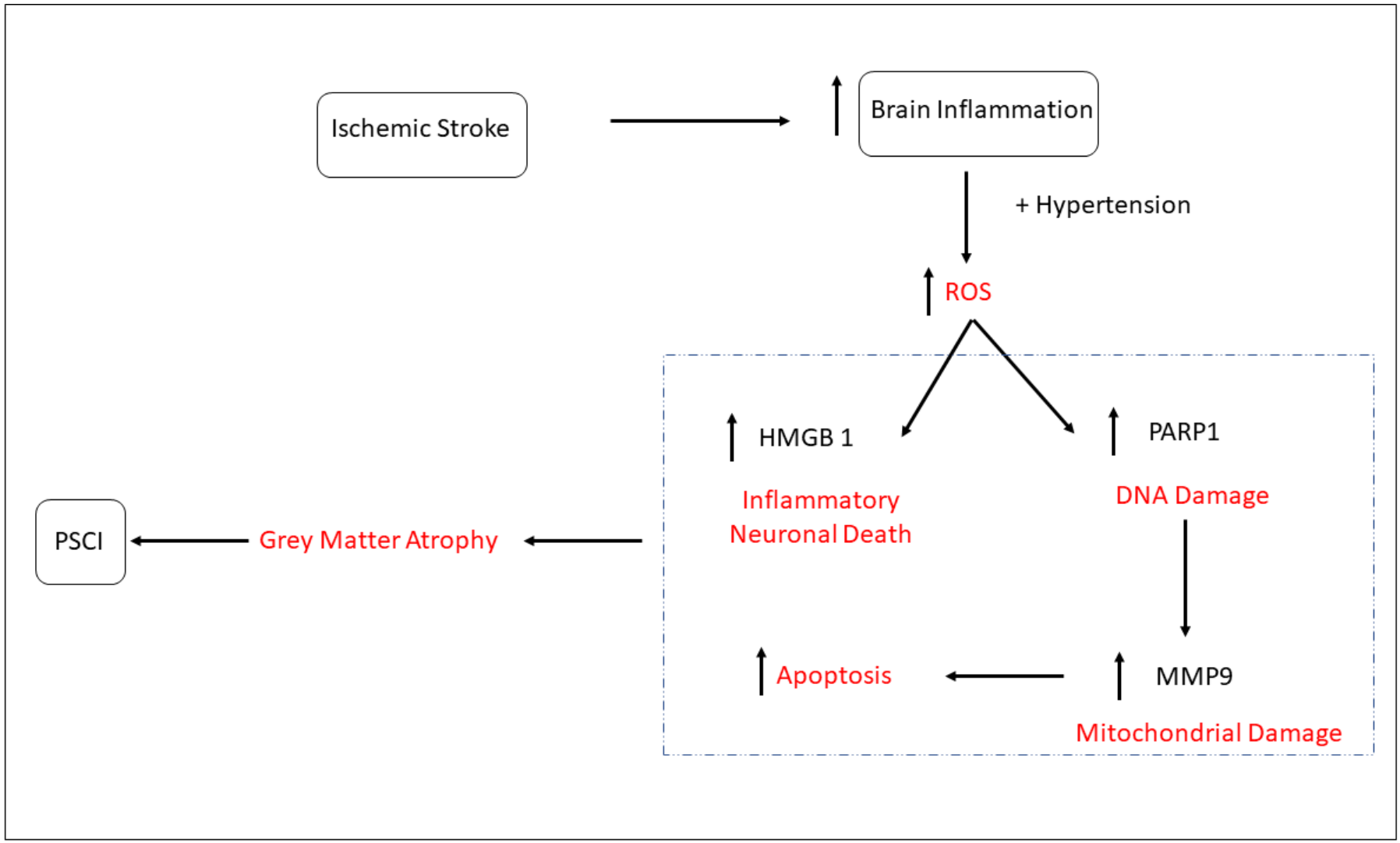

A
W-sham
W-stroke
S-sham
S-stroke
PARP1 (116 KDA)
MMP9 (82 KDA)
MMP9 (67 KDA)
Beta actin (42 KDA)
HMGB1 (30 KDA)
B
HMGB1
Wistar
SHR
Strain p=0.71
Surgery p=0.21
Interaction p=0.013
C
Activated MMP9 upper band
Strain p=0.40
Surgery p=0.79
Interaction p=0.0039
D
Activated MMP9 lower band
Strain p=0.34
Surgery p=0.38
Interaction p=0.0009
E
Full Length PARP1
Strain p=0.56
Surgery p=0.43
Interaction p=0.0092
Sham
Stroke

**Figure (2.7): Stroke induces long term neurodegeneration in hypertensive animals.** Ischemic stroke causes a marked inflammation in stroked animals. In hypertensive animals, which already suffer from an increase in ROS production, the ischemic insult and the oxidative stress result in chronic increase in DNA damage and neuronal cell death, marked with an increase in PARP1 and HMGB1. PARP1 increase induces the transcription of MMP9, which triggers apoptosis. The result of these processes is chronic neuronal loss in the form of grey matter atrophy.

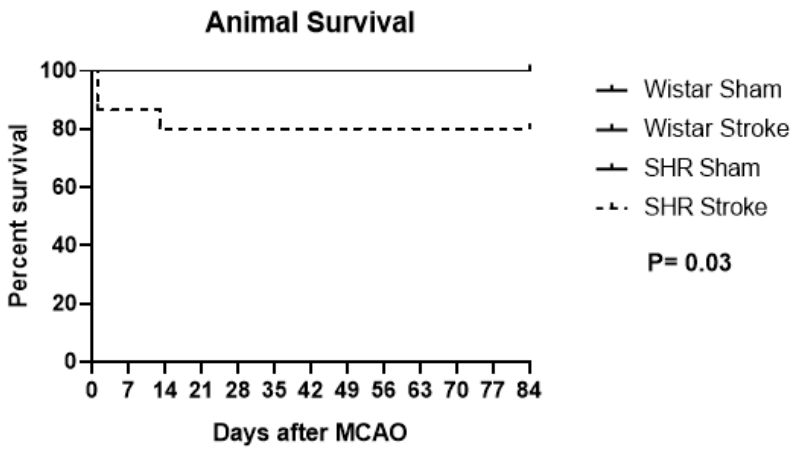
Fig. 2.7


Ischemic Stroke
Brain Inflammation
+ Hypertension
ROS
HMGB 1
PARP1
Inflammatory
Neuronal Death
DNA Damage
PSCI
Grey Matter Atrophy
Apoptosis
MMP9
Mitochondrial Damage

**Supplementary Figure (2.1): Survival curve.** 3 animals died in the stroke SHR group, non from the other groups.

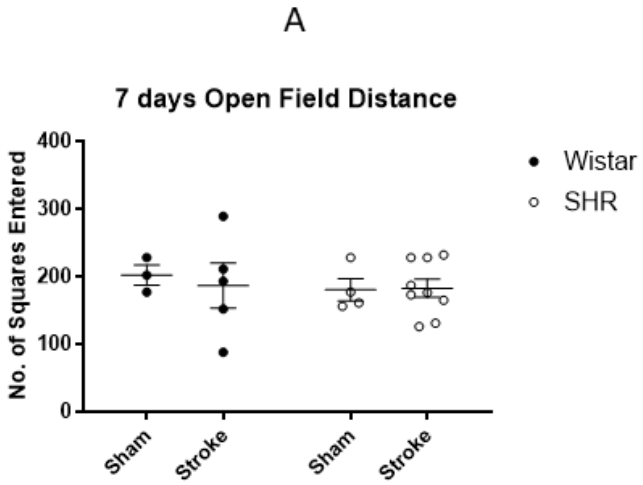
Supp. Fig 2.1

A


Animal Survival
Percent survival
100
80
60
40
20
0
0 7 14 21 28 35 42 49 56 63 70 77 84
Days after MCAO
Wistar Sham
Wistar Stroke
SHR Sham
SHR Stroke
P= 0.03

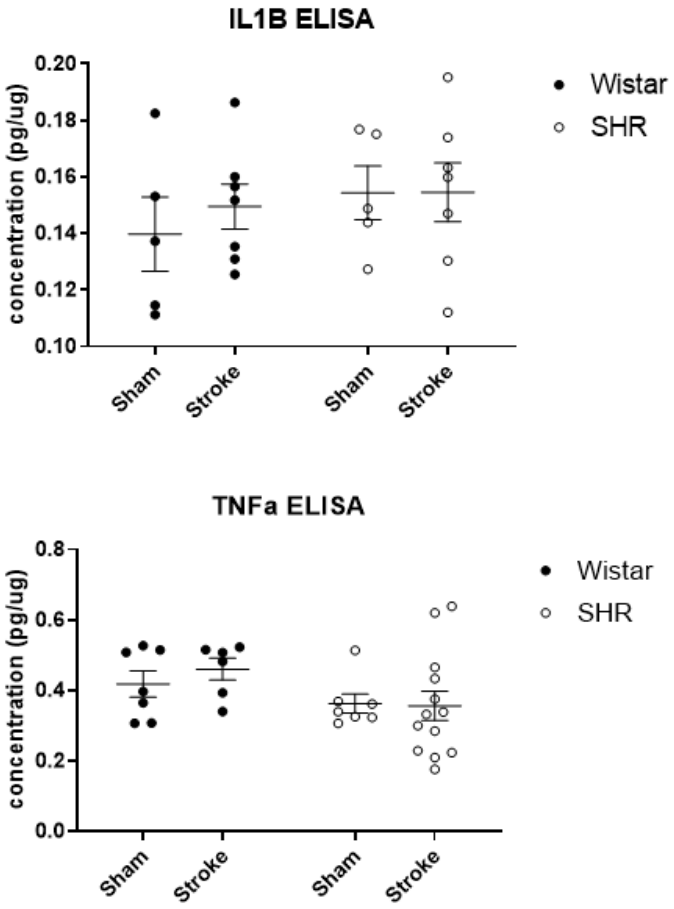
**Supplementary Figure (2.2): Open field distance.** No significant differences between groups.

Supp. Fig 2.2


A
7 days Open Field Distance
No. of Squares Entered
400
300
200
100
0
Sham
Stroke
Sham
Stroke
Wistar
SHR

**Supplementary Figure (2.3): IL-1β and TNFα ELISA.** No significant differences between groups.

Supp. Fig 2.3


IL1B ELISA
concentration (pg/ug)
0.20
0.18
0.16
0.14
0.12
0.10
Sham
Stroke
Sham
Stroke
Wistar
SHR
TNFa ELISA
concentration (pg/ug)
0.8
0.6
0.4
0.2
0.0
Sham
Stroke
Sham
Stroke
Wistar
SHR

## References


1. Benjamin, E.J., et al., *Heart Disease and Stroke Statistics-2018 Update: A Report From the American Heart Association.* Circulation, 2018. **137**(12): p. e67-e492.
2. Towfighi, A. and J.L. Saver, *Stroke declines from third to fourth leading cause of death in the United States: historical perspective and challenges ahead.* Stroke, 2011. **42**(8): p. 2351-5.
3. Sun, J.H., L. Tan, and J.T. Yu, *Post-stroke cognitive impairment: epidemiology, mechanisms and management.* Ann Transl Med, 2014. **2**(8): p. 80.
4. Woitzik, J., T. Back, and C. Thome, *Flow-dependent versus spreading-like impairment of brain tissue integrity during focal cerebral ischemia and its consequences for neuroprotective strategies.* Front Biosci, 2008. **13**: p. 1500-6.
5. Leys, D., et al., *Poststroke dementia.* Lancet Neurol, 2005. **4**(11): p. 752-9.
6. Obaid, M., et al., *Long-Term Outcomes in Stroke Patients with Cognitive Impairment: A Population-Based Study.* Geriatrics (Basel), 2020. **5**(2).
7. Pendlebury, S.T., et al., *Underestimation of cognitive impairment by Mini-Mental State Examination versus the Montreal Cognitive Assessment in patients with transient ischemic attack and stroke: a population-based study.* Stroke, 2010. **41**(6): p. 1290-3.
8. Bour, A., et al., *How predictive is the MMSE for cognitive performance after stroke?* J Neurol, 2010. **257**(4): p. 630-7.
9. Arba, F., et al., *Enlarged perivascular spaces and cognitive impairment after stroke and transient ischemic attack.* Int J Stroke, 2018. **13**(1): p. 47-56.

10. Delavaran, H., et al., *Cognitive function in stroke survivors: A 10-year follow-up study.* Acta Neurol Scand, 2017. **136**(3): p. 187-194.

11. Rouch, L., et al., *Antihypertensive drugs, prevention of cognitive decline and dementia: a systematic review of observational studies, randomized controlled trials and meta-analyses, with discussion of potential mechanisms.* CNS Drugs, 2015. **29**(2): p. 113-30.

12. Wiesmann, M., A.J. Kiliaan, and J.A. Claassen, *Vascular aspects of cognitive impairment and dementia.* J Cereb Blood Flow Metab, 2013. **33**(11): p. 1696-706.

13. Filipska, K., et al., *Are Depression and Dementia a Common Problem for Stroke Older Adults? A Review of Chosen Epidemiological Studies.* Psychiatr Q, 2020.

14. Jellinger, K.A., *The pathology of "vascular dementia": a critical update.* J Alzheimers Dis, 2008. **14**(1): p. 107-23.

15. Sahathevan, R., A. Brodtmann, and G.A. Donnan, *Dementia, stroke, and vascular risk factors; a review.* Int J Stroke, 2012. **7**(1): p. 61-73.

16. Tu, J.V., *Reducing the global burden of stroke: INTERSTROKE.* Lancet, 2010. **376**(9735): p. 74-5.

17. Qiu, C., B. Winblad, and L. Fratiglioni, *The age-dependent relation of blood pressure to cognitive function and dementia.* Lancet Neurol, 2005. **4**(8): p. 487-99.

18. O'Rourke, M.F. and M.E. Safar, *Relationship between aortic stiffening and microvascular disease in brain and kidney: cause and logic of therapy.* Hypertension, 2005. **46**(1): p. 200-4.

19. Au, R., et al., *Association of white matter hyperintensity volume with decreased cognitive functioning: the Framingham Heart Study.* Arch Neurol, 2006. **63**(2): p. 246-50.

20. de Groot, J.C., et al., *Cerebral white matter lesions and subjective cognitive dysfunction: the Rotterdam Scan Study.* Neurology, 2001. **56**(11): p. 1539-45.

21. Eldahshan, W., et al., *Angiotensin II type 2 receptor stimulation with compound 21 improves neurological function after stroke in female rats: a pilot study.* Am J Physiol Heart Circ Physiol, 2019. **316**(5): p. H1192-h1201.

22. Ahmed, H.A., et al., *RAS modulation prevents progressive cognitive impairment after experimental stroke: a randomized, blinded preclinical trial.* Journal of neuroinflammation, 2018. **15**(1): p. 229-229.

23. Ahmed, H.A., et al., *Angiotensin receptor (AT2R) agonist C21 prevents cognitive decline after permanent stroke in aged animals-A randomized double- blind pre-clinical study.* Behav Brain Res, 2019. **359**: p. 560-569.

24. Ahmed, H.A., et al., *Role of angiotensin system modulation on progression of cognitive impairment and brain MRI changes in aged hypertensive animals - A randomized double- blind pre-clinical study.* Behavioural brain research, 2018. **346**: p. 29-40.

25. Jackson, L., et al., *Delayed Administration of Angiotensin II Type 2 Receptor (AT2R) Agonist Compound 21 Prevents the Development of Post-stroke Cognitive Impairment in Diabetes Through the Modulation of Microglia Polarization.* Transl Stroke Res, 2019.

26. Wang, Y., et al., *Comparison of invasive blood pressure measurements from the caudal ventral artery and the femoral artery in male adult SD and Wistar rats.* PLoS One, 2013. **8**(4): p. e60625.

27. Ba, X. and N.J. Garg, *Signaling mechanism of poly(ADP-ribose) polymerase-1 (PARP-1) in inflammatory diseases.* Am J Pathol, 2011. **178**(3): p. 946-55.

28. Bai, P. and L. Virag, *Role of poly(ADP-ribose) polymerases in the regulation of inflammatory processes.* FEBS Lett, 2012. **586**(21): p. 3771-7.

29. Ha, H.C., *Defective transcription factor activation for proinflammatory gene expression in poly(ADP-ribose) polymerase 1-deficient glia.* Proc Natl Acad Sci U S A, 2004. **101**(14): p. 5087-92.

30. Masson, G.S., et al., *Aerobic training normalizes autonomic dysfunction, HMGB1 content, microglia activation and inflammation in hypothalamic paraventricular nucleus of SHR.* Am J Physiol Heart Circ Physiol, 2015. **309**(7): p. H1115-22.

31. Barbay, M., et al., *Vascular cognitive impairment: Advances and trends.* Rev Neurol (Paris), 2017. **173**(7-8): p. 473-480.

32. Kalaria, R.N., R. Akinyemi, and M. Ihara, *Stroke injury, cognitive impairment and vascular dementia.* Biochim Biophys Acta, 2016. **1862**(5): p. 915-25.

33. Levine, D.A., et al., *Trajectory of Cognitive Decline After Incident Stroke.* Jama, 2015. **314**(1): p. 41-51.

34. Ishitsuka, K., et al., *High blood pressure after acute ischemic stroke is associated with poor clinical outcomes: Fukuoka Stroke Registry.* Hypertension, 2014. **63**(1): p. 54-60.

35. Ergul, A., et al., *Impact of Comorbidities on Acute Injury and Recovery in Preclinical Stroke Research: Focus on Hypertension and Diabetes.* Transl Stroke Res, 2016. **7**(4): p. 248-60.

36. Nam, H., et al., *Learned helplessness and social avoidance in the Wistar-Kyoto rat.* 2014. **8**(109).

37. Zorzo, C., et al., *Adult social isolation leads to anxiety and spatial memory impairment: Brain activity pattern of COx and c-Fos.* Behav Brain Res, 2019. **365**: p. 170-177.

38. Kono, S., et al., *Neurovascular protection by telmisartan via reducing neuroinflammation in stroke-resistant spontaneously hypertensive rat brain after ischemic stroke.* J Stroke Cerebrovasc Dis, 2015. **24**(3): p. 537-47.

39. Sato, K., et al., *Telmisartan reduces progressive oxidative stress and phosphorylated alpha-synuclein accumulation in stroke-resistant spontaneously hypertensive rats after transient middle cerebral artery occlusion.* J Stroke Cerebrovasc Dis, 2014. **23**(6): p. 1554-63.

40. Ritter, S., et al., *Cerebroventricular dilation in spontaneously hypertensive rats (SHRs) is not attenuated by reduction of blood pressure.* Brain Res, 1988. **450**(1-2): p. 354-9.

41. Halmosi, R., et al., *Effect of poly(ADP-ribose) polymerase inhibitors on the ischemia-reperfusion-induced oxidative cell damage and mitochondrial metabolism in Langendorff heart perfusion system.* Mol Pharmacol, 2001. **59**(6): p. 1497-505.

42. Li, W.H., et al., *Baicalein attenuates caspase-independent cells death via inhibiting PARP-1 activation and AIF nuclear translocation in cerebral ischemia/reperfusion rats.* Apoptosis, 2020. **25**(5-6): p. 354-369.

43. Eros, K., et al., *Chronic PARP-1 inhibition reduces carotid vessel remodeling and oxidative damage of the dorsal hippocampus in spontaneously hypertensive rats.* PLoS One, 2017. **12**(3): p. e0174401.

44. Besson, V.C., et al., *Deleterious poly(ADP-ribose)polymerase-1 pathway activation in traumatic brain injury in rat.* Brain Res, 2003. **989**(1): p. 58-66.

45. Noh, M.Y., et al., *Regulatory T cells increase after treatment with poly (ADP-ribose) polymerase-1 inhibitor in ischemic stroke patients.* Int Immunopharmacol, 2018. **60**: p. 104-110.

46. Turner, R.J. and F.R. Sharp, *Implications of MMP9 for Blood Brain Barrier Disruption and Hemorrhagic Transformation Following Ischemic Stroke.* Front Cell Neurosci, 2016. **10**: p. 56.

47. Mechtouff, L., et al., *Matrix Metalloproteinase-9 and Monocyte Chemoattractant Protein-1 Are Associated With Collateral Status in Acute Ischemic Stroke With Large Vessel Occlusion.* Stroke, 2020. **51**(7): p. 2232-2235.

48. Gao, Y., et al., *Effect of Telmisartan on Preventing Learning and Memory Deficits Via Peroxisome Proliferator-Activated Receptor-gamma in Vascular Dementia Spontaneously Hypertensive Rats.* J Stroke Cerebrovasc Dis, 2018. **27**(2): p. 277-285.

49. Chaitanya, G.V., A.J. Steven, and P.P. Babu, *PARP-1 cleavage fragments: signatures of cell-death proteases in neurodegeneration.* Cell Commun Signal, 2010. **8**: p. 31.

50. Candelario-Jalil, E., Y. Yang, and G.A. Rosenberg, *Diverse roles of matrix metalloproteinases and tissue inhibitors of metalloproteinases in neuroinflammation and cerebral ischemia.* Neuroscience, 2009. **158**(3): p. 983-94.

51. Mandler, R.N., et al., *Matrix metalloproteinases and tissue inhibitors of metalloproteinases in cerebrospinal fluid differ in multiple sclerosis and Devic's neuromyelitis optica.* Brain, 2001. **124**(Pt 3): p. 493-8.

52. Nicolescu, A.C., et al., *Inhibition of matrix metalloproteinase-2 by PARP inhibitors.* Biochem Biophys Res Commun, 2009. **387**(4): p. 646-50.

53. Christensen, J. and V.P. Shastri, *Matrix-metalloproteinase-9 is cleaved and activated by cathepsin K.* BMC Res Notes, 2015. **8**: p. 322.

54. Bi, J., et al., *Critical role of matrix metallopeptidase 9 in postoperative cognitive dysfunction and age-dependent cognitive decline.* Oncotarget, 2017. **8**(31): p. 51817-51829.

55. Wang, H., et al., *HMG-1 as a late mediator of endotoxin lethality in mice.* Science, 1999. **285**(5425): p. 248-51.

56. Klune, J.R., et al., *HMGB1: endogenous danger signaling.* Mol Med, 2008. **14**(7-8): p. 476-84.

57. Gao, H.M., et al., *HMGB1 acts on microglia Mac1 to mediate chronic neuroinflammation that drives progressive neurodegeneration.* J Neurosci, 2011. **31**(3): p. 1081-92.

58. Paudel, Y.N., et al., *HMGB1: A Common Biomarker and Potential Target for TBI, Neuroinflammation, Epilepsy, and Cognitive Dysfunction.* Front Neurosci, 2018. **12**: p. 628.

59. Famakin, B.M., et al., *HMGB1 is a Potential Mediator of Astrocytic TLR4 Signaling Activation following Acute and Chronic Focal Cerebral Ischemia.* Neurol Res Int, 2020. **2020**: p. 3929438.

60. Singh, V., et al., *HMGB1 as a Key Mediator of Immune Mechanisms in Ischemic Stroke.* Antioxid Redox Signal, 2016. **24**(12): p. 635-51.

# CHAPTER 3

# DELAYED ADMINISTRATION OF COMPOUND 21 FOR THE TREATMENT OF ISCHEMIC STROKE IN HYPERTENSION: A RANDOMIZED, BLINDED PRECLINICAL TRIAL IN A HYPERTENSIVE RAT MODEL OF MIDDLE CEREBRAL ARTERY OCCLUSION[1]

[1] - Sayed MA, Abdelbary M, Pillai B, Alshammari A, Jahan A, Ergul A and Fagan SC. To be submitted to [Translational Stroke Research]

**Delayed Administration of Compound 21 for the Treatment of Ischemic Stroke in Hypertension: A Randomized, Blinded Preclinical Trial in a Hypertensive Rat Model of Middle Cerebral Artery Occlusion**

Mohammed A. Sayed[1,2], Mahmoud Abdelbary[3], Bindu Pillai[1,2], Abdulkarim Alshammari[1,2], Asmat Jahan[1,2], Adviye Ergul[4,5] and Susan C. Fagan* [1,2]

[1]Clinical and Experimental Therapeutics, College of Pharmacy, University of Georgia, Augusta, GA; [2]Charlie Norwood VA Medical Center, Augusta, GA; [3]Department of Physiology, Medical College of Georgia, Augusta, GA; [4]Department of Pathology and Laboratory Medicine, Medical University of South Carolina, Charleston, SC; and [5]Ralph H. Johnson VA Medical Center, Charleston, SC

**Corresponding Author: Susan C. Fagan, Pharm.D., FAHA**

914 New Baillie Street, HM Building Room 116, Augusta, GA 30901

Tel.: 706-721-0130 Email: sfagan@uga.edu

**Abstract**

The aim of this translational, randomized, controlled, blinded preclinical trial was to determine the effect of delayed administration of compound 21 (C21) in ischemic stroke in hypertensive rats. Spontaneously hypertensive rats (SHR) were subjected to 60-min middle cerebral artery occlusion (MCAO) or sham surgery. They received C21 (0.12 mg/kg/d) or water (orally) for 8 weeks, with the first dose given at 3 days post-MCAO. Adhesive removal task (ART), Novel Object Recognition (NOR), Passive Avoidance Test (PAT) and Sucrose Preference Test (SPT) were utilized to test sensorimotor and cognitive function. After performing the behavior tests, brains were collected for analyses. Markers of inflammation, cell-death and DNA damage were quantified in the brain lysates. Stroked animals suffered significant sensorimotor deficits that improved overtime and cognitive deficits compared to sham animals. Exploration time was reduced due to stroke and there was evidence of depressive-like symptoms at 8 weeks after stroke. Delayed treatment with C21 was not effective in improving the rate of sensorimotor recovery or preventing cognitive deficits. More studies are needed to determine the cellular effects on brain histology.

**Keywords**: Compound C21; middle cerebral artery occlusion; AT2R; ischemic stroke; hypertension; cognitive impairment.

## Introduction

The rate of death due to ischemic stroke has been falling continuously over the last decade in the United States, due to continuous improvement in healthcare.[1, 2] As a result of the decrease in stroke mortality, however, residual disability is steadily increasing among stroke survivors.[3] Ischemic stroke is known to produce irreversible brain damage and subsequent motor and cognitive impairment.[4] Recent estimates show that post-stroke cognitive impairment (PSCI) affects up to two-thirds of patients following ischemic stroke, with up to one third eventually developing dementia.[5, 6] A very substantial portion of this impairment results from neuronal pathogenesis.[7]

Hypertension is being increasingly recognized as a risk factor for the development of PSCI.[8, 9] The Renin-Angiotensin system (RAS) is a chief therapeutic target in the treatment of hypertension. Recent research found that in addition to the systemic RAS, there is a local RAS that affects the brain, where angiotensin II (Ang II), the most studied vasoactive substance, has been found to exert a direct effect at the cellular level impacting cell survival, differentiation and inflammation, in addition to its vascular and renal actions.[10, 11] Ang II binds two major receptors, AT1R and AT2R.[12] Over-activation of AT1R is known to result in vasoconstriction systemically, and is linked to the development of cognitive impairment, possibly through an increase in inflammation and cell death.[13-16] On the other hand, AT2R is known to play an important role in vasodilation, and within the brain, and

to specifically enhance cognition, cell survival and has both antioxidant and anti-inflammatory properties.[13, 16-18] Activation of cell surface AT2R can lead to the promotion of cell survival and can counteract the AT1R activation of pathways such as Src Homology Region 2 Domain-Containing Phosphatase-1 (SHP–1), serine-threonine phosphatase (PP2A) and Peroxisome Proliferator-activated Receptors (PPARγ).[12] The protective effects mediated by the activation of AT2R were found to play a role in alleviating cognitive impairment. In rodents, AT2R activation was associated with an increased VEGF production, enhanced survival of cortical neurons and improved neurological deficits after ischemic injury.[17] On the other hand, reduced AT2R activation induced dendritic spine abnormalities in the hippocampus and led to spatial memory deficits.[18] In the basal ganglia, AT2R downregulation was associated with dysfunctional signaling of dopaminergic neurons in animal models of Parkinson's disease.[19]

Among the best AT2R agonist candidates for use in humans is compound 21 (C21). This is a relatively novel non-peptide AT2R agonist, that was discovered in 2004, and has the benefits of both systemic and oral activity. [20] In the last few years, our lab has worked extensively on validating the therapeutic potential of C21 in the treatment of cerebral ischemia. Acutely, a single IP injected dose of C21 reduced infarct size and improved behavioral outcome at 24 h without affecting blood pressure. On the molecular level it decreased brain hemoglobin content, down-regulated apoptotic, inflammatory and oxidative markers, and increased pro-survival molecules in the brain. [21, 22] The acute beneficial effects of C21 post-

stroke was not limited to male rats. In females, a single IP dose of C21 resulted in an improvement in sensorimotor scores, one day after MCAO.[23]

More recently, our lab garnered more evidence about the neurorestorative effects of C21 when administered in longer term studies. We demonstrated the beneficial effects of C21 on the cognitive function of aged hypertensive rats with chronic cerebral hypoperfusion and permanent focal ischemia, as well as young hypertensive rats following ischemic stroke.[13, 24, 25] These protective effects were independent of blood pressure and β-amyloid accumulation. In diabetic rats, it was found that diabetes exacerbated the development of PSCI and increased inflammation and demyelination. Delayed administration of C21, starting 3 days post-stroke, reduced mortality and improved sensorimotor and cognitive deficits.[26] It was still unclear whether hypertensive animals would react similarly to the delayed administration of C21 after stroke and whether similar mechanisms are at work. In this study, we evaluated the impact of C21 as an experimental treatment for PSCI in hypertensive animals, with careful attention to blinding, randomization and relevant sham operated controls.

**Methods**

The data that support the findings of this study are available from the corresponding author on reasonable request. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Charlie

Norwood VA Medical Center, Augusta, Georgia. Experimental design can be found in (Fig. 3.1).

**Data Analysis**

Statistical analyses were performed using GraphPad Prism version 8.2 (GraphPad Software, San Diego, CA). All data are presented as means ±SEM. Unless otherwise mentioned, all results presented were analyzed by 2-way ANOVA (2 Treatment (Vehicle vs. C21) X 2 Surgery (sham vs. stroke)) and 3-way mixed model ANOVA (Treatment X Surgery X Time), with p-values for the tests presented as tables on the graphs. Letters in the graphs represent the results of Tukey post-hoc multiple comparisons tests for significant ANOVA results where pairs of means with different letters are significantly different. Otherwise, comparisons of two groups were analyzed by student's t-test for parametric values, and Wilcoxon's signed rank test for non-parametric values. Results are considered significant at a Type I error rate of 5%.

**Experimental Animals**

Male SHR animals were purchased from Charles River (Wilmington, MA) and were housed (1 rat per cage) in a pathogen free, temperature-controlled facility (24 ±1 C; 12-12-hour light-dark cycle) with access to standard chow and water ad libitum.

**Transient Middle Cerebral Artery Occlusion (tMCAO) surgery**

25 SHR rats (age: 10-14 weeks) (body weight: 320-400 g) were subjected to 60-min of tMCAO using 4–0 silicon coated nylon suture (Doccol 403756)) as previously described [22]. The original source of method description is our previously published work, Eldahshan et al. [23] Briefly, the animals were anesthetized using 2-5% isoflurane, a ventral mid-line neck incision was made, the right common carotid artery (CCA) was exposed, and the external carotid artery (ECA) was ligated and cut. The suture was advanced from a small nick at the ECA into the internal carotid artery (ICA) until a mild resistance was encountered, indicating the branching of the anterior and middle cerebral artery. The suture was tied in place for the duration of the occlusion and the animals were allowed to recover from anesthesia. Several minutes before the end of the occlusion time, the animals were re-anesthetized, the suture was removed for reperfusion and the small nick at the ECA was permanently ligated. In sham surgeries, the CCA was isolated and manipulated without cutting or insertion of the suture and the skin was closed.

We defined a failed MCAO surgery as: “Insertion of the suture that did not succeed in producing an ischemic damage as evident from the lack of motor deficits after reperfusion, possibly due to an incomplete occlusion of the origin of the MCA”.

## Treatment

*Dose and timing justification:*

We chose Day 3 to start administering C21 because it is well out of the neuroprotective window of 6 hours and acute infarct evolution is likely to be complete.[27] We chose an 8-week follow-up in an attempt to capture the progressive development of PSCI over time. The oral dose of 0.12 mg/kg was calculated based on an oral bioavailability of 0.25, so is equivalent to the intravenous (IV) dose of 0.03 mg/kg [21].

## Randomization and Blinding

The treatment and vehicle groups were prepared by an individual not involved in the surgery or assessments and labeled as group A and group B. Each animal was numbered before baseline behavioral assessments were taken. After MCAO surgery, the animals that met the pre-set inclusion criteria were assigned to group A and B using a random number generator. All behavioral and histological assessments were coded and conducted by a blinded investigator. Drinking water groups were also blinded.

### Inclusion criteria

In order to ensure inclusion of animals with a significant degree of ischemic injury, we implemented strict inclusion criteria. The inclusion criteria included assessment of sensorimotor function and weight loss. 3 days after MCAO, animals underwent the adhesive removal task (ART). Animals that had an ART above 30 seconds and had more than 5% weight loss were randomly assigned into C21 treatment and vehicle treatment groups.

## Assessment of Functional Outcome

### Body Weight

As we described in our previously published work Ahmed et al. [24], weight monitoring is an extremely important tool that serves as an independent and unambiguous measure of an animal's overall health and welfare, specifically after stroke. For our studies, animals were weighed before surgery and then daily after stroke for the first 14 days, then once a week until the day of sacrifice. All animals selected for the study were in the range of 300-400 grams body weight at baseline.

### Neurobehavioral Testing

All neurobehavioral tests were conducted, recorded, and analyzed in a blinded manner.

## Sensorimotor Testing

In addition to the adhesive removal task (ART) mentioned above, animals underwent an 8-point modified neurological assessment modified from the Bederson protocol [23] at days 1, 3, 5, 7 and 14 post surgery.

## Modified Bederson Score

Animals were assessed neurologically on an 8-point scale measuring 4 basic functions (spontaneous rotation, resistance to lateral push and fore and back paw flexion) with a score of 2 points awarded in each category for an animal exhibiting a natural response, a score of 1 point for mildly impaired animals and a score of 0 for strongly impaired animals. Higher scores indicate better performance with a maximum possible score of 8/8 and a minimum possible score of 0/8.

## Adhesive Removal Test

Method description is adapted from our previously published work, Jackson et al.[26] For the ART, the animals were trained for 4 days and then baseline measurements were recorded prior to stroke. 3 days post-stroke, measurements were recorded to determine eligibility for inclusion in the study. If the rat was included in the study, then subsequent ART measurements were recorded at

weeks 1, 2, 4, and 8 post-stroke. ART was carried out as previously described with modification [28]. Contact and removal latency of the adhesive paper dot was recorded, and the average was taken from 3 trials with a maximum removal latency of 180 seconds per trial.

**Cognitive Testing**

Cognitive tests were performed according to the design. Special consideration was taken for cognitive tests to allow sufficient period of time to prevent different tests from affecting one another. No cognitive tests were allowed to be performed on the same day to reduce interference between tests. The NOR was performed to evaluate non-spatial working memory at day 10, week 4 and week 8 [24-27], while the passive avoidance test (PAT) assessed associative learning and reference memory at week 4 and 5 poststroke. [28, 29]

**The Novel Object Recognition (NOR) test**

The original source of method description is our previously published work, Ahmed et al.[24] The NOR test was performed to evaluate non-spatial working memory related to frontal-subcortical circuits. This test was based on the spontaneous tendency of animals to interact with a novel object more than a familiar one and consisted of 2 trials separated by a retention period. On the designated test day, animals were first subjected to an acquisition/sample trial, where the animal is

presented with 2 identical (sample) objects and allowed to explore for 15 min. Following sample object exposure, the animal was returned to its home cage for a 1-h retention period. The 2nd preference trial/test session (5 min), which follows the retention period, was conducted in the same manner as the 1st trial, except that a new/novel object replaces one of the familiar/sample objects. The arena and objects were cleaned after each session with 70% ethanol. The time spent in exploring each object during the preference trial/test session was recorded and the discrimination index, which is the difference in exploration time for the objects divided by total time of exploration, was calculated. The discrimination index (DI) and the recognition index (RI), which is the time spent exploring the novel object relative to the total time of exploration, were taken as indicators of working memory.

Discrimination index (DI) = (TN − TF)/(TN + TF)

Recognition index (RI) = TN/(TN + TF)

- Time spent interacting with the familiar object (TF)
- Time spent interacting with the novel object (TN)

The required exploratory criteria was that animals should spend between 20-80% of the time exploring the objects out of the 5 min. Objects used were chosen as previously described.[30] Briefly, objects used were unified between animals and chosen according to recommendations of Heyser and Chemero in that they were

symmetrical and transparent, and made of odorless, durable, and easy to clean plastic and glass.

**The Passive Avoidance Test (PAT)**

The original source of method description is our previously published work, Ahmed et al.[22] The passive avoidance test was used to assess aversive associative learning and related reference memory. For this test, one of the compartments of a Y-maze was equipped with a metal floor connected to an electric circuit box, adjusted to deliver brief, moderate intensity electric shocks (3 s duration, 0.75 mA). For the acquisition trial, the shock compartment/arm was blocked, and the animal placed in one of the “safe” arms and allowed 5 min to explore the 2 open arms. Upon completion of 5 min, the door blocking the shock arm was opened allowing the animal to enter. Once the animal had fully entered the shock arm, its initial latency was recorded, and it received a brief electric shock before being returned to its cage. After a 72-h and a 7-day retention periods, the test trial was conducted. This was performed in a manner similar to that of the acquisition trial except that the foot shock was omitted, and all 3 arms were accessible to the animal from the start. The difference, between training and test sessions, the latency to enter the shock arm was used as a measure of retention. This latency was recorded for up to 300 s, as the index of long-term aversive associative memory consolidation.

## The Sucrose Preference Test (SPT)

The original source of method description is Serchov et al. [29] The sucrose preference test (SPT) is a reward-based test, used as in indicator of anhedonia. Anhedonia, or the decreased ability to experience pleasure, represents one of the core symptoms of depression. Rodents are born with an interest in sweet foods or solutions. Reduced preference for sweet solution in SPT represents anhedonia.[29] SPT was carried out in the animal's home cage, 24 hours before sacrifice. Animals are presented with 2 sipper bottles replacing the treatment bottle. One bottle contains plain drinking water, and the second contains a 2% sucrose solution. Water and sucrose solution intake are measured after 24 hours, and the positions of two bottles is switched between cages to reduce any confound produced by a side bias. Sucrose preference is calculated as a percentage of the volume of sucrose intake over the total volume of fluid intake.

## Animal Sacrifice and Tissue Collection

The original source of method description is our previously published work, Ahmed et al.[24] At week 12, animals were anesthetized with IP ketamine/xylazine and transcardially perfused with 300 ml of PBS. Animals were decapitated, and their brains collected. Brains were sliced into 2 mm coronal sections, using a glass slicer matrix (Braintree Scientific, Braintree, MA, USA) and sections were labeled from A to F, anterior to posterior. Sections A and B, from brain matrix, were snap frozen

and kept for molecular testing. The remaining brain tissue was immersed in 10% formalin (Fischer Scientific, Waltham, MA, USA) for 48 h and then transferred to a 30% sucrose solution until taken for frozen sectioning.

## Results

### Stroke induces the development of sensorimotor deficits that spontaneously recover with a rate unaffected by C21 treatment

In an effort to ensure inclusion of animals with a significant degree of ischemic injury a set of strict inclusion criteria was employed (see methods). 3 days after the animals were stroked, those that met the weight loss and sensorimotor deficit criteria were included in the study (1 animal was excluded). From day 3 to week 8, of the included animals, one animal experienced early mortality due to excessive weight loss from the vehicle treated group, while no animals from the C21 treated group experienced early mortality. The neurological score of the stroked animals from both treatment groups was at its lowest 24 hours post-stroke and improved with time ($p<0.001$), with no significant effect of the treatment ($p>0.05$). (Fig 3.2A). The pre-set inclusion criteria resulted in maximum sensory deficits in ART contact time (Fig. 3.2B) and fine motor deficits in ART removal time (Fig. 3.2C) within both the vehicle and C21 treated groups at day 3 that improved with time ($p<0.0001$), with no effect for the treatment ($p>0.05$).

**Stroke induces the development of long-term memory deficits and stroke-induced depression in hypertensive rats that is unaffected by C21 treatment**

The long-term memory function of SHRs was assessed at week 4 post stroke using the passive avoidance test (PAT) (Fig. 3.3). Stroked animals showed a strong trend of long-term memory impairment compared to sham animals after a 72-hour retention period (Fig. 3.3A, $p=0.06$), that was exacerbated when tested after a 7-day retention period (Fig. 3.3B, $p<0.05$). Treatment with C21 did not result in an improvement in the long-term memory function.

The working memory of the animals and the exploration behavior were tested at day 10, week 4 and week 8 post-stroke. (Fig. 3.4 A-B) There was a significant reduction for stroked animals compared to non-stroked animals in their preferential tendency to explore the novel object and their overall exploratory tendency ($p<0.05$). C21 showed a trend to have a beneficial effect on the working memory, but not on the exploratory behavior of stroked animals. Interestingly, sham but not stroked animals showed a reduced exploration time over time, indicating loss of interest in the task, possibly due to repetition.

Anhedonia, a sign of post-stroke depression was measured using the sucrose preference test (SPT). Stroked animals showed a significant decrease in their sucrose preference compared to sham animals. (Fig. 3.4C, $p<0.01$) C21 treatment did not affect sucrose preference.

**Stroke increased the levels of both pro- and anti-inflammatory cytokines in the brains of hypertensive rats that is unaffected by C21 treatment**

Interleukin 1 beta (IL-1β) is a pro-inflammatory cytokine that is known to be secreted in response to ischemic damage. High levels of IL-1β is associated with worse stroke outcome.[30] We measured the levels of IL-1β in the brain lysates using ELISA. The levels of brain IL-1β were found to be significantly increased in stroked animals, regardless of C21 treatment. (Fig. 3.5A, $p<0.05$).

Tumor necrosis factor alpha (TNFα) is another pro-inflammatory cytokine whose level is known to rise immediately after ischemic stroke, correlated with the size of ischemic damage.[31] We measured the levels of TNFα in the brain lysates using ELISA. The levels of brain TNFα were found to be similar between stroked and sham animals, regardless of C21 treatment. (Fig. 3.5B, $p>0.05$).

Interleukin 10 (IL-10) is an anti-inflammatory cytokine that is produced in the brain as a protective mechanism in response to ischemic stroke-induced damage. Higher IL-10 levels seen post-ictus have been associated with worse stroke outcomes in clinical trials.[32] We measured the levels of IL-10 in the brain lysates using ELISA. The levels of brain IL-10 were found to be significantly increased in stroked animals, regardless of C21 treatment. (Fig. 3.5C, $p<0.05$).

**Stroke increases the expression of markers of cell-death, DNA damage and oxidative stress in the brains of hypertensive animals, unaffected by C21 treatment.**

Cyclin-dependent kinase 5 (Cdk5) is an essential kinase that is considered to be one of the principle players mediating neuronal cell-death in response to stroke-induced ischemic damage in rodent brains.[33] We measured the expression levels of Cdk5 in the brain lysates using Western blotting. The levels of brain Cdk5 were found to be significantly increased in stroked animals, regardless of C21 treatment. (Fig. 3.6A, $p<0.01$).

Poly(ADP-ribose) polymerase-1 (PARP-1) is an abundant nuclear enzyme that acts at the center of cellular stress. Oxidative stress causes DNA damage and consequently activates PARP-1 to repair the damaged DNA.[34] The cleaved form of PARP-1 is the inactive form. We measured the expression levels of total and cleaved PARP-1 in the brain lysates using Western blotting. The ratio of total (active) PARP-1 to cleaved (inactive) PARP-1 were found to be significantly increased in stroked animals, compared to non-stroked ones (Fig. 3.6B, $p<0.01$) These results were not affected by C21 treatment.

High mobility group box 1 protein (HMGB1) expression has been shown to be associated with neuronal damage, increased cell death and increased inflammation.[35] We measured the expression levels of HMGB1 in the brain lysates using Western blotting. The levels of brain HMGB1 were found to be

significantly increased in stroked animals, regardless of C21 treatment. (Fig. 3.6C, $p<0.01$).

## Discussion

This study was designed to conform with the STAIR and RIGOR recommended scientific guidelines for developing effective translational stroke research[36-38], that were developed in response to the failure of numerous clinical trials to reproduce the results of preclinical trials in humans. Therefore, a great deal of care was utilized for proper blinding and randomization.

We and others have shown the beneficial effects of C21 in improving stroke outcome acutely when C21 was given immediately after stroke.[39-43] However, when C21 was used in a large, blinded preclinical trial of embolic MCAO, the results on sensorimotor recovery were disappointing.[27] Therefore, our focus shifted from utilizing C21 for neuroprotection in the acute phase, to neurorestoration and prevention of cognitive impairment after long term treatment. To isolate the acute effects of C21, we delayed the start of the treatment to day 3 post-stroke and only in animals that matched a tight set of inclusion criteria, to ensure result reproducibility.

In one of the most recently published studies of our group, this approach succeeded in producing protective effects in an experimental model of diabetes.[26] In that study, diabetic animals suffered from a significantly worse

stroke outcome mediated by inflammatory processes, where C21 succeeded in producing a potent anti-inflammatory effect. We utilized the same treatment regimen, similar inclusion criteria and some of the same endpoints. In addition, we included more cognitive endpoints and more endpoints related to oxidative stress and DNA damage.

This study is the first study to examine the long-term effects of delayed C21 treatment for the treatment of stroke in hypertensive animals. This is clinically relevant, as a great proportion of stroke patients suffer from hypertension. Although maximum motor recovery in humans is usually achieved within 6 months to one year after stroke, stroke survivors can be prone to the development of cognitive impairment and dementia for decades after the initial event.[44] It is now believed that this is likely due to long term progressive neurodegeneration, which is exacerbated by comorbidities, such as hypertension.[45]

In this study, the development of long-term post-stroke cognitive impairment was found to be robust even at 8 weeks post stroke. This study is one of the first to examine the development of long-term post-stroke depression in hypertensive rats. The results of the sucrose preference test strongly indicated the presence of anhedonia long after the near-complete motor recovery of the animals. This opens more doors to study this phenomenon that can affect up to one third of human stroke survivors.[46]

As expected, our animals suffered significant post-stroke deterioration to their sensorimotor function that resolved over the course of 8 days. We were expecting

C21 to accelerate the rate of improvement as we saw with our diabetic animals, however this was not the case.

Similarly, our animals suffered from a very robust impairment to their long-term memory function at 4 weeks post-stroke. This is consistent with the results of our previously published studies. In one such study [24], long-term therapy with C21, started immediately after stroke, succeeded in rescuing this cognitive impairment. Although we used here the same dosing regimen, the only difference was the delayed administration in our case. This denotes that the aforementioned beneficial effect of C21 was likely due to its positive effect on earlier physiologic processes, reducing the impact of the initial insult.

When examining short-term working memory, our stroked animals showed an overall negative effect due to stroke on their performance in the NOR. However, there was no effect for time. This casts a shadow of doubt on the usefulness of this test as a measure of progressive post-stroke cognitive impairment in rodents, as the test was not initially designed to be done repeatedly. Sham animals in this test showed a continuous reduction of total exploration time with each time the test is repeated, even when the objects used were changed and with more than 2 weeks between each time point. In any case, delayed C21 failed to produce a beneficial effect in either the NOR or the exploration time.

The positive anti-inflammatory results of delayed C21 in our diabetic study[26] gave us hope that we would see a similar effect in hypertensive animals. Indeed, the amount of long-term inflammation found in the diabetic animals was much

higher than our relatively healthier hypertensive animals. This can be evident in the increased rate of mortality in the diabetic, compared to the almost non-existent mortality in our animals with similar insults. Delayed C21 failed to produce any anti-inflammatory effect in our young animals. A more clinically relevant approach for studying the long-term deleterious effects of post-stroke hypertension lies perhaps in using aged SHR animals, in which hypertension has more opportunity to produce long-lasting inflammatory damage.

As expected, our SHR animals showed a marked increase in the markers of oxidative-stress, cell-death and DNA damage following stroke. This will need to be further examined using immunohistochemistry through evaluating direct cellular damage, apoptosis, microgliosis and astrogliosis.

In summary, this rigorously conducted, randomized, blinded, controlled preclinical trial was essentially negative in supporting the future development of C21 as a therapeutic option for the prevention of long-term complications of ischemic stroke in young hypertensive animals. However, the results of this study open the door to further develop this animal model for studying long-term cognitive impairment and post-stroke depression, which would likely be magnified in aged animals.

**Sources of Funding:**

This study was supported by National Institute of Health (NIH) R01 NS104573 to Adviye Ergul and Susan C. Fagan (MPI).

**Competing Interests:**

No conflicts of interest, financial or otherwise, are declared by the authors.

**Figure (3.1): Experimental study design.** Experimental study design showing different time points for behavioral testing. Neuro-score: Modified Bederson Score for Neurological Assessment. ART: Adhesive Removal Test. NOR: Novel Object Recognition. PAT: Passive Avoidance Test. SPT: Sucrose Preference Test. SAC: Sacrifice and Tissue Collection.

Fig. 3.1

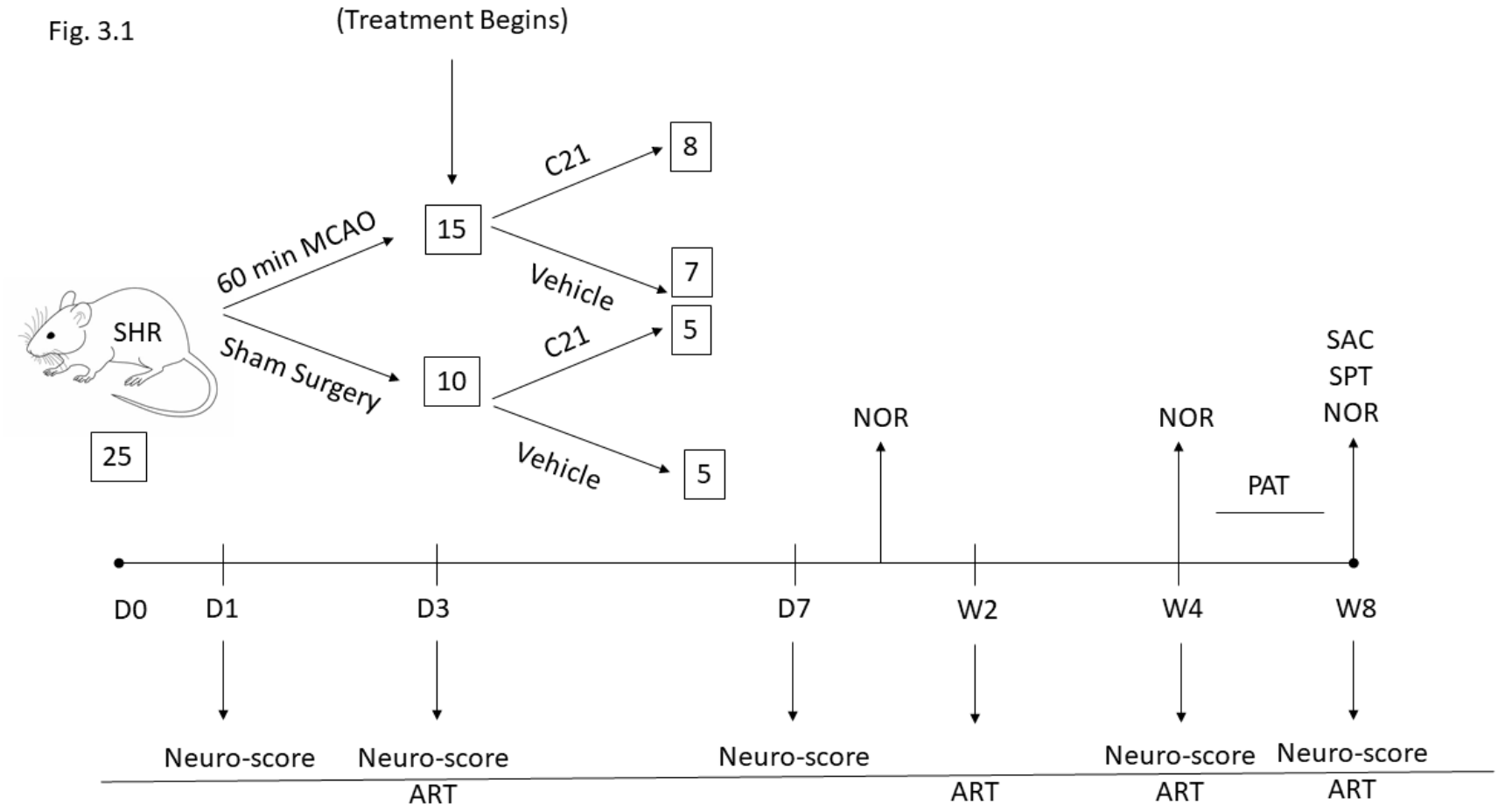
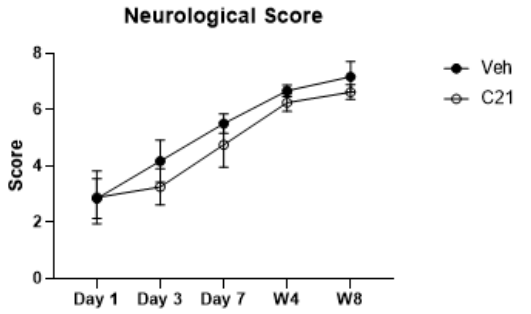

(Treatment Begins)
C21
8
15
60 min MCAO
Vehicle
7
SHR
Sham Surgery
C21
5
10
Vehicle
5
25
NOR
NOR
SAC
SPT
NOR
PAT
D0
D1
D3
D7
W2
W4
W8
Neuro-score
Neuro-score
ART
Neuro-score
ART
Neuro-score
ART
Neuro-score
ART

**Figure (3.2): Stroke induces sensorimotor deficits in SHRs that spontaneously recover, with no effect for delayed C21 treatment.** (A) Stroked treatment groups showed significantly reduced neurological scores after stroke that recovered over time. (2-way repeated measures ANOVA, $p(Time)<0.0001$, n=5-8 per group) (B) Stroked treatment groups showed significantly increased contact times in ART that improved over time compared to shams. (3-way mixed model ANOVA, $p(Time)<0.001$, $p(Surgery)<0.0001$, $p(Surgery \times Time)<0.0001$, n=5-8 per group). (C) Stroked treatment groups showed significantly increased contact times in ART that improved over time compared to shams. (3-way mixed model ANOVA, $p(Time)<0.0001$, $p(Surgery)<0.0001$, $p(Time \times Surgery)<0.0001$, n=5-8 per group).

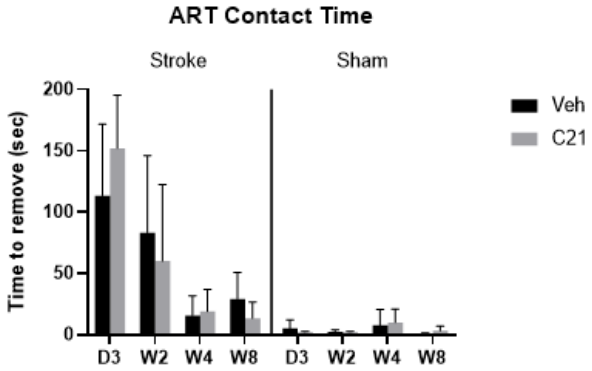
Fig. 3.2

**A**



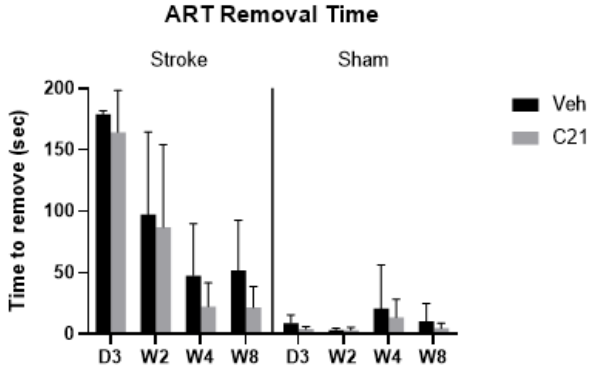

| | | | |
|---|---|---|---|
| Source of Variation | % of total variation | P value | P value summary |
| Time x Treatment | 0.5479 | 0.9137 | ns |
| Time | 49.03 | <0.0001 | **** |
| Treatment | 1.362 | 0.3963 | ns |

**B**




| | | |
|---|---|---|
| Fixed effects (type III) | P value | P value summary |
| Time | 0.0003 | *** |
| Surgery | <0.0001 | **** |
| Treatment | 0.9411 | ns |
| Time x Surgery | <0.0001 | **** |
| Time x Treatment | 0.4669 | ns |
| Surgery x Treatment | 0.9700 | ns |
| Time x Surgery x Treatment | 0.3634 | ns |

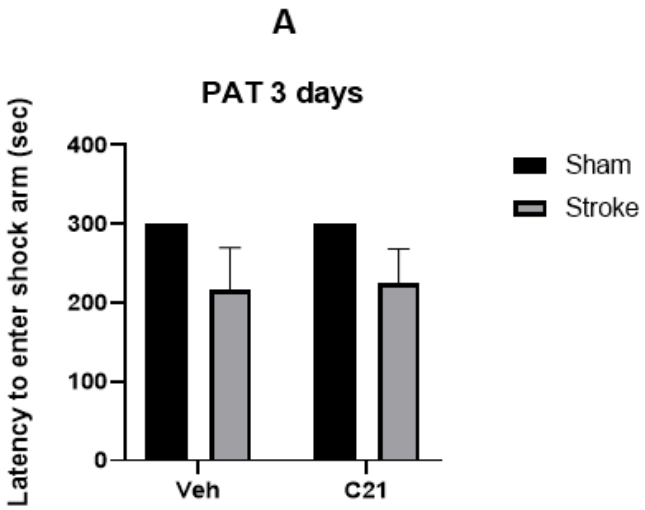
**C**



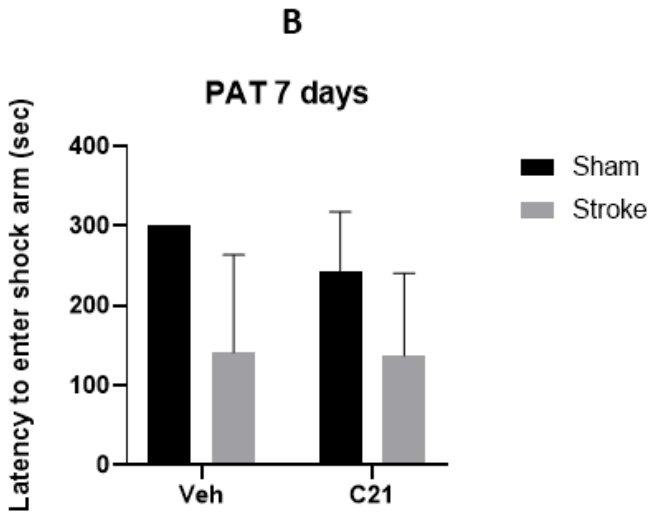

| | | |
|---|---|---|
| Fixed effects (type III) | P value | P value summary |
| Time | <0.0001 | **** |
| Surgery | <0.0001 | **** |
| Treatment | 0.1847 | ns |
| Time x Surgery | <0.0001 | **** |
| Time x Treatment | 0.9248 | ns |
| Surgery x Treatment | 0.4114 | ns |
| Time x Surgery x Treatment | 0.9859 | ns |

**Figure (3.3): Stroke induces the development of long-term memory impairments in SHRs that are not affected by delayed C21 treatment.** (A) Stroked treatment groups showed a trend of reduced latency to enter the shock arm 72-hours after the initial shock. (2-way ANOVA, p(Surgery)=0.06, n=5-8 per group) (B) Stroked treatment groups showed a significantly reduced latency to enter the shock arm 7 days after the initial shock. (2-way ANOVA, p(Surgery)<0.05, n=5-8 per group)

Fig. 3.3




| | | | |
|---|---|---|---|
| Source of Variation | % of total variation | P value | P value summary |
| Interaction | 0.04825 | 0.9156 | ns |
| Treatment | 0.04825 | 0.9156 | ns |
| Surgery | 16.16 | 0.0637 | ns |

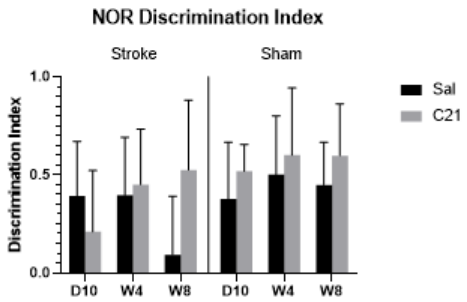



| | | | |
|---|---|---|---|
| Source of Variation | % of total variation | P value | P value summary |
| Interaction | 1.359 | 0.5952 | ns |
| Treatment | 1.798 | 0.5418 | ns |
| Surgery | 32.72 | 0.0184 | * |

**Figure (3.4): Stroke induces the development of working memory impairments and anhedonic behavior in SHRs, with no effect for delayed C21 treatment.** (A) Stroked treatment groups showed significantly reduced recognition index in NOR that did not change over time compared to shams. (3-way mixed model ANOVA, $p(Surgery)<0.05$, n=5-8 per group). (B) Stroked treatment groups showed significantly reduced object exploration time in NOR that did not change over time compared to shams. Sham animals showed a reduction of object exploration time over time. (3-way mixed model ANOVA, $p(Surgery)<0.05$, $p(Time \times Surgery)<0.05$, n=5-8 per group). (C) Stroked treatment groups showed a significantly reduced sucrose preference in SPT. (2-way ANOVA, $p(Surgery)<0.01$, n=5-8 per group)

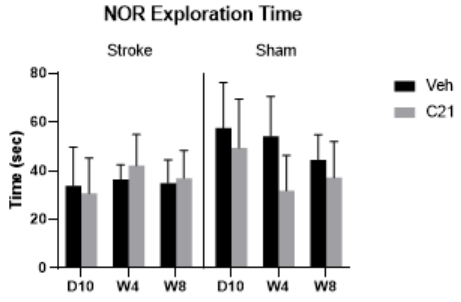
Fig. 3.4

**A**



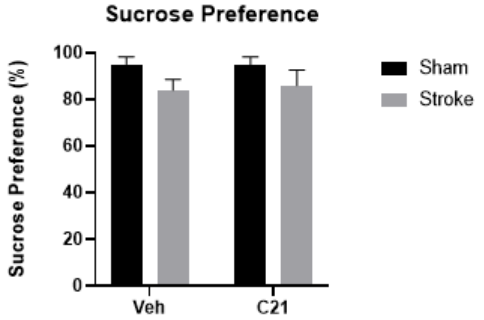

| | | |
|---|---|---|
| Fixed effects (type III) | P value | P value summary |
| Time | 0.4637 | ns |
| Surgery | 0.0326 | * |
| Treatment | 0.1254 | ns |
| Time x Surgery | 0.8760 | ns |
| Time x Treatment | 0.2207 | ns |
| Surgery x Treatment | 0.8516 | ns |
| Time x Surgery x Treatment | 0.2590 | ns |

**B**




| Source of Variation | % of total variation | P value | P value summary |
|---|---|---|---|
| Time | 1.442 | 0.3895 | ns |
| Surgery | 10.51 | 0.0348 | * |
| Treatment | 3.244 | 0.2227 | ns |
| Time x Surgery | 6.796 | 0.0172 | * |
| Time x Treatment | 0.5758 | 0.6852 | ns |
| Surgery x Treatment | 5.272 | 0.1243 | ns |
| Time x Surgery x Treatment | 2.622 | 0.1889 | ns |

**C**

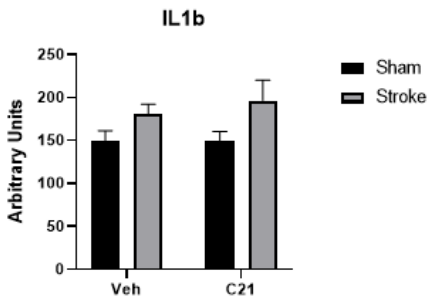



| Source of Variation | % of total variation | P value | P value summary |
|---|---|---|---|
| Interaction | 0.6911 | 0.6654 | ns |
| Treatment | 0.3829 | 0.7472 | ns |
| Surgery | 50.09 | 0.0021 | ** |

**Figure (3.5): Stroke increases cytokine concentration in brain homogenates, with no effect for delayed C21 treatment.** (A) Stroked treatment groups showed a significantly increased IL-1β levels in ELISA compared to shams. (2-way ANOVA, p(Surgery)<0.05, n=5-8 per group) (B) No difference between groups in TNFα levels in ELISA. (2-way ANOVA, n=5-8 per group) (C) Stroked treatment groups showed a significantly increased IL-10 levels in ELISA compared to shams. (2-way ANOVA, p(Surgery)<0.05, n=5-8 per group).

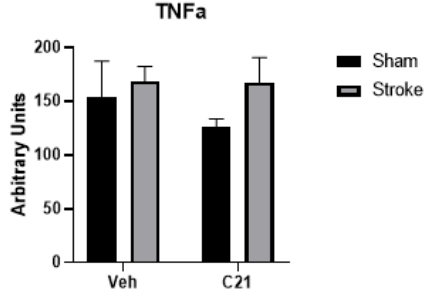
Fig. 3.5

A



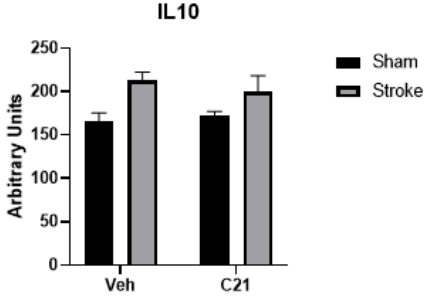

| Source of Variation | % of total variation | P value | P value summary |
|---|---|---|---|
| Interaction | 0.6123 | 0.6988 | ns |
| Treatment | 0.7026 | 0.6786 | ns |
| Surgery | 17.81 | 0.0470 | * |

B




| Source of Variation | % of total variation | P value | P value summary |
|---|---|---|---|
| Interaction | 1.527 | 0.5679 | ns |
| Treatment | 1.866 | 0.5283 | ns |
| Surgery | 6.474 | 0.2458 | ns |

C

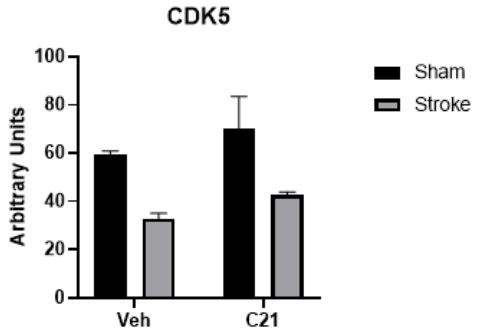



| Source of Variation | % of total variation | P value | P value summary |
|---|---|---|---|
| Interaction | 1.638 | 0.5136 | ns |
| Treatment | 0.2273 | 0.8069 | ns |
| Surgery | 24.84 | 0.0175 | * |

**Figure (3.6): Stroke increases the expression levels of markers of oxidative stress, cell-death and DNA damage, with no effect for delayed C21 treatment.** (A) Stroked treatment groups showed a significantly reduced levels of CDK5 in WB. (2-way ANOVA, $p(Surgery)<0.01$, n=3-4 per group) (B) Stroked treatment groups showed a significantly increased ratio of active/inactive PAPR1 in WB. (2-way ANOVA, $p(Surgery)<0.01$, n=3-4 per group) (C) Stroked treatment groups showed a significantly increased levels of HMGB1 in WB. (2-way ANOVA, $p(Surgery)<0.05$, n=3-4 per group) (D) Representative images of Western blots.

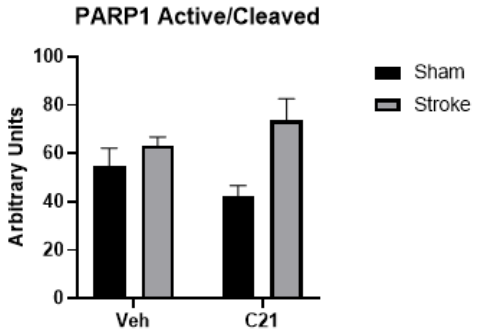
Fig. 3.6

A



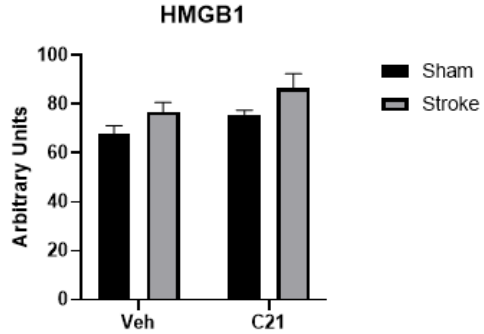

| Source of Variation | % of total variation | P value | P value summary |
|---|---|---|---|
| Interaction | 0.005662 | 0.9695 | ns |
| Treatment | 6.712 | 0.2052 | ns |
| Surgery | 49.27 | 0.0038 | ** |

B



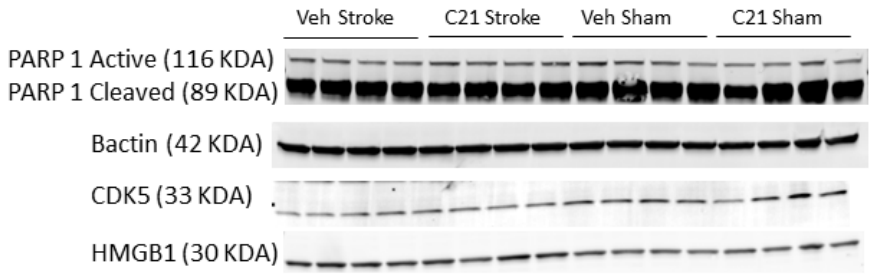

| Source of Variation | % of total variation | P value | P value summary |
|---|---|---|---|
| Interaction | 12.79 | 0.1014 | ns |
| Treatment | 0.08950 | 0.8839 | ns |
| Surgery | 39.14 | 0.0096 | ** |

C




| Source of Variation | % of total variation | P value | P value summary |
|---|---|---|---|
| Interaction | 0.3490 | 0.7952 | ns |
| Treatment | 20.51 | 0.0662 | ns |
| Surgery | 26.89 | 0.0396 | * |

D



## References


1. Benjamin, E.J., et al., *Heart Disease and Stroke Statistics-2018 Update: A Report From the American Heart Association.* Circulation, 2018. **137**(12): p. e67-e492.
2. Towfighi, A. and J.L. Saver, *Stroke declines from third to fourth leading cause of death in the United States: historical perspective and challenges ahead.* Stroke, 2011. **42**(8): p. 2351-5.
3. Sun, J.H., L. Tan, and J.T. Yu, *Post-stroke cognitive impairment: epidemiology, mechanisms and management.* Ann Transl Med, 2014. **2**(8): p. 80.
4. Woitzik, J., T. Back, and C. Thome, *Flow-dependent versus spreading-like impairment of brain tissue integrity during focal cerebral ischemia and its consequences for neuroprotective strategies.* Front Biosci, 2008. **13**: p. 1500-6.
5. Leys, D., et al., *Poststroke dementia.* Lancet Neurol, 2005. **4**(11): p. 752-9.
6. Obaid, M., et al., *Long-Term Outcomes in Stroke Patients with Cognitive Impairment: A Population-Based Study.* Geriatrics (Basel), 2020. **5**(2).
7. Arba, F., et al., *Enlarged perivascular spaces and cognitive impairment after stroke and transient ischemic attack.* Int J Stroke, 2018. **13**(1): p. 47-56.
8. Sahathevan, R., A. Brodtmann, and G.A. Donnan, *Dementia, stroke, and vascular risk factors; a review.* Int J Stroke, 2012. **7**(1): p. 61-73.
9. Tu, J.V., *Reducing the global burden of stroke: INTERSTROKE.* Lancet, 2010. **376**(9735): p. 74-5.

10. Fouda, A.Y., et al., *Renin-angiotensin system as a potential therapeutic target in stroke and retinopathy: experimental and clinical evidence.* Clin Sci (Lond), 2016. **130**(4): p. 221-38.

11. Paul, M., A. Poyan Mehr, and R. Kreutz, *Physiology of local renin-angiotensin systems.* Physiol Rev, 2006. **86**(3): p. 747-803.

12. Jackson, L., et al., *Within the Brain: The Renin Angiotensin System.* International journal of molecular sciences, 2018. **19**(3): p. 876.

13. Ahmed, H.A., et al., *Role of angiotensin system modulation on progression of cognitive impairment and brain MRI changes in aged hypertensive animals - A randomized double- blind pre-clinical study.* Behav Brain Res, 2018. **346**: p. 29-40.

14. Xia, H. and E. Lazartigues, *Angiotensin-converting enzyme 2 in the brain: properties and future directions.* J Neurochem, 2008. **107**(6): p. 1482-94.

15. Zhang, T.L., et al., *The neuroprotective effect of losartan through inhibiting AT1/ASK1/MKK4/JNK3 pathway following cerebral I/R in rat hippocampal CA1 region.* CNS Neurosci Ther, 2012. **18**(12): p. 981-7.

16. Labandeira-Garcia, J.L., et al., *Brain Renin-Angiotensin System and Microglial Polarization: Implications for Aging and Neurodegeneration.* Front Aging Neurosci, 2017. **9**: p. 129.

17. Mateos, L., M.J. Perez-Alvarez, and F. Wandosell, *Angiotensin II type-2 receptor stimulation induces neuronal VEGF synthesis after cerebral ischemia.* Biochim Biophys Acta, 2016. **1862**(7): p. 1297-308.

18. Maul, B., et al., *Impaired spatial memory and altered dendritic spine morphology in angiotensin II type 2 receptor-deficient mice.* J Mol Med (Berl), 2008. **86**(5): p. 563-71.

19. Villar-Cheda, B., et al., *Nigral and striatal regulation of angiotensin receptor expression by dopamine and angiotensin in rodents: implications for progression of Parkinson's disease.* Eur J Neurosci, 2010. **32**(10): p. 1695-706.

20. Steckelings, U.M., et al., *AT2 receptor agonists: hypertension and beyond.* Current opinion in nephrology and hypertension, 2012. **21**(2): p. 142-146.

21. Alhusban, A., et al., *Compound 21 is pro-angiogenic in the brain and results in sustained recovery after ischemic stroke.* J Hypertens, 2015. **33**(1): p. 170-80.

22. Fouda, A.Y., et al., *Role of interleukin-10 in the neuroprotective effect of the Angiotensin Type 2 Receptor agonist, compound 21, after ischemia/reperfusion injury.* Eur J Pharmacol, 2017. **799**: p. 128-134.

23. Eldahshan, W., et al., *Angiotensin II type 2 receptor stimulation with compound 21 improves neurological function after stroke in female rats: a pilot study.* Am J Physiol Heart Circ Physiol, 2019. **316**(5): p. H1192-h1201.

24. Ahmed, H.A., et al., *RAS modulation prevents progressive cognitive impairment after experimental stroke: a randomized, blinded preclinical trial.* Journal of neuroinflammation, 2018. **15**(1): p. 229-229.

25. Ahmed, H.A., et al., *Angiotensin receptor (AT2R) agonist C21 prevents cognitive decline after permanent stroke in aged animals-A randomized double- blind pre-clinical study.* Behav Brain Res, 2019. **359**: p. 560-569.

26. Jackson, L., et al., *Delayed Administration of Angiotensin II Type 2 Receptor (AT2R) Agonist Compound 21 Prevents the Development of Post-stroke Cognitive Impairment in Diabetes Through the Modulation of Microglia Polarization.* Transl Stroke Res, 2019.

27. Ishrat, T., et al., *Dose-response, therapeutic time-window and tPA-combinatorial efficacy of compound 21: A randomized, blinded preclinical trial in a rat model of thromboembolic stroke.* J Cereb Blood Flow Metab, 2019. **39**(8): p. 1635-1647.

28. Chen, J., Y. Li, and M. Chopp, *Intracerebral transplantation of bone marrow with BDNF after MCAo in rat.* Neuropharmacology, 2000. **39**(5): p. 711-6.

29. Serchov, T., D. van Calker, and K. Biber, *Sucrose Preference Test to Measure Anhedonic Behaviour in Mice.* Bio-protocol, 2016. **6**(19): p. e1958.

30. Sobowale, O.A., et al., *Interleukin-1 in Stroke: From Bench to Bedside.* Stroke, 2016. **47**(8): p. 2160-7.

31. Wilkins, H.M. and R.H. Swerdlow, *TNFα in cerebral ischemia: another stroke against you?* Journal of neurochemistry, 2015. **132**(4): p. 369-372.

32. Garcia, J.M., et al., *Role of Interleukin-10 in Acute Brain Injuries.* Frontiers in neurology, 2017. **8**: p. 244-244.

33. Meyer, D.A., et al., *Ischemic stroke injury is mediated by aberrant Cdk5.* The Journal of neuroscience : the official journal of the Society for Neuroscience, 2014. **34**(24): p. 8259-8267.

34. Krietsch, J., et al., *PARP activation regulates the RNA-binding protein NONO in the DNA damage response to DNA double-strand breaks.* Nucleic Acids Res, 2012. **40**(20): p. 10287-301.

35. Masson, G.S., et al., *Aerobic training normalizes autonomic dysfunction, HMGB1 content, microglia activation and inflammation in hypothalamic paraventricular nucleus of SHR.* Am J Physiol Heart Circ Physiol, 2015. **309**(7): p. H1115-22.

36. Lapchak, P.A., J.H. Zhang, and L.J. Noble-Haeusslein, *RIGOR guidelines: escalating STAIR and STEPS for effective translational research.* Translational stroke research, 2013. **4**(3): p. 279-285.

37. Lapchak, P.A., *Scientific rigor recommendations for optimizing the clinical applicability of translational research.* Journal of neurology & neurophysiology, 2012. **3**.

38. Albers, G.W., et al., *Stroke Treatment Academic Industry Roundtable (STAIR) recommendations for maximizing the use of intravenous thrombolytics and expanding treatment options with intra-arterial and neuroprotective therapies.* Stroke, 2011. **42**(9): p. 2645-2650.

39. Fouda, A.Y., et al., *Role of interleukin-10 in the neuroprotective effect of the Angiotensin Type 2 Receptor agonist, compound 21, after ischemia/reperfusion injury.* European journal of pharmacology, 2017. **799**: p. 128-134.

40. Alhusban, A., et al., *Compound 21 is pro-angiogenic in the brain and results in sustained recovery after ischemic stroke.* Journal of hypertension, 2015. **33**(1): p. 170-180.

41. Joseph, J.P., et al., *The angiotensin type 2 receptor agonist Compound 21 elicits cerebroprotection in endothelin-1 induced ischemic stroke.* Neuropharmacology, 2014. **81**: p. 134-141.

42. McCarthy, C.A., et al., *Direct angiotensin AT2 receptor stimulation using a novel AT2 receptor agonist, compound 21, evokes neuroprotection in conscious hypertensive rats.* PloS one, 2014. **9**(4): p. e95762.

43. Schwengel, K., et al., *Angiotensin AT2-receptor stimulation improves survival and neurological outcome after experimental stroke in mice.* Journal of Molecular Medicine, 2016. **94**(8): p. 957-966.

44. Levine, D.A., et al., *Trajectory of Cognitive Decline After Incident Stroke.* Jama, 2015. **314**(1): p. 41-51.

45. Wiesmann, M., A.J. Kiliaan, and J.A. Claassen, *Vascular aspects of cognitive impairment and dementia.* J Cereb Blood Flow Metab, 2013. **33**(11): p. 1696-706.

46. Filipska, K., et al., *Are Depression and Dementia a Common Problem for Stroke Older Adults? A Review of Chosen Epidemiological Studies.* Psychiatr Q, 2020.

# CHAPTER 4

# INTEGRATED DISCUSSION

The principle aim of this dissertation was to examine the role played by hypertension, the most common comorbidity to ischemic stroke, in the development of post-stroke cognitive impairment (PSCI), one of the most common and yet under-diagnosed complications of ischemic stroke. In addition to that, we were aiming to uncover some of the cellular and molecular mechanisms that contribute to the progressive accumulation of cognitive impairment, contrasting that to the self-resolving sensorimotor deficits. Lastly, we aimed to examine the therapeutic potential of delayed AT2R stimulation with the novel non-peptide agonist, compound 21, in neurorestoration and stopping or delaying the progression of post-stroke cognitive impairment. We hypothesized that hypertensive animals would develop PSCI at a rate higher than their normotensive controls, even as the animals recovered from their sensorimotor deficits. We believed that these phenomena would be mitigated by delayed RAS modulation, by a mechanism involving reduction in chronic inflammation and reduced neurodegeneration.

Our hypotheses were developed based on robust preliminary data from multiple different animal models and treatment regimes. In both male and female young SHRs, we

demonstrated preserved cognition at 30 days after stroke when animals were treated with an AT2R agonist (C21), starting at 24 hours after stroke.[1] This protection was associated with a reduction in microglial activation in the males and an increased vascular density in the ovariectomized females (Eldahshan et al., currently under review). In rats made diabetic by streptozotocin injection and a high fat diet, the same treatment initiated at 3 days after stroke, robustly reduced microglial and macrophage polarization to the inflammatory phenotype and prevented delayed cognitive impairment at 8 weeks.[2] When microglia were depleted genetically in the same model, PSCI was reduced in the diabetic animals, proving a causal relationship between microglia and cognitive impairment.[3] The diabetic model we used was limited by a high early mortality, however, lessening the translatability of our findings. Since hypertension is the most common risk factor for ischemic stroke, we needed to know whether delayed progressive PSCI occurs and whether it can be prevented with intervention.

First, we have confirmed the work of others suggesting that hypertensive animals are uniquely vulnerable to ischemic damage, compared to normotensive animals. Our study was unique, however, in that we were able to document the longer-term effects of these differences in our animals. We clearly demonstrated long-term gross changes to brain morphology, including enlarged ventricles and loss of grey matter volume, 3 months after an initial insult of a size comparable to that of normotensive animals. We also showed that these changes in brain morphology reflected deeper changes in inflammation, oxidative stress, cell-death and DNA damage, both on the cellular and the molecular levels. This was reflected in a unique pattern of delayed apoptosis in the peri-ischemic

areas in hypertensive animals, as well as an increase in HMGB1, active PARP1 and active MMP9 protein levels in brain lysates of hypertensive animals that did not appear in normotensive animals. We have interpreted these changes as evidence of ongoing neurodegeneration. We were unable to link these findings to worsened or progressive post-stroke cognitive impairment in hypertensive animals compared to normotensive animals, however. Although we did see evidence of cognitive impairment at 4 weeks after stroke in the hypertensive animals, it was not different from that experienced by the normotensive controls. Our repeated measurement of spatial memory, using the NOR, did not reveal impairment in either of the groups at any time. Our experience with the NOR revealed that the assessment of memory relies entirely on the animals' interest in the novel objects, which wanes with each repetition (even when the objects are changed between trials) and their overall mood. In our hypertensive animals, we saw a dramatic reduction in exploration times after stroke, which could have been magnified by our protocol of requiring single-housing (attempting to reduce wound injury). The NOR was never intended to be used to monitor cognitive function changes over time and is likely not sensitive enough to do so. We saw similar limitations in the MWM, however, where repeated assessments failed to identify any cognitive impairment. The only test that has reliably proved sensitive to cognitive impairment after stroke, is the passive avoidance task (PAT). In all of the experiments we have conducted, we are able to show defects in PAT at 4 weeks after stroke when compared to sham controls. This test involves an aversive stimulus that is remembered for a long time in normal animals and is not amenable to repetition.

One of the other main limitations of our first study was our utilization of young hypertensive animals instead of using aged ones. We expect the accumulating damage as a result of untreated hypertension to translate into worsening of progressively accumulating post-stroke cognitive deficits. Indeed, many of the cognitive deficits that we were able to observe in aged hypertensive animals, under a much weaker ischemic insult (namely, unilateral common carotid artery occlusion),[4] were not visible in this model that suffered from a much stronger ischemic insult, pointing towards the role aging and accumulation of hypertensive damage play in the development of post-stroke cognitive impairment.

In the second part of these series of investigation we turned the focus towards the development of C21 as a therapeutic neurorestorative agent for the treatment of post-stroke cognitive impairment in hypertensive animals. Over the last few years, AT2R activation has shown promising results in producing neuroprotective and pro-angiogenic effects in different models of cerebral ischemia by our lab and others (for example, [5-8] reviewed in detail in chapter 1), however, when a large, blinded, pre-clinical trial was carried out to examine different doses and different time windows of C21 treatment in embolic stroke, the results were disappointing, finding very little evidence of benefit for C21 treatment alone or in combination with tPA.[9] Therefore, our focus shifted to investigating the effects of C21 in preventing or slowing the accumulating neurodegenerative and neuroinflammatory damage post-stroke.

In our second blinded, controlled, pre-clinical trial, delayed treatment with C21 failed to show any benefit in improving the stroke outcome in young hypertensive animals. There was no improvement in sensorimotor deficits measured by neurological scoring and adhesive removal test, neither was there any improvement in post-stroke cognitive impairment measured by novel object recognition, passive avoidance and sucrose preference tests (the latter being a newly implemented model in our lab to investigate post-stroke depression). These results were affirmed by an accompanying lack of positive effects on the inflammatory profile, measured through the levels of IL1-β, TNFα and IL10. Interestingly, a previous acute study of C21 in our lab showed that C21 produced an anti-inflammatory effect, reducing TNFα through IL10.[10] In addition to that, C21 did not produce a beneficial effect on markers of cell-death, oxidative stress and DNA damage (Cdk5, PARP1 and HMGB1).

However, while the results of our second study were disappointing in developing a new therapeutic strategy for post-stroke cognitive impairment, it succeeded in establishing the presence of long-term post-stroke cognitive deficits in SHRs. Over the course of our 8-week study, our animals developed significant sensorimotor deficits that spontaneously resolved. By the end of the study, the sensorimotor abilities of the animals recovered to near baseline. However, these same animals suffered from substantial impairment to their cognitive abilities that remained until the end of the study, mainly in long-term memory and post-stroke depression, and to a lesser extent to their working memory.

In conclusion, this dissertation provides a better understanding to the role hypertension plays in the development of post stroke cognitive impairment. It is now clear that ongoing,

accumulating cell death and gray matter atrophy, as a result of accumulating oxidative stress and DNA damage, uniquely affects hypertensive animals in the chronic post-stroke period. It also sheds the light on post-stroke depression, a phenomenon that affects a great number of stroke survivors and its ties to hypertension. We have also shown the difficulty in demonstrating the progressive nature of cognitive deficits in experimental animals with the tools available. Most have been developed as a one-time test with learning effects and rapid decreases in sensitivity limiting repeatability. The development of more sensitive tests, amenable to repeated testing, is of utmost importance. Otherwise, large cohort studies, with a single follow-up time in each cohort, would be needed to definitely prove the progressive nature of post-stroke cognitive impairment in experimental stroke models.

It shows that the beneficial non-acute effects of AT2R stimulation after stroke might be more valuable, as a therapeutic strategy, in patients suffering from high levels of background systemic inflammation, such as diabetic and older hypertensive patients, as opposed to young hypertensive patients. It proves that there is still a long way to go to produce better treatments to reduce post-stroke disability, beyond treatments that target reducing the initial ischemic insult.

## References


1. Ahmed, H.A., et al., *RAS modulation prevents progressive cognitive impairment after experimental stroke: a randomized, blinded preclinical trial.* Journal of neuroinflammation, 2018. **15**(1): p. 229-229.
2. Jackson, L., et al., *Delayed Administration of Angiotensin II Type 2 Receptor (AT2R) Agonist Compound 21 Prevents the Development of Post-stroke Cognitive Impairment in Diabetes Through the Modulation of Microglia Polarization.* Transl Stroke Res, 2019.
3. Jackson, L., et al., *Microglia knockdown reduces inflammation and preserves cognition in diabetic animals after experimental stroke.* Journal of Neuroinflammation, 2020. **17**(1): p. 137.
4. Ahmed, H.A., et al., *Role of angiotensin system modulation on progression of cognitive impairment and brain MRI changes in aged hypertensive animals - A randomized double-blind pre-clinical study.* Behavioural brain research, 2018. **346**: p. 29-40.
5. Alhusban, A., et al., *Compound 21 is pro-angiogenic in the brain and results in sustained recovery after ischemic stroke.* J Hypertens, 2015. **33**(1): p. 170-80.
6. Fouda, A.Y., et al., *Renin-angiotensin system as a potential therapeutic target in stroke and retinopathy: experimental and clinical evidence.* Clin Sci (Lond), 2016. **130**(4): p. 221-38.
7. McCarthy, C.A., et al., *Direct angiotensin AT2 receptor stimulation using a novel AT2 receptor agonist, compound 21, evokes neuroprotection in conscious hypertensive rats.* PloS one, 2014. **9**(4): p. e95762.

8. McCarthy, C.A., et al., *Angiotensin II type 2 receptor stimulation initiated after stroke causes neuroprotection in conscious rats.* Hypertension, 2012. **60**(6): p. 1531-1537.

9. Ishrat, T., et al., *Dose–response, therapeutic time-window and tPA-combinatorial efficacy of compound 21: A randomized, blinded preclinical trial in a rat model of thromboembolic stroke.* Journal of Cerebral Blood Flow & Metabolism, 2018. **39**(8): p. 1635-1647.

10. Fouda, A.Y., et al., *Role of interleukin-10 in the neuroprotective effect of the Angiotensin Type 2 Receptor agonist, compound 21, after ischemia/reperfusion injury.* European journal of pharmacology, 2017. **799**: p. 128-134.