The Neuroprotective Compound P7C3-A20 Promotes Neurogenesis and Improves Functional Outcomes After Focal Cerebral Ischemia

Zachary Balmuth-Loris
University of Miami, zbalmuth@gmail.com

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THE NEUROPROTECTIVE COMPOUND P7C3-A20 PROMOTES NEUROGENESIS AND IMPROVES FUNCTIONAL OUTCOMES AFTER FOCAL CEREBRAL ISCHEMIA

By
Zachary Balmuth-Loris

A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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THE NEUROPROTECTIVE COMPOUND P7C3-A20 PROMOTES NEUROGENESIS AND IMPROVES FUNCTIONAL OUTCOMES AFTER FOCAL CEREBRAL ISCHEMIA

Zachary Balmuth-Loris

Approved:

W. Dalton Dietrich, Ph.D.  
Professor of Neurological Surgery, Neurology, and Cell Biology and Anatomy

Peter Larsson, Ph.D.  
Professor of Physiology and Biophysics

Thomas Sick, Ph.D.  
Professor of Neurology and Physiology/Biophysics  
Basic Science Division

Miguel Perez-Pinzon, Ph.D.  
Professor of Neurology/Neuroscience

Robert Keane, Ph.D.  
Professor of Physiology and Biophysics

Guillermo Prado, Ph.D.  
Dean of the Graduate School

Tatjana Rundek, M.D., Ph.D.  
Professor of Neurology
Ischemic stroke is the second leading cause of death worldwide and the leading cause of adult long-term disability in the United States. Despite its prevalence, there are few therapeutic interventions available. The neuroprotective compound P7C3-A20 (A20) has been shown to reduce mature neuronal cell death while also increasing the net magnitude of postnatal neurogenesis in models of neurodegeneration and acute brain injury. A20 compounds demonstrate protection by enhancing the flux of nicotinamide adenine dinucleotide (NAD) in mammalian cells, a proposed therapeutic approach to treating cerebral ischemia. The studies carried out in this dissertation sought to investigate the effectiveness of A20 treatment after focal cerebral ischemia by assessing subacute and chronic histopathological and behavioral outcomes, as well as ischemia-induced neurogenesis. In the first series of experiments, rats underwent a weeklong course of A20 or vehicle treatment, beginning immediately after a 90 minute unilateral transient middle cerebral artery occlusion (tMCAO). A20-treated rats performed significantly better than vehicle-treated controls in sensorimotor cylinder and grid-walk tasks, and in a chronic test of spatial learning and memory. These behavioral improvements with A20 treatment were correlated with significantly decreased cortical and hippocampal atrophy as well as increased neurogenesis in the subventricular zone and hippocampal dentate gyrus subgranular zone. Furthermore, cerebral ischemia
significantly depleted NAD in the cortex, but treating with A20 restored cortical NAD levels.

After demonstrating efficacy of A20 treatment at an early, post-ischemic timepoint, we then sought to examine A20’s treatment window of opportunity. Due to a limited therapeutic window, current stroke pharmacological treatment is rarely administered to ischemic patients. Therefore, we investigated a more clinically relevant time point and again treated tMCAO rats for one week with A20, beginning either immediately (iA20) or at a delayed point (dA20) 6 hours post-reperfusion. dA20 treatment significantly reduced ischemia-induced sensorimotor deficits in motor coordination and limb-use asymmetry as well as cognitive deficits in hippocampal-dependent spatial learning, memory retention, and working memory. In the cerebral cortex, dA20 treatment significantly increased tissue sparing 7 weeks after stroke and reduced infarct volumes 48 hours after reperfusion compared to vehicle-treated animals. At 48 hours after injury, there was no change in striatal infarct volumes between tMCAO groups. However, when tissue volume was reassessed at 7 weeks, A20-treated animals had a significant increase in striatal tissue volume, suggesting that A20’s protection in the ischemic striatum requires an extended treatment regimen. In the hippocampus, only iA20-treated animals had a significant increase in tissue sparing compared to vehicle-treated stroke animals. This translated into minimal hippocampal-dependent behavioral improvements with dA20 treatment. However, all rats treated with dA20 did demonstrate a significant improvement in both sensorimotor tasks compared to vehicle controls, suggesting a somatosensory driven recovery.
Overall, our studies show that A20 treatment is an effective strategy against focal cerebral ischemia by mitigating chronic neurodegeneration, enhancing repair, and rescuing stroke-induced behavioral deficits when treated at a clinically relevant time point. Therefore, treatment with A20 compounds represent a novel therapeutic approach to safely augment NAD tissue levels, promoting two independent processes critical to protecting the brain from ischemic stroke; mature neuron survival and postnatal hippocampal neurogenesis throughout the post-ischemic brain.
To my family for their unwavering support, my friends for putting up with me, and my fianceé Rebecca for not leaving me.
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Chapter 1

Introduction

1.1 Cerebral Ischemia and Stroke

Prevalence

Stroke is the second leading cause of death worldwide, afflicting 15 million people annually \(^1\). Of those 15 million people, 6 million will die and 5 million will be left permanently disabled, making stroke the principal cause of adult long-term disability in the United States (US) \(^2\). Unsurprisingly, stroke incurs a huge financial burden, costing the US taxpayers $71.55 billion in 2010 \(^3\). With an aging population, the American Heart Association estimates that the annual stroke related medical costs will increase 129% to $183.13 billion by 2030 \(^3\). The incidence of stroke in developing countries is even greater than in developed countries, resulting in 3 out of 4 patients living with some level of disability \(^4\). To make matters worse, the occurrence of stroke is suspected to rise in Latin America, the Middle East, and sub-Saharan Africa, which has tripled the estimated mortality rate over the next 20 years \(^1\). Risk factors are a major reason for the increased prevalence of stroke. Hackman and Spence estimate that the chance of suffering a stroke would drop nearly 80% by just targeting risk factors such as diet, tobacco use, diabetes, and lack of exercise \(^5\).

A stroke occurs when there is severely reduced cerebral blood flow to an area of the brain, resulting in ischemic cell death and infarction. This can manifest as either an ischemic stroke (cerebral ischemia) or as a hemorrhagic stroke. Ischemic stroke occurs when a vessel is occluded, whereas hemorrhagic stroke occurs when there is bleeding into or around the brain \(^6\). Ischemic stroke accounts for 87% of all strokes in the United
States and is caused by an embolism or thrombosis. The location of the blocked artery will dictate which brain tissue will be hypoperfused and thus, what functional deficits will emerge. Since the middle cerebral artery (MCA) is the largest and most often occluded cerebral artery, it generates the largest infarct. The MCA supplies blood flow to the inferior regions of the frontal and parietal lobes, superior regions of the temporal and occipital lobes, and the striatum. Therefore, a MCA occlusion (MCAO) can cause a myriad of deficits including sensory, motor, and cognitive dysfunction.

Due to collateral circulation, blood flow is not uniform throughout the injury site, which causes varying degrees of damage. The center of the brain ischemia is the infarct core, which will have pan-necrosis because blood flow is reduced below 20%. In contrast, the peri-infarct (penumbra) area, can have enhanced cell survival because blood flow is increased in a graded fashion. The blood flow in the penumbra is sufficient that neurons can maintain their ion gradients, yet reduced enough to impede normal neuronal function. Cell in the penumbra are still incredibly vulnerable and over time may die, thus expanding the ischemic core. One pathophysiological process that contributes to the expansion of the ischemic core is cortical spreading depression (CSD). Initiated by cerebral ischemia, a CSD is a wave of depolarizing cells that initiates from the fringes of the ischemic core and propagates through tissue in the penumbra and into non-ischemic cerebral gray matter. A consequence of CSD is that these waves substantially reduce blood flow. CSD is an energy consuming event that will exacerbate tissue damage during focal ischemia because blood flow cannot be restored. Moreover, the frequency and duration of CSDs directly correlate to core volume. If blood flow is not restored,
the penumbra may become incorporated into infarcted tissue, emphasizing the importance of time-sensitive treatments.  

Pathophysiology of ischemic stroke

During focal cerebral ischemia, severely reduced blood flow initiates a complex series of events known as the ischemic cascade. Although the cascade appears to be sequential, it does not always occur in a linear progression. One event can trigger multiple actions, can occur in different progressions, or can even initiate previous events.

The ischemic cascade begins when cerebral blood flow is significantly reduced, which inhibits energy production. Without receiving oxygen and glucose, cells cannot generate enough ATP to maintain energy homeostasis. The metabolic demands of normal neuronal function are so great, that within 4-5 minutes of cerebral ischemia, neurons will have exhausted all their ATP supplies. Oxygen is depleted within seconds because it cannot be stored, whereas glucose reserves can last up to 4 minutes. Without oxygen, cells will begin to switch to anaerobic metabolism, which produces lactic acid and a buildup of H+. Cells will try to restore the local pH by first using their Na+/H+ antiporters to remove excess H+, and then by using their Na+/Ca+ antiporters to replace Na+ ions. This exchange of ions causes intracellular calcium levels to rise. Since ATP production is inhibited, cells cannot remove the excess calcium with their ATP-dependent transport pumps, which are crucial to maintaining ion homeostasis, especially during ischemic stress. Homeostasis is so critical to cellular function that 50-75% of all energy production is used exclusively for maintaining ion gradients across the cellular membrane under basal conditions.
With Na+/K+ ATPase pumps inoperative, extracellular potassium begin to steadily rise, reducing the transmembrane K+ gradient. Prolonged ischemia will lead to a large increase in extracellular potassium, causing a total depolarization\textsuperscript{22,23}. This increased membrane potential activates voltage-dependent calcium channels, which release excitatory amino acids such as glutamate into the extracellular space\textsuperscript{16}. Under normal conditions, the glutamate transporters would quickly remove excess glutamate from the postsynaptic cleft\textsuperscript{24}. However, since these active transport pumps requires ATP, glutamate will accumulate and cause its \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and \(N\)-methyl-D-aspartate (NMDA) receptors to be continuously stimulated, causing an influx of Ca+ ions\textsuperscript{25}. With already high levels of intracellular calcium, additional Ca+ ions now flood into the cell via the calcium permeable NMDA receptors\textsuperscript{26}. This massive influx of Ca+ through glutamate stimulation results in excitotoxicity\textsuperscript{27}. In order to combat this rising intracellular calcium, cells will try to sequester excess Ca+ in various organelles such as the mitochondria\textsuperscript{29}.

During excitotoxicity, harmful cytotoxic chemicals are produced such as reactive oxygen species (ROS)\textsuperscript{28}, free radicals\textsuperscript{29}, and calcium-dependent phospholipases, endonucleases, and proteases\textsuperscript{30}. These enzymes degrade essential proteins, making the cell membrane more permeable for ions to penetrate, causing damage to DNA, the cytoskeleton, and the cellular membrane\textsuperscript{31}. The mitochondria will sequester the majority of the calcium during excitotoxicity, which will eventually open the permeability transition pore (PTP) and depolarize the mitochondria\textsuperscript{32}. Opening the PTP will release pro-apoptotic molecules, such as cytochrome c, activating caspases and apoptotic signaling\textsuperscript{30,33}. If cells undergo necrosis, they will release toxic chemicals into the cellular
environment, exciting or harming nearby cells. Due to the severe drop in blood flow and reduced metabolic substrates, cells located in the ischemic core will undergo necrosis, whereas cells in the penumbra will initiate multiple types of cell death including apoptosis.

Even if blood flow is restored, cells might undergo delayed cell death because reperfusion injury can lead to a host of additional deleterious effects. For example, large molecules can enter the blood brain barrier (BBB) from blood vessels, which results in edema. These molecules pull in water via osmosis, which applies physical stressors on nearby cells and exacerbates recovery. Reperfusion can also increase free radical formation, which harms the vasculature locally as well as globally, leading to tissue damage throughout the brain. In addition, reperfusion can amplify the local inflammatory response, which will aggravate tissue damage and contribute to the ischemic cascade. For example, peripheral leukocytes can cross the BBB and enter the cerebral parenchyma, releasing cytokines that will in turn activate microglia. ROS’, which are more pronounced during reperfusion, will stimulate ischemic cells to also secrete cytokines, which leads to further recruitment of leukocytes. These activated inflammatory cells will then enter the BBB and release additional cytotoxic agents that further damages the ischemic brain tissue. Inflammatory cells can also exacerbate injury by phagocytosing healthy cells, in addition to dead cellular debris.

Experimental stroke models

The MCA territory is most the prevalent location for ischemic stroke, and therefore, MCAO is the most common type of focal cerebral ischemia used in animal preclinical studies. The most common method of MCAO is the intraluminal suture
model, which uses a suture with a coated tip to block cerebral blood flow in the MCA. The suture is advanced from the common carotid artery (CCA) into the internal carotid artery (ICA) until it reaches the MCA. Changing the suture tip will alter the effectiveness of the occlusive event and thus, the perfusion rate, ischemic severity, and region profile of the infarcted tissue. Suture tips vary in the type of coating used, diameter of the coating, and length of the tip. The intraluminal suture model has relatively low mortality because it avoids performing a craniotomy. However, variability between animal strains is a common occurrence. A major disadvantage to this model is that animals may lose a large amount of weight because the external carotid artery (ECA) is permanently severed. The ECA supplies blood to the muscles involved in mastication and swallowing food, therefore food consumption can be reduced after surgery.

Using a similar set up as the intraluminal suture model, the embolic model induces focal ischemia with intravascular coagulated blood. Blood is coagulated either ex-vivo, with 300-400um diameter spheres, or during the occlusion. Using a catheter, thrombin or coagulated blood is slowly inserted into the ICA where it will ultimately block blood flow in the MCA. The major advantage to this model is that thrombolytic agents, such as tPA, can be utilized to test combinatorial treatment strategies. However, infarct volumes are more variable than the intraluminal suture model because the coagulated blood can branch off and occlude additional arteries.

The photothrombosis model was first developed to limit variables introduced during surgery and incorporate thrombotic events into ischemic stroke pathogenesis. In this model, photosensitive dyes are first injected intravenously in the animal. Ischemia then occurs by irradiating multiple or single vessels for a designated amount of time,
which provides control over the size and location of the thrombotic occlusion. The irradiation induces ischemic lesions by photooxidation, which generates singlet oxygen, endothelia damage, and platelet aggregation to the endothelium. A disadvantage with the photothrombosis model is the damage produces a relatively small ischemic penumbra, which limits treatment strategies except for thrombolytic approaches. Oxidative damage is localized to the infarct core rather than distributed throughout the peri-infarct. In addition, by disrupting the local endothelia integrity, photothrombosis increases vasogenic edema.

Much like the photothrombosis model, venal and arterial vasoconstrictors can also be used to target specific areas of the brain. Compounds such as Endothelin-1 (Et-1) are long lasting vasoconstrictors that will produce a dose dependent infarct size while limiting ischemia edema. ET-1 can be applied to the superficial or subcortical layers of the brain. A disadvantage of using ET-1 to occlude a vessel is that its converting enzyme and receptors are located not just on endothelia cells but also on neurons and astrocytes. Therefore, administering ET-1 may directly induce neuronal damage and astrogliosis, which could alter the reparative process, especially when an additional treatment is utilized. It is clear that no one model of focal cerebral ischemia completely mimics the clinical conditions of stroke. However, available models do allow specific pathomechanisms to be critically investigated and treatment strategies evaluated. The ultimate goal of preclinical studies is to develop and test novel therapies that can target specific ischemic mechanisms and improve functional outcomes.
1.2 Endogenous Neurogenesis

Neurogenesis is an active process of proliferation, differentiation, migration, and integration of neural progenitor cells (NPC) into the local circuitry. The long-time dogma posited that neurogenesis stops after development. However, it is now well accepted that neurogenesis occurs throughout the adult life, even in humans.\(^55\). Despite cell proliferation first being observed in the rat hippocampi in 1965, it took nearly 30 years to reveal how ubiquitous neurogenesis was. Not until the advent of bromodeoxyuridine (BrdU)\(^56\), were scientists finally able to document how pervasive neurogenesis was in the mammalian family.\(^57\). BrdU is a thymidine analog that only becomes incorporated when cells divide, making it a common and accepted strategy to measure neurogenesis.\(^56\). In adults, neurogenesis is limited to 2 discrete zones; the subgranular zone (SGZ) in the dentate gyrus (DG) of the hippocampus, and the subventricular zone (SVZ) of the lateral ventricles. It was not until recently were the kinetics of neurogenesis elucidated in humans by utilizing radiolabeled \(^{14}\text{C}\).\(^58\). Since above ground nuclear testing only existed between the years 1955-1963 (the International Test Ban Treaty was signed in 1963), scientists were able to evaluate cell turnover dynamics by measuring the cellular levels of \(^{14}\text{C}\). Spalding and colleagues (2013) found that just like rodents, hippocampal neurogenesis declines with age, but at a much smaller rate. They also demonstrated that 1/3 of human’s hippocampal neurons turnover, which is 3 times as many neurons that turnover in mice.\(^58\).

**Subgranular zone (SGZ)**

Within the hippocampus, NPCs proliferate in the SGZ and migrate a short distance to the granule cell layer.\(^59\). Some cells differentiate into glia, whereas the
majority of NPCs differentiate into dentate granule cells (DGC) $^{59}$. Neurogenesis is a very dynamic process in rodents as 9,000 DGCs are estimated to be generated daily, comprising 6% of the total hippocampus population $^{60}$. Over the course of 3 weeks in rodents, DGCs will migrate to the granular cell layer and begin to project axons (mossy fibers) to the hippocampal CA3 pyramidal cells. However, it is not until 4-6 weeks, that DGCs will display enhanced synaptic plasticity during long term potentiation (LTP), a technique utilized to measure synaptic strength $^{55}$.

Since the hippocampus is critical for spatial and episodic memory $^{55}$, hippocampal neurogenesis is also thought to play a critical role in learning and memory $^{61}$. SGZ neurogenesis is considered necessary in pattern separation, an important step for processing multiple memories $^{55}$. If neurogenesis is ablated chronically, a rat’s ability to perform a spatial discrimination task and discriminate between two objects that are close together will be impaired $^{62}$. SGZ neurogenesis has also been shown to extinguish old memories. Newborn DGCs will directly interfere with the existing hippocampal circuits and thus induce ‘forgetting’ $^{63}$. In addition, learning has also been shown to be a key regulator in many aspects of SGZ neurogenesis. Hippocampal-dependent tasks produce a 2-fold increase in DGC proliferation, which is absent in a hippocampal-independent task $^{64}$. Furthermore, learning has been reported to tightly regulate neurogenesis by increasing both the survival of older DGCS and elimination of younger DGCs by inducing apoptosis $^{65}$. This activity is heightened during consolidation since the late phases of learning will increase apoptosis and decrease the number of viable DGCs $^{66}$. If cell death is inhibited, then the animal’s performance during learning and memory behavioral tasks, such as the Morris Water Maze (MWM), will be impaired $^{66}$.
**Subventricular zone (SVZ)**

Neurogenesis in the SVZ is a more dynamic process than the SGZ since migrating NPCs travel further distances, 5mm in mice, to the olfactory bulb (OB) than migrating SGZ NPCs. Cells begin their migration in the lateral ventricles and travel tangentially down a well-defined, glial tube network called the rostral migratory stream (RMS). Overall, 60% of NPCs will die, 25% will migrate, and 15% will stay within the SVZ. In the OB, neuroblasts will differentiate into granule cells and migrate to the granule cell layer, or they will differentiate into periglomerular cells (PG) and migrate to the glomerular layer. The cellular fate of SVZ NPCs appears to be determined by the neurogenic niche and not by an endogenous signal. When hippocampal NPCs were transplanted in the SVZ, they migrated down the RMS and differentiated into olfactory interneurons in the OB.

NPCs reside in the subependyma zone of the SVZ, the penultimate layer of the ventricle, as slowly proliferating, radial-like glial (type-B) cells. Type-B cells will then give rise to transient dividing (type-C) cells, which eventually give rise to neuroblasts (type-A). At the front of the RMS, neuroblasts will form chain-like structures by gap or adheren junctions and begin to migrate towards the OB over the course of 9-13 days in mice. This migration is not unidirectional since cells can change direction, speed up, slow down, or even stop migrating all together. However, SVZ migration is faster than other types of migration as cells travel at speeds of 70-80 μm/hr. Blood vessels help provide a scaffold in the RMS, allowing specialized astrocytes to form the glial tubes that surround the migrating neuroblasts. Throughout the RMS, neuroblasts will travel in close proximity to endothelia cells, thus, their migration path is determined in part by the...
vasculature 73. Overall, SVZ neurogenesis is regulated by a myriad of different factors that are released by endothelial cells, ependymal cells, astrocytes, and neurons along the SVZ, RMS, and OB 71,74.

Neurogenesis and cerebral ischemia

There is a significant increase in SGZ and SVZ proliferation at 2, 6, and 16 weeks after cerebral ischemia, which can last for several months 75. In order to rapidly expand the number of neuroblasts, cells will switch from asymmetric to symmetric cell division by shortening the cell cycle length in the G1 phase of the cell cycle 76. Using various labeling techniques, NPCs have been observed away from the SVZ and near or in the infarct area. These cells have also been reported to form chain-like structures around endothelial cells in the penumbra, where most of the remodeling occurs after stroke 77. Eight years ago, it was suggested that these newborn cells might not be stem cells but instead were ependymal cells that act as a reservoir for neuroblasts and astrocytes after cerebral ischemia. Carlen and colleagues (2009) demonstrated that under normal conditions, ependymal cells stay quiescent via canonical notch signaling, however, ischemic stroke depletes their numbers, thus removing the inhibition signal 78. Moreover, inhibiting notch signaling in uninjured animals causes ependymal cells to differentiate into olfactory interneurons, whereas forced notch signaling after stroke keeps ependymal cell quiescent 78.

Many investigators speculate that SVZ neurogenesis is an endogenous repair response to stroke because inhibiting it worsens outcome. Jin and colleagues (2010) demonstrated that transgenically ablating neurogenic cells post-ischemia increases the infarct size, neurological score, and sensorimotor deficits 79,80. However, despite this
surge in post-ischemic neurogenesis, the majority of newly proliferated cells will not survive to maturation ⁸¹. In the striatum, 80% of stroke-generated NPCs will die within 2 weeks after injury through activation of various caspases ⁸². Thus, protecting these vulnerable cells presents a novel approach to aid in repair after ischemic stroke.

1.3 Experimental Treatment for Ischemic Stroke

If cerebral blood flow is not restored for an extended period of time, the ischemic core may expand into the penumbra. Therefore, vascular recanalization is the most imperative strategy to combat focal cerebral ischemia. To date, the only FDA approved treatment for ischemic stroke is tissue plasminogen activator (tPA). tPA is a serine protease that converts plasminogen to plasmin, thus restoring tissue perfusion ⁸³. In phase III clinical trials, patients treated with tPA were 30% more likely than the placebo group to have no or minimal disability by 3 months post ischemia ⁸⁴. Although, tPA is not always effective; only 20-66% of ischemic patients treated with systematic tPA experienced complete recanalization ⁸⁵. Another issue is that tPA can only be administered within 4.5 hours of stroke symptoms, which drastically reduces eligible patients ⁸⁶. The majority of people suffering ischemic stroke take more than 3 hours after stroke onset to reach an emergency department ⁸⁷, and the average door to needle (DTN) time is over an hour ⁸⁸. When tPA was administered past 4.5 hours, patients showed greater risks for hemorrhaging with only minimal benefits ⁸⁶,⁸⁹.

A major reason for the delayed arrival time is the public’s general knowledge about stroke is still lacking ⁹⁰,⁹¹. In order to reduce the arrival time, hospitals and organizations have started to create public awareness campaigns to educate people on the
signs and symptoms of having a stroke. In order to tackle the high DTN time, hospitals have begun to equip ambulances with both a stroke team and a CT scanner. Since tPA cannot be administered to hemorrhagic stroke patients, being able to confirm the stroke is ischemic in an ambulance has significantly decreased the DTN time without observing any adverse effects.

**Vascular approaches**

There have been many different therapies developed to treat ischemic stroke, but to date, only vascular strategies have been successfully translated into the clinic. In a study examining all clinical controlled stroke trials between the years 1995-1999, only 3 treatments (out of 178) had a “positive” outcome. Of those 3, all were vascular approaches, including tPA. In the last 10 years, endovascular thrombectomy, which mechanically remove clots, has also become an effective and common vascular approach to combat focal cerebral ischemia. Thrombectomy devices operate by either coil retrievers, which use a stent to create a hole in the center of the thrombus; aspiration devices, which remove the thrombus by vacuum aspiration; or stent retrievals, which use a combination of the other two. Using a stent to entangle the thrombus, stent retrievers will slowly retract the stent and thrombus until it can be aspirated into the catheter. In five randomized clinical trials using endovascular thrombectomy, patients who received treatment had a significant reduction in disability compared to the control group at 90 days post-ischemia. The success of endovascular thrombectomy procedure can be attributed in part to their extended therapeutic window. These approaches can be utilized up to 12 hours of patient symptoms, and when combined with tPA, endovascular thrombectomy demonstrated increased efficacy.
Neurogenesis approaches

Since the majority of newborn cells die after an ischemic insult, there has been an interest in developing therapeutics that augment this potentially important endogenous repair response in order to improve stroke outcome. Treatments aim to enhance neurogenesis by targeting the regulatory factors that modulate NPC proliferation, migration, differentiation, and survival 69.

Proliferation

Less than 0.5% of cells in the SVZ are stem cells, therefore boosting their numbers presents the first potential target for neurogenesis 97. However, increasing proliferation requires caution because proliferation also increases the risk of developing aberrant cell growth and tumor formation 69. Since NPCs will only proliferate in a neurogenic niche, proliferation is associated with astrocytes, ependymal cells, and endothelia cells 98. Astrocytes and ependymal cells release soluble factors that promote proliferation, such as pigment epithelium-derived factor (PEDF) 99,100. Astrocytes have also been reported to directly regulate NPC proliferation via Wnt signaling. For example, overexpressing Wnt3 in astrocytes increases proliferation, whereas inhibiting Wnt3 abolishes neurogenesis 101. Additionally, vascular endothelia growth factor (VEGF) is suspected to play a key role in proliferation since its receptor VEGFR2, colocalizes with the immature neuronal marker doublecortin (DCX) on neural progenitor cells 102. Furthermore, intraventricular injection of VEGF binds to VEGFR2 and promotes proliferation in both the SVZ and SGZ, which is subsequently blocked with a VEGFR2 inhibitor 102. There are also several trophic factors that have been shown to increase proliferation, such as brain-derived neurotrophic factor (BDNF), insulin-like growth
factor 1 (IGF-1), epidermal growth factor (EGF)\textsuperscript{103}, and glial cell derived neurotrophic factor (GDNF)\textsuperscript{69}. In the SVZ, erythropoietin (EPO) is critical to SVZ proliferation since knocking down EPO reduces NPC proliferation and subsequently, migration to the penumbra\textsuperscript{104}.

**Migration**

In order to enhance repair, NPCs need to arrive with ample numbers at the damaged area, presenting another potential target for stroke therapies. In uninjured animals, chemoattractants and chemorepulsive signals are provided by cells as well as extracellular matrix proteins to direct migration of neuroblasts along the RMS\textsuperscript{69}. For example, polysialated neural cell adhesion molecule (PSA-NCAM) is a chemoattractant necessary to correctly organize in the RMS, whereas ephrin B2 or B4 are chemorepulsive signals necessary to orchestrate proper chain migration\textsuperscript{100}. Other secreted cues that are important for migration include integrins, receptor tyrosine kinase ErB4, and Slit1 or Slit2\textsuperscript{105}. Interestingly, diffusible factors are only effective when they are released close to the NPCs. If the OB is disconnected\textsuperscript{106} or removed\textsuperscript{107}, or if the RMS\textsuperscript{108} is severed, neuroblast migration is unaffected. Along the RMS, astrocytes can only regulate neuroblast migration by direct contact\textsuperscript{109}. Astrocytes control the speed at which neuroblasts migrate by releasing GABA to slow down or glutamate to speed up\textsuperscript{100}.

Angiogenesis is also critical for proper migration since blood vessels provide the scaffold for astrocytes along the RMS\textsuperscript{110}. While traveling in close proximity to endothelia cells, NPCs will migrate to areas with increased vascularization\textsuperscript{111}. After injury, there are multiple released angiogenic factors that indirectly guide neuroblast migration, such as VEGF, stromal cell-derived factor 1 (SDF-1), angiopoietin-1, and
EPO \textsuperscript{112}. EPO regulates the production of red blood cells, and knocking down EPO impairs NPC migration after ischemic stroke \textsuperscript{104}. In the SGZ, disrupted-in-schizophrenia 1 (DISC1) has been shown to be an important guidance cue. If DISC1 is inhibited, SGZ cells will migrate a further distance than they normally do \textsuperscript{113}. Similarly, SVZ-derived immature neurons also migrate a greater distance than do under basal conditions as a direct result of focal cerebral ischemia \textsuperscript{114}.

Limited information is known about the molecular mechanisms involved in guiding neuroblasts, specifically to the infarct, after acute ischemic stroke. However, one suspected pathway involves SDF-1\textalpha and its receptor CXCR4, which are expressed by stroke-generated neuroblasts. When SVZ NPCs were transplanted on the surface of organotypic brain slices from ischemic rats, they migrated further distances and had higher levels of both SDF-1\textalpha and CXCR4 than NPCs that were placed on normal organotypic brain slices \textsuperscript{115}. Moreover, blocking CXCR4 interrupted NPC migration, whereas increasing SDF-1\textalpha enhanced neuroblast migration in a dose-dependent manner \textsuperscript{115}. After cerebral ischemia, reactive astrocytes and microglia have been reported to secrete the monocyte chemoattractant protein-1 (MPC-1), which helps guide neuroblasts to the striatum \textsuperscript{116}. When MPC-1 was knocked out, migration was impaired and NPCs did not travel to the penumbra after tMCAO \textsuperscript{116}. The severity of the ischemic insult also directly correlates with the number of migrating neuroblasts in the infarct. For example, there were markedly fewer striatal neuroblasts after a 30 minute MCAO than there were following a 2 hour insult \textsuperscript{117}. Thus, there is a relationship between the amount of tissue damage and the activation of specific cellular mechanisms used to guide neuroblasts to the injury site \textsuperscript{117}. 
Differentiation

In adult neurogenesis, there are multiple cells that influence NPC differentiation, such as astrocytes, ependymal cells, and additional neurons. In the hippocampus, astrocytes will direct stem cell neuronal fate and neuroblast integration by releasing soluble factors into the surrounding environment. These astrocyte-secreted diffusible molecules regulate synapse formation or elimination as well as synaptic transmission. Ependymal cells regulate progenitor cell differentiation in the SVZ by expressing Noggin, which antagonizes bone-morphogenetic proteins (BMPs). If BMPs are expressed, cells will differentiate into glial cells rather than neurons. Neurons can direct progenitor cell differentiation by releasing glutamate or GABA. When NPCs receive a GABAergic input from the hippocampus, cells will depolarize and increase their expression of the neuronal differentiation marker NeuroD.

The process of NPC differentiation and maturation is poorly understood after cerebral ischemia, therefore most therapies do not target this aspect of neurogenesis. A mature NPC is typically identified using various differentiation or maturation markers, such as NeuN/PV, BrdU/NeuN, and BrdU/Tuj1. However, it is unclear from functional studies if these stroke-generated NPCs actually become integrated into the local circuitry. In the striatum, neuroblasts have been reported to receive only excitatory and not inhibitory input, suggesting these cells might have not fully matured. Although, new evidence indicates that these cells may simply be providing support by releasing trophic factors to injured cells rather than actually replacing them. For example, if growth factors were suppressed after ischemic injury, mice developed larger stroke cavities and performed worse on functional assessments. Moreover, if growth
factors were administered in immunosuppressed mice, only glial cells appeared in the infarct tissue, suggesting that the immune system might also direct differentiation \(^{125}\).

**Survival**

Since the majority of NPCs will not survive and reach maturation, protecting these vulnerable cells presents an appealing approach to enhance neurogenesis \(^{126}\). During neurogenesis, there appears to be a critical window for NPC survival. Between 1-3 weeks, immature neurons begin to demonstrate specific physiological properties that are critical to cell maturation, such as the formation of dendritic spines. If cells receive glutamate signaling in the third week via NMDA receptors, NPCs will have enhanced survival \(^{127}\). Neuroprotective strategies that inhibit apoptotic signaling pathways, such as p53, have also shown to increase the NPC pool and lead to functional improvements after cerebral ischemia \(^{128}\). Similar strategies to enhance survival have been employed with granulocyte colony-stimulating factor (G-CSF), a hematopoietic factor, and VEGF, which both improve NPC survival \(^{123,129}\).

**External stimuli**

In addition to endogenous factors that regulate neurogenesis, there are many exogenous factors that affect cellular proliferation or survival. Learning has been reported to enhance neurogenesis but only if the stimulus is associated with the neurogenic niche \(^{100}\). For example, in the SGZ, hippocampal-dependent learning increases NPC survival, whereas a hippocampal-independent tasks, such as active shock avoidance or training, had no effect \(^{130}\). Cellular maturation also appears to be an important aspect to learning-induced cell survival. If olfactory learning occurred between days 14 and 28, NPCs had increased survival, whereas olfactory learning past 30 days had no effect \(^{131,132}\). Innate
biological factors, such as age, sex, and sleep, will also modulate neurogenesis. For example, as animals age, neurogenesis will decline in both the SGZ and SVZ, which is thought to be a contributor to age-related cognitive decline 133. Interestingly, age only influences cell proliferation since NPCs demonstrate normal migration and maturation in aged rodents 134. In addition, aging changes the endocrinology of the brain, which directly effects neurogenesis by reducing neurotrophic factors, growth factors, and hormone levels 135. Hormones such as progesterone or testosterone have been reported to be neuroprotective, and both have independently demonstrated they will increase either cellular proliferation or survival 135. For example, in both overiectomised female rats and male rats, progesterone has been shown to increase SGZ proliferation 136,137.

There are also negative environmental stimuli that can suppress neurogenesis, such as multiple types of stress. Stress can be either psychosocial or physical, but any duration will reduce proliferation and increase apoptosis of NPCs 138. Since sleep is critical to maintaining homeostasis, sleep disruption can also act as a physical stressor. Disturbed sleep increases blood pressure, inflammatory cytokines, and cortisol levels, which impair brain function and neurogenesis 139. Although, only chronic sleep disruption has been shown to negatively regulate neurogenesis 140, if disturbed sleep lasts a short period of time, there will be no change in the rate of proliferation or cell survival 140.

Despite not fully understanding the mechanisms by which sleep deprivation suppresses hippocampal neurogenesis, there are many contributing factors such as the neurotransmitter serotonin (5-HT). Normally serotonin heightens neurogenesis in both neurogenic niche by activating the 5-HT1A receptor 141. During sleep deprivation, the 5-
HT1A receptor becomes gradually desensitized \(^{142}\); however, treating sleep deprived animals with a 5-HT4 receptor agonist restored neurogenesis levels \(^{143}\). Voluntary running, enriched environments (EE), and diet restrictions have also been shown to reverse the deleterious effects of chronic stress, and at the same time, enhance neurogenesis. Animals showed increased survival of 1-3 week old SGZ NPCs when they exercised or were exposed to an EE, likely due to BDNF or VEGF \(^{144}\). Interestingly, transgenically ablating BDNF reduced immature neuron survival even if animals were also exposed to an EE \(^{145}\).

**Neuroprotective approaches**

A common approach to treating ischemic stroke is the use of neuroprotective agents to prevent vulnerable neurons from undergoing cell death. Neuroprotective strategies aim to reduce the infarct size, reperfusion injury, and inflammatory responses, as well as prolong the time window until direct, vascular therapeutic strategies can be administered \(^{146}\). Instead of reducing brain injury by restoring blood perfusion, neuroprotective therapies target aspects of the ischemic cascade, such as excitotoxicity and free radical production. Excitotoxicity can be prevented or delayed by modulating the effects of neurotransmission, such as reducing glutamate release or increasing inhibitory activity. Other therapeutic approaches that target excitotoxicity have included inhibiting magnesium or blocking glutamatergic receptors, calcium channels, and glycine channels, as well as increasing GABA receptors \(^{147}\). Another example includes NXY-059, which demonstrated efficacy and improved patient outcomes in phase 1 clinical trials, by trapping free radicals after ischemic stroke \(^{148}\).
Due to the complexity of the ischemic cascade, neuroprotective strategies that target multiple mechanisms could demonstrate the greatest efficacy. For example, therapeutic hypothermia targets many protective pathways against brain injury such as reducing excitotoxicity, cerebral oxygen demand, lipid peroxidation, ROS’, MMPs, inflammation, and endogenous plasminogen, as well as stabilizing the BBB. Since hypothermia demonstrates greater efficacy during a temporary MCAO than a permanent MCAO, a large amount of hypothermia’s neuroprotective effects are likely a result of reducing reperfusion injury. In experimental stroke models, hypothermia reduced infarct volume, edema, and stress response gene induction, which improved behavioral outcomes. Unfortunately, clinical trials have produced mixed results with most of the clinical evidence for the therapeutic benefit of hypothermia being anecdotal. For example, when hypothermia was given to comatose survivors of cardiac arrest and neonatal hypoxic ischemia, they had improved outcomes. However, since most ischemic stroke patients are awake when arriving at the hospital, there are feasibility concerns such as patient discomfort. In this regard, there have been practical improvements when administering hypothermia by combining anti-shivering regimens with advancements in mechanical cooling devices. After demonstrating success in phase 2 clinical trials, hypothermia combined with thrombolysis is currently underway in phase 3 clinical trials investigating whether hypothermia can extend tPA’s therapeutic window up to 6 hours after stroke symptoms emerge.

With over 1000 compounds tested in preclinical settings, neuroprotective agents have had difficulty translating to the clinic and have only demonstrated efficacy in experimental models. Despite, more than 200 neuroprotective compounds going to
clinical trials, none have been successful. Possible explanations for this lack of translation from the bench to bedside include having a biased approach, unknown specific mechanisms for neuroprotection, lack of rigorous translatable outcome measures in preclinical models, and pharmacokinetic shortcomings of candidate therapeutic agents. Unfortunately, this poor preclinical evidence will culminate as proof for the initiation of a clinical trial. In order to combat this, stroke treatment academic industry roundtable (STAIR) was created in 1999. STAIR is a consortium made up of the leading stroke experts in academia, government, and industry who meet every 2 years to develop recommendations of all aspects of stroke research.

Underscoring the importance of increasing neuroprotective translation, the first STAIR meeting were developed specifically to create recommendations for neuroprotective compounds. These recommendations were designed to better represent the human patient population as well as to improve aspects of experimental design such as blinding. For example, most experimental stroke studies are conducted in young, healthy rodents with treatment beginning immediately after a consistent occlusive insult. However, patients who suffer cerebral ischemia are typically much different. Most ischemic strokes have variable occlusion times, occur in a heterogeneous, elderly population, and the time until treatment is inconsistent. In many early preclinical studies, animal studies only assessed infarct volume 24 hours after injury, which may not translate to functional improvement. Moreover, acute infarct reductions do not necessarily correlate to chronic atrophy reductions. Current studies now rely on more delayed treatment protocols combined with long-term functional outcome measures.
1.4 Nicotinamide Phosphoribosyltransferase (NAMPT) as a Target for Ischemic Stroke

For a long time, stroke was treated as a purely vascular or neuronal event; however recently, scientists have begun to examine multiple cell types involved in the ischemic cascade. When blood flow is occluded, cells making up the neurovascular unit will undergo a complex interaction between one another that ultimately determines if that cell is salvaged or killed. Moreover, its fate will then directly impact neighboring cells in the surrounding environment. Due to this complexity, combination approaches or therapies that have multiple targets (pleotropic), including the endogenous repair response, will likely demonstrate the greatest efficacy when combating the deleterious effects of stroke.

Nicotinamide phosphoribosyltransferase (NAMPT)

NAMPT has roles in energy production, mitochondrial biogenesis, neurogenesis, and inflammation, making it an attractive therapeutic target for stroke therapies. NAMPT is the rate limiting enzyme in the predominant cellular pathway (salvage pathway) used to generate nicotinamide adenine dinucleotide (NAD). NAMPT salvages excess nicotinamide and converts it into nicotinamide mononucleotide (NMN), which is subsequently converted to NAD by nicotinamide mononucleotide adenylyltransferase (Nmnat). NAMPT is conserved throughout nature, appearing from sea sponges to mammals. Furthermore, it is thought to be necessary for mammalian survival since genetically ablating NAMPT is lethal in mice.
NAMPT is found in most cell types in the mammalian body with the brain being most dependent on NAMPT, particularly for NAD-driven production\textsuperscript{168,171}. In addition to intracellular NAMPT (iNAMPT), many cells types can also generate and release extracellular NAMPT (eNAMPT). Circulatory eNAMPT is thought to mainly occur with cells in the periphery such as adipocytes, hepatocytes, and leukocytes\textsuperscript{172-174}. Since eNAMPT can cross the BBB, it is also thought to help maintain cerebral biogenetic demands by regenerating NAD. This process is especially relevant and active during cerebral ischemia\textsuperscript{175,176}. Within the cell, NAMPT is predominantly found in the cytosol but does exist in the mitochondria and nucleus\textsuperscript{177}.

NAMPT activity is modified by numerous stimuli, such as diet, stress, exercise, disease, aging, and circadian rhythm\textsuperscript{168}. Since NAMPT and NAD both display circadian oscillations throughout the day, they are suspected to coordinate energy storage and usage in mammals. Ramsey and colleagues (2009) demonstrated that increasing intracellular NAD suppresses the transcription factor circadian locomoter output cycles protein kaput (CLOCK), which reduces NAMPT RNA levels in the liver\textsuperscript{178}. However, inhibiting NAMPT releases CLOCK and drives NAMPT-mediated NAD biosynthesis, thus creating a positive feedback loop controlled by circadian rhythms\textsuperscript{178}. This relationship is even evident in humans as NAMPT expression and NAD are lowest at noon every day\textsuperscript{178,179}. Moreover, more awake strokes occur between 10am and noon than any other 2 hour time point in awake humans, suggesting there is an interplay between circadian rhythm, NAMPT, NAD, and stroke\textsuperscript{180}.

In addition, NAMPT is involved in cellular energy metabolism and many biological signaling pathways. Since NAMPT is a regulator of NAD, it is an important
component in redox metabolism and ATP production during oxidative phosphorylation. In the electron transport chain (ETC), NAD is used to generate the electrochemical gradient required for energy production. The malate-aspartate shuttle reduces NAD to NADH in the mitochondria matrix, and it also oxidizes NADH in the cytosol, which generates the potential energy across the mitochondria inner membrane. NADH can then supply the ETC with its extra electron, which will then be utilized to generate ATP. NAMPT signaling also has roles in angiogenesis and neurogenesis. eNAMPT has been shown to increase production of VEGF, and iNAMPT is required for neural stem cell proliferation. NAD is also required for progression in the G1 to S phase of the cell cycle.

NAMPT has a big role in the cellular defense system via the NAMPT-NAD-Sirt cascade. Under stressful conditions, poly ADP-ribose polymerase 1 (PARP1) and CD38 are the major source of NAD depletion. During cerebral ischemia, NAD gradually becomes depleted and eventually initiates apoptotic signaling pathways. Administering NAMPT or NAD reverses this process by suppressing the translocation of apoptosis-inducing factor, a mitochondrial intermembrane flavoprotein, from the mitochondria. This crosstalk between NAMPT and PARP/CD38 occurs via the Siruts (Sirts), which deacylase their activity based on NAD fluctuations. Therefore, NAD function as a currency used between NAMPT, SIRTs, PARP1, and CD38 that together represent a cellular feedback mechanism. This feedback is evident during cytotoxic or ischemic stress because cells will activate PARP1, which subsequently consumes NAD and increases cell death. However, treating with NAMPT restores NAD and therefore enhances cell survival.
During inflammation, NAMPT has a somewhat conflicting role. In both *in vivo* and *in vitro* studies, eNAMPT has shown to exacerbate injury after stroke by releasing tumor necrosis factor α (TNF-α) and activating proinflammatory factors. However, inhibiting iNAMPT reduces TNF-α and neuronal injury. Interestingly, inhibiting iNAMPT in neurons does not affect survival, suggesting that TNF-α is released from another cell type. In contrast, eNAMPT was also shown to have a positive therapeutic effect during inflammation by polarizing monocytes into M2 macrophages, which will then produce and release immunosuppressive cytokines.

**NAD and stroke**

After cerebral ischemia, NAD levels become depleted at 30 minutes after the initial MCAO, as well as 6 and 25 hours after reperfusion in rats. The first drop is thought to be due to excess ROS generation caused by reperfusion injury, which damages DNA and activates PARPs. The second and third drop in NAD levels represent when necrosis and apoptosis are most pronounced. Since NAD levels are rapidly reduced after stroke, there have been many effective therapies aimed at targeting NAMPT in order to restore intracellular NAD. For example, overexpressing NAMPT prevents cell death during oxygen glucose deprivation (OGD), an *in vitro* model of stroke, as well as reduces infarct volume and improves behavioral outcomes after MCAO. In addition, administering eNAMPT after focal cerebral ischemia protects against white matter injury by increasing the amount of myelinated fibers in the striatum and corpus callosum. Conversely, inhibiting NAMPT increases glutamate excitotoxicity via Sirt1, which expands the infarct volume and the number of degenerating neurons after
photothrombosis\textsuperscript{197,200}. Ultimately, NAMPT’s pleotropic targets, makes it a potentially effective strategy to treat acute ischemic stroke.

1.5 P7C3-A20 (A20)

The neuroprotective compound P7C3-A20 (A20), is an attractive treatment strategy for cerebral ischemia because it activates NAMPT, a proposed therapeutic target for stroke. A20 was first identified in 2010 by Pieper and colleagues using an unbiased, target-agnostic, \textit{in vivo} drug screen\textsuperscript{201}. One thousand different compounds were examined for their ability to increase the net magnitude of postnatal hippocampal neurogenesis in wild type mice. Initially, a library of 200,000 drug-like chemicals were reduced down to 1,000 computationally by their chemical diversity, complexity, potential toxicity, location of their chiral center, ability to form hydrogen bonds, and electrostatic contacts with potential receptors\textsuperscript{201}. Compounds were then randomly assigned into pools of 10 (100 pools in total) and injected intracerebroventricularly (ICV) in mice via osmotic pumps for 7 days. Each pool was tested on two different mice at a concentration of 10uM. In order to measure neurogenesis, mice also received daily intraperitoneally (IP) injections of BrdU (50mg/kg). Fibroblast growth factor 2 (FGF-2) was used as a positive control and artificial cerebral spinal fluid (aCSF) was used as a negative control. After 1 week, animals were sacrificed and neurogenesis was assessed by examining the total BrdU+ cells in the SGZ.

Of the 100 initial pools, only 10 demonstrated enhanced neurogenesis when compared to FGF-2. These 10 positive pools were then broken down into individual components and retested on the same assay. In these studies, 2 concentrations (10uM and
100uM) of every compound were administered to 2 different mice. Of the 10 positive pools, only eight compounds demonstrated a pro-neurogenic effect. These compounds then underwent *in silico* predictions for absorption, distribution, metabolism, and excretion (ADME), wherein pool 7 compound 3 (P7C3) displayed the best pharmacological properties. P7C3 had the highest half-life, rate of clearance, bioavailability, and penetration of the BBB. Moreover, it had phenomenal molecular stability and could be administered IP, IV, or orally (AUC<sub>brain</sub>/AUC<sub>plasma</sub> = 3.7 oral, 0.61 IP; AUC: area under the curve)\(^{202}\). In order to determine P7C3’s chemical structure, 37 derivatives were created and tested using an *in vivo* structure activity relationship study. Using the same assay to assess proneurogenic ability, only the 20<sup>th</sup> derivative (A20) exhibited elevated neurogenesis. A20 is identical to its parent compound P7C3 except, the hydroxyl group in the chiral center is replaced with a fluoride (Figure 1). In addition, A20 also demonstrated a lower toxicity profile and more favorable pharmacokinetics \(^{201,203}\).

*Figure 1. Molecular representation of P7C3-A20.* P7C3-A20 is an aminopropyl carazole with three-ring heterocycles. It differs from its parent compound P7C3 by replacing the hydroxyl group in the chiral center with a fluoride (adapted from Pieper at al. 2010).
Although A20 was originally thought to increase the rate of proliferation, the enhanced neurogenesis is most likely the result of neuroprotection. Since the majority of newborn cells die in the hippocampus basally, preventing apoptosis would also increase the number of BrdU+ cells $^{69}$. To test this hypothesis, two different pulse-chase experiments were conducted $^{201}$. In the first experiment, BrdU was pulsed once in concert with P7C3. At 7 days, the same number of BrdU+ cells were observed in the treatment and control groups. In the second experiment, BrdU was pulsed once, but animals were also administered P7C3 continuously for one month. After 30 days, the treatment group had a 500% increase in BrdU+ cells than the control group. Taken together, this suggests that P7C3 exhibits its proneurogenic effect by improving cell survival $^{201}$.

A20 is an aminopropyl carazole with three-ring heterocycles (Figure 1), which retains many structural similarities to the compound Dimebon, a non-selective, anti-histamine treatment used in Russia for many years $^{204}$. Dimebon is anti-apoptotic by blocking NMDA receptor signaling pathways, which inhibited the mitochondria PTP from opening $^{205}$. Since its neuroprotective effects extended to enhancing memory, Dimebon was also tested as a possible therapy for Alzheimer’s disease in 2001 $^{205,206}$. In clinical trials, long-term treatment with Dimebon demonstrated a significant improvement in symptoms, however there was no significant difference compared to the placebo control group in phase 3 clinical trials $^{207,208}$. One likely reason for this failure was the small Dimebon dosage (20mg) used. In order to be efficacious, a 20mg dose was determined necessary, however 20mg was administered in the clinical trial, which resulted in a concentration of less than 0.6uM $^{205,209}$. Fortunately, A20 was shown to be more efficacious than Dimebon during neurotoxic conditions $^{201,210,211}$. 
Recently, it was determined that A20 was neuroprotective by binding to NAMPT, enhancing its activity, and increasing intracellular levels of NAD. Wang et al., performed 2D gel electrophoresis on cell lysates that were pretreated with A20. For protein identification, lysates next underwent shotgun mass spectrometry in which NAMPT yielded the highest spectral counts. To verify NAMPT was the correct target, NAD levels were depleted using doxorubicin, a DNA damaging toxin, which activates PARP1. When cells were treated with A20, NAD was restored in a dose-dependent manner. The salvage pathway was specifically targeted next by administering cells radiolabeled nicotinamide in addition to doxorubicin. Treatment with A20 restored both NAD and its intermediate, NMN, in a dose dependent manner.

P7C3 compounds have been successfully used as a treatment for multiple neurodegenerative diseases, but it first demonstrated efficacy in a mouse model of Parkinson disease (PD) and amyotrophic lateral sclerosis (ALS). A20 prevented cell death of dopaminergic neurons in the substantia nigra during PD and motor neurons in the ventral horn of the spinal cord during ALS. Since its creation, P7C3 compounds have shown protective efficacy in preclinical models of cognitive decline due to aging, depression associated with neurodegeneration, cell death associated with genetic susceptibility to mental illness, neonatal nerve injury, visual deficits associated with blast-mediated traumatic brain injury (TBI), and axonal degeneration after TBI. Furthermore, Blaya and colleagues (2014) demonstrated that after moderate fluid percussion injury, A20 reduced contusion volume, improved sensorimotor and learning and memory behavior, and increased neurogenesis in the SGZ.
The efficacy of A20 has recently been examined after cerebral ischemia using both OGD and electrocoagulation, a permanent model of focal stroke. During 1.5 hours of OGD, A20 increased neuronal viability in a dose-dependent manner with a dose of 30uM demonstrating the greatest efficacy. When the dosage was increased to 100uM, A20 extended protection up to 12 hours of OGD and exhibited higher Tuj-1 expression, a marker of axon stability, than the vehicle experimental group. After electrocoagulation of the MCA, A20-treated ischemic rats had reduced infarct volumes and elevated NAD levels in the cortex at 24 hours after injury. However, despite A20 demonstrating efficacy, many STAIR recommendations were not addressed, raising additional questions. First and foremost, does the degree of acute neuroprotection translate into chronic functional improvements? Due to the complex circuitry involved in basic behavior, enhancing neuronal survival and reducing infarct volume does not always translate to improved functional ability in the clinic. In addition, using electrocoagulation to permanently occlude an artery requires a craniotomy and thus is a highly variable model of cerebral ischemia that may produce inconsistent results. Moreover, permanent occlusive models do not address difficult clinical scenarios, such as reperfusion injury. Additionally, since NAMPT targets multiple cell types and processes, it is unclear if A20 is protective via other mechanisms, such as neurogenesis. Finally, previous studies using MCAO did not investigate if these acute changes would be sustained chronically, or if treatment could be delayed to a clinically relevant, post-ischemic time point.
1.6 Concluding Remarks and Hypotheses

Cerebral ischemia induces a complex set of reactions and secondary injury mechanisms in which many potential therapeutic targets exist. One possible target is NAMPT because it has multiple roles in neuroprotection, such as cellular defense, repair, inflammation, and neurogenesis. Since neurogenesis is heightened after stroke, but the majority of newly generated cells undergo apoptosis, protecting these vulnerable cells presents a unique strategy to combat cerebral ischemia. Moreover, ample evidence suggests that whatever the mechanism of action, protecting brain tissue after stroke may ultimately lead to improvements in chronic behavioral outcomes. Therefore, my central hypothesis is that A20 treatment reduces infarct volume and promotes neurogenesis, which improves functional recovery following stroke. A20’s efficacy after cerebral ischemia will be evaluated using numerous clinically relevant outcome measures, such as functional ability on behavioral tasks, tissue damage, and neurogenesis. Furthermore, acute as well as chronic assessments will be conducted in order to evaluate progressive injury. Finally, the therapeutic window for A20 will be explored in order to increase the likelihood for clinic translation.
Chapter 2

Materials and Methods

2.1 Transient Focal Cerebral Ischemia

All procedures involving the use of animals were in accordance with the guidelines set by the Institutional Animal Care and Use Committee at the University of Miami, Miller School of Medicine, and the NIH Guide for the Care and Use of Laboratory Animals. Adult, male Sprague-Dawley rats (280-330g) were anesthetized with 3% isoflurane/70% N₂O/ 30% O₂ and then maintained with 2% isoflurane. Brain and body temperatures were monitored and regulated with heating lamps to maintain normothermic temperatures (36.5°C to 37.5°C) during the induction of cerebral ischemia.

Cerebral ischemia was induced using a 90 minute tMCAO. First, the right CCA was exposed with a midline neck incision and dissected from the surrounding nerves. Next, the ECA and superior thyroid artery were ligated. A small incision was then made in the ECA and a 4-0, 20mm, nylon monofilament with a silicone-rubber coated tip (Doccol Corporation, Sharon, MA) was inserted at the proximal end. The suture was advanced into the CCA and then the ECA was severed. In all animal groups, including Sham, the ECA was permanently severed. In order to manipulate the suture tip into the ICA, the ECA was then maneuvered parallel to the CCA, and the suture was retracted. Once in the ICA, the suture could slowly be advanced until it reached and blocked blood flow of the MCA.

After the MCA was occluded, the ECA was temporary ligated. The animal was then placed back in their cage and allowed to awake during the occlusion. Sham surgeries were identical to tMCAO except no suture was used. Before re-anesthetizing the rats, a
neurological assessment score was obtained in order to determine the severity of the ischemic insult. The assessment protocol uses an established 5-point scale where a score of 0 indicates no neurological deficit, and a 5 indicates a severe neurological deficit. A score of 1 indicates a mild neurological deficit in which the animal could not extend their left forepaw fully. A score of 2 indicates a moderate neurological deficit in which the rat would only circle to the left in their cage. A score of 3 indicates a severe neurological deficit in which the animal would fall to the left. Finally, a score of 4 indicates that the animal had a depressed level of consciousness since they did not move in their cage. If animals had a mild to moderate neurological deficit, they were included in the experiment.

Animals that passed the neurological score were re-anesthetized using 3\% isoflurane/70\% N\textsubscript{2}O/ 30\% O\textsubscript{2}. At 90 minutes, the suture was slowly retracted, thus initiating reperfusion. Once the suture was completely removed from the animal, the ECA was permanently closed with a suture knot, and the rat was sutured shut. Immediately following reperfusion, rats were administered buprenorphine 0.03 mg/kg (s.c.). Then, animals were returned to their cage and placed on soft bedding. Cages were placed on a heated pad (Peco Services Ltd, England, UK) at 37°C while animals recovered. Rats were monitored twice daily and daily weight assessments were done to insure no animals lost more than 15\% body weight.

Consistent with animal research reporting and STAIR guidelines, a power analysis was performed prior to the experiment to determine the numbers of animals required for each experimental group\textsuperscript{161,225}. Before treating, animals were randomly
allocated to each experimental group, injury versus sham as well as vehicle versus drug
treatment. A total of 75 animals were used in the first study and 148 in the second study.

2.2 P7C3 Compound

Drug was prepared by dissolving A20 in DMSO, Kolliphor, and 5% Dextrose. Following reperfusion, drug or vehicle (DMSO, Kolliphor, and 5% Dextrose) was injected IP (10 mg/kg) into rats. Treatment continued twice daily for 7 days in concert with daily BrdU labeling reagent (10:1; 1mL / 100g of rat bodyweight, ThermoFisher Scientific, Waltham, MA). This treatment dose and strategy was based on previous neurodegenerative studies that demonstrated efficacy of A20. In order to remove treatment bias, vehicle or A20 was selected by a researcher not associated with the study. In this series of studies, A20 or vehicle treatment was initiated immediately or in a delayed fashion after tMCAO. These specific treatment methods and results are described in Chapter 3 of the dissertation proposal.

2.3 Behavioral Analysis

The investigator was blinded to the experimental groups while performing and evaluating all behavioral tests, and only after data analysis had been completed were the treatment groups decoded.

Sensorimotor

To evaluate stroke-induced sensorimotor deficits, the cylinder and grid-walk tasks were conducted one week post-injury. This post-ischemic time period was chosen based on previous studies in vehicle non-treated tMCAO rats. Both behavioral tests lasted 5
minutes, and animals were recorded. During data analysis, video tapes were played back in slow motion at 1/5th the normal speed in order to accurately quantify sensorimotor ability.

For the cylinder task, animals were placed in a clear Plexiglas cylinder (20cm diameter and 30cm height), which encouraged spontaneous forelimb use as animals vertically explored their environment. During the task, independent forelimb paw placements were assessed and asymmetry was calculated. Asymmetry was quantified by: 

\[
\left(\frac{\text{number of paw placements of the affected forelimb}}{\text{number of paw placements of both forelimbs}}\right) \times 100
\]

Prior to injury, a baseline test was performed to verify no animal had a previous forelimb bias. The cylinder task was then repeated one week post injury and the asymmetry index was normalized to baseline.\textsuperscript{228,229}

For the grid-walk task, rats were placed on an elevated platform made of 11×11 cm diameter chicken wire, and since it was a novel environment, they quickly traversed the grid. During the test, animals walked along the outside grid and tried to not fall through. Rats used their somatosensory stimuli to determine if the wire was below before placing the full weight on their paws. If a paw penetrated through the grid, it was considered a misstep as it did not provide support. The percentage of missteps was calculated by: 

\[
\left(\frac{\text{number of missteps}}{\text{total number of paw placements}}\right) \times 100
\]

\textit{Learning and memory}

All hippocampal-dependent spatial memory assessments were conducted using the MWM. Animals were recorded and later analyzed using Ethosvision software (Noldus Information Technology, Leesburg, VA)\textsuperscript{223} for multiple parameters, such as
time spent in each quadrant, path length, and swim speed. Swim speed was calculated by: 
\[
\frac{\text{Swim distance}}{\text{escape latency}}
\]
Based on the temporal maturation of newly generated hippocampal neurons, MWM experiments began 4 weeks after injury. The MWM examines acquisition, memory retention, and working memory.

Beginning at 28 days, animal’s memory acquisition was evaluated over four training days. Before initiating the test, the rat’s dorsum was painted black to facilitate video tracking. Once rats were placed in the pool, they quickly tried to escape by locating a hidden platform, which was slightly submerged underneath the water. In order to keep the platform location hidden from the animal, the water was rendered opaque by using white paint. Using only visual cues located along the wall, rat’s escape latencies were recorded (maximum of 60 seconds) as animals searched for the platform. After 10 seconds on the platform, animals were placed back in their cage. If animals could not find the platform after 1 minute, they were guided to it. Rats were tested on 4 different starting locations with a minimum of 4 minute intervals between trials. The order of the starting locations varied daily, but the platform remained in the NE quadrant throughout training.

After 4 days, rat’s memory retention was evaluated using a probe trial. The platform was removed prior to the trial, and animals were placed in the pool for 60 seconds. Memory retention was determined by measuring the percentage of time animals spent in the target quadrant (quadrant that previously housed the platform). Since animals do not want to swim in the pool they actively search for the hidden platform. Therefore, the longer the animal spends in the target quadrant searching for the platform, the better their memory retention.
Starting on the 6\textsuperscript{th} day, working memory was assessed using paired trials (location trial and match trial). At the beginning of each paired trial, the platform was moved, and the rat’s starting location was changed. Therefore, both the start and end locations were novel. For the location trial, animals had 60s to find the platform. On the platform, animals were given 10 seconds to rest and determine their location within the pool. Immediately after, animals were released in the same starting location and again had 60 seconds to re-find (match) the unmoved platform\textsuperscript{234}. Escape latency was measured for each trial and the percent improvement on the match trial was calculated by: \[
\frac{\text{(location trial-match trial)}}{\text{location trial}} \times 100\] \textsuperscript{223}. There were 5 paired trials performed (5 different starting and ending locations) over 2 days with the first day considered to be a training day.

\textbf{2.4 Triphenyl Tetrazolium Chloride (TTC) Staining}

Infarct volume assessments were performed 2 days post-injury using a triphenyl tetrazolium chloride (TTC) assay. TTC is a redox indicator of cellular respiration used to differentiate between metabolically active and inactive tissue. In healthy mitochondria, TTC becomes enzymatically reduced to 1,3,5-triphenylformazan (TPF) by various dehydrogenases, turning the tissue red. Since these enzymes are absent in regions of necrosis, TTC will remain unchanged and the tissue will appear white \textsuperscript{235}.

Forty-eight hours after tMCAO, rats were deeply anesthetized with 3\% isoflurane/70\% N\textsubscript{2}O/ 30\% O\textsubscript{2} and transcardially perfused with saline (0.9\% NaCl; 75ml). Brains were quickly dissected and placed in a brain mold. To improve cutting, the mold was placed in a -75\textdegree C freezer for 7 minutes to lightly harden the tissue. Next, 9 sections
of tissue were cut coronally (every 2mm) and incubated in 1.5% warmed (37°C) triphenyl tetrazolium chloride for 7 minutes. The sections were then flipped to the other side and incubated for 7 additional minutes. Sections were then placed in formaldehyde for 5 minutes to slightly preserve the tissue. Cerebral cortical and striatal infarct volumes were quantified on ImageJ 1.50b (NIH) using the direct method: 

\[
\left( \frac{\text{infarct}}{\text{infarct + live tissue}} \right) \times 100
\]

2.5 Immunohistochemistry

Tissue processing

Seven weeks post injury, animals were perfused for chronic tissue assessments. Rats were first deeply anesthetized (3% isoflurane/70% N₂O/ 30% O₂.) and then transcardially perfused with saline (0.9% NaCl; 75ml) followed by 4% paraformaldehyde (PFA) in 0.1M phosphate buffer pH 7.4 (150ml). Brains were then dissected and post-fixed in PFA for 48 hours. Next, tissue was emerged in a gradient of sucrose (15-30%) over 4 days. Brains were then embedded in optimal cutting temperature (OCT) Tissue-Trek (VWR, Radnor, PA), flash frozen for 1 minute in isopentane, and placed on dry ice. Using a cryostat (Leica CM1900; Leica Microsystems, Inc., Buffalo Grove, IL), tissue was coronally sectioned at 30µm and mounted in a stereological series on superfrost charged slides (Thermo Fisher Scientific, Waltham, MA). Slides were then placed on a slide warmer at 37°C for 20 minutes. If tissue was not immediately used, it was stored at -80°C.
**Hematoxylin and eosin (H&E) staining**

For chronic tissue volume assessments, slides were stained with hematoxylin and eosin (H&E) and then were imaged using PathScan Enabler IV (Meter Instruments, Houston, TX). The ipsilateral cortex, hippocampus, and striatum were contoured, and the volumes were quantified with StereoInvestigator (MicroBrightField Bioscience, Williston, VT). White matter tracts were used as boundaries for each brain region. The cortex and striatum were contoured between bregma level 1.2 and -4.3mm, and the entire hippocampus was contoured. Healthy intact tissue volume was then normalized to the sham experimental group. Ipsilateral tissue volume was calculated by: 

\[
\frac{\text{total ipsilateral live tissue volume}}{\text{average of all sham animal’s ipsilateral tissue volume}} \times 100
\]

**Immunofluorescence**

For neurogenesis studies, frozen slides were first washed in 0.1m PBS. Slides were then treated with 100°C citrate buffer for 10 minutes in order to expose antigens. Next, tissue was washed in PBST (1% tween-20 in 0.1m PBS) to remove excess citrate buffer. Sections were blocked for 30 minutes (3% normal donkey serum in PBST) at room temperature. Overnight at 4°C, tissue was then incubated in blocking buffer with primary antibodies: mouse anti-Neuronal Nuclei (Merck Millipore, Darmstadt, Germany; 1:500) and rat anti-BrdU (Novus, Littleton, CO; 1:100). The following day, tissue was washed in PBST to remove excess primary antibodies. Tissue was then developed for 2 hours at room temperature using blocking buffer with secondary antibodies: donkey anti-mouse 488 (Life Technologies, Carlsbad, CA; 1:500) and donkey anti-rat 568 (Life Technologies, Carlsbad, CA; 1:200). Finally, slides were mounted and cover slipped.
For all sections, antibody penetration was confirmed through the entire tissue at 100X magnification using a Zeiss Axiovert 200M microscope (Carl Zeiss Microscopy, LLC, Thornwood, NY). All quantifications and analyses were performed by a blinded experimenter at 20X magnification using StereoInvestigator software. For both the SGZ and SVZ, a contour was created around the region of interest and every double labeled BrdU+:NeuN+ cell was counted within the contour. In the SGZ, the entire dentate gyrus was contoured between bregma levels -2.3 to -3.5mm. In the SVZ, a radius was extended 500μm around the entire ventricle between bregma levels 0.5 through -0.5mm.

2.6 Tissue Homogenization

A new batch of animals (n=6 per group) was used for the NAMPT and NAD assessments. The three groups were sham, tMCAO+Vehicle, and tMCAO+A20. At 48 hours post injury, animals were anesthetized for 5 minutes with 3% isoflurane/70% N2O/30% O2 and then sacrificed. Rat’s cortical and hippocampal tissue were quickly dissected and flash frozen in liquid nitrogen. Frozen tissue was then homogenized using a dounce homogenizer in cocktail buffer (0.2N NaOH, PBS, 0.125 ug/ml pepstatin, 1 ug/ml leupeptin and 2.5 ug/ml aprotiruin). After homogenization, tissue was flash frozen in liquid nitrogen again. A Bradford assay was then performed to quantify tissue protein levels.

NAD quantification

NAD+ was quantified for cortical tissue, using a modified version of the NAD/NADH-Glo Assay provided by Promega (Promega Corporation, Madison WI). The assay uses a ratio of NAD/NADH to produce a bioluminescent readout. NAD
cycling enzyme is first used to reduce NAD to NADH. The enzyme reductase then reduces proluciferin reductase substrate into luciferin using the newly available NADH. Luciferin is measured using a FLUOstar Omega (BMG Labtech, Ortenberg, Germany) bioluminescent reader. 

*NAMPT quantification*

NAMPT quantifications were determined via western blotting. First, homogenized cortical and hippocampal tissue were resuspended in 4x Laemmli buffer and boiled for 10 minutes at 100°C. Samples were then run on 4-15% mini protean PAGE gels (#456-1086, Hercules, CA) for 2.5 hours at 100V. Next, tissue was transferred to a PVDF membrane (EMD Millipore, Billerica, MA) overnight in 4°C. The transfer ran for 15 hours at 20V. Membranes were then washed in TBST (0.1% Tween-20 in Tris-buffered saline) to remove unbound proteins. The following night, membranes were incubated at 4°C in blocking buffer (5% milk in PBS), β-actin (Sigma-Aldrich, St. Louis, MO; 1:100,000), and PBEF (Bethyl Laboratories, Montgomery, TX; 1:10,000). Next, membranes were washed with TBST to remove any excess primary antibodies. Membranes were incubated for 2 hours at room temperature in blocking buffer with mouse secondary antibody (Cell Signaling Technologies, Danvers, MA; 1:10,000) using HRP-conjugated rabbit (Cell Signaling Technologies, Danvers, MA; 1:1000). Western blots were then developed with luminol chemiluminescence (Cell Signaling Technologies, Danvers, MA) and x-ray film (Phenix Research Products, Hayward, CA). Finally, blots were densitized using ImageJ 1.50b (NIH), and the integrated density measurements were normalized to β-actin for each sample.
2.7 Statistical Analysis

Since there is sizable physiological variability after tMCAO, a power analysis was performed. Twelve animals were deemed necessary for the behavioral, histopathological, and immunocytochemical experiments. For the biochemical NAD and NAMPT assessments, 6 animals were required. Using our laboratories working memory preliminary data and published data, the power analysis was calculated using a power of 0.8 and an alpha of 0.05.

Tissue volume assessments were expressed as mean ± standard deviation. All other data were expressed as mean ± standard error of the mean (SEM). Before assessing significance, a normal distribution was performed on all experimental groups. For comparison of three or more groups with one independent variable, a one-way analysis of variance (ANOVA) with Tukey’s honest significance difference (HSD) correction was used. For comparison of three or more groups with two independent variables, a two-way repeated measures ANOVA with Bonferroni correction was utilized. To directly assess possible correlations between histopathological outcome measures and behavioral parameters, a Pearson Correlation was used.
Chapter 3

Immediate A20 Treatment after Ischemic Stroke

3.1 Introduction to the Experiment

As discussed in Chapter 1, stroke is a leading worldwide cause of adult long-term disability \(^{16}\), and over the last decade tremendous advancements in acute stroke therapy have meaningfully reduced the mortality rate \(^{239,240}\). Currently, the thrombolytic agent tPA is the only FDA-approved drug that has shown a significant benefit in Phase III clinical trials; improving neurological function up to 30\%\(^{84}\). However, due to the limited time window of therapeutic efficacy, only a small percentage of ischemic stroke patients in the United States are able to receive this drug \(^{86,241}\). While restoring blood flow is critical to combating stroke, cells undergo a complex ischemic cascade until reperfusion can be established, which ultimately results in their death \(^{16}\). This underscores the need to develop neuroprotective drugs to preserve brain function after stroke in parallel with advanced reperfusion strategies.

As previously discussed, there is a physiologic surge in neurogenesis after cerebral ischemia in both the SGZ of the hippocampal dentate gyrus and the SVZ of the lateral ventricles \(^{242}\), which promotes recovery from neurological deficits \(^{79,80}\). However, despite this increased proliferation of progenitor cells, the majority of newborn cells do not survive to full maturation \(^{81}\). Therapeutic strategies that promote survival of these newborn neurons might provide a new treatment for patients \(^{243}\). Indeed, a therapeutic agent that can increase the survival of both mature and newborn neurons after cerebral ischemia may present an ideal acute approach to mitigate long-term neurological dysfunction after stroke.
To date, there have been many clinical trials with putative neuroprotective agents for stroke, none of which have been successful. Possible explanations for this lack of successful translation to patient care include the limitations of a biased approach, unknown specific mechanisms for neuroprotection or pharmacokinetic shortcomings of candidate therapeutic agents. Being mindful of this, a target-agnostic in vivo screen of more than 1,000 compounds revealed a highly active neuroprotective aminopropyl carbazole that was named A20. To date, A20 has demonstrated protective efficacy in multiple preclinical models of neurodegeneration but has never been tested in transient ischemic stroke.

A20 is suspected to be neuroprotective by enhancing NAMPT activity, which subsequently increases the flux of NAD through the salvage pathway in normal mammalian cells. NAD is important for mitochondrial metabolism, the cellular defense system, and neurogenesis, as well as an essential substrate for ATP production. During cerebral ischemia, NAD is rapidly depleted and augmentation of NAD has been proposed as a potential therapeutic target for treating patients suffering from stroke. In one recent cerebral ischemia study, A20 treatment 30 minutes after permanent electrocoagulation MCAO in mice increased NAD levels and reduced infarct size 24 hours after injury. However, this study had three major limitations. First, it did not address the clinically relevant scenario of cerebral ischemia followed by reperfusion. Second, it did not investigate if A20 treatment enhanced neurogenesis and reduced brain damage chronically. Third, it did not evaluate any behavioral tasks. The study did assess infarct volume 24 hours post injury, however acute tissue damage correlates to neither
chronic tissue damage nor functional ability\textsuperscript{161}. We designed our experiment to improve upon these limitations by addressing STAIR recommendations.

In this chapter we evaluate A20’s efficacy as an immediate treatment for transient focal cerebral ischemia, using many outcome measures including: tissue volume, neurogenesis in the SGZ and SVZ, and sensorimotor and learning and memory behavior. Furthermore, we specifically investigate A20’s proposed mechanism by examining NAMPT and NAD levels, at 48 hours post ischemia. This is the first study to report chronic and functional reduction in stroke-deficits using A20.

3.2 Results

**Inclusion, Exclusion, and Mortality**

There were 12 animals used in each of the three experimental groups for histopathological and behavioral assessment, and 6 animals were used in each group for NAD and NAMPT assessments. After surgery, a neurological score was performed to assess injury, and 4 animals were excluded because they did not show behavioral deficits consistent with focal ischemia. A box plot was used to determine statistical outliers. Three animals were excluded from the cylinder task, and 3 animals were excluded from the water maze task. There were a total of 11 animals that died within 24 hours after tMCAO. Six of these rats were vehicle treated and 5 were A20 treated.
Figure 2. Experimental paradigm. Rats were acclimated to their new environment one week before surgery. After tMCAO, rats were administered either A20 or vehicle immediately after reperfusion followed by twice daily injections for one week in concert with daily BrdU injections. After one week, rats underwent sensorimotor behavioral assessments using the cylinder and grid-walk task. One month after tMCAO, learning and memory behavioral tasks were examined using the Morris water maze. At 6 week post-injury, rats were sacrificed and tissue was processed for pathological and immunohistochemical analysis.

A20 Treatment Improves Sensorimotor Recovery 1 week after Stroke

To investigate whether A20 would have a beneficial behavioral effect after stroke, we administered drug twice daily for one week starting immediately after reperfusion (90 minutes after the initial occlusion; Figure 2). All animals underwent every stage of the experimental paradigm, which includes sensorimotor behavior tasks, learning and memory behavior tasks, and histology. After one week, sensorimotor function was assessed using the cylinder and grid-walk tasks. The cylinder task evaluates weight bearing shifts of both forepaws during vertical exploratory behavior. After ischemic injury, rats rarely bear weight on their affected forelimb when exploring the novel environment, which was observed with vehicle-treated animals (p < 0.001) (Figure 3A). However, A20-treated rats utilized their affected forelimb nearly twice as much as vehicle-treated rats (p < 0.001) (Figure 3A).
In the grid-walk task, rats use somatosensory stimuli and only step along the grid prior to placing their full weight on their paws. All experimental groups showed some level of missteps (~10% in sham rats), likely due to not having trained in the task before. There was a significant difference amongst the three groups (p=0.014). Vehicle-treated rats performed significantly worse than both sham (p < 0.01) and A20-treated animals (p < 0.05) (Figure 3B). A20-treated animals had significantly improved motor coordination and performed at a similar ability as sham animals. The decreased missteps observed in the grid-walk task, along with increased use of the contralateral forelimb in the cylinder task, indicates A20 treatment significantly improved somatosensory function, likely by protecting the vulnerable somatosensory cortex after stroke.

**Figure 3. A20 treatment improves sensorimotor function 1 week after stroke.**
Before undergoing surgery, a baseline cylinder test was performed to confirm that rats did not have a symmetry bias. **A,** The cylinder test was then repeated one week after stroke and asymmetry was normalized to the baseline. Ischemia produced a significant deficit in symmetry for vehicle-treated rats (p < 0.001) as compared with sham animals. However, rats treated with A20 utilized their ischemic forelimb significantly more than vehicle-treated rats (p < 0.001). **B,** On the same day, animal’s motor coordination was examined with the grid-walk task. Sham animals had missed steps 10% of the time. However, the number of missed steps was significantly heightened after injury with vehicle-treated rats (p < 0.01). Rats that were treated with A20 had a significant reduction in missed steps (p < 0.05). For all groups, n=12; mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 versus sham. One-way ANOVA with Tukey’s HSD correction for multiple comparisons.
A20 Treatment Improves Learning and Memory 4 Weeks after Stroke

At 28 days after stroke, rats underwent learning and memory behavioral testing in two different paradigms of the MWM in order to examine both long-term and working memory. To assess long-term memory, (Figure 4A), rats underwent 4 trials a day in which they were allowed up to 60 seconds to find a hidden platform. Over the course of 4 days, all groups demonstrated acquisition learning of the task. There was no significant interaction between animal treatment and days (Figure 4A), however there was a significant main effect for animal treatment ($F(2,33) = 4.48, p = 0.019$). Specifically, vehicle-treated stroke animals had significantly increased escape latencies compared to sham animals ($p < 0.001$). On the final day of acquisition, there was a significant improvement ($p < 0.05$) in escape latency in A20-treated rats compared to the vehicle-treated group (Figure 4B). There was only a significant effect on day 4 ($p=0.006$).

The rat’s ability to recall the platform location was assessed on day 5 using a probe trial. The hidden platform was removed, and the total time spent in the target quadrant was measured. Both sham ($p < 0.001$) and A20-treated rats ($p < 0.05$) spent significantly more time in the target quadrant as compared to vehicle-treated rats (Figure 4C). Since all animals spent the same amount of time in the probe trial (60s), the total path length was used to assess swimming deficits caused by tMCAO. Between treatment groups, there were no significant difference in total path length (Figure 4D) in the probe trial or swim speed (Figure 4E) in the 4 training days, suggesting that the stroke itself did not affect swimming ability.
Figure 4. A20 treatment improves learning and memory 1 month after stroke. A, All treatment groups had a significant reduction in escape latency during acquisition. B, On day 4, vehicle-treated animals showed significantly worse escape latencies than both sham (p < 0.001) and A20-treated (p < 0.05) rats. Each data point represents the latency for an individual animal; wider horizontal bars represent the group average; smaller horizontal bars represent one standard error from the mean. C, During the probe trial, both sham (p < 0.001) and A20-treated (p < 0.05) rats spent significantly more time in the target quadrant than vehicle-treated animals. D, E, None of the treatment groups differed in their swim speed over the entire behavior paradigm or path length during the probe trial. F, To examine working memory, animals were tested using 5 paired trials; location trial and match trial. G, All groups had significant reductions in escape latency on the
match trial when compared to the location trial. During the match trial, vehicle-treated animal’s latency was significantly higher than that of sham (p < 0.05) and A20-treated (p < 0.05) animals. There was no significant difference between sham animals and A20-treated animals. H, tMCAO significantly impaired working memory compared to sham (p < 0.001), however A20 treatment was associated with near full recovery (p < 0.01). For all groups, n=12; mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 versus sham. A, G, Two-way ANOVA with Bonferroni correction for multiple comparisons. B-E, H, One-way ANOVA with Tukey’s HSD correction for multiple comparisons.

To assess working memory, rats were tested in the MWM with 5 paired trials, consisting of a location trial and match trial. The location of the hidden platform remained the same within each pair of trials, and the difference in escape latency between the location and match trials was used to measure working memory (Figure 4F). There were no significant differences in escape latency between experimental groups during the location trial, but there was a significant decrease in escape latency in all groups between the two trials (Figure 4G). Within the match trial, sham (p < 0.05) and A20-treated rats (p < 0.05) showed significantly greater improvements as compared to vehicle-treated rats after stroke. In addition, there was no significant difference between sham and A20-treated rats during the match trial (Figure 4H). In summary, A20 treatment significantly improved spatial learning, memory retention, and working memory after stroke.

A20 Treatment Increases Cortical and Hippocampal Tissue Volume after Stroke

To investigate the effects of A20 treatment on progressive cortical and hippocampal atrophy after stroke, coronal brain sections were stained with H&E 42 days after ischemia, and tissue volumes were quantified (Figure 5A,C). Rats that received treatment with A20 after stroke had significantly increased cortical volume (p < 0.05).
than rats that were treated with vehicle (Figure 5B). Within the hippocampus, a significant increase in tissue volume (p < 0.05) was also observed in A20-treated animals as compared to vehicle treatment after stroke (Figure 5D). Ipsilateral cortical and hippocampal tissue volume assessments were normalized to the average volume of the sham experimental group.

**Figure 5. A20 treatment increases tissue sparing after stroke.** Representative images of cortical A and hippocampal C tissue sparing six weeks after stroke. B, D, A20-treated animals had increased cortical and hippocampal tissue volume compared with vehicle controls after tMCAO (p < 0.05). Tissue volume was normalized to sham experimental group. Each point represents the calculated volume for an individual animal; wider horizontal bars represent the group average; smaller horizontal bars represent one standard error from the mean. For all groups, n = 12; mean ± SEM. *p < 0.05 versus tMCAO+vehicle. One-way ANOVA with Tukey’s HSD correction for multiple comparisons.

**A20 Treatment Increases Postnatal Neurogenesis in the SVZ and SGZ after Stroke**

Cerebral ischemia increases the number of proliferating progenitor cells, in both the SVZ and SGZ, for many weeks after injury. However, the majority of these cells do
not survive to maturation. We have previously shown that treatment with A20 of the SGZ, however some were localized within the granular cell layer. In the SVZ,

**Figure 6.** A20 treatment increases neurogenesis after stroke, as illustrated by representative images of neurogenesis in the SGZ of the hippocampus A and SVZ of the lateral ventricles C. NeuN+ cells were labeled with green (Alexa 488), and BrdU+ cells were labeled with red (Alexa 594). Neurogenesis occurred basally in both neurogenic niches as demonstrated in sham animals. After focal cerebral ischemia, there was a significant increase in co-labeled BrdU+:NeuN+ cells in the SGZ (p < 0.001) B and SVZ (p < 0.001) D in vehicle-treated animals. A20 treatment further enhanced neurogenesis as significantly more co-labeled cells were identified in the SGZ (p < 0.01) and SVZ (p < 0.05) then both vehicle and sham animals. For all groups, n=12; mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 versus sham. One-way ANOVA with Tukey’s HSD correction for multiple comparisons.
neurogenesis was most prevalent at the edges of the ventricles along the rostral migratory stream (Figure 6C). As in the SGZ, there was a significant increase in BrdU+:NeuN+ cells in the SVZ after stroke (p < 0.001), which was further increased with A20 treatment (p < 0.05) (Figure 6D).

**Increased Tissue Sparing with A20 Treatment was Positively Correlated with Improved Behavioral Measurements**

The relationship between histopathology and behavioral performance was investigated in order to demonstrate similar trends for improved outcomes with A20 treatment. The relationship between sensorimotor performance and histopathology was examined first with the cylinder task (Figure 7A). There was a strong positive relationship with sensorimotor ability and cortical tissue sparing (Pearson’s r=0.83; p<0.001) as well as SVZ neurogenesis (Pearson’s r=0.68; p<0.05) with A20 treated rats. There was no correlation between SGZ neurogenesis and sensorimotor ability (Pearson’s r=0.53), but hippocampal tissue sparing did have a significant, but weak, relationship with sensorimotor ability (Pearson’s r=0.86; p<0.001).

The relationship with memory retention and tissue sparing or neurogenesis, was evaluated next with the MWM probe trial (Figure 7B). In the hippocampus, there was a positive relationship between hippocampal tissue sparing and time in the target quadrant (Pearson’s r=0.21; p<0.05) with A20 treatment. There was no significant correlation between SGZ neurogenesis and memory retention, however the data was trending (Pearson’s r=0.56; p=0.057) for drug treated rats. In the cerebral cortex, there was a
Figure 7. Tissue sparing strongly correlates with A20-induced behavioral improvements. Scatter plots of histopathological measures in relation to behavioral outcomes. A, Correlations of tissue sparing in the cortex and hippocampus as well as neurogenesis in the SVZ and SGZ with asymmetry during the cylinder sensorimotor task. B, Correlations of total time spent in the target quadrant during the MWM probe trial with tissue sparing in the cortex and hippocampus as well as neurogenesis in the SVZ and SGZ. C, Correlations of the amount of cortical tissue
sparing versus SVZ neurogenesis as well as the amount of hippocampal tissue sparing versus SGZ neurogenesis. For all groups, n=12. *p<0.05, **p<0.01, ***p<0.001, two-tailed Pearson Correlation coefficients. Lines represent regression analyses.

A moderate relationship between cortical tissue sparing and latency in the probe trial (Pearson’s r=0.62; p<0.05), but the probe trial improvements were not correlated to increased SVZ neurogenesis (Pearson’s r=0.062). To determine if there was a positive correlation between tissue sparing and neurogenesis, these two outcome measures were also examined (Figure 7C). There was a moderate correlation between SVZ neurogenesis and cortical tissue sparing (Pearson’s r=0.66; p<0.05) as well as between SGZ neurogenesis and hippocampal tissue sparing with A20 treatment (Pearson’s r=0.68; p<0.05).

**A20 Treatment Increases Cortical NAD Levels after Stroke**

A20 enhances flux of NAD via the salvage pathway in normal mammalian cells. To determine whether A20 treatment might also help maintain NAD levels *in vivo*, we assessed NAD levels in the brain 48 hours after cerebral ischemia. This post-ischemic time point was chosen based on a previous publication that demonstrated NAD was upregulated in the cortex 48 hours after administering A20. In the present study, there was a significant reduction in NAD levels (p < 0.01) in the cortex of injured animals compared to sham animals (Figure 8A), which was rescued in the A20-treatment group (p < 0.05). Although there was no significant change in NAD levels between experimental groups in the hippocampus, the trend was similar to cortical NAD levels (Figure 8B). Since active variants of A20 have been shown to bind to NAMPT and
enhance its activity, protein levels for NAMPT were analyzed via western blotting. At 48 hours after cerebral ischemia, there was no significant change in cortical (Figure 8C) or hippocampal (Figure 8D) NAMPT protein levels.

Figure 8. A20 treatment preserves NAD levels in the brain after stroke. 48 hours after stroke, NAD and NAMPT levels were assessed from cortical and hippocampal tissue. A. Stroke significantly reduced NAD levels in the ipsilateral cortex of vehicle-treated animals (p < 0.01), but NAD was restored to normal levels after A20 treatment (p < 0.05). B. There was no significant change in NAD levels in the hippocampus after tMCAO. C, D, NAMPT levels were unchanged after focal cerebral ischemia. For all groups, n=6; mean ± SEM. *p<0.05, **p<0.01 versus sham. One-way ANOVA with Tukey’s HSD correction for multiple comparisons.

3.3 Major Findings

The experiments conducted in this study demonstrate that treatment with the neuroprotective compound A20 after transient ischemic stroke increases neurogenesis in the SGZ and SVZ as well as tissue volume in the hippocampus and cortex. Furthermore, this neuroprotection and neurogenesis directly correlate to the improvements observed in
sensorimotor ability, using the cylinder task, and hippocampal-dependent spatial memory, using the MWM. This protection also improved motor coordination during the grid-walk task as well as acquisition memory, memory retention, and working memory during the MWM. Finally, this recovery was likely due to A20’s ability to enhance NAMPT activity, which increased NAD levels in the cortex. This study is the first to show the treatment with A20 improves chronic outcomes after transient focal cerebral ischemia.

3.4 Limitations for Immediate A20 Treatment

Despite demonstrating a reduction in cerebral ischemia-induced deficits with A20, treating within 90 minute of an occlusion may not be a practical strategy for the clinic. tPA is already an effective FDA approved drug for ischemic stroke, but less than 5% of ischemic patients receive treatment because it can only be administered within 4.5 hours of symptoms 86. The majority of patients take more than 3 hours just to enter to a hospital building 87, which limits the available time for scheduling and conducting a CT scan. Furthermore, most people have difficulty identifying the precise time their symptoms emerge and instead provide a time range. Treating physicians typically air on the side of caution and select the greater time point when deciding if a patient qualifies for treatment with tPA, which excludes many people 247. Therefore, tPA’s shortcomings demonstrate how limiting a 90 minute therapeutic window is in a common clinical situation.

STAIR understood the repercussions of not examining the window of opportunity, specifically for neuroprotective agents, and consequently incorporated it as a recommendation in their first published conference proceedings in 1999 161. STAIR-1
even suggested that the NMDA antagonist’s selfotel and cerestat failed clinical trials because the therapeutic window was not known. Since NMDA antagonists block the flux of calcium, they were mostly effective when excitotoxicity was most prevalent, which was up to 1 hour after the initial occlusion in animals\textsuperscript{248}. Future studies are now required to determine if A20 treatment can be delayed to a practical, clinically relevant time point.
Chapter 4

Beneficial Effects of Delayed A20 Treatment after tMCAO in Rats

4.1 Introduction to the Experiment

Ischemic stroke is a devastating condition caused by obstructing or reducing cerebral blood flow, which results in cell death\textsuperscript{15}. In the penumbra, cells can survive longer than the ischemic core due to enhanced blood perfusion. Restoring blood flow is critical to treating stroke because 1.9 million neurons are estimated to die for every minute cerebral ischemia persists\textsuperscript{249}. Underscoring the importance of tissue perfusion, tPA is effective by disintegrating clots and thus reperfusing the brain\textsuperscript{84}. In clinical trials, tPA was shown to be effective when administered early, but when it was administered past 4.5 hours, there were greater risks for hemorrhagic transformation with only limited benefits\textsuperscript{86,89}. To aggravate matters, the majority of ischemic patients take more than 3 hours after stroke onset to reach an emergency department\textsuperscript{87}, and the average door to needle time is over an hour\textsuperscript{88}. These factors drastically reduce the number of patients eligible for tPA therapy. Endovascular thrombectomy has also demonstrated success as a therapy for ischemic stroke due in part to their prolonged therapeutic window. These procedures can be utilized up to 12 hours of patient symptoms and when combined with tPA demonstrate increased efficacy\textsuperscript{96}. Although, even if blood flow is restored by spontaneous or therapeutic interventions, neurons may still undergo delayed cell death\textsuperscript{37}. There is therefore a critical need to develop new cytoprotective agents for patients that directly support the survival of injured neurons after ischemic stroke.

A promising approach to treat ischemic stroke is the use of combination therapies, including neuroprotective strategies, that aim to extend the time window for thrombolytic
intervention. Neuroprotective drugs could delay cell death until blood flow is restored, and also counteract the deleterious effects of reperfusion injury. For example, in experimental models, positive synergistic effects have been observed when thrombolysis was combined with AMPA and NMDA receptor antagonists, MMP inhibitors, immunosuppressants, scavengers of ROS, and therapeutic hypothermia. However, few neuroprotective compound’s therapeutic window have been established, which has contributed to the poor translation of neuroprotective agents.

In Chapter 3, A20 demonstrated therapeutic efficacy when it was administered immediately after a 90 minute tMCAO. A20 was selected because it has attractive pharmacological properties and was efficacious in multiple preclinical injury models in the nervous system. A20 protected both mature and immature neurons from degeneration, which translated into preservation of sensorimotor and cognitive behavioral function as well as maintenance of brain tissue volume at chronic time points. Improved functional recovery directly correlated with increased tissue sparing and increased neurogenesis in both the subventricular zone and hippocampal dentate gyrus subgranular zone. Although, tPA’s narrow therapeutic window revealed how limiting it would be if P7C3-A20 could only be administered during the early periods after stroke.

Encouraged by these positive effects of immediate A20 treatment, we next sought to determine whether delayed treatment with A20 beginning 6 hours after reperfusion, a more clinically relevant time point, would also provide significant protection from chronic deficits. In the current chapter, a one week course of A20 treatment was initiated immediately (iA20) or delayed by 6 hours (dA20) after a 90 minute tMCAO in rats.
Behavioral and histopathological outcome were then assessed chronically over 7 weeks. Both iA20- and dA20-treated animals displayed reduced sensorimotor deficits on the cylinder and grid-walk tasks as well as improved cognitive performance in the MWM. In addition, there was a reduction in cortical infarct volume 48 hours after cerebral ischemia with both immediate and delayed A20 treatment. Both groups of A20-treated stroke animals also displayed increased cortical tissue volume compared to vehicle-treated stroke controls at 7 weeks after injury, suggesting this protection was sustained chronically. These results support our previous findings and demonstrate for the first time a persistent therapeutic effect with delayed administration of A20 after stroke in mitigating neurodegeneration and promoting chronic functional recovery.

4.2 Results

Inclusion, exclusion and mortality

There were two sets of animals used in the experimental paradigm (Figure 9); 60 animals for chronic behavioral and tissue volume assessments and 48 animals for acute infarct volume (TTC) assessments. A neurological score was performed prior to MCA reperfusion to assess ischemic-induced functional deficits, and a total of 14 animals were excluded because they did not demonstrate an acute behavioral deficit. In addition, eight statistical outliers were determined using a box plot, which eliminated 4 rats based on performance in the cylinder task and 4 rats based on performance in the water maze task. Also, within 3 days of cerebral ischemia, 18 animals died or were excluded from future testing because they lost more than 20% of their body weight.
**Figure 9. Experimental paradigm for delayed A20 treatment.** Rats were acclimated to the new environment for one week before surgery. Stroke was induced using a 90 min tMCAO, and rats were then administered vehicle or A20 either immediately or 6 hours post reperfusion. Forty-eight hours after reperfusion, infarct volume was assessed in the cortex and striatum. At 7 days, sensorimotor ability was determined with the cylinder and grid-walk task. Hippocampal-dependent spatial memory tasks began 4 weeks post injury and lasted for one week. Six weeks after ischemia, rats were sacrificed for cortical, striatal, and hippocampal histological assessments.

**Immediate (iA20) and delayed (dA20) A20 Treatment Reduces Acute Cortical Infarct Volume**

Infarct volumes were first assessed 48 hours after reperfusion using TTC staining, and representative images are presented in Figure 10A. In the cortex, there was a significant reduction in cortical infarct volume with iA20 treatment as compared with tMCAO+Vehicle (p < 0.001) (Figure 10B). This protection with A20 was sustained when treatment was delayed 6 hours, as dA20-treated animals also had significantly reduced infarct volumes compared to tMCAO+Vehicle animals (p < 0.01). Furthermore, there was no significant difference in cortical infarct volume between iA20 and dA20 treatment groups. Since the infarct core involves the striatum with the filament-MCAO model, striatal TTC volumetric assessments were also performed. At 48 hours, there was no significant difference between any experimental groups in striatal infarct volumes (Figure 10C).
Figure 10. iA20 and dA20 treatment reduce cortical infarct volume. Forty-eight hours after reperfusion, rats were sacrificed and cortical and striatal infarct volumes were determined using TTC staining (A). B, Immediate (iA20) and 6 hour delayed (dA20) A20 treatment significantly reduced cortical infarct compared to tMCAO+Vehicle. C, There was no significant change in striatal infarct volumes. For all groups, n=12; mean ± SEM. **p<0.01, ***p<0.001 versus tMCAO+Vehicle. One-way ANOVA with Tukey’s HSD correction for multiple comparisons.

Sensorimotor Deficits are Reduced by Both iA20 and dA20 Treatment

Since dA20 treatment reduced infarct volumes acutely, we next investigated whether this tissue protection translated into functional improvements. At 7 days after tMCAO, sensorimotor ability was assessed using the cylinder and grid-walk task. As expected, sham animals had no deficit in symmetry (Figure 11A). After cerebral ischemia, however, there was a significant reduction in symmetry in the tMCAO+Vehicle treatment group as compared to sham animals (p < 0.001). dA20 treatment significantly increased symmetry as animals used their contralateral forelimb more than
tMCAO+Vehicle animals (p<0.01). There was no significant difference between iA20 and dA20-treated animals (Figure 11A).

On the same day, impairments in limb function were assessed using the grid-walk task. Motor coordination was determined by measuring the percentage of missteps that occurred in the rat’s contralateral forelimb. Due to the unfamiliarity of the task, all animals will make some degree of misstep (Figure 11B). Cerebral ischemia significantly impaired motor coordination as all treatment groups had an elevated number of missteps as compared to sham rats. There was a significant reduction in missteps, however, with both iA20 and dA20 treatment as compared with tMCAO+Vehicle (p < 0.05). Sham, iA20 treatment, and dA20 treatment were not significantly different (Figure 11B).

**Figure 11.** iA20 and dA20 treatment both improve sensorimotor ability 1 week after stroke. One week after injury, sensorimotor ability was assessed using the cylinder and grid-walk test. A, In the cylinder task, sham animals demonstrated normal symmetry. However, cerebral ischemia significantly impaired forelimb use in tMCAO+Vehicle rats. There was significant improvement in symmetry with dA20 treatment as compared with vehicle treatment after tMCAO (p<0.01). Between iA20 and dA20 treatment, there was no significant difference in symmetry. B, On the same day, motor coordination was assessed with the grid-walk task. Cerebral ischemia significantly impaired motor coordination in all experimental groups, and treatment with both iA20 and dA20 significantly reduced the percentage of missteps (p<0.05) compared to tMCAO+Vehicle. For all groups, n=12; mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 versus sham. One-way ANOVA with Tukey’s HSD correction for multiple comparisons.
iA20 and dA20 Treatment Improves Hippocampal-Dependent Spatial Memory

Based on our previous findings that iA20 treatment protects rats from learning and memory deficits after tMCAO, we next tested whether this same therapeutic benefit would be documented with delayed treatment. Four weeks after cerebral ischemia, cognitive ability was examined using a hippocampal dependent spatial test, the MWM. Acquisition was assessed over 4 training days as animals learned the location of a hidden platform using spatial cues located around the room. All group’s escape latencies were reduced daily, demonstrating acquisition learning in all animals. There was no significant interaction between escape latency and days (Figure 12A). There was a significant main effect on treatment ($F_{(4,44)} = 13.06, p < 0.001$) as tMCAO+Vehicle rats were significantly different than sham, iA20, and dA20 treatment. On the final day of acquisition, both sham and iA20-treated animals found the hidden platform fastest, whereas tMCAO+Vehicle rats took twice as long ($p<0.01$) (Figure 12B). There was no significant difference between dA20 and tMCAO+Vehicle animals or between iA20-treated, dA20-treated, and sham animals.

Memory retention was evaluated next by removing the hidden platform and measuring the time spent in the quadrant that previously housed the platform in a probe trial (Figure 12C). Focal ischemia impaired memory retention as tMCAO+Vehicle rats performed significantly worse than sham animals ($p < 0.01$). Treatment with iA20 improved memory retention as rats spent significantly more time in the target quadrant ($p < 0.01$). There was no improvement with dA20 treatment, although the results were trending ($p =0.058$). Total path length and swim velocity were also examined during the probe trial, and there was no difference between treatment groups (Figure 12D,E).
Figure 12. iA20 and dA20 treatment reduces stroke-induced cognitive deficits. Four weeks after stroke, learning, memory retention and working memory were assessed using the MWM. A, Over the course of four training days, all experimental groups improved in their escape latencies. There was no significant interaction between treatment and days, but there was a significant main effect of treatment between sham, iA20 treatment and dA20 treatment compared to tMCAO+Vehicle. B, On the final day of acquisition, sham animals reached the target quadrant in 10 seconds, whereas tMCAO+Vehicle spent twice as long. Administering iA20 significantly reduced escape latency compared to tMCAO+Vehicle (p<0.01). There was no improvement with dA20 treatment. C, After training days, memory retention was assessed with a probe trial. tMCAO+Vehicle animals spent significantly less time in the target quadrant as compared to sham (p<0.001) and iA20 treated (p<0.01) animals. dA20 did not significantly improve memory retention, but the results were trending (p=0.058). D, E, There was no change in swim velocity or path length on the probe trial between all groups. F, Working memory was determined using paired
trials, consisting of a location trial and then a match trial. G, There was no significant interaction between treatment group and trial. H, Between the two trials, there was a significant reduction in working memory after cerebral ischemia (p<0.001). Both iA20 (p<0.001) and dA20 (p<0.05) treatment significantly improved working memory on the match trial as compared to tMCAO+Vehicle. For all groups, n=12; mean ± SEM. A, **p<0.01, ***p<0.001 versus Vehicle. B-G, *p<0.05, **p<0.01, ***p<0.001 versus sham. A, G, Two-way ANOVA with Bonferroni correction for multiple comparisons. B-E, H, One-way ANOVA with Tukey’s HSD correction for multiple comparisons.

After the probe trial, working memory was evaluated with a MWM paradigm that used 5 paired trials, consisting of a location and match trial (Figure 12F). The starting location of the rat and hidden platform location were changed at the start of each location trial. Working memory was determined as the difference between the match and location trial. There was no significant interaction between treatment groups and trials, but it was trending (p=0.068) (Figure 12G). All treatment groups performed better on the match trial (Figure 12H), however, sham animals demonstrated the greatest improvement (62%). There was a significant reduction in working memory ability with tMCAO+Vehicle animals as compared to sham (p < 0.001), which was recovered with iA20 treatment (p < 0.001). There was no significant improvement with dA20 treatment as compared with vehicle treatment (p < 0.05). Sham, iA20 and dA20 were not significantly different in the MWM working memory paradigm.

Cortex and Striatum are Protected Chronically with both iA20 and dA20 Treatment

We next speculated that A20’s ability to increase cell survival could be responsible for the sensorimotor and cognitive improvements observed in treated animals. Seven weeks after injury, rat brains were stained with H&E in order to quantify
cortical, striatal, and hippocampal tissue volume (Figure 13A,D). In the cortex, both iA20 and dA20-treated animals had significantly more cortical volume as compared to tMCAO+Vehicle (Figure 13B). There was a similar trend in the striatum, as iA20 (p < 0.01) and dA20 (p < 0.01) treated animals had significantly more tissue volume then

**Figure 13. iA20 and dA20 treatment increases cortical and striatal tissue volume.** Seven weeks after tMCAO, H&E staining was performed in order to calculate cortical, striatal and hippocampal tissue volume (A, D). B, There was a significant increase in cortical tissue volume with iA20 (p<0.001) and dA20 treatment (p<0.05) when compared with tMCAO+Vehicle. C, In the striatum, there was a significant increase in tissue volume with both A20 treatment groups (p<0.01) compared to tMCAO+Vehicle. E, Only iA20 treatment had significantly (p<0.01) increased tissue volume compared to tMCAO+Vehicle in the hippocampus. Percent tissue volume was normalized to the average of all sham animals for each region. Each point represents the calculated volume for an individual animal; wider horizontal bars represent the group average; smaller horizontal bars represent one standard error from the mean. For all groups, n=12. *p<0.05 versus tMCAO+Vehicle. B, C, D, One-way ANOVA with Tukey’s HSD correction for multiple comparisons.
tMCAO+Vehicle (Figure 13C). In the hippocampus there was minimal tissue loss in the tMCAO+Vehicle animals compared to sham animals. There was only significant tissue preservation with iA20 treatment (p < 0.05) and not dA20 treatment (Figure 13D). In all histopathological assessments, there was no significant difference between iA20 and dA20 treatment groups.

4.3 Major Findings

The major goal of the present study was to determine whether A20 was efficacious when treatment was initiated 6 hours after a 90 minute tMCAO. iA20 and dA20 treatment significantly reduced stroke-induced sensorimotor deficits in the cylinder and grid walk task as well as learning and memory deficits in the MWM. In addition, both iA20 and dA20 treatment significantly reduced cortical infarct volume acutely and increased cortical tissue volume chronically. Despite no difference in acute striatal infarct volume with both A20 treatment groups, there was a significant increase in striatal tissue volume chronically. At 7 weeks post injury, there was only a significant difference in hippocampal tissue volume with iA20 treatment and not with dA20 treatment. This reduced hippocampal protection with dA20 treatment correlated to the diminished cognitive recovery observed during hippocampal-dependent spatial learning, memory retention, and working memory behavioral tests. Finally, dA20 treatment demonstrated functional improvements in every sensorimotor test but only in a few learning and memory tests.
Chapter 5

Discussion

5.1 Introduction to Experimental Treatments for Stroke

The first documentation of stroke dates back to 460 BCE by Hippocrates\textsuperscript{251}. Noticing that victims typically experience sudden paralysis, Hippocrates appropriately named the condition “apoplexy,” which translates to “struck down by violence”\textsuperscript{252}. However, despite 2,400 years of medical advancements, people today who suffer a stroke still have limited treatment options. That being said, there have been advancements in immediate stroke care, such as improving thrombolytic and antiplatelet agents, which has increased overall patient survival after ischemic stroke. But as mortality has decreased, disability has subsequently increased. Cerebral ischemia is currently the leading cause of long-term disability, emphasizing the need to develop novel experimental strategies to treat ischemic stroke\textsuperscript{253}.

Some emerging therapies have reported efficacy as a treatment for ischemic stroke, including intra-arterial tPA, stent retrievers, and combinatorial approaches that utilize neuroprotective agents. Neuroprotective treatments are effective by preventing or activating specific cellular mechanisms in the ischemic cascade, and they could be utilized to delay cell death until thrombolysis can be implemented. The studies in this dissertation project sought to explore, for the first time, the effects of protecting both mature and immature neurons to promote recovery after focal cerebral ischemia using the neuroprotective compound A20. In addition, we wanted to investigate if A20 reduced stroke-induced histopathological damage and functional deficits, such as sensorimotor ability and hippocampal dependent spatial memory. Finally, we wanted to evaluate A20’s
window of opportunity to determine whether treatment could be administered at a clinically relevant time point.

5.2 Immediate Treatment with A20 Promotes Neurogenesis and Improves Cognitive Function after Ischemic Stroke

The objective of the first study was to evaluate the efficacy of prolonged administration of the aminopropyl carbazole A20 in a model of transient cerebral ischemia using long-term behavioral, biochemical, and immunocytochemical outcomes related to both mature neuron survival and postnatal neurogenesis. One week post-stroke, A20-treated animals demonstrated significant improvements in both the grid-walk and cylinder tasks, demonstrating that sensory ability and motor control were improved with A20 treatment after stroke. Improvements in both short-term and long-term memory at a later chronic time point were also observed with A20-treated animals, using two different MWM paradigms. A20-treated animals had significantly reduced latency to locate the hidden platform, spent significantly increased time in the target quadrant during the probe trial, and showed significantly reduced time in the location trial of the working memory paradigm. A20 treatment thus improved both learning acquisition and memory retention after stroke, which was associated with increased neurogenesis within the SVZ and dentate gyrus SGZ.

A benefit of A20 that made it particularly well-suited for this study is its excellent pharmacological properties. It can be administered orally, has a long half-life, crosses the blood-brain barrier and is nontoxic even at very high doses 201. Here, we demonstrate that A20 has a robust and sustained therapeutic effect on chronic stroke outcome in rats, in
terms of promoting sensorimotor and cognitive recovery, as well as limiting histologic
signs of neurodegeneration at chronic time points. In this study we used the MWM to
assess cognitive function after focal ischemia using methods similar to previous studies
\cite{226,254,255}. However, it has recently been suggested that the MWM has some limitations,
possibly due to the resulting motor dysfunction \cite{233}. Since tMCAO rats might have
difficulty swimming due to the unilateral deficit from the ischemia, it could be
challenging to separate a purely cognitive deficit from a possible sensorimotor deficit.
However, we demonstrate that there was no significant difference in swim speed or path
length during the probe trial, which suggests that swimming ability was not a contributor
to the cognitive performance deficit observed in tMCAO animals.

A20 has been shown to enhance flux of the NAD salvage pathway in normal
mammalian cells. Moreover, active variants of A20 have been shown to bind to NAMPT
\cite{212}, the rate limiting enzyme in NAD salvage, which converts nicotinamide to NAD.
During ischemia, NAD-dependent PARP-1 becomes activated and rapidly leads to
plummeting NAD levels that trigger apoptosis \cite{178,246,256,257}. Conversely, administering
NAD or overexpressing NAMPT reduces infarct size in the striatum and corpus callosum
\cite{175,196}. Here, we observed no difference in cortical NAMPT levels between the
experimental groups, supporting A20’s mechanism to increase the activity and not the
amount of NAMPT. Moreover, since we also observed an A20-dependent preservation of
NAD levels only in ischemic animals, we suspect this proposed mechanism could explain
the significant increase in cortical volume after stroke. This region is normally perfused
by the middle cerebral artery (MCA), which was transiently occluded to induce the
stroke. Furthermore, we conclude that A20 treatment rescued the surrounding area
around the cortical infarct region, which typically has greater collateral blood circulation than the ischemic core, and thus exhibits delayed initiation of cell death. In addition, the motor and somatosensory cortex are most affected by tMCAO, which correlates with the improvements we observed in sensorimotor behavior as a function of A20 treatment.

Although the hippocampus is not directly perfused by the MCA, we observed a significant reduction in hippocampal tissue volume after tMCAO and protection when we treated A20. This hippocampal degeneration in the vehicle-treated stroke animals may be due to secondary damage to local circuits, synaptic structures, gap junctions, or hippocampal denervation. Moreover, reinnervation by corticospinal neurons near the infarct has also been observed in stroke, and thus the increase we observed in hippocampal volume in A20-treated stroke animals might also be due to enhanced survival of newborn neighboring neurons or circuits that foster this reinnervation. It has also been reported that lateral ventricle dilation occurs with hippocampal atrophy after focal ischemia, and thus might be a potential result of hippocampal degeneration. In the present study, evidence for ventricular enlargement at 6 weeks after tMCAO was observed possibly due to the reduced tissue volume in the ipsilateral cerebral cortex and hippocampus.

In addition to its role in mature neuron survival, NAD and NAMPT play an important role in postnatal neurogenesis. NAMPT activation is required for G1/S progression of the cell cycle, and NAD is an important energy source for progenitor cell proliferation. Ablating NAMPT reduces stem cell pools and proliferation in the adult hippocampus, whereas increasing NAD rescues this deficit. Furthermore, it was
recently reported that transgenic mice that overexpress NAMPT showed an increase in the number of neural stem cells after MCAO compared with controls. In the present study, postnatal neurogenesis was examined by quantifying the number of BrdU+ NeuN+ cells in the SGZ of the hippocampus and the SVZ of the lateral ventricles. Since BrdU incorporates into dividing cells and neurogenesis peaks 7 days after ischemia, BrdU was injected for one week post-injury. Therefore, any co-labeled cells in a neurogenic niche were identified as having been generated during the one week time period after injury. Co-expression of the mature neuronal protein NeuN indicates that the surviving newborn cells became mature neurons. After cerebral ischemia, both the SGZ and SVZ experience a surge of neurogenesis, with the majority of newborn cells dying before maturation. Newborn neurons from the SGZ travel from the inner blade region into the granule cell layer of the dentate gyrus where they become functionally mature, and this integration correlates with improved cognition. In the SVZ, immature neurons will migrate down the RMS and differentiate into olfactory interneurons in the OB. However after cerebral ischemia, NPCs migrate away from the well-defined RMS and have been reported to reside in or near the damaged areas where they functionally mature into spiny neostriatal projection neurons and interneurons.

In our study, focal cerebral ischemia increased the number of BrdU+ NeuN+ cells in the SVZ, and this was further enhanced with A20 treatment. It is suspected that SVZ neurogenesis has a role in stroke recovery because preventing NPC migration towards the infarct worsens functional outcomes in the beam-walking and water maze behavioral task. In our experiments, we also observed a relationship between SVZ neurogenesis and augmented stroke recovery. In both tMCAO experimental groups, there was a significant
correlation with SVZ neurogenesis and cortical tissue sparing, as well as SVZ neurogenesis and sensorimotor function using the cylinder task. SVZ neurogenesis could be contributing to the improved motor ability observed with A20 treatment because NPCs migrate to the striatum and neocortex, two regions that are heavily relied upon during the cylinder task. Currently, it is unknown if these migrating NPCs are replacing damaged cells or just providing trophic support, however, these data suggest that SVZ neurogenesis may be part of an endogenous reparative response to cerebral ischemia.

We observed a similar trend in the SGZ; cerebral ischemia significantly heightens neurogenesis, and treating with A20 further expands the NPC pool. There was also a significant correlation between SGZ neurogenesis and hippocampal tissue sparing, as well as SGZ neurogenesis and memory retention using the probe trial. Since the probe trial is a hippocampal-dependent spatial memory task, the behavioral improvements are most likely due to treatment-induced tissue sparing. Taken together, the significant correlation between SVZ neurogenesis and cortical tissue volume, as well as SGZ neurogenesis and hippocampal tissue volume supports a beneficial role of post-ischemic neurogenesis in chronic tissue preservation. In this regard, Jin and colleagues (2010) demonstrated that ablating doublecortin expressing cells following MCAO, results in increased tissue damage and neurological deficits. Moreover, these findings are consistent with previous studies showing that increased SVZ and SGZ neurogenesis are associated with increased tissue sparing and improved functional ability, respectively.

In previous neuroprotective studies, many compounds that have performed well in preclinical models of cerebral ischemia have later failed in clinical trials. In response
to this crisis, experts in academia, industry and government created STAIR to establish standards and benchmarks for aiding in efficacious translation of new therapies from the bench to the clinic. Therefore, we designed our study implementing several STAIR recommendations, including physiological monitoring, randomization of animal treatment groups, blinding of the experimenter to treatment group, utilization of quantitative histological and functional outcome measures, and assessment of outcome measures at more chronic periods after the initiation of the ischemic insult. These results indicate that A20 provides significant protective effects when tested under a rigorous study design, potentially raising the likelihood of successful translation to the clinic.

Summary

In summary, our studies demonstrate for the first time the beneficial effects of A20 treatment in advancing clinically relevant outcomes after stroke, including chronic pathological and behavioral recovery. We also report that A20 promotes neurogenesis in both the SGZ and SVZ neurogenic niches after stroke. The ability of A20 to simultaneously protect against progressive atrophy and promote restorative processes involving newborn neurons in postnatal neurogenesis makes it an attractive strategy for improving outcome after focal ischemic stroke.

5.3 Delayed A20 Treatment after Stroke

The major goal of this study was to determine whether the therapeutic benefits of A20 would be sustained if the treatment window was extended beyond immediate initiation of treatment after tMCAO. We show for the first time that delaying treatment of
A20 to 6 hours after a 90 min tMCAO significantly reduces cortical infarct volume 48 hours after reperfusion, as well as increases cortical and striatal tissue sparing 7 weeks after injury. These reductions in tissue damage translated into significant improvements in sensorimotor and hippocampal-dependent learning and memory ability. In each outcome measured, there was no significant difference between either iA20 or dA20 treatment, suggesting that the beneficial effects of A20 did not significantly diminish if treatment was delayed 6 hours post reperfusion. Taken together, this study support and extends previous publications demonstrating that A20 is efficacious as a treatment for focal cerebral ischemia.

Despite iA20’s ability to ameliorate many stroke-induced deficits, initiation of treatment immediately after reperfusion is not a practical expectation for treating the majority of acute stroke patients. This limitation is especially evident in regard to the use of tPA, which the majority of ischemic patients do not receive treatment for because it can only be administered up to 4.5 hours after the onset of stroke-related neurological symptoms. However, evidence in the literature hinted an extended therapeutic window for A20 treatment might exist. In blast-mediated TBI, 24 hour delayed P7C3 treatment blocked axonal degeneration and preserved learning and memory. A20’s mechanism of action provided additional support for extending A20’s therapeutic window after focal stroke. By enhancing flux of the NAD salvage pathway via stimulation of NAMPT, A20 stabilizes levels of NAD after injury. Interestingly, the coenzyme NAD becomes rapidly depleted after 30 minutes of ischemia, predominantly due to DNA damage and activation of PARPs, as well as 6 hours and 25 hours after reperfusion when necrosis and
apoptosis are most prominent, respectively. Therefore, salvaging NAD at 6 hours after focal stroke could have a significant beneficial effect on recovery.

As previously discussed, enhancing NAMPT activity and increasing intracellular NAD pools decreases infarct volume and prevents apoptosis after stroke. In chapter 3 we observed that NAD levels were recovered at 48 hours after reperfusion with iA20 treatment, therefore, we suspected A20 would also reduce infarct volumes at that same time point. In this study, we detected a significant reduction in cortical infarct with both iA20 and dA20 treatment, which suggests A20’s ability to protect the cortex was preserved when treatment was delayed 6 hours after reperfusion. By contrast, we observed no significant change between iA20 or dA20 treatment in striatal infarct volumes 2 days post-injury. Chronically however, there was a significant increase in tissue sparing in the cortex and striatum with both A20 treatment groups compared to tMCAO+Vehicle animals. In this regard, we were somewhat surprised to observe chronic protection in the striatum considering there was no difference in the striatal infarct acutely. One possible explanation is that animals that underwent infarct assessments had a curtailed treatment regimen. Since infarct volume was assessed only 48 hours after injury, the same time point that NAD was quantified in our previous A20 stroke publication, rats only received 1/3 the dose of A20 that chronic rats received. Therefore, A20’s striatal protection may require an extended treatment paradigm. Moreover, since the striatum represents the ischemic core of the insult, it is particularly vulnerable to a lack of collateral circulation after focal stroke. Cells in the striatum might simply require longer treatment regimens to attenuate the mechanisms underlying progressive atrophy and augment cell survival. In addition, dA20 treatment did not
significantly protect the hippocampal volume chronically after stroke, which correlated with minimal improvements observed in hippocampal-dependent spatial memory tests. Only in a working memory paradigm did dA20 treatment reduce any stroke-induced cognitive deficits. However, the results in memory retention with dA20 treatment were trending towards a protective effect.

Since we previously reported that iA20 treatment enhanced motor functional recovery 223, we also wanted to determine if this was seen in the dA20 group. In the grid-walk tasks, both iA20 and dA20 treated rats demonstrated significant improvements over tMCAO+Vehicle animals, indicating that delaying treatment of A20 had a sustained therapeutic effect. In the cylinder task, tMCAO significantly impaired symmetry and use of the animal’s contralateral forelimb, which was subsequently recovered with both iA20 and dA20 treatment. Despite observing no improvement with iA20 animals compared with tMCAO+Vehicle animals, there was also no significant difference between iA20 and dA20 rats.

Delayed treatment of A20 also reduced learning and memory behavioral deficits, which are commonly reported after focal cerebral ischemia. In the MWM, dA20-treated rats showed improvement in spatial learning, memory retention, and working memory. To confirm that the cognitive improvements observed with A20 treatment were not due to the impairments in motor ability, swimming performance was also evaluated using total path length and swim velocity during the probe trial 226,254,255. There was no significant difference in path length or swim velocity between the experimental groups, suggesting that tMCAO did not contribute to the observed MWM learning and memory deficits. Interestingly, the improvements in hippocampal-dependent learning were not as
great as the gains observed in sensorimotor function, possibly indicating a somatosensory-driven recovery with dA20 treatment.

**Summary**

In summary, our studies demonstrate for the first time that delaying the initiation of A20 treatment to 6 hours after cerebral reperfusion has a significant beneficial effect on long-term stroke outcome. A20 treatment reduced acute and chronic tissue damage, which was associated with improvements in sensorimotor ability and cognitive performance. In addition, this study replicated our original findings, which established the beneficial role of iA20 treatment after transient focal cerebral ischemia \(^{221,223}\). Ultimately, since the majority of ischemic patients take more than 4 hours after stroke onset to receive treatment \(^{87,88}\), being able to treat at 7.5 hours after the initial ischemic insult makes A20 an appealing strategy for acute ischemic stroke.

**5.4 Limitations, Risk Factors, and Adverse Effects**

Despite A20 demonstrating efficacy in preclinical settings, there are always limitations to any experimental study. One of the biggest disadvantages of pharmacotherapies is that experimental treatment paradigms do not usually reflect clinical treatment paradigms. For example, most medications prescribed outside a hospital are administered orally and not injected IP, unlike the animals in our study. Besides the obvious practical benefits to treating orally, these medications are not usually as effective at crossing the BBB and therefore, typically require a higher dose, which might cause adverse side effects \(^{268}\). Moreover, these potentially deleterious effects could potentially be unnoticed in a preclinical setting because the dosage could be lower. In
addition, clinical dosages often have to be readjusted throughout the treatment regimen and on an individual basis because there is always variability between people, just like rodents. The exact same medication might require a higher dosage or a shorter treatment course then originally prescribed, and therefore, physicians may have to alter the dose in order to maximize the drug’s desired effect. In our study, treatment remained consistent throughout our experimental ischemic stroke study even though A20 is also unlikely to have identical therapeutic efficacy between animals.

In this dissertation, rats underwent a previously established dosage and treatment regimen of A20. Beginning either immediately or 6 hours after reperfusion, rats were administered 10 mg/kg of A20 IP, followed by twice daily injections for 7 days. Although, we did not performed a dose-response curve in these series of studies, previous studies did investigate the optimal dose needed to demonstrate maximal pro-neurogenic efficacy\textsuperscript{201,211,215}. Our study also observed this enhanced neurogenesis, which was sustained 7 weeks post-injury with A20 treatment. The dose of A20 used throughout these studies appears to be adequate to observe efficacy because treating ischemic mice with A20 reduced sensorimotor and learning and memory behavioral deficits. However, studies should now be conducted to determine what the optimal dosage and duration of A20 is in order to maximize its therapeutic benefits for focal ischemic stroke.

It would also be advantageous to determine whether A20 is efficacious in other models of experimental stroke, especially because most rodent models do not accurately resemble human stroke\textsuperscript{44}. Since the majority of ischemic stroke patients do not have thrombolysis, A20 treatment should be evaluated in hemorrhagic stroke or permanent cerebral ischemia. Previously, A20 was assessed after a permanent MCAO using
electrocoagulation \(^{221}\), however since electrocoagulation requires a craniotomy, it has considerable variability \(^{44}\). Therefore, A20’s efficacy should be reevaluated using additional permanent occlusive ischemic stroke models, such as the intraluminal suture model or embolic model. Another issue with tMCAO, particularly in rodents, is that the occlusion typically generates larger infarcts than what is observed in the patient population \(^{44}\). Therefore, additional studies are now required to determine whether A20 treatment will produce significant, quantifiable improvements when the occlusion time is reduced. Moreover, it would also be beneficial to examine different durations of tMCAO, which could alter the infarct size and thus, treatment paradigm needed in order to observe therapeutic efficacy.

Even though A20 has been examined in many different neurodegenerative and acute injury models, it has only been evaluated in rodents \(^{203}\). Unfortunately, many therapies have demonstrated efficacy in rodent models, yet failed to successfully translate into other species \(^{269}\). Because of this, STAIR recommends \(^{161}\), as well as the FDA requires, that compounds be evaluated in higher order animals before beginning a clinical trial \(^{270}\). Studies are now needed to determine whether A20 could also improve recovery after cerebral ischemia in other model organisms including larger animals.

Finally, although we did not observe any change in seizure activity with A20 treatment, stroke does increase the incidence of epilepsy, and enhanced neurogenesis has been shown to increase the prevalence of seizures \(^{271}\). Furthermore, epilepsy heightens neurogenesis, which further increases the frequency of chronic seizures, thus creating a positive feedback loop \(^{272}\). Therefore, the occurrence of seizures post-ischemia with A20 treatment should now be investigated.
5.5 Future Directions

The following projects presented in this dissertation demonstrate that A20 substantially reduces the deleterious consequences of ischemic stroke. However, the mechanism by which A20 protects the brain after cerebral ischemia has not been fully elucidated. Wang and colleagues (2014) proposed that A20 enhances NAMPT activity and elevates levels of NAD\(^{212}\), so future studies should now be employed to directly verify this pathway. By utilizing transgenic animals to knock down or overexpress NAMPT, we can specifically evaluate if A20’s therapeutic mechanism is dependent on NAMPT. For example, if the previously observed therapeutic benefits of A20 are gone when NAMPT is ablated, then NAMPT is likely required, or responsible, for the improvements observed after ischemic stroke. Conversely, if A20 is still effective as a treatment for cerebral ischemia despite NAMPT being knocked down, then A20’s protection is likely through a different or additional mechanisms. In addition, even though A20-treated cells yielded the highest spectral counts for NAMPT when conducting mass spectrometry, it also yielded counts for other targets\(^{212}\). Most drugs do not typically bind to only one location, which is why therapies can produce multiple side effects\(^{273}\). Moreover, there are always limitations to any assay scientists use. For example, the quality or homogeneity of the sample, as well as the dissociation efficiency will directly affect any results obtained with mass spectrometry\(^{274}\). It would therefore not be surprising if A20 also has off-target effects.

Since we previously observed that iA20 treatment increased neurogenesis at 7 weeks post-injury, future studies are also now needed to determine the role of neurogenesis with dA20 treatment\(^{223}\). Specifically, does dA20 treatment also increase
neurogenesis in the SGZ and SVZ, and if so, does this phenomenon participate in the
functional recovery that we observed? Since cerebral ischemia increases SVZ
proliferation and NPCs migrate towards the penumbra, many scientists believe this is an
endogenous repair response. However, even though there is increased NPC proliferating,
there is conflicting evidence whether this elevated neurogenesis improves stroke recovery
Some groups have demonstrated that ablating neurogenesis exacerbates infarct
volume, whereas other have demonstrated it does not change functional recovery. In
the future, we plan to utilize available transgenic models to help clarify whether the
benefits with A20 are primarily dependent on reducing adult neuronal vulnerability to
ischemia, or may also involve the generation of newborn neurons.

Future studies are also required to determine if A20 is efficacious in female as
well as male animals. There are many physiological differences in sex that can
substantially change the pharmacokinetics of a compound, such as hormone levels. For example, ischemic stroke is more common in males, however after women reach
menopause this trend completely reverses. Hormones such as estrogen and progesterone
have demonstrated neuroprotective efficacy after cerebral ischemia, and these hormone
levels are strikingly different after menopause. Furthermore, hormones will also affect
physiological difference such as body fat, muscle weight, and metabolic enzymes, which
can alter drug efficacy. Women typically need lower doses than men of lipophilic drugs
because they have higher body fat and thus larger distributions. Not investigating
differences in sex could also have dangerous consequences. For example, when Zolipem
(Ambien) was administered during clinical trials, its dosage was based on weight and not
sex. Consequently, women had twice the level of Ambien in their body than an
equivalent dose for a male, which resulted in a substantial rise in car accidents the morning after women took Ambien\textsuperscript{275}. The NIH now requires both females and males be utilized in experimental models, and they will not fund any grant that does not include female animals\textsuperscript{275}.

In addition, it would be useful to determine if A20 has an additive effect when combined with other treatment methods, such as tPA and thrombectomy. For example, tPA in combination with a stent retrieval protocol, performed better than tPA alone in the SWIFT PRIME trial for large vessel occlusions\textsuperscript{247}. Neuroprotective agents such as A20 might have a synergistic effect by protecting vulnerable neurons from acute and more progressive injury mechanisms. In the past, many neuroprotective compounds have been combined with thrombolysis and evaluated as a treatment strategy for ischemic stroke, such as AMPA and NMDA receptor antagonists, MMP inhibitors, immunosuppressants, scavengers of ROS and hypothermia\textsuperscript{250}. However, since the therapeutic window was not established beforehand, it generated mixed results\textsuperscript{147}. In our study, A20 demonstrated a persistent therapeutic effect when treated 7.5 hours after the initial ischemic insult.

Ultimately, if A20 is shown to demonstrate efficacy after hemorrhagic stroke, and has a synergistic effect with the current stroke standard of care treatments, it would be an ideal therapy for first responders. Paramedics could utilize A20 to protect and delay vulnerable cells from dying before patients arrive at the hospital and blood flow is restored. In addition, A20 could quickly be adopted in the clinic because it can be administered at a delayed, practical time point. This makes A20 an attractive, potential therapy to mitigate neurodegeneration and promote chronic functional recovery after ischemic stroke.
References


