The Problem

Stroke is the third leading cause of death in the US and the leading cause of adult disability. Yet, the treatment options for stroke are extremely limited. Much stroke research has focused on trying to salvage dying neurons or promote neurogenesis. These studies have yielded useful information, but have not produced new treatments[1]. The failure of multiple clinical and pre-clinical trials resulted in the development of a set of guidelines for translational stroke research. These are referred to as the STAIR Guidelines[2] and you should be sure to follow them in your proposed studies.

Your PI has identified a drug that seems to reduce neuronal death in the cortex and striatum after an ischemic stroke, the compound is called Moneymaker. Your NRSA application should focus on testing the therapeutic potential of Moneymaker and identifying the mechanism by which Moneymaker has its effect. Moneymaker could reduce neuronal death by apoptosis, it could also alter the inflammatory response or increase neurogenesis or vascluogenesis / angiogenesis. You are free to choose what type of compound Moneymaker is and what type of receptor it binds to. Potential drug classes are steroids, free radical scavengers, anti-inflammatory agents, statins or growth factors. Information about the type of Moneymaker compound and its receptors should be provided in the background section of your NRSA.

Your Task

Your task is to write an NRSA type proposal which should contain the following elements:

1. A specific aims page - this should include no more than 2 aims and clear testable hypotheses associated with each of these aims (your aims page should be no more than 1 single spaced page)
2. A background with section detailing the significance of your proposed research and why your studies are innovative (2-3 pp)
3. A preliminary data section - you have been provided with a PowerPoint presentation containing some images that you can incorporate into this section as your preliminary data. You should also feel free to generate your own graphs for this section. Consider carefully what data would be necessary to support your hypothesis. Be sure to describe how you collected this data and what it means in relation to your proposed studies. (2-4 pp)
4. A description of the studies you propose to do this should contain the following elements (6-7pp):
    - a description of the animals / cells you intend to use
    - a detailed description of treatments and experimental endpoints (in vivo or in vitro studies and or behavioral outcomes)
    - a discussion of the expected outcomes and why you expect these
    - a discussion of alternative outcomes and approaches
    - a description of the statistical analysis you plan to use
5. You should include references in your proposal, the reference list will not count towards the page limit.

Remember, NRSA grants fund one person's research for 2 years, do not propose more studies than can be done in that time. Your proposal should adhere to the NIH NRSA format (consult NIH webpage if you have questions about the requirement, look for the predoctoral fellowship F31 mechanism). The Aims Page should be provided single spaced, the rest of the text body should be double spaced (10 - 12 pages).

1. Donnan GA. The 2007 Feinberg lecture: a new road map for neuroprotection. *Stroke* 2008; **39**(1)**:** 242.
2. Fisher M, Feuerstein G, Howells DW, Hurn PD, Kent TA, Savitz SI, Lo EH. Update of the stroke therapy academic industry roundtable preclinical recommendations. *Stroke* 2009; **40**(6)**:** 2244-2250.

Specific Aims

Ischemic stroke is the third leading cause of death in adults in the United States, with a mortality rate of around 30%. Surviving stroke victims are often left disabled with major deficits in quality of life. Ischemic stroke occurs from an occlusion of one or more cerebral arteries, reducing cerebral blood flow. The areas of the brain receiving restricted cerebral blood flow are transiently or persistently deprived of oxygen and glucose, causing neurons and other cellular elements to die. This area of death is called an infarction, and consequences may include peripheral weakness, sensory loss, language disturbance, visual field impairment, or impaired spatial perception, depending on the area of infarction. Despite countless efforts, there is no adequate therapy that gives hope to stroke victims with promise of preventing neuronal injury after stroke.

Injury to the brain resulting from ischemic stroke occurs through a complex cascade of pathogenic mechanisms such as excitotoxicity, peri-infarct depolarizations, programmed cell death, and inflammation. In order to develop effective and targeted therapeutics, we must hone in on specific pathogenic mechanisms. There is growing evidence that post-ischemic inflammation is playing a significant negative role in brain injury. Inflammation itself results in a cascade of events eventually leading to apoptosis surrounding the infarct. These negative effects may last for several days, contributing to further brain injury.

Recent work has pointed to the molecule adenosine as having neuroprotective therapeutic potential for attenuating inflammation-induced neuronal injury following stroke. Adenosine exists in all mammalian systems and is involved in many aspects of homeostasis. There are four G-protein coupled adenosine receptors that it can bind to (A_1, A_{2A}, A_{2B}, and A_3). Although adenosine looks promising, there are significant limitations to its therapeutic potential: *(i)* it has a very short half-life *(ii)* it is rapidly metabolized by adenosine kinase and adenosine deaminase *(iii)* it has difficulty crossing the blood brain barrier (BBB).

Our lab has recently discovered a drug, Moneymaker, which is an analogue of adenosine, and has already shown promise for reducing neuronal death post-stroke (**Figure 1**). Moneymaker is not degraded by adenosine deaminase or adenosine kinase and is easily transported via the BBB adenosine carrier, allowing it to target the sight of infarction. Moneymaker has a characteristically long half-life that long outlasts the half-life of adenosine, allowing Moneymaker to have prolonged effects at the site of injury. Moneymaker also has a very high affinity for the A_1 adenosine receptor, which has a widespread distribution in the brain, and A_{2A} adenosine receptor, which is highly expressed in the striatum. These two receptors have currently gained attention for their regulatory roles in inhibiting inflammatory events following stroke. These essential benefits of Moneymaker make it a prime candidate for post-stroke neuroprotection via attenuating the inflammatory response. **The overall hypothesis of this proposal is that Moneymaker, an adenosine analogue, will attenuate the post-stroke inflammatory response around the sight of infarction, resulting in reduced neuronal death in the cortex and striatum, and a corresponding increase in behavioral functioning and mobility.** This hypothesis will be tested in two Specific Aims in this proposal:

Specific Aim 1: Determine the optimal time course for administration of Moneymaker as well as observe and quantify behavioral outcomes correlated with reductions in the size of infarction. Behavioral outcomes will be measured post-stroke to determine long-term effectiveness of Moneymaker. Triphenyltetrazolium chloride staining will be used to assess reductions of infarct volume. This aim will test the therapeutic potential of Moneymaker in reducing neuronal death and increasing function after stroke.

Specific Aim 2: Examine Moneymaker's histological effects on inflammation, angiogenesis, apoptosis, and neurogenesis. Specific biomarkers for each of these events will be quantified using protein assay (inflammation and angiogenesis) or immunofluorescence (apoptosis and neurogenesis) to determine the effect that Moneymaker has on each of these phenomena. This will allow us to determine the specific mechanisms by which Moneymaker offers neuroprotection.

Following completion of these specific aims we will better understand the roles of Moneymaker and inflammation in post-stroke neuroprotection, and the mechanisms by which these effects occur. This knowledge will lead to more targeted therapeutics in the prevention of brain injury occurrence through ischemic stroke. With a greater understanding of Moneymaker's neuroprotective mechanisms, we will hopefully be able to advance to the next step of trials on the road to clinical testing.

Research Strategy

Significance

Stroke is one of the most debilitating and prominent neurological disorders, and is the third leading cause of death in American adults. Currently there are no adequate therapeutics to prevent brain injury after stroke, leaving stroke victims debilitated and still searching for hope. Stroke results from a lack of oxygen to the brain caused by blockage (ischemic) or bleeding (hemorrhagic) of cerebral arteries, leading to neuronal and cellular death of the affected brain area, called an infarction. The infarct volume is maximal after around six hours of permanent ischemia (Aronowski et al., 1999). The area surrounding the infarction with *potentially salvageable tissue* is known as the ischemic *penumbra* (Auriel and Bornstein, 2010). Depending on where the infarction occurs in the brain, peripheral weakness or paralysis may occur in various motor and sensory systems (Kandel et al., 2000).

Injury to the brain from ischemic stroke is the result of a complex sequence of pathogenic, cascading mechanisms such as excitotoxicity, peri-infarct depolarizations, programmed cell death, and inflammation (Dirnagl et al., 1999). Growing evidence suggests ischemia-induced inflammation has negative effects on brain injury, and actually contributes to it. This chronic type of inflammation can direct itself against inappropriate targets, such as healthy neurons. A consequence of the inflammatory response is the production of neurotoxins, such as nitric oxide, from local leukocytes responding to injury. These neurotoxins can easily result in cell death, adding to the injury. Preventing neutrophils, crucial inflammatory mediators, and adhesion molecules and their receptors all effectively reduce cerebral ischemic damage (Connolly et al., 1996). This proposal will test a novel drug therapeutic that will target and interfere with the inflammatory response, help salvage tissue at the penumbra, and reduce neuronal death. This could be one of the first effective therapeutics to prevent brain injury after stroke, giving hope to stroke victims everywhere. Successful completion of this project may shift the field of stroke research in a new direction, encouraging more targeted therapeutics to the inflammatory cascade.

Innovation

Adenosine and its Receptors: In order to understand the innovation of this proposal, I must first introduce some background on a current therapeutic avenue, adenosine, which has potential, but also has

some concerning limitations. Current research is highlighting adenosine, a purine ribonucleoside that forms from the breakdown of adenosine triphosphate (ATP), and its receptors as a new avenue for neuroprotection after stroke. Adenosine levels greatly increase following ischemia, hypoxia, and inflammation, indicating a potential role in neuroprotection at the penumbra (Lopes et al., 2011). Adenosine is present in every mammalian organ system and is involved in the control homeostasis. It helps regulate numerous mechanisms such as sleep/arousal, locomotion, analgesia, drug/alcohol effects, and neuroprotection (Boison and Shen, 2010; Dunwiddie and Masino, 2001). Adenosine has four receptors, A_1, A_{2A}, A_{2B}, and A_3, all of which are G-protein-coupled and contain seven transmembrane domains. The adenosine receptors display a variety of transduction mechanisms, and each has unique characteristics. *The A_1 and A_{2A} receptors are highly expressed on immune cells and are able to decrease their inflammatory response through cAMP signaling*.

The A_1 receptor is $G_{i/o}$-protein coupled, involved in inhibition of adenylyl cyclase, and linked to various kinase pathways through the cAMP-dependent pathway. A_1 receptor distribution in the brain is widespread and it is involved in the inhibition of transmitter release (glutamate in particular) and Ca^{2+} channels, as well as activation of K^+ channels. The presynaptic inhibition and hyperpolarizing effects from activated A_1 receptors reduce excitotoxicity by limiting Ca^{2+} entry, thus playing a large role in ischemic preconditioning. Pretreatment with an A_1 antagonist worsens ischemic damage (Boison and Shen, 2010; Haselkorn et al., 2010). As mentioned above, the A_1 receptor is an endogenous inhibitor of microglial immune response post-injury. In a mouse model of traumatic brain injury, A_1 receptors reduced microglia by up to 50% (Haselkorn et al., 2010).

The $G_{s/olf}$-coupled A_{2A} receptor is primarily concentrated in the striatum, but is also expressed in other brain areas such as cortex and hippocampus, as well as endothelial and smooth muscle cells of cerebral vasculature (Chen et al., 1999). When activated, it exerts excitatory effects, facilitating glutamate and acetylcholine release. A_{2A} receptors also have inhibitory effects through release of GABA, which contributes to the complex evidence that the A_{2A} receptor can both protect against ischemic damage as well as worsen it (Boison and Shen, 2010; Chen et al., 1999). Deleterious effects of the A_{2A} receptor result from an increase in glutamate release, activation of mitogen activated protein kinase (MAPK), and activation of pro-inflammatory cytokines. Some studies report A_{2A} knock-out mice have attenuated infarct volumes (Chen et al., 1999; Dai and Zhou, 2011), while others report that A_{2A} agonists convey neuroprotective effects. These neuroprotective

effects are attributed to increased vasodilation, inhibition of platelet aggregation, suppression of superoxide generated by neutrophils, elevation of cAMP, and anti-inflammatory actions (Boison and Shen, 2010; Chen et al., 1999; Ohta and Sitkovsky, 2001). During the inflammatory response, neutrophils are recruited to the injury site through altered expression of adhesion molecules on the endothelium, allowing neutrophils to leave circulation to adhere to the epithelium. A_{2A} receptor activation prevents neutrophils from adhering to the endothelium, helping to suppress the inflammatory response. A_{2A} also mediates the inhibition of tumor necrosis factor-α (TNF-α) along with other macrophage-derived cytokines crucial to the inflammatory response. As mentioned above, A_{2A} receptors are expressed on cytokines such as lymphocytes and directly regulate their response during inflammation (Hasko et al., 2008).

Receptor A_{2B} (G_s-coupled) and A_3 ($G_{i3/q}$-coupled) are both widely distributed in the brain, and both have much lower affinities for adenosine than the previous two receptors. They are the least studied, but both seem to play a small role in neuroprotection (for a review see (Fredholm et al., 2011)). For the purposes of this proposal we will focus on receptors A_1 and A_{2A}, which offer the most therapeutic potential.

Innovation: Moneymaker and its Benefits: We have recently discovered a drug, Moneymaker, which is an adenosine analogue with a very high affinity for A_1 and A_{2A} adenosine receptors (**Figure 3)**. Both A_1 and A_{2A} receptors are located on the cell body, as well as the axon (Cunha, 2001). This is promising for translational research, as the cascade of detrimental effects after ischemic stroke is mainly confined to the gray matter in animal models, but also occurs in the white matter in humans (Donnan, 2008). Moneymaker has exciting therapeutic potential because it offers all of the neuroprotective benefits of adenosine, without the physiological limitations. The first limitation of adenosine is its short half-life (<10 seconds) and rapid metabolism, with the endogenous surge after ischemia reaching its peak three hours post-stroke. Levels dwindle back to baseline less than twenty-four hours later, creating a delay in neuronal loss (Boison and Shen, 2010; Laubach et al., 2011). Moneymaker has a prolonged half-life of 30 minutes, allowing its neuroprotective effects to span for longer periods of time. Whereas adenosine has difficulty crossing the blood-brain barrier (BBB) because it is rapidly metabolized by adenosine deaminase and adenosine kinase, the molecular structure of Moneymaker allows it to cross the BBB through the adenosine CNT2 transporter without being rapidly metabolized by adenosine enzymes (Pardridge et al., 1994). Because of its ability to

cross the BBB, Moneymaker may effectively be given exogenously through the blood stream, providing a huge therapeutic advantage. After an ischemic episode, the A_1 receptor is rapidly upregulated in the brain (Boison and Shen, 2010). We have evidence to show that exogenous administration of Moneymaker has an additive effect to this upregulation of A_1 receptors in the brain (**Figure 2**), further increasing its ability to bind and cause a cessation on cerebral inflammatory events. Overall, this drug offers hope and exciting possibilities for stroke victims, with its neuroprotective potential just waiting to be tapped.

Preliminary Data

Moneymaker Reduces Infarction Size

To determine a dose-response curve of the drug Moneymaker, twelve 12-week old healthy male Sprague Dawley rats were used in a pilot study. Cerebral ischemia was induced through middle cerebral artery occlusion (MCAO) (see research plan for detailed methods). Thirty minutes after occlusion, rats were subcutaneously injected in the back of the neck with either 0.1, 0.5, 1, 5, or 10 mg/kg of Moneymaker concentrated in a saline. Two control rats received only saline injection, and were used for a baseline for infarction volume. 72 hours after injection rats were rapidly decapitated and brains were removed and sliced into 2mm thick sections. In order to assess the infarct volume brains were stained using triphenyltetrazolium chloride (TTC), and infarct volume was determined as a percentage as in (Pires et al., 2011). The dose-response curve and brain sections are characterized in Figure 1, with 5 mg/kg being the optimal threshold.

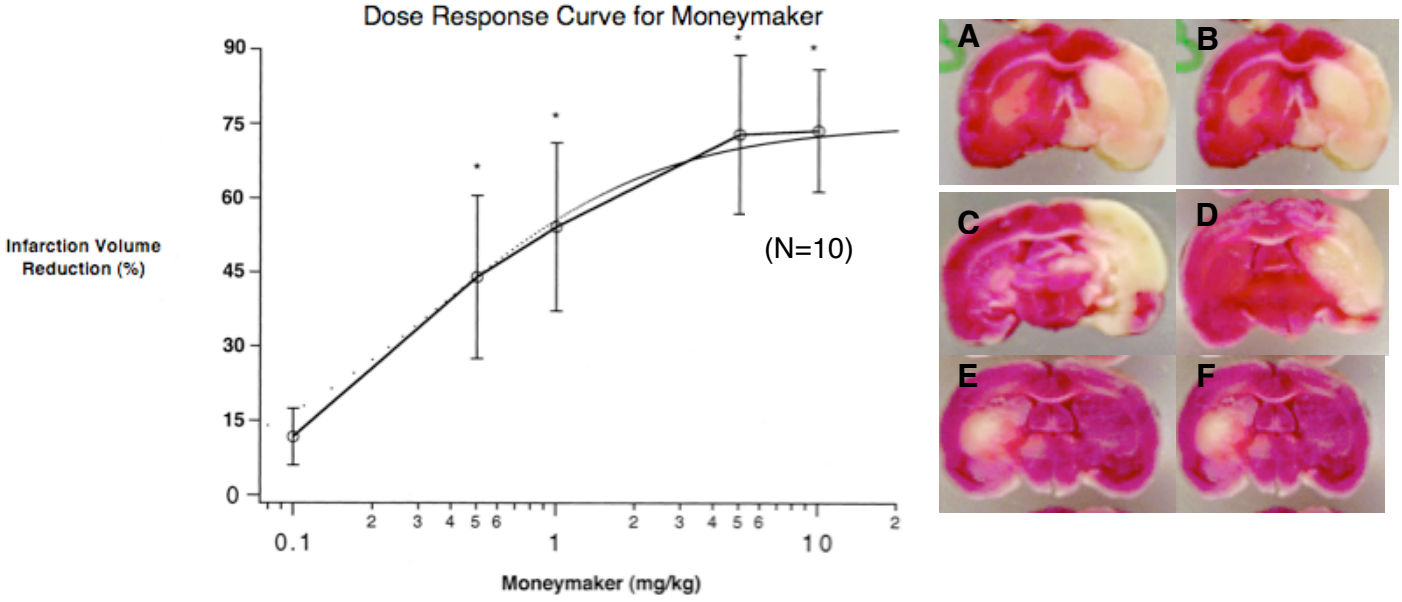


Figure 1. *Left* is the dose response curve for the percentage of reduction in infarction volume after induced ischemia for the adenosine analogue Moneymaker. Graph does not include control animals. Reduction of infarction volume by 0.1. 0.5, 1, 5, and 10 mg/kg Moneymaker was 12 ± 5% ($P < 0.05$), 43 ± 13% ($P < 0.04$), 47 ± 13% ($P < 0.03$), 68 ± 9% ($P < 0.01$), and 67 ± 7% ($P < 0.002$), correspondingly. Graph modified from (Chen and van den Pol, 1997). *Right* are sections stained with TTC, with **A** being a control brain, and **B, C, D, E,** and **F** corresponding to 0.1, 0.5, 1, 5, or 10 mg/kg of Moneymaker, respectively.

The data represented in Figure 1 indicates that Moneymaker effectively reduces the size of infarct volume following ischemic stroke. It acts in a dose-dependent manner, with 5 mg/kg providing an optimal reduction in the size of the infarction. Doubling the dose provided no further benefit, so 5 mg/kg will be the standard dose used for future studies. These results look very promising, with almost a 70% reduction in the size of the infarct.

Moneymaker Upregulates Receptor Expression

From the same pilot study animals, we took brain punches of 500μm from the cortex and striatum in the afflicted hemisphere from both a control and a 5 mg/kg treated animal. From each of these samples protein was individually extracted in RIPA Lysis Buffer (Santa Cruz Biotechnology) per manufacturer's instructions. Total protein concentration was determined using a Bio-Rad Protein Assay with absorbance at 595 nm using a GeneQuant Pro Spectrophotometer (Amersham). For each sample, 30ug of total protein was run on a Western Blot using an anti-adenosine receptor A_1 antibody (Pierce, PA1-041A), and an HRP-conjugated secondary (Thermo-Fisher). Results can be seen in Figure 2. Two(treatment) x two(brain region) ANOVA indicated that both the cortex and striatum tissue that was treated with Moneymaker had higher mean optical densities than tissue treated with saline. This indicates that Moneymaker acts to upregulate the A_1 adenosine receptor even more so than when it is upregulated in response to ischemic injury. This may be one mechanism for Moneymaker's neuroprotective effects; upregulated receptor expression allows for more binding opportunities for the drug. Shown in **Figure 3,** Moneymaker has a very high affinity for both the A_1 and A_{2A} adenosine receptors, and rather low affinity for the A_{2B} and A_3 receptors.

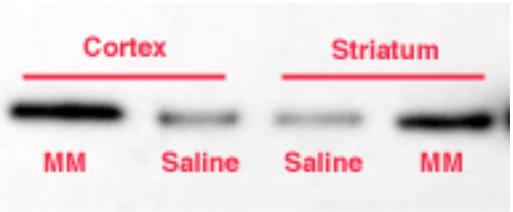
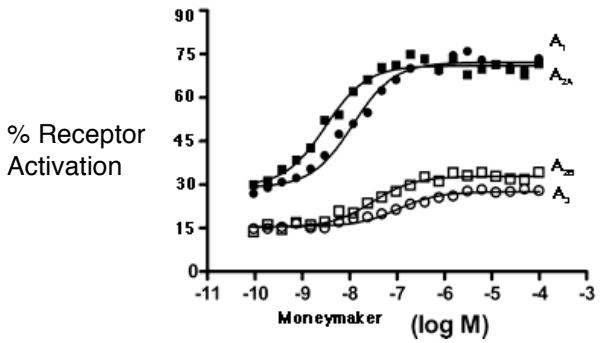


Figure 2. Western Blot image displaying mean optical density of protein levels of the A_1 adenosine receptor from cortex and striatum of both the saline injected animal and the animal treated with 5 mg/kg of Moneymaker (MM). Moneymaker significantly upregulated receptor expression compared to control in both the cortex and the striatum (both $p<0.001$). β-actin was used as a loading control.

Figure 3. The percentage of receptor activation by administration of Moneymaker; indicates the affinity of Moneymaker for each of the adenosine receptors. Assay of G protein-coupled receptor activation of G proteins (Bidlack & Parkhill, 2004)



Approach

Specific Aim 1: Determine the optimal time course for administration of Moneymaker as well as observe and quantify behavioral outcomes correlated with reductions in the size of infarction.

Rationale: As we have already shown that Moneymaker effectively reduces infarct volume in a dose-dependent manner (**Figure 1**), it is essential to determine the most effective time course of administering the drug to adhere with STAIR guidelines (For a full review see (Fisher et al., 2009)). As mentioned previously, A_1 adenosine receptors, when activated *before* the onset of stroke, precondition the brain and protect it from further damage (Boison and Shen, 2010). Also mentioned previously, the body's endogenous adenosine system peaks at three hours after the ischemic event, and declines shortly thereafter (Boison and Shen, 2010), which creates a need for adenosine receptors to continue to be activated after this short three hour window. STAIR guidelines also recommend that reduction in infarct volume and improved neurological scores are considered as effective outcome measures to determine therapeutic potential.

Method: Induced Ischemic Stroke: Forty-eight 12-week old healthy Sprague Dawley rats will be used for this experiment. Each of the animals will have an ischemic event induced at an appointed Time 0 using the middle cerebral artery occlusion (MCAO) method, as described in (Pires et al., 2011). Rats will be anaesthetized using 2% isoflurane in oxygen and body temperature maintained at 37°C. The right common carotid artery and external carotid artery will be exposed through incision and a 3.0 monofilament will be inserted into the common carotid artery and internally advanced to block blood flow to the middle cerebral artery. The monofilament will be tied in permanently. The occlusion will be verified using a scanning laser Doppler and flow probe to measure the blood flow to the brain region supplied by the middle cerebral artery; a drop in flow will indicate successful occlusion. Animals who do not display the drop in flow will be excluded from the study. Following occlusion, animals will be sutured and returned to their cage. Unfortunately, no analgesic will be given because it would interfere with the inflammation process and confound our results. Animals will be given heating pads under their cages and will be monitored every hour to ensure their health.

Treatment Groups: *Full randomization and blinding was adhered throughout the duration of this proposal.* Eight of the animals will serve as a negative control, and will receive no treatment at all. Another eight animals will serve as an internal positive control, and hypothermia specific to the affected hemisphere will be induced using a 20G cooling coil with PE50 tubing surgically placed between the temporalis muscle and skull. Immediately after the ischemic event, cool water will run through the coil and maintain the brain temperature at 34°C for 12 hours. This method of hypothermia reliably reduces damage following ischemia (Fingas et al., 2009; Yenari and Hemmen, 2010), and will serve as an internal control to validate that success or failure is not due to technical errors. The final three groups of eight animals will all receive a subcutaneous injection in the back of the neck of Moneymaker concentrated at 5 mg/kg (see dose response curve in preliminary data) in saline solution. One group will receive the injection three hours before MCAO, one group immediately after MCAO, and the third group three hours after MCAO.

Behavioral Outcomes: Behavioral tests will be performed two and four weeks after MCAO to measure long term recovery in behavioral performance after occlusion. Three behavioral measures (spontaneous activity, elevated body swing test, and the step test) will be performed as previously described in (Vendrame et al., 2004) to determine any effects of treatment on motor function behavioral outcomes.

Infarction Size Quantification: Upon completion of behavioral testing, rats will be rapidly decapitated and brains removed and sliced into 2mm thick sections. In order to assess the infarct volume, brains will be stained using TTC as in **Figure 1**, and infarct volume determined as a percentage, as in (Pires et al., 2011). The percentage of reduction in the infarction volume will be compared across groups.

Statistics: Group means will be calculated for each of the behavioral measures and a five(treatment group) x two(time after MCAO) between-subjects ANOVA will be used for each behavioral measure to determine which time window most effectively reduces long-term behavioral deficits. A Tukey's HSD post-hoc test will be used to determine which groups are statistically significant from each other. A one-way ANOVA will be performed to determine any significant effects of treatment group on the reduction in infarct volume. Finally, a Pearson correlation will be determined for reduction in infarct volume and behavioral deficits.

Expected Results: First and foremost, I do not expect to see a difference in behavioral measures between weeks two and four. This is because Moneymaker will be given in a single, transient dose that will

have its maximum effects within the first few days after stroke. By preventing the inflammatory response, this drug will reduce neuronal death at the penumbra in a more immediate fashion. Any reduction in behavioral deficit seen at four weeks should also be seen at two weeks. If there is any greater reduction in deficit at four weeks, it is not likely due to the drug. Moneymaker's neuroprotective effects will be evident long-term, but further research will be needed to determine if continuous and/or long-term dosing will provide greater benefits.

I expect to see the greatest reduction in behavioral deficits, as well as reduction in infarct volumes, in the group that was injected with Moneymaker three hours before MCAO, given that the A_1 adenosine receptors are thought to have a preconditioning effect, as mentioned in the rationale. I would expect this to be due, in part, to the fact that Moneymaker acts to upregulate receptor expression in the brain, increasing binding opportunities (**Figure 2**). Because of its remarkably longer lifespan than adenosine, Moneymaker should still be present at high levels when endogenous adenosine peaks in response to the occlusion. It is possible that Moneymaker and adenosine could even have a synergistic effect. I also expect to see a large reduction in behavioral deficits in the group injected three hours after MCAO. Given that endogenous adenosine levels have already peaked and receptors will begin to be inactivated, Moneymaker can swoop in to bind and have its effect. I believe that both of these groups will have a greater reduction in infarct volume than the group treated with the reliably neuroprotective hypothermia, due to the active effect of reversing the inflammatory response. If we see this effect, this would be good cause to move on to the next phase of experimental trials.

I do not expect to see as big of a reduction in behavioral deficits or infarction volume in the group treated with Moneymaker directly after occlusion. Although Moneymaker upregulates adenosine receptors, the increase in gene transcription would not be immediate. While this process takes place, Moneymaker and adenosine may be competitively binding to available receptors. The abundance of ligand and shortage of receptors would likely do nothing to further facilitate neuroprotection. The other two time points of drug administration still allow the brain to capitalize on the endogenous adenosine response after the ischemic event, as well as its exogenously delivered, longer lasting analogue.

Possible Pitfalls: Likely the greatest concern of the proposed experiment is the role of adenosine and its receptors in every organ of the body and in the many mechanisms of homeostasis. A major function of adenosine that poses particular concern is its ability to slow the heart rate. It is very possible that Moneymaker could disrupt homeostasis throughout the body and even slow the heart to dangerous levels. If this is the case, the mechanism by which Moneymaker is administered will have to be reconsidered. It would certainly be beneficial for patients to receive the drug by subcutaneous or intraperitoneal injection; however, if this threatens the patients' homeostatic regulation in the periphery, I would recommend that the drug be administered through lateral cerebroventricular injection. This would ensure Moneymaker reaches its target in the brain, while minimizing its effects in the periphery.

Specific Aim 2: Examine Moneymaker's histological effects on inflammation, angiogenesis, apoptosis, and neurogenesis.

Rationale: Inflammation is the result of a downstream cascade of pathological events caused from ischemic stroke. Inflammation itself causes its own cascade of events that have negative effects at the penumbra. Because inflammation begins almost immediately after ischemic stroke, it is difficult to tease apart the negative effects that are caused by both events. While we want to ensure that Moneymaker is decreasing inflammation as we think, we also want to determine the effects that subduing the inflammatory cascade has on angiogenesis, apoptosis, and neurogenesis. In a series of two experiments, we will determine the individual effect that Moneymaker has on inflammation, angiogenesis, neurogenesis, and apoptosis to determine the mechanisms (whether they be directly decreasing inflammation, or a downstream consequence of that) by which Moneymaker offers its neuroprotective effects.

Experiment 1: Inflammation and Angiogenesis

Method: Animals will be randomly assigned to either a negative control group (n=8) receiving no treatment before or after an induced stroke event, a positive control group (n=8) receiving hypothermia treatment for six hours after stroke (same method but half the time as in **Specific Aim 1**), or a treatment group (n=8) receiving one 5 mg/kg injection of Moneymaker (at the optimal time window determined in **Specific Aim 1**) before/after stroke. Ischemic stroke will be induced using the same MCAO methods from

Specific Aim 1. It takes approximately six hours for the infarct volume to reach its maximum after permanent ischemia (Aronowski et al., 1999), so rats will be rapidly decapitated at six hours post-stroke. Brains will be frozen in 2-methylbutane and 2mm sections will be generated. Punches will be taken from both the cortex (two punches) and striatum (two punches) of the affected hemisphere. From each of these samples protein will be individually extracted in RIPA Lysis Buffer (Santa Cruz Biotechnology) per manufacturer's instructions. Total protein concentration will be determined using a Bio-Rad Protein Assay.

One set of punches (cortex and striatum) from each animal will be used in a Rat Inflammation ELISA Strip Assay from Signosis (per manufacturer's instructions found on their website Signosisinc.com, product EA-1201), which will assess the protein expression of eight different cytokines: TNFa, IL-6, IFNr, IL-1a, IL-1b, MCP-1, Rantes and MIP. Cytokines are immunomodulating proteins, and major determinants of the inflammatory response. We can measure these different cytokines and quantify them as biomarkers determining the induction or suppression of the inflammatory response in treated and control rats.

The other set of punches from each animal will be used to quantify angiogenesis with a Rat Angiogenesis ELISA Strip Assay (per manufacturer's instructions found on their website Signosisinc.com, product EA-1211). Angiogenesis is governed by multiple angiogenic factors, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (FGFb) and IL-6. These three factors, along with five others (TNFa, IFNr, MCP, Rantes, & Leptin), will be measured and quantified as biomarkers of angiogenesis to determine Moneymaker's effects on the phenomenon.

Statistics: For each of the assays I will perform a three(treatment group) x two(brain area) between-subjects ANOVA for each of the cytokines (inflammation assay) and angiogenic factors (angiogenesis assay). This will allow us to determine how each of these cytokines/factors are increasing or decreasing based on the treatment group they were in, as well as differences across brain regions. I will also use a Tukey's HSD post hoc test.

Expected Results: In the inflammation assay I expect to see a drastic reduction in all eight of the cytokines in the group treated with Moneymaker, given that its proposed neuroprotection is through decreasing the inflammatory response. I also expect to see reductions in the hypothermia group, although not as much as the Moneymaker group. I do not expect to see differences across brain areas. Given that we now

know angiogenesis and inflammation have common signaling pathways, and that angiogenesis can actually upregulate the inflammatory response (Imhof and Aurrand-Lions, 2006), I expect to see decreases in the pro-angiogenic factors and increases in the anti-angiogenic factors in the treated group. Due to their common signaling pathways, it is likely that Moneymaker will decrease the formation of new blood vessels while it decreases inflammation. I expect to see a similar decrease in angiogenesis in the hypothermic positive control group, but to a lesser extent, and higher levels of angiogenesis in the negative control group. As in the inflammation assay, I expect to see no differences across brain area.

Possible Pitfalls: There may be large levels of variance due to individual differences in the subjects, potentially masking real effects. If individual variances are high, I propose redoing the experiment as a within-subjects design. The healthy hemisphere would serve as the control to the hemisphere with the infarction in animals treated with Moneymaker. Another possible pitfall is a decreased sensitivity of the Signosis assays. In this case, I would propose doing quantitative polymerase chain reaction (qPCR) on the above-mentioned cytokines and angiogenesis factors. This method would allow us to amplify and quantify the relative amount of a targeted DNA molecule and normalize it to a housekeeping gene, such as GAPDH.

Experiment 2: Apoptosis and Neurogenesis

Method: Animals (N=24) will be in the same randomized treatment groups and will receive the MCAO method of induced ischemic stroke as described above in **Experiment 1**. Six hours post-stroke animals will be rapidly decapitated, brains frozen in methyl butane, and sliced at 40μm into two series (A and B). The first series (A) will be used for double-label immunofluorescence staining to examine apoptosis. An anti-Caspase-3, cleaved (Asp175) antibody conjugated to Alexa Fluor 488 (Cell Signaling Technology, 9669) will be used to visualize cells undergoing apoptosis; they will fluoresce green when excited. Caspase-3 is a critical executioner of apoptosis. Sections will be counterstained with anti-NeuN antibody (NEUronal Nuclei; clone A60) conjugated to Alexa Fluor 594 (red fluorescence, Invitrogen); this will serve as a neuronal marker. Colocalization of red and green fluorescence will indicate a neuron undergoing apoptosis.

The second series of tissue (B) will also be used for double-label immunofluorescence, but will examine the occurrence of neurogenesis. An anti-bromodeoxyuridine (BrdU) antibody conjugated to Alexa Fluor 488 (Invitrogen, A21303) will be utilized. BrdU is readily incorporated into newly synthesized DNA, thus

marking cell proliferation. Counterstain with the same anti-NeuN antibody as above will act as a marker for neurons. Colocalization of red and green fluorescence will indicate neurogenesis in this instance. Both neurogenesis and apoptosis will be calculated using unbiased stereology, by an investigator blind to treatment group, on the hemisphere affected by stroke in both control and treated animals in both cortex and striatum.

Statistics: A three(treatment group) x two(brain region) between-subjects ANOVA will be performed separately for both apoptosis and neurogenesis to determine if treatment with Moneymaker either reduces or increases apoptosis or neurogenesis compared to controls. A Tukey's HSD post hoc analysis will also be performed.

Expected Results: I expect to see a reduction in the amount of apoptosis occurring in the brains of animals treated with Moneymaker compared to controls. Moneymaker serves to decrease inflammation, and should thus decrease the ensuing cascade of neuronal death. Although acute inflammation can actually stimulate neurogenesis in some instances, the inflammation occurring following a stroke is chronic, and detrimental to neurogenesis in the brain (Ekdahl et al., 2003; Whitney et al., 2009). I therefore expect an increase in neurogenesis in animals treated with Moneymaker compared with controls. I do not expect to see differences across brain region in either apoptosis or neurogenesis.

Possible Pitfalls: The same concern as in Experiment 1 of large individual differences creating high variance applies to this experiment as well. The same solution of a within-subjects design using the healthy brain hemisphere as a control to the afflicted brain hemisphere would eliminate this problem. If the caspase-3 antibody is not effective, a TUNEL assay could also be performed to assess apoptosis.

Upon completion of the proposed experiments, we will not only have an understanding of the therapeutic potential of the drug, Moneymaker, on reducing neuronal death at the penumbra following stroke, but we will also have a clearer picture of the mechanisms by which this drug offers its neuroprotective effects. Logical future directions for this research include examining Moneymaker's neuroprotection *in vitro* with cell culture studies, as well as its efficacy in higher animal models of stroke, such as primates. Determining whether systematic long term dosing of Moneymaker offers additive neuroprotection should also be investigated.

Literature Cited

Aronowski, J., Cho, K.H., Strong, R., and Grotta, J.C. (1999). Neurofilament proteolysis after focal ischemia; when do cells die after experimental stroke? J Cereb Blood Flow Metab *19*, 652-660.

Auriel, E., and Bornstein, N.M. (2010). Neuroprotection in acute ischemic stroke--current status. J Cell Mol Med *14*, 2200-2202.

Bidlack, J.M., and Parkhill, A.L. (2004). Assay of G protein-coupled receptor activation of G proteins in native cell membranes using [35S]GTP gamma S binding. Methods Mol Biol *237*, 135-143.

Boison, D., and Shen, H.Y. (2010). Adenosine kinase is a new therapeutic target to prevent ischemic neuronal death. The Open Drug Discovery Journal, 108-118.

Chen, G., and van den Pol, A.N. (1997). Adenosine modulation of calcium currents and presynaptic inhibition of GABA release in suprachiasmatic and arcuate nucleus neurons. J Neurophysiol *77*, 3035-3047.

Chen, J.F., Huang, Z., Ma, J., Zhu, J., Moratalla, R., Standaert, D., Moskowitz, M.A., Fink, J.S., and Schwarzschild, M.A. (1999). A(2A) adenosine receptor deficiency attenuates brain injury induced by transient focal ischemia in mice. J Neurosci *19*, 9192-9200.

Connolly, E.S., Jr., Winfree, C.J., Springer, T.A., Naka, Y., Liao, H., Yan, S.D., Stern, D.M., Solomon, R.A., Gutierrez-Ramos, J.C., and Pinsky, D.J. (1996). Cerebral protection in homozygous null ICAM-1 mice after middle cerebral artery occlusion. Role of neutrophil adhesion in the pathogenesis of stroke. J Clin Invest *97*, 209-216.

Cunha, R.A. (2001). Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: different roles, different sources and different receptors. Neurochem Int *38*, 107-125.

Dai, S.S., and Zhou, Y.G. (2011). Adenosine 2A receptor: a crucial neuromodulator with bidirectional effect in neuroinflammation and brain injury. Rev Neurosci *22*, 231-239.

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

Donnan, G.A. (2008). The 2007 Feinberg lecture: a new road map for neuroprotection. Stroke *39*, 242.

Dunwiddie, T.V., and Masino, S.A. (2001). The role and regulation of adenosine in the central nervous system. Annu Rev Neurosci *24*, 31-55.

Ekdahl, C.T., Claasen, J.H., Bonde, S., Kokaia, Z., and Lindvall, O. (2003). Inflammation is detrimental for neurogenesis in adult brain. Proc Natl Acad Sci U S A *100*, 13632-13637.

Fingas, M., Penner, M., Silasi, G., and Colbourne, F. (2009). Treatment of intracerebral hemorrhage in rats with 12 h, 3 days and 6 days of selective brain hypothermia. Exp Neurol *219*, 156-162.

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

Fredholm, B.B., Ijzerman, A.P., Jacobson, K.A., Linden, J., and Muller, C.E. (2011). International Union of Basic and Clinical Pharmacology. LXXXI. Nomenclature and classification of adenosine receptors--an update. Pharmacol Rev *63*, 1-34.

Haselkorn, M.L., Shellington, D.K., Jackson, E.K., Vagni, V.A., Janesko-Feldman, K., Dubey, R.K., Gillespie, D.G., Cheng, D., Bell, M.J., Jenkins, L.W.*, et al.* (2010). Adenosine A1 receptor activation as a brake on the microglial response after experimental traumatic brain injury in mice. J Neurotrauma *27*, 901-910.

Hasko, G., Linden, J., Cronstein, B., and Pacher, P. (2008). Adenosine receptors: therapeutic aspects for inflammatory and immune diseases. Nat Rev Drug Discov *7*, 759-770.

Imhof, B.A., and Aurrand-Lions, M. (2006). Angiogenesis and inflammation face off. Nat Med *12*, 171-172.

Kandel, E.R., Schwartz, J.H., and Jessel, T.M. (2000). Principles of Neural Science. 1305-1309.

Laubach, V.E., French, B.A., and Okusa, M.D. (2011). Targeting of adenosine receptors in ischemia-reperfusion injury. Expert Opin Ther Targets *15*, 103-118.

Lopes, L.V., Sebastiao, A.M., and Ribeiro, J.A. (2011). Adenosine and related drugs in brain diseases: present and future in clinical trials. Curr Top Med Chem *11*, 1087-1101.

Ohta, A., and Sitkovsky, M. (2001). Role of G-protein-coupled adenosine receptors in downregulation of inflammation and protection from tissue damage. Nature *414*, 916-920.

Pardridge, W.M., Yoshikawa, T., Kang, Y.S., and Miller, L.P. (1994). Blood-brain barrier transport and brain metabolism of adenosine and adenosine analogs. J Pharmacol Exp Ther *268*, 14-18.

Pires, P.W., Rogers, C.T., McClain, J.L., Garver, H.S., Fink, G.D., and Dorrance, A.M. (2011). Doxycycline, a matrix metalloprotease inhibitor, reduces vascular remodeling and damage after cerebral ischemia in stroke-prone spontaneously hypertensive rats. Am J Physiol Heart Circ Physiol.

Vendrame, M., Cassady, J., Newcomb, J., Butler, T., Pennypacker, K.R., Zigova, T., Sanberg, C.D., Sanberg, P.R., and Willing, A.E. (2004). Infusion of human umbilical cord blood cells in a rat model of stroke dose-dependently rescues behavioral deficits and reduces infarct volume. Stroke *35*, 2390-2395.

Whitney, N.P., Eidem, T.M., Peng, H., Huang, Y., and Zheng, J.C. (2009). Inflammation mediates varying effects in neurogenesis: relevance to the pathogenesis of brain injury and neurodegenerative disorders. J Neurochem *108*, 1343-1359.

Yenari, M.A., and Hemmen, T.M. (2010). Therapeutic hypothermia for brain ischemia: where have we come and where do we go? Stroke *41*, S72-74.

GRADE: PASS

Student 2

This application was extremely clear and well thought out. The addition of behavioral studies was considered a plus and the focus on adenosine was novel. The PI had really thought about the preliminary data required and the addition of the receptor activation data was a plus. The experimental design with an internal control for all the studies was excellent. The only negative was that the student did not suggest measuring blood pressure, adenosine acts as a dilator in some vascular beds and could lower blood pressure. The PI proposed within-subject design to address possible pitfalls, but it was not clear why the actual experiment cannot use this design, if it is superior (and possibly money-saving). And, strictly speaking, the student exceeded the page limit (albeit little – but still one cannot do this in a 'real' grant application!).