NEUROPROTECTIVE EFFECTS OF A NOVEL APPLE PEEL EXTRACT AF4 IN A MOUSE MODEL OF HYPOXIC-ISCHEMIC BRAIN INJURY

by

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The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "NEUROPROTECTIVE EFFECTS OF A NOVEL APPLE PEEL EXTRACT AF4 IN A MOUSE MODEL OF HYPOXIC-ISCHEMIC BRAIN INJURY" by Kate E. Dunlop in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

The neuroprotective effects of AF4, a flavonoid-enriched extract derived from the peel of Northern Spy apples (containing quercetin-3-O-glucoside, quercetin-3-O-galactoside, quercetin-3-O-rhamnoside, quercetin-3-O-rutinoside, epicatechin, and cyanidin-3-O-galactoside) were examined by assessing neuronal loss and motor impairment resulting from hypoxic-ischemic (HI) brain injury in adult C57BL/6 mice. Relative to vehicle treatment (dH_2O, 10mL/kg/day), oral administration of AF4 (50 mg/kg/day) for 3 days reduces HI-induced neuronal loss in the striatum and hippocampus, motor impairments, and reduces the ability of LPS to stimulate the production of TNF-α in whole blood. Pretreatment with AF4 (1 ug/mL) decreased the death of mouse primary cortical neurons subjected to oxygen glucose deprivation (12 hours) in comparison to vehicle (DMSO) or the same concentration of quercetin or its metabolites. Taken together these findings indicate that AF4 reduces HI-induced brain injury and motor deficits by increasing the resistance of vulnerable neurons to ischemic cell death and decreasing the production of inflammatory cytokines.

LIST OF ABBREVIATIONS AND SYMBOLS USED

AF4	Apple Fraction 4
Akt/PKB	Akt/protein kinase B
ANOVA	Analysis of variance
ARE	Anti-oxidant response element
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
Ca^{2+}	Calcium ion
cm	Centimetre
CO_2	Carbon dioxide
CREB	cAMP responsive element binding protein
DAB	Diaminobenzidine
dH_2O	Distilled water
DIV	Days in vitro
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E16	Embryonic Day 16
ELISA	Enzyme-Linked Immunosorbent Assay
g	Gram
HBSS	Hank's Balanced Salt Solution
HCL	Hydrochloric Acid

HI	Hypoxia-ischemia
HMG-CoA reductase	3-hydroxy-3-methyl-glutaryl-CoA reductase
H_2O_2	Hydrogen Peroxide
IL6	Interleukin 6
INT	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium
IR3G1cA	Isoshamnetin-3-glucuronic acid
kg	Kilogram
L	Litre
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MAP kinase	Mitogen activated protein kinase
MCA	Middle cerebral artery
MCAO	Middle cerebral artery occlusion
mg	Milligram
min	Minute
mL	Millilitre
mm	Millimetre
mM	Millimolar
N_2	Nitrogen
Na^+	Sodium ion
NeuN	Neuronal Nuclei
nm	Nanometres
OGD	Oxygen glucose deprivation

p.o.	Per os
PB	Phosphate buffer
PBS	Phopsphate buffered saline
PBS-TX	Phosphate buffered saline with Triton X
PComA	Posterior communicating artery
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PDE4	Phosphodiesterase type 4
PDL	Poly-D-lysine
PFA	Paraformaldehyde
pg	Picogram
PI 3-kinase	Phosphoinositide 3-kinase
PKA	Protein Kinase A
PKB	Protein Kinase B
PKC	Protein kinase C
Q3G	Quercetin-3-O-glucoside
Q3G1cA	Q-3-O-glucuronic acid
Q3'S	Q-3'-O-sulphate
RNA	Ribonucleic acid
rot	Rotations
RT-PCR	Reverse transcription polymerase chain reaction
s	Second
SCD1	Stearoyl-Coenzyme A desaturase 1

SEM	Standard error of mean
SREBP-2	Sterol response element binding factor 2
TNF-α	Tumour Necrosis Factor Alpha
TTC	2,3,4-Triphenyltetrazolium chloride
WT	Wild type
µg	Microgram
µm	Micrometer
µM	Micromolar
%	Percent
°C	Degrees Celsius
4-VO	Four-vessel occlusion

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Chapter 1: Introduction

The interruption in blood flow to the brain during cerebral ischemia results in a disruption in oxygen and nutrient supply to the brain, as well as the removal of waste products. This inhibits the ability of the brain to meet metabolic demands and results in cell death within minutes, producing a condition known as stroke. Stroke is the third leading cause of death in North America and the leading cause of adult disability (Johansen et al., 2006). Depending on the severity, location of brain injury and duration of flow reduction after stroke, persistent impairments of sensory/motor function, speech, reasoning and memory may occur. These deficits result primarily from the permanent loss of electrically excitable brain cells, known as neurons, that process and transmit information by electrochemical signaling. Furthermore, stroke survivors often require extensive rehabilitation and life-long assistance from family members and health professionals to perform tasks necessary for day-to-day living, causing a major cost to the health care system. Currently there are no approved pharmacological treatments to protect neurons from degeneration or to promote the recovery of neurological function following a stroke. Therefore dietary inclusion of a natural product such as apple-derived flavonoids produced in Nova Scotia would be a simple, inexpensive, and safe method for preventing a condition that represents a major unmet medical need. The focus of the present study was to determine whether pretreatment with an apple-derived flavonoid fraction (AF4) effectively decreases motor deficits and brain damage following an experimental stroke in mice.

My first goal was to assess the neuroprotective effects of AF4 pretreatment in reducing neuronal injury and motor deficits two weeks following cerebral ischemia.

Transient forebrain ischemia was produced by hypoxic-ischemic (HI) brain injury resulting in damage primarily in the ipsilateral hippocampus and striatum with occasional loss of neurons in the ipsilateral cortex (Olson and McKeon, 2004). Asymmetric motor deficits resulting from cerebral ischemia-induced damage to the striatum are a major cause of long-term disability in stroke patients. Therefore it is crucial to find a treatment that reduces the risk of motor deficits and brain damage following a stroke. In the present study C57Bl/6 adult male mice were used to determine if pretreatment with AF4 attenuated HI-induced neuronal cell loss in the striatum and hippocampus, as well as motor deficits in comparison to vehicle treated animals.

My second goal was to assess the ability of AF4 to attenuate the upregulation of inflammatory cytokines (i.e. TNF-α, IL6) following ischemic brain injury. This is important as the inflammatory response initiated in the brain following necrotic cell death leads to further brain injury following a stroke (Ernsley et al., 2008). Flavonoids, such as quercetin, have been shown to possess anti-inflammatory properties that Nair et al. (2006) has shown to inhibit the expression of pro-inflammatory cytokine TNF-α in blood. In the present study C57Bl/6 adult male mice were pretreated with AF4 or vehicle prior to HI injury in order to determine if this flavonoid-enriched fraction reduces the expression of inflammatory cytokines in whole blood, including TNF-α, following ischemic brain injury.

1.1 Anatomy of the Cerebral Vasculature System

Although the brain accounts for only 2% of our body weight it utilizes 20% of our body's oxygen and 25% of our body's glucose (Zauner et al., 2002). To accommodate the

high volume of blood flow, and the rapid response time necessary to sustain this high-energy demand in the brain, an intricate and dynamic cerebral vasculature system is required. The main arteries responsible for maintaining the blood flow and nutrient supply to the brain are the two internal carotid arteries and the basilar artery. The internal carotid arteries are predominantly responsible for the nutrient supply to the brain as they have a higher perfusion rate of 530 mL/min in humans, compared to 170 mL/min flowrate for the basilar artery (Schoning et al., 1994). Both the internal carotid arteries and the basilar artery feed into the circle of Willis, which is an enclosed arterial system located below the ventral surface of the brain. This structure provides redundancy so that if blood flow via one artery is compromised the circle of Willis will enable perfusion to the brain to be maintained. There are three arteries that are responsible for maintaining the arterial flow between hemispheres, forming the circle of Willis; the anterior communicating artery, and the two posterior communicating arteries (PComA). In some rodents such as the gerbil the PComA is not patent. In the case of C57Bl/6 mice the patency of the PComA is significantly reduced due to a small PComA with a diameter of less than one third of that of the basilar artery found only on one side of the brain (Kitagawa et al., 1998). This reduction in patency of the PComA restricts blood flow between cerebral hemispheres. The main arteries branching from the circle of Willis that supply the brain are, from anterior to posterior, the anterior cerebral artery, the anterior choroidal artery, the middle cerebral artery (MCA), the posterior cerebral artery and the superior cerebellar artery (Dorr et al., 2007).

1.2 Rodent Models of Ischemic Brain Injury

Rodent models of ischemic brain injury have been crucial in studying the pathophysiology of stroke and potential therapeutic interventions for this disease. The two main models used to experimentally induce cerebral ischemia are the focal and global cerebral ischemia models.

Middle cerebral artery occlusion (MCAO) is the most frequently utilized model for focal ischemia and involves either the insertion of an intraluminal thread at the origin of the MCA or the coagulation of the MCA in combination with the occlusion of one or both common carotid arteries (Carmichael, 2005). The MCAO method is commonly used to model cerebral ischemia as it represents the most frequent cause of stroke in humans and results in damage in the frontal, parietal and temporal lobes of the neocortex and the lateral striatum, while sparing the hippocampus (Carmichael, 2005).

Global ischemic models involve the transient occlusion of blood flow to the forebrain either unilaterally (one hemisphere) in the case of the HI model or bilaterally in the case of the four-vessel occlusion (4-VO) model. The HI model of brain injury relies on the poor development of the posterior communicating artery in C57Bl/6 mice that restricts blood flow between hemispheres via the circle of Willis (Yang et al., 1997). Therefore cauterization of a single carotid artery decreases blood flow to the MCA and reduces blood flow to the brain territory perfused by the MCA in the ipsilateral hemisphere. When combined with exposure to a low oxygen environment (8% oxygen) this treatment is sufficient to induce a state of cerebral ischemia that damages primarily the ipsilateral hippocampus and striatum with occasional loss of neurons in the ipsilateral cortex (Olson and McKeon, 2004). As a result, rodents display motor deficits in the

contralateral side of their body due to neuronal loss following HI (Olson and McKeon, 2004). The advantage of the HI model over MCAO models is that the rodent is only anaesthetized for a short period of time (approximately 10 minutes) during cauterization of the common carotid artery and the anaesthetic is not administered during the ischemic episode. This is beneficial as inhalation anaesthetics may confound the interpretation of studies aimed at testing the efficacy of neuroprotective strategies, as anaesthetics themselves may be neuroprotective (Matchett et al., 2009).

1.3 Cellular Damage Following Cerebral Ischemia

An interruption of blood flow to the brain resulting in cerebral ischemia injury produces a shortage of glucose and oxygen available to tissue in the ischemic core. As adenosine triphosphate (ATP) is produced exclusively in the brain through aerobic respiration involving oxygen and glucose, termed oxidative phosphorylation, cerebral ischemia causes a marked reduction in the amount of ATP produced. This reduces the amount of ATP available to provide the cells with energy to fuel metabolic processes essential for normal function. The most energy demanding process in the brain is the maintenance of the electrochemical gradient of axonal membranes by the sodium/potassium ATPase membrane pump (Astrup et al., 1981b). The sodium/ potassium ATPase pump utilizes ATP to extrude Na^+ and Ca^{2+} from inside the cell. Due to this reduction in ATP production during an ischemic event neurons are unable to sufficiently maintain this electrochemical gradient, rendering them unable to perform processes integral to cellular homeostasis such as the maintenance of the resting membrane potential.

The loss of ion homeostasis during cerebral ischemia causes neuronal depolarization, releasing excessive amounts of excitatory neurotransmitters such as glutamate into the synapse that further exacerbates depolarization resulting from energy failure. This increase in glutamate release in combination with reduced glutamate uptake by ATP-dependent pumps leads to an over-stimulation of glutamate receptors, known as excitotoxicity. As glutamate receptors facilitate the entry of Na^+ and Ca^{2+} into the cell, excitotoxicity can result in an one thousand to ten thousand fold increases in intracellular Ca^{2+} concentration (Silver and Erecinska, 1990). Over a prolonged period of time this can lead to the activation of a number of Ca^{2+} dependent enzymes, i.e. phospholipase A2 and calpain (Arai et al., 2001), which mediate cellular destruction during ischemia. Elevated intracellular Ca^{2+} levels are also responsible for the activation of both the intrinsic and extrinsic apoptotic pathways by inducing the release of cytochrome c from the mitochondria or stimulating the production of pro-inflammatory cytokines that activate tumour necrosis factor and Fas ligand receptors, respectfully (Sugawara et al., 2004; Martin-Villalba et al., 1999).

Cerebral ischemia also leads to an increase in free radical generation, combined with a failure in cellular processes responsible for free radical scavenging, exacerbating injury following an ischemic event (Piantadosi and Zhang, 1996). Free radicals are known to exacerbate the injury by causing lipid peroxidation, protein denaturation, and deoxyribonucleic acid (DNA) damage (Slemmer et al., 2008).

During cerebral ischemia a sequence of gene signaling events is initiated, beginning with the expression of immediate early genes that occurs within minutes of induction of ischemia. This is followed by the expression of genes encoding for heat

shock, pro-inflammatory and pro-apoptotic proteins, and finally genes encoding growth factors are induced (Iadecola, 1999). Immediate early genes are expressed within minutes of cerebral ischemia and are known to encode for DNA binding proteins and transcription factors that activate the expression of additional genes which may attribute to cellular injury (Akins et al., 1996). Heat shock proteins are induced in response to metabolic stress in the viable tissue surrounding the ischemic core and are thought to play a protective role. The next wave of gene expression involves genes encoding for inflammatory cytokines (i.e. TNF-α, IL6) and adhesion molecules that are responsible for inflammation in the ischemic region. Inflammatory responses triggered by necrotic cell death lead to further brain injury following a stroke (Ernsley et al., 2008). The expression of genes related to apoptosis peak at 12-24 hours after the induction of cerebral ischemia and result in programmed cell death. Twenty-four hours following the ischemic event genes encoding growth factors are induced, that promote the reorganization and repair of neural networks necessary for functional recovery.

Following an ischemic event a region of rapid cell death (necrosis) occurs in the tissue that is most severely hypoperfused, termed the ischemic core. The region surrounding the ischemic core that contains viable, but electrically quiescent, neurons is known as the penumbra (Astrup et al., 1981a). Cell death in the penumbra is the result of apoptosis as well as necrosis caused by a persistent reduction in cerebral blood flow (Wityk and Restrepo, 2003). The ischemic core experiences the most severe reduction in blood flow and therefore undergoes rapid and irreversible damage, whereas damage in the ischemic penumbra is thought to be potentially reversible if blood flow is restored to the area and neuroprotective measures are taken (Carmichael, 2005). Thus the ischemic

penumbra is the major target of many neuroprotective strategies. Approaches to improve the outcome following stroke would ideally attenuate both the acute and chronic damaging effects of cerebral ischemia, as well as aid in the recovery of damaged brain tissue following a stroke.

1.4 Flavonoids: Structural Properties

Flavonoids, a class of polyphenolic compounds, are among the most potent dietary free radical scavengers identified to date and are present in many sources including wine, fruits, vegetable and teas (Youdim et al., 2002). Flavonoids are subdivided into different subclasses based on their chemical structure, which are predictive of their pharmacological properties. All flavonoids contain the same basic chemical structure, shown in Figure 1, compromised of three six-membered rings: an aromatic A-ring fused to a heterocyclic C-ring that is attached through a single carbon-carbon bond to an aromatic B-ring. Subclasses of flavonoids differ mainly in the addition of functional groups and changes in the degree of unsaturation in the C ring. Flavonoids "classical" antioxidant nature is defined by the presence of a dihydroxylated B-ring capable of donating an electron, in the form of a hydrogen, to stabilize a radical species, as well as the presence of 2,3 unsaturation in conjugation with a carbonyl group on the C4 carbon in the C-ring and the presence of functional groups capable of binding transition metal ions (Rice-Evans et al., 1996). However, it has been speculated that this "classical" antioxidant activity is not the sole mechanism by which flavonoids offer protection and it is proposed that flavonoids are involved in the modulation of a number of protein kinase and lipid kinase signaling cascades. This bioactivity is thought to be

contingent on the structure of the flavonoid, including the number and position of hydroxyl functional groups on the B-ring and the degree of unsaturation of the bond between the C2 and C3 carbons in the C-ring (Williams et al., 2004). By comparison to the parent compound, the metabolism of these compounds can often result in a reduction of their ability to protect cells from oxidative stress, as a result of metabolic modifications that alter chemical structure.

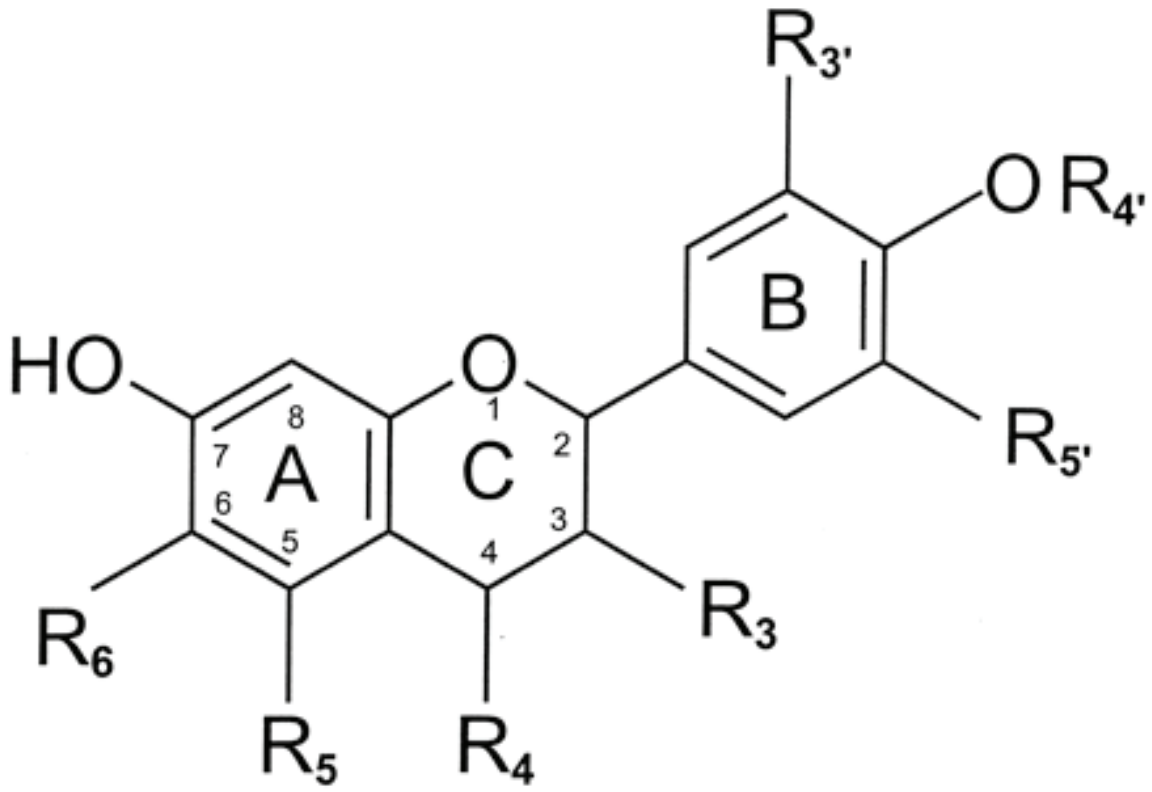


Figure 1: General structure and numbering pattern for flavonoids. The core structure is compromised of three six-membered rings: an aromatic A-ring fused to a heterocyclic C-ring that is attached through a single carbon-carbon bond to an aromatic B-ring. The subclasses of flavonoids differ mainly in the addition of functional groups (R moieties shown above) and changes in the degree of unsaturation in the C ring. This figure was adapted from Beecher (2003).

There are six main classes of flavonoids, which include the flavanones, flavonols, flavones, flavonols, anthocyanidins, and isoflavonoids. The main differences in chemical structure between subclasses, shown in Figure 2, comprise the addition of a carbonyl group on the C4 carbon in the C-ring of flavanones, a hydroxyl group on the C3 carbon of the C-ring in flavanols, a carbonyl group on the C4 carbon and a double bond between C2 and C3 carbons on the C-ring in flavones, a carbonyl group on the C4 carbon, a double bond between the C2 and C3 carbons, and a hydroxyl group on the C3 carbon of the C-ring in flavonols, and the B ring being connected to the C-ring at the C2 carbon rather than the C3 carbon (Gutierrez-Merino et al., 2011). Arora and Nair (1998) have shown that the ability of flavonoids to scavenge free radicals is based on their capacity to form stable radicals, which is increased by the addition of hydroxyl groups to the B ring and the presence of a carbonyl group at the C4 position on the C-ring. Flavonols have been shown to be the most potent class of flavonoids in scavenging free radicals (Husain et al., 1987). This finding suggests that AF4 should be neuroprotective as the main components of this apple peel extract, such as quercetin-3-O-glucoside are flavonols and thus potent anti-oxidants.

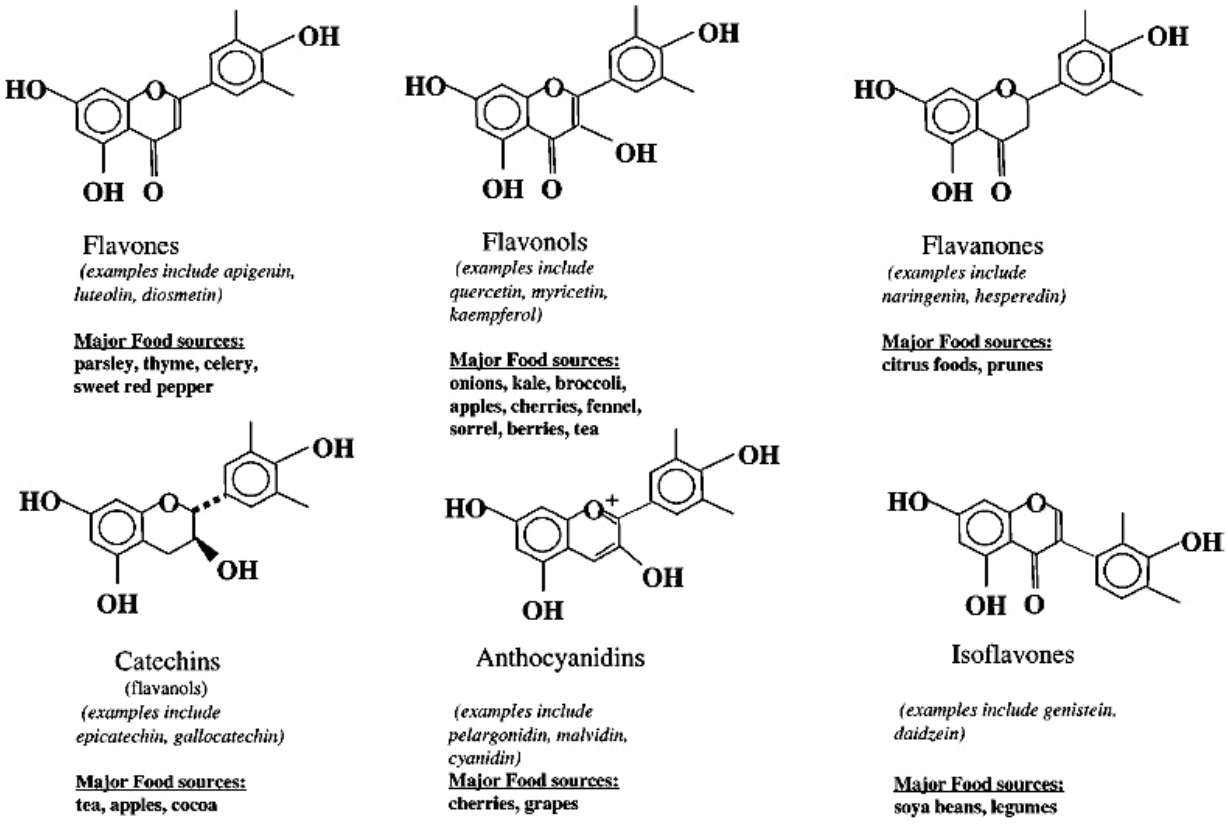


Figure 2: Structures of the six main flavonoid subclasses: Flavanones, flavonols, flavones, flavonols, anthocyanidins, and isoflavonoids. The subclasses of flavonoids differ mainly in the addition of functional groups and changes in the degree of unsaturation in the C ring. These differences in chemical structure are predictive of their pharmacological properties. This figure was adapted from Ross & Kasum (2002).

1.5 Flavonoids as Neuroprotective Agents

Flavonoids are contained in a variety of sources consumed in our normal diets, including wine, fruits, vegetables and teas. Quercetin and its glycoside derivatives are the most abundantly consumed flavonoids in the diet, reaching levels of 30-40 mg/day (Spencer et al., 2004). Flavonoids are known to scavenge free radicals, inhibit a variety of kinases, reduce lipid peroxidation, inhibit apoptosis, prevent platelet aggregation and exhibit anti-inflammatory effects (Williams et al., 2004; Peng & Kuo, 2003; Molina et al., 2003). Several flavonoids have been documented to cross the blood-brain barrier and to protect neurons from cell death in both *in vitro* and *in vivo* models of neurodegenerative diseases (Mandel et al., 2006; Youdim et al., 2004; Soundararajan et al., 2008).

It has been proposed that flavonoids have utility in the prevention or treatment of various conditions and diseases associated with oxidative stress (Williams et al., 2004). This hypothesis is supported by the fact that free radical generation has been implicated in numerous neurodegenerative disorders and flavonoids are excellent free radical scavengers (Williams et al., 2004). There is an emerging view that flavonoids and their *in vivo* metabolites may exert modulatory actions in cells through actions at protein kinase and lipid kinase signaling pathways (Williams et al., 2004). Flavonoids, and more recently their metabolites, have been reported to act at phosphoinositide 3-kinase (PI 3-kinase), Akt/protein kinase B (Akt/PKB), tyrosine kinases, protein kinase C (PKC), and mitogen activated protein kinase (MAP kinase) signaling cascades. Inhibitory or stimulatory actions of these pathways may profoundly impact cellular function by altering the phosphorylation state of target molecules and by modulating gene expression

(Williams et al., 2004). Thus, in addition to being radical scavengers, flavonoids may also modulate various kinase pathways that control oxidative stress and cell death. Furthermore, synergies may also exist between the particular flavonoids present in a given extract. This is supported by epidemiological studies showing that dietary supplementation with flavonoid-rich fruits and vegetables decreases the risk of developing chronic vascular, metabolic and neurological diseases, while clinical studies involving the administration of an individual antioxidant have not consistently demonstrated preventive effects, suggesting that a combination of phytochemicals found naturally in fruit and vegetables are necessary for health benefits (Liu, 2003).

1.6 Apple Flavonoids and Neurodegenerative Disorders

Apples, particularly apple skins, are a rich source of phenolic compounds, such as flavonoids, which have potent antioxidant potential. Apple skins are a waste product of the apple processing industry (e.g. apple sauce, juice and pie) in many countries and are typically available at high quantity and low cost. For example, in Nova Scotia, it has been estimated that 2-3 million kg of apple peels are generated each year (Rupasinghe, 2003). Therefore, phenolic compounds isolated from apple skin represent an ideal source of natural antioxidants for the food, natural health product, pharmaceutical and cosmetic industries. Since the raw source material is readily available at low cost, an economic benefit may be realized that can be passed on to consumers, thereby having the potential to decrease the cost of health care.

Epidemiological data suggest that apple flavonoids reduce the risk of cancer, cardiovascular disease, and neurological disorders (Boyer & Liu, 2004). Apples are a

good dietary source of phenolic compounds, and are the second highest source of dietary antioxidants, next to oranges, in the North American diet. An apple peel has 3- to 6-fold higher flavonoid content than apple flesh and has unique flavonoids, such as quercetin glycosides, not found in the flesh (Wolfe, Wu & Liu, 2003; Wolfe & Liu, 2003). Apple peel extracts have been shown to possess powerful free radical scavenging activity (Kondo et al., 2002). Oxidative stress induced by reactive oxygen species is linked to a large number of diseases (Gorman et al., 1996). Plant-derived antioxidants may therefore provide dietary modulators useful in preventing or treating a number of such diseases, including certain neurodegenerative diseases (Kaur and Kapoor, 2001; Heo et al. 2004). A ubiquitous flavonoid, quercetin 3-*O*-glucoside (Q3G), was recently demonstrated to have neuroprotective effects *in vitro* (Soundararajan et al., 2008). Currently, there are no registered natural products with health claims for protecting against any neurodegenerative disorders. As our overall population ages, there is increased desire to maintain health as well as to use alternative medications derived from natural or biological sources. This has driven the identification and development of safe and effective natural products to treat, prevent or reduce the risk of oxidative stress-mediated diseases, such as neurodegenerative disorders.

1.7 AF4 as a Neuroprotectant

Peels from the apple cultivar, Northern Spy, were collected from a commercial pie manufacturer, Apple Valley Foods Inc., Kentville, NS, Canada. These flavonoid-enriched fractions are derived from the skin rather than the flesh of the apple, as quercetin glycosides are found exclusively in the skin and there are a higher percentage of total

phenolics in the skin compared to the flesh. Analyses of major individual phenolic compounds present in 15-apple peel fractions (Tables 1a and 1b) were performed according to the procedure reported by Rupasinghe et al. (2010). The apple peel fractions were numbered AF1 through AF15, with each fraction having different phenolic profiles based on the relative amounts of each component in the fraction. Fractions AF4 and AF5 had the highest concentration of phenolic compounds, with AF4 having 12138.3 mg/L, and AF5 having 13138.6 mg/L total phenolics concentration per 800 ml of eluted fraction. For the purpose of this study apple fraction 4 (AF4) was chosen due to availability, and high total concentrations of phenolics and cyanidin-3-O-galactoside. AF4 is novel apple peel fraction enriched in quercetin-3-O-glucoside, quercetin-3-O-galactoside, quercetin-3-O-rhamnoside, quercetin-3-O-rutinoside, epicatechin, and cyanidin-3-O-galactoside.

Quercetin-3-O-glucoside (Q3G), a component of AF4, has been shown to be protective *in vitro* as SH-SY5Y cells, a human derived neuroblastoma cell line, pretreated with Q3G prior to being subjected to oxidative stress were more resistant to H_2O_2-induced cell death in a manner associated with an up-regulation of the expression of genes that increase lipid and cholesterol biosynthesis (Soundararajan et al., 2008). Soundararajan et al. (2008) found that this flavonoid-induced cytoprotection in SH-SY5Y cells was mediated by increased SREBP-2-induced sterol synthesis that decreased lipid peroxidation by maintaining membrane integrity in the presence of oxidative stress.

Sun et al. (2002) proposed that phytochemical extracts derived from fruits have powerful antioxidant and antiproliferative effects that may reduce the risk of chronic diseases when consumed on a regular basis. It is thought that the additive and synergistic

effects of the phytochemicals contained in fruit are responsible for the potent activity of flavonoid-enriched extracts, as they preserve the natural combination of phytochemicals in the fruit (Liu, 2003). This may partially explain why diet supplementation with flavonoid-rich fruits and vegetables reduces the risk of chronic diseases, while clinical studies involving the administration of an individual antioxidant have not been found to consistently offer preventive effects (Liu, 2003). This indicates the synergistic effect of combining individual phytochemicals is necessary to achieve health benefits.

Taken together these findings suggest AF4 may exhibit protective effects in many neurodegenerative disorders due to the potential synergistic effects of several flavonoids found in this extract. Furthermore, AF4 contains high levels of Q3G that potently protects the neuronal-like cell line SH-SY5Y from oxidative stress-induced cell death (Soundararajan et al., 2008). The combined anti-oxidative and anti-inflammatory properties of AF4 suggest that this extract has the potential to be used as dietary supplement to protect against neurodegeneration and motor deficits resulting from a stroke. The following hypotheses were tested to determine whether pretreatment with AF4 reduced neuronal cell loss and motor deficits in experimental models of stroke.

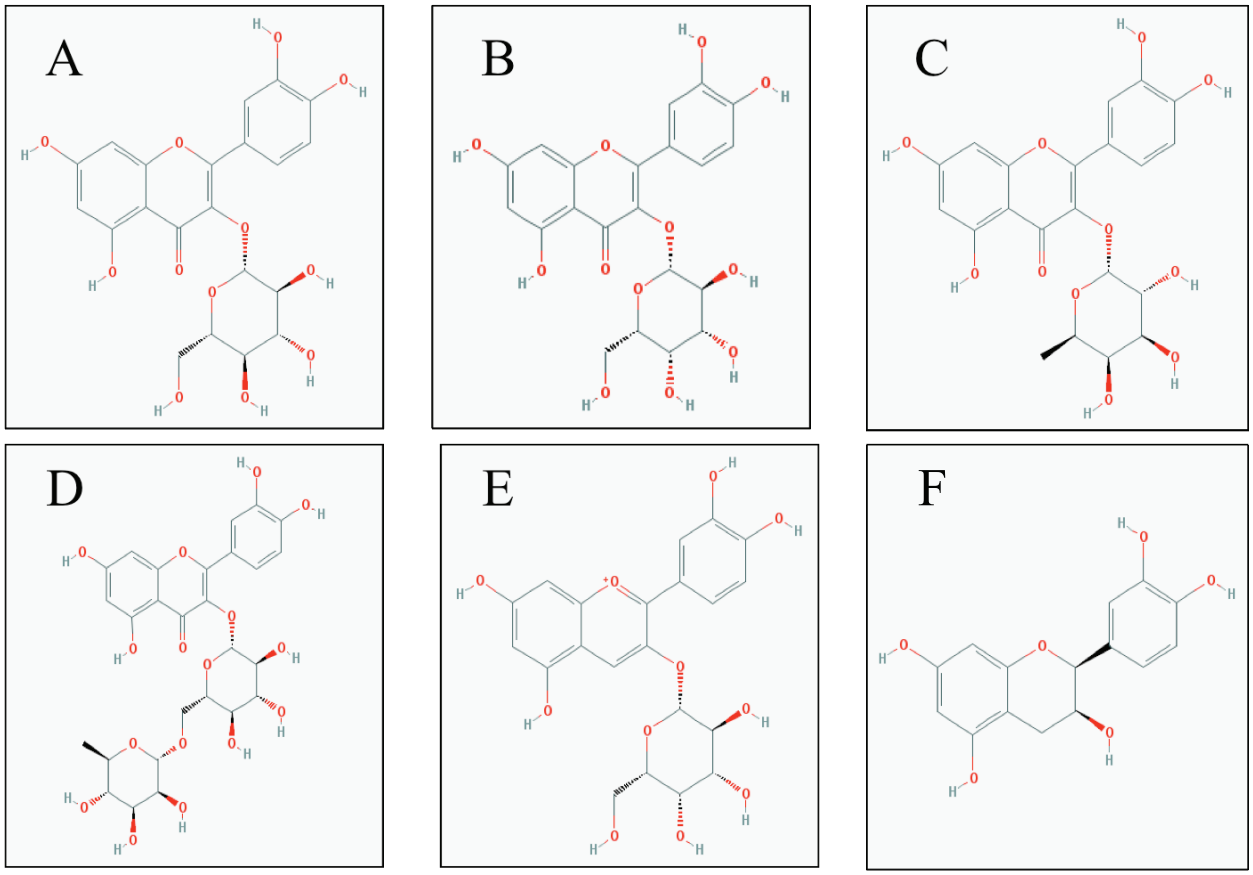


Figure 3: Structures of AF4 flavonoid components. (A) quercetin-3-O-glucoside, (B) quercetin-3-O-galactoside, (C) quercetin-3-O-rhamnoside, (D) quercetin-3-O-rutinoside, (E) cyanidin-3-O-galactoside and (F) epicatechin.

Phenolic compound	Fraction number						
	AF1	AF2	AF3	AF4	AF5	AF6	AF7
Quercetin (Q)	0.1	0.1	0.6	9.9	20.6	11.9	50.2
Q-3-*O*- paltoside	0	0	7.2	63.8	29.0	2.3	0
Q-3-*O*- rutinoside	0	0	293.3	1535.7	1105.0	285.1	30.3
Q-3-*O*- galactoside	2.0	1.2	566.8	2914.9	3914.7	2346.1	575.8
Q-3-*O*- glucoside	0.5	0.3	101.6	1474.8	1657.1	721.5	101.7
Q-3-*O*- rhamnoside	1.8	1.0	86.9	2771.6	4339.2	3112.8	973.6
Total Flavonols	***4.4 (48.4%)***	***2.6 (1.6%)***	***1056.40 (23.1%)***	***8770.7 (72.3%)***	***11065.6 (84.2%)***	***6479.7 (86.9%)***	***1731.6 (85.7%)***
Cyanidin-3-O-galactoside	***0 (0%)***	***0 (0%)***	***527.6 (11.5%)***	***559.4 (4.6%)***	***167.4 (1.2%)***	***29.2 (0.3%)***	***6.3 (0.3%)***
Phloridzin	1.3	0.9	7.7	386.8	711.5	614.1	239.9
Phloritin	0.8	0	0.8	1.0	1.2	1.2	1.8
Total dihydrochalcone	***2.1 (23.1%)***	***0.9 (0.56%)***	***8.5 (0.1%)***	***387.8 (3.1%)***	***712.7 (5.4%)***	***615.3 (8.2%)***	***241.7 (11.9%)***
Chlorogenic acid	1.9	99.8	1663.0	1221.1	502.8	97.7	10.4
Cafeic acid	0.7	0.9	2.1	43.6	25.1	7.0	1.4
Ferulic acid	0	0	1.2	0	13.3	20.3	5.8
Isoferulic acid	0	0	0	3.7	23.5	13.9	4.3
Total phenolic acids	***2.6 (28.6%)***	***100.7 (62.8%)***	***1666.3 (36.4%)***	***1268.4 (10.4%)***	***564.7 (4.3%)***	***138.9 (1.9%)***	***21.9 (1.1%)***
Epigallocatechin	0	0.9	7.4	0.9	2.9	1.3	0
Catechin	0	15.7	210.4	106.8	46.0	13.2	1.7
Epicatechin	0	39.4	1104.8	1044.3	579.3	178.2	16.8
Total Flavan-3-ol	***0 (0%)***	***56.0 (34.9%)***	***1322.6 (28.9%)***	***1152.0 (9.5%)***	***628.2 (4.8%)***	***192.7 (2.6%)***	***18.5 (0.9%)***
Total phenolics	**9.1**	**160.2**	**4578.4**	**12138.3**	**13138.6**	**7455.8**	**2020**

Table 1a: Concentration (mg/L) of phenolic compounds in Fractions 1 to 7. The values shown in brackets represent the percentage of each component relative to the total phenolic content measured in that fraction, such that the total phenolic amount shown at the bottom of each row represents 100% for that fraction. It can be seen that different fractions may have distinct phenolic profiles based on the relative amounts of each component in the fraction. The various fractions may contain additional unidentified phenolic components.

Phenolic compound	Fraction number							
	AF8	AF9	AF10	AF11	AF12	AF13	AF14	AF15
Quercetin (Q)	45.0	53.8	68.2	1.4	32.1	10.7	7.7	0.5
Q-3-*O*- paltoside	0	0	0	0	0	0	0	0
Q-3-*O*-rutinoside	4.9	1.5	0	0	0	0	2.4	0
Q-3-*O*-galactoside	75.8	21.7	11.7	6.8	5.5	7.5	33.4	10.6
Q-3-*O*-glucoside	14.2	3.7	2.4	1.2	1.2	1.2	5.4	2.1
Q-3-*O*-rhamnoside	176.4	34.3	11.9	5.7	3.9	3.2	19.4	19.5
Total Flavonols	***316.3 (82.4%)***	***115 (84.4%)***	***94.2 (89.6%)***	***15.1 (70.6%)***	***42.7 (65.8%)***	***22.6 (45.9%)***	***68.3 (77.4%)***	***32.7 (74.7%)***
Cyanidin-3-O-galactoside	***1.9 (0.4%)***	***1.1 (0.8%)***	***0.8 (0.7%)***	***0.5 (2.3%)***	***0.6 (0.9%)***	***0.6 (1.2%)***	***0.7 (0.8%)***	***0.5 (1.1%)***
Phloridzin	53.6	12.0	4.3	2.6	2.2	1.7	6.5	7.9
Phloritin	3.1	3.3	1.8	1.0	0.1	0.9	0.9	0.8
Total dihydrochalcone	***56.7 (14.7%)***	***15.3 (11.2%)***	***6.1 (5.8%)***	***3.6 (16.8%)***	***3.2 (4.9%)***	***2.6 (5.3%)***	***7.4 (8.4%)***	***8.7 (19.9%)***
Chlorogenic acid	3.3	2.3	2.0	1.7	10.5	9.5	4.2	1.2
Cafeic acid	0.8	0	0.7	0	0.8	0.8	0.8	0.7
Ferulic acid	1.7	1.1	0	0	0	0	0	0
Isoferulic acid	0.9	0	0	0	0	0	0	0
Total phenolic acids	***6.7 (1.7%)***	***3.4 (2.5%)***	***2.7 (2.6%)***	***1.7 (7.9%)***	***11.3 (17.4%)***	***10.3 (20.9%)***	***5.0 (5.7%)***	***1.9 (4.3%)***
Epigallocatechin	0	0	0	0	0	0	0	0
Catechin	0	0	0	0	1.9	1.8	0.9	0
Epicatechin	2.3	1.5	1.3	0.5	5.2	11.3	5.9	0
Total Flavan-3-ol	***2.3 (0.6%)***	***1.5 (1.1%)***	***1.3 (1.2%)***	***0.5 (2.3%)***	***7.1 (10.9%)***	***13.1 (26.6%)***	***6.8 (7.7%)***	***0***
Total phenolics	**383.9**	**136.3**	**105.1**	**21.4**	**64.9**	**49.2**	**88.2**	**43.8**

Table 1b: Concentration (mg/L) of phenolic compounds in Fractions 8 to 15. The values shown in brackets represent the percentage of each component relative to the total phenolic content measured in that fraction, such that the total phenolic amount shown at the bottom of each row represents 100% for that fraction. It can be seen that different fractions may have distinct phenolic profiles based on the relative amounts of each component in the fraction. The various fractions may contain additional unidentified phenolic components.

1.8 Rationale: Use of AF4 as a Neuroprotective Agent Prior to Transient Forebrain Ischemia

Flavonoids are potent inhibitors of a variety of kinases, inflammatory cytokines, and apoptotic mechanisms, and also reduce oxidative stress by scavenging free radicals and reducing lipid peroxidation (Williams et al., 2004; Peng and Kuo, 2003; Molina et al., 2003). Several flavonoids found in AF4 have been reported to cross the blood-brain barrier and to protect neurons from cell death in both *in vitro* and *in vivo* models of neurodegenerative disorders (Mandel et al., 2006; Youdim et al., 2004; Soundararajan et al., 2008). Sun et al. (2002) proposed that phytochemical extracts derived from fruits have powerful antioxidant and antiproliferative effects and when consumed on a regular basis can play a role in the prevention of chronic diseases. Furthermore it has been hypothesized that the additive and synergistic effects of the phytochemicals contained in fruit are responsible for the potent activity of flavonoid-enriched extracts, as it preserves the natural combination of phytochemicals in the fruit (Liu, 2003). Therefore AF4 may exhibit protective effects in many neurodegenerative disorders due to the potential synergistic effects of these flavonoids administered in combination. Also, Q3G, a major component of AF4, has been shown *in vitro* to protect SH-SY5Y cells from oxidative stress-induced cell death (Soundararajan et al., 2008). As a result of the combined anti-oxidative and anti-inflammatory properties of AF4 it has the potential to be used as a dietary supplement to protect against neurodegeneration and motor deficits in an animal model of stroke.

To test the potential neuroprotection of AF4 against ischemic cell death, the HI-method was used. Transient forebrain ischemia induced by HI primarily injures the

ipsilateral hippocampus and striatum with occasional loss of neurons in the ipsilateral cortex. The HI model relies on the poor development of the posterior communicating artery in C57Bl/6 mice that restricts blood flow between hemispheres via the circle of Willis (Yang et al., 1997), allowing the cauterization of a single carotid artery combined with exposure to a low oxygen environment (8% oxygen) to induce a state of cerebral ischemia. The advantage of the HI model over MCAO models is that the rodent is only anaesthetized for a short period of time and the anaesthetic is not administered during the ischemic episode. This is beneficial as inhalation anaesthetics can confound the interpretation of studies aimed at testing neuroprotective strategies since anaesthetics themselves may be neuroprotective (Matchett et al., 2009). In the present study, I dosed adult C57Bl/6 mice with either AF4 (5, 10, 25 or 50 mg/kg/day) or vehicle (dH_2O, 10mL/kg) for 3 days prior to being subjected to 50 minutes of HI and then assessed neuronal cell damage in the hippocampus and striatum, and motor deficits following HI-injury.

To assess motor deficits the rotarod was used. The rotarod is a rotating cylinder with an acceleration of 100 rot/min^2 that the mice walk along. The amount of time spent on the rotarod was recorded as a measure of motor performance, with longer times indicative of better motor performance. The advantages of the rotarod behavioral test are that it is reproducible and easy to quantify performance and does not involve an excessively complex task or invoke a stressful response in comparison to other behavioral tests.

1.9 Hypothesis 1: AF4 Will Reduce the Death of Primary Cortical Neurons Subjected to Oxygen Glucose Deprivation (OGD)

Oxygen glucose deprivation (OGD) is an *in vitro* model for cerebral ischemia that replicates the insufficient availability of oxygen and nutrients for cells to meet metabolic demands in brain tissue affected by an ischemic event. I therefore hypothesize that pretreatment of primary cortical neurons with AF4 prior to oxygen glucose deprivation (OGD) will protect cells from necrotic cell death induced by OGD, as compared to vehicle treated cells.

1.10 Hypothesis 2: Mice Pretreated with AF4 Will Show Less Neuronal Cell Loss, in Comparison to the Vehicle Group, Following Hypoxic-Ischemic (HI) Brain Injury

As flavonoids have been shown to exhibit anti-inflammatory and anti-oxidant properties and to exert synergistic effects when administered in combination, I hypothesize that adult male mice pretreated with AF4 will show a reduction in neuronal cell loss following HI in comparison to mice treated with vehicle (dH_2O).

1.11 Hypothesis 3: Pretreatment with AF4 Prior to Hypoxia-Ischemia (HI) Injury Will Protect Against Motor Deficits Following Cerebral Ischemia

Transient forebrain ischemia produced by HI results primarily in damage of the ipsilateral hippocampus and striatum, and occasional loss of neurons in the ipsilateral cortex (Olson and McKeon, 2004). As the striatum plays a crucial role in motor control, unilateral HI-induced damage in the striatum often results primarily in motor deficits on the side contralateral to the ischemic event. I therefore hypothesize that adult male mice pretreated with AF4 will show a reduction in motor deficits following HI in comparison

to mice treated with vehicle (dH_2O) due to protection against HI-induced cell loss in the striatum.

1.12 Hypothesis 4: AF4 Pretreatment Before Hypoxia-Ischemia (HI) Injury Will Attenuate HI-Induced Inflammation

The expression of genes encoding inflammatory cytokines, such as TNF-α, is increased following necrotic cell death leading to further brain injury following a stroke (Ernsley et al., 2008). Since flavonoids are known to have potent anti-inflammatory effects the pretreatment with a flavonoid-enriched fraction (AF4) may be able to suppress this inflammatory response to ischemic injury and thus reduce the severity of an experimental stroke. To test this hypothesis I used an *ex vivo* assay to assess whether AF4 reduced inflammation following HI. This *ex vivo* assay utilizes lipopolysaccharide (LPS) to induce the release of TNF-α from whole blood derived from mice treated with AF4 or vehicle. I hypothesis that LPS-induced TNF-α release will be reduced in mice treated with AF4 relative to animals that receive vehicle.

Chapter 2: Methods

2.1 Animal Care

All experiments involving the use of animals were approved by the University Committee on Laboratory Animals and done in accordance with guidelines for the Canadian Council on Animal Care. The animal holding rooms were on a 12 hour dark/light cycle and water and food were provided *ad libitum*.

2.2 AF4 Treatment

An apple-derived flavonoid-enriched fraction (AF4), composed of quercetin-3-O-glucoside, quercetin-3-O-galactoside, quercetin-3-O-rhamnoside, quercetin-3-O-rutinoside, epicatechin, and cyanidin-3-O-galactoside, was extracted from Northern Spy apple skins. The AF4 dose for each experiment was standardized based on the concentration of the total non-polymeric phenolics in AF4, which include quercetin-3-O-glucoside, quercetin-3-O-galactoside, quercetin-3-O-rhamnoside, quercetin-3-O-rutinoside, epicatechin, and cyanidin-3-O-galactoside. The extract was administered orally (p.o.) to C57/Bl6 mice (6-8 weeks old) at a dose of 50 mg/kg/day for 3 consecutive days. Mice in the control group were given vehicle (water, dH_2O) at a dose of 10 mL/kg/day body weight orally for 3 consecutive days. Twenty-four hours after the final dose of AF4 or vehicle, all mice were subjected to 50 minutes of hypoxia-ischemia (HI) and sacrificed 2 weeks later.

2.3 Unilateral Common Carotid Artery Occlusion Combined with Exposure to a Low Oxygen Environment (HI)

The procedure used to induce cerebral ischemia in adult mice was adapted from the hypoxia-ischemia (HI) method developed by Levine (Levine, 1960) for rats. Mice were anaesthetized using isoflurane (Baxter Corporation, Mississauga, ON) in an induction chamber (3% vaporized with medical oxygen at a flow-rate of 3 L/min). The ventral portion of the neck was shaved and then sterilized with Soluprep (SoluMed Inc., Laval, PC) and Betadine (Purdue Frederick Inc., Pickering, ON). Anesthesia was maintained with 2% isoflurane vaporized with oxygen at a flow-rate of 1.5 L/min. A small ventral incision was made on the neck of the mouse with a pair of scissors to expose the sternohyoid and sternomastoid muscles. The left carotid artery was located beneath the intersection point of the sternohyoid and the sternomastoid muscles. The left carotid artery was carefully separated from the vagus nerve and permanently occluded using a high-temp elecrocautery pen (Bovie Instruments, St. Petersburg, FL). If the common carotid artery was not completely occluded or exhibited blood loss the mouse was immediately euthanized. Following a 2-3 hour recovery period the mice were placed in a hypoxia-chamber, consisting of a glass cylinder vented with 8% oxygen balanced with nitrogen flowing at a rate of 6 L/min. The chamber was placed in a water bath at 36.5°C to maintain normal body temperature. After 50 minutes of exposure to the low oxygen environment (8% oxygen balanced with nitrogen) the mice were removed from the chamber and returned to their home cage. The mice were allowed to survive for 2 weeks following HI to permit the brain infarct in the ipsilateral hemisphere to develop before harvesting the brain tissue for histological analysis.

2.4 Assessment of Rotarod Performance

Time spent on a rotarod (ACCURotor Rotarod, ACCUScan Instruments Inc., Columbus, OH) was measured to assess motor performance of the mice. The apparatus consists of a rotating cylinder that mice walk along. The rotational speed increases at a constant acceleration making it more difficult for mice to continue walking. The amount of time spent on the rod was recorded as a measure of performance, with longer times indicative of better motor performance. The acceleration of the rotarod was set to 100 rot/min^2. Mice were tested on the third day of AF4 treatment (24 hours pre-HI) and 2 weeks following HI (14 days post-HI) (Figure 13). On each of these days the mice were tested with 3 sessions and the average time spent on the rotarod was calculated for that day. The difference in performance 14 days post-HI and 24 hours pre-HI was determined and compared between the two treatment groups.

Figure 4: Experimental timeline used to assess motor performance in mice treated with either AF4 (50 mg/kg p.o.) or vehicle (dH_2O, 10 mL/kg p.o.) once a day for 3 consecutive days and then subjected to 50 minutes of HI. The mice were first tested on the rotarod (ACCURotor Rotarod, ACCUScan Instruments Inc., Columbus, OH) on day 3 after the final dose of AF4 or vehicle. There were 3 trials on day 3. On day 18 the mice were tested again on the rotarod. There were 3 trials on day 18.

2.5 Preparation of Tissue for Histology

The mice were humanely euthanized by intraperitoneal (i.p.) administration of sodium pentobarbital (Scherung-Plough, Pointe-Claire, PQ) at a dose of 240 mg/kg. The mice were than transcardially perfused with 0.9% saline, followed by 4% paraformaldehyde (PFA) in phosphate buffer at pH 7.4. Brains were removed and post-fixed by storing in 4% PFA for 48-72 hours. Next, the tissue was cryoprotected by submersion in a solution of 30% sucrose in 0.1 M phosphate buffer for 24 hours. Free floating coronal sections were cut on a freezing microtome at a thickness of 30 μm and placed in a solution of phosphate with 0.06% sodium azide for long-term storage.

2.6 Nissl Staining

Sections 360 μm apart were mounted onto superfrost glass slides (Fisher Scientific, Nepean, ON) and allowed to dry for 24 hours. Once dry the sections were dehydrated using a graded series of increasing concentrations of ethanol (2 minutes of 50%, 70%, 95%, 100%) and then placed in xylenes for 5 minutes. Then the tissue was rehydrated using another graded series of ethanol of decreasing dilution (100%, 95%, 70%, 50%). The brain sections were then rinsed with distilled water and incubated in 1% cresyl violet solution (Sigma-Aldrich, Oakville, ON) for 10-15 minutes. The sections were rinsed in water and destained in a 1% acetic acid solution. The sections were then dehydrated through a series of graded ethanol solutions of increasing concentrations (50%, 70%, 95%, 100%) and cleared in xylenes before coverslipping using Cytoseal (Stephens Scientific, Riverdale, NJ).

Images of the sections were captured using PixeLink software with a 1X objective and a 10X ocular lens. The images were analyzed using ImageJ software. The area of the hemisphere ipsilateral to the common carotid artery occlusion and the area of the contralateral hemisphere were measured for all sections and a ratio of ipsilateral/ contralateral area was calculated to determine a measure of relative tissue loss in the ipsilateral hemisphere. A ratio of 1.0 indicated no tissue loss, while a ratio of less than 1.0 indicated tissue loss in the ipsilateral hemisphere.

2.7 Regressive Haematotoxylin & Eosin (H&E) Staining

Sections 360 μm apart were mounted onto superfrost glass slides (Fisher Scientific, Nepean, ON) and allowed to dry for 24 hours. Sections were then placed in Wheaton jars and put under a gentle flow of tap water for ~1 minute and placed in Harris' haematoxylin (Cat No:HHS16, Sigma-Aldrich, Oakville, ON) for 5 minutes. The sections were rinsed off in tap water to remove the excess stain. This was followed by rapid immersion in a 1% acid alcohol solution (70 mL of 100% ethanol, 30 mL distilled water, and 1 mL of 1 M HCL) and then rinsed under running tap water for 10-15 seconds. The sections were then placed in Scott's tap water solution (1.75 g of sodium bicarbonate, 10 g of magnesium sulfate, and 500 mL of tap water to make a 500 mL solution) for 2 minutes, or until the sections turn blue. Slides underwent repeated cycles of dipping slides in 1% acid alcohol, plunging into tap water and placing in Scott's tap water solution until the background of the tissue was no longer blue. Sections were washed for 5 minutes in running tap water and then stained in 1% acidified eosin (2 g in 200 mL of a solution of 600 mL 95% ethanol, 4 mL glacial acetic acid) for 5-10 seconds. Sections

were then rinsed in running tap water and treated with 70% ethanol for a few seconds to remove excess eosin. The sections were then dehydrated through a series of graded ethanol solutions of increasing concentrations (50%, 70%, 95%, 100%) and cleared in xylenes before coverslipping using Cytoseal (Stephens Scientific, Riverdale, NJ).

2.8 Neuronal Nuclei (NeuN) Immunohistochemistry

Sections were first rinsed three times with PBS containing 0.1% Triton X (PBS-TX) for 10 minutes at room temperature. Then the tissue was placed in 1% H_2O_2 in PBS-TX for 30 minutes to quench endogenous peroxidases. The tissue was then rinsed three times in PBS-TX, as previously described, and incubated in 5% horse serum in PBS-TX for 30 minutes. Following incubation in serum sections were incubated with primary antibody, monoclonal anti-NeuN antibody raised in mouse (Cat No: MAB377, Millipore, Etobicoke, ON), at a dilution of 1:2000 in PBS-TX for one hour at room temperature and then left over night at 4°C on the shaker. After incubating the tissue in primary antibody overnight the tissue was rinsed three times in PBS-TX and incubated in secondary antibody, biotinylated anti-mouse raised in horse (Vector Laboratories Inc., Burlingame, CA), at a dilution of 1:500. Following another series of washes in PBS-TX, the tissue was incubated in an Avidin-Biotin complex in PBS-TX at a dilution of 1:1000 for 1 hour to amplify the signal from the secondary antibody. The sections were then washed and placed in a solution of 0.5 mg/mL diaminobenzidine (DAB) (Sigma-Aldrich, Oakville, ON) with nickel, glucose oxidase, ammonium chloride and D-glucose in PBS. The tissue was reacted with the DAB solution for 5-10 minutes until the desired staining intensity was achieved. No primary, no secondary and no ABC controls were used to confirm

staining specificity. Next, the tissue was washed and mounted onto superfrost glass slides (Fisher Scientific, Nepean, ON) and left overnight to dry. Once dry the sections were dehydrated in a graded ethanol series of 50%, 70%, 95%, and 100%, cleared in xylenes, and coverslipped using Cytoseal (Stephen's Scientific, Riverdale, NJ).

2.9 Image Analysis of Sections Processed for NeuN Immunohistochemistry

Sections stained for NeuN immunoreactivity from the striatum 0.1 mm anterior to bregma and the hippocampus 1.8 mm posterior to bregma were captured on a light microscope using PixeLink software at 50X (10X ocular lens and a 5X objective). The images were analyzed using ImageJ software by an observer blind to the treatment group of the animals.

The cell counts in the striatum were determined by first converting the images to an 8-bit grey scale and setting the pixel threshold so only pixels that are 3X background were black on a white foreground by selecting the binary tool. Then the striatum was outlined and the analyze particle function was used to count positively labeled cells in the striatum. An index of neuronal cell survival was determined by dividing the number of NeuN positive cells in the ipsilateral striatum by the number of NeuN positive cells in the contralateral striatum, where a value of 1.0 indicated no injury in the ipsilateral striatum while a value of less than 1.0 indicated neuronal loss.

Neuronal cell loss in the hippocampus was determined by converting the images to 8-bit grey scale. The binary tool was selected to set the pixel threshold so that only positively labeled cells were black on a white foreground. The hippocampus was outlined and the area of positively labeled cells was measured with the measurement functions.

Neuronal cell loss in the hippocampus was determined by measuring the area occupied by NeuN positive cells in the entire hippocampus of sections cut at 1.8 mm posterior to bregma, as the dense packing of pyramidal neurons in the dorsal hippocampus precludes cell counts in sections 30 μm thick. Dividing the area of the NeuN positive neurons in the ipsilateral hippocampus by the area of the NeuN positive neurons in the contralateral hippocampus to obtain an index of neuronal cell loss. Where a value of 1.0 indicated no injury in the ipsilateral hippocampus while a value of less than 1.0 indicated neuronal loss.

The mortality group included mice that did not survive HI or had to be sacrificed following HI due to seizures or paralysis. The number of mice that met this criteria was divided by the total number of mice in the study to yield the mortality rate.

2.10 2,3,4-Triphenyltetrazolium Chloride (TTC) Staining

6-8 week old male C57Bl/6 mice were given a single dose of either vehicle (dH_2O, 10 mL/kg) or AF4 (50 mg/kg). Then 24 hours following the dose the mice were subjected to 50 minutes of HI. The mice were then sacrificed 24 hours following HI. The brains were removed and sliced using an acrylic mouse brain slicer (Zivic Instruments, Pittsburgh, PA, USA). 2,3,4-Triphenyltetrazolium chloride (TTC) (Sigma-Aldrich, Oakville, ON) staining was performed to measure the infarct volume, as the tetrazolium salts reacts with the succinate dehydrogenase in viable cells staining healthy tissue red whereas infarcted tissue remains unstained (white) as it lacks these enzymes. The infarct volume was quantified by taking a ratio of ipsilateral viable tissue over contralateral viable tissue to give the percent viability. The average was taken of the 6 brain sections

for each animal and then an average viability for each treatment group was calculated and compared statistically between treatment groups.

2.11 TNF-α ELISA

6-8 week old C57Bl/6 mice were dosed orally with either vehicle (10 mL/kg) or AF4 (50 mg/kg) once a day for 3 days. Sixteen hours after the last dose, mice were sacrificed and whole blood, from cardiac puncture, was collected into lithium heparin tubes (Cat No: 02675182, Becton, Dickinson and Company, Franklin Lakes, NJ). Lipopolysaccharide (LPS)(Sigma-Aldrich, Oakville, ON) was added to the blood at a concentration of 100 μg LPS/mL of blood to induce the release of TNF-α. The blood was incubated for 4 hours in a tissue culture incubator at 37°C. The tubes were then centrifuged at 1400 x g for 10 minutes at 4°C, and the top layer of plasma was collected and stored at -80°C. A mouse TNF-α ELISA kit (Cat No: KMC3011, Invitrogen, Amarillo, CA) was used to assess the LPS-induced TNF-α released in the whole blood. The amount of TNF-α in the sample was determined by measuring the intensity of the colored product at an absorbance of 450 nm, using an ELx800 UV spectrophotometer (Bio-tek Instruments Inc., Winooski, VT), that is directly proportional to the amount of TNF-α in the sample.

The experiment was repeated using 6-8 week old C57Bl/6 mice that were dosed orally with vehicle (10 mL/kg) or AF4 (50 mg/kg) once a day for 3 consecutive days and subjected to HI. Twenty-four hours after the last dose the mice were subjected to 50 minutes of HI. Six hours following HI the mice were sacrificed and whole blood was collected into lithium heparin tubes (Cat No: 02675182, Becton, Dickinson and

Company, Franklin Lakes, NJ). Lipopolysaccharide (LPS) (Sigma-Aldrich, Oakville, ON) was added to the blood at a concentration of 100 μg LPS/mL of blood to induce the release of TNF-α. The blood was incubated for 4 hours in a tissue culture incubator at 37°C. The tubes were then centrifuged at 1400 x g for 10 minutes at 4°C, and the top layer of plasma was collected and stored at -80°C. A mouse TNF-α ELISA kit (Cat No: KMC3011, Invitrogen, Amarillo, CA) was used to measure the LPS-induced TNF-α released in the whole blood. The amount of TNF-α in the sample was determined by measuring the intensity of the colored product at an absorbance of 450 nm, using an ELx800 UV spectrophotometer (Bio-tek Instruments Inc., Winooski, VT), which is directly proportional to the amount of TNF-α in the sample.

2.12 Preparation of Mouse Primary Cortical Neuron Cultures

Primary cortical neuron cultures were prepared from cerebral cortices of wild type (WT) CD1 mouse embryos as described previously (Katchanov et al., 2001), with the following modifications. Pregnant CD1 females were heavily anaesthetized with isoflurane vapor (Benson Medical Industries, Inc., Markham, ON) before being euthanized by decapitation. The embryonic day 16 fetuses were immediately removed from the sacrificed pregnant females by cesarean section and placed in a medium sized Petri dish filled with ice-cold Hank's Balanced Salt Solution (HBSS) (GIBCO; Invitrogen, Amarillo, CA). The meninges were removed from the brains and cortices were isolated under a dissecting microscope. The cortices from each embryo were placed in individual wells of a 24-well plate (Corning, Lowell, MA), containing 1 mL of ice-cold sterile dissecting solution (phosphate-buffered saline (PBS) (GIBCO; Invitrogen,

Amarillo, CA) with 1 mM Mg^{2+}, 13 mM glucose and 0.3% w/v bovine serum albumin (BSA) (Invitrogen, Amarillo, CA). Under sterile conditions, the tissue was briefly minced, transferred to 15 mL sterile conical tubes (Corning, Lowell, MA) and centrifuged at 350 x g for 3 minutes at room temperature. The dissecting solution was discarded and the cortical neurons were then dissociated by a 15 minute incubation in 1 mL of 0.1% trypsin solution (0.1% w/v trypsin (Invitrogen, Amarillo, CA) in PBS with 1 mM Mg^{2+} and 13 mM glucose) at 37 $^{\circ}$C. The trypsinization was inhibited by the addition of 0.5 mL of trypsin inhibitor solution (0.06% w/v trypsin inhibitor (Invitrogen, Amarillo, CA) and 0.01% DNase I (Invitrogen, Amarillo, CA) in PBS with 1 mM Mg^{2+}, 13 mM glucose and 0.3% w/v BSA). The tubes were mixed briefly and then cells were centrifuged at 350 x g for 3 minutes at room temperature. The trypsin and inhibitor solutions were discarded and each cell pellet was suspended in 1 mL of cortical neuron plating medium (Neurobasal medium (Invitrogen, Amarillo, CA) with 10% fetal bovine serum (GIBCO; Invitrogen, Amarillo, CA), 2% B27 supplement (Invitrogen, Amarillo, CA), 1 mM L-glutamine (Invitrogen, Amarillo, CA), and 1% Gentamycin (Invitrogen, Amarillo, CA)), triturated 10 times and counted using trypan blue exclusion and a hemocytometer.

The cortical neurons were plated in 24-well plates (Corning, Lowell, MA) that were pre-coated with poly-D-lysine (PDL) (Sigma-Aldrich, Oakville, ON), according to the procedure described by the manufacturer. The plates were briefly coated with 100 μg/mL PDL for 5-10 min (250 μL/well) and then washed three times with tissue-culture grade water and left to dry for 2 hours before cells were introduced. Cortical neurons were plated at a concentration of 1 x 10^6 cells/mL. The medium was completely changed

the day after plating and every 3 days in culture thereafter. Cultures were maintained in serum-free cortical culture medium (Neurobasal medium (Invitrogen, Amarillo, CA) with 2% B27 supplement (Invitrogen, Amarillo, CA), 1mM L-glutamine (Invitrogen, Amarillo, CA), and 1% Gentamycin (Invitrogen, Amarillo, CA)) in a humidified, 37 $^{\circ}$C incubator with 5% CO_2. Experiments were performed on the eighth day *in vitro* (DIV8).

2.13 Treatment of AF4, Quercetin and Quercetin Metabolites on Primary Cortical Neuron Cultures

AF4, quercetin (Q) and quercetin metabolites (Q-3-O-glucoside, Q-3'-sulphate, Q-3-glucuronic acid, and isoshamnetin-3-glucuronic acid) were tested on primary cortical neuron cultures to determine their individual effects on the survival of cultured neurons in response to oxygen glucose deprivation (OGD). The fractions were incubated in serum free medium on DIV8 cells (E16 Cortical Cultures) for 12 hours proceeding, as well as during the 12-hour period of OGD on DIV9. Cell death was assayed by LDH release into serum free/aglycaemic/anoxic (OGD) medium.

The fractions were tested at 3 concentrations, 0.01 μg/mL, 0.1 μg/mL and 1.0 μg/mL (where 0.1 μg/mL is equivalent to a flavonoid concentration of about 2 μM) against their respective dimethyl sulfoxide (DMSO) vehicle controls (0.1%, 0.01% and 0.001% DMSO respectively). Primary cortical neuron cultures were prepared as described previously. Cortical neuron cultures were exposed to vehicle (0.1%, 0.01% or 0.001% DMSO) or AF4 at concentrations of 0.01 μg/mL, 0.1 μg/mL and 1.0 μg/mL in serum-free cortical neuron medium for a period of 12 hours on DIV8 before they were subjected to OGD. Glucose-free Dulbecco's Modified Eagle Medium (Invitrogen, Amarillo, CA) containing AF4 (0.01, 0.1, or 1.0 μg/mL) or the corresponding DMSO

control was placed in a 96-well plate and equilibrated to 0% oxygen in a modular chamber incubator (Billups-Rothenberg, Del Mar, CA). The chamber was flushed for 4 minutes at 20 L/min with a 5% CO_2 anoxic gas mixture balanced with N_2 (PraxAIR, Dartmouth, NS) using a step-down pressure system and placed in a humidified incubator for 12 hours at 37°C. Then the cortical neuron medium was replaced with this OGD-medium (anoxic and glucose-free) containing the flavonoids or DMSO control and the cultures were placed in the modulator chamber incubator. The chamber was flushed again with anoxic gas and placed in the humidified incubator for 12 hours at 37°C. Cell culture supernatants were then collected on DIV9 (Figure 5).

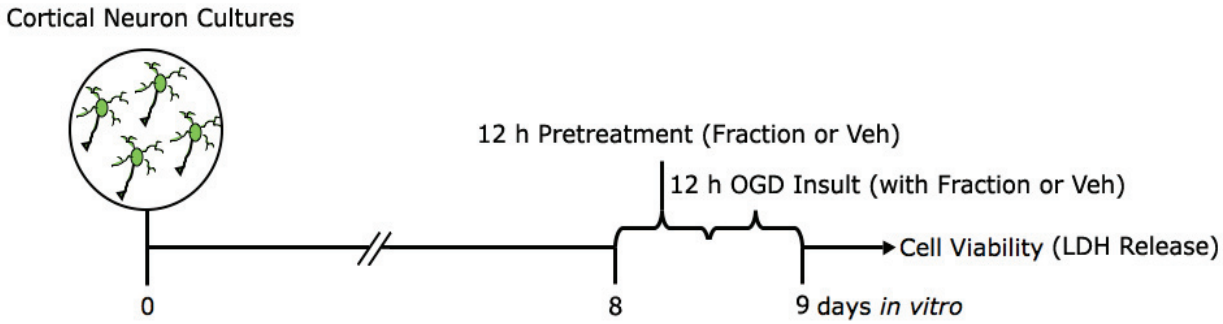


Figure 5: Experimental timeline used to test AF4, quercetin and quercetin metabolites (Q-3-O-glucoside, Q-3'-sulphate, Q-3-glucuronic acid, and isoshamnetin-3-glucuronic acid) in an OGD model on mouse primary cortical neurons. Flavonoid fractions were tested at 3 concentrations 0.01 ug/mL, 0.1 ug/mL, and 1.0 ug/mL (where 0.1 ug/mL is about 2 μM flavonoid) against their corresponding DMSO controls (0.1%, 0.01% and 0.001% DMSO respectively) in serum free medium on E16 Cortical Cultures on Day 8 for 12 hours proceeding, as well as during a 12 hours period of OGD on day 9 in culture (DIV9). Cell death was assayed by LDH release into serum free/aglycaemic/anoxic (OGD) medium on day 9.

2.14 Lactase Dehydrogenase (LDH) Assay

Lactate dehydrogenase is a stable cytosolic enzyme that is released by necrotic cells upon cell membrane damage. The membrane integrity of cortical neurons was assayed by measuring the release of LDH using the Cytotoxicity Detection Kit^{PLUS} (Roche Applied Science, Indianapolis, IN). This assay kit detects LDH released into culture supernates by a coupled enzymatic reaction that converts a tetrazolium salt into a red formazan product. Resulting formazan was detected using an ELx800 UV spectrophotometer (Bio-tek Instruments Inc., Winooski, VT). Positive (100% LDH release) and negative (spontaneous LDH release) controls were prepared in triplicate according to the manufacturer's instructions. This assay was used to access the difference in the percentage of cell death between treatment groups. The LDH assay is a colorimetric assay that detects the amount of lactate dehydrogenase that is released from the cytosol of damaged cells into the supernatant to give a quantification of cell death and cell lysis.

The catalyst (Diaphorase/NAD+ mixture) and the dye solution (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium (INT) and sodium lactate) were combined and then added 100 μL to each well and left on the cell for 25 minutes at room temperature in the dark. Then add 50 μL of stop solution and shake the plate for 30 seconds. Absorbance was measured at 490 nm with a reference wavelength of 620 nm. The percentage of total LDH release was calculated according to the manufacturers instructions. Background was subtracted and the LDH release in each sample and expressed as a percentage of the positive control included in the Cytotoxicity Detection Kit^{PLUS} (Roche Applied Science, Indianapolis, IN).

2.15 Quantitative RT-PCR

Quantitative reverse transcription (RT)-PCR was used to assess changes in stearoyl-Coenzyme A desaturase 1 (SCD1), 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase), and sterol response element binding factor 2 (SREBP-2) message in the hippocampus of vehicle (10 mL/kg) and AF4 (50 mg/kg) treated C57Bl/6 mice following 50 minutes of HI. Twenty-four hours after HI the hippocampus was dissected on ice. RNA was extracted from the hippocampus ipsilateral to the common carotid artery occlusion and hippocampus contralateral to the common carotid artery occlusion (uninjured) using the Qiagen RNeasy Lipid Tissue Mini Kit (Qiagen Sciences, MD). RNA was reversed transcribed to form first-strand cDNA and amplified using Taqman EZ RT-PCR reagents (Applied Biosystems, Foster City, CA). Mouse primers and probes were purchased from Applied Biosystems (Foster City, CA) and were designed to amplify genes SREBP-2 (AssayID# Mm01306283_m1), SCD1 (AssayID# Mm00772290_m1), and HMG-CoA reductase (HMGR forward GGGAGCATAGGCGGCT, reverse TGCGATGTAGATAGCAGTGACA, probe 6FAMCAACGCCCACGCAGCAACATAMRA) as well as β-actin as an endogenous control gene. Results are stated as a fold expression increase relative to a calibrator sample obtained from pooled contralateral hippocampus (uninjured) tissue of vehicle treated C57Bl/6 mice. Relative increases from the calibrator were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.16 Statistical Analyses

All t-tests, analysis of variance (ANOVA) statistical tests, and non-parametric tests statistics were computed using GraphPad Prism 4 software. An alpha level of less than 0.05 was chosen to determine significance.

Chapter 3: Results

3.1 Oral Administration of AF4 (50 mg/kg/day for 3 days) Prior to 50 Minutes of Hypoxia-Ischemia (HI) Reduces Brain Damage Relative to Vehicle Controls (dH_2O, 10 mL/kg)

Two groups of C57Bl/6 mice (6-8 weeks old) were dosed by oral gavage (p.o.) once a day for 3 consecutive days with either AF4 (50 mg/kg/day) or vehicle (10 mL/kg/day). Twenty-four hours after the last dose all mice were subjected to 50 minutes of HI. All mice were sacrificed two weeks after HI. The relative amount of tissue loss in the ipsilateral hemisphere was assessed to determine the ability of pretreatment with AF4 to prevent HI-induced tissue loss relative to the vehicle treatment group. The area of the hemisphere ipsilateral to the common carotid artery occlusion and the area of the contralateral hemisphere were measured for all coronal brain sections. A ratio of ipsilateral/contralateral area was calculated to give a measure of the measure of tissue loss in the ipsilateral hemisphere (Figure 6). The mean ± SEM tissue loss ratios were 0.80 ± 0.03 for the vehicle treatment group and 0.90 ± 0.03 for the AF4 treatment group. A one-sided student's t-test revealed a significant difference between treatment groups ($t(45)=2.121$; $p=0.0197$). This result indicates that pretreatment with 3 doses of AF4 (50 mg/kg/day, p.o.) prior to 50 minutes of HI reduces tissue loss in the ipsilateral hemisphere following HI in comparison to the vehicle treated group (10 mL/kg/day, p.o.). Figure 7 shows a representative photomicrograph of the coronal sections from vehicle and AF4 treated mice following 50 minutes of HI cut at the mid level (-0.10 mm to bregma) of the striatum (Paxinos & Franklin, 2001).

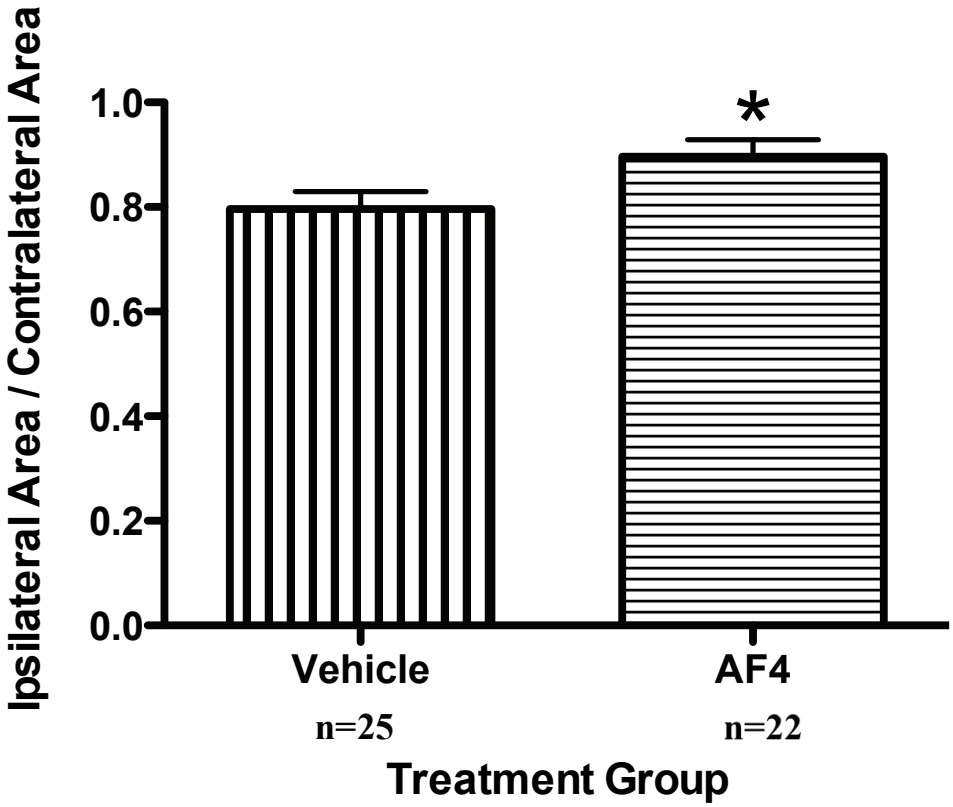


Figure 6: Administration of 3 oral doses of AF4 (50 mg/kg/day) prior to HI injury showed protection against HI reduced tissue loss in the ipsilateral hemisphere in comparison to the vehicle group (10 mL/kg/day). A one-sided student's t-test determined a significant difference between treatment groups ($t(45)=2.121$; $p=0.0197$). *$P< 0.05$

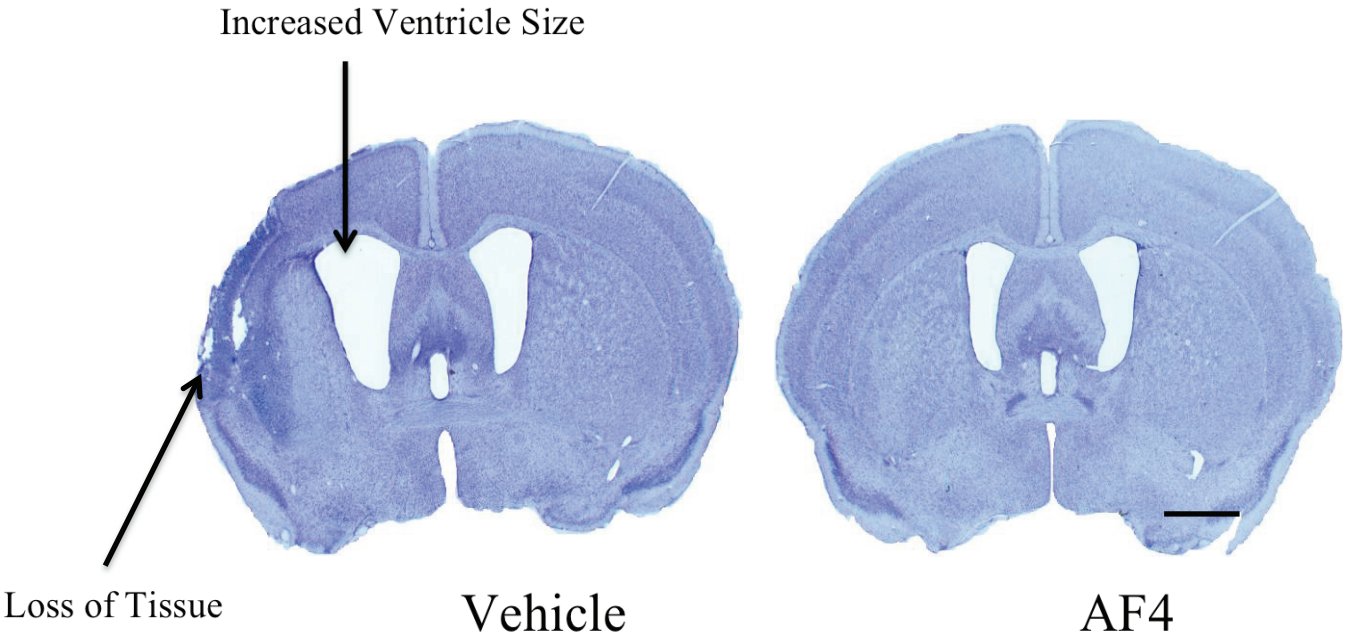


Figure 7: Representative photomicrographs of Nissl-stained coronal sections of C57Bl/6 mice 2 weeks following 50 min of HI. The coronal sections were stained with cresyl violet to visualize nissl bodies. Notice the loss of tissue and the increased ventricle size in the ipsilateral hemisphere in vehicle treated mice, in comparison to the ipsilateral hemisphere of the AF4 treated group. The scale bar on the right of the panel represents 1.0 mm.

3.2 Oral Administration of AF4 (50 mg/kg/day for 3 days) Prior to HI Reduces Neuronal Cell Loss in the Striatum and Hippocampus in Comparison to the Animals Treated with Vehicle (dH_2O, 10 mL/kg/day for 3 days)

Two groups of C57Bl/6 mice (6-8 weeks old) were dosed by oral gavage once a day for 3 consecutive days with either AF4 (50 mg/kg/day) or vehicle (10 mL/kg/day). Twenty-four hours after the last dose all mice were subjected to 50 minutes of HI. All mice were sacrificed two weeks after HI and neuronal cell loss in the striatum and hippocampus assessed to determine the protective effects of pre-dosing with AF4 against HI-induced damage in the striatum and hippocampus, in comparison to the vehicle treatment group. The number of NeuN positive cells (neurons) in the striatum approximately 0.1 mm anterior to bregma was counted in both the ipsilateral and contralateral hemisphere, with respect to carotid cauterization, to calculate a cell survival ratio (Figure 8). The mean ± SEM cell survival ratios were 0.42 ± 0.07 for the vehicle treatment group and 0.69 ± 0.07 for the AF4 treatment group. A one-sided student's t-test determined a significant difference between treatment groups ($t(58)=2.690$; $p=0.0047$). In the hippocampus, the area of positive NeuN labeling was measured in both the ipsilateral and contralateral hemisphere in coronal sections at approximately 1.8 mm posterior to bregma to yield an index of neuronal loss (Figure 10). The mean ± SEM indices of neuronal loss were 0.43 ± 0.07 for the vehicle treatment group and 0.64 ± 0.07 for the AF4 treatment group. A one-sided student's t-test determined a significant difference between treatment groups ($t(59)=2.022$; $p=0.0239$). These results indicate that pretreatment with 3 doses of AF4 (50 mg/kg, p.o.) prior to HI reduces neuronal cell loss following HI in comparison to the vehicle group (10 mL/kg, p.o.; once a day for 3 days).

Figures 9 and 11 show a representative photomicrograph of the striatum and hippocampus, respectively, of vehicle and AF4 treated mice following 50 minutes of HI.

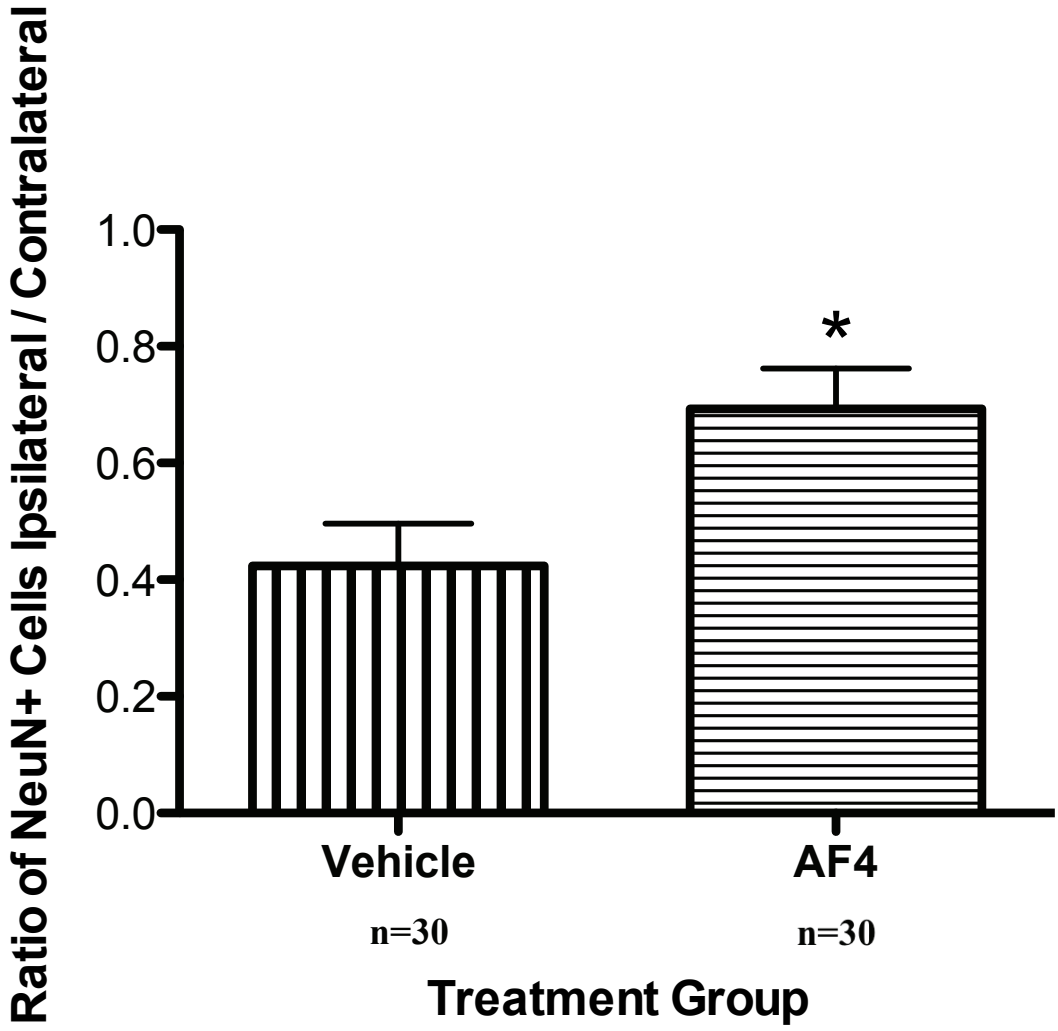


Figure 8: Neuronal survival ratios for the striatum 2 weeks following 50 minutes of HI for C57Bl/6 mice treated orally with either AF4 (50 mg/kg/day) (n=30) or vehicle (10 mL/kg/day) (n=30) once a day for 3 days prior to HI injury. Administration of 3 doses of AF4 (50 mg/kg) prior to HI reduced HI-induced neuronal cell loss in the ipsilateral striatum compared to vehicle treatment. A one-sided student's t-test determined a significant difference between treatment groups ($t(58)=2.690$; $p=0.0047$). *$P< 0.05$

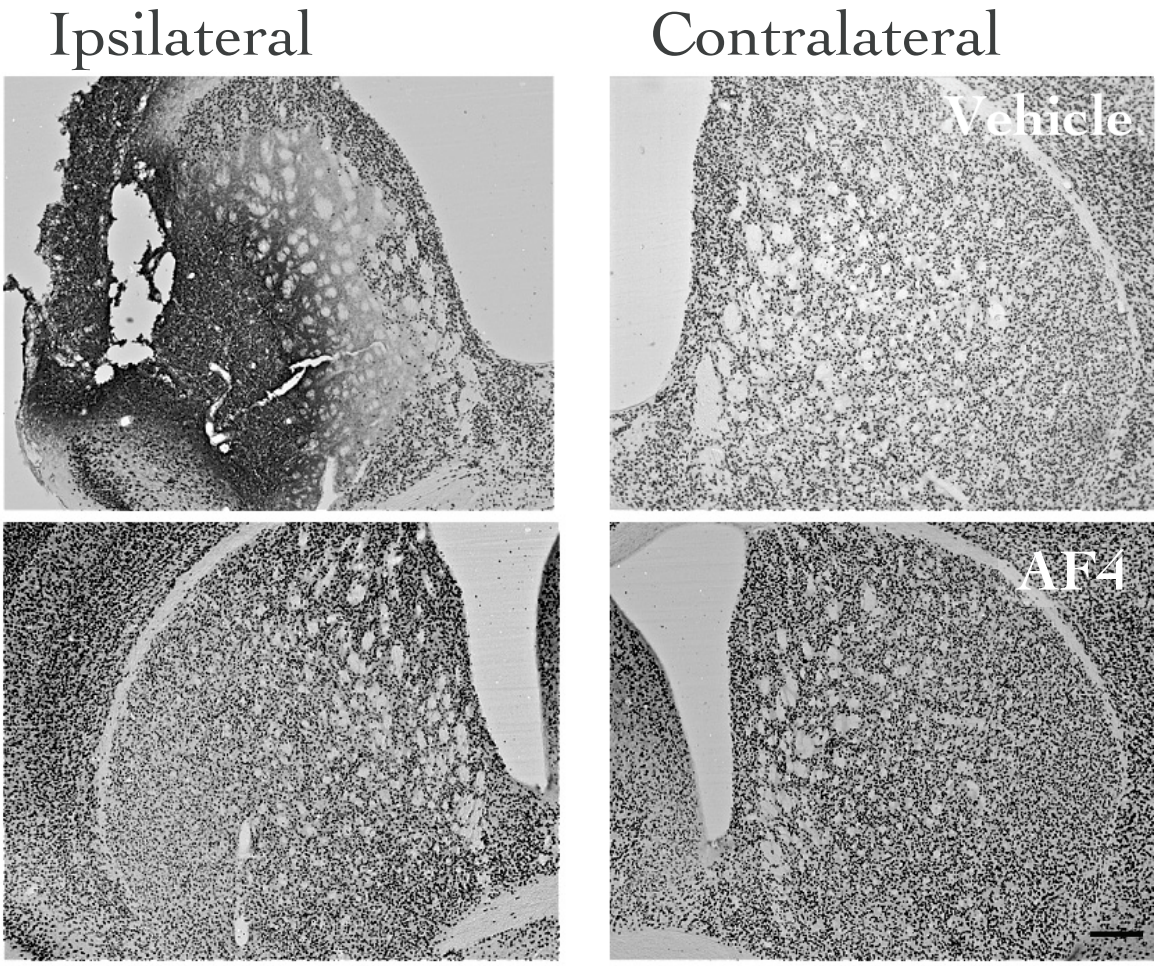


Figure 9: Representative photomicrograph of the ipsilateral and contralateral striatum of C57Bl/6 mice 2 weeks following 50 minutes of HI stained with a NeuN antibody to visualize viable neurons. Notice the loss of neurons in the ipsilateral striatum of mice in the vehicle treatment group in comparison to the ipsilateral striatum of mice treated with AF4 (50 mg/kg/day for 3 days). The scale bar in the bottom right panel represents 50 μm.

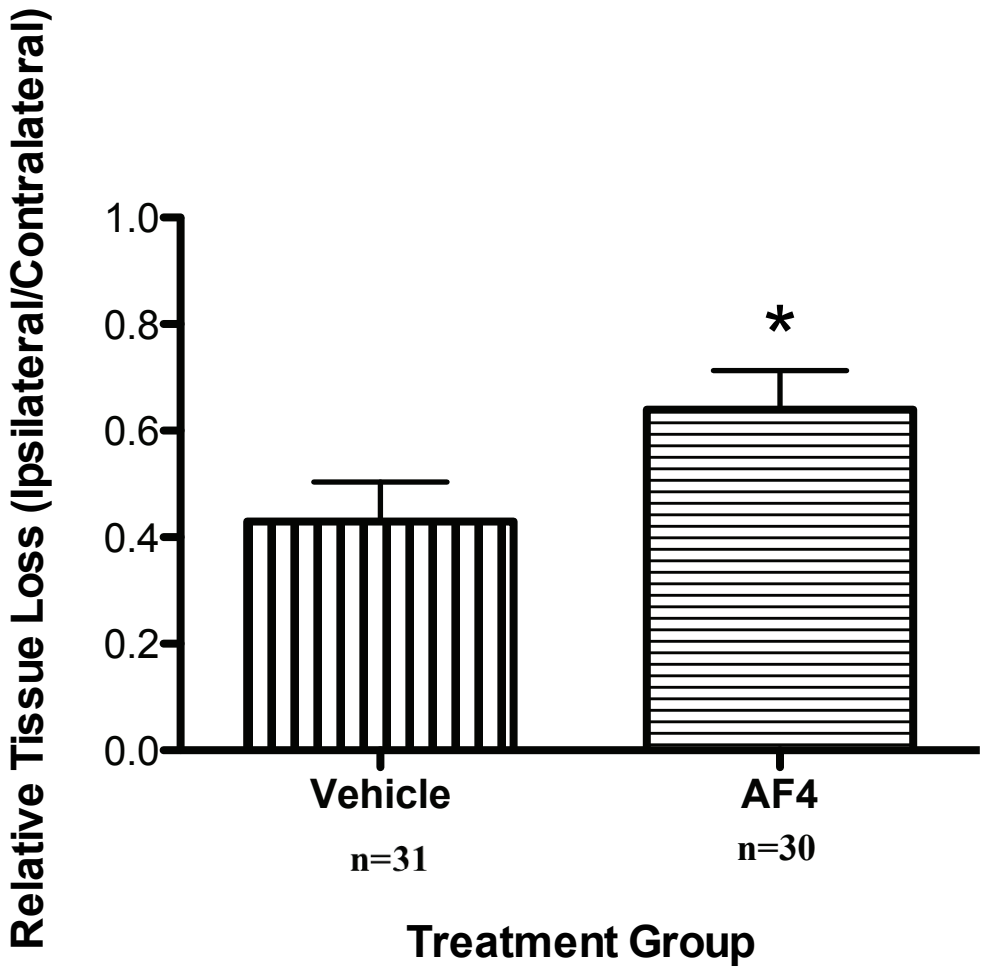


Figure 10: Neuronal survival ratios for the hippocampus 2 weeks following 50 minutes of HI for C57Bl/6 mice treated orally with either AF4 (50 mg/kg) (n=30) or vehicle (dH_2O, 10 mL/kg) (n=31) once a day for 3 days prior to HI injury. Administration of 3 doses of 50 mg/kg AF4 prior to HI reduces HI-induced injury in the ipsilateral hippocampus. A one-sided student's t-test determined a significant difference between treatment groups ($t(59)=2.022$; $p=0.0239$). *$P< 0.05$

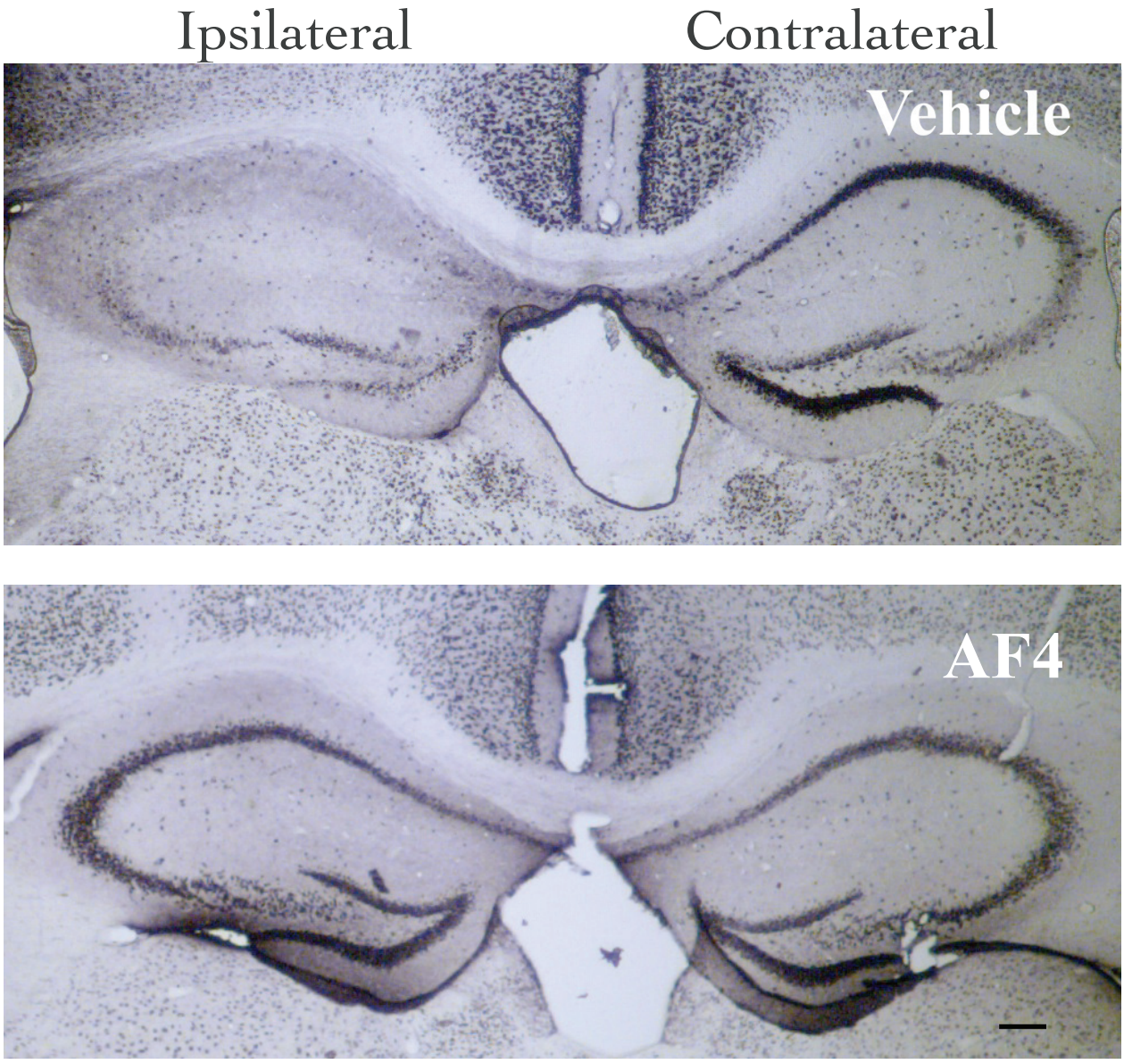


Figure 11: Representative photomicrographs of the ipsilateral and contralateral hippocampus of C57Bl/6 mice 2 weeks following 50 minutes of HI stained with a NeuN antibody to visualize viable neurons. Notice the loss of neurons in the ipsilateral hippocampus of mice in the vehicle group in comparison to the ipsilateral hippocampus of mice treated with AF4 (50 mg/kg/day for 3 days, p.o.). The scale bar in the bottom right panel represents 50 μm.

3.3 HI-Induced Mortality Rates of AF4 or Vehicle Treated C57Bl/6 Mice Subjected to HI

Mortality and injury rates were determined for C57Bl/6 mice treated with either AF4 (50 mg/kg, p.o.; once a day for 3 days) or vehicle (10 mL/kg, p.o.; once a day for 3 days) following 50 min HI. The mortality rates were 3/28 (11%) for vehicle treated mice and 1/23 (4%) for AF4 treated mice following HI (Figure 12). A Fischer's exact test comparing mortality rates revealed no significant difference between groups ($p=0.6173$). These results indicated that mice treated with AF4 or vehicle did not differ in their susceptibility to death as a consequence of 50 minutes of HI.

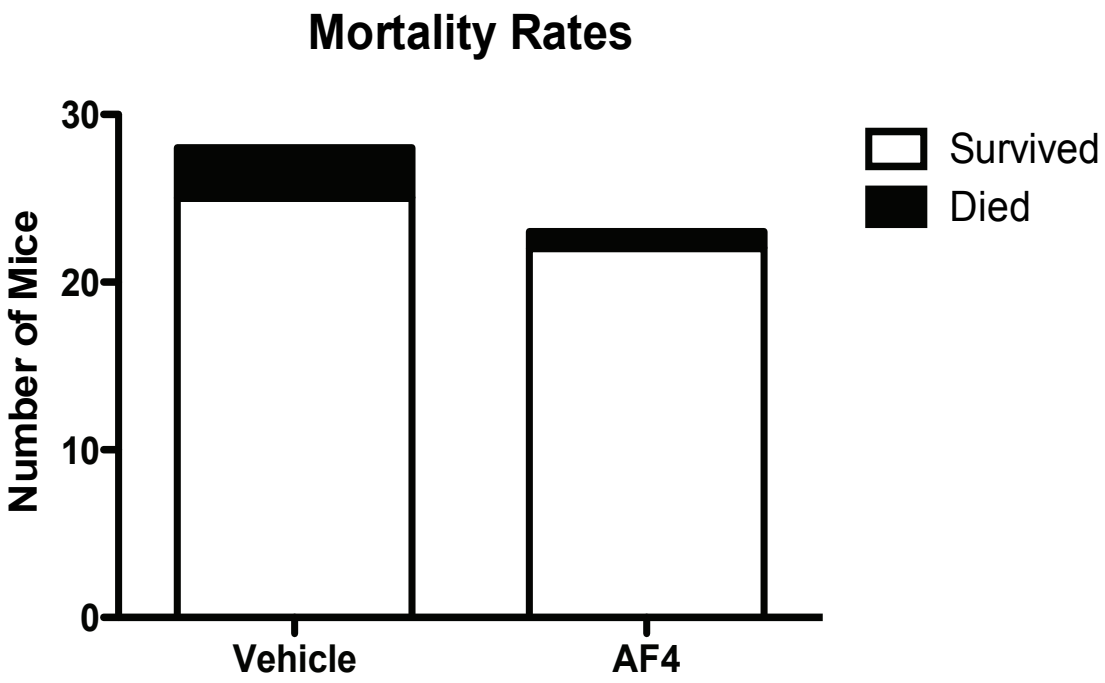


Figure 12: Bar graph displaying the number of mice that died or survived for two weeks following 50 minutes of HI. No statistical difference was found between the two treatment groups, AF4 (n=23) and vehicle (n=28), using the Fischer's exact test (p=0.6173).

3.4 Pretreatment with 3 Doses of AF4 (50 mg/kg, p.o.) Prior to 50 minutes of HI Reduced Motor Deficits Following HI

Two groups of C57Bl/6 mice (6-8 weeks old) were dosed by oral gavage once a day for 3 consecutive days with either AF4 (50 mg/kg) or vehicle (10 mL/kg). Twenty-four hours after the last dose all mice were subjected to 50 minutes of HI. All mice were sacrificed two weeks after HI. Mice were tested on the rotarod, a behavioral test for motor performance, on the third day of AF4 treatment (24 hours pre-HI) and 2 weeks following HI (14 days post-HI). On each of these days the mice were tested 3 times, with 2 hours between each successive trial and the average time spent on the rotarod was calculated for that day. The difference between performance on 14 days post-HI and 24 hours pre-HI was determined and used as a measure for motor performance (Figure 13). The mean ± SEM motor performance difference scores were -5.71 ± 2.72 seconds for the vehicle treatment group and 3.72 ± 2.49 seconds for the AF4 treatment group. A one-sided student's t-test revealed a significant difference between treatment groups ($t(45)=2.530$; $p=0.0075$). These results indicate that pretreatment with 3 doses of AF4 (50 mg/kg, p.o.) prior to 50 minutes of HI reduced HI-induced motor deficits in comparison to the vehicle treated group (10 mL/kg, p.o.).

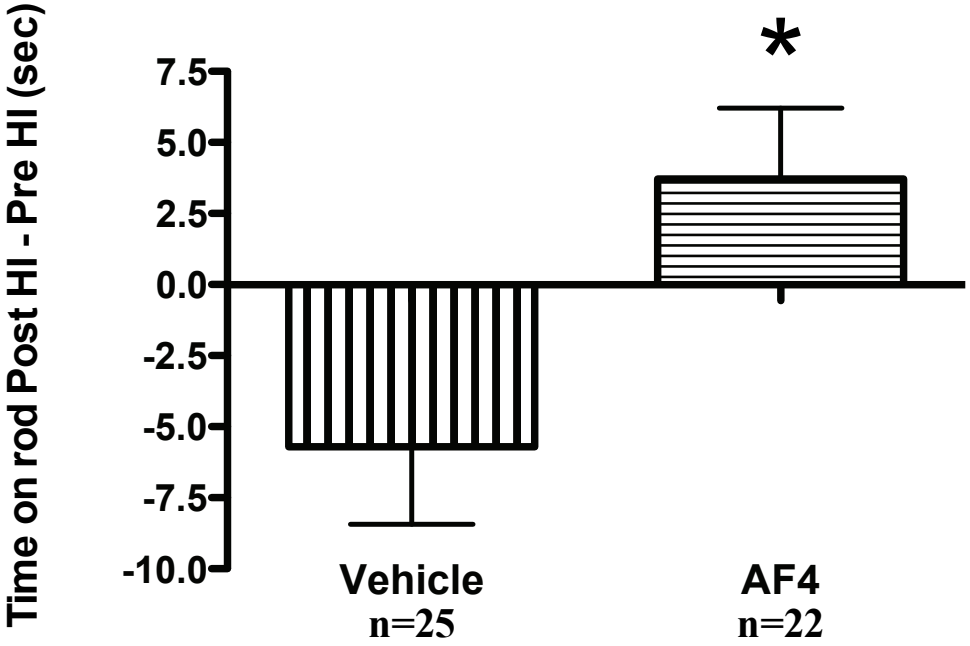


Figure 13: The ability of AF4 (50 mg/kg/day for 3 days, p.o.) to reduce HI-induced motor performance deficits was determined by subtracting the time spent on a rotarod 24 hours before HI and two weeks after HI. Negative value for this difference indicated that HI impaired co-ordination resulting in motor deficits, while positive values suggested that AF4 pretreatment prevented HI-induced motor deficits. A one-sided student's t-test determined a significant difference between treatment groups ($t(45)=2.530$; $p=0.0075$). *$P< 0.05$

3.5 Pretreatment with One Dose of AF4 (50 mg/kg) Prior to 50 minutes of HI was Insufficient to Reduce Infarct Volume Resulting From HI

C57Bl/6 mice (6-8 weeks old) received a single dose (p.o.) of AF4 (50 mg/kg) or vehicle (10 mL/kg). Twenty-four hours after dosing, all mice were subjected to 50 minutes of HI and sacrificed 24 hours later. Coronal brain sections were stained with 2,3,4-triphenyltetrazolium chloride (TTC) and a ratio of ipsilateral viable tissue over contralateral viable tissue was determined to yield the relative amount of viable tissue following HI injury (Figure 14). The mean ± SEM indices of relative viable tissue were 0.55 ± 0.06 for the vehicle treatment group and 0.61 ± 0.05 for the AF4 treatment group. A one-sided student's t-test revealed no difference between treatment groups ($t(40)=0.6747$; $p=0.2519$). These results demonstrated that pretreatment with a single dose of AF4 (50 mg/kg, p.o.) prior to 50 minutes of HI did not reduce neuronal cell loss following HI by comparison to the vehicle treated group (10 mL/kg, p.o.).

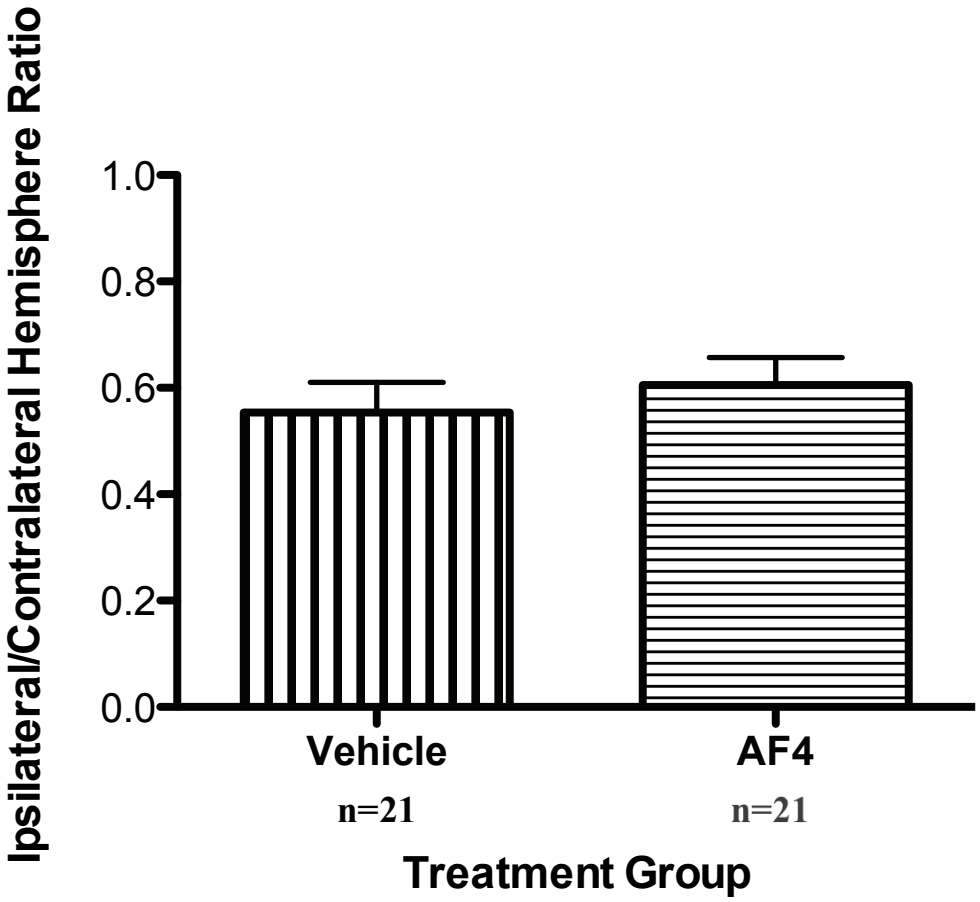


Figure 14: Relative amount of viable tissue following HI injury in C57Bl/6 mice dosed orally with AF4 (50 mg/kg) (n=21) or vehicle (10 mL/kg) (n=21) 1 day prior to 50 minutes of HI. Brain tissue was stained with TTC to measure the infarct volume following HI by determining the ratio of ipsilateral viable tissue over contralateral viable tissue. Administration of a single dose of AF4 (50 mg/kg) 24 hours prior to HI did not reduce infract volume caused by HI in comparison to the vehicle treatment group. No statistical difference was found between treatment groups using a one-sided student's t-test ($t(40)=0.6747$; $p=0.2519$).

3.6 Dose-Dependent Reduction in HI-Induced Hippocampal and Striatal Neuronal Cell Loss by Pretreatment with 3 Doses of AF4

Five groups, composed of 7-9 adult male C57Bl/6 mice were dosed orally once a day with vehicle (10 mL/kg) or AF4 (5, 10, 25 or 50 mg/kg) for 3 consecutive days. All animals received 50 minutes of HI 24 hours after the last administration of water or AF4 and sacrificed 2 weeks following HI. Neuronal cell loss in the striatum and hippocampus was assessed to determine the protective effects of pretreatment with increasing dosage of AF4 against HI-induced damage relative to vehicle treated animals. The number of NeuN positive cells in the striatum approximately 0.1 mm anterior to bregma was counted in both the ipsilateral and contralateral hemisphere, with respect to carotid cauterization, to calculate a cell survival ratio (Figure 15). The mean ± SEM cell survival ratios were 0.17 ± 0.04 for the vehicle treatment group, 0.20 ± 0.03 for the 5 mg/kg AF4 treatment group, 0.60 ± 0.07 for the 10 mg/kg AF4 treatment group, 0.66 ± 0.14 for the 25 mg/kg AF4 treatment group, and 0.60 ± 0.12 for the 50 mg/kg AF4 treatment group. A one-way ANOVA revealed a significant difference between the vehicle treatment group and the 10 mg/kg, 25 mg/kg and 50 mg/kg AF4 treatment groups ($F(5,32)= 6.264$; $p= 0.0011$). A one-way ANOVA also revealed a significant difference between the 5 mg/kg AF4 treatment group and the 10 mg/kg, 25 mg/kg and 50 mg/kg AF4 treatment groups ($F(5,32)= 6.264$; $p= 0.0011$). No significant difference was found between the vehicle treatment group and the 5 mg/kg AF4 treatment group. In the hippocampus, the area of positive NeuN labeling was measured in both the ipsilateral and contralateral hemisphere at a level approximately 1.8 mm posterior to bregma to yield an index of neuronal loss (Figure 17). The mean ± SEM cell survival ratios were 0.35 ± 0.09 for the vehicle

treatment group, 0.34 ± 0.02 for the 5 mg/kg AF4 treatment group, 0.62 ± 0.09 for the 10 mg/kg AF4 treatment group, 0.73 ± 0.09 for the 25 mg/kg AF4 treatment group, and 0.68 ± 0.10 for the 50 mg/kg AF4 treatment group. A one-way ANOVA revealed a significant difference between the vehicle treatment group and the 25 mg/kg and 50 mg/kg AF4 treatment groups ($F(5,35)= 5.083$; $p= 0.0030$). A one-way ANOVA also revealed a significant difference between the 5 mg/kg AF4 treatment group and the 25 mg/kg and 50 mg/kg AF4 treatment groups ($F(5,32)= 5.083$ $p= 0.0030$). No significant difference was found between the vehicle treatment group and the 5 mg/kg or 10 mg/kg AF4 treatment groups. These data suggest that pretreatment with 3 doses of AF4 (25 mg/kg/day, p.o.) prior to 50 minutes of HI is neuroprotective against neuronal cell loss in the hippocampus and the striatum following HI in comparison to the vehicle treated group (10 mL/kg/day, p.o.). Figures 16 and 18 show representative photomicrographs of the striatum and dorsal hippocampus, respectively; for vehicle (10 mL/kg/day, p.o.) treated mice and AF4 (5, 10, 25 or 50 mg/kg; p.o.) treated mice subjected to 50 minutes of HI.

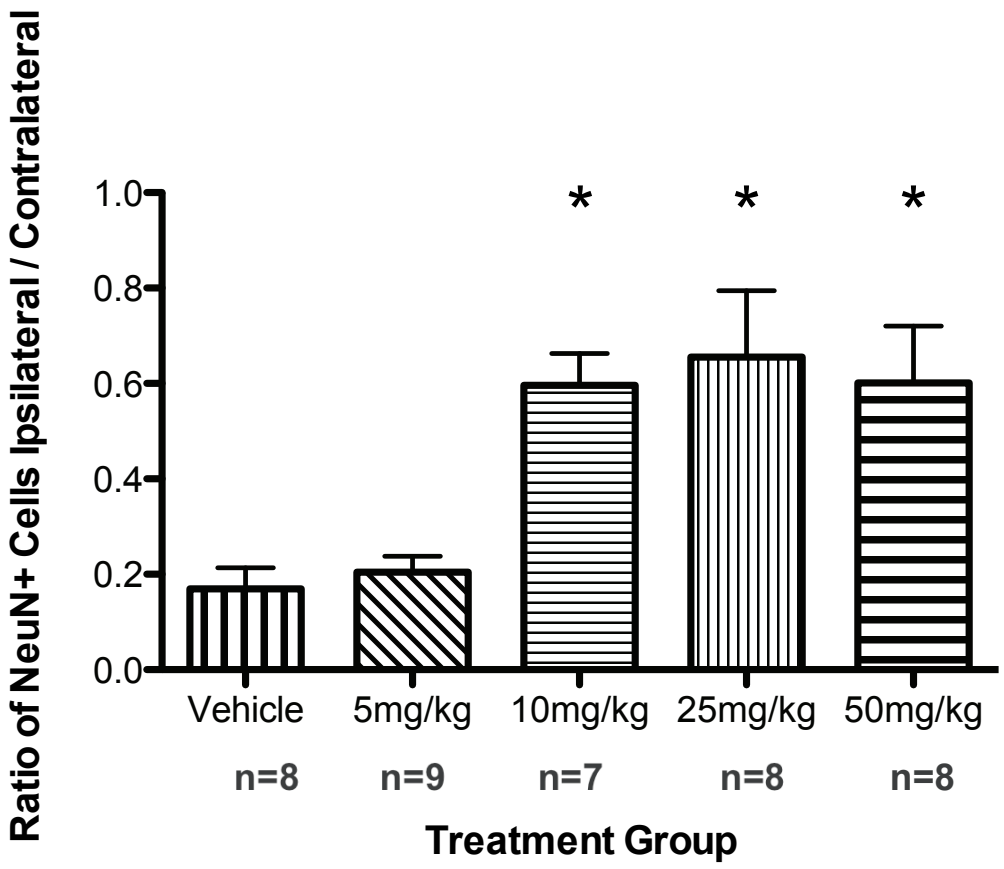
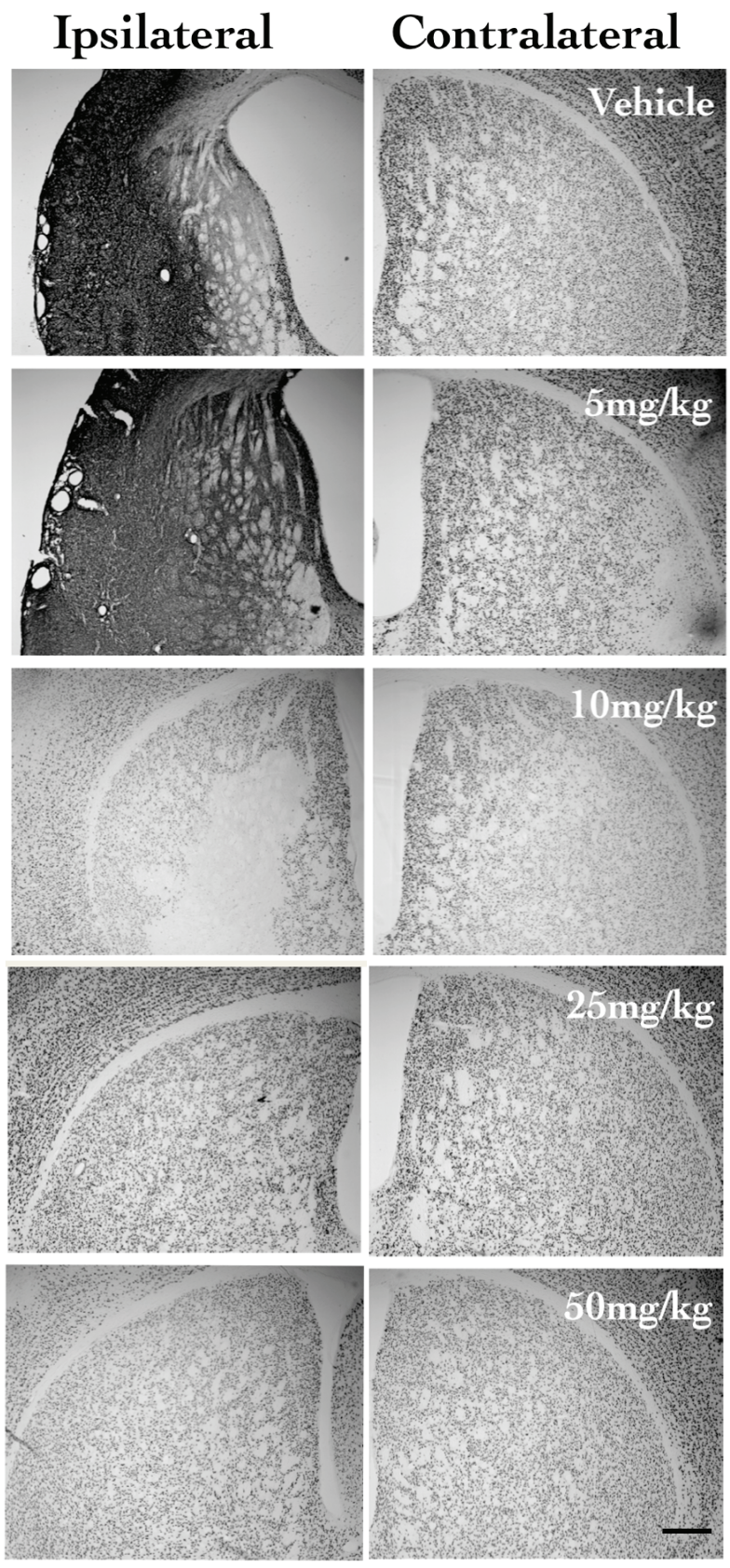


Figure 15: Neuronal survival ratios for the striatum of C57Bl/6 mice treated with either vehicle (10 mL/kg; p.o.) or increasing doses of AF4 (5, 10, 25 or 50 mg/kg; p.o.) administered once a day for 3 days prior to 50 minutes of HI. AF4 produced a dose dependent reduction in striatal cell loss. *P< 0.05 Relative to vehicle or 5 mg/kg AF4 groups. One way ANOVA followed by Newman-Keuls post-hoc test.

Figure 16: Representative photomicrographs of the ipsilateral and contralateral striatum stained with an antibody against the neuron specific marker NeuN to visualize viable neurons of C57Bl/6 mice subjected to 50 minutes of HI that were treated orally with vehicle (10 mL/kg/day for 3 days) or increasing doses of the AF4 (5, 10, 25 or 50 mg/kg/day for 3 days). Administration of AF4 at a dose of 5 mg/kg did not reduce the damaging effects of HI in the ipsilateral striatum. Administration of the 10 mg/kg dose of AF4 partially protected the ipsilateral striatum against brain injury caused by HI. Administration of 25 mg/kg or 50 mg/kg AF4 was protective against HI-induced striatal injury. The scale bar in the bottom right panel represents 50 μm.


Ipsilateral
Contralateral
Vehicle
5mg/kg
10mg/kg
25mg/kg
50mg/kg

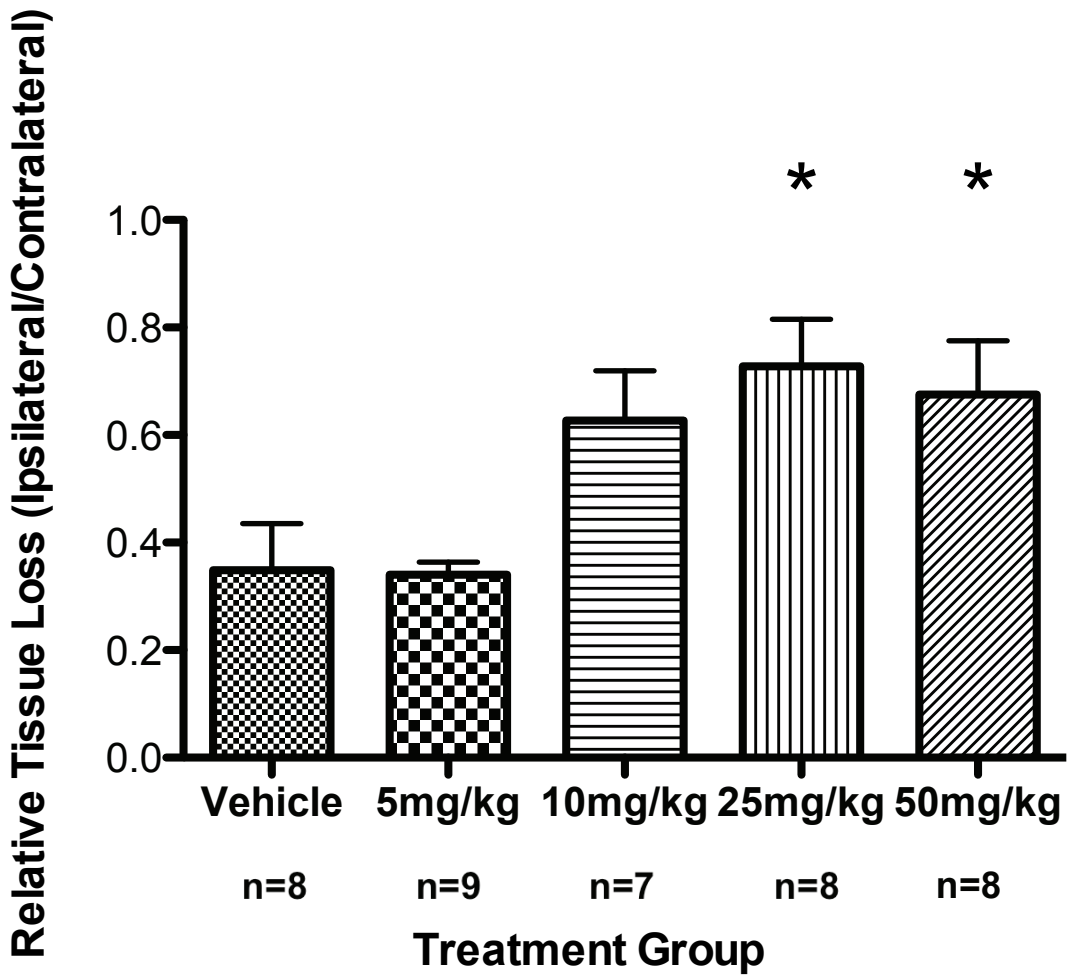
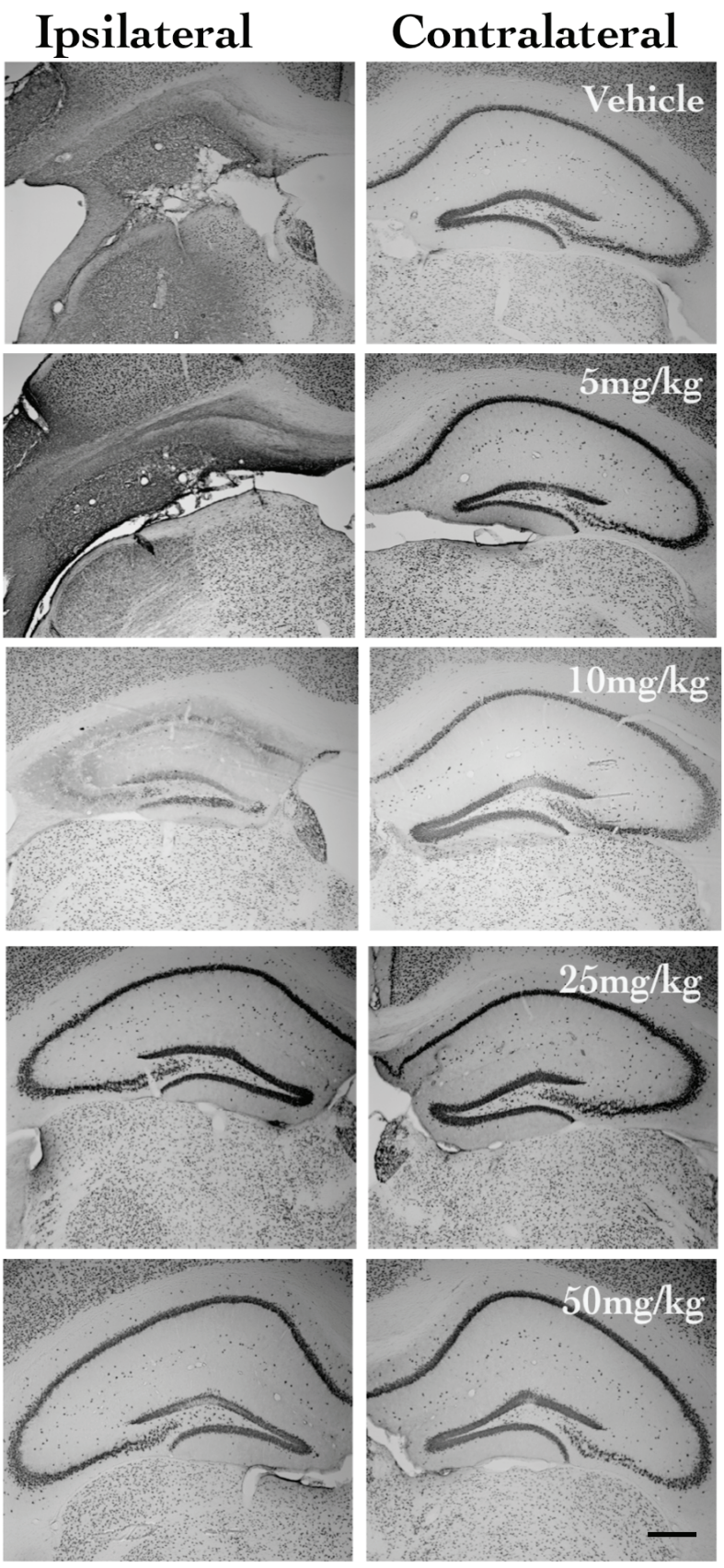


Figure 17: Neuronal survival ratios for the hippocampus of C57Bl/6 mice treated with either vehicle (10 mL/kg; p.o.) or increasing doses of AF4 (5, 10, 25 or 50 mg/kg; p.o.) once a day for 3 days prior to 50 minutes of HI. AF4 produced a dose dependent reduction in hippocampal cell loss. *P< 0.05 Relative to vehicle and 5 mg/kg AF4 groups. One wave ANOVA followed by Newman-Keuls post-hoc test.

Figure 18: Representative photomicrographs of the ipsilateral and contralateral hippocampus stained with an antibody against the neuron specific marker NeuN to visualize viable neurons of C57Bl/6 mice exposed to 50 minutes of HI and treated orally with vehicle (10 mg/kg/day for 3 days) or increasing doses of the AF4 (5, 10, 25 or 50 mg/kg/day for 3 days). Administration of AF4 at a dose of 5 mg/kg or 10 mg/kg did not reduce the damaging effects of HI in the ipsilateral hippocampus. Administration of 25 mg/kg or 50 mg/kg AF4 was reduced HI-induced hippocampal injury HI. The scale bar in the bottom right panel represents 50 μm.


Ipsilateral
Contralateral
Vehicle
5mg/kg
10mg/kg
25mg/kg
50mg/kg

3.7 Reduction in HI-Induced Hippocampal and Striatal Neuronal Cell Loss Produced by Increasing the Number of Pretreatments with AF4 (25 mg/kg, p.o.)

Four groups, composed of 4-9 adult male C57Bl/6 mice each, were dosed orally once a day with vehicle (10 mL/kg/day) or AF4 (25 mg/kg/day) for 1, 3, or 7 days. Twenty-four hours after the final dose of AF4 or vehicle all animals were subjected to 50 minutes of HI and sacrificed 2 weeks later. Neuronal cell loss in the striatum and hippocampus was assessed to determine effects of increasing the number of pretreatment doses of AF4 (25 mg/kg) on HI-induced neuronal cell loss in comparison to the vehicle treatment group. The number of NeuN positive cells in the striatum approximately 0.1 mm anterior to bregma was counted in both the ipsilateral and contralateral hemisphere with respect to carotid cauterization to calculate a cell survival ratio (Figures 19, 21 and 23). The mean ± SEM cell survival ratios were 0.60 ± 0.17 for the vehicle treatment group (n=7) and 0.49 ± 0.14 for the AF4 treatment group (n=9) for mice that received a single pretreatment. A one-sided student's t-test determined no significant difference between treatment groups ($t(14)=0.4865$; $p=0.3171$) after 1 day of dosing pre-HI injury. The mean ± SEM cell survival ratios were 0.19 ± 0.08 for the vehicle treatment group (n=10) and 0.69 ± 0.10 for the AF4 treatment group (n=9) for mice dosed once a day for 3 days. A one-sided student's t-test determined a significant difference between treatment groups ($t(17)=4.113$; $p=0.0004$) after 3 days of pretreatment with AF4 prior to HI injury. The mean ± SEM cell survival ratios were 0.21 ± 0.07 for the vehicle treatment group (n=5) and 0.69 ± 0.17 for the AF4 treatment group (n=5) for mice dosed once a day for 7 days. A one-sided student's t-test determined a significant difference between treatment groups ($t(8)=2.539$; $p=0.0174$) after 7 days of pretreatment with AF4 prior to HI injury.

In the hippocampus, the area of positive NeuN labeling was measured in both the ipsilateral and contralateral hemisphere at approximately 1.8 mm posterior to bregma to yield an index of neuronal loss (Figures 25, 27 and 29). The mean ± SEM cell survival ratios were 0.73 ± 0.10 for the vehicle treatment group (n=7) and 0.64 ± 0.09 for the AF4 treatment group (n=7) for mice dosed for 1 day. A one-sided student's t-test determined no significant difference between treatment groups (t(12)=0.7141; p=0.2444) after 1 day of dosing before HI. The mean ± SEM cell survival ratios were 0.45 ± 0.08 for the vehicle treatment group (n=11) and 0.70 ± 0.07 for the AF4 treatment group (n=11) for mice dosed once a day for 3 days. A one-sided student's t-test determined a significant difference between treatment groups (t(20)=2.334; p=0.0.151) after 3 days of pretreatment with AF4 (25 mg/kg/day, p.o.) prior to HI injury. The mean ± SEM cell survival ratios were 0.35 ± 0.14 for the vehicle treatment group (n=6) and 0.79 ± 0.09 for the AF4 treatment group (n=5) for mice dosed once a day for 7 days. A one-sided student's t-test revealed a significant difference between treatment groups (t(9)=2.590; p=0.0146) after 7 days of pretreatment with AF4 prior to HI injury. Although three doses of AF4 produced a reduction of HI-induced hippocampal atrophy but some neuronal loss was still apparent, however, seven doses of AF4 (25 mg/kg/day, p.o.) prevented the loss of hippocampal neurons. Taken together, these results suggest that pretreatment with at least 7 doses of AF4 (25 mg/kg/day, p.o.) prior to 50 minutes of HI is necessary to produce optimal protection against neuronal cell loss in both the striatum and hippocampus following HI, in comparison to the vehicle treated group (10 mL/kg/day). Figures 20, 22, and 24 show representative photomicrographs of the striatum of vehicle and AF4 treated mice following 1, 3, and 7 days of dosing, respectively, prior to 50

minutes of HI. Figures 26, 28, and 30 show representative coronal sections of the hippocampus for vehicle and AF4 treated mice that received 1, 3, and 7 days, respectively, of dosing prior to 50 minutes of HI. See Figure 31 for representative photomicrographs of the striatum and hippocampus of vehicle and AF4 treated mice following 1, 3, and 7 days of dosing prior to 50 minutes of HI subjected to a regressive haematoxylin eosin stain.

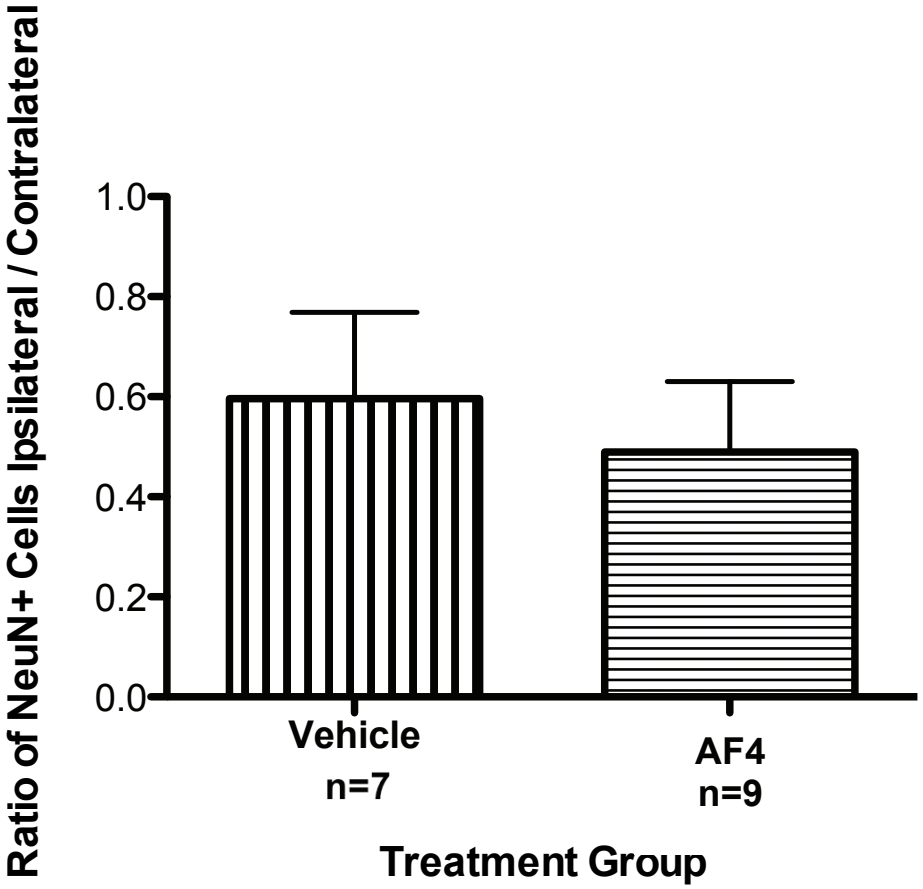


Figure 19: A single predose of AF4 (25 mg/kg, p.o.) failed to reduce neuronal cell loss in the ipsilateral striatum after HI. This graph depicts neuronal survival ratios, determined by the ratio of the number of NeuN positive cells in the ipsilateral striatum divided by the number of NeuN positive cells in the contralateral striatum. No statistical difference was found between treatment groups using a one-sided student's t-test ($t(14)=0.4865$; $p=0.3171$).

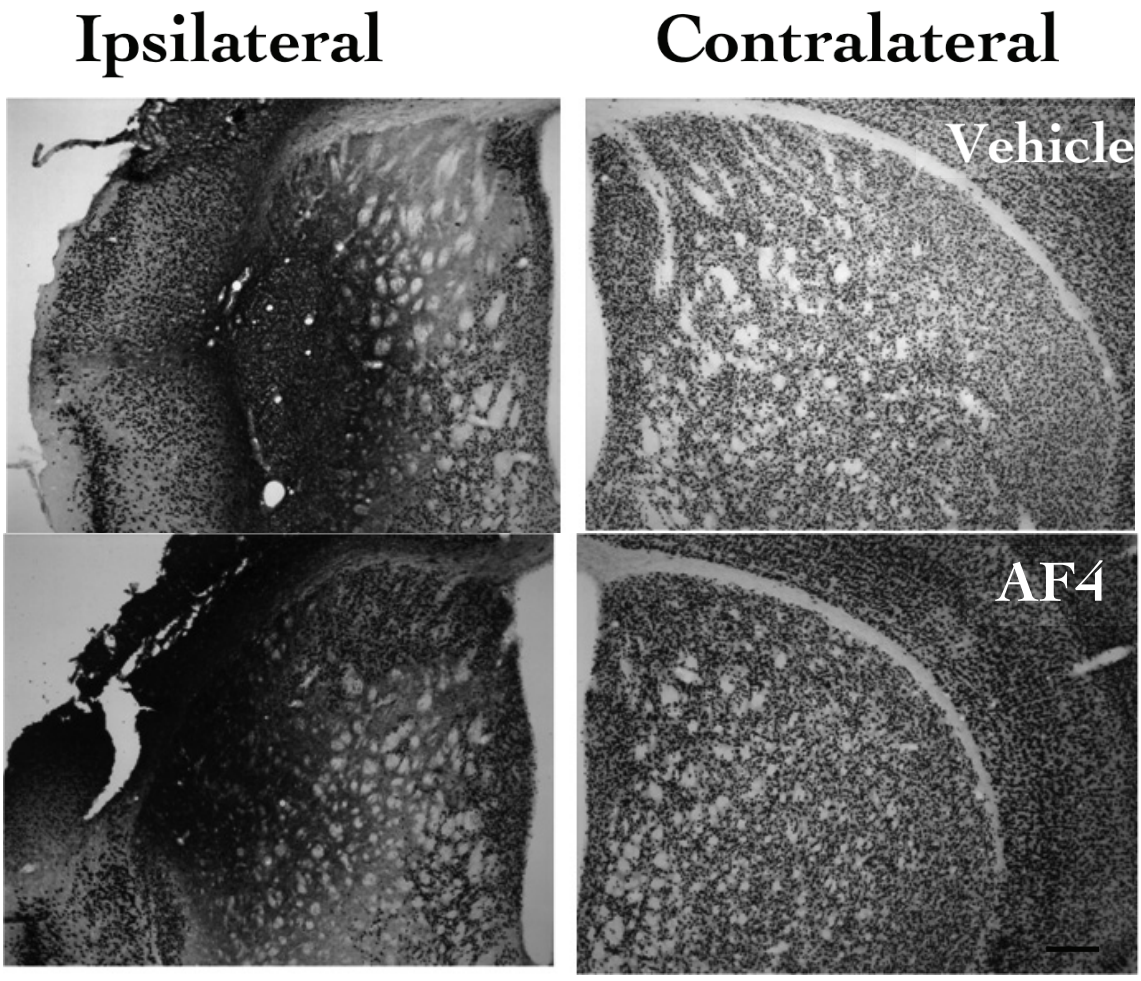


Figure 20: Representative photomicrographs of the ipsilateral and contralateral striatum of C57Bl/6 mice dosed orally with AF4 (25 mg/kg) or vehicle (10 mL/kg) for 1 day prior to 50 minutes of HI. Administration of a single dose of AF4 (25 mg/kg) 24 hours prior to HI did not reduce the damaging effects of HI in the ipsilateral striatum. This figure shows coronal brain sections cut at the level of 1.0 mm anterior to bregma and stained with an antibody that recognizes the neuron specific protein NeuN. These figures are representative of the results obtained from 7-9 animals per group. The scale bar in the bottom right panel represents 50 μm.

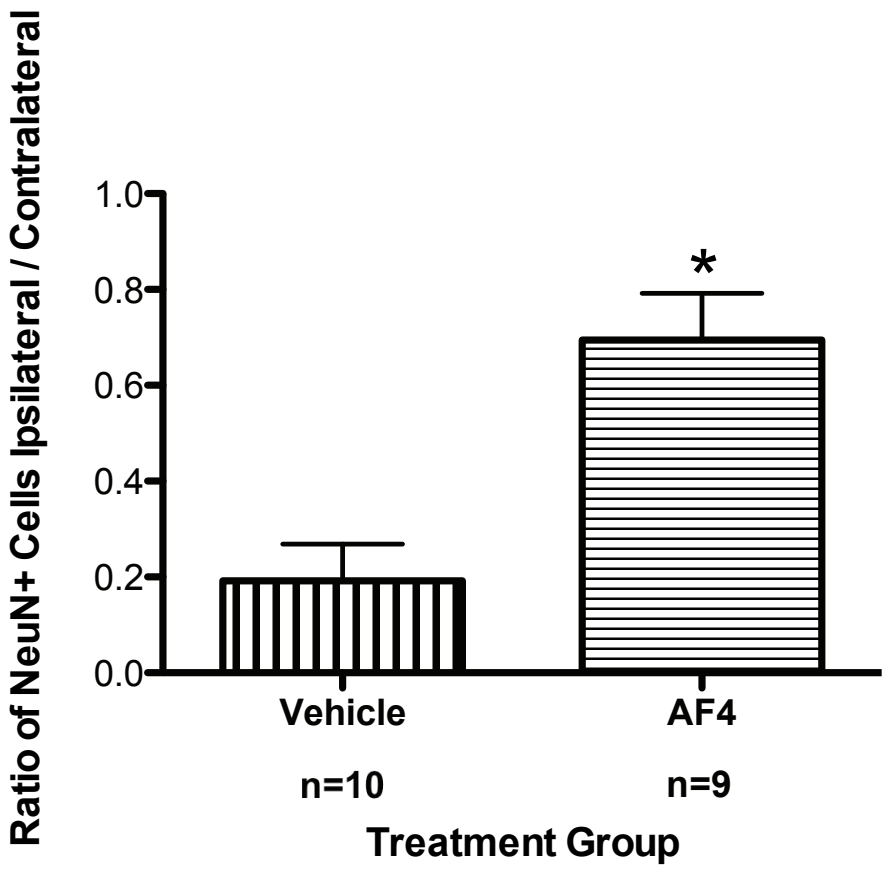


Figure 21: Three pre-doses of AF4 (25 mg/kg/day for 3 days; p.o.) reduced neuronal loss in the ipsilateral striatum after HI. This graph depicts neuronal survival ratios, determined by the ratio of the number of NeuN positive cells in the ipsilateral striatum divided by the number of NeuN positive cells in the contralateral striatum. A one-sided student's t-test revealed a significant difference between treatment groups ($t(17)=4.113$; $p=0.0004$). *$P< 0.05$

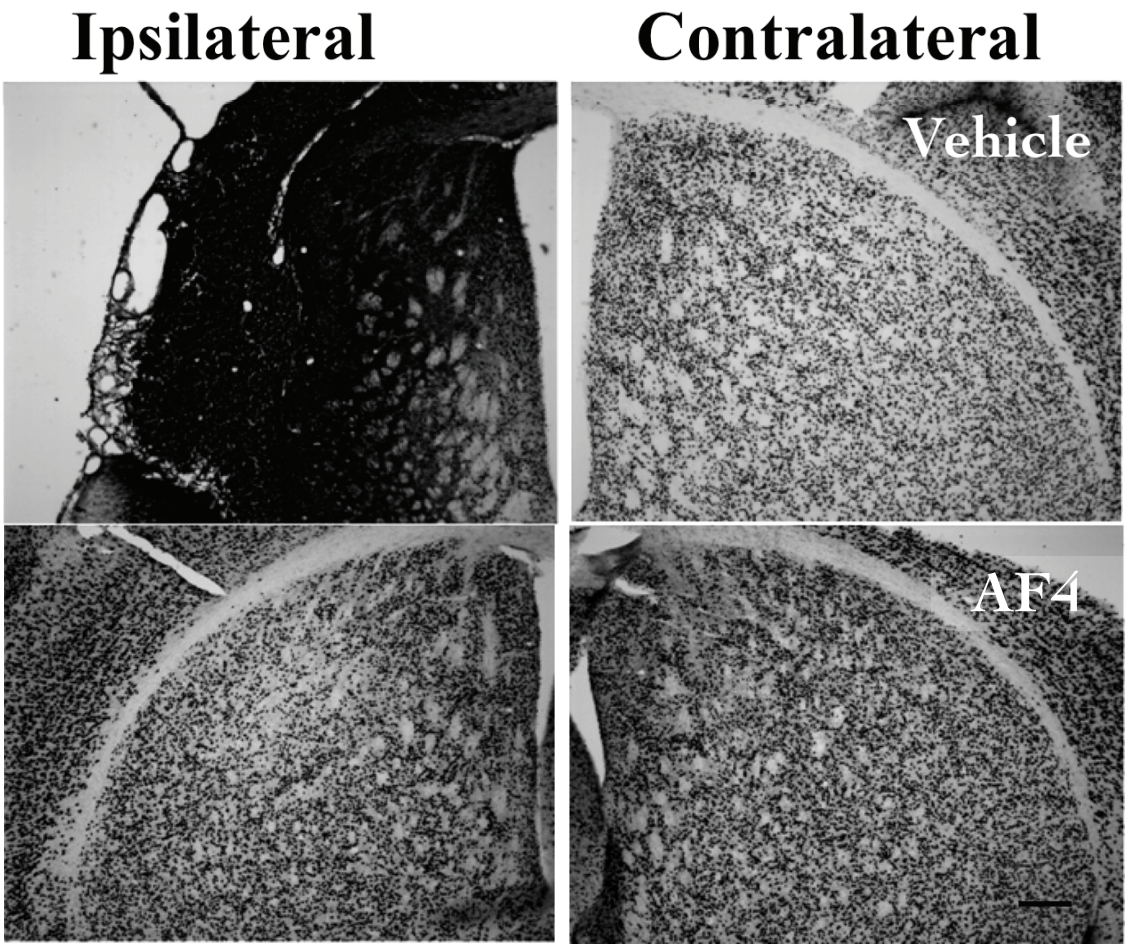


Figure 22: Representative photomicrographs of the ipsilateral and contralateral striatum of C57Bl/6 mice dosed orally with AF4 (25 mg/kg/day) or vehicle (10 mL/kg/day) once a day for 3 consecutive days prior to 50 minutes of HI. Administration of 3 doses of AF4 (25 mg/kg/day) prior to HI produced near complete protection against HI-induced injury in the ipsilateral striatum. This figure shows coronal brain sections cut at 1.0 mm anterior to bregma and stained with an antibody that recognizes the neuron specific protein NeuN. These figures are representative of the results obtained from 11 animals per group. The scale bar in the bottom right panel represents 50 μm.

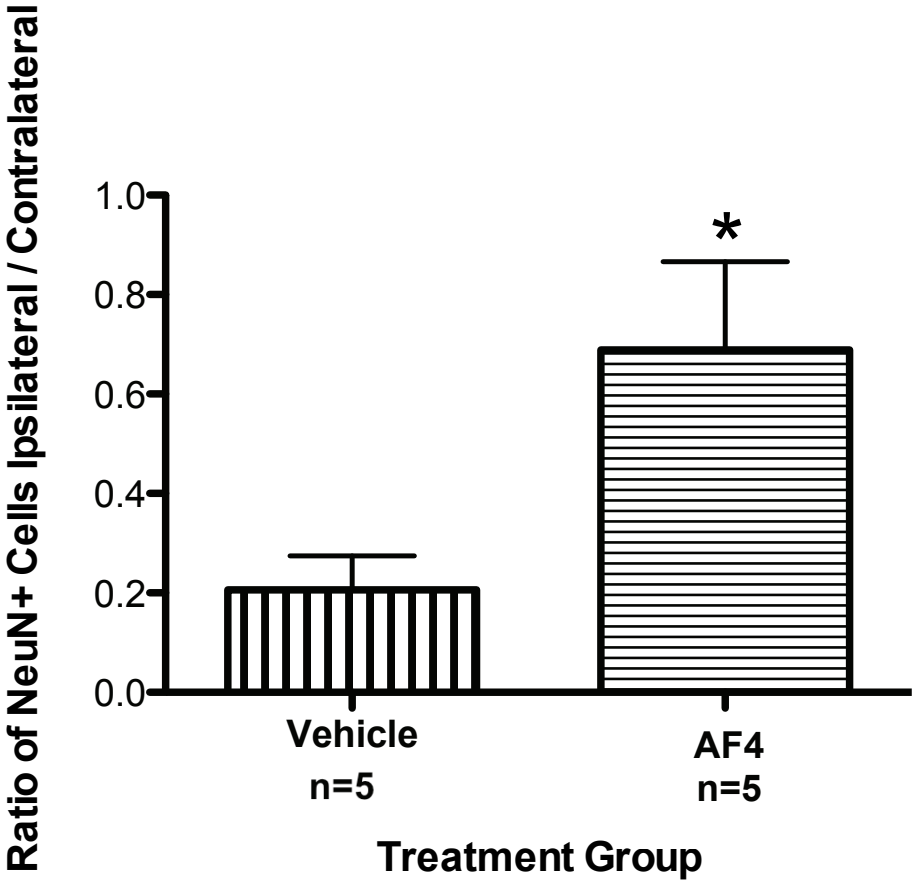


Figure 23: Seven predoses of AF4 (25 mg/kg/day, p.o.) reduced neuronal loss in the ipsilateral striatum after HI. This graph depicts neuronal survival ratios, determined by the ratio of the number of NeuN positive cells in the ipsilateral striatum divided by the number of NeuN positive cells in the contralateral striatum. A one-sided student's t-test determined a significant difference between treatment groups ($t(8)=2.539$; $p=0.0174$). *$P< 0.05$

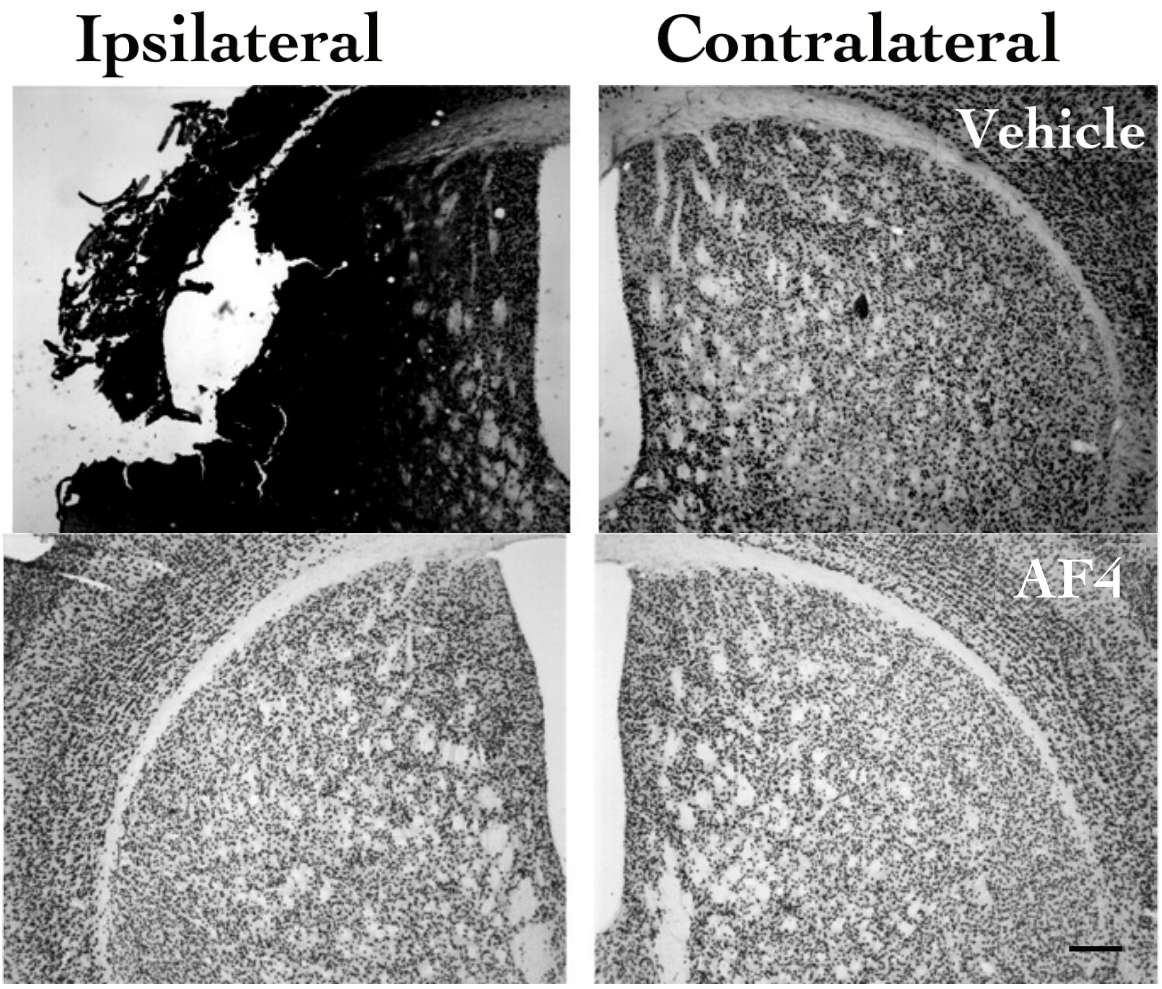


Figure 24: Representative photomicrographs of the ipsilateral and contralateral striatum of C57Bl/6 mice dosed orally with AF4 (25 mg/kg) or vehicle (10 mL/kg) once a day for 7 consecutive days prior to 50 minutes of HI. Administration of 7 doses of AF4 (25 mg/kg)/day prior to HI produced a near complete protection against HI-induced injury in the ipsilateral striatum. This figure shows coronal brain sections cut 1.0 mm anterior to bregma and stained with an antibody that recognizes the neuron specific protein NeuN. These figures are representative of the results obtained from 5-6 animals per group. The scale bar in the bottom right panel represents 50 μm.

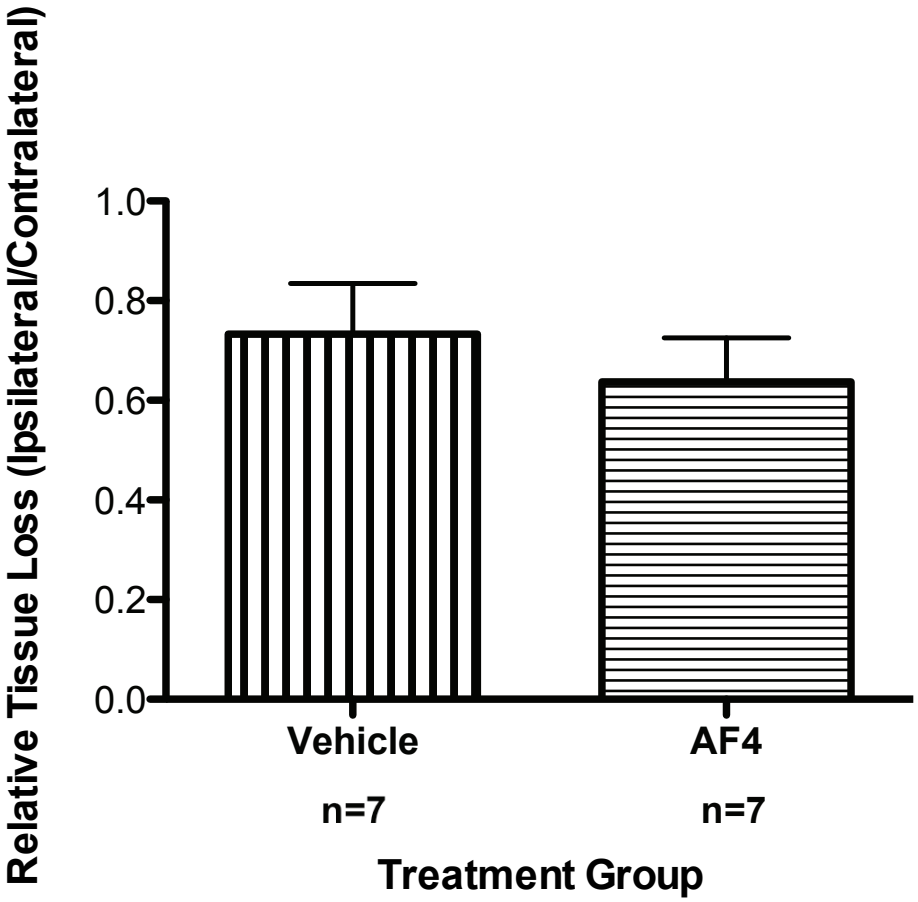


Figure 25: A single predose of AF4 (25 mg/kg, p.o.) failed to prevent hippocampal injury in the ipsilateral hemisphere after HI. This graph depicts neuronal survival ratios, determined by the ratio of the number of NeuN positive cells in the ipsilateral hippocampus divided by the number of NeuN positive cells in the contralateral hippocampus. No statistical difference was found between treatment groups using a one-sided student's t-test ($t(12)=0.7331$; $p=0.2444$).

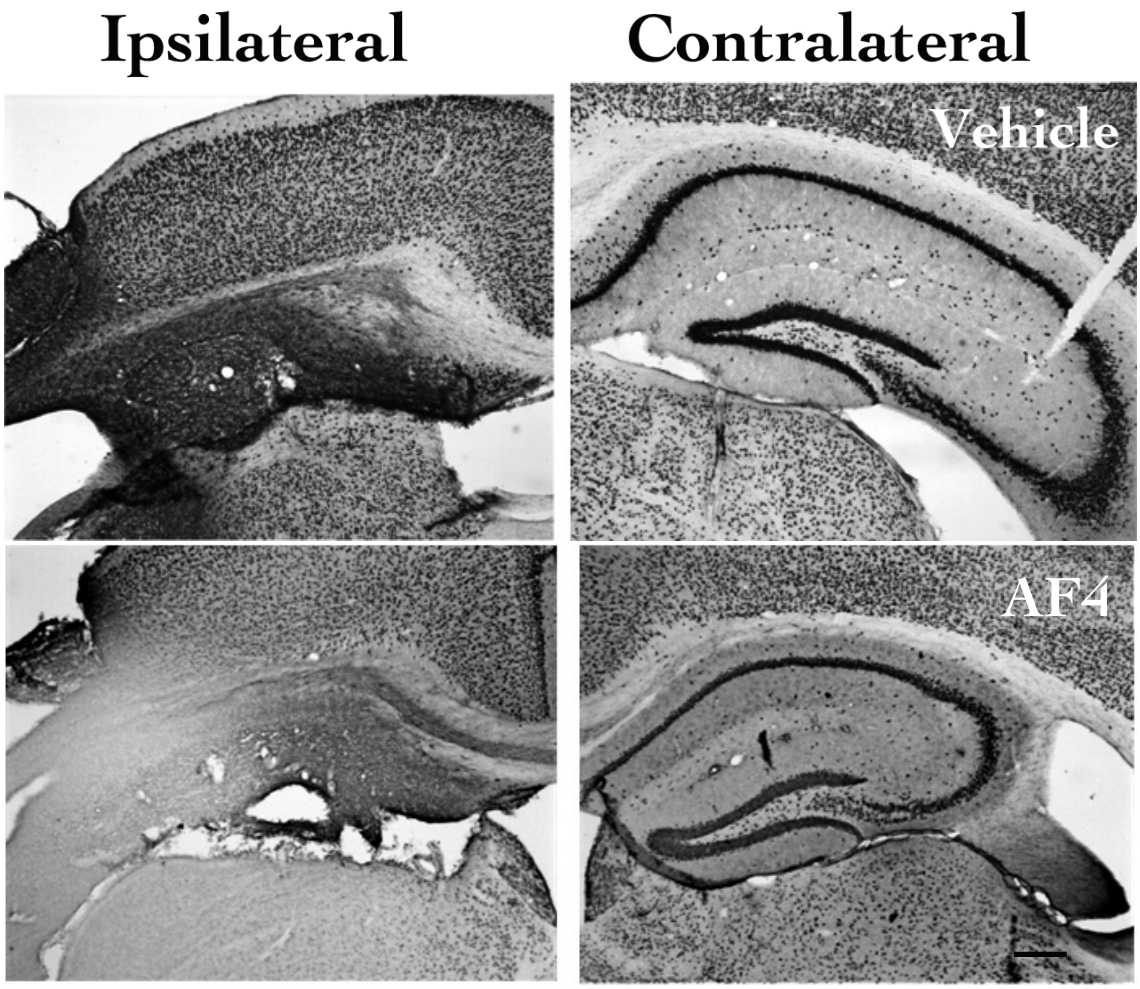


Figure 26: Representative photomicrographs of the ipsilateral and contralateral hippocampus of C57Bl/6 mice dosed orally with AF4 (25 mg/kg) or vehicle (10 mL/kg) for 1 day prior to 50 minutes of HI. Administration of a single dose of AF4 (25 mg/kg) 24 hours prior to HI did not reduce the damaging effects of HI in the ipsilateral hippocampus. This figure shows coronal brain sections cut at 1.8 mm posterior to bregma and stained with an antibody that recognizes the neuron specific protein NeuN. These figures are representative of the results obtained from 7-9 animals per group. The scale bar in the bottom right panel represents 50 μm.

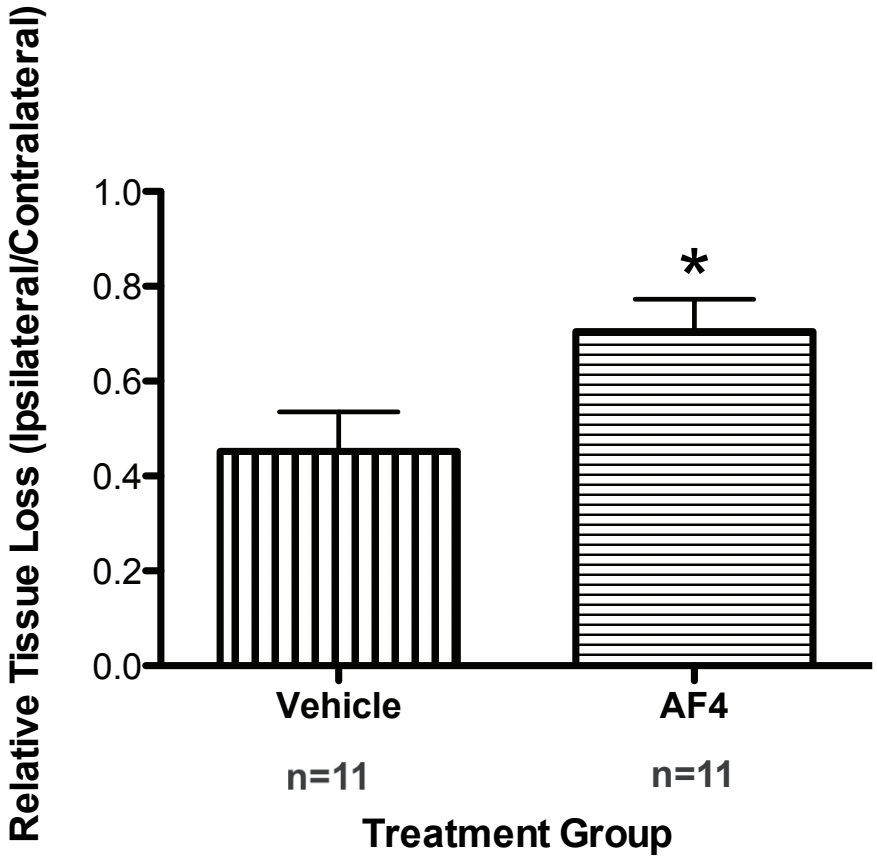


Figure 27: Three predoses of AF4 (25 mg/kg, p.o.) reduced hippocampal injury in the ipsilateral hemisphere after HI. This graph depicts neuronal survival ratios, determined by the ratio of the number of NeuN positive cells in the ipsilateral hippocampus divided by the number of NeuN positive cells in the contralateral hippocampus. A one-sided student's t-test determined a significant difference between treatment groups ($t(20)=2.334$ $p=0.0151$). *$P< 0.05$

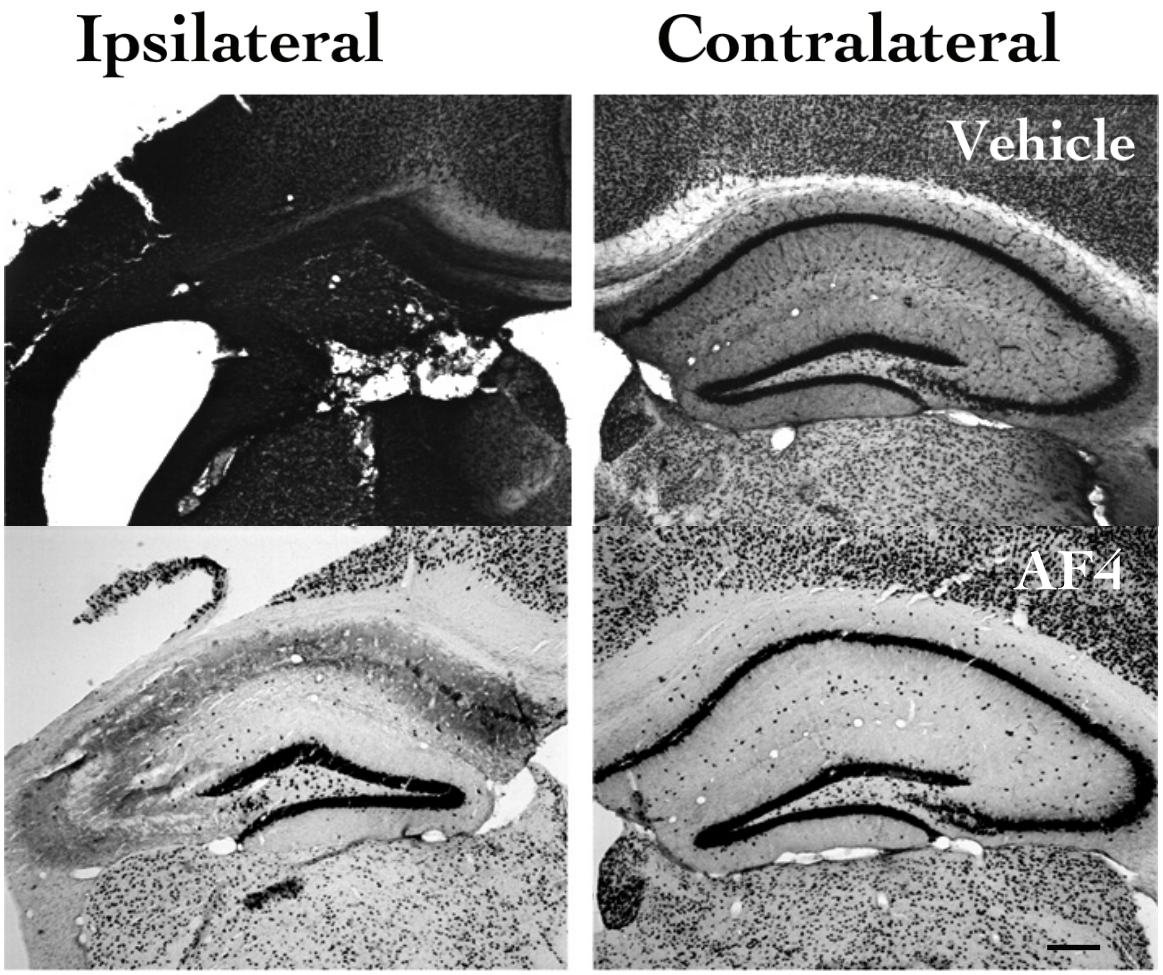


Figure 28: Representative photomicrographs of the ipsilateral and contralateral hippocampus of C57Bl/6 mice dosed orally with AF4 (25 mg/kg) or vehicle (10 mL/kg) once a day for 3 consecutive days prior to 50 minutes of HI. Administration of 3 doses of AF4 (25 mg/kg/day, p.o.) prior to HI showed protection against HI-induced injury in the ipsilateral hippocampus. This figure shows coronal brain sections cut at 1.8 mm posterior to bregma and stained with an antibody that recognizes the neuron specific protein NeuN. These figures are representative of the results obtained from 11 animals per group. The scale bar in the bottom right panel represents 50 μm.

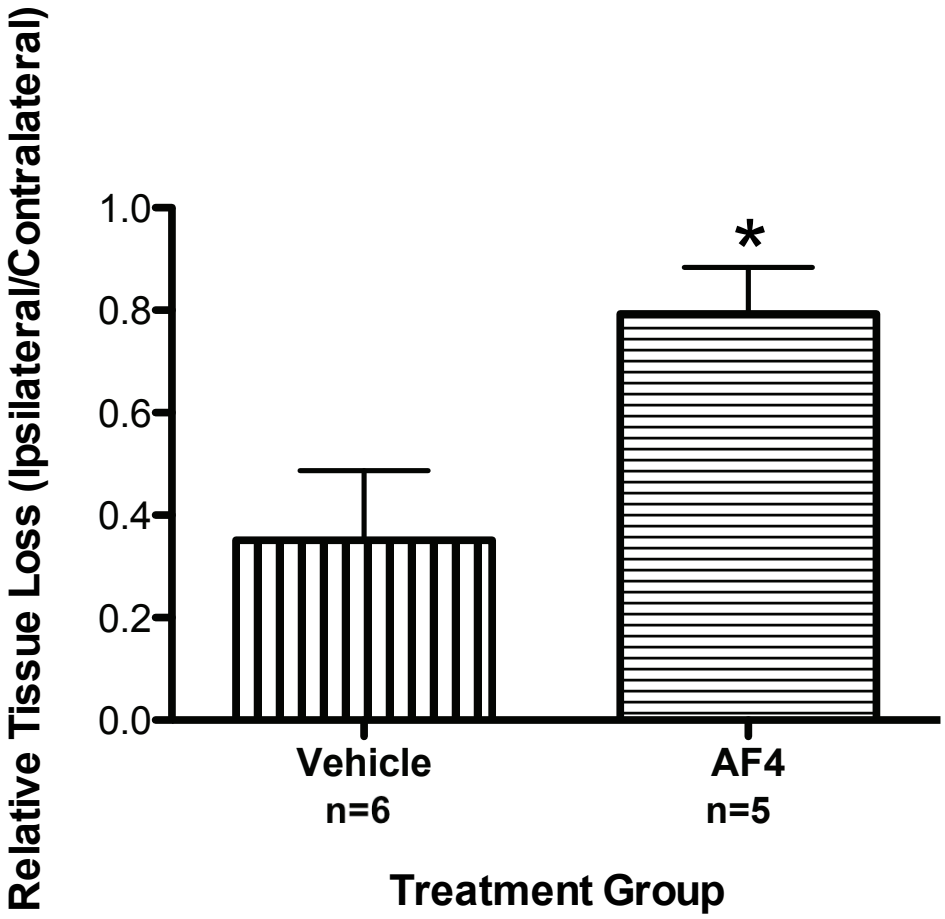


Figure 29: Seven predoses of AF4 (25 mg/kg, p.o.) reduced hippocampal injury in the ipsilateral hemisphere after HI. This graph depicts neuronal survival ratios, determined by the ratio of the number of NeuN positive cells in the ipsilateral hippocampus divided by the number of NeuN positive cells in the contralateral hippocampus. A one-sided student's t-test determined a significant difference between treatment groups ($t(9)=2.590$; $p=0.0146$). *$P< 0.05$

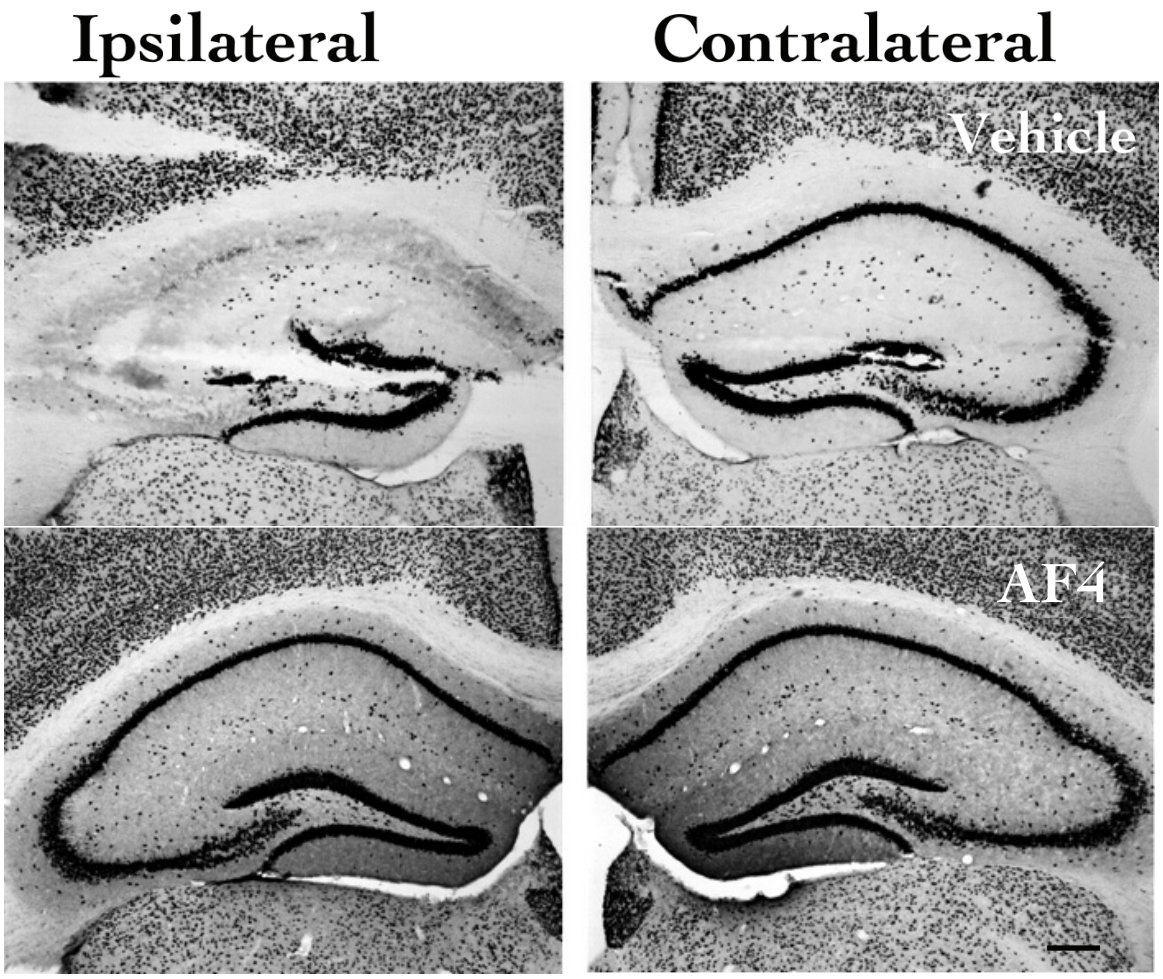
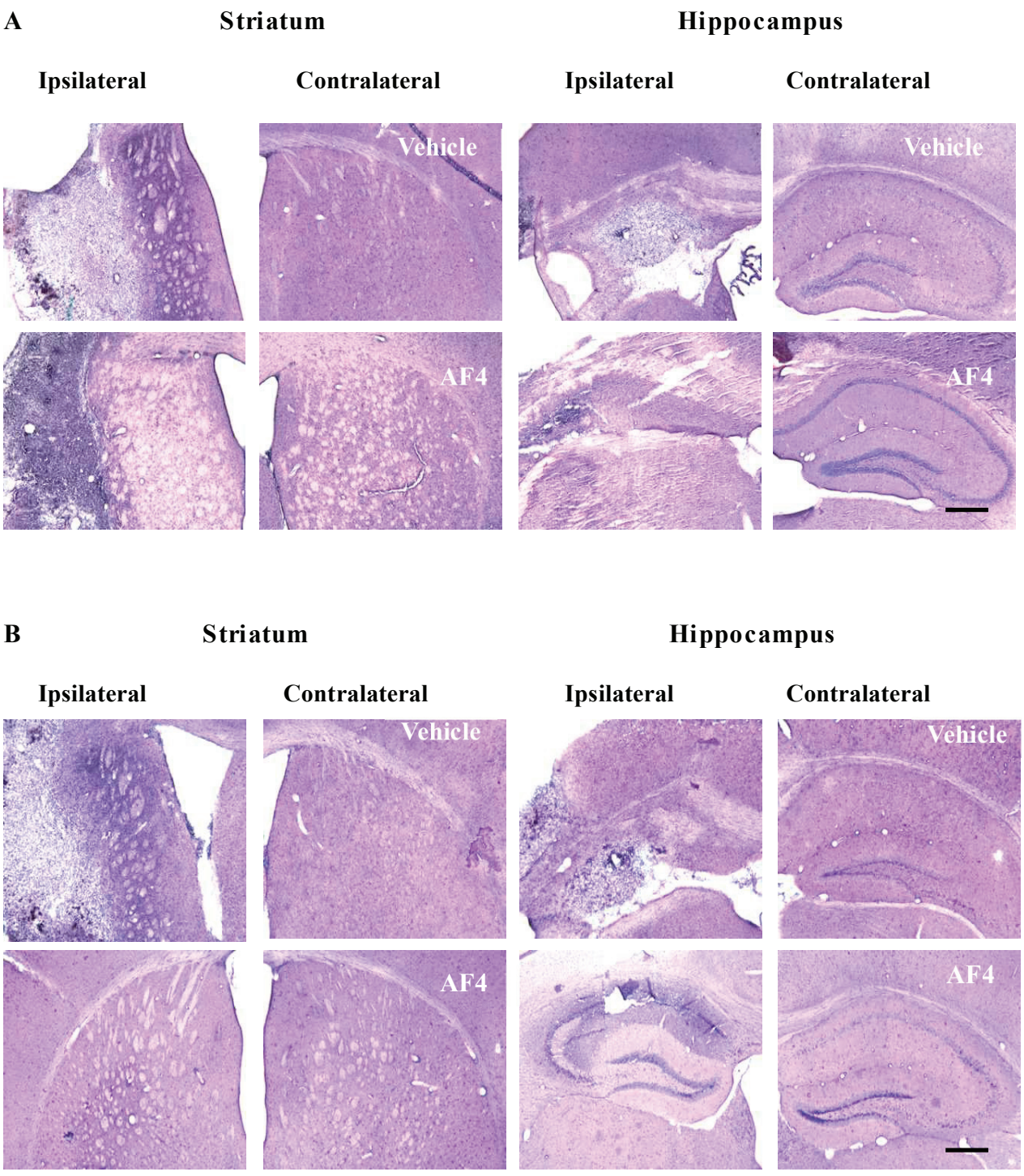


Figure 30: Representative photomicrographs of the ipsilateral and contralateral hippocampus of C57Bl/6 mice dosed orally with AF4 (25 mg/kg) or vehicle (10 mL/kg) once a day for 7 consecutive days prior to 50 minutes of HI. Administration of 7 doses of AF4 (25 mg/kg/day, p.o.) prior to HI produced a near complete protection against HI-induced injury in the ipsilateral hippocampus. This figure shows coronal brain sections cut at 1.8 mm posterior to bregma and stained with an antibody that recognizes the neuron specific protein NeuN. These figures are representative of the results obtained from 5-6 animals per group. The scale bar in the bottom right panel represents 50 μm.

Figure 31: Representative photomicrographs of the ipsilateral and contralateral striatum and hippocampus of C57Bl/6 mice treated with AF4 (25 mg/kg/day) or vehicle (10 mL/kg/day) for 1, 3 or 7 days prior to HI (Figures 30 A, B and C respectively). The coronal sections were histochemically processed for regressive haematoxylin & eosin staining. This stain utilizes a haematoxylin solution for nuclear staining (blue) and an eosin solution for cytoplasmic staining (pink) to provide an overview of tissue structure. A) Comparable tissue damage in the ipsilateral striatum and hippocampus of mice treated with vehicle (10 mL/kg) or AF4 (25 mg/kg) for 1 day prior to HI injury. B) Reduced tissue damage in the ipsilateral striatum of mice in the vehicle treatment group in comparison to the ipsilateral striatum of mice treated with AF4 (25 mg/kg/day) for 3 days prior to HI injury. C) Reduction in tissue damage in the ipsilateral striatum and hippocampus of mice treated with AF4 (25 mg/kg/day) for 7 days prior to HI injury in comparison to mice treated with vehicle (10 mL/kg/day). The scale bar in the bottom right panel represents 50 μm.


A
Striatum
Hippocampus
Ipsilateral
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Ipsilateral
Contralateral
Vehicle
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B
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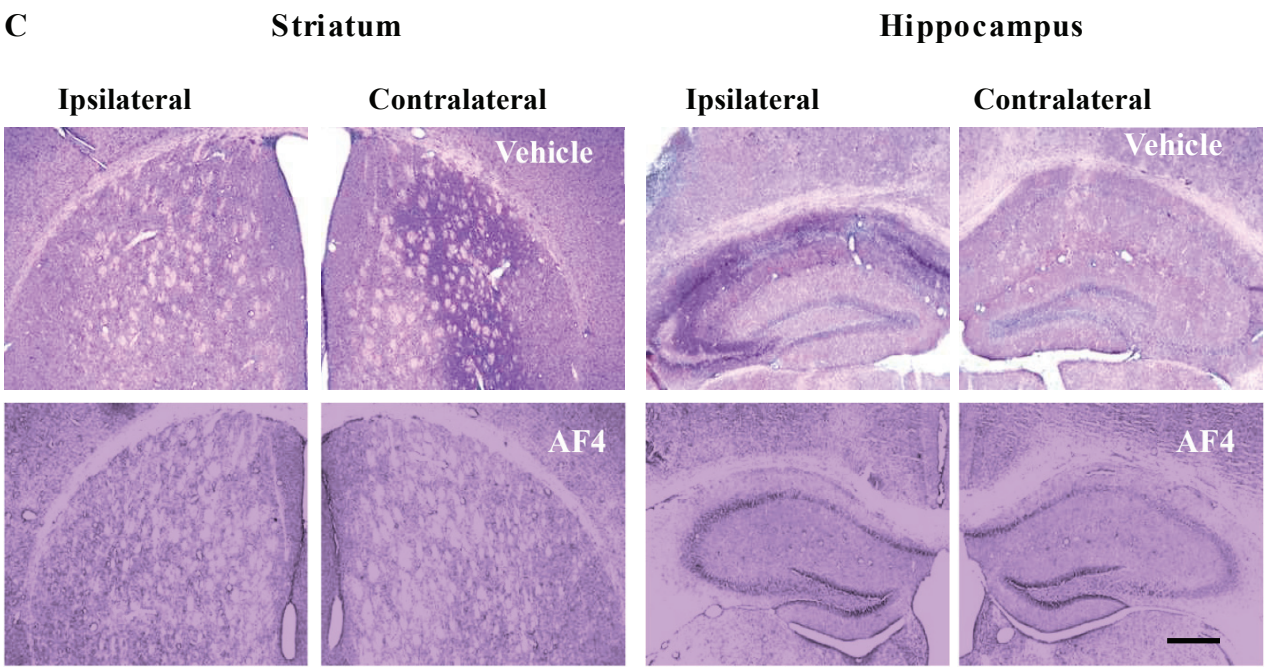
AF4
AF4


C
Striatum
Hippocampus
Ipsilateral
Contralateral
Ipsilateral
Contralateral
Vehicle
Vehicle
AF4
AF4

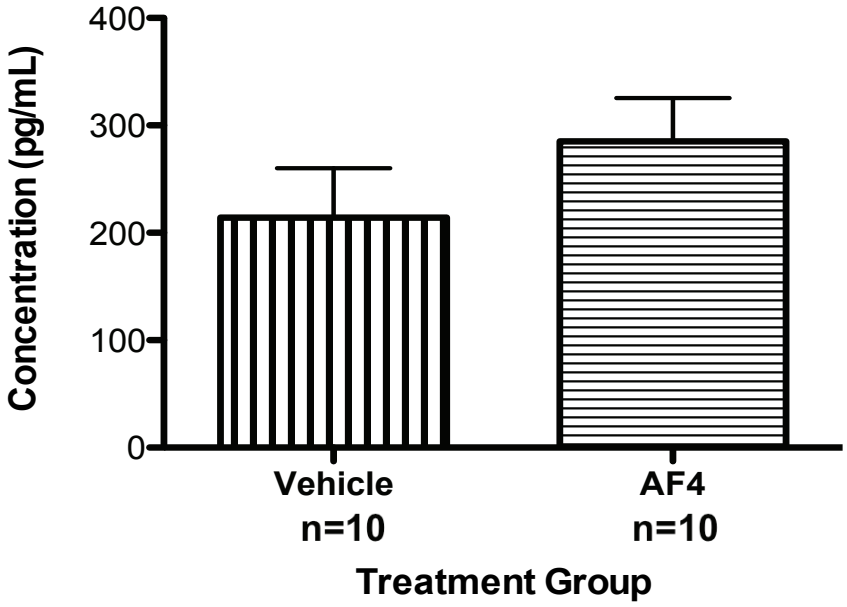
3.8 Oral Administration of AF4 (50 mg/kg/day for 3 days) Reduces Production of the Inflammatory Cytokine Tumour Necrosis Factor Alpha (TNF-α) in LPS-Stimulated Whole Blood From Mice Subjected to HI

Two groups, composed of 15 adult male C57BL/6 mice each, were dosed orally with vehicle (10 mL/kg) or AF4 (50 mg/kg) once a day for 3 consecutive days. Animals from these two treatment groups were subjected to either sham HI surgery (n=10) or 50 minutes of HI (n=5). Twenty-four hours after the last administration of vehicle or AF4, mice in the HI surgery group (n=5) were subjected to 50 minutes of HI, sacrificed 6 hours later and whole blood collected. Mice in the sham surgery group (n=10) were sacrificed 16 hours after the last dose of water or AF4 and whole blood collected. LPS-induced TNF-α release was reduced in whole blood by HI injury relative sham surgery.

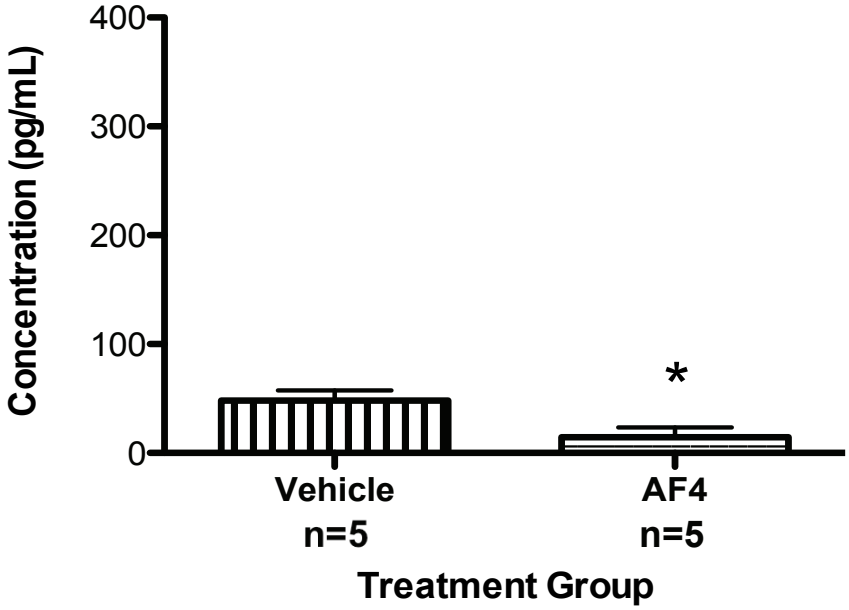
Administration of 3 doses of AF4 did not produce a reduction in TNF-α release in whole blood of mice subjected to sham HI surgery compared to the vehicle treatment group (Figure 32A). No statistical difference was found between treatment groups using a one-sided student's t-test ($t(18)=1.165$; $p=0.1297$). These data indicate that LPS-induced TNF-α release was comparable in whole blood from sham treated animals (no brain injury) that received 3 doses of AF4 or water. Administration of 3 doses of AF4 (50 mg/kg) prior to HI reduced TNF-α production in comparison to the vehicle treatment group (Figure 32B). A one-sided student's t-test revealed a significant difference between treatment groups ($t(8)=2.607$; $p=0.0156$). These data indicate that LPS-induced TNF-α release was the greater in whole blood from HI animals (brain injury) that received oral administration of water compared to mice that received AF4.

Figure 32: Oral administration of AF4 (50 mg/kg/day for 3 days) reduces production of the inflammatory cytokine TNF-α in LPS-stimulated whole blood from mice subjected to HI. These graphs depict the concentration (pg/mL) of LPS-induced TNF-α release from whole blood collected from C57Bl/6 mice that were treated with either AF4 (50 mg/kg/day, p.o.) or vehicle (10 mL/kg/day, p.o.) for 3 days prior to HI or sham surgery. A) Administration of 3 doses of AF4 did not produce a reduction in TNF-α release in whole blood compared to the vehicle treatment group. No statistical difference was found between treatment groups using a one-sided student's t-test ($t(18)=1.165$; $p=0.1297$). B) Administration of 3 doses of 50 mg/kg AF4 prior to HI was shown to reduce TNF-α production in comparison to the vehicle treatment group. A one-sided student's t-test determined a significant difference between treatment groups ($t(8)=2.607$; $p=0.0156$). *$P< 0.05$

A)


400
300
200
100
0
Concentration (pg/mL)
Vehicle
n=10
AF4
n=10
Treatment Group

B)


400
300
200
100
0
Concentration (pg/mL)
*
Vehicle
n=5
AF4
n=5
Treatment Group

3.9 AF4 Reduced the Death of Mouse Primary Cortical Neurons Subjected to Oxygen Glucose Deprivation (OGD)

The neuroprotective effects of AF4, quercetin and quercetin metabolites (Q-3-O-glucoside, Q-3'-O-sulphate, Q-3-O-glucuronic acid, isoshamnetin-3-glucuronic acid), relative to vehicle were examined using primary cultures of mouse cortical neurons subjected to OGD. Mouse primary mouse cortical neuron cultures (DIV8 cells, E16 Cortical Cultures) were incubated with either 1 μg/mL, 0.1 μg/mL or 0.01 μg/mL of AF4, quercetin, Q-3-O-glucoside, Q-3'-O-sulphate, Q-3-O-glucuronic acid, isoshamnetin-3-glucuronic acid, or the corresponding DMSO control (0.1%, 0.01% or 0.001% DMSO, respectively) for 12 hours proceeding, as well as during the 12-hour period of OGD on DIV9. The % of total possible LDH (100% cell death) release into serum free/aglycaemic/anoxic (OGD) medium was determined and used as a measure of cell death. Treatment with AF4, quercetin or quercetin metabolites at a concentration of 0.01 μg/mL did not reduce % LDH release in comparison to the vehicle treatment group (One-way ANOVA ($F(8,96)=1.183$: $p=0.3207$)) (Figure 33). Treatment with AF4, quercetin or quercetin metabolites at a concentration of 0.1 μg/mL also did not reduce % LDH release in comparison to the vehicle treatment group (One-way ANOVA ($F(8,96)= 0.4256$; $p= 0.8839$)) (Figure 34). By contrast, treatment with AF4 but not quercetin or its metabolites, at a concentration of 1.0 μg/mL, produced a 65% reduction in LDH release in comparison to the vehicle treatment group (Figure 35). A one-way ANOVA demonstrated a statistical difference in % LDH release between the groups ($F(8,96)= 7.420$; $p<0.0001$). Post hoc testing revealed that a reduction in LDH release for AF4 treated cultures was responsible for this difference (Dunnett's Multiple Comparison test,

$p < 0.05$). These data demonstrate that AF4, but not quercetin or its metabolites, directly protected mouse primary cortical neurons from OGD-induced neuronal cell loss. neuroprotective effect on cortical neurons under oxidative stress produced by OGD.

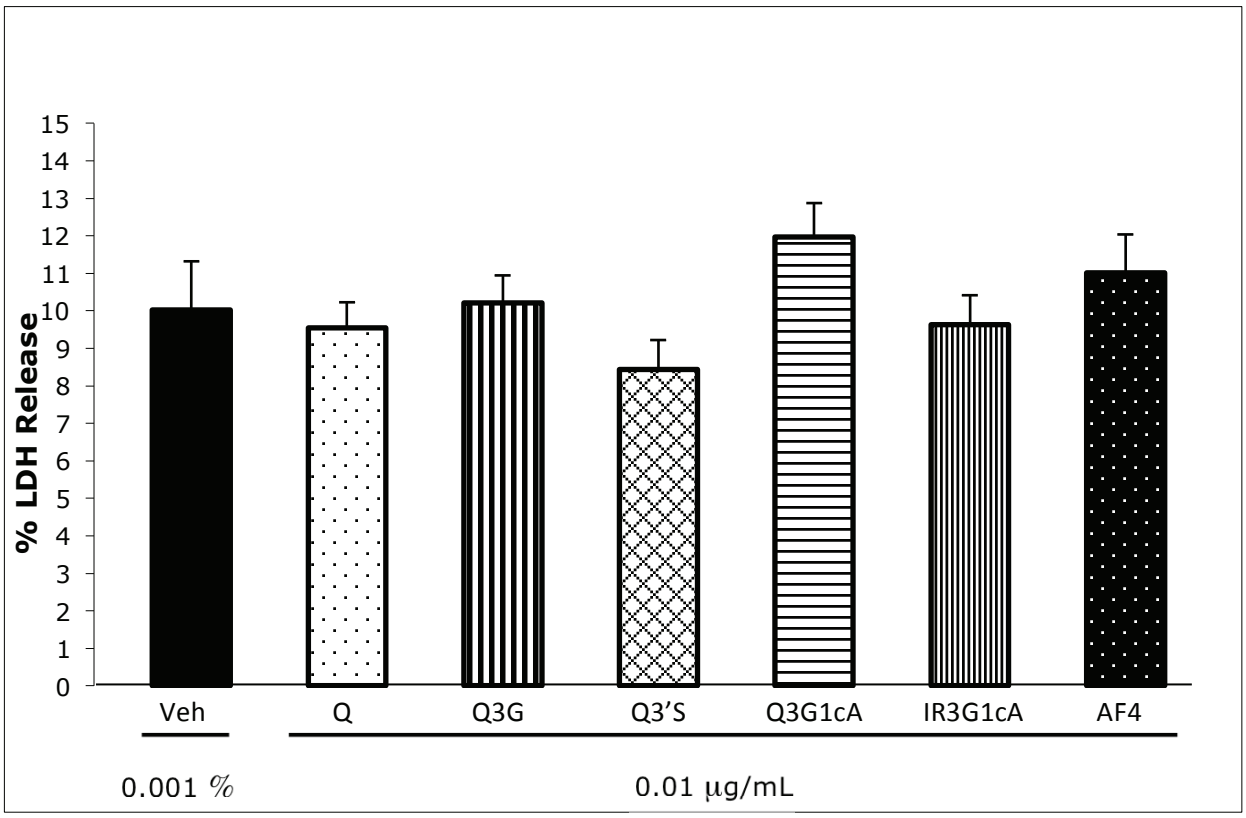


Figure 33: AF4, quercetin, and metabolites failed to reduce OGD-induced death of mouse primary cortical cultures at a concentration of 0.01 µg/mL. Percentage of total possible LDH release from mouse primary cortical cultures treated with vehicle (0.001% DMSO), quercetin (Q) or quercetin metabolites (Q-3-O-glucoside (Q3G), Q-3'-O-sulphate (Q3'S), Q-3-O-glucuronic acid (Q3G1cA), isoshamnetin-3-glucuronic acid (IR3G1cA)) or AF4 (n=12) at a concentration of 0.01 µg/mL and subjected to 12 hours of OGD. The % LDH release was the same for all groups. One-way ANOVA ($F(8,96)= 1.183$; $p= 0.3207$).

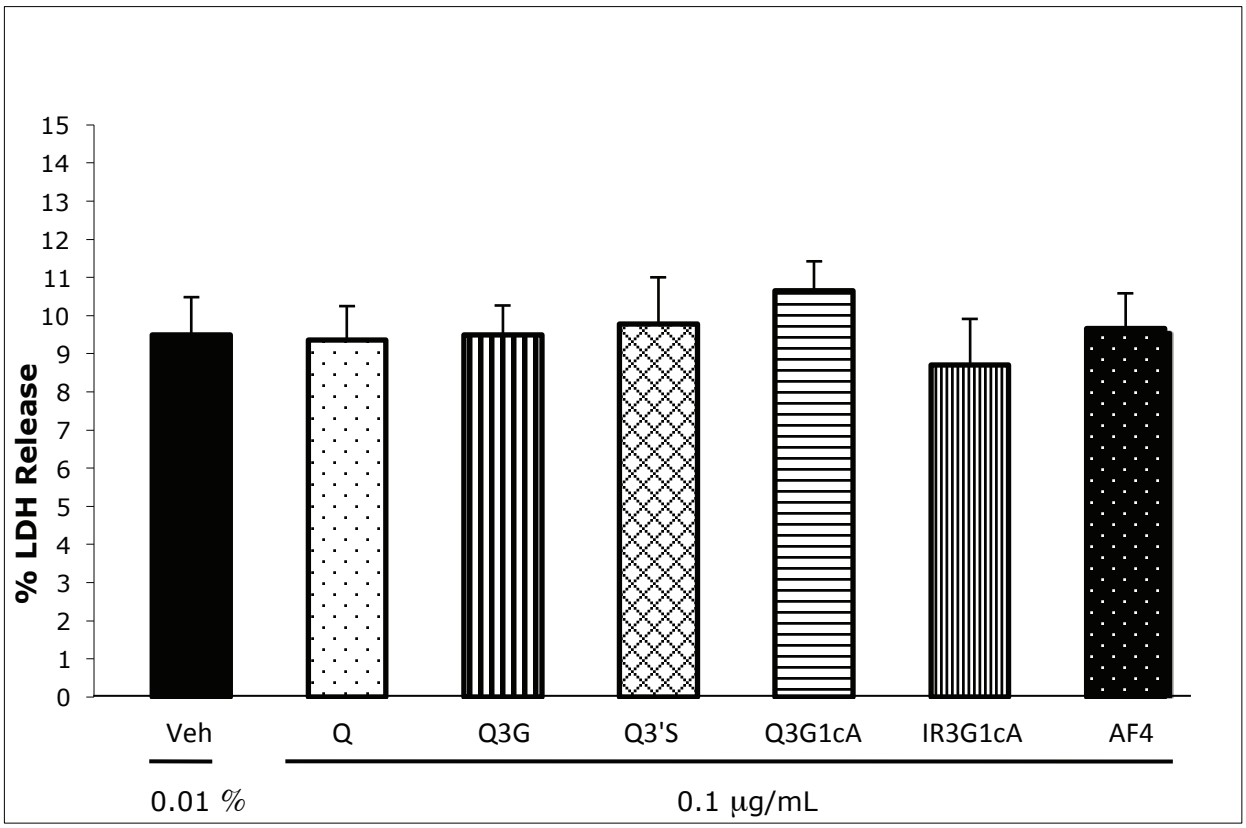


Figure 34: AF4, quercetin, and metabolites failed to reduce OGD-induced death of mouse primary cortical cultures at a concentration of 0.1 μg/mL. Percentage of total possible LDH release from mouse primary cortical cultures treated with vehicle (0.01% DMSO), quercetin (Q) or quercetin metabolites (Q-3-O-glucoside (Q3G), Q-3'-O-sulphate (Q3'S), Q-3-O-glucuronic acid (Q3G1cA), isoshamnetin-3-glucuronic acid (IR3G1cA)) or AF4 (n=12) at a concentration of 0.1 μg/mL and subjected to 12 hours of OGD. The % LDH release was the same for all groups. One-way ANOVA ($F(8,96)= 0.4256$; $p= 0.8839$).

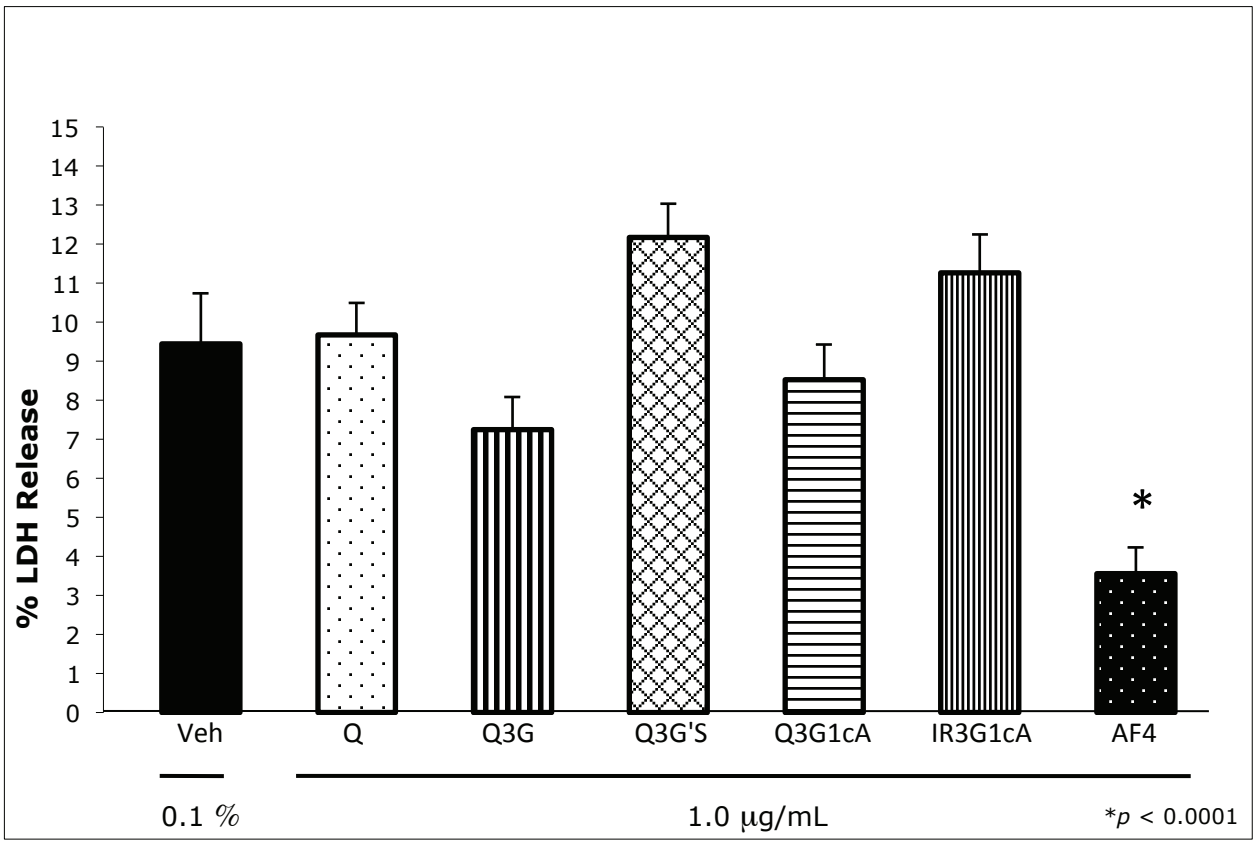


Figure 35: AF4, but not quercetin, and metabolites was able to reduce OGD-induced death of mouse primary cortical cultures at a concentration of 1.0µg/mL. Percentage of total possible LDH release from mouse primary cortical cultures treated with vehicle (0.1% DMSO), quercetin (Q) or quercetin metabolites (Q-3-O-glucoside (Q3G), Q-3'-O-sulphate (Q3'S), Q-3-O-glucuronic acid (Q3G1cA), isoshamnetin-3-glucuronic acid (IR3G1cA)) or AF4 (n=12) at a concentration of 1.0 µg/mL and subjected to 12 hours of OGD. Treatment with AF4 but not quercetin or quercetin metabolites showed a significant reduction in % LDH release in comparison to the vehicle treatment group at a concentration of 1.0 µg/mL. One-way ANOVA revealed a statistical difference in % LDH release between all groups ($F(8,96)= 7.420$; $p<0.0001$). A Dunnett's Multiple Comparison post-hoc test demonstrated that reduced LDH release from culture treated with AF4 was responsible for the main effect of groups ($p<0.05$).

3.10 AF4 Administration Failed to Increase Hippocampal Expression of Genes Associated with the Protective Effects of Q3G Against Oxidative Stress-Induced Cell Death

C57Bl/6 adult male mice were dosed orally with either AF4 (50 mg/kg) (n=6) or vehicle (10 mL/kg) (n=6) once a day for 3 consecutive days and then subjected to 50 minutes of HI 24 hours after the final dose. Each animal was sacrificed 24 hours after HI and the brain removed and the hippocampus was rapidly dissected on ice. Total RNA was extracted from the hippocampus ipsilateral (injured) and contralateral (uninjured) to the common carotid artery occlusion. Quantitative RT-PCR was performed to determine the levels of SCD1, HMG-CoA reductase and SREBP-2 mRNAs relative to β-actin mRNA. The expression of SCD1, HMG-CoA reductase and SREBP-2 mRNAs in mice following treatment with 3 doses of AF4 or vehicle and exposure to 50 minutes of HI was expressed as a fold change from pooled contralateral hippocampus (uninjured) tissue of vehicle treated C57Bl/6 mice. Changes in fold expression of SCD1, HMG-CoA reductase and SREBP-2 mRNAs was assessed in response to AF4 treatment and AF4 treatment in combination with HI injury. HMG-CoA reductase expression was not increased by AF4 treatment or AF4 treatment in combination with HI in the hippocampus. The mean ± SEM fold increase of HMG-CoA reductase mRNA expression in the hippocampus was 1.05 ± 0.11 for the vehicle contralateral hippocampus group, 1.20 ± 0.17 for the AF4 contralateral hippocampus group, 1.16 ± 0.09 for the vehicle ipsilateral hippocampus group and 1.34 ± 0.13 for the AF4 ipsilateral hippocampus group relative to pooled contralateral hippocampus (uninjured) tissue of vehicle treated C57Bl/6 control mice (Figure 36). One-way ANOVA showed no statistical difference between treatment groups ($F(4,6)= 0.8479$; $p= 0.4839$). SCD1 expression was not increased by AF4

treatment or AF4 treatment in combination with HI in the hippocampus. The mean ± SEM fold increase of SCD1 mRNA expression in the hippocampus was 1.01 ± 0.06 for the vehicle contralateral hippocampus group, 1.18 ± 0.15 for the AF4 contralateral hippocampus group, 1.23 ± 0.17 for the vehicle ipsilateral hippocampus group and 0.99 ± 0.10 for the AF4 ipsilateral hippocampus group relative to pooled of the contralateral hippocampus (uninjured) tissue of vehicle treated C57Bl/6 control mice (Figure 37). One-way ANOVA showed no statistical difference between treatment groups ($F(4,6)= 0.9277$; $p= 0.4455$). SREBP2 expression was not increased by AF4 treatment or AF4 treatment in combination with HI in the hippocampus. The mean ± SEM fold increase of SREBP-2 mRNA expression in the hippocampus was 1.12 ± 0.15 for the vehicle contralateral hippocampus group, 1.24 ± 0.12 for the AF4 contralateral hippocampus group, 0.98 ± 0.16 for the vehicle ipsilateral hippocampus group and 1.00 ± 0.10 for the AF4 ipsilateral hippocampus group relative to pooled contralateral hippocampus (uninjured) tissue of vehicle treated C57Bl/6 control mice (Figure 38). One-way ANOVA showed no statistical difference between treatment groups ($F(4,6)= 0.7546$; $p= 0.5326$). In summary, these results demonstrate that SCD1, HMG-CoA reductase and SREBP-2 mRNA levels are not affected following treatment with AF4 (50 mg/kg) or AF4 (50 mg/kg) treatment in combination with HI compared to the vehicle treated mice.

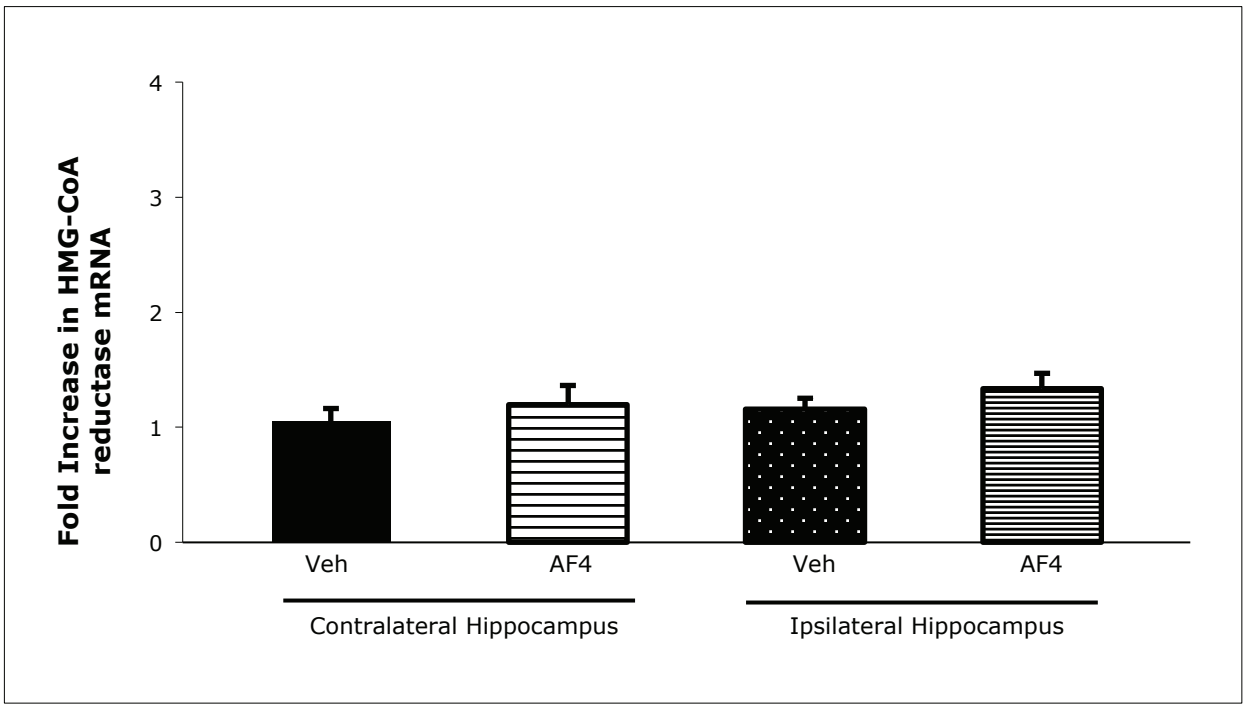


Figure 36: AF4 failed to increase the expression of HMG-CoA reductase in the hippocampus. This graph depicts fold increases in HMG-CoA reductase mRNA expression in the hippocampus of C57Bl/6 adult male mice dosed orally with either AF4 (50 mg/kg) (n=6) or vehicle (10 mL/kg) (n=6) once a day for 3 consecutive days and subjected to 50 minutes of HI. No difference in fold increase in HMG-CoA reductase mRNA levels was found between the four experimental conditions: ipsilateral and contralateral hippocampi of AF4 and vehicle treated mice, One-way ANOVA ($F(4,6)= 0.8479$; $p= 0.4839$).

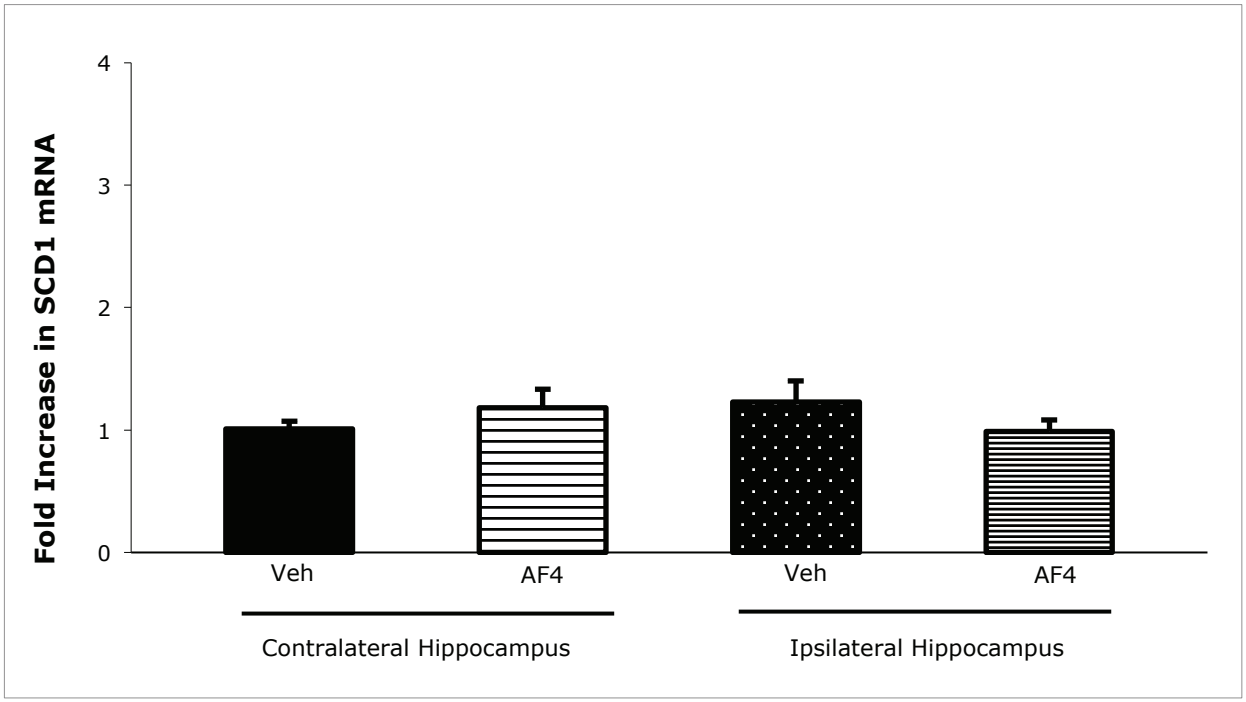


Figure 37: AF4 failed to increase the expression of SCD1 reductase in the hippocampus. This graph depicts fold increases in SCD1 mRNA expression in the hippocampus of C57Bl/6 adult male mice dosed orally with either AF4 (50 mg/kg) (n=6) or vehicle (10 mL/kg) (n=6) once a day for 3 consecutive days and subjected to 50 minutes of HI. No difference in fold increase in SCD1 mRNA levels was found between the four experimental conditions: ipsilateral and contralateral hippocampi of AF4 and vehicle treated mice, One-way ANOVA ($F(4,6)= 0.9277$; $p= 0.4455$).

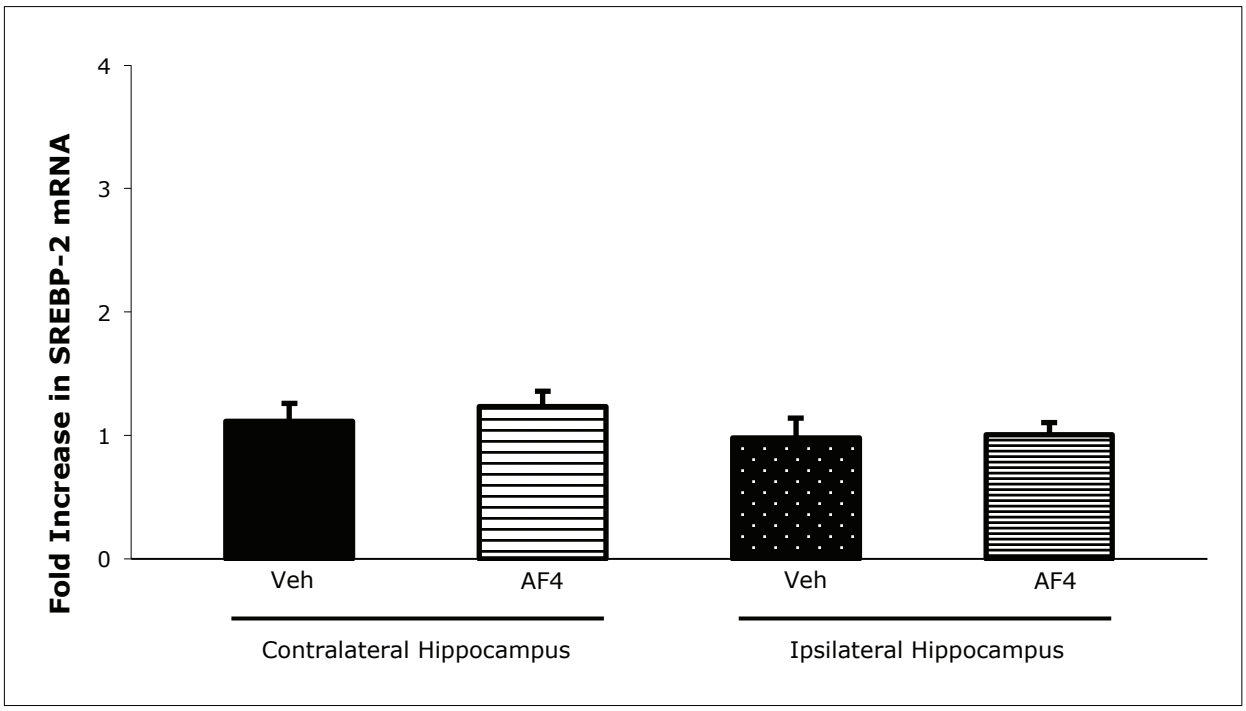


Figure 38: AF4 failed to increase the expression of SREBP-2 reductase in the hippocampus. This graph depicts fold increases in SREBP-2 mRNA expression in the hippocampus of C57Bl/6 adult male mice dosed orally with either AF4 (50 mg/kg) (n=6) or vehicle (10 mL/kg) (n=6) once a day for 3 consecutive days and subjected to 50 minutes of HI. No difference in fold increase in SREBP-2 mRNA levels was found between the four experimental conditions: ipsilateral and contralateral hippocampi of AF4 and vehicle treated mice, One-way ANOVA ($F(4,6)= 0.7546$; $p= 0.5326$).

Chapter 4: Discussion

4.1 AF4 Prevents Motor Deficits and Neuronal Cell Loss in a Model of Hypoxic-Ischemic Brain Injury

The effects of pre-dosing with AF4 on motor deficits and neuronal cell loss were assessed in a mouse model of hypoxic-ischemic (HI) brain injury. Relative to control mice that received oral administration of vehicle (10 mL/kg/day for 3 days), AF4-treated mice (50 mg/kg/day for 3 days) displayed a marked attenuation of motor deficits and reduced neuronal cell loss in brain regions normally injured by HI (striatum, cortex and hippocampus). Motor deficits experienced on the contralateral side following ischemic brain injury is known to result from neuronal loss in brain regions ipsilateral to the MCA occlusion implicated in the control of movement, such as the dorsal lateral hippocampus and motor cortex (Olson and McKeon, 2004). Motor deficits normally observed following HI injury, as assessed by rotarod performance, were completely prevented by pre-treatment with 3 doses of AF4 (50 mg/kg, p.o.) prior to 50 minutes of HI (Figure 13). This finding is supported by the ability of pretreatment with AF4 (50 mg/kg/day for 3 days) to markedly attenuate neuronal cell loss in the ipsilateral striatum and hippocampus following HI injury, in comparison to the vehicle (10 mL/kg/day for 3 days) treatment group. By contrast, a single predose of AF4 (50 mg/kg/day) was not effective in preventing neuronal cell loss in the ipsilateral striatum or hippocampus (shown in Figure 14). This indicates that multiple doses of AF4 over a period of several days was necessary to offer protection against HI-induced brain injury. This suggests that a transcriptional mechanism resulting in the gradual alteration of proteins levels may be responsible for the beneficial effects of AF4 pretreatment.

4.2 Pretreatment with Increasing Amounts of AF4 (5, 10, 25 or 50 mg/kg/day for 3 days) Produced a Dose-Dependent Reduction in HI-induced Hippocampal and Striatal Neuron Cell Loss

Separate groups of adult C57Bl/6 mice were dosed orally with increasing amounts of AF4 (5, 10, 25, or 50 mg/kg) to determine the optimal dose of AF4 required to reduce the damaging effects of HI brain injury in two vulnerable forebrain structures: the dorsal hippocampus and striatum. AF4 produced a dose-dependent reduction of tissue atrophy and neuronal cell loss in the ipsilateral striatum and hippocampus (Figures 15 and 17, respectively). Oral administration of 25 mg/kg/day for 3 days of AF4 was the lowest dose that produced maximal protection against HI-induced injury in both the striatum and hippocampus (Figure 21 and 27 respectively). Lower doses of AF4 (5-10 mg/kg/day for 3 days, p.o.) were found to be less neuroprotective than 25 mg/kg (once a day for 3 days, p.o.) AF4, while a dose of 50 mg/kg/day for 3 days (p.o.) AF4 was no more effective than the 25 mg/kg (once a day for 3 days, p.o.) dosing regime.

Administration of AF4 at a dose of 5 mg/kg/day for 3 days (p.o.) was not found to reduce the damaging effects of HI in the ipsilateral striatum or hippocampus, while 10 mg/kg/day for 3 days (p.o.) AF4 produced a partial reduction in HI-induced damage in the ipsilateral striatum and hippocampus. Oral administration of 25 mg/kg/day or 50 mg/kg/day for 3 days of AF4 appeared to produce a near complete protection against HI-induced striatal and hippocampal injury HI. Since a dose of 50 mg/kg/day for 3 days (p.o.) of AF4 was no more effective than 25 mg/kg/day for 3 days (p.o.) of AF4 in reducing brain injury following HI, 25 mg/kg/day for 3 days of AF4 was determined to be the lowest dosage that reliably produced maximal neuroprotection.

4.3 Comparison of the Effects of 1, 3 or 7 Doses of AF4 (25 mg/kg/day) Prior to HI Revealed that Optimal Neuroprotection was Produced by 7 Pre-doses of AF4

In terms of dose frequency, a single oral administration of AF4 (25 mg/kg) 24 hours prior to HI was not found to attenuate HI-induced injury in the striatum (Figure 19). Oral administration of AF4 (25 mg/kg/day) for 3 days produced a large reduction in neuronal cell loss in the striatum (Figure 21) that was not further enhanced by increasing the number of doses to 7 (Figure 23). In the case of the hippocampus, a single dose of AF4 (25 mg/kg, p.o.) did not reduce atrophy of this structure (Figure 25). Three doses of AF4 (25 mg/kg, p.o.) produced a modest reduction of HI-induced hippocampal atrophy but in the surviving tissue neuronal loss was still evident (Figure 27). By contrast, seven pre-doses of AF4 (25 mg/kg, p.o.) prevented both hippocampal atrophy and neuronal loss in this structure (Figure 29). These differences likely reflect the fact that the hippocampus is more vulnerable to HI damage than the striatum. Hence, relative to the striatum, longer treatment with AF4, 25 mg/kg/day (p.o.) for 7 days rather than 3 days, was required to maximally protect the hippocampus.

Taken together these findings indicate that for AF4-induced neuroprotection, 25 mg/kg (p.o.) given once a day for 7 days is the optimal dosing regime in a mouse model of HI-induced brain injury. The increasing neuroprotection produced by repeated administration of AF4 suggests this extract protects against HI-induced brain injury by a transcriptional mechanism. Flavonoids have a fused ring system consisting of an aromatic ring and a benzopyran ring with a phenyl substituent (Williams et al., 2004). These structural features are common to a variety compounds that are kinase inhibitors, which modulate neuronal cell survival and reduce inflammation. For instance phosphodiesterase

3 and 4 inhibitors, which decrease inflammation; benzodiazepine ligands that oppose excitotoxicity; agonists and antagonist for various subtypes of adenosine receptors that reduce inflammation and xenobiotics that activate the anti-oxidant response element (ARE) found in a wide variety of genes which combat oxidative stress and inflammation (Williams et al., 2004). This suggests that AF4 may be working through several different mechanisms to achieve its beneficial effects in reducing inflammation and neuronal cell injury following cerebral ischemia.

4.4 AF4 Reduced the Death of Primary Cortical Neuron Cultures Exposed to Oxygen-Glucose Deprivation (OGD)

The ability of AF4 to directly protect neurons from ischemic injury was examined using the OGD model. Mouse primary cortical neuron cultures treated with this flavonoid-enriched fraction were rendered more resistant to cell death produced by OGD than cultures incubated with an equivalent concentration of DMSO (vehicle). This model is known to induce the death of cortical neurons by a variety of mechanisms including excitotoxicity, oxidative stress, calcium over-load, protease activation and apoptosis (Newcomb-Fernandez et al., 2001; Beck et al., 2003; Aksenova et al., 2005). OGD is therefore a highly relevant model to study the cytoprotective effects of AF4 against a variety of pathological mechanisms implicated in neurodegenerative disease, such as stroke, multiple sclerosis, Alzheimer's disease, and Parkinson's disease (Aksenov et al., 2005).

Relative to primary cortical neuron cultures treated with vehicle, the incubation with AF4 at a concentration of 1 μg/mL reduced OGD-induced cell death (LDH release) (Figure 36). This result suggested that the neuroprotective effects of AF4 in the mouse HI

model for stroke might be mediated by a direct effect on the brain. Quercetin and its major metabolites (quercetin-3-o-glucuronic acid, isorhamnetin-3-gluronic acid, and quercetin-3-O-sulphate), as well as quercetin-3-O-glucoside (component of AF4), individually failed to protect cortical neurons against OGD-induced injury at the same concentration of AF4 that was neuroprotective (1 μg/mL). These results suggest that the ability of AF4 to protect cortical neurons against OGD-induced injury may derive from the combined effects of multiple flavonoids contained within this fraction that work in synergy. This hypothesis is supported by findings by Ha et al. (2003) indicating that a much higher concentration of quercetin (40 μg/mL) was only moderately protective, producing only a 30% decrease in cell death, against a considerably shorter period of OGD (50 minutes), compared to 12 hours in the present study. In other models of oxidative stress-induced cell death, including exposure to hydrogen peroxide (Kim et al., 2009) or oxidized low-density lipoproteins (Schroeter et al., 2000), protection with individual flavonoids has only been achieved at concentrations 10-15 times higher (10-15 μg/mL) than AF4 (1 μg/mL). In contrast, combining different flavonoids such as catechin (5 μg/mL), epicatechin (5 μg/mL) and epigallocatechin gallate (0.5 μg/mL) reduces biochemical correlates of cell injury in the OGD model that are not observed with 10-15 times higher concentrations of these flavonoids individually (Panickar et al., 2009). Taken together, these findings suggest that the efficacy of AF4 may derive from optimization of the flavonoid composition necessary to prevent the injurious effects of OGD.

4.5 Anti-inflammatory Effects of AF4: Reduction of LPS-Induced TNF-α Release in Whole Blood from Animals Treated with AF4 and Subjected to HI Brain Injury

Incubation of whole blood with lipopolysaccharide (LPS) potentially triggers a production of the powerful inflammatory cytokine tumour necrosis factor alpha (TNF-α) that is readily detected by a mouse ELISA. Given that flavonoids have been shown to block inflammatory enzymes such as phosphodiesterase 4 (PDE4) (Peluso, 2006) and PDE4 inhibitors are known to potently block LPS-induced TNF-α release from whole blood I have used this *ex vivo* assay to determine the anti-inflammatory properties of AF4. My results indicated that LPS-induced TNF-α release in whole blood is reduced in animals subjected to HI brain injury compared to animals that receive no injury (See Figures 32 and 33). This finding is consistent with clinical studies in which LPS-induced TNF-α release is also reduced in whole blood from stroke patients (1 week after cerebral ischemia) compared to healthy controls, indicating a possible compensatory response to brain inflammation (Ernsley et al., 2008). Relative to vehicle treated HI mice, LPS-induced TNF-α release in whole blood from HI mice treated with AF4 was reduced by 70% (Figure 33). By contrast AF4 did not reduce LPS-induced TNF-α release in whole blood from normal mice, suggesting that this extract is not a general immunosuppressant but rather has immunomodulatory actions that are context specific, as AF4 only reduced TNF-α levels in the presence of excessive inflammation and not at basal levels. This evidence also supports a transcriptional mechanism underlying AF4's anti-inflammatory properties, indicating cellular changes are needed to invoke neuroprotection. To the best of my knowledge this highly desirable therapeutic property is unique to AF4.

A large body of evidence has implicated TNF-α in the initiation of inflammatory events secondary to necrotic cell death which further contribute to brain damage after a stroke (Ernsley et al., 2008). The ability of AF4 to reduce the expression of TNF-α following ischemic brain injury (Figure 33) may therefore attribute to the neuroprotective properties of this apple peel-derived extract. This hypothesis is supported by studies showing that flavonoids, such as quercetin, that possess anti-inflammatory properties also inhibit the production of the pro-inflammatory cytokine TNF-α in blood (Nair et al., 2006). An anti-inflammatory mechanism underlying the neuroprotective properties of AF4 has been proposed by Warford, Dunlop & Robertson, unpublished data. Warford, Dunlop & Robertson showed that oral administration of AF4 (25 mg/kg/day) for 20 days, beginning at first signs of paralysis, reduced disease progression in a mouse model of experimental autoimmune encephalomyelitis (EAE). Since EAE is a model of T cell-mediated autoimmune disease these findings suggest the notion that AF4 reduced HI-induced brain damage by suppressing inflammation.

4.6 AF4 Failed to Increase the Expression of Genes that Mediate the Cytoprotective Effects of Quercetin-3-O-glucoside (Q3G) Against Oxidative-Stress Induced Cell Death

Previous work by Soundararajan et al. (2008) showed that pretreatment with Q3G, a main component of AF4, protected SH-SY5Y cells against H_2O_2-induced cell death. SH-SY5Y cells pretreated with Q3G prior to being subjected to H_2O_2-induced oxidative stress showed an up-regulation in the expression of genes associated with lipid and cholesterol biosynthesis (Soundararajan et al., 2008). Inhibitors of HMG-CoA reductase, a SREBP-2-driven gene, blocked Q3G-mediated cytoprotection in SH-SY5Y

cells (Soundararajan et al., 2008). These findings suggest that this flavonoid-induced cytoprotection in SH-SY5Y cells involved SREBP-2-mediated sterol synthesis that decreased lipid peroxidation by maintaining membrane integrity in the presence of oxidative stress. This lead us to examine whether the neuroprotective effects of AF4, a flavonoid-enriched fraction containing Q3G, are associated with increased expression of genes that promote cholesterol biosynthesis. C57Bl/6 mice were dosed orally with either vehicle (10 mL/kg) or AF4 (50 mg/kg) once a day for three consecutive days and then subjected to 50 minutes of HI 24 hours after the final dose. Mice were sacrificed 24 hours after the final dose and RNA was extracted from the hippocampus and used to determine the expression of SREBP-2 and two SREBP-2 driven genes, SCD1 and HMG-CoA reductase, by quantitative RT-PCR. The expression of SCD1, HMG-CoA reductase and SREBP-2 mRNAs in mice following treatment with 3 doses of AF4 or vehicle and exposure to 50 minutes of HI was expressed as a fold change from pooled contralateral hippocampus (uninjured) tissue of vehicle treated C57Bl/6 mice. Changes in fold expression of SCD1, HMG-CoA reductase and SREBP-2 mRNAs was assessed in response to AF4 treatment and AF4 treatment in combination with HI injury. My results (Figures 37, 38 and 39) showed no difference in fold increase of SREBP-2, SCD1 or HMG-CoA reductase mRNA levels after HI or any effect of AF4 pretreatment in the expression of genes in the hippocampus. These results suggest an alternate mechanism must be responsible for the neuroprotective effects of AF4 *in vivo*.

4.7 AF4 Mechanistic Possibilities

My future goal is to elucidate a possible mechanism whereby AF4 is able to offer protection. Flavonoids are known to reduce the risk of in many chronic disorders involving oxidative stress or inflammation by various mechanisms including scavenging free radicals, inhibiting a variety of kinases, reducing lipid peroxidation, inhibiting apoptosis, preventing platelet aggregation and exhibiting anti-inflammatory effects (Williams et al., 2004; Peng and Kuo, 2003; Molina et al., 2003). Several flavonoids have been documented to cross the blood-brain barrier and to protect neurons from cell death in both *in vitro* and *in vivo* models of neurodegenerative disorders (Mandel et al., 2006; Youdim et al., 2004; Soundararajan et al., 2008). Understanding the mechanism of action of AF4 will encourage the possibility of it being used as a safe, effective and novel natural health product for the prevention of diseases in which oxidative stress and inflammatory mechanisms have been implicated.

Several flavonoids are known to be inhibitors of different phosphodiesterase (PDE) isoforms based on the positions of hydroxylation on the A-ring, as these are potential sites of hydrogen bonding with protein residues (Peluso, 2006). For example, hydroxylation at carbons C5 and C7 are important for PDE4 inhibition, as this configuration adheres electrostatically and sterically with the catalytic site of PDE4 (Peluso, 2006). Quercetin, a component of AF4, has been shown to be a steric and electrostatic match for the cyclic nucleotide binding pocket in the catalytic site of human PDE4, due to the positioning of the hydroxyl groups (Peluso, 2006). The PDE4 isoform is found in inflammatory and immune cells that controls cAMP levels. PDE4 inhibitors have been shown to have potential as anti-inflammatory drugs, as they suppress the

release of cytokines and inhibit the production of reactive oxygen species. Given that flavonoids have been shown to block inflammatory enzymes such as phosphodiesterase 4 (PDE4) (Peluso, 2006) and PDE4 inhibitors are known to potently block LPS-induced TNF-α release from whole blood, we tested AF4's ability to suppress LPS-induced TNF-α release from whole blood. As inhibition of PDE4 is known to enhance the effects of physiological processes mediated by cAMP by inhibiting degradation of this second messenger. I would like to test the hypothesis that AF4 may prevent the activation of this enzyme after HI.

Another possible mechanism by which AF4 may be exerting its neuroprotective effects is through the nuclear protein, cAMP response element binding protein (CREB), which activates the transcription of genes containing cAMP responsive elements in their promoter region. CREB is a prosurvival factor that opposes cell death. Activation of CREB is triggered by an increase in the concentrations of Ca^{2+} or cAMP. Genistein, a soy isoflavone, at a dose of 10 nM has been shown to elevate cellular cAMP levels, activating protein kinase A (PKA), CREB, and CREB-mediated gene expression (Liu, et al., 2005). Quercetin, a component of AF4, has also been suggested to increase the activation of the cAMP driven transcription factor CREB (Williams et al., 2004). Unphosphorylated CREB can be detected by immunohistochemistry in brain sections. However, the activated form is phosphorylated on Ser133 inducing a conformational change detectable with an antibody that selectively recognizes pCREB. Therefore in the future I would like to use an antibody against pCREB to detect changes in the activated form of CREB by immunohistochemistry in fresh frozen tissue or by Westerns, using whole cell extracts from whole brain or primary neuron cultures treated with AF4. If treatment with AF4

prior to the hypoxic-ischemic event results in an increase in the expression of the activated form of CREB this pathway will be further examined to confirm this mechanism of action for AF4.

Gene expression profiling will also be used to gain insights into the mechanism of action of AF4. Gene expression profiling permits the activity (expression) of thousands of genes to be measured at once in order to generate a global picture of cellular function. This has become possible through the development of DNA microarray technology that permits the expression of thousands of genes to be measured simultaneously in the same biological sample. In brief, a glass slide (1.28 X 1.28 cm) is spotted with thousands of microscopic DNA oligonucleotides called features, each containing picomoles (10^{-12} moles) of a specific DNA sequence, known as probes. Each probe encodes a region of DNA unique to a specific gene that will hybridize to a complementary RNA or DNA (called the target) under high-stringency conditions. Probe-target hybridization is usually detected and quantified by detection of fluorophore, silver-, or chemiluminescence-labeled targets to determine relative abundance of nucleic acid sequences in the target. Modern cDNA microarrays contain tens of thousands of probes (40,000) enabling many genetic tests to be run at the same time. As a result, cDNA microarray technologies have dramatically accelerated many types of investigation.

I therefore propose to use DNA microarray technology to determine differences in gene expression produced by AF4 in both *in vitro* (OGD) and *in vivo* (HI) models of ischemic cell injury. In the case of the former, primary cortical cultures will be incubated with either AF4 (1 μg/mL) or vehicle containing an equivalent amount of DMSO (0.1%) for 12 hours before exposure to OGD for 12 hours. Total RNA will be harvested at

varying times after OGD to assess differences in the patterns and kinetics of gene expression (2, 4 8 and 16 hours later) between AF4 and vehicle treated cultures using a mouse DNA microarray (GeneChip® Mouse Genome 430 2.0 Array, Affymetrix Inc., Santa Clara, CA) which measures 39,000 transcripts and variants from over 34,000 well characterized mouse genes. In the case of the later, two groups of C57Bl/6 mice will receive either AF4 (25 mg/kg/day, p.o.) or vehicle (10 mL/kg/day, p.o.) for 7 days prior to 50 minutes of HI and sacrificed 2, 4, 8 or 16 hours later. Total RNA will be extracted from the ipsilateral hippocampus and striatum and gene expression profiling performed using the GeneChip® Mouse Genome 430 2.0 Array and bioinformatic software (www.sbgn.org). The advantage of the former approach is the use of a near pure population of neurons (95-99% cortical neurons). However, the disadvantage of this approach is that changes in gene expression observed in primary cultures may not be relevant to the effects of AF4 *in vivo*. Consequently, by comparing changes in gene expression between the OGD and HI models it is more likely pathways relevant to the neuroprotective effects of AF4 will be identified. This will also assist in the identification of gene expression patterns, or perhaps even specific genes that will serve as surrogate blood markers for efficacy and toxicity for AF4 in mouse and human subjects necessary for the development of this extract as a Natural Health Product.

4.8 Conclusions

The present study explored the neuroprotective potential of AF4, a flavonoid-enriched extract derived from the peel of Northern Spy apples (containing quercetin-3-O-glucoside, quercetin-3-O-galactoside, quercetin-3-O-rhamnoside, quercetin-3-O-rutinoside, epicatechin, and cyanidin-3-O-galactoside) by assessing neuronal cell loss and

motor impairment resulting from HI-induced brain injury in adult C57BL/6 mice. Using this experimental model of stroke, I demonstrated that pretreatment with 3 doses of AF4 (50mg/kg/day) prior to 50 minutes of HI reduced the overall decrease of hemispheric volume, neuronal cell loss in the striatum and hippocampus and motor impairments resulting from ischemic brain injury. A dose-dependent reduction in HI-induced neuron cell loss was established, with 25 mg/kg of AF4 being the lowest dose that achieved maximal neuroprotection. The comparison of 1, 3 and 7 doses of AF4 (25 mg/kg/day, p.o.) prior to HI revealed that optimal neuroprotection was produced by 7 pre-doses of AF4. Using an *ex vivo* blood assay, I demonstrated that AF4 mediates its effects at least in part through an anti-inflammatory mechanism, as oral administration of AF4 (50 mg/kg/day for 3 days), relative to vehicle treatment, prior to HI reduced the ability of LPS to stimulate the production of TNF-α in whole blood. AF4 was also found to have direct protective effects on cortical neurons as pretreatment with 1 ug/mL AF4 decreased the death of primary cultures of mouse cortical neurons subjected to oxygen glucose deprivation (OGD, 12 hours) in comparison to cultures exposed to vehicle (DMSO) or the same concentrations of quercetin or its metabolites. Taken together these findings suggest a transcriptional mechanism may attribute to the ability of AF4 to reduce HI-induced brain injury and motor deficits by direct protection of vulnerable neurons against OGD-induced damage and decreasing the production of inflammatory cytokines such TNF-α. Also as AF4 did not reduce LPS-induced TNF-α release in whole blood from normal mice, it suggests that this extract is not a general immunosuppressant but rather has immunomodulatory actions that are context specific, which is an ideal feature for a therapeutic.

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