"I do not know what I may appear to the world; but to myself I seem to have been only like a boy playing on the seashore, and diverting myself in now and then finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me".

- Sir Issac Newton

University of Alberta

Mechanisms of injury and recovery after an intracerebral hemorrhage

by

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This thesis is dedicated to my dear Appa (Father), Amma (Mother), Sister (Geetha) and Sampath

Abstract

Bleeding within the brain parenchyma causes a severe form of stroke named intracerebral hemorrhagic stroke (ICH). An understanding of how brain injury occurs after an ICH may suggest alternative therapies. For this reason, the current dissertation focuses on two important questions of how the brain injury and recovery occurs after ICH. In these experiments we studied the 2 putative mechanisms of injury, thrombin and iron. Moreover we also evaluated whether recovery after ICH occurs by ameliorating iron toxicity.

Chapter 2 assesses the role of thrombin by injecting thrombin directly into the rat striatum. After thrombin was given, the surviving neurons were scrutinized in the peri-infarct region using Golgi-Cox stain. We also assessed the short and long term effects of thrombin in causing tissue loss. Even a small dose of thrombin caused surviving neurons to atrophy; however thrombin did not cause long-term tissue loss. In chapter 3 a similar experimental method was employed to evaluate the role of iron. Iron caused remarkable neuronal atrophy, short and long term tissue loss and neurodegeneration. Furthermore, to attenuate the toxicity of iron, we administered a ferrous iron chelator (bipyridine) in three different models of ICH (collagenase, whole blood and ferrous chloride model) with multiple behavioural testing (neurological score, walking and turning bias) and histological endpoints (tissue loss, neurodegeneration, chapter 4). Despite testing the drug with multiple models and end points we could not find any beneficial effect of bipyridine in ameliorating iron toxicity.

The experiments described in chapter 5 aimed to address the mechanism by which rehabilitation promotes functional recovery after an ICH. Skilled reaching therapy

combined with enriched environment was given as rehabilitation treatments after ICH induced by collagenase. Rehabilitation promoted behavioural recovery and showed a neuroprotective effect by reducing neurodegeneration (Fluoro-Jade stained cells), but did not influence the iron toxicity and inflammation after ICH. In summary, our results suggest that thrombin contributes to an acute phase of injury and iron causes both acute and chronic injury after ICH. The data also suggest that rehabilitation therapy improves functional recovery and neuroprotection without influencing iron toxicity and inflammation.

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List of Abbreviations

ANOVA	analysis of variance
BBB	blood brain barrier
BrdU	bromodeoxyuridine
BP	blood pressure
BWC	brain water content
BDNF	brain derived neurotrophic factor
CIMT	constraint-induced movement therapy
EE	environmental enrichment
ER	enhanced rehabilitation
FGF	fibroblast growth factor
GAP 43	growth associated protein 43
GFAP	glial fibrillary acidic protein
GSH	glutathione
GSSG	glutathione disulfide
GST	glutathione-S-transferase
GPx	glutathione peroxidase
HO-1	heme oxygenase 1
ICH	intracerebral hemorrhage
Iba1	ionized calcium binding adaptor molecule 1
i.p.	intraperitoneal
LDL	low density lipoprotein
MMP	matrix metalloproteases

NADPH	nicotinamide adenine dinucleotide phosphate
NMDA	N methyl D aspartate
NDS	neurological deficit score
NeuN	neuronal specific nuclear protein
NGF	nerve growth factor
PAR-1	proteases activated receptor-1
PBS	phosphate buffered saline
rFVIIa	recombinant factor VIIa
SAL	sterile saline
SAH	subarachnoid hemorrhage
TBARS	thiobarbituric acid reactive substances
tPA	tissue plasminogen activator
TIA	transient ischemic attack
TNF α	tumor necrosis factor

Chapter 1

General Introduction

1. Introduction to Stroke

Blood supply interruption to any part of the brain can lead to a debilitating neurological condition termed “stroke”. According to Heart and Stroke Foundation of Canada, stroke is the third leading cause of death and the primary cause of neurological disability. There are two common types of stroke, ischemic stroke caused by blockage of blood flow (typically a clot) and hemorrhagic stroke caused by a breakage of a blood vessel. Symptoms of stroke are mainly associated with early warning signs including: weakness, speech difficulties, vision problems, headache, dizziness and impaired consciousness. Immediately after stroke the type (ischemic versus hemorrhagic) has to be identified using diagnostic imaging tools (CT, MRI etc.). However the strategies for treating the two class of stroke are essentially opposite. In treating a hemorrhage, the primary aim is to stop bleeding from a ruptured artery, whereas in ischemic stroke treatments are mainly to promote blood flow through the blocked artery.

The primary aim of stopping the bleeding in hemorrhagic stroke is achieved by treating the patients with therapies to improve hemostasis (Goldstein and Gilson, 2011, Morgenstern, et al., 2010). The next goal is to provide treatments that could lessen the secondary damage that occurs due to the ongoing cell death. Hence my thesis contribution is towards studying the mechanisms of secondary injury, and investigating ways to improve recovery after intracerebral hemorrhagic stroke.

2. History Of Hemorrhagic Stroke

Hippocrates observed the existence of stroke about 2400 years ago. Stroke was initially named as “apoplexy” which means “struck down by violence” in Greek. Much later, Johann Jacob Wepfer observed the existence of bleeding in the autopsy of patients who had apoplexy in 1658 (Schiller, 1970). Hence, he named bleeding inside the brain as hemorrhagic stroke. Wepfer also identified the main blood vessels that supply the brain such as the vertebral and carotid arteries. He also identified the cause of ischemic stroke, which was the blockage of a blood vessel by a clot. Thus, Wepfer contributed significantly in the early stroke literature by providing the basic difference between hemorrhagic and ischemic stroke (Thompson, 1996). Later in 1848, Thompson and other authors described the main symptoms of cerebral hemorrhage, which include decreased level of consciousness, headache and dilated pupils. Two of Thompson’s treatments that he listed in 1848 are still used in present day. Thompson’s treatment involved head elevation, cooling and bloodletting (Thomson, 1848). Head elevation was done to decrease the intracranial pressure in hemorrhagic patients. Blood letting showed a decrease in mean arterial pressure and lowered intracranial pressure (Fox, et al., 1890). Hypothermia (cooling) was done assuming that it would aid blood clotting (Thomson, 1848). Presently hypothermia treatment is used for cardiac arrest and neonatal hypoxic ischemic injury but not routinely for ICH (Benson, et al., 1959, Correia, et al., 2000, Jacobs, et al., 2007); although there is some clinical data suggesting it may improve outcome by attenuating severe edema (Kollmar, et al., 2010). Later in 1932, Gunewardene studied the role of hypertension after ICH in about 150 patients. The study reported that most of the patients had above 135mm/hg of diastolic

and 200 mm/hg systolic blood pressure (Cobban, 1932). The report mainly highlighted that ICH incidence can be reduced by controlling the comorbid conditions like hypertension. Later decades established various animal models to study the pathology of ICH. These animal models of ICH are further discussed in the section titled animal models.

3. Stroke Types

Other than ischemic and hemorrhagic stroke, there are other types of stroke, for example TIAs (transient ischemic attack). TIAs or mini-strokes are similar to ischemic stroke, however the symptoms resolve within 24 hours. They are important warning signs of stroke and can give the patient a chance to prevent recurrent stroke (Easton, et al., 2009).

3.1. Ischemic stroke

Ischemic stroke accounts for about 80% of major strokes in Canada. This type of stroke occurs when the blood clot has occluded the cerebral vessel. Ischemic stroke is mainly caused by deposition of plaque (fat and cholesterol deposition) resulting in reduced blood flow and clot formation in that region. The most common blood vessels that are affected in the brain are the branches of middle cerebral artery, which leads to motor disabilities (Kaste and Waltimo, 1976). The motor disabilities can include impaired eye, facial, hand, arm and leg paresis (Jorgensen, et al., 1995). Currently there are a few treatments available for focal ischemia. One acute treatment is tPA (tissue plasminogen activator) that degrades the clots within the blood vessel when provided

within 4.5 hours of stroke (Sakurama, et al., 1994, The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group, 1995). This treatment is beneficial (reduces death and dependency) because it prevents brain damage by opening the occluded blood vessel (Otwell, et al., 2010). The use of tPA has saved the lives of many people across the world (Gorelick and Ruland, 2010). However, a common problem with using thrombolytic drugs (e.g. tPA) is that it can cause hemorrhagic transformation from ischemia, with greater risk if administration is delayed (i.e. 6 or more hours post-stroke) (Font, et al., 2011). About 9% of patients usually show hemorrhagic transformation and the incidence has been increasing in the past few years (Paciaroni, et al., 2008).

3.2. Hemorrhagic Stroke

Hemorrhage stroke accounts for 20% of stroke in Canada, which occurs in ~15% for intracerebral hemorrhage (ICH) and ~5% for subarachnoid hemorrhage (SAH) (Sacco, et al., 2009). Subarachnoid hemorrhage involves bleeding in the area between the skull and the brain, whereas bleeding from a blood vessel inside the brain causes ICH. Two-thirds of spontaneous ICH cases occur in deep structures of the brain, which includes the basal ganglia and internal capsule (35-70% of cases), brain stem (5-10%) and cerebellum (5-10%) (Xi, et al., 2006). For example, ICH in striatum (basal ganglia) can result in multiple deficits pertaining to motor, oculomotor, cognitive, associative, and limbic functions. The most common symptoms of striatal ICH include loss of fine skilled motor function, walking disability etc. (Su, et al., 2007).

Pre-existing modifiable risk factors such as high blood pressure, diabetes, obesity, excessive alcohol consumption, smoking etc. increase the likelihood of hemorrhagic stroke (Qureshi, et al., 2001). Other un-modifiable risk factors include increased age, male gender and ethnicity (Ayala, et al., 2002). About 80-85% primary spontaneous ICH are caused by arterial hypertension and 5-20% are due to cerebral amyloid angiopathy (Gebel and Broderick, 2000, Hanggi and Steiger, 2008, Jüttler and Steiner, 2007). Secondary spontaneous ICH is attributed to oral anticoagulation (4-20%), tumors (5%), vascular malformations (1-2%) and other uncommon reasons such as cerebral vasculitis, drugs and others (1%) (Fewel, et al., 2003, Qureshi, et al., 2001). Combined risk factors such as age and hypertension, also increases the severity of spontaneous ICH (Qureshi, et al., 2001). Hence, controlling modifiable risk factors can be a preventive measures to reduce the risk of stroke. Even though we still do not have targeted therapy for ICH, the management of stroke in hospitals has improved significantly and saved many lives (Broderick, et al., 1999, Morgenstern, et al., 2010). The medical care management (e.g. airways stabilization, blood pressure management, etc.) has reduced morbidity and mortality (Morgenstern, et al., 2010).

4. Intracerebral Hemorrhagic Stroke Pathophysiology

The bleeding of blood vessels in the brain causes two forms of damage to occur. The primary type of damage results from mechanical injury as blood dissects through the tissue (Xi, et al., 2006). The secondary (delayed) type of damage is caused by various factors such as edema (Xi, et al., 2002), thrombin (blood clotting factor), iron (released from ruptured erythrocytes) (Xi, et al., 2004), inflammation (e.g. immune-cell

activation, release of cytotoxic factors, etc.) (Wang and Dore, 2007) and other blood components (e.g. heme oxygenase 1 and bilirubin) (Qureshi, et al., 2001). The study of mechanisms of secondary damage is therefore clinically important, and may provide insight into novel therapeutic targets.

4.1 Edema formation

The excess accumulation of fluid inside the tissue is termed edema (Xi, et al., 2002)**.** Perihematomal edema occurs immediately and peaks for many days post-ICH. In humans edema develops within 3 hours of ICH onset, increases during the first 24 hours (Gebel, et al., 2002) and peaks again between 10 - 20 days (Suzuki, et al., 1995). The main adverse effect of edema is an increase in intracranial pressure (ICP) resulting in tissue herniation, and thereby contributing to mortality (Ropper, 1986, Xi, et al., 2006). Klatzo (1967) classified edema into two types: vasogenic and cytotoxic. Vasogenic edema is a result of an increase in the permeability of cerebral blood vessels to protein and fluid, caused by a breakdown of the blood brain barrier. Cytotoxic edema is caused by cellular swelling, which results from cell-injury, and abnormal homeostatic ion-pump function (Frosch 2004). The diverse chemical and cellular reactions occurring after hemorrhage contribute to cytotoxic edema. After ICH, edema commonly occurs in three phases: the first phase occurs within a few hours of ICH onset, and is due to hydrostatic pressure and clot retraction caused by serum proteins released from the clot into surrounding tissues (Xi, et al., 2002). The coagulation factors such as thrombin causes the second phase of edema formation, which occurs several days post-ICH (Xi, et al., 2002). The third phase of edema formation occurs after a period of days to weeks

post-ICH, and is mainly caused by erythrocyte lysis leading to the release of hemoglobin breakdown products such as iron, carbon monoxide and biliverdin (Xi, et al., 2006). Lysed erythrocytes induce edema, brain damage, cell death and blood brain barrier breakdown (Xi, et al., 2001). Hence in chapter 2, we evaluated the edema levels for different doses of iron. In summary, the volume of bleed and blood components are some of the main factors of injury after ICH.

4.2. Thrombin

Thrombin, an essential clotting factor, is produced immediately after ICH to stop the bleeding in the brain (Hua et al., 2007). Thrombin is a serine protease that converts soluble fibrinogen into insoluble fibrin (Xue and Del Bigio, 2001). It has been noted in one-third of patients that the production of thrombin for clotting is insufficient to stop the bleeding. Thus, clinical trials have focused on early treatment with activated factor VIIA, which increases production of thrombin. Unfortunately, this treatment failed to significantly improve patient outcomes (Mayer, et al., 2005, Tuhrim, 2008). Even though the treatment failed to benefit, there is still ongoing research on factor VIIa in addressing its potential benefits for a subset of ICH patients (Mayer, et al., 2009).

Thrombin is an important factor with a biphasic role. Based on the concentration of thrombin, it can be either beneficial or deleterious. At a low concentration thrombin improves recovery by promoting neurogenesis (i.e. by increasing the expression of double cortin and BrdU levels) (Yang, et al., 2008), whereas higher concentrations causes edema, blood brain barrier breakdown, inflammation, etc. (Xi, et al., 2001, Xi, et al., 2006, Xi, et al., 2003). The harmful effect is exerted when thrombin acts on

proteases activated receptor (PAR). The PARs family (PAR-1, PAR-3, PAR-4) is a GPCR (G protein coupled receptor) that are activated by the proteolytic cleavage of thrombin (Noorbakhsh, et al., 2003). The overall schematics of activation of thrombin and PAR-1 receptors in microglia, astrocytes and neurons are shown in figure 1.1. The PAR-1 signaling mechanism is represented in figure 1.2. The coupling of PAR-1 with other G-proteins can lead to different responses such as nerve growth factor (NGF), PAR-1 upregulation, astrocyte proliferation, neurite retraction and neural cell-survival or apoptosis.

Although the brain has endogenous thrombin inhibitors such as serine protease inhibitors and thrombomodulin, their expression pattern after ICH is not well known. Many rat studies have shown that anticoagulant therapies such as hirudin and argatroban improved ICH induced neurological deficits (Ohnishi, et al., 2007). Hirudin reduces edema after ICH (Lee, et al., 1996). Argatroban has also been shown to reduce edema and inflammation in the rat collagenase ICH model (Nagatsuna, et al., 2005). However, most of the antithrombin drug studies have only looked at acute administration, so there remains a need for long-term survival studies to evaluate whether these drugs impede plasticity and provide a long-term neuroprotective effect. Additionally, studying the dichotomous roles of thrombin will improve our understanding of the balance between the deleterious versus beneficial effects of thrombin, and allow for the design of better therapeutic strategies.

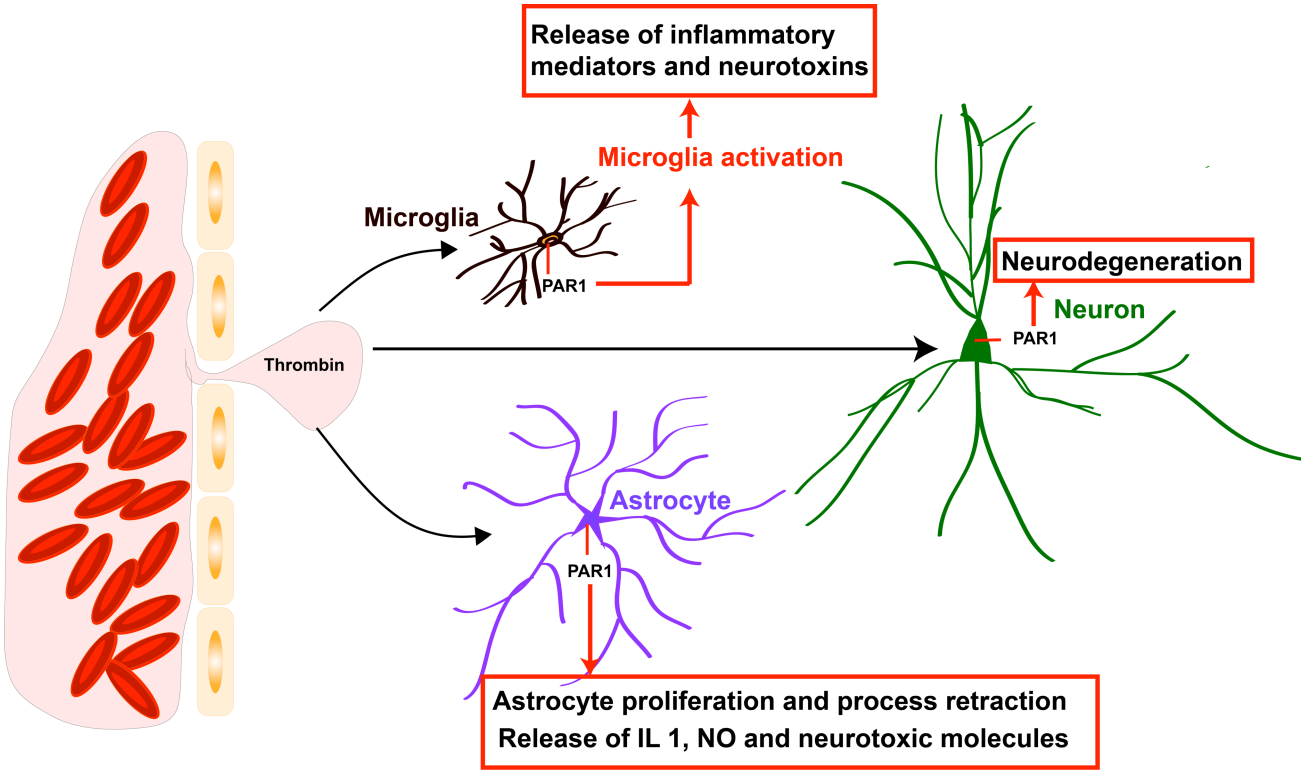

Figure 1.1. Adapted and simplified from: (Noorbakhsh, et al., 2003). PAR-1 release from astrocytes and microglia can be harmful to the neurons by producing neurotoxins. The neuroprotective and neurotoxic effect of PAR-1 receptor is dependent on the amount of thrombin activation. A lower dose of thrombin is neuroprotective whereas higher doses are neurotoxic.

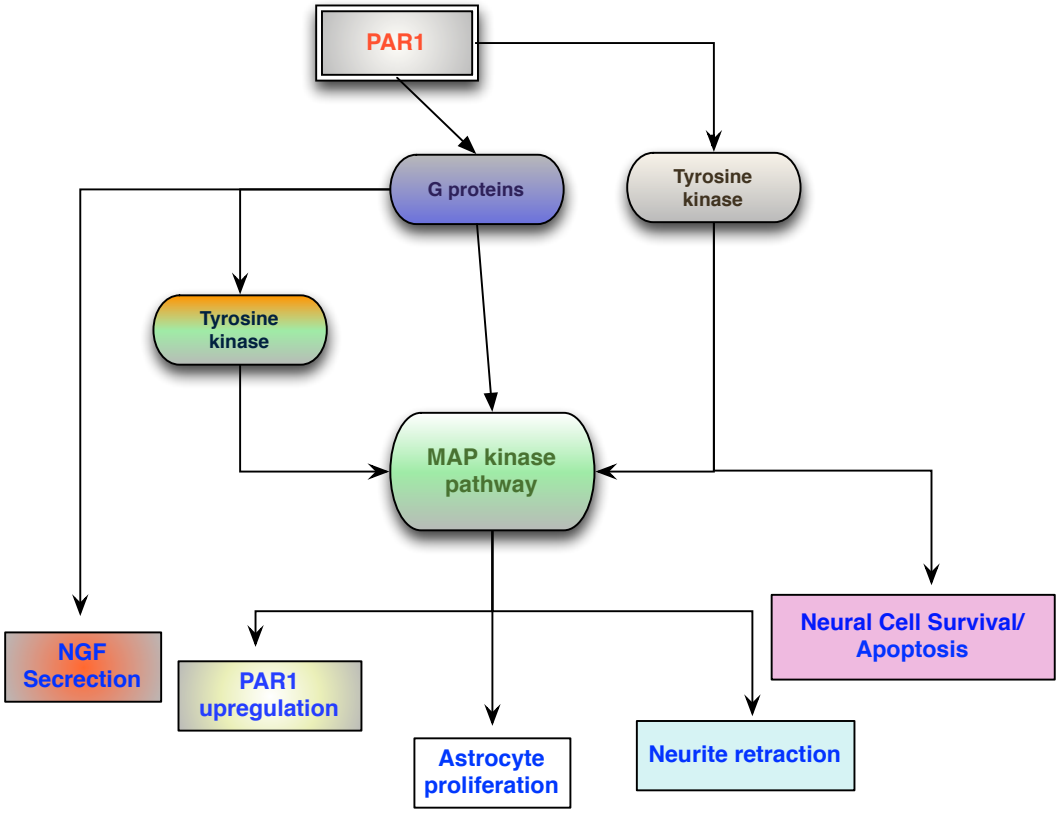

Figure 1.2. Adapted and simplified from: (Noorbakhsh, et al., 2003). This pathway represents the signaling cascade of PAR-1 receptor activation. The activation of PAR 1 different pathway mediated by G proteins can lead to the following: nerve growth factor (NGF) secretion, PAR 1 upregulation, astrocyte proliferation, neurite retraction and neural cell survival or apoptosis.

4.3. Iron

Iron is an essential metal that is normally required for many biological functions, including oxygen transport, DNA transport and mitochondrial oxidation (Schulz, et al., 2011). However after ICH, degrading erythrocytes release the non-protein bound iron (free iron - Fe^{2+}) from hemoglobin. The ferrous (Fe^{2+}) form of free iron can generate free radicals such as the hydroxyl radical ($OH^{\cdot}$). This occurs by the Fenton reaction and Haber-Weiss reaction leading to oxidative damage (Wu, et al., 2002). Free radicals are very toxic and can damage all the components of cells including proteins, lipids and DNA (Aronowski and Zhao, 2011, Siesjö, et al., 1989). The overall detrimental effect of free iron release from erythrocytes is explained in Figure. 1.3. Iron is one of the key molecules that are involved in secondary injury after ICH. The role of iron toxicity is further discussed in chapter 3, 4 and 5.

4.4. Iron and Oxidative Stress

Iron has the capacity to accept and donate electrons, which makes it physiologically available as an oxygen-binding molecule (hemoglobin) (Emerit, et al., 2001). However free iron in pathological condition damages tissue by catalyzing conversion of superoxide to hydrogen peroxide to free radical species (Emerit, et al., 2001). Our body deals with oxidative stress by using enzymes and non-enzymatic mechanisms of defense (Jørgensen and Nielsen, 2004). Glutathione (GSH) is a non-enzymatic endogenous anti-oxidant that is vital for iron metabolism and neutralization of free radicals and reactive oxygen species (Connor, et al., 2001). The ratio of oxidized and reduced glutathione (GSSH/GSSG) is important to measure the cellular toxicity.

Glutathione peroxidase (GPx) is an enzyme that requires GSH and NADPH to reduce hydrogen peroxide to water, thereby scavenging the free radical production. GSH is the main non-enzymatic defense of the brain (Haddad and Harb, 2005) and together with GPx and other enzymes and antioxidants it can attenuate lipid peroxidation. There are antioxidant rich drugs (e.g. sulforaphane) available to increase the antioxidant effect and promote neuroprotection after ICH (Zhao, et al., 2009). Apart from drugs environmental enrichment in rodents have been shown to reduce oxidative stress in ischemic stroke (Briones, et al., 2011). Knowing the significance of oxidative stress caused by iron, we further evaluated the role of iron in causing secondary damage (chapter 2).

4.5. Other Blood Components

There are other blood components that are involved in pathophysiology of ICH, which includes bilirubin, heme oxygenase-1, etc. The lysis of heme in hemoglobin molecule is degraded by an enzyme called heme oxygenase 1 (HO-1) and converts biliverdin to bilirubin. Bilirubin is a neurotoxin, which disrupts the plasma membrane of the cells (Brito, et al., 2008). *In vitro* experiments have shown that bilirubin leads to NMDA (N-methyl-D-aspartate) activation, which causes nitric oxide mediated excitotoxicity (Rodrigues, et al., 2002). Heme oxygenase 1 is an enzyme that is involved in oxidative stress induced cell injury post-ICH (Wang, et al., 2011). In summary, there are multiple factors that are involved in secondary injury post-ICH. However, understanding each blood components is essential to design a therapeutic drug that can act on multiple pathways of injury.

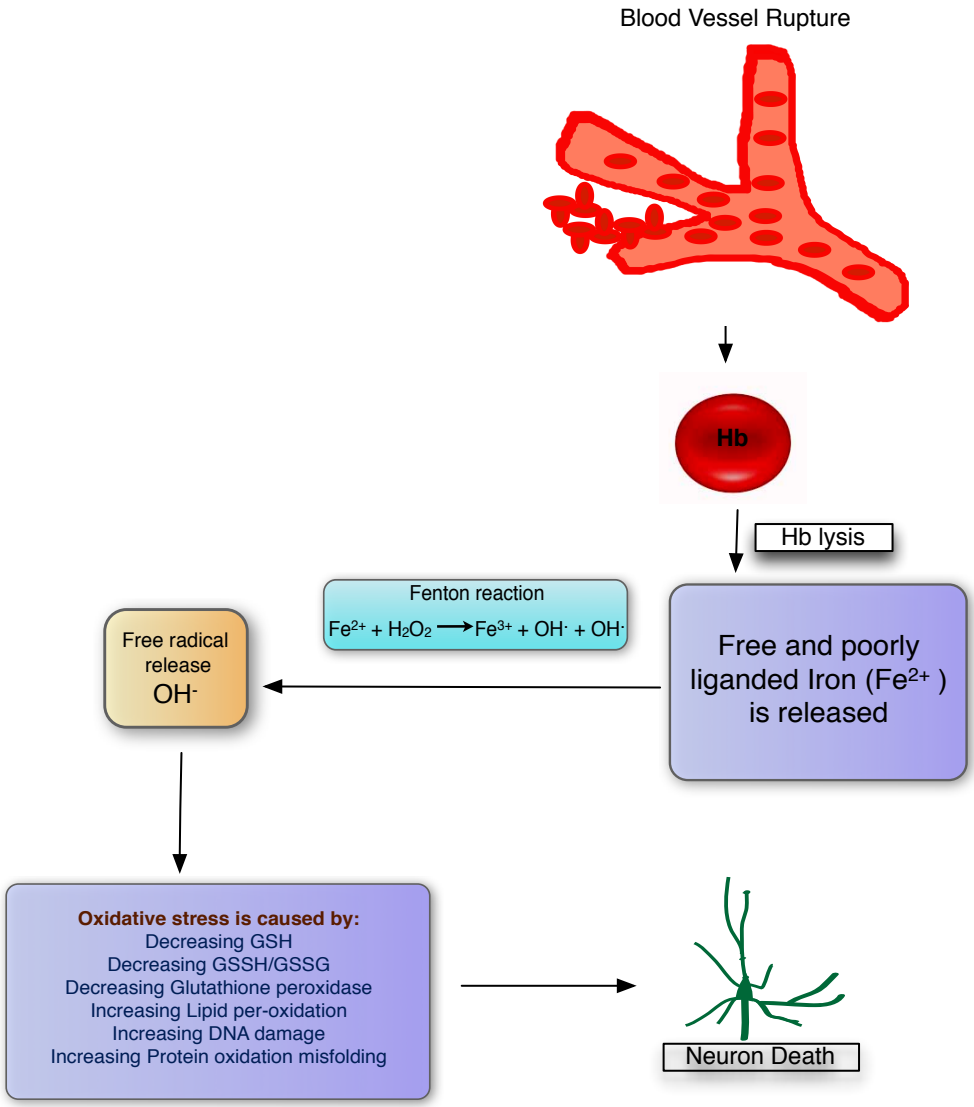

Figure 1.3. Overall Schematic Representation of the ways by which iron is toxic to the brain after an ICH (Adapted and modified from: Aronowski J and Zhao X, 2011).

4.6. Inflammation

Inflammatory reactions occur immediately after ICH, as soon as the blood components, including erythrocytes, leukocytes and macrophages enter the brain (Wang, 2010). Leukocytes are an important cell type that perform normal immunological defense. In patients leukocyte counts have been used as a predictor of early neurological deterioration in spontaneous ICH (Lee, et al., 1975, Leira, et al., 2004). Neutrophils also contribute to blood brain barrier damage, white matter damage (axons and myelin), ROS generation and secretion of pro-inflammatory proteases after ICH (Moxon-Emre and Schlichter, 2011, Weiss, 1989). Furthermore, depletion of neutrophils decreases white matter damage, blood brain barrier damage and inflammation (Moxon-Emre and Schlichter, 2011).

Microglia cells play a major role in clearing the hematoma (Schwartz, et al., 2003, Wang, 2010) through the induction of phagocytotic activity that clears debris, as well as releasing neurotrophic factors (Schwartz, et al., 2003, Wang, 2010). Inhibiting microglia activation with tuftsin fragment 1-3 has significantly reduced hematoma size and improved neurological function (Wang, et al., 2003, Wang and Tsirka, 2005). Minocycline is a drug that is commonly used to reduce microglia activation in rodent models of ICH. Delayed minocycline treatment (6 hours post-collagenase ICH) has been shown to reduce edema, microvessel loss, TNFα and MMP-12 expression, blood brain barrier breakdown and neurological deficit (Wasserman and Schlichter, 2007, Wasserman, et al., 2007, Wang, 2010, Xue, et al., 2010). The efficacy of minocycline remains controversial, however, because other studies show no beneficial effect of this drug on reducing neuronal death, infarct volume or functional impairment (Szymanska,

et al., 2006, Wasserman and Schlichter, 2007). Conversely TNFα is neuroprotective after ischemic infarction (Nawashiro, et al., 1997). MMPs are also involved in neuroplasticity mechanisms following stroke (Lo, 2008). Furthermore, we do not know whether reducing TNFα and MMP's are impeding plasticity. Thus, further efficacious studies are needed to better understand the positive and negative roles of anti-inflammatory drugs (Diguet, et al., 2004).

Astrocytes are important for the formation and maintenance of blood brain barrier, ionic balance and metabolism of toxins and also play a central role in the repair process (Kimelberg and Nedergaard, 2010, Wang and Dore, 2007). It has been shown post-ICH that astrocytes reduce oxidative stress by releasing glutathione (GSH) and preventing neuronal death (Pyo, et al., 2003, Wang and Dore, 2007). Thus, understanding the role of astrocytes, and finding ways to increase their activation could present a novel therapeutic strategy after ICH.

5. Stroke Recovery

Brain plasticity is crucial to the recovery of function in stroke patients. A variety of adaptive structural and functional changes occur within the brain following ICH. These recovery mechanisms are further explained in this section.

5.1 Factors Improving Recovery

After stroke, patients show spontaneous recovery due to blood flow reperfusion to the injured brain region that occurs after hours to days (Fieschi, et al., 1989, Jorgensen, et al., 1995, Zanette, et al., 1995). Other reasons for spontaneous recovery include the

resolution of edema, diaschisis and inflammation. Diaschisis occurs immediately after stroke when neurons far from injury are functionally impaired (i.e., a remote functional depression in areas connected to by not directly damaged by the stroke). It occurs due to the disruption of functional input from the injured region (Nudo, et al., 2001). Another important phenomenon in functional recovery mainly occurs because of behavioural compensation and neuroplasticity. Compensation is learning new strategies to perform a particular behavioural task, resulting from adaptation of motor elements or substitution of new motor patterns (Metz, et al., 2005, Takeuchi and Izumi, 2012).

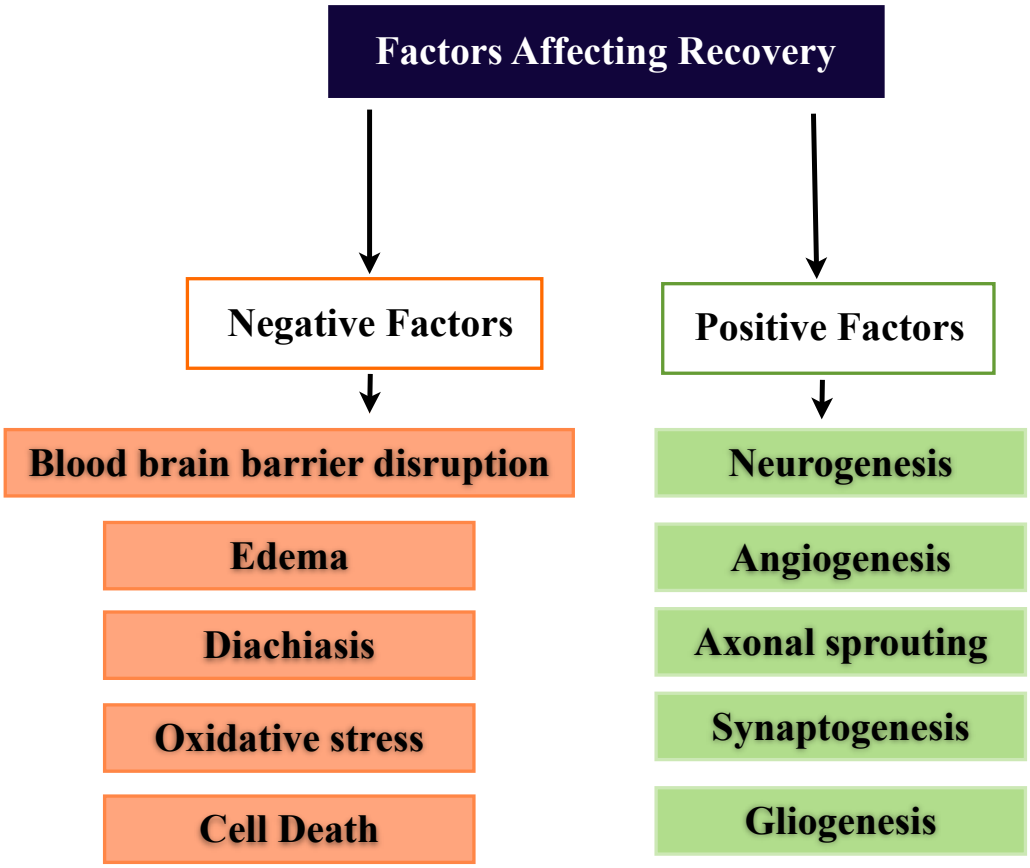

Figure 1.4. Schematics of factors affecting recovery

Factors that affect recovery include blood brain barrier disruption, edema, diachiasis, oxidative stress and cell death (Wieloch and Nikolich, 2006). The above-mentioned negative factors lead to cell death and exacerbate disease pathology by worsening the disability. Inhibiting the negative factors and promoting the cell survival is an effective way to promote recovery (Murphy and Corbett, 2009). Plasticity is an important positive factor of recovery, which occurs by alteration in synaptic network leading to strengthening of synaptic connection (Murphy and Corbett, 2009). Neuroplasticity is crucial for rewiring lost connections and helping the brain to recover after an insult. Synaptogenesis, angiogenesis, neurogenesis, gliogenesis and axonal sprouting, among other events, are important underlying neuroplasticity mechanisms contributing to behavioural recovery. Synaptogenesis is formation of new synaptic connections between neurons (Stroemer, et al., 1995). Axonal sprouting is caused by undamaged axons growing new nerve endings to reconnect neurons through forming new pathways (Stroemer, et al., 1995). The other factors that are involved in restorative process are angiogenesis and neurogenesis. Angiogenesis is formation of new blood vessel from the preexisting vessels and it leads to increased blood flow after stroke. Angiogenesis helps to increase the formation of new cells (neurogenesis). Some proteins like thrombospondins 1 and 2 cause the motor recovery in ischemic stroke (Jason, et al., 2008). Thrombospondins 1 and 2 are also involved in angiogenesis, synaptogenesis and axonal sprouting (Jason, et al., 2008). Gliogenesis is formation of new glial cells such as astrocytes, microglia, oligodendrocytes and ependymal cells. Microglia secretes various factors such as ions, proteases, cytokines, growth factors and neuromodulators that are necessary for synaptic plasticity (Tremblay and Majewska,

2011). Some important functions of astrocytes include maintenance of blood brain barrier and nervous system repair mechanisms by regulating blood flow and synapse formation through releasing growth factors (Sofroniew and Vinters, 2010). In summary the above-mentioned positive factors are very important for regulating neuroplasticity. Neuroplasticity can be up regulated by rehabilitation treatment. Therefore, in chapter 5 I evaluated the role of rehabilitation in promoting functional recovery after ICH.

5.2. Rehabilitation and Neuroplasticity

As mentioned earlier, rehabilitation is an effective treatment for mild and moderately suffering patients. In some cases even patients with severe stroke benefit from this therapy (Teasell, et al., 2005, Yagura, et al., 2005). Depending on the lesion location, severity, symptoms and preexisting comorbid conditions, the patients recover after stroke (Horn, et al., 2005). Comorbidity conditions can worsen stroke damage and lead to poor and slower recovery (Weimer, et al., 2005). The first goal of rehabilitation therapy is to help the patients return to normal life by allowing them to regain or relearn the skills of everyday living. The second goal of rehabilitation is to prevent the recurrence of a stroke or cardiovascular events that occur with greater frequency in hemorrhagic patients (Shah, 2006).

The importance of time in recovery is apparent in animal stroke research. Early rehabilitation has been shown to maximize the recovery and plasticity mechanisms after stroke (Biernaskie, et al., 2004). A study in ischemic stroke rats compared rehabilitation started after 5, 14 and 30 days post stroke. The results showed that a 5-day delay post-stroke provided the maximum functional recovery with increased dendritic plasticity in

the brain, whereas with the 14-day delay this benefit was decreased and with the 30-day delay there was no benefit (Biernaskie, et al., 2004). Like the animal data, clinical data suggests that early rehabilitation is associated with better outcome (Paolucci, et al., 2000). Nudo and colleagues in 1996 found that early training in skilled reaching prevents the loss of movement representation in peri-lesion cortex after focal cortical infarction in squirrel monkeys (Nudo and Milliken, 1996).

After an ICH, there is an increase in neuroplasticity if rehabilitation therapy is provided (Auriat, et al., 2010). However, less is known about the specific ways neuroplasticity can influence recovery after ICH. The data suggest that rehabilitation increases dentritic plasticity and astrocytic plasticity after ICH (Auriat, et al., 2010, Mestriner, et al., 2011). Furthermore, voluntary exercise has been shown to increase neural progenitor cells in the subventricular zone after ICH (Jin, et al., 2010). Similarly, in ischemic stroke, it has been shown that rehab increases dentritic plasticity, angiogenesis, growth factors (BDNF, NGF, etc.), neurogenesis and the formation of new synaptic connections (Gobbo and O'Mara, 2004, Komitova, et al., 2005, Komitova, et al., 2005, Murphy and Corbett, 2009, Nithianantharajah and Hannan, 2006, Zhao, et al., 2001). Given the state of knowledge regarding neuroplasticity following ischemia, additional studies are needed to verify whether the same mechanisms of recovery occur in hemorrhagic stroke.

5.3 Rehabilitation Intensity

The optimal intensity of rehabilitation therapy is unclear. The amount and duration of rehabilitation needed after stroke varies considerably. It is mostly dependent

on the patient's impairment, functional deficits and the rate of improvement (Kwakkel, et al., 2004, Schaechter, 2004). Animal studies have shown that early intensity therapy is detrimental after ischemic stroke (Humm, et al., 1998, Kozlowski, et al., 1996). This detriment from intense motor activity is due to an increase in tissue loss and excitotoxicty caused by hyperexcitability in areas surrounding the lesions (Risedal, et al., 1999). However, we also do not know whether the same excitotoxicty occurs after hemorrhagic insult. It has also been shown that early rehabilitation causes hyperthermia and aggravates the injury (DeBow, et al., 2004). However, clinical data suggests that when rehabilitation is started after the patients are stable, it provides an improved outcome (Kwakkel, et al., 1997). Patients who received 5 days a week therapy showed improved recovery and shortened length of hospital stay (Kwakkel, et al., 1997). The same conclusions are observed in animal data: 5 days a week of combined task-specific therapy (skilled reaching task), with enriched environment, is beneficial in improving functional recovery, decreasing tissue loss and dendritic plasticity after hemorrhagic stroke (Auriat and Colbourne, 2009).

5.4. Rehabilitation Types

5.4.1. Exercises

After stroke, impaired walking greatly contributes to functional disability in 63% of patients (Jorgensen, et al., 1999). Exercises are important to reduce the motor and sensory deficits occurring in patients suffering from stroke (Jorgensen, et al., 1999). The common exercises given to the stroke patients include aerobic exercises such as walking and swimming (Gordon, et al., 2004). The usual method to help patients walk is by

training them in treadmill exercise with or without body weight support, which allows for increase in their aerobic capacity and sensorimotor functions (Gordon, et al., 2004). It has been shown to increase independence in ADLs (activities of daily living), increase walking speed and efficiency, increase tolerance for improved physical activity, and finally reduce the risk of cardiovascular events (Gordon, et al., 2004). Task-specific treadmill exercise has shown to improve recovery by activating subcortical neural networks in patients who suffered from stroke (Luft, et al., 2008).

5.4.2. Environmental Enrichment

Enriched environment (EE) is widely used to induce brain plasticity after brain injury. The idea for enriched environment comes from report published by a Canadian scientist named Donald Hebb in 1940. Donald Hebb brought home laboratory rats as a pet for his children and he observed better problem-solving ability in those rats compared to rats housed in the lab (Hebb, 1947).

Environmental enrichments therapies have improved recovery in various types of brain damages such as hippocampal and cortical lesions, traumatic brain injury, global and focal ischemia and Alzheimer's disease (Belayev, et al., 2003, Biernaskie, et al., 2004, Biernaskie and Corbett, 2001, Einon, et al., 1980, Galani, et al., 1997, Herring, et al., 2010, Johansson, 1996, Kolb and Gibb, 1991). The key findings from animal research in global and focal ischemia suggests that EE decrease oxidative damage and ongoing neurodegeneration in hippocampus (Briones, et al., 2011), others found that EE promotes compensatory movements after global ischemia (Knieling, et al., 2009). Apart from improving the motor function EE also improves cognitive functions by improving

the learning impairment in morris water maze task after focal ischemia (Dahlqvist, et al., 2004). Additionally, EE also increases the expression of neurotrophic factors such as nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) (Johansson, 2004). There are plenty of studies, which evaluate EE on ischemic stroke, however few studies are available for ICH (Auriat, et al., 2010, Auriat, et al., 2009, Nguyen, et al., 2008). Pilot studies done on EE alone after ICH did not show any benefit (unpublished data), hence we combine the EE with skilled reaching task.

A very recent randomized clinical study has suggested that enriching the environment of patients by increasing their physical, cognitive and social activities is beneficial to patients (Janssen, et al., 2013). The study also recommended using environmental enrichments in all rehabilitation units. EE was effective in increasing activity in stroke patients and reduced the time spend inactive and alone. There has also been a lot of growing evidence on stroke units, which are encouraging patients to engage in various activities involving playing and singing among patients.

Stroke units may be a closest resemblance to environmental enrichment for stroke patients. Stroke units are dedicated units with specialized clinicians (e.g. neurologist) and paramedical professional (e.g. physiotherapist, speech therapist)) working for stroke patients (Sinha and Warburton, 2000). It has been shown that stroke units are beneficial to the patients in number of ways such as by improving functional outcome, decreasing the length of stay in hospital and reducing the mortality rate (Rønning, et al., 2001). The length of stay in general ward is 19 days, whereas in stroke units it is decreased to 15 days. This saves 240 million dollars a year based on the current rate of 50,000 stroke patients (Heart and Stroke Foundation, Canada) (Evans, et al., 2002). Stroke units

reduce the time spent inactive when compared to general ward (Jorgensen, et al., 1999, Jorgensen, et al., 2000).

5.4.3. Constraint-Induced Movement Therapy (CIMT)

Dr. Edward Taub developed the constraint induced movement therapy (Taub, et al., 1993). This therapy is used in stroke survivors who are affected by hemiparesis. In CIMT treatment the patients are allowed to restrain the unimpaired limb after injury and are in turn forced to use the impaired limb during normal daily activities and rehabilitation exercises (Taub, et al., 1999). The CIMT treatment is a solution to avoid further deterioration of affected upper limb; termed as "learned non-use". CIMT patients are often engaged to repetitive exercises with the impaired limb during the therapy (Taub, et al., 1999). As a result the brain develops new neural pathways and is referred to as cortical reorganization. This therapy mainly works by improving the function of impaired limb. It has been shown that 20-25% of ischemic stroke survivors benefit from CIMT (Taub and Morris, 2001). Clinically the main limitation of using CIMT is the need of minimum 10 degrees of active finger and wrist extension. Therefore it cannot be used for severe stroke patients. Furthermore this treatment also needs therapists to always be with the patients, which is expensive (Dombovy, 2009).

Animal studies also suggest that CIMT when combined with exercises beginning 7 days post-ICH significantly improves skilled reaching ability in rats (DeBow, et al., 2003). CIMT also promotes motor recovery by increasing the cortical organization after stroke in primates (Taub, et al., 1999). Though only few animal studies and clinical data

are available in ICH literature, they clearly suggest that combining CIMT with other exercises (tray task, running, walking) therapy is beneficial (DeBow, et al., 2003).

6. Animal Models of ICH

The patho-physiology of ICH is studied by mimicking ICH in various animal models using pigs and rodents. Common methods of inducing ICH in animals are injections of either bacterial collagenase or autologous blood into the striatum. Other methods include balloon infusion models and the spontaneously hypertensive model. These models have contributed significantly to our knowledge of ICH-induced injury mechanisms especially about the role of edema formation, BBB disruption and specifically about blood components. Most importantly these models are providing us with knowledge about ICH induced biochemical and molecular events and enabling us to test potential pharmacological and rehabilitative therapies (Wagner, 2007).

The main advantages of using rodent models of ICH include commonly used species, well developed neurobehavioural paradigms, extensive studies on immunocytochemistry and molecular biology can be performed, transgenic and knock out animals are available to study the role of specific gene and protein functions (Kirkman, et al., 2011, Wagner, 2007). The main disadvantages include paucity of white matter compared to humans. Hence using a pig or primate would be advantageous to study the white matter injury after ICH, however these species are expensive to purchase and housing them requires special facilities and veterinary care. For my thesis, I have used different models of ICH in rats, which include iron and thrombin infusion models, the whole blood model and the collagenase ICH model. Hence I will discuss

briefly the advantages and disadvantages of each model in this section. No models of ICH are perfect, which is the reason we tried to use multiple models of ICH to study the injury and recovery mechanisms after ICH.

6.1. Autologous Blood Model

The autologous blood model was one of the earliest animal models of stroke and was developed in 1980's (Bullock et al, 1984). It involves injecting blood taken from the tail artery of the rat to the rat striatum and mimics ICH. This model is widely used to mimic a single large bleed that occurs in patients. This model is ideal for studying the mechanisms of mechanical injury after ICH injury (MacLellan, et al., 2008). The disadvantage of the autologous blood model is that it does not replicate the small vessel rupture scenario along with microvascular breakdown effects (Kirkman, et al., 2011). It does not cause spontaneous bleeding and is also not suitable to study the rebleeding situation. This model also causes ventricular rupture during the infusion and also causes back flow along the needle. These problems can lead to intraventricular and subarachnoid leakage of blood.

6.2. Collagenase ICH Model

The Collagenase ICH model was developed in the 1990's (Rosenberg, et al., 1990). This model is widely used because it mimics the spontaneous bleeding that occurs in patients. Collagenase is a bacterial enzyme that dissolves the basal lamina and extracellular matrix of blood vessels, which causes spontaneous bleeding to occur. This model has been shown to imitate the bleeding and rebleeding phenomenon that occurs in patients. The reproducible spontaneous hemorrhage can be correlated with the

amount of collagenase injected and the blood vessel leakage does not develop needle track backflow. This model is also useful to study the long-term functional outcomes following ICH (MacLellan, et al., 2008). The main disadvantage of this model is that collagenase dissolves the extracellular matrix around capillaries to produce hemorrhage, this model differs from the arterial rupture that occurs in human ICH (Del Bigio, et al., 1996, Del Bigio, et al., 1999).

6.3. Simple Models of Injury

Simple models are best suited to isolate and study the individual component of ICH such as thrombin and iron (Nakamura, et al., 2006, Xi, et al., 1999, Xue and Del Bigio, 2001, Yang, et al., 2008). Hence for my study I used the simplified model to study the pathophysiology of iron and thrombin. Some disadvantages of these models are that they can mimic only components of ICH such as thrombin and iron (Kirkman, et al., 2011). Iron or thrombin is injected into the brain, which of course does not exactly replicate what happens ICH patients (e.g., time course of iron release after bleeding is considerably slower than just injecting iron). However, we can improve this method by using osmotic infusion pumps and causing slow release of thrombin and iron (MacLellan, et al., 2012).

6.4. Other Models

The inflation of a needle mounted microballoon causes mechanical damage that occurs in ICH. This model is used to study the mass effect of hematoma and also for examining the isolated role of physical injury (Sinar, et al., 1987). In addition, the

relationship between elevations of ICH and local perfusion pressure can be mimicked by inflatable microballoon (Kingman, et al., 1988). The main disadvantage of the balloon inflation model is that it does not produce blood and hence the role of blood and its breakdown products cannot be studied using this model.

Okamoto and colleagues developed the Spontaneously Hypertensive Rat (SHR) in the 1960's by breeding Wister-Kyoto rats with high blood pressure. They developed two breeds, (1) SHR – Spontaneous Hypertensive Rats with elevated blood pressure of about 180 mmHg systolic and (2) SHRSP – Spontaneous Hypertensive Stroke Prone Rats (SHSPR) with highly elevated blood pressure of about 220 mmHg systolic. The SHRSP is a sub strain of SHR, however they are more efficient in producing signs of hemorrhagic stroke and malignant hypertension. The SHR and SHRSP have higher face validity for studying the pathology process of human stroke (Bailey, et al., 2011). The main disadvantage of using SHSPR model is that the hemorrhage occurs only after 20 weeks. However, it can be hastened by providing the animals with a high salt diet. One other drawback of SHR rats is that they are very expensive. The difficulty of using this model in research is because location and timing of injury and severity is not well controlled. Hence it is not a suitable model for doing behaviour studies and injury progression and it is hard to state what behaviour deficits will occur and when it occurred.

7. Basic research versus translational research issues and recommendations

For developing clinically effective neuroprotective treatments, understanding the complex network of events that occur after ICH is very important. Basic research plays

an important role to unravel these complex events. Listed below are some main issues in basic research (Kirkman, et al., 2011, MacLellan, et al., 2012).

One major issue is that most stroke experiments are conducted in young healthy animals, whereas clinical ICH occurs mainly in the aged population. Furthermore, the majority of experimental ICH studies are performed exclusively in male rats. Hence, there is less understanding of the pathophysiology of female gender, which may exhibit inherent differences. As a corollary, more studies are needed to elucidate the role played by sex hormones in ICH susceptibility and recovery. Another issue is that studies are carried out mainly in rodents, which possess less white matter when compared to humans. The comorbid conditions of diabetes, obesity, hypertension, cholesterol, which are known to be the main contributing factors to ICH, are also usually ignored. As no model of ICH perfectly mimics human ICH, there is a need to study the pathophysiology and neuroprotective treatment in multiple models of ICH. Studies need to incorporate physiological variables such as measurement of temperature, blood gas (pO2), glucose levels and blood pressure. Furthermore, journals should encourage researchers to publish negative results to ensure that the best treatments are selected for clinical trials. In basic research, most of the treatments are only tested for short-term effects, whereas long-term studies are needed to determine if a treatment affects the neuroplasticity mechanisms of recovery.

8. Objectives of the Present Thesis

As outlined above, with the exception of supportive rehabilitation care, there are no approved treatments for ICH. Therefore, the 3 main objectives of this thesis are to

explore the mechanisms of secondary pathophysiology, discover treatment options and promote recovery mechanisms post-ICH. To this end I have divided the thesis into the following 4 sections:

(1) **Thrombin causes striatal neurons to atrophy and acute injury but does not trigger progressive tissue loss: implication for intracerebral hemorrhage.**

(2) **Intra-parenchymal ferrous iron infusion causes neuronal atrophy, cell death and progressive tissue loss: implications for intracerebral hemorrhage.**

(3) **Bipyridine, an iron chelator, does not lessen intracerebral iron-induced damage or improve outcome after intracerebral hemorrhagic stroke in rats.**

(4) **Rehabilitation improves behavioural recovery and lessens cell death without affecting iron, ferritin, transferrin or inflammation after intracerebral hemorrhage in rats.**

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Chapter 2

Thrombin causes striatal neurons to atrophy and acute tissue damage but does not trigger progressive tissue loss: implication for intracerebral hemorrhage

Manuscript in preparation.

Abstract

Intracerebral hemorrhage (ICH) is a devastating stroke causing considerable tissue damage from blood vessel disruption. Thrombin is released immediately to stop the bleeding. However increased thrombin expression after ICH has been related to secondary injury post-ICH. Hence we evaluated the role of thrombin in causing injury after intracerebral hemorrhage. The rats were given infusions of thrombin versus saline control followed by behavioural assessment using the corner turn test. At day 7 the rats were euthanized to perform the Golgi-Cox staining to measure the neuronal morphology (dendritic branches and length). Last, rats survived 7 or 60 days post-thrombin injection for histological assessment. Thrombin caused the surviving neurons in the peri-injury zone considerable atrophy (vs. contralateral side and controls). Finally, tissue loss occurred at 7 and 60 days. However thrombin caused acute tissue loss but not chronic damage. In summary thrombin causes surviving neurons to atrophy along with acute tissue damage but does not cause long-term tissue loss.

Introduction

Intracerebral hemorrhage (ICH) is caused by extravasation of blood after a vessel ruptures into the brain parenchyma. This devastating stroke causes higher mortality rate than ischemic stroke, and in survivors, long-term neurological problems (Sacco et al., 2009). The primary damage after ICH results from mechanical trauma as blood dissects through tissue (Frantzias et al., 2011). Conversely, secondary damage results from various factors, including thrombin and iron. Iron that is released from rupturing erythrocytes causes cell death, chronic neurodegeneration, edema, behavioural dysfunction and oxidative stress (Caliaperumal, et al., 2012, Gu, et al., Wu, et al., 2011, Wu, et al., 2002, Xi, et al., 2006). The study described in here was conducted to evaluate the role of thrombin in mediating secondary injury following ICH.

After an ICH thrombin is released immediately to stop the bleeding. Thrombin is a blood-derived serine protease essential for coagulation of blood and is abundantly present in hematoma after ICH. The secondary injury due to thrombin occurs due to protease activated receptors (PARs) activation (Babu, et al., 2012). High concentrations of thrombin activity have been noted after ICH (Gong, et al., 2008). High thrombin concentration causes neuronal cell death as well as brain edema and behavioural dysfunction (Hua, et al., 2007). For example, cultured neurons and astrocytes experiments have shown that thrombin induces cells death mediated by apoptosis (Vaughan, et al., 1995). Similarly an intrastriatal injection of thrombin also increases apoptotic cells, edema formation, blood brain barrier disruption and causes brain damage (Lee, et al., 1996, Lee, et al., 1997, Xue, et al., 2009). Both the *in vitro* and *in vivo* studies have shown that over-activation of thrombin leads to deleterious effects.

The effects of thrombin are mediated by protease-activated receptors, PAR1, PAR3, and PAR4 (Xi, et al., 2003). Expression of thrombin receptor is seen in neurons and astrocytes and has been reported in human brain tissue (Weinstein, et al., 1995). Findings suggest that PAR1 mediates some pathological effects of thrombin and participates in pathophysiological events induced by intracerebral hemorrhage (Xue, et al., 2009). Additional evidence of thrombin-mediated damage after ICH comes from studies that limited toxicity by thrombin inhibitors such as hirudin and argatoban (Nagatsuna, et al., 2005, Ohnishi, et al., 2010, Xue, et al., 2009). Argatroban has been shown to reduce the ICH induced injury when delayed and systemic administration was performed (Nagatsuna, et al., 2005). Agatroban treatment (3 hours) after intracerebral blood infusion causes significant reduction in edema (Kitaoka, et al., 2003). After collagenase-induced ICH argatroban treatment has shown to decrease the edema formation and inflammation (Nagatsuna, et al., 2005). Hirudin has also been shown to reduce perihematomal brain edema and improve neurological deficits (Hua, et al., 2002).

Apart from causing brain injury, lower doses of thrombin are neuroprotective *in vitro* and *in vivo*. *In vitro* studies have shown that thrombin protects rat astrocytes and hippocampal neurons from oxidative stress and hypoglycemia-induced cell death (Striggow, et al., 2000, Vaughan, et al., 1995). *In vivo* studies have shown that pretreatment of thrombin prevents cell damage induced by large dose of thrombin (Yajun, et al., 2002). Low dose of thrombin also causes neurogenesis and angiogenesis, which improves recovery after ICH (Yang, et al., 2008). However, inhibiting thrombin using hirudin blocks the neurogenesis (Yang, et al., 2008, Tsopanoglou and

Maragoudakis, 2007). Thrombin activates vascular endothelial growth factor, hypoxia inducible factor-1 and angiopoiten to induce angiogenesis (Neveu, et al., 1993). The current study shows the effect of thrombin on neuroplasticity, which is important for cell survival and recovery (Murphy and Corbett, 2009). Thus assessing whether thrombin causes dendritic changes, will give us a better understanding on their role in ICH.

Presently we used the intra-striatal infusion of thrombin as a simplified model (Lee, et al., 1995, Lee, et al., 1997, Xue and Del Bigio, 2001, Xue and Del Bigio, 2005) to test the role of thrombin in causing behavioural deficits, dendritic atrophy and early and late tissue loss. We evaluated turning bias at baseline and at 7 days post-thrombin infusion after 1U of thrombin was infused into the striatum of rats (Experiment 1). We deliberately used a moderate dose to avoid extensive striatal damage. A 1 U thrombin dose was subsequently used to produce a moderate lesion and using this dose we evaluated dendritic structure (Experiment 1). We used the golgi-cox stain to examine dendritic arborization. Finally, we compared histological outcome at 7 and 60 days after a 1U thrombin infusion (Experiment 2). We hypothesized that protracted neuronal death would occur leading to significant enlargement of tissue lost over time associated with thrombin accumulation.

Material and Methods

Animals

All procedures followed the guidelines of the Canadian Council of Animal Care and were approved by the Biosciences Animal Care and Use Committee at the University of Alberta. We used 50 male Sprague – Dawley rats (250-350g, ~3 months old) randomly assigned to 2 experiments (n = 9-15 each) with blinded assessment. Rats were single housed in standard polycarbonate cages (wood chip bedding), in both experiment 1 and 2. The influence of social housing on dendritic shape was avoided by single housing (Kolb et al., 1998). Food and water were provided ad lib and rats were kept in a temperature and humidity controlled room (lights on from 7 am – 7 pm).

Surgery (All Experiments)

Surgical procedures were performed aseptically. Rats were anesthetized with isoflurane (4% induction, 1.5-2.5% maintenance in 60% N_2O, balance O_2). Body temperature was maintained at 37°C during anesthesia with a heated water blanket and a rectal temperature probe. After placing the animals in a stereotaxic frame a hole was drilled 3.5 mm right and 0.2 mm anterior to Bregma. A 26 gauge needle was inserted 6.5 mm into the striatum to infuse 1 U thrombin contained in 30 ml of solution or saline alone over 10 minutes using an infusion pump (Nakamura et al., 2006). The needle was removed following an additional 10 minutes. A small metal screw was inserted in the hole, bupivacaine was applied and clips were used to close the wound (Caliaperumal, et al., 2012).

Behavioural Evaluation (Experiment 1)

Two groups (n = 9 and 11 each) were used (1 U thrombin infusion). Both groups were subjected to behavioural evaluation followed by Golgi-Cox stain at a 7-day survival. Previously it has been noted that dendritic atrophy occurs after 7 days after collagenase ICH (Nguyen, et al., 2008), hence we used the same survival time for our study.

Corner Turn Test

The turning bias was measured by corner turn test by placing rats in front of two angled Plexiglas walls (41 cm in height; 30.5 cm in length). The number of times the rats turn away after entering a 30° corner was counted (Hua et al., 2002; Warkentin et al., 2010). The striatal injured rat's turn in ipsilateral direction when exiting. Testing was done on the day prior to thrombin infusion and 7 days post-injection (Caliaperumal, et al., 2012).

Golgi-Cox Staining (Experiment 1)

Animals were euthanized (pentobarbital) at 7 days for Golgi-Cox staining. We evaluated the peri-lesional dendritic atrophy (i.e., enough striatum remained to obtain sufficient numbers of surviving striatal neurons). For this study, rats were euthanized at 7 days after thrombin (1 U, n=11) or a saline infusion control (n=9). Rats were transcardially perfused with saline and the brains were removed and immersed in Golgi-Cox solution. After 14 days of golgi cox incubation, sections were cut at 200 μm using vibrotome (Leica VT 1200S). The staining was performed according to established

procedures (Gibb and Kolb 1998; MacLellan et al., 2011). Five to six medium spiny neurons in the peri-insult and contralateral striatum were chosen and drawn per animal (20 × camera lucida). A clearly stained neuron with no obstruction by blood vessel was chosen and drawn. Sholl analysis and branch order analysis was performed, which estimates the dendritic length and branches. The above mentioned analysis evaluates the complexity of a neuron (Kolb et al., 1998; MacLellan et al., 2011). These data were averaged per region per animal (Caliaperumal, et al., 2012).

Lesion Volume (Experiment 2)

Rats were euthanized with pentobarbital (100 mg/kg IP) and transcardially perfused with 0.9% saline, then formalin. Coronal frozen section at 40 μm thickness were cut and stained with cresyl violet staining. Lesion volume was determined with Scion Image J (4.0; Scion Corporation, Frederick, MD) as routinely done on digitized images of coronal brain sections taken so that they extended from anterior, through and beyond the sections with obvious tissue damage (Auriat et al., 2012; MacLellan et al., 2006). The volume of tissue lost was calculated: Tissue lost (mm^3) = volume of normal hemisphere - volume of injured hemisphere. Hemisphere volume = average (area of the complete coronal section of the hemisphere – area of ventricle – area of damage if any) × interval between sections × number of sections.

Statistical Analysis (All Experiments)

Data are presented as mean ± standard deviation (SD). Data were analyzed by analysis of variance (ANOVA) with Tukey HSD post-hoc testing when appropriate

(SPSS v.17.0, SPSS Inc, Chicago, IL).

Results

Exclusions

One rat from experiment 2 died during surgery. No other unplanned mortality occurred.

Experiment 1

Behavioural data were collected before and after thrombin infusion (Fig. 1). All animals had normal baseline corner turn test scores. Repeated-measures ANOVA on the corner turn test revealed a significant time effect ($p < 0.001$), time by group interaction ($p = 0.002$) but no group effect ($p = 0.07$). There was significant impairment 7 days post-thrombin infusion ($p < 0.0001$ vs. baseline) and also there was a significant difference between groups ($p = 0.003$). Thus, post-infusion scores were higher than baseline (i.e., turn bias). The saline controls, showed normal behavioural scores before and after infusion ($p = 0.507$) (e.g., no bias in the corner turn test).

In the Golgi-Cox study, the striatal neurons (Fig. 2 B) near the lesion were markedly atrophied compared to the saline control (Fig. 2 D). Sholl analysis (Fig. 3 A) estimates dendritic length, showed that surviving peri-lesion neurons had considerably shorter dendrites ($p < 0.001$). The comparison of ipsi- and contralateral neurons within the thrombin group was significant ($p < 0.001$). Whereas the comparison of ipsi and contralateral neurons within saline group; was not significant ($p = 0.109$). For the branch order analysis (Fig. 3 B), which reflects dendritic complexity, we found that

thrombin showed significantly fewer branches compared to saline group ($p < 0.001$). Thrombin group had fewer lower order branches (1 through 6, $p \leq 0.022$ vs. saline). The 7th order branch was near zero normally and thus was not significantly different ($p = 0.111$). There was a branch order main effect ($p < 0.001$) group effect ($p < 0.001$) and interaction ($p < 0.001$). The contralateral side (normal side) was not significantly different between thrombin and saline group in the order of branches (1 through 7, $p \geq 0.111$). Thus, thrombin infusion significantly reduced dendritic length and number of branches in the surviving neurons in peri-lesion zone. The saline control infusion did not have any injury and did not affect the dendritic structure.

Experiment 2

A 1 U infusion of thrombin caused considerable tissue loss at 7 (Fig. 4 A) and 60 days (Fig. 4 B). However, these groups were not significantly different from each other (Fig. 4 C, $p = 0.803$).

Discussion

An intracerebral infusion of thrombin caused cell death and behavioural dysfunction as found by other studies (Babu, et al., 2012, Lee, et al., 1996, Lee, et al., 1997, Xue and Del Bigio, 2001). Our study is the first to find that thrombin, unlike iron, does not cause chronic injury. This suggests then that the thrombin produced after an ICH does not contribute to chronic degeneration, such as that found in the collagenase model (Caliaperumal, et al., 2012, Nguyen, et al., 2008). This striking dendritic atrophy likely contributes to behavioural dysfunction, but given that chronic cell death was not

significant it suggests that the initial neuronal atrophy is not lethal. Furthermore, the resolution of this atrophy over time is perhaps a mechanism of behavioural recovery.

Thrombin activates various deleterious pathways. For example, infusion of thrombin activates complement C9 on neuronal membranes, which is an immune reaction for cell lysis and inflammation. Inhibition of complement C9 by N-acetylheparin attenuates edema and neurological deficits (Gong, et al., 2005). Thrombin can also increase the pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α) and interleukin β (IL-β). Tumor necrosis factor α (TNF−α) has been increased after ICH and is implicated to cause edema (Rosenberg, et al., 1995). The knock out of TNF−α reduces brain edema and neurological deficits in mice (Barone, et al., 1997). Activation of Interleukin β (IL-β) by thrombin has deleterious role by causing neurotoxicity, blood brain barrier disruption and induction of apoptosis (Barone and Feuerstein, 1999).

So far studies have shown that low doses of thrombin are neuroprotective, and higher doses are neurotoxic. The present study suggests that a moderate dose causes short-term tissue loss but no long-term damage. There are several limitations of the present study. Firstly, we did not measure whether dendritic atrophy would persist beyond the short-term survival time we used (day 7). Previously it has been shown that after collagenase ICH there is initial atrophy of dendrites and they are recovered at day 60 (Nguyen, et al., 2008). Secondly, we did not evaluate the role of thrombin receptors such as PAR1, PAR2, PAR3 and their temporal expression pattern after ICH. Future experiments are needed on PAR receptors and their role after thrombin infusion. Thirdly, a typical bleed of 100 μl of blood produces about ≈ 36 U of thrombin (Lee, et

al., 1996). Experimental studies have shown that there is over-expression of PAR-1 receptor and thrombin production in the hematoma after ICH (Guan, et al., 2004, Lee, et al., 1997, Xi, et al., 2006, Xi, et al., 2003). However we do not know whether there are enough thrombin inhibitors to reduce the thrombin overproduction. So future studies are needed to evaluate the time course of thrombin and thrombin inhibitors (e.g. serpins, thrombomodulin) expression pattern after an ICH.

In summary, our study shows that thrombin causes substantial dendritic damage, and acute tissue loss but does not cause ongoing chronic injury. These findings strongly support that thrombin contributes to short-term damage leading to secondary degeneration occurring after striatal ICH. Therefore, future studies must evaluate the deleterious and neuroprotective effect of thrombin after ICH.

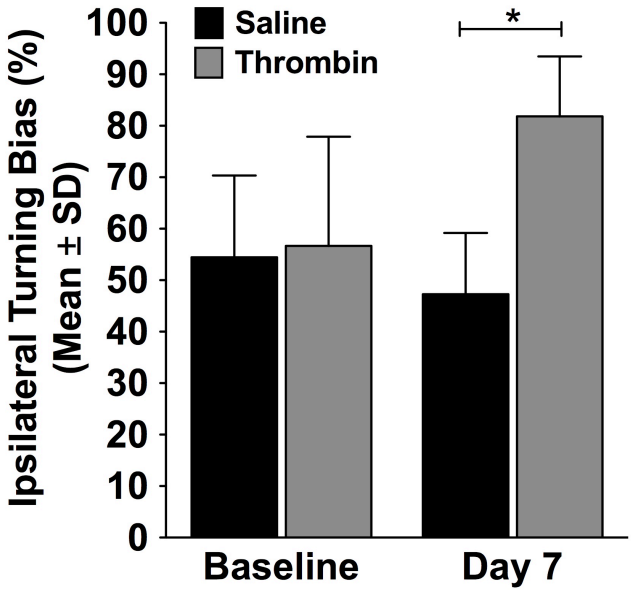

Figure 1. Thrombin infusion (1 U) caused significant impairment (bias) on the corner turn test. After post-thrombin infusion scores were significantly different from baseline and saline group (*$p < 0.05$).

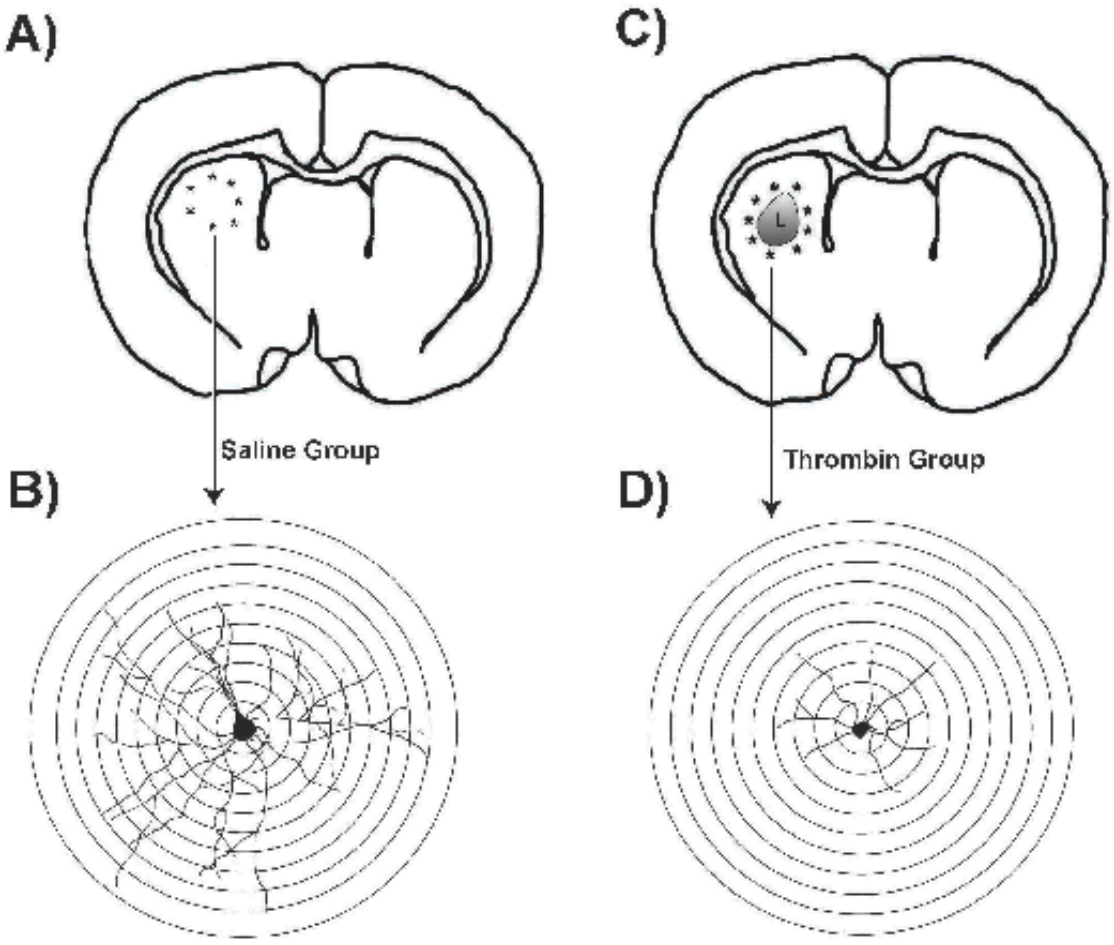

Figure 2. The lesion was indicated by ('L') and (*) indicates the surrounding cells that were drawn after Golgi-Cox staining (A). Contralateral striatal neurons were also drawn; saline group was used as a control. Representative drawings of peri-lesion zone atrophied striatal neuron (D) and a normal cell from the contralateral striatum (B). The surrounding concentric circles in the Sholl analysis method (B & D), is a standard measure of dendritic length.

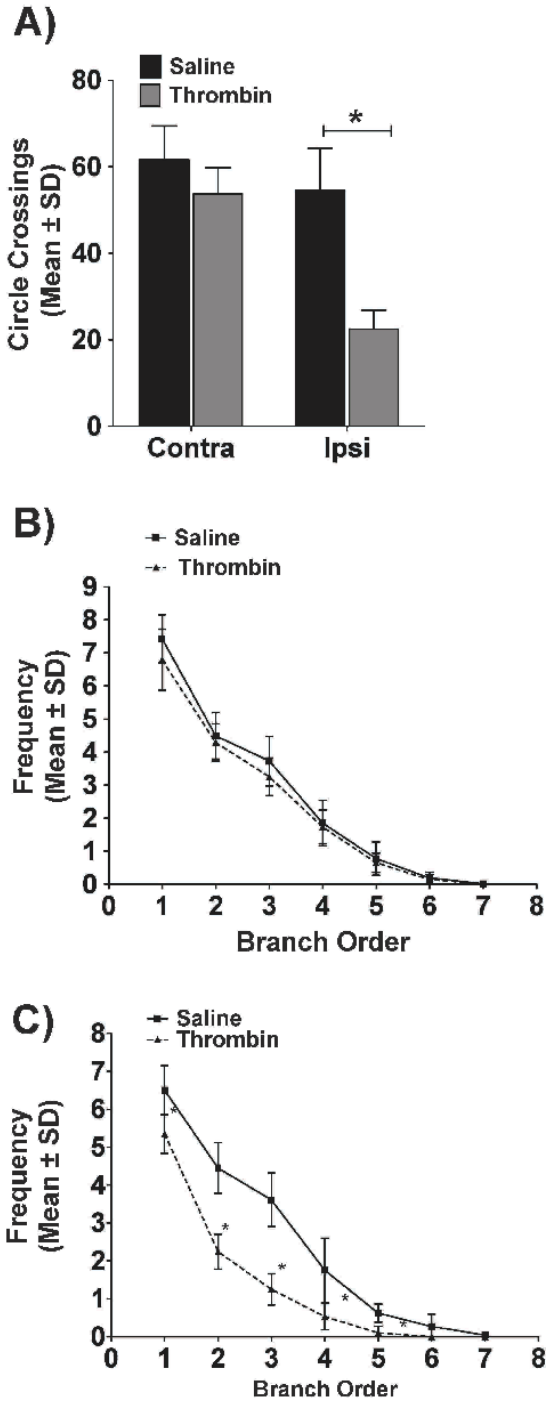

Figure 3. Sholl analysis showed a considerably reduced dendritic length in the peri-lesion zone (*$p < 0.05$). As well, branch order analysis (1st, 2nd, etc.) shows a significant reduction in the number of neuronal branches of thrombin group on the injected side compared to the saline control group (C, *$p < 0.05$).

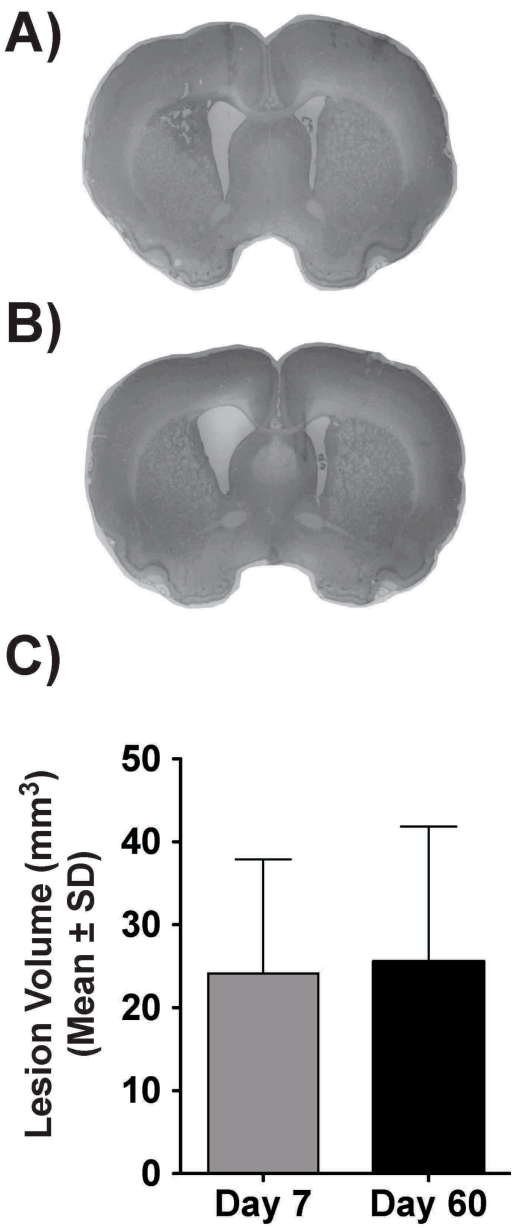

Figure 4. Representative photomicrographs showing the typical injury at 7 (A) and 60 days (B) after thrombin infusion. The quantification of the lesion volume showed no difference between days 7 versus 60.

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Chapter 3

Intra-parenchymal ferrous iron infusion causes neuronal atrophy, cell death and progressive tissue loss: implications for intracerebral hemorrhage

A version of this chapter has been published. Caliaperumal *et al*. 2012. *Experimental Neurology*. 15:363-9.

Abstract

Intracerebral hemorrhage (ICH) is a devastating stroke causing considerable tissue destruction from mechanical trauma and secondary degeneration. Free iron, released over days from degrading erythrocytes, causes free radicals that likely contribute to delayed injury. Indeed, an intracerebral injection of iron rapidly kills cells and causes cerebral edema. We expanded upon these observations by: determining a dose-response relationship of iron infusion, examining the structural appearance of surviving striatal neurons, and evaluating injury over months. First, we measured 24-h edema in rats given 3.8, 19.0 or 38.0 μg infusions of $FeCl_2$ (i.e., 30 μL of a 1, 5 or 10 mmol/L solution). Second, rats were given these infusions (vs. saline controls) followed by behavioural assessment and histology at 7 days. Third, dendritic structure was measured in Golgi-Cox stained neurons at 7 days after a 0.95-μg dose (30 μL of a 0.25 mmol/L solution). Last, rats survived 7 or 60 days post-injection (19.0 μg) for histological assessment. Larger doses of iron caused greater injury, but this was generally not reflected in behaviour that indicated similar deficits among the 3.8 – 38.0 μg groups. Similarly, edema occurred but was not linearly related to dose. Even after a low iron dose the surviving neurons in the peri-injury zone were considerably atrophied (vs. contralateral side and controls). Finally, continuing tissue loss occurred over weeks with prominent neuronal death and iron-positive cells (e.g., macrophages) at 60 days. Iron alone may account for the chronic degeneration found after ICH in rodent models.

Introduction

Intracerebral hemorrhage (ICH) is a particularly devastating stroke causing higher death rates than ischemia, and in survivors, long-term neurological problems (Sacco et al., 2009). A better understanding of disease pathophysiology will improve treatment options. Since the initial damage results from mechanical trauma as blood dissects through tissue, investigators have been targeting secondary (delayed) damage, which is thought to result from factors such as thrombin, inflammation, and especially iron (Frantzias et al., 2011; Wang 2010; Xi et al., 2006).

After an ICH ferrous iron is released from lysed erythrocytes after hemoglobin breakdown (Wu et al., 2003). Parenchymal levels of non-heme and total iron increase over a few days and persist for months in animal models (Auriat et al., 2012; Hua et al., 2006; Wu et al., 2003) and patients (Wu et al., 2010). Ferrous iron reacts with H_2O_2 to generate reactive hydroxyl radicals ($OH\cdot$), and this oxidative stress damages proteins, lipids and DNA (Nakamura et al.,2005; Triggs and Willmore 1984). Apart from inducing cell death, iron also causes blood brain barrier dysfunction and cerebral edema (Huang et al., 2002). Indeed, direct proof of iron mediated toxicity was shown by injecting $FeCl_2$ (ferrous iron) into rat brain and observing DNA damage (Nakamura et al., 2005). Cell death and neuronal atrophy of cortical neurons have also been clearly demonstrated following $FeCl_2$ (Willmore et al., 1980) and $FeCl_3$ infusions (Reid et al., 1979). Additional evidence for iron-mediated secondary damage after ICH comes from studies that limited toxicity with free radical scavengers such as NXY-05 (Peeling et al., 2001), and iron chelators, such as deferoxamine (Gu et al., 2009; Hua et al., 2006; Huang et al., 2002; Song et al., 2008; Wan et al., 2006) and 2,2'-dipyridyl (Nakamura et

al., 2006; Wu et al., 2012). These promising data have lead to a clinical trial with deferoxamine, which is currently underway (Selim et al., 2011). It should be noted, however, that not all animal studies find that deferoxamine improves outcome after ICH (Auriat et al., 2012; Warkentin et al., 2010; Wu et al., 2011).

In animal models, secondary damage occurs over the first few days but sometimes for much longer. The extent and timing of injury has been studied in the whole blood and collagenase models. For instance, significant tissue loss occurs over weeks after collagenase infusion (MacLellan et al., 2008; Nguyen et al., 2008). Noting the persistence of iron in the brain after ICH, we hypothesized that such delayed tissue loss is mediated by iron. As well, striatal neurons in the peri-hematoma region have atrophied dendrites after ICH (Nguyen et al., 2008), which we hypothesize is partly caused by iron toxicity as shown, for example, to occur after cortical infusion of ferric iron in cat (Reid et al., 1979). One must distinguish this dendritic atrophy from the common usage of 'atrophy' to describe a general loss of tissue over time, as evidence by ventriculomegaly. The latter can be due to dendritic atrophy, cell death and other reasons (e.g., increased pressure within the ventricles). Dendritic arborization is a key measure of neuroplasticity and clearly an important factor in behavioural recovery (Kleim and Jones 2008; Kolb et al., 1998; Murphy and Corbett 2009). Thus, assessing dendritic atrophy as well as total atrophy is important.

Presently we used the intra-striatal infusion of $FeCl_2$ as a simplified model (Nakamura et al., 2006; Willmore et al., 1980; Willmore and Rubin 1982) to test the role of iron in edema, early and late tissue loss, and dendritic atrophy. We measured edema 24 h after 3.8 – 38.0 μg infusions (in 30 μL of saline) of $FeCl_2$ was infused into

the striatum of rats (Experiment 1). A range in doses was used to mimic the range in hematoma volume, edema and brain damage commonly encountered in ICH research, and as done by others (Nakamura et al., 2006). Next, we evaluated behaviour and lesion size at 7 days in groups given various doses of $FeCl_2$ or saline controls (Experiment 2). A 0.95 μg dose was subsequently used to produce a small lesion and using this dose we evaluated dendritic structure (Experiment 3). We used the Golgi-Cox stain to examine dendritic arborization. Finally, we compared histological outcome at 7 and 60 days after a 19.0 μg infusion of iron (Experiment 4). We hypothesized that protracted neuronal death would occur leading to significant enlargement of tissue lost over time associated with iron deposition.

Material and Methods

Animals

All procedures followed the guidelines of the Canadian Council of Animal Care and were approved by the Biosciences Animal Care and Use Committee at the University of Alberta. We used 84 male Sprague – Dawley rats (250-350g, ~3 months old) randomly assigned to 4 experiments (n=18-23 each) with blinded assessment. Rats were grouped 4/cage in standard polycarbonate cages (wood chip bedding), except for single housing used in experiment 2. There we wished to avoid any influence of social housing on dendritic shape (Kolb et al., 1998). Food and water were provided ad lib and rats were kept in a temperature and humidity controlled room (lights on from 7 am – 7 pm).

Surgery (All Experiments)

Surgical procedures were performed aseptically. Rats were anesthetized with isoflurane (4% induction, 1.5-2.5% maintenance in 60% N_2O, balance O_2). Body temperature was maintained at 37°C during anesthesia with a heated water blanket and a rectal temperature probe. After placing the animals in a stereotaxic frame a hole was drilled 3.5 mm right and 0.2 mm anterior to Bregma. A 26 gauge needle was inserted 6.5 mm into the striatum to infuse FeCl2 (0.95, 3.8, 19.0 or 38.0 μg contained in 30 μl of a 0.25, 1, 5 and 10 mmol/L solution of $FeCl_2$ in saline, respectively) or saline alone (at pH of 4 or 5.2) over 10 minutes (Nakamura et al., 2006). The needle was removed following an additional 10 minutes. A small metal screw was inserted in the hole, bupivacaine was applied and clips were used to close the wound.

Brain Water Content (BWC) (Experiment 1)

The BWC was measured 24 hours after infusing 3.8, 19.0 or 38.0 μg of iron (n=5, 5, 8 each). Briefly, animals were anesthetized with isoflurane and quickly decapitated. The brain was blocked 2 mm anterior to 2 mm posterior to the injection and separated into cortex and striatum. The cerebellum served as a control. The wet-dry weight method was used. Basically, sample wet

weight was taken before and after 24 h at 100°C. We calculated BWC as (Wet Weight – Dry Weight) / Wet Weight)*100 (Wu et al., 2003).

Behavioural Evaluation (Experiment 2)

Three groups (n=6, 6 and 5 each) were initially done (3.8, 19.0 or 38.0 μg

infusions). Subsequently, we added two control groups (saline at pH of 4 and 5.2; n= 4 each) to approximate the pH range of $FeCl_2$ groups. This allowed us to test whether the injury was due to acidity, which previous studies indicate is not the case (Willmore et al., 1980; Willmore and Rubin 1982). All of these groups were subjected to behavioural evaluation followed by lesion volume determination at a 7-day survival.

Neurological Deficit Scale

A neurological deficit scale (NDS) that is sensitive to striatal damage (Del Bigio et al., 1996; Hua et al., 2002; MacLellan et al., 2006; Peeling et al., 2001) was used at 1, 4 and 7 days post injection (vs. day before injection). Briefly, the rats were evaluated on: spontaneous circling, hind limb retraction, bilateral forepaw grasp, contralateral forelimb flexion, and beam walking. A maximum score of 14 denotes greatest impairment.

Forelimb Asymmetry

Rats were placed in the vertical cylinder (45 cm in height and 20 cm in diameter) and video recorded for ~10 minutes on the day prior to iron injection and 7 days afterwards. This 'cylinder test' is used to evaluate forelimb contact during wall exploration, and is sensitive to striatal injury. The % ipsilateral forelimb use was: (ipsilateral forelimb contact + ½ both) / (contralateral forelimb contact + ipsilateral forelimb contact + both) × 100 (Hua et al., 2002; MacLellan et al., 2006).

Corner Turn Test

The corner turn test measures turning bias when rats turn away after entering a 30° corner (Hua et al., 2002; Warkentin et al., 2010). Rats are placed in front of two angled Plexiglas walls (41 cm in height; 30.5 cm in length) and those with striatal injury turn ipsilateral when exiting. Testing was done on the day prior to iron injection and 7 days post-injection.

Lesion Volume (Experiment 2 and 4)

Based upon Experiment 2, we selected the 3.8-µg dose for the last experiment to allow for injury progression to occur and to avoid possible ceiling effects with severe insults or floor effects with very mild insults. Rats were euthanized with pentobarbital (100 mg/kg IP) and transcardially perfused with 0.9% saline, then formalin. Coronal frozen sections (40 µm) were stained with cresyl violet. Lesion volume was determined with Scion Image J (4.0; Scion Corporation, Frederick, MD) as routinely done on digitized images of coronal brain sections taken so that they extended from anterior, through and beyond the sections with obvious tissue damage (Auriat et al., 2012; MacLellan et al., 2006). The volume of tissue lost was calculated: Tissue lost (mm^3) = volume of normal hemisphere - volume of injured hemisphere. Hemisphere volume = average (area of the complete coronal section of the hemisphere – area of ventricle – area of damage if any) × interval between sections × number of sections.

Golgi-Cox Staining (Experiment 3)

We first conducted a pilot study with a low dose of FeCl2 (0.95 µg, n= 3) where

animals were euthanized (pentobarbital) at 7 days for assessing lesion volume (as in Experiments 2 and 4). We deliberately used a low dose to avoid causing extensive striatal damage that would prevent us from evaluating peri-lesional dendritic atrophy (i.e., enough striatum remained to obtain sufficient numbers of surviving striatal neurons). For this study, rats were euthanized at 7 days after iron (0.95 μg, n=11) or a saline infusion control (n=9). Rats were transcardially perfused with saline and the brains were removed and immersed in Golgi-Cox solution for 14 days. Vibratome sections were cut at 200 μm (Leica VT 1200S) and stained according to established procedures (Gibb and Kolb 1998; MacLellan et al., 2011). Medium spiny neurons in the peri-insult and contralateral striatum were drawn (20 × camera lucida). Neurons (n=5/6 per region / animal) had to be fully impregnated and unobstructed (e.g., by blood vessels) to be drawn. These were quantified using Sholl analysis, which estimates the dendritic length, and branch order analysis to evaluate complexity (Kolb et al., 1998; MacLellan et al., 2011). These data were averaged per region per animal. Note that it is not possible to accurately determine lesion volume from Golgi-Cox stained tissue due to the nature of the stain and the use of thick sections necessary for determining dendritic arborization.

Fluoro-Jade Stain (Experiment 4)

The Fluoro Jade B stain marks degenerating neurons (Schmued and Hopkins 2000). Slides were rinsed in water and then dehydrated using alcohols and incubated with 0.06% potassium permanganate for 15 minutes. Slides were then washed with water, incubated in 0.001% FluoroJade (Chemicon, Temecula, CA) for 30 minutes in

the dark, and cover slipped. Sections at

the level of significant injury were qualitatively evaluated to determine whether there was recent neuronal death or not.

Perls' Stain (Experiment 4)

Perls' stain (Wu et al., 2003) was used to identify iron-positive cells (e.g., macrophages). 4% potassium ferrocyanide was mixed with 4% HCl into which sections were incubated for 60 minutes. The sections were then washed with water for 10 minutes. 0.4% DAB solution was prepared and slides were incubated for 45-60 minutes. Then the slides were quickly dehydrated with alcohol and kept in Citrosolv (Fisher Scientific, USA) before cover slipping.

Statistical Analysis (All Experiments)

Data are presented as mean ± standard deviation (SD) except NDS, which is reported as median. Data were analyzed by analysis of variance (ANOVA) with Tukey HSD post-hoc testing when appropriate. For NDS we used Kruskal Wallis or Mann Whitney tests (SPSS v.17.0, SPSS Inc, Chicago, IL).

Results

Exclusions

One rat from experiment 1 died during surgery. No other unplanned mortality occurred. Also, whereas other studies have noted the occurrence of seizures following

cortical injections of iron, we observed none in our striatal infusion paradigm. Finally, two rats in experiment 4 were excluded because of technical problems with surgery.

Experiment 1

Significant edema (24 h survival), comparable to that commonly reported in ICH models, occurred in ipsilateral striatum and cortex for all groups ($p \leq 0.004$ vs. contralateral side, Fig. 1). Edema in the injured cortex and striatum was lowest in the 3.8 µg group ($p \leq 0.005$) compared to 5 and 10 mmol/L groups, which were similar ($p \geq 0.841$).

Experiment 2

Iron infusion caused noticeable striatal damage with additional injury to the corpus callosum and cortex at higher doses (Fig. 2B). While there was a dose-dependent increase in tissue loss ($p < 0.001$), it was not linear (Fig. 2C). Specifically, the 3.8 µg dose caused less damage than the higher doses ($p \leq 0.002$), but the two highest doses were not statistically different from each other ($p = 0.594$). The two saline controls, which were added after the iron experiments were completed, sustained only minimal injury from the needle insertion and infusion procedure (Fig. 2A and C). These groups did not differ significantly from each other ($p = 0.235$), and they had considerably less damage than the iron groups (e.g., the 19.0 µg $FeCl_2$ group had many times more damage than the acidity matched saline control).

Behavioural data were collected for the 3.8 to 38.0 µg groups for the NDS (Fig. 3A), cylinder (Fig. 3B) and corner turn tests (Fig. 3C). All animals had normal baseline

NDS scores. Groups were not significantly different on days 1 ($p = 0.358$) and 7 ($p = 0.090$), but on day 4 the 38.0 μg group was slightly more impaired than the other two ($p \leq 0.005$). Repeated-measures ANOVA on the cylinder task revealed a time effect ($p < 0.001$), but no interaction ($p = 0.068$) or group effect ($p = 0.400$). Thus, all groups showed a bias for using the ipsilateral (non-impaired) forelimb after the lesion, but there were no difference among groups at baseline or day 7. A repeated-measures ANOVA on the corner turn test revealed a significant time effect ($p < 0.001$), but no interaction ($p = 0.509$) or group effect ($p = 0.707$). Thus, post-infusion scores were higher than baseline (i.e., turn bias), but there was no difference among the $FeCl_2$ groups. The additional saline controls, which were analyzed separately (added after the 3.8 – 38 μg groups were completed), also showed normal behavioural scores before and after infusion (e.g., NDS scores of 0, and no bias in the corner turn test) that did not differ when comparing baseline with post-infusion scores (data not shown).

Experiment 3

The pilot study showed that the 0.95 μg $FeCl_2$ infusion caused only a very small amount of tissue loss at 7 days as expected (8.55 ± 3.07 mm^3). Nonetheless, for the Golgi-Cox study, the striatal neurons (Fig. 4A) near the lesion were markedly atrophied compared to the normal side (Fig. 4B). Sholl analysis (Fig. 4C), which estimates overall dendritic length, showed that surviving peri-lesion neurons had considerably shorter dendrites ($p < 0.001$). Conversely, the contralateral side was normal (vs. saline, $p = 0.599$). The comparison of ipsi- and contralateral neurons within the iron group was significant ($p < 0.001$). Thus, iron caused remarkable dendritic atrophy (~50%) of peri-

lesion striatal neurons. For the branch order analysis (Fig. 4D), which reflects dendritic complexity, we found a significant interaction ($p < 0.001$) in the 2-factor design for the peri-lesion neurons. Thus, further analysis showed that the iron group had fewer lower-order branches (1 through 5, $p \leq 0.002$ vs. saline). The 6 and 7 orders were near zero normally and thus not expected to be different ($p \geq 0.232$). There was a branch order main effect ($p < 0.001$) for the contralateral neurons (i.e., fewer distal bifurcations), but no group effect ($p = 0.991$) nor interaction ($p = 0.827$, data not shown). Thus, iron infusion significantly reduced dendritic length and number of branches indiscriminately only in the peri-lesion zone. The saline control infusion did not have a noticeable effect on dendritic structure and there was minimal injury (e.g., needle tract) as seen in Experiment 2.

Experiment 4

A 3.8-μg infusion of iron caused considerable tissue loss at 7 (Fig. 5A) and 60 days (Fig. 5B). Damage was ~50% greater at 60 days (Fig. 5C, $p = 0.017$). Fluorojade staining in the ipsilateral hemisphere showed that at least some of this continuing tissue loss resulted from ongoing neuronal death observed at 7 (Fig. 6A) and 60 days (Fig. 6B), but not in the contralateral side. Cell counts were not performed, but examining several sections per animal revealed that there were considerable (hundreds per coronal section) numbers of Fluorojade positive cells at day 7 whereas at day 60 there were mild to moderate number of cells in every animal as shown in representative photomicrographs. The Perls' stain was used to qualitatively show the number and spatial distribution of iron-positive cells (macrophages, microglia). Sample

photomicrographs bordering the lesion cavity are shown at 7 (Fig. 6C) and 60-day survival times (Fig. 6D). Intense staining was observed at both times in all animals. There was no labeling further away from the lesion (e.g., ventral cortex) or in the contralateral side (not shown). Thus, the spatial localization of iron overlaps with atrophied and degenerating cells.

Discussion

An intracerebral infusion of iron is well known to cause edema and rapid cell death (Huang, et al., 2002, Nakamura, et al., 2006, Willmore, et al., 1980, Willmore and Rubin, 1982), as the present findings confirm and extend upon by documenting the relationship between increasing iron dose and greater cell death / edema. Beyond this, our study primarily sought to determine whether ferrous iron causes protracted cell death and tissue loss in striatum, which did occur. Thus, our data supports the belief that iron released from degrading erythrocytes accounts for much of the delayed, secondary damage observed in animal models of ICH (Del Bigio, et al., 1996, Felberg, et al., 2002, MacLellan, et al., 2008, Nguyen, et al., 2008), which typically target the striatum and rarely the cortex (MacLellan, et al., 2012). As well, we sought to determine if surviving striatal neurons would show dendritic atrophy as others have shown in cortex after iron infusion in rat (Willmore, et al., 1980) and cat (Reid, et al., 1979) and we have seen following collagenase-induced ICH in striatum of rat (Nguyen, et al., 2008). Indeed, we found that the surviving striatal neurons in the peri-lesion zone were strikingly atrophied after a low-dose $FeCl_2$ infusion, which caused a relatively minor lesion.

Accordingly, this supports the hypothesis that iron released into brain parenchyma after an ICH contributes to neuronal atrophy.

Our conclusions about the importance of iron in mediating secondary (chronic) degeneration are in line with numerous studies showing that free radical scavengers (Nakamura, et al., 2008, Peeling, et al., 2001) and iron chelators (Gu, et al., 2009, Hua, et al., 2006, Huang, et al., 2002, Song, et al., 2008, Wan, et al., 2006, Wu, et al., 2012) limit injury, edema and behavioural dysfunction after ICH. In most cases chelators were given acutely, but our findings suggest that delayed chelation therapy might provide additional protection against protracted cell death and atrophy, perhaps because not all iron is sufficiently liganded (Kell, 2010). However, some studies find that iron chelators do not improve outcome. For example, we (Warkentin, et al., 2010) reported that deferoxamine failed to affect cerebral edema, tissue loss or behavioural outlook in the collagenase model despite using protocols found effective in the whole blood model. More recently we reported that deferoxamine lowers total iron levels (measured by rapid scanning x-ray fluorescence imaging – RS-XRF) after collagenase-induced ICH while failing to improve neurological recovery (Auriat, et al., 2012). Differences among studies, including model and hematoma size, which influences inflammation, the timing and amount of iron released, changes in ferritin levels, etc., may contribute to study discrepancies. As well, chelators or free radical scavengers alone may not be sufficient in some cases, but require combination treatment to maximally reduce oxidative stress (Kell, 2010) and to noticeably attenuate lesion size.

The prolonged injury occurring after $FeCl_2$ infusion does not explain differences between the whole blood and collagenase models with regard to the extent of delayed

injury. Specifically, infusing collagenase can cause considerable delayed tissue loss over weeks (MacLellan, et al., 2008, Nguyen, et al., 2008), as we find with $FeCl_2$, and much more than we have observed in the whole blood model. Differences among ICH models, such as the extent of inflammation, the size of the initial insult, and the extent of parenchymal infiltration by blood (and damage it causes), must be considered (Frantzias, et al., 2011, MacLellan, et al., 2012). For instance, little secondary damage appears to occur in very small collagenase-induced insults (Wasserman and Schlichter, 2007). As well, there appears to be differences between the whole blood and collagenase models with regard to dendritic atrophy (Auriat, et al., 2010, MacLellan, et al., 2011, Nguyen, et al., 2008), but again this might be influenced by initial lesion size. Similar model differences have been reported in the ischemia literature (Gonzalez and Kolb, 2003). Regardless, there are undoubtedly other factors important in delayed injury that are not modeled with $FeCl_2$ infusion.

Several additional observations deserve consideration. First, there was a non-linear relationship at the higher iron doses. This is potentially due to limited diffusion distances at the higher doses (e.g., barriers such as the corpus callosum), or alternatively by differences in how inflammatory cells respond to limit extreme injury. Linear relationships might also be found had we evaluated other survival times (e.g., edema at 3 days or histology at 4 weeks). Second, the behavioural tests show similar levels of dysfunction despite a considerable range in injury, which also overlaps with that produced in ICH models. Some behavioural tests are effective "lesion detectors" but are sometimes insensitive to variations in lesion size (MacLellan, et al., 2006) as the present findings also indicate. This is concerning as some treatments may significantly reduce

injury without being behaviourally noticeable on many routine tests. Third, because dendritic arborization is strongly tied to functional performance (Kleim and Jones, 2008, Kolb, et al., 1998, Murphy and Corbett, 2009) and clearly affected by ICH (Nguyen, et al., 2008) and iron (present findings), one ought to consider dendritic damage in ICH studies – not only rehabilitation studies (Auriat, et al., 2010, Takamatsu, et al., 2010) but also when testing neuroprotectants. For instance, iron chelators and / or free radical scavengers may partly work to improve behavioural recovery by attenuating cellular atrophy and not just by reducing cell death.

There are several methodological limitations that warrant consideration. First, while the infusion of $FeCl_2$ is clearly toxic, the release of iron from degrading erythrocytes occurs slowly after ICH allowing for protective measures to be initiated, such as increasing ferritin production to limit iron toxicity (Wu, et al., 2003). We opted to use the simplified commonly used method to determine whether iron alone (vs. mechanical trauma, etc.) causes striatal neuronal atrophy and progressive tissue loss including from neuronal death. However, future studies might consider more protracted release (e.g., cannula and mini-pump) to better mimic ICH.

Second, we did not count dead neurons because this data cannot be used to calculate its contribution to tissue loss (mm^3), which is our primary endpoint. Cell counts in the peri-lesion zone are also biased by tissue changes (loss of neuropil and lesion expansion) making it difficult to compare survival times. Thus, our findings only show that prominent and lasting neuronal death occurred. Likely, dendritic atrophy and delayed neuronal death both contribute to the increasing volume of tissue lost with time,

but one cannot determine the exact contribution of each to tissue loss or functional impairment.

Third, with the Golgi-Cox method we could not determine whether the dendritic atrophy caused by $FeCl_2$ infusion would have recovered or eventually led to cell death. We suspect that many neurons would have eventually succumbed based upon prominent FluoroJade staining in this zone and the remarkable atrophy found. Our previous collagenase study showed neuronal atrophy at 7 but not 60 days after ICH (Nguyen, et al., 2008). This implies that atrophied cells recover, but changes in lesion size over time and the use of a between subjects design must be considered. Notably, atrophied neurons observed soon after an ICH may eventually die off (contributing to lesion expansion) leaving only more peripheral, healthy-looking neurons at later survival times (i.e., sampling location bias). While the Golgi method is well suited to striatal insults, an alternative would be to evaluate atrophy after cortical hemorrhage with 2-photon microscopy. Ischemia studies using this method show dendritic atrophy near the infarct, which is balanced by growth of these cells' dendrites that are located away from the infarct (Brown, et al., 2010). Presently, all branches of striatal neurons appeared markedly atrophied after $FeCl_2$ infusions, but it is possible that there is a distance-to-lesion effect in this model and after ICH.

Fourth, total iron levels cannot be quantified with Perls' staining, which was the histochemical method presently used. An alternative is to use RS-XRF, which we recently used to show that total iron levels are significantly increased in the hematoma and immediate peri-hematoma region, but not in areas farther away from the ICH (Auriat, et al., 2012). This, of course, fits with the sequestering of iron by microglia and

macrophages (Perls' positive cells) that migrate to the hematoma and peri-hematoma areas after ICH (Wang, 2010), and similarly, injured areas caused by $FeCl_2$ infusion. Presently, we can only visually relate the extent of injury to the localization of iron in the damaged and immediate surrounding zone. Even with RS-XRF to quantify total iron, we would still need to know the type (Fe^{2+} vs. Fe^{3+}) and molecular localization of iron to draw stronger conclusions. For instance, one would have to determine how much of the total iron load is bound to ferritin and other proteins that render it harmless. Thus, further mechanistic studies are needed to relate iron-mediated oxidative stress and other potential mechanisms (e.g. abnormal electrical activity, deafferentation and inflammation) to atrophy and cell death. Unfortunately, the RS-XRF method is not compatible with Golgi-Cox staining and Perls' histochemistry cannot be used to accurately quantify iron levels. Thus, directly relating iron load to the distribution to neuronal death and dendritic atrophy is difficult to do.

In summary, our study proves that iron overload leads to edema, substantial dendritic damage, extended neuronal death and protracted tissue loss in the striatum of rat. These findings strongly support the assertion that iron toxicity contributes to delayed, secondary degeneration occurring after striatal ICH. Therefore, treatments targeting iron overload and its consequences are logical therapeutic targets, especially considering that the progression of cell death with iron toxicity is quite long and beyond the timeframe commonly targeted in neuroprotection trials. Future studies must also evaluate other ICH specific factors contributing to very delayed injury and neuronal atrophy (e.g., thrombin).

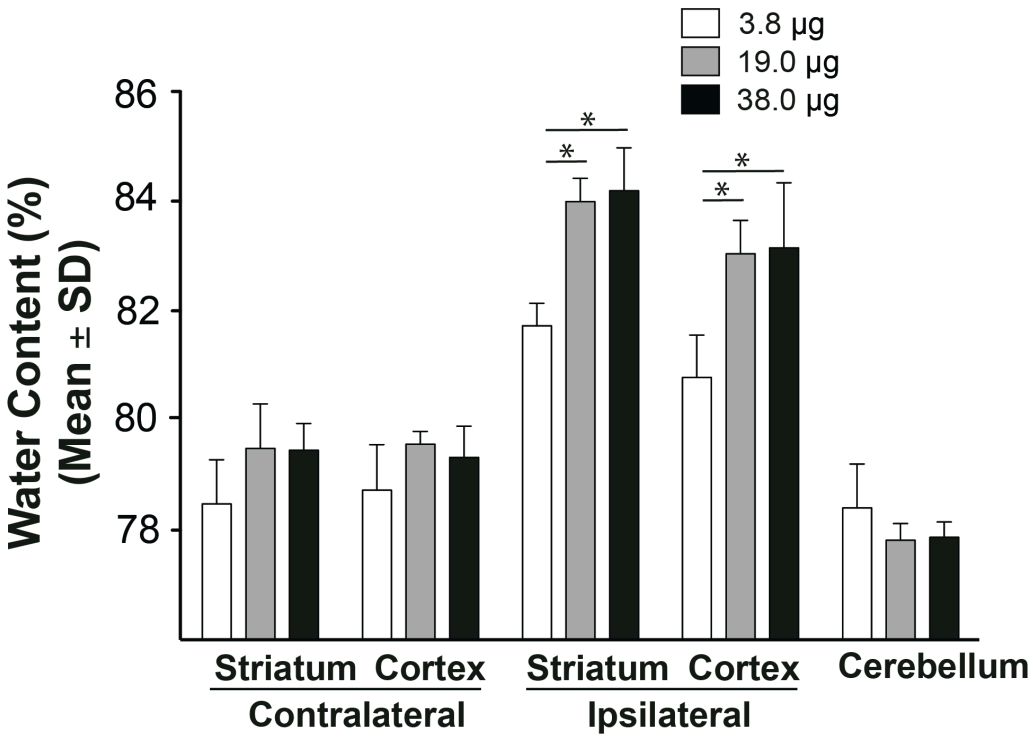

Figure 1. Significant edema occurred on the side of iron injection. The 19.0 and µg groups had greater swelling than the 3.8-µg group (*$p < 0.05$). There was no edema (normal BWC) on the contralateral side or cerebellum.

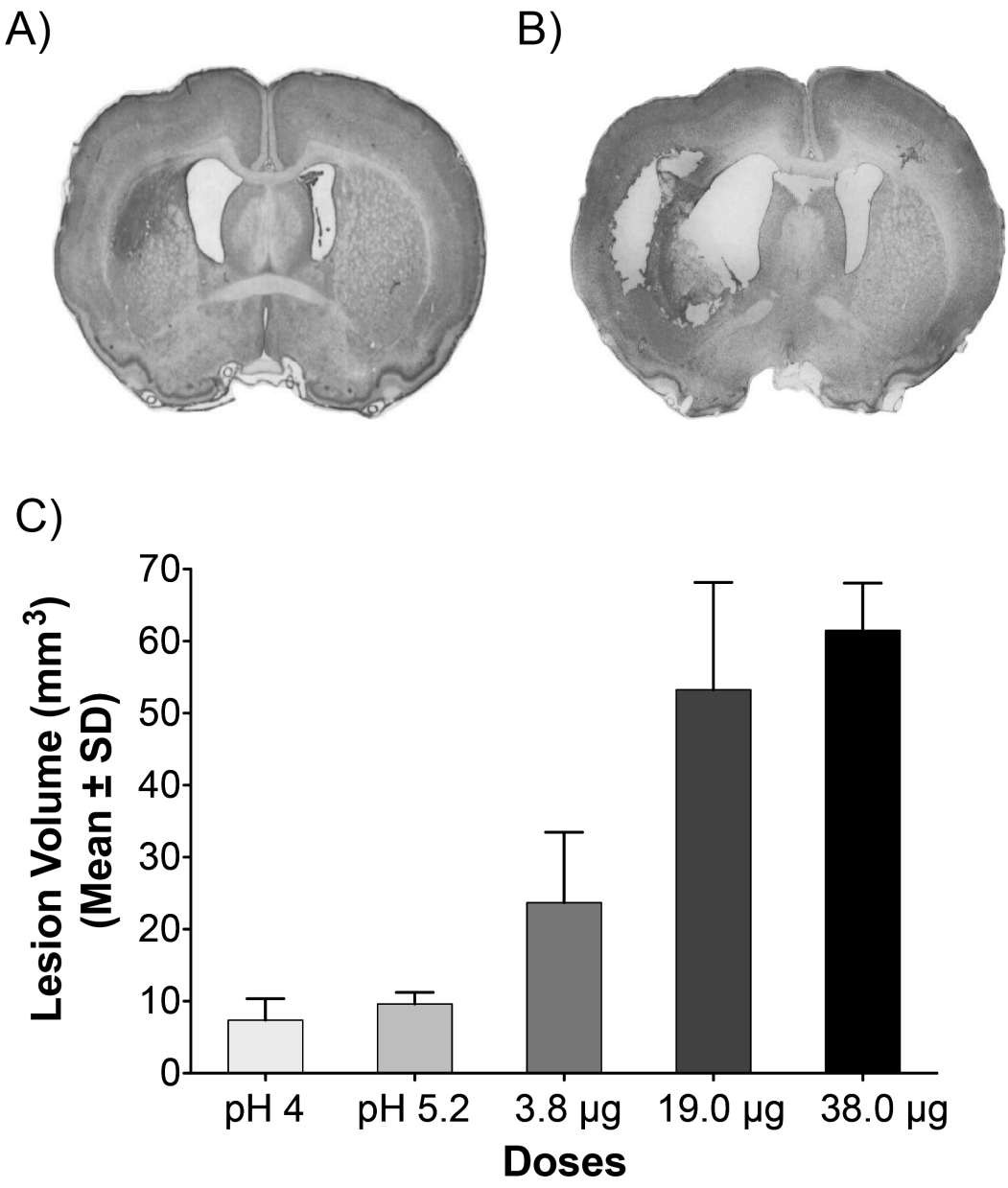

Figure 2. Representative sections illustrating damage caused by saline infusion (A) and the 38.0 µg iron infusion (B). Control groups had damage limited to the injection tract whereas extensive destruction affecting striatum, corpus callosum, and cortex occurred with iron infusion. Tissue lost at 7 days is shown in C (*$p<0.05$ vs. 3.8 µg group, #$p<0.05$ vs. combined saline controls, which were not different).

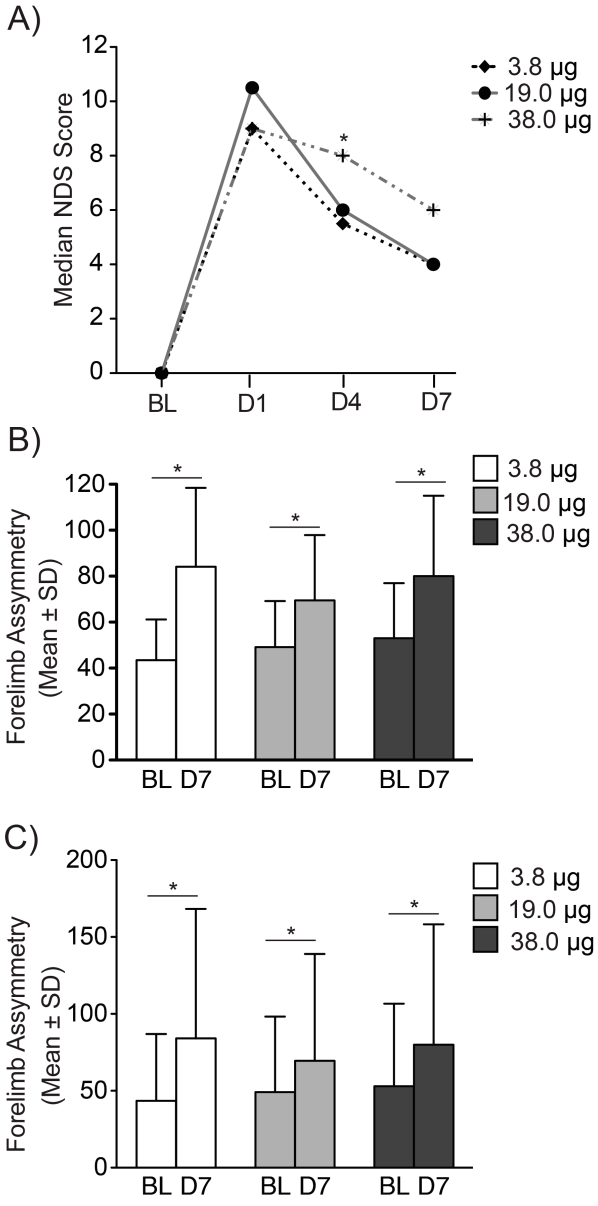

Figure 3. Iron infusion (3.8, 19.0 or 38.0 µg) caused significant impairment on the NDS (A), cylinder (B), and corner turn tests (C). Only the 38.0 µg group was worse than others on day 4 NDS (*$p<0.05$). For the other tests, post-lesion scores were different from baseline (*$p < 0.05$), which was normal, but the groups were not different. Saline controls were normal (data not shown).

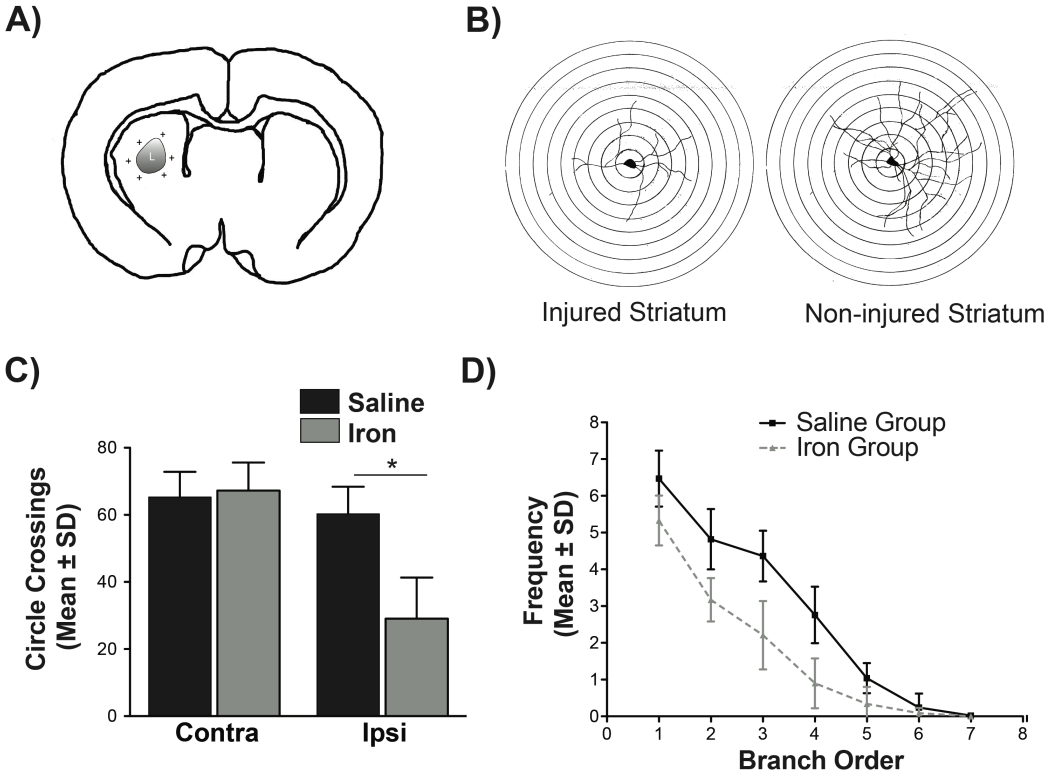

Figure 4. Illustration (A) showing the lesion ('L') and the surrounding cells (+) that were drawn after Golgi-Cox staining. Contralateral striatal neurons were also drawn. Representative drawings of are shown of an atrophied striatal neuron in the peri-lesion zone and a normal cell from the contralateral striatum (B). Surrounding concentric circles illustrate the Sholl analysis method (D), a standard measure of dendritic length. Dendritic length was considerably reduced in the peri-lesion zone (*$p < 0.05$). As well, branch order analysis (1st, 2nd, etc.) shows a significant reduction in the number of neuronal branches of iron group on the injected side compared to the saline control group (D, *$p < 0.05$).

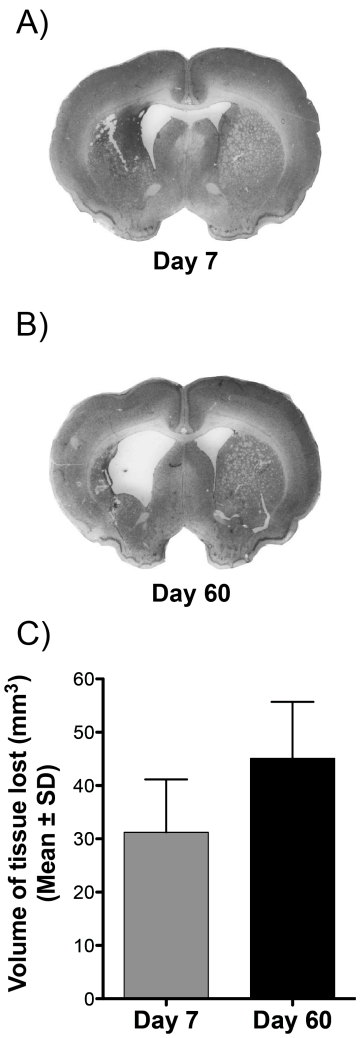

Figure 5. Photomicrograph of typical injury at 7 (A) and 60 days (B) after iron injection (3.8 μg) showing significantly more tissue loss at 60 days (C, *$p<0.05$). Abnormal looking tissue, including areas with only cellular debris and inflammatory cells (arrow in A points to a large area of destroyed tissue), was excluded from our tracings of 'normal' tissue. Thus, the continuing loss of tissue over time, measured as a smaller volume of normal brain in the damaged hemisphere, was not erroneously due to natural removal of damaged tissue (by phagocytes) seen more extensively at 7 days.

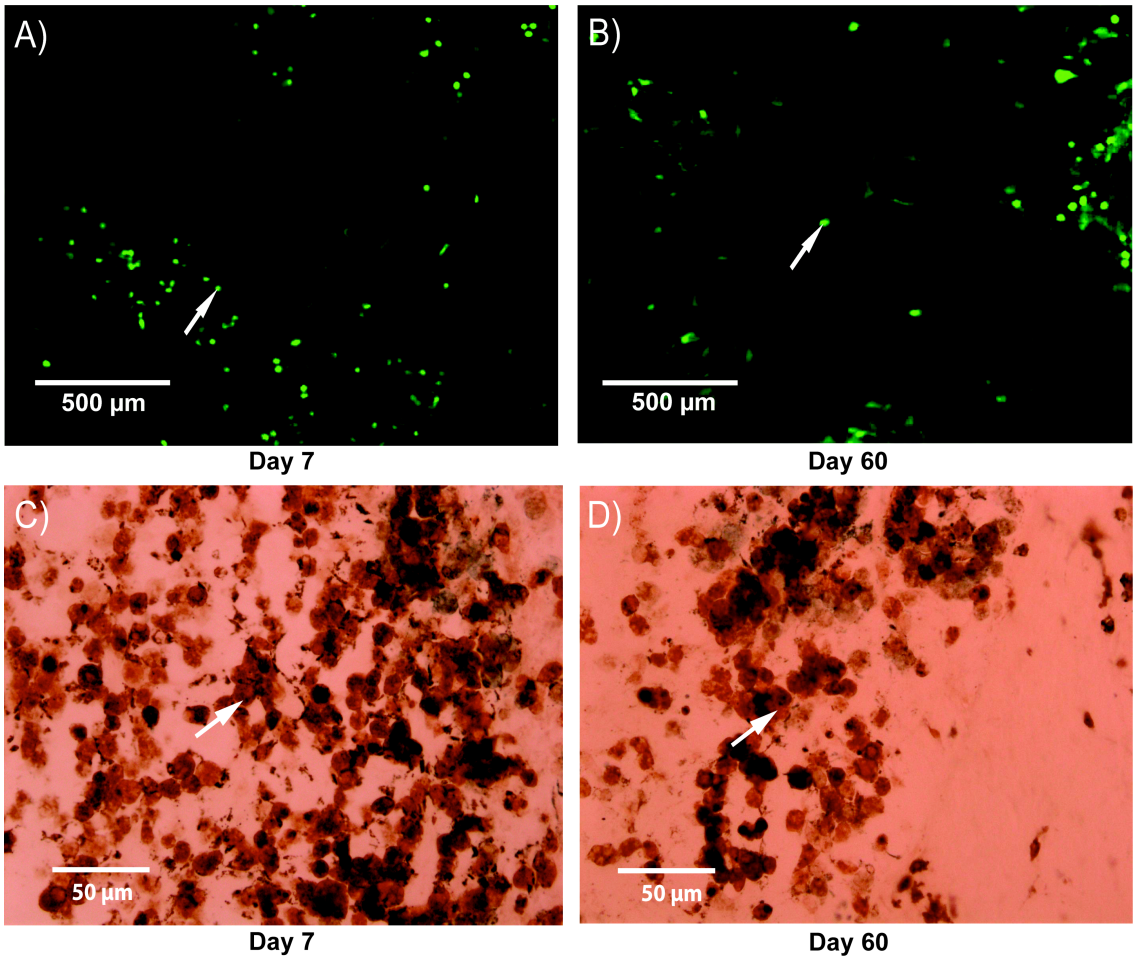

Figure 6. Representative photomicrographs showing the many degenerating neurons (FluoroJade positive cells, arrows) that were found around the iron infusion (3.8 µg) site at 7 (A) and 60 days (B). Representative photomicrographs also show heavy Perls' staining at 7 (C) and 60 days (D) after iron infusion. The contralateral side was always normal.

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Chapter 4

Bipyridine, an iron chelator, does not lessen intracerebral iron-induced damage or improve outcome after intracerebral hemorrhagic stroke in rats

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Abstract

Iron chelators, such as the intracellular ferrous chelator 2,2'-bipyridine, are a potential means of ameliorating iron-induced injury after intracerebral hemorrhage (ICH). We evaluated bipyridine against the collagenase and whole-blood ICH models and a simplified model of iron-induced damage involving a striatal injection of $FeCl_2$ in adult rats. First, we assessed whether bipyridine (25 mg/kg, 12 hours post-ICH and every 12 hours for 3 days) would attenuate non-heme iron levels in the brain and lessen behavioural impairments (neurological deficit scale, corner turn test, horizontal ladder) 7 days after collagenase-induced ICH. Second, we evaluated bipyridine (20 mg/kg, 6 hours post-ICH and then every 24 hours) on edema 3 days after collagenase-infusion. Body temperature was continually recorded in a subset of these rats beginning 24 hours prior to ICH until euthanasia. Third, bipyridine was administered (as per experiment 2) after whole-blood infusion to examine tissue loss, neuronal degeneration, and behavioural impairments at 7 days post-stroke, as well as body temperature for 3 days post-stroke. Finally, we evaluated whether bipyridine (25 mg/kg, 2 hours prior to surgery and then every 12 hours for 3 days) lessens tissue loss, neuronal death, and behavioural deficits after striatal $FeCl_2$ injection. Bipyridine caused a significant hypothermic effect (maximum drop to 34.6°C for 2-5 hours after each injection) in both ICH models; however in all experiments, bipyridine treated rats were indistinguishable from vehicle controls on all other measures (e.g., tissue loss, behavioural impairments, etc.). These results do not support the use of bipyridine against ICH.

Introduction

Intracerebral hemorrhage (ICH) has a high mortality rate and greatly impairs survivors (Sacco, et al., 2009). Currently, no clinically approved neuroprotective treatments exist. The primary damage of an ICH (i.e., mechanical injury from extravasated blood) occurs rapidly making it difficult to treat. Mechanisms causing secondary damage occur over hours to weeks and accordingly are more suitable therapeutic targets. Considerable research into secondary damage has identified possible treatments targeting inflammation, blood brain barrier disruption, edema, thrombin, and the breakdown products of erythrocytes (Keep, et al., 2012, Rincon and Mayer, 2012, Selim, 2009, Wagner, et al., 2003).

Over the initial days following an ICH, erythrocytes lyse releasing hemoglobin into the hematoma and surrounding parenchyma (Wagner, et al., 2003, Wu, et al., 2003). The heme from hemoglobin is degraded causing increased levels of labile ferrous iron, which promotes oxidative stress (Nakamura, et al., 2006, Wagner, et al., 2003, Wu, et al., 2012, Wu, et al., 2003). This oxidative stress stems from labile iron's involvement in the Haber-Weiss reactions (i.e., the Fenton reaction) and the production of highly reactive hydroxyl radicals. As labile ferric iron catalyzes the conversion of superoxide to molecular oxygen the ferric iron is reduced to ferrous iron. Ferrous iron catalyzes the production of hydroxyl radicals from hydrogen peroxide (Kell, 2009, Kell, 2010, Wagner, et al., 2003). Reactive oxygen species, such as hydroxyl radicals, damage macromolecules (e.g., DNA), generate other reactive species, and increase inflammation all of which can kill neurons (Kell, 2009, Kell, 2010, Wagner, et al., 2003). Indeed, a $FeCl_2$ injection into the brain causes oxidative stress (Nakamura, et al.,

2006, Willmore and Rubin, 1982), edema (Willmore and Rubin, 1982), epileptic activity, lesions, and an inflammatory response (Willmore, et al., 1980). Furthermore, a striatal injection of $FeCl_2$ in rats causes significant, progressive lesion enlargement, neuronal degeneration, and dendritic atrophy (Caliaperumal, et al., 2012), which mirror findings in the collagenase ICH model (Nguyen, et al., 2008).

Numerous animal studies have evaluated the efficacy of iron chelators (Okauchi, et al., 2010, Selim, 2009, Wu, et al., 2011) and deferoxamine, an intra- and extracellular ferric iron chelator, has completed phase 1 clinical trials (Selim, et al., 2011). Deferoxamine has shown neuroprotective (Okauchi, et al., 2010, Wu, et al., 2011), antioxidant and anti-inflammatory effects (Selim, 2009, Wu, et al., 2011); however, there are other studies that find no short-term (Auriat, et al., 2012, Warkentin, et al., 2010, Wu, et al., 2011) or long-term neuroprotective benefit (Warkentin, et al., 2010). Deferoxamine binds to ferric iron and although both ferric and ferrous iron catalyze the Haber-Weiss reaction, it is ferrous iron that contributes to the formation of hydroxyl radicals. Recently there has been interest in 2,2'-bipyridine, an intracellular ferrous iron chelator. An *in vitro* comparison between deferoxamine and bipyridine found that bipyridine has better cell membrane permeation and intracellular iron sequestering ability than deferoxamine (Kress, et al., 2002). Bipyridine binds the potentially more harmful ferrous iron and has superior cell permeation; therefore, it may be a more suitable treatment for ICH.

Bipyridine has been studied as a treatment for several forms of stroke. In models of subarachnoid hemorrhage, bipyridine reduced vasospasm and cell death (Yu, et al., 2010) whereas in ischemia, bipyridine diminished blood brain barrier disruption

(Methy, et al., 2008). A recent study of ICH found pre-treatment with bipyridine reduced neuronal degeneration, reactive oxygen species formation, microglia reactivity, and white matter damage whereas post-treatment decreased edema, lesion volume, and behavioural impairments in mice (Wu, et al., 2012). This study used the whole blood and collagenase models of ICH to comprehensively investigate bipyridine. Another study using the whole blood model in rats found a reduction in protein oxidation and DNA damage with bipyridine post-treatment (Nakamura, et al., 2006).

The current study evaluated bipyridine in three rat models of striatal injury. An ICH was produced either via injecting collagenase, a bacterial enzyme that degrades blood vessel walls causing a spontaneous bleed and progressive tissue loss (Del Bigio, et al., 1996, MacLellan, et al., 2008, Nguyen, et al., 2008, Rosenberg, et al., 1990), or whole blood into the striatum. Also, we used a striatal injection of $FeCl_2$ to isolate iron-mediated damage (Caliaperumal, et al., 2012). First, we measured non-heme iron levels and behavioural deficits 7 days after collagenase-induced ICH using a previously reported dose and injection regimen (25 mg/kg beginning 12 hours after ICH and then twice a day for three days (Nakamura, et al., 2006)). Second, based on a recent study we used a smaller dose of bipyridine injected less frequently (20 mg/kg beginning 6 hours after ICH and then once every 24 hours for two days (Wu, et al., 2012)) and measured edema 3 days after collagenase-induced ICH as this is the typical peak of edema in rodents. The whole-blood ICH model was used to determine the effect of bipyridine (20 mg/kg beginning 6 hours after ICH and then once every 24 hours for two days (Wu, et al., 2012)) on tissue loss, cell death, and behavioural impairments a week post-ICH. We also determined whether bipyridine caused hypothermia, a common experimental

confound in stroke research, in both ICH models. Finally, we investigated the effect of bipyridine pre-treatment (25 mg/kg beginning 2 hours prior to $FeCl_2$ injection and then once every 12 hours for three days) on iron-induced tissue loss, neuronal degeneration, and behavioural impairments.

Materials and Methods

Animals

All procedures complied with the Canadian Council on Animal Care guidelines and were approved by the University of Alberta's Animal Care and Use Committee: Biosciences. We used 90 male Sprague – Dawley rats (~11 weeks old) single housed in polycarbonate cages (width 38 cm, length 49 cm, height 20 cm) with wood chip bedding. Animals were kept in a humidity and temperature-controlled room on a 12-hour light/dark schedule with ad-lib food (Rodent Diet 5001) and water. In each experiment, animals were randomly assigned (n=10/group, except in experiment 3 with n=15/group) to either the bipyridine group (BIP) or the saline control group (SAL). All behavioural testing and data analysis were conducted by a researcher unaware of treatment identity.

Experiment 1:

Experiment Groups

Injections of bipyridine (25 mg/kg i.p.) or an equivalent volume of saline began 12 hours after ICH followed by an injection every 12 hours for 3 days (Nakamura, et

al., 2006). Rats underwent baseline behaviour prior to ICH and behavioural testing 7 days post-ICH, after which they were euthanized for non-heme iron level analysis.

Collagenase ICH Surgery

Surgical procedures were performed aseptically as previously described (Del Bigio, et al., 1996, MacLellan, et al., 2008, Nguyen, et al., 2008, Rosenberg, et al., 1990). Briefly, rats were anesthetized with isoflurane (4% induction, 2-2.5% maintenance, 60% N_2O balance O_2) and a hole was drilled 3.5 mm right and 0.5 mm anterior to Bregma into the skull. A 26-gauge needle was inserted 6.5 mm deep from the surface of the skull and 0.7 μl of collagenase (0.14 U) was injected. The wound was closed and Marcaine was applied to diminish post-operative pain.

Neurological Deficit Scale (NDS)

Neurological deficit scales are sensitive to striatal ICH (Del Bigio, et al., 1996, MacLellan, et al., 2006). Rats were scored on the following tasks: spontaneous circling, hind limb retraction, bilateral forepaw grasp, contralateral forelimb flexion, and beam walking ability. Each task had a score range of 0-3 except contralateral forelimb flexion, which had a range of 0-2. The sum of the score ranges from 0-14 with 14 denoting maximum impairment.

Corner Turn Test (CTT)

The CTT evaluates an animal's turning bias out of a corner and is sensitive to striatal injury (Schallert, 2006, Warkentin, et al., 2010). The rats were placed in front of two walls (41 cm height, 30.5 cm length) that made a 30° angle and the direction of

turning out of the corner was recorded. Baseline testing was completed over 2 consecutive days and the average performance over 10 trials for each day was determined. Ten trials were done and averaged at the day 7 test time. Data for this test was excluded for rats that exhibited a turning bias (>70% or <30% for one direction) at baseline.

Ladder Test

The walking ability of the rats was assessed using a horizontal ladder, a measurement sensitive to striatal ICH (MacLellan, et al., 2006, Metz and Whishaw, 2002). Animals crossed the 1 m ladder made of steel rungs spaced 1–4 cm apart (3 mm diameter). Four crosses for each rat was video recorded at baseline and 4 at testing at day 7. These were then analyzed for the average number of steps and slips. At any testing time, rats that crossed fewer than two times were excluded from analysis for this test.

Non - heme Iron Assay

Seven days after ICH rats were euthanized (isoflurane anesthesia and decapitation) and a 6 mm thick coronal section of both forebrains was taken from each animal (2 mm anterior to 4 mm posterior the site of collagenase injection). Tissue taken from the ipsilateral forebrain, contralateral forebrain and cerebellum (Cb) was homogenized (Auriat, et al., 2012, Rebouche, et al., 2004). Briefly, tissue proteins were precipitated and the samples were centrifuged. The supernatant was collected and reacted with a Ferrozine chromogen solution for a colorimetric assessment of the tissue's non-heme iron concentration.

Experiment 2:

Experiment Groups

Rats were given bipyridine (20 mg/kg i.p.) or saline 6 hours after ICH and then once every 24 hours until euthanasia. This change in dose regimen was due to a recent publication on bipyridine after ICH (Wu, et al., 2012). Most of the rats (BIP n=8, SAL n=7) underwent a core probe implantation to measure body temperature. Rats were euthanized 72 hours after ICH for edema measurement.

Core Temperature Probe Implantation

Four days before ICH, rats were implanted with core temperature telemetry probes (TAT10TA-F20 or F40, Transoma Medical, St. Paul, MN). Briefly, animals were anesthetized with isoflurane and the probe was surgically implanted into the peritoneal space (MacLellan, et al., 2006). Marcaine was applied to the wound and the animals recovered in a clean cage. Temperature data, sampled every 30 seconds, was taken beginning 24 hours prior to ICH surgery (baseline) and until euthanasia.

ICH Surgery

Surgical procedures were the same as in experiment 1.

Edema or Brain Water Content (BWC)

The rats were euthanized 72 hours post-ICH (decapitation under isoflurane) to measure BWC (MacLellan, et al., 2006, Warkentin, et al., 2010). A 6 mm coronal section of the ipsilateral and contralateral forebrains was taken (2 mm anterior to 4 mm

posterior the injection site) along with the Cb as a control. The cortex and striatum were separated in each forebrain for individual weight measurement before and after being baked at 100°C for 24 hours.

% BWC = ((Wet Weight - Dry Weight) / Wet Weight) × 100.

Experiment 3:

Experimental Groups

The same bipyridine treatment used in experiment 2 was used in this experiment. We chose this treatment as opposed to the greater dosage in experiment 1 since there was no discoloration of rat feces in experiment 2 (i.e., side effects). Rats were subjected to a whole-blood ICH and tested for behavioural deficits followed by euthanasia 7 days post-insult. Most rats underwent core temperature probe implantation 4 days prior to ICH (n=7/group).

Whole-blood ICH Surgery and Core Probe Implantation

Surgery was the same as in experiment 1 except instead of collagenase, 100 μL of whole-blood (from tail vein) was infused into the striatum over 10 minutes and the needle was left in place for an additional 10 minutes (MacLellan, et al., 2006, MacLellan, et al., 2008). Same surgical procedures for probe implantation used in experiment 2 were used here.

Behavioural Testing

The same behavioural testing was done as in experiment 1.

Lesion Volume

The rats were euthanized with pentobarbital (100 mg/kg i.p.) and transcardially perfused with 0.9% saline followed by 10% formalin. Brain sections (40 μm) were stained with cresyl violet. One in every 10 sections was analyzed for lesion analysis using ImageJ (MacLellan, et al., 2006) as follows:

Volume of a hemisphere = average (area of the complete coronal section of the hemisphere – area of ventricle – area of damage) × interval between sections × number of sections.

Tissue lost = remaining volume of normal hemisphere - remaining volume of injured hemisphere.

Fluoro-Jade Stain

Degenerating neurons were identified using Fluoro-Jade B (Caliaperumal, et al., 2012, Schmued and Hopkins, 2000). The slides were prepared and incubated with 0.001% Fluoro-Jade (Chemicon, Temecula, CA). Fluoro-Jade positive cells (FJ+) were visualized with a fluorescein filter and the total number of cells for one section per rat was counted at the level of the maximum hematoma size.

Experiment 4:

Experiment Groups

This experiment tested a pre-treatment of bipyridine on iron-induced damage. Two hours before $FeCl_2$ injection rats received bipyridine (25 mg/kg i.p.) or saline and

then an injection every 12 hours for 3 days. Animals underwent behavioural testing 7 days after $FeCl_2$ injection and were euthanized to determine lesion volume and neurodegeneration.

$FeCl_2$ Surgery

Surgery was the same as experiment 1 except we infused 3.8 μg of $FeCl_2$ in a 30 μl solution of sterile unbuffered saline over 10 minutes (Caliaperumal, et al., 2012, Nakamura, et al., 2006). This solution was mixed and kept frozen until used.

Behavioural Testing

The same 3 tests used in experiment 1 were used here.

Lesion Volume and Fluoro-Jade Stain

The same procedures used in experiment 3 were used here.

Experiment 5:

A spectrometry assay was performed to confirm that our preparation of bipyridine bound iron at a 3:1 mole ratio (Pedersen, 2009). The concentration of $FeCl_2$ was kept constant (12.68 μg $FeCl_2$ in 10 ml of saline) and mixed with increasing concentrations of bipyridine (prepared 10 mM of bipyridine which contained 97.6125 mg of bipyridine in 62.5 ml of saline and then diluted to 1, 2, 3, 4, 5, 6, and 7 mM). The readings were measured at a wavelength of 556 nm. Saline + bipyridine, saline + $FeCl_2$, and saline alone were used as controls.

Statistical analysis

The data are presented as mean ± standard deviation (SD), except for NDS scores that are presented as medians. Data was analyzed via repeated measures and/or 1-way analysis of variance (ANOVA) using SPSS, except the NDS scores that were analyzed by Mann Whitney and Wilcoxon tests (SPSS v.17.0, SPSS Inc, Chicago, IL).

Results

There was no mortality in any experiment. One rat was excluded from the SAL group in experiment 3 due to surgical error.

Experiment 1

The percent drop in weight (from surgery) in the BIP group ranged from 10.7%-11.6% on days 1- 3 post-surgery, while the SAL group lost 2.7% on day 1 and gained 2.1% by day 3. There was a significantly greater loss in the BIP group on all 3 days ($p \leq 0.001$). Animals treated with bipyridine had orange discoloration of their feces.

Behavioural Outcome

Three rats were excluded from CTT due to a baseline turning bias (two from BIP and one from SAL). There were no exclusions in NDS and the ladder test. Baseline NDS scores were normal and there was no difference between groups ($p=0.549$, Fig. 1A). There was significant impairment 7 days post-ICH ($p<0.0001$ vs. baseline); however, no difference between groups ($p=0.646$). For the CTT, there was a significant Time effect ($p<0.0001$, Fig. 1B) but no Group effect ($p=0.718$) or interaction ($p=0.222$).

In ladder, there was a significant Time effect ($p<0.0001$, Fig. 1C) but no Group effect ($p=0.259$) or interaction ($p=0.538$).

Non-heme Iron Levels:

There was a significant increase in non-heme iron levels (μg iron/g of brain tissue) in the injured forebrain (BIP 35.43±18.45, SAL 30.40±4.49) compared to the non-injured forebrain (BIP 13.87 ±1.93, SAL 14.65±2.9, $p<0.0001$) and the cerebellum (BIP 14.08±1.78, SAL 15.64±3.05, $p\leq0.002$) but no difference between groups ($p=0.413$). Thus, bipyridine did not alter iron levels after ICH.

Experiment 2

Body weight dropped in the BIP group (1.8-4.5%) over the first 3 days post-ICH whereas for the SAL group there was a 1.7% drop on the first day but a 2.3% increase by the third day. The group differences in weight change were significant ($p\leq0.002$).

BWC

Edema was measured 3 days post-ICH (Fig. 2). There was a significant increase in BWC in the injured striatum ($p<0.0001$) and cortex ($p=0.009$) compared to the uninjured side and the Cb ($p<0.0001$). Bipyridine did not reduce edema in the ipsilateral cortex or striatum ($p\geq0.593$).

Body Temperature

Bipyridine transiently reduced temperature (Fig. 3A) but there was no difference between groups at baseline ($p=0.828$). The first injection 6 hours after ICH caused the

greatest cooling ($p \leq 0.029$) that lasted 5 hours. The second and third injections also caused significant but shorter hypothermia ($p \leq 0.037$ vs. SAL on hourly averaged data).

Experiment 3

Although the whole-blood model caused weight loss over the first three days, bipyridine did not worsen it ($p=0.922$).

Behavioural Outcome

One SAL rat was excluded from the CTT analysis due to a baseline turning bias in this test. In all behavioural tests ICH caused significant impairment ($p<0.0001$ vs. baseline). There was no difference between groups in NDS ($p = 0.804$, Fig. 4A). In CTT there was no Group effect ($p=0.683$, Fig. 4B) or interaction ($p=0.336$) and again for ladder there was no Group effect ($p=0.318$) or interaction ($p=0.860$, Fig. 4C).

Lesion volume and Neurodegeneration

Bipyridine did not lessen tissue loss ($p = 0.097$, Fig. 5A) or the number of FJ+ cells ($p=0.790$ Fig. 5B) a week post-hemorrhage.

Body Temperature

Bipyridine caused transient cooling after each injection ($p \leq 0.001$, Fig. 3B) but there was no difference between groups at baseline ($p=0.871$).

Experiment 4

The BIP group lost greater weight ($p \leq 0.011$) on all 3 days post-$FeCl_2$ injection while the SAL group gained weight (drop of 3.5% vs. gain of 1.8% at day 3).

Behavioural Outcome

Three rats in each group were excluded from the CTT due to a turning bias and one rat from the SAL group was excluded from the ladder test because of insufficient crossing. Baseline NDS scores were normal. At 7 days after $FeCl_2$ injection there was significant impairment ($p<0.0001$ vs. baseline, Fig. 6A) but no difference between groups ($p=0.098$). Results for CTT showed a Time effect ($p=0.001$) but no Group effect ($p=0.879$) or interaction ($p=0.802$, Fig. 6B). In the ladder test there was a significant Time effect ($p<0.0001$) but no Group effect ($p=0.243$) or interaction ($p=0.238$, Fig. 6C). Thus, a striatal injection of $FeCl_2$ caused impairment, which bipyridine did not attenuate.

Lesion Volume and Neurodegeneration

Bipyridine did not ameliorate tissue loss ($p=0.332$, Fig. 5C) or the number FJ+ cells ($p=0.657$, Fig. 5D) at a week post-insult.

Experiment 5

Bipyridine chelated increasing concentrations of $FeCl_2$ until saturation at a ratio of 3 moles of bipyridine per mole of iron (data not shown). At least in a test tube, bipyridine was chemically active, as expected.

Discussion

Despite using three models, two drug doses, pre- and post-injury regimens, and multiple endpoints we failed to find any effect of bipyridine. Specifically, post-treatment did not impact parenchymal non-heme iron levels, behavioural impairments, or edema after a collagenase-induced ICH. Nor did bipyridine lessen tissue loss, cell death, or behavioural impairment in the whole-blood ICH model. Finally, pre- and post-treatment did not influence the $FeCl_2$-induced lesion, neurodegeneration, or behavioural impairment. Thus, these results are contrary to the considerable literature supporting the use of iron chelators for ICH (Nakamura, et al., 2006, Okauchi, et al., 2010, Selim, et al., 2011, Wu, et al., 2012).

Our study has several, general limitations. First, in any negative study it remains possible that other drug doses or treatment regimens could provide neuroprotection. Thus, we cannot exclude this possibility even after conducting four efficacy experiments using 2 different drug doses and treatment regimens. Second, despite considerable methodological overlap with previous studies (e.g., dose regimens, models, and endpoints) (Nakamura, et al., 2006, Wu, et al., 2012), some factor or combinations of factors may differ among studies and be critically important to drug-induced neuroprotection. Unfortunately, the present findings do not explain why others find a neuroprotective effect whereas we do not. Similarly, our lab's failure to obtain benefit with deferoxamine (Auriat, et al., 2012, Warkentin, et al., 2010) stands in contrast to a considerable amount of work supporting neuroprotective effects. Again, there was overlap in drug dosage, endpoints, etc. Third, small treatment effects may have gone unnoticed in our studies despite using above-average group sizes and

multiple endpoints that are widely used and well established in the field (MacLellan, et al., 2012). Notably, there were no trends suggesting benefit in our study. Fourth, we did not prove that bipyridine entered and chelated iron in the brain. There is no simple way to do this and while we could have assessed whether bipyridine affected oxidative stress we did not owing to the lack of a neuroprotective and behavioural effect. However, we did show that bipyridine, mixed fresh prior to each injection, was chemically active in a dish. As well it was clear the drug affected the animals (i.e., discoloured feces, weight loss, hypothermia). Of note in experiment four, some animals pre-treated with bipyridine displayed significant movement abnormalities (e.g., high amplitude shaking including aberrant tremulous movement of head, trunk, and limbs). Once they awoke from anesthesia, which we have never observed previously including in rats given striatal ICH or $FeCl_2$ infusion. This behaviour subsided within an hour but suggests that bipyridine does reach the striatum and thus interacts with the $FeCl_2$. Besides general limitations common to most negative studies, each experiment here has its own limitations.

The first study confirmed the increase in non-heme iron levels beginning a few days after ICH (Auriat, et al., 2012, Wu, et al., 2003). Bipyridine did not lower iron levels (i.e., improve iron clearance), but it is possible the non-heme assay is not sensitive enough to detect a treatment effect, which may have been observed with other methods such as x-ray fluorescence imaging (Auriat, et al., 2012). Also, while we did not assess cell death in this model, the behavioural data, as well as the histology and behaviour of our other experiments, suggests (but do not prove) that there would be no neuroprotection in the collagenase model either.

The second study showed that bipyridine did not reduce edema 3 days after collagenase-induced ICH. Possibly, edema may have been affected at other times but this time point was chosen as it corresponds to the peak edema time in rats and is commonly used. As well, Wu and others (Wu, et al., 2012) found bipyridine to reduce edema in the whole-blood model in mice. Thus, species or model differences likely account for study discrepancies. It is also possible that the bipyridine-induced hypothermia contributed to the reduction in edema found in mice, especially if the cooling was more pronounced or occurred at the time of euthanasia in that study. Hypothermia reduces edema after ICH without consistently conferring neuroprotection or improving behavioural impairments (MacLellan, et al., 2006). Note that at euthanasia, our animals were no longer colder than controls (Fig. 3A).

As we only assessed the histological and behavioural effect of bipyridine in the third and fourth experiments, some benefit may have gone unmeasured. However, owing to the lack of histological and behavioural effect, as well as the null results on edema and iron levels in the collagenase-model we did not see reason to pursue other endpoints (e.g., oxidative stress).

Bipyridine failed to attenuate iron-induced neuronal damage and behavioural impairment in experiment four despite pre- and post treatment. An injection of $FeCl_2$ is used as a simplified model to mimic ICH and traumatic injury, but there are limitations. For instance, a single bolus dose in an acidic solution (pH 5) does not mimic the slow release of iron from degrading erythrocytes at physiological pH. However, this model has been previously used in mechanistic studies of ICH (Caliaperumal, et al., 2012, Nakamura, et al., 2006) and we have shown that it is the iron not the acidity that causes

injury in this model (Caliaperumal, et al., 2012). While pH may influence bipyridine's chelating abilities this is likely a transient effect as the body buffers the solution. Still this model has limited face validity and although we attempted to study whether a striatal injection of bipyridine and $FeCl_2$ solution would cause injury, rats displayed similar behavioural abnormalities as described and we terminated the study owing to health concerns. Finally, while this model resulted in similar behavioural deficits produced by a whole-blood injection, $FeCl_2$ caused a slightly milder turning bias and somewhat worse walking impairment compared to collagenase. This may relate to subtle differences in lesion size and placement or to other factors (edema, plasticity responses, etc.). However, as we have previously shown even matching the whole blood and collagenase models is exceedingly difficult as each model has markedly different outcomes despite matching initial hematoma volumes (MacLellan, et al., 2008).

There are several reasons to explain why bipyridine did not prevent the iron-induced damage. Conditions within the brain after injury (e.g., pH changes) may impede the chelator's ability to bind iron. It is also possible that bipyridine did not reach the injured striatum. Yet, we used similar treatment protocols that others have reported benefit with and the abnormal behaviour immediately post-surgery suggest that bipyridine does reach the striatum. Alternatively, injections lasting only 3 days after injury may not be sufficient to provide neuroprotection and improve behavioural recovery. Okauchi and others (Okauchi, et al., 2010) found that only prolonged periods of deferoxamine treatment (7-14 days) provided lasting histological and behavioural benefit. However, a longer duration of deferoxamine treatment (i.e., 7 days) has not always provided protection (Warkentin, et al., 2010). Finally, it is also possible that

chelators bind only a portion of the labile iron pool and there is enough left unbound to generate potent hydroxyl radicals. Hydroxyl radicals, along with damaging macromolecules, generate a cascade of reactive species leading to a snowballing effect of oxidative damage (Kell, 2009). Of note, a possible adverse effect of iron chelators is that they may interfere with the brain's endogenous response to increased non-heme iron levels (i.e., up-regulating ferritin) thereby making the brain vulnerable to un-chelated iron (Pedersen, et al., 2009).

Iron from degrading erythrocytes poses a significant risk to surrounding tissue (Caliaperumal, et al., 2012, Keep, et al., 2012, Nakamura, et al., 2006, Wagner, et al., 2003, Wu, et al., 2011, Wu, et al., 2003) and thus is an attractive treatment strategy. Animal studies show a persistent increase in non-heme iron levels from 3 to 7 days after ICH and these levels remain elevated up to 28 days (Auriat, et al., 2012, Wu, et al., 2003). In patients, magnetic resonance imaging suggests that iron deposits expand out from the initial hematoma into the surrounding parenchyma over 3 months (Wu, et al., 2010). As iron from an ICH is not readily cleared, the brain is vulnerable to iron-induced oxidative injury. Even protein-bound iron (e.g., ferritin) may be liberated by free radicals (e.g., superoxide) thereby propagating formation of more reactive species (Kell, 2009). Although iron causes secondary cell death, there are several other secondary mechanisms of injury after ICH. Thus, while bipyridine may chelate ferrous iron this may not be enough to improve overall outcome. Combination treatments or those with pleiotropic effects are more likely to provide neuroprotection. For example, antioxidants (e.g., NXY-059) have failed clinical trials (Lyden, et al., 2007) possibly because these drugs (e.g., ascorbate in the presence of molecular oxygen) when

catalyzed by insufficiently bound iron, can become pro-oxidants (Kell, 2009). Therefore, it has been suggested that combinations of iron chelators with antioxidants will provide better protection than either alone (Kell, 2009). Sulforaphane, an isothiocyanate, promotes the activity of nuclear factor E2-factor related factor, a transcription factor that acts on the antioxidant response element (Fitch, et al., 1999) thereby increasing expression of several different antioxidants (Fitch, et al., 1999, Zhao, et al., 2007) and attenuating behavioural impairments, inflammation, and oxidative stress after ICH (Zhao, et al., 2007). Sulforaphane also promotes expression of haptoglobin, a hemoglobin-binding protein, thereby preventing release of iron (Zhao, et al., 2009).

In summary, our results do not support the use of bipyridine as a single treatment approach for ICH. This study comprehensively evaluated bipyridine using several models and found the drug ineffective for all endpoints. Thus, iron chelators do not accomplish enough on their own to provide neuroprotection and improve behavioural impairments. Many have argued for the publication of negative animal data in order to provide a more thorough picture prior to clinical research (Lapchak and Zhang, 2011). In this regard, our results suggest further experimentation is needed on iron chelators before further movement through clinical ICH studies.

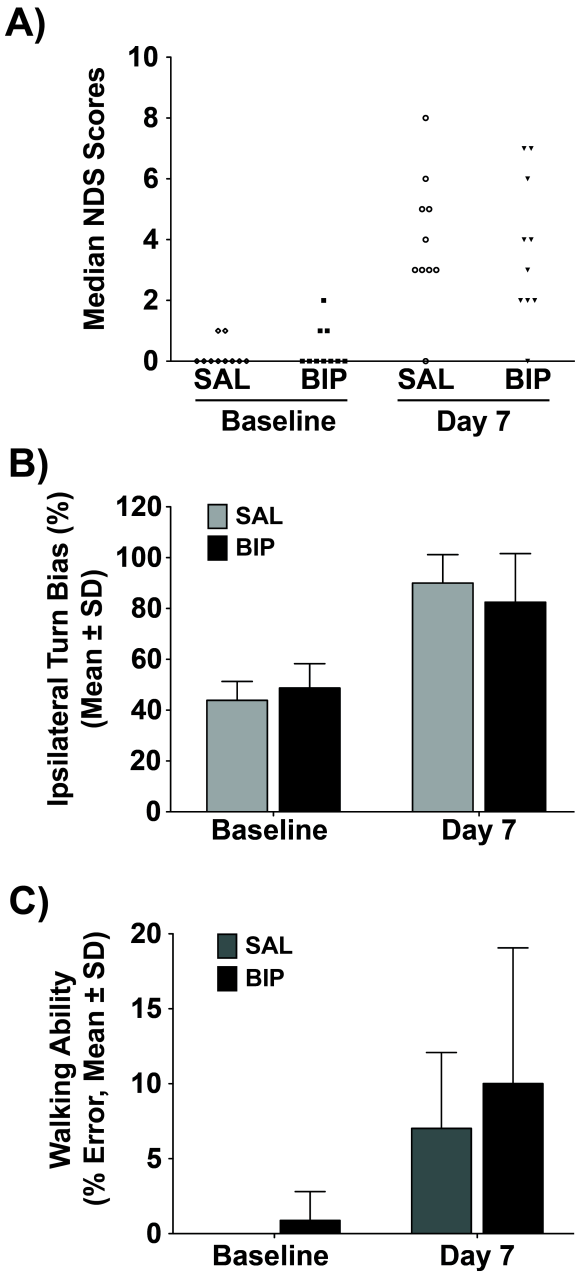

Figure 1. The collagenase-induced ICH caused significant impairments on the A) NDS, B) CTT, and C) ladder tests, but there was no significant effect of bipyridine treatment.

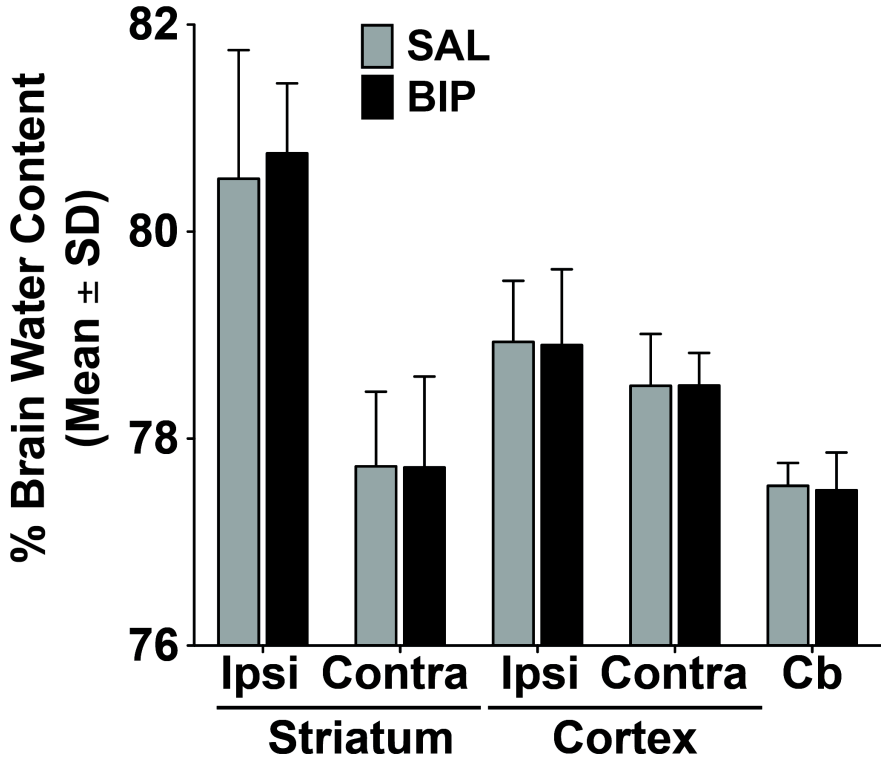

Figure 2. Collagenase-induced ICH significantly elevated striatal and cortical BWC at 3 days (vs. contralateral side and Cb). Bipyridine had no significant effects.

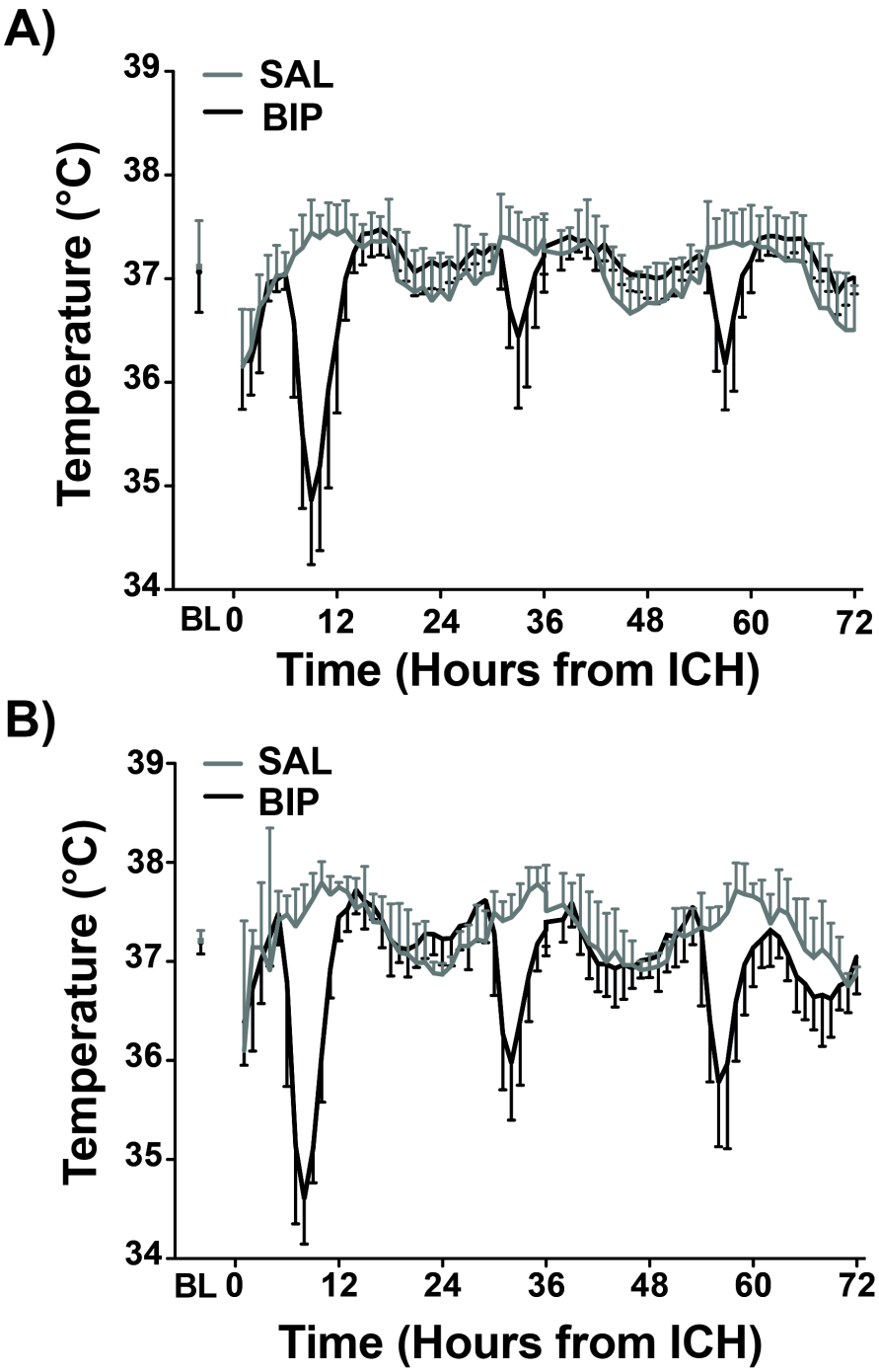

Figure 3. Body temperature (°C) was measured using telemetry probes before and after collagenase (A) and blood infusions (B). There was no difference in baseline temperature (BL) but there was significant, transient cooling after each bipyridine injection (6, 30, and 54 hours after ICH).

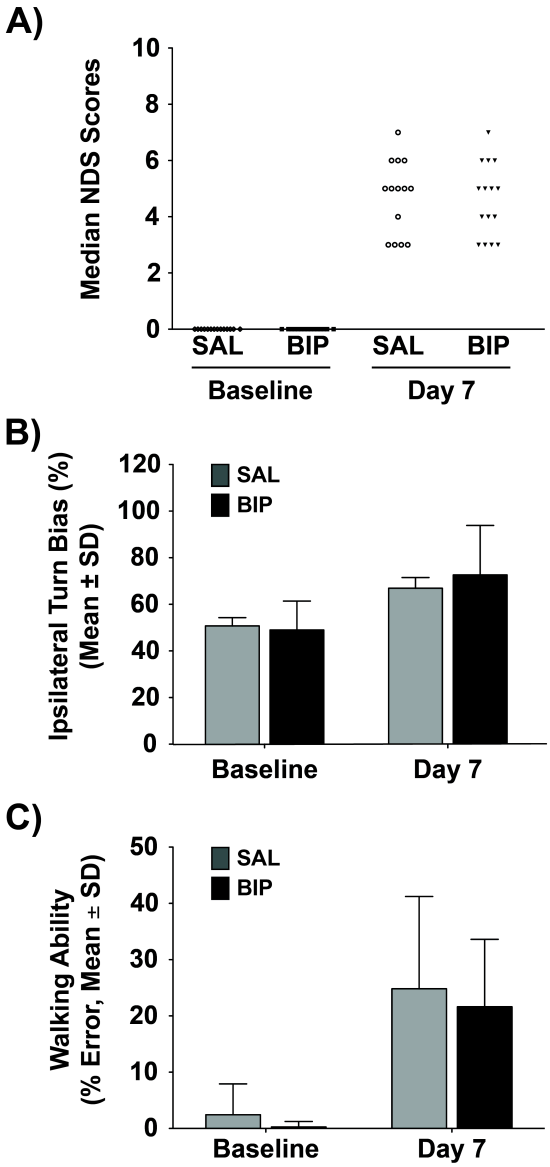

Figure 4. The whole-blood infusion caused significant impairments on the A) NDS, B) CTT, and C) ladder tests, which bipyridine did not treat.

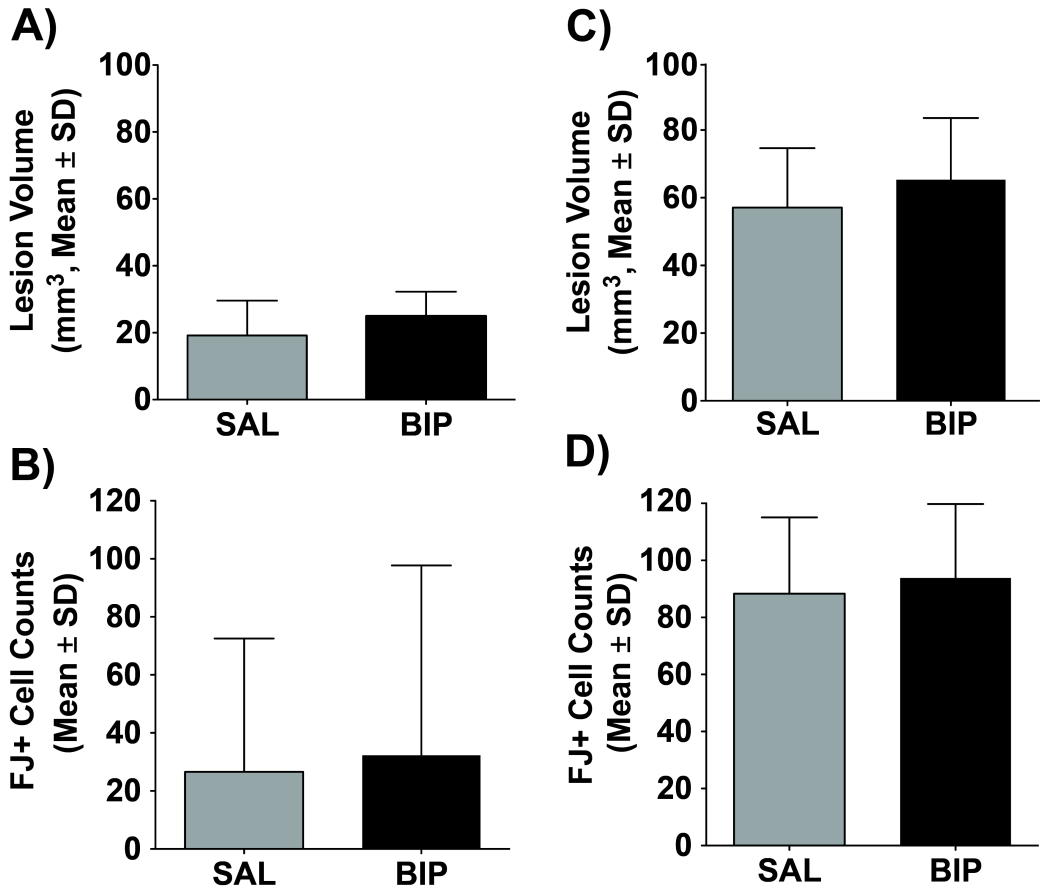

Figure 5. Lesion volume and neuronal degeneration was assessed 7 days after blood (A&B) and $FeCl_2$ striatal infusion (C&D). Lesion volume (A&C) and number of FJ+ cells (B&D) were similar between groups.

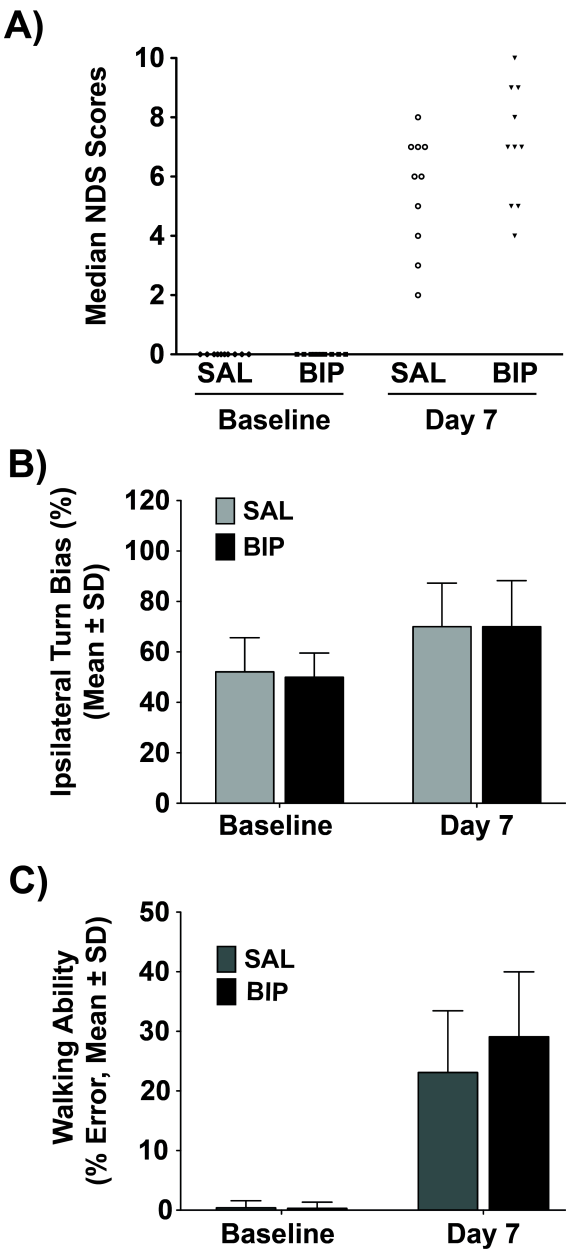

Figure 6. A striatal $FeCl_2$ infusion caused significant deficits on A) NDS, B) CTT, and C) ladder tests, which were not influenced by bipyridine.

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Chapter 5

Rehabilitation Improves Behavioural Recovery and Lessens Cell Death Without Affecting Iron, Ferritin, Transferrin or Inflammation after Intracerebral Hemorrhage in Rats

A version of this chapter is under review. Caliaperumal J and Colbourne F. *Neurorehabilitation and Neural Repair, 2013*.

Abstract

Background. Rehabilitation aids recovery from stroke in animal models, including intracerebral hemorrhage (ICH). Sometimes, rehabilitation lessens brain damage. *Objective*. We tested whether rehabilitation improves recovery and reduces peri-hematoma neuronal death. We also evaluated whether rehabilitation influences iron toxicity and inflammation – mediators of secondary degeneration after ICH. *Methods*. Rats were trained to retrieve food pellets in a staircase apparatus and later subjected to a striatal ICH (via collagenase infusion). After one week they were given either enhanced rehabilitation (ER), including reach training with group housing and environmental enrichment, or Control treatment (group housing). Rats in the first experiment were treated for two weeks, functionally assessed, and euthanized at one month to determine brain levels of non-heme iron. A second experiment used a similar approach except that animals were euthanized at 14 days to evaluate peri-hematoma neuronal death (FluoroJade), iron distribution (Perls), as well as astrocyte (GFAP) and microglia (Iba-1) activity. A third experiment measured levels of iron binding proteins (ferritin and transferrin) at 14 days. *Results*. Striatal ICH caused functional impairments, which were significantly improved with ER. The ICH caused delayed peri-hematoma neuronal death, which ER significantly reduced. Hemispheric iron levels, the amount of iron binding proteins, and peri-hematoma astrocytes and microglia numbers were significantly elevated after ICH (vs. normal side), but were not affected by ER.

Conclusions. Rehabilitation is an effective behavioural and neuroprotective strategy for ICH. Neither effect appears to stem from influencing iron toxicity or inflammation. Thus, additional work must identify underlying mechanisms to help further therapeutic gains.

Introduction

Intracerebral hemorrhage (ICH) is a devastating stroke that accounts for 10-20% of all cases. Mortality rates are high and long-term neurological outcome is often poor (Chiu, et al., 2010, Sacco, et al., 2009). For instance, those with an ICH affecting the basal ganglia are often left with significant motor impairments that limit activities of daily living (Miyai, et al., 1997, Paolucci, et al., 2003). Animal ICH models have been instrumental in identifying mechanisms underlying brain damage and for testing putative neuroprotectants (Frantzias, et al., 2011, Kirkman, et al., 2011, MacLellan, et al., 2012), although none have yet been clinically proven to work. Unfortunately, few studies have examined recovery mechanisms after ICH (Auriat, et al., 2010, Jin, et al., 2010, Mestriner, et al., 2011, Nguyen, et al., 2008, Takamatsu, et al., 2010). Many reviews (Cramer, 2008, Krakauer, et al., 2012, Murphy and Corbett, 2009, Teasell, et al., 2006) also focus on ischemia assuming perhaps that the mechanisms and timing of repair processes and the effectiveness of rehabilitation strategies are similar between stroke types – an unproven assumption.

Compared to ischemic stroke, it is rare for clinical studies to specifically target ICH patients either to test efficacy or mechanisms of action of a rehabilitation treatment (Kwak, et al., 2010). However, several clinical studies have compared recovery after ischemic and hemorrhagic stroke with mixed results (Chiu, et al., 2010, Kelly, et al., 2003, Paolucci, et al., 2003), which has also been recently evaluated in a rat model of ICH (Mestriner, et al., 2013). A greater number of animal studies have proven that rehabilitation promotes behavioural recovery after ICH (Auriat, et al., 2010, DeBow, et al., 2003, Lee, et al., 2003, Mestriner, et al., 2011), although such therapies are not

always effective (Auriat, et al., 2006, MacLellan, et al., 2005). As with ischemia (Biernaskie and Corbett, 2001, Murphy and Corbett, 2009), rehabilitation has been shown to promote growth of neuronal dendrites (Auriat, et al., 2010, MacLellan, et al., 2011, Takamatsu, et al., 2010), and astrocyte processes and density (Mestriner, et al., 2011) following ICH. As in some situations with ischemic stroke (Yang, et al., 2003), rehabilitation can mitigate tissue damage when initiated either early or later after ICH (Auriat and Colbourne, 2009, DeBow, et al., 2003, Lee, et al., 2003). For example, DeBow and colleagues (DeBow, et al., 2003) were the first to show that forced use therapy started 1 week after ICH significantly lessened the final volume of brain damage in a rat ICH model. The mechanisms of this remarkable neuroprotective effect are not known. However, given that rehabilitation was given after 1 week, it makes sense to evaluate how rehabilitation affects pathophysiological mechanisms contributing to chronic cell death.

Unlike ischemia, a significant amount of damage after ICH results from mechanical trauma as blood dissects through tissue. This occurs rapidly whereas secondary damage occurs over days, even weeks. For instance, injecting collagenase into the brain, which damages the basal lamina of blood vessels causing a spontaneous bleed, leads to both rapid and chronic cell death (Del Bigio, et al., 1996, Rosenberg, et al., 1990). Indeed, in our lab this model causes significant tissue loss occurring beyond a week post-ICH (Auriat, et al., 2012, MacLellan, et al., 2008, Nguyen, et al., 2008). Secondary damage results from many factors including iron toxicity and excessive inflammation (Aronowski and Zhao, 2011, Keep, et al., 2012, Wang, 2010). Elevated ‘free’ iron in the brain, originating from degrading erythrocytes, promotes free radical

formation (e.g., OH^-) by Fenton reactions thereby damaging proteins, lipids and DNA (Aronowski and Zhao, 2011, Keep, et al., 2012, Nakamura, et al., 2006, Wagner, et al., 2002, Wu, et al., 2002). Along with these findings, chelator studies also strongly suggest that iron contributes to a considerable portion of secondary degeneration (Aronowski and Zhao, 2011, Huang, et al., 2002, Nakamura, et al., 2003). Furthermore, injecting iron directly into the brain mimics many features of ICH (e.g., edema, rapid cell death) including the occurrence of protracted cell death (Caliaperumal, et al., 2012, Huang, et al., 2002, Willmore and Ueda, 2009). Thus iron toxicity is widely accepted to cause acute and chronic cell death.

Iron toxicity does not go unchecked. After ICH there is a substantial and prolonged increase in iron binding proteins such as ferritin (iron storage) and transferrin (iron transfer) (Aronowski and Zhao, 2011, Wu, et al., 2003). Ferritin plays an important role in iron homeostasis through its two subunits, ferritin heavy chain (iron utilization within cells) and ferritin light chain (iron storage) (Aronowski and Zhao, 2011). Transfer of iron within or outside the cell is a function of transferrin and thus it plays an important role in iron clearance. Furthermore, inflammatory cells such as microglia and macrophages are essential for neutralizing iron and clearing debris (Aronowski and Zhao, 2011, Wang, 2010). Unfortunately, these defenses are overwhelmed after ICH. Thus, a more complete understanding of these and other processes are needed to develop effective neuroprotective strategies, and perhaps too for optimizing and understanding rehabilitation therapies.

Presently, we evaluated two general objectives: 1) whether rehabilitation lessens peri-hematoma neuronal death, and 2) whether changes in iron levels, iron binding

proteins or inflammation accounts for the neuroprotective effects of rehabilitation after ICH. We used the rat collagenase model of ICH because it causes spontaneous bleeding from multiple vessels, which has high face validity. As discussed, this model results in a well-defined profile of chronic cell death, behavioural impairment (walking and reaching deficits after striatal ICH), and responsiveness to rehabilitation. Enhanced rehabilitation (ER), which consists of environmental enrichment and a skilled reaching task (Biernaskie and Corbett, 2001), was used because it improves functional recovery and has been shown to decrease tissue loss after ICH (Auriat and Colbourne, 2009) as does a similar therapy (DeBow, et al., 2003). A one-week delay was used to mimic the delays encountered clinically (vs. ultra-early interventions commonly used in animal research). We evaluated whether ER affected non-heme iron levels 32 days after ICH (weeks after the 2-week ER treatment). Similarly the ongoing neuronal degeneration, iron-positive cells, lesion volume, astrocytes and microglia were also evaluated 14 days post-ICH (i.e., after 1 week of ER). Finally, protein expressions of ferritin H and L chain and transferrin were assessed 14 days post-ICH (i.e., after 1 week of ER).

Materials and Methods

Animals

All procedures followed the Canadian Council of Animal Care guidelines and were approved by the Animal Care and Use Committee for Biosciences at the University of Alberta. Eighty-four male Sprague-Dawley rats weighing 200–225g (~7 weeks old) were randomly assigned to Control or ER treatment in 3 experiments. Rats survived for 32, 14, and 14 days post-ICH in experiment 1 (n = 16 each), 2 (n = 16 in

Control, n = 20 in ER), and 3 (n = 8 each), respectively. Rats were kept in a temperature and humidity controlled room under diurnal light cycles (on 7 am - 7 pm). They were housed 4 rats/cage in standard polycarbonate cages (width: 38 cm, length: 49 cm, height: 20 cm) containing wood chip bedding with ad lib water and food, except as noted below.

Skilled Reaching (Experiment 1)

The staircase test (Montoya, et al., 1991), which measures skilled reaching for sugar pellets (dextrose and fructose, 45 mg, Bio-Serv, Frenchtown, NJ), was used because it is highly sensitive to striatal ICH (DeBow, et al., 2003, MacLellan, et al., 2006). Rats were trained over 4 weeks before ICH (two 15-minute sessions / day, 5 days / week). They were tested on day 6 and on days 28-32 post-ICH. The rats that did not reach an average of ≥8 pellets per session over the final three days of baseline testing were excluded. Rats were food deprived to ≈90% of their free feeding during these times, and their weight was slowly increased to accommodate natural growth. Note that behavioural training was conducted for Experiments 2 and 3 in order to maintain procedural consistency with Experiment 1. However, as there was no post-treatment assessment, which was due to the timing of euthanasia, we did not evaluate the baseline data for the staircase or ladder.

Walking Ability (Experiment 1)

The horizontal ladder test (Metz and Whishaw, 2009), which is sensitive to striatal ICH (DeBow, et al., 2003, MacLellan, et al., 2006), measures a rat's ability to

traverse a 1 m long horizontal ladder with rungs randomly spaced 1–4 cm apart (3 mm diameter). Walking ability was assessed for 2 days (4 crosses/day) and video recorded 2 days prior to ICH (all experiments) and on 6 (all experiments) and 32 days (experiment 1 only) post-ICH. Percent success was calculated as: successful steps / total steps ×100 (total of all ladder traverses in a day).

Intracerebral Hemorrhage

The collagenase model of ICH was used (Rosenberg, et al., 1990) as we have repeatedly done (Auriat and Colbourne, 2009, DeBow, et al., 2003, MacLellan, et al., 2006, MacLellan, et al., 2008). Rats were anesthetized with isoflurane (4% induction, 2% maintenance, 60% N_2O, remainder O_2) for aseptic stereotaxic surgery. Body temperature was maintained between 36.5-37.5°C by a rectal temperature probe and a warm water blanket. A hole was drilled at 3.5 mm lateral and 1.0 mm anterior to Bregma on the side contralateral to the more successful paw in the staircase test (number of pellets retrieved at end of training). Overall, 45% of rats included in this study had ICH on the left side. A 0.7 μL solution of sterile saline containing 0.14 U of collagenase (Type IV–S, Sigma–Aldrich, Oakville, ON) was infused over 5 minutes through a 26-gauge needle (Hamilton syringe; Hamilton, Reno, NV) at a depth 6.5 mm below the skull. The needle was removed after 10 minutes to avoid back flow. A small metal screw was inserted in the hole, bupivacaine was applied, and the wound was closed.

Enhanced Rehabilitation (ER)

The ER treatment, initially developed for ischemic stroke (Biernaskie and Corbett, 2001), started 7 days after ICH and lasted for 14 (experiment 1) or 7 days (experiments 2 and 3). The latter time was because animals were euthanized at 14 days post-ICH, a time we expected delayed cell death to occur based upon our experience with this model (Auriat, et al., 2012, MacLellan, et al., 2008, Nguyen, et al., 2008). The treatment consisted of reach training in a modified staircase apparatus and environmental enrichment housing for 5 consecutive days / week (Auriat and Colbourne, 2009, Auriat, et al., 2010, MacLellan, et al., 2011). There were four 15-minute reach training sessions (2 hour intervals) during the day. Essentially, rats were allowed unlimited access to sugar pellets (45 mg size) located in one large well on the side of their impaired limb. The amount of sugar pellets obtained was recorded for each session so that controls could receive the same amount in their home cages. Enrichment cages (width: 35 cm, length: 75 cm, height: 75 cm) contained ramps, tunnels, and various objects (e.g., toys changed twice weekly) and were used from 5:30 p.m. to 8:30 a.m. with free access to water and their daily ration of food to keep them at 90% of free feeding weight. During the day, ER rats were returned to their standard cages without food.

Non-Heme Iron Assay (Experiment 1)

Rats were euthanized 32 days after ICH to measure the total amount of ipsi- and contralateral-to-ICH (2 mm anterior to 4 mm posterior from the site of collagenase injection) non-heme iron using a Ferrozine based colorimetric assay that used freshly

prepared standard curves (Auriat, et al., 2012, Rebouche, et al., 2004). Cerebellum and liver samples were also analyzed as controls.

Histology (Experiment 2)

At 14 days post-ICH the rats were administered pentobarbital (100 mg/kg IP) and perfused transcardially with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde. Brains were fixed overnight and then transferred to 30% sucrose in PBS. We took 40 μm coronal sections to determine lesion volume (cresyl violet), peri-hematoma cell death (Fluoro-Jade B), Fe^{3+} accumulation (Perls), and astrocyte (GFAP) and microglia activation (Iba1). The volume of tissue lost in serial sections (every 400 μm) extending through and beyond the lesion, and including overt tissue damage and atrophy (e.g., ventriculomegaly), was calculated as routinely done (DeBow, et al., 2003, MacLellan, et al., 2006, MacLellan, et al., 2008):

a) Hemisphere volume = average (area of the complete coronal section of the hemisphere – area of ventricle – area of damage if any) × interval between sections × number of sections.

b) Tissue lost (mm^3) = volume of normal hemisphere - volume of injured hemisphere.

The number of Fluoro-jade B positive (FJ+, degenerating) neurons (Schmued and Hopkins, 2000) was determined in 3 coronal sections (200 μm apart) around the level of maximal injury (Caliaperumal, et al., 2012). Owing to the very large number of Perls' positive cells, we counted these in 1 coronal section at the level of maximum injury. Perls largely identifies ferric iron (Meguro, et al., 2007). While the number of these cells can be counted, the amount of iron per cell cannot be accurately determined with this method. Thus, counting Perls positive cells do not replace quantitative

methodology, such as that used in Experiment 1.

For immunofluorescence, the slides were boiled in 0.1 mol/L citrate buffer (pH 6.3) for 15 minutes in microwave (i.e., improved antigen retrieval). After cooling the slides for 10 minutes, the tissue was blocked for 1 h in room temperature in PBS-T (0.025% Triton X-100 in PBS) with 10% horse serum. The slides were incubated overnight in the following primary antibodies: mouse anti GFAP (1:400, Sigma, Canada) and rabbit anti-Iba1 (1:1000, Wako, Richmond, VA). The slides were subjected to three 5 min washes using PBS and PBS-T, followed by 1 h incubation with 1:1000 dilution Alexa 488- conjugated donkey anti-mouse and goat anti-rabbit antibody (Abcam, USA). The slides were washed again and then cover slipped. One section per brain was counted at 20× for both Iba-1 and GFAP (Wasserman, et al., 2008). The microglia was counted based on their morphology as ramified, activated and macrophage like (method adopted from (Wasserman, et al., 2008)).

Western Blot (Experiment 3)

Rats were euthanized as above and perfused with saline. Brains were quickly removed and frozen (-80°C). Subsequently they were dissected into ipsilateral (containing remnants of the ICH) and contralateral striatum (−2 to + 4 mm to injection site). These samples were homogenized quickly in 1× RIPA buffer containing protease inhibitors. Protein concentration was determined using Bradford assay. We used 8% and 12% (w/v) acrylamide gels for transferrin and Ferritin H / L chain, respectively (Wu, et al., 2003). Fifty µg of total proteins was loaded in each well during SDS PAGE electrophoresis. The proteins were electrotransferred onto nitrocellulose membranes and then blocked using tris buffered saline (TBS) with tween 20 in 5% non-fat milk (TBS-

TM) for 1 hour. The membranes were incubated overnight at 4°C with gentle rocking in the presence of mouse anti-ferritin H chain, anti-ferritin L chain, anti-transferrin, or β anti-actin at 1:1000 dilution in TBS-TM (Wu, et al., 2003). After successive washes with TBS and TBS-T, the membranes were incubated with a 1:2000 dilution of the appropriate HRP-conjugated secondary antibodies in TBS-TM for 1 h at room temperature and further washed with TBS and TBS-T. Proteins were detected using Western Lightning chemiluminescence reagent plus (Invitrogen, Canada) and visualized using a Kodak Image Station 440CF (Kodak, NY, USA)(Vilas, et al., 2012). Quantitative densitometry analyses were performed using Scion Image J (4.0; Scion Corporation, Frederick, MD).

Statistical Procedures

Surgical procedures were done blinded to group identity. It was not possible to be blinded to treatment administration. However, behavioural and histological assessments were done blinded.

The data are presented as mean ± standard deviation (SD) and were analyzed by repeated measures and/or 1-way analysis of variance (ANOVA) using SPSS (SPSS v.17.0, SPSS Inc., Chicago, IL).

Results

Exclusions

In experiment 1, 10 rats were excluded (n = 5 in each group) due to a technical error. Two rats in experiment 2 were excluded in ER group because they did not reach

the baseline criteria in staircase training. One Control rat was excluded from experiment 3 because of a technical error.

Experiment 1

An analysis of reaching success with the contralateral-to-ICH forelimb in the staircase test showed significant Day ($P < 0.001$) and Group ($P = 0.007$) main effects with a significant interaction ($P = 0.001$; Figure1A). Groups were not different prior to ICH ($P = 0.753$) or 6 days after ICH ($P = 0.760$), but the ICH did cause significant impairment (i.e., day 6 scores lower than baseline, $P < 0.001$). As predicted, ER rats retrieved almost twice as many pellets at 32 days post-ICH ($P = 0.003$).

The contralateral-to-ICH forelimb (Figure 1B) stepping success was evaluated for the ladder task revealing significant Day ($P < 0.001$) and Group ($P = 0.004$) main effects with a significant interaction ($P < 0.001$). Analysis of the contralateral-to-ICH hind limb stepping success (Figure 1C) revealed a significant Day main effect ($P < 0.001$) and interaction ($P = 0.007$) with a non-significant Group effect ($P = 0.406$). Owing to significant interactions, comparisons were made at each time for both limbs. At baseline, the groups had similar forelimb stepping success ($P = 0.677$), whereas the hind limb was statistically different ($P = 0.045$). There were no significant differences on day 6 after ICH for both fore ($P = 0.510$) and hind limbs ($P = 0.271$). The ER treatment significantly improved fore ($P < 0.001$) and hind limb walking abilities ($P = 0.008$) at 32 days post-ICH. Owing to the statistically significant baseline hind limb difference, we also expressed post-operative data as % baseline, but this did not meaningfully change our results, which showed no difference between groups on day 6

($P = 0.190$) whereas the ER group were significantly better on day 32 ($P = 0.031$, data not shown).

The ICH hemisphere had approximately twice as much non-heme iron (Figure 2) as the normal side ($P < 0.001$), but there was no effect of ER including in the cerebellum ($P = 0.450$) or liver controls (not shown, average value ~107 μg of iron / g of brain, $P = 0.773$).

Experiment 2

There were many FJ+ (recently dead) neurons at 14 days post-ICH. The ER treatment reduced, by approximately half, their numbers ($P = 0.040$; Figure 3A-C). The total volume of tissue loss was not significantly different at this time ($P = 0.394$; Fig. 3D,E).

All rats exhibited numerous Perls' positive cells (presumptive macrophages and microglia) at 14 days post-ICH (Figure 4A), which was not affected by ER ($P = 0.474$; Figure 4B). Similarly, the number of GFAP positive astrocytes were obviously increased in the peri-hematoma region (astrogliosis), but not affected by ER ($P = 0.214$, Figure 5A-C). Finally, the numbers of ramified, activated and macrophage-like microglia were substantial in the peri-hematoma / cavity region, but not different between groups ($P \geq 0.351$, Figure 5 D-H).

Experiment 3

The expression of ferritin H and L chain and transferrin in the ICH hemisphere was significantly higher than in the contralateral hemisphere ($P < 0.001$), but the groups were not significantly different ($P \geq 0.174$; Figure 6).

Discussion

Enhanced rehabilitation training substantially improved recovery of walking and skilled reaching following a striatal ICH of moderate severity. Benefit occurred despite using only a 2-week regimen given after a 1-week delay. Furthermore, chronic neuronal death in the peri-hematoma striatum was found and it was significantly attenuated with only 1 week of treatment. Thus, a modest rehabilitation therapy rescues many injured peri-hematoma neurons. Our study is the first to evaluate whether rehabilitation mitigates injury through affecting iron and inflammation. Although these are known effectors of peri-hematoma neuronal death, our results suggest they are not involved in the neuroprotective effects of rehabilitation, but they may be potential targets to further augment behavioural therapies.

Compared to ischemia, there are relatively few studies examining the effectiveness of rehabilitation treatments for ICH. Furthermore, the diversity among therapies (type, timing and intensity) and models makes it difficult to identify those treatments that work best. Perhaps it is telling that in our lab, forced use therapies with and without environmental enrichment work well (Auriat and Colbourne, 2009, Auriat, et al., 2010, DeBow, et al., 2003), whereas simple exercises (running) or enrichment alone are less effective (Auriat and Colbourne, 2008, Auriat, et al., 2006). Others have

also reported that skilled training is more effective than unskilled training after ICH (Mestriner, et al., 2011). Furthermore, the present findings confirm the remarkable efficacy of this ER regimen after ICH (Auriat and Colbourne, 2009, Auriat, et al., 2010, MacLellan, et al., 2011), which is based upon the seminal work of Corbett and colleagues in ischemic stroke (Biernaskie and Corbett, 2001). Further work is needed to determine if the mechanisms of action are similar. In this regard, it is clear that ER and other rehabilitation therapies increase dendritic complexity after ischemia (Biernaskie and Corbett, 2001, Murphy and Corbett, 2009), and this is true for ICH too (Auriat, et al., 2010, MacLellan, et al., 2011, Takamatsu, et al., 2010). However, delayed ER does not appear to be neuroprotective after ischemia, unlike for ICH. Certainly, this relates to differences between these stroke types with respect to the timing, extent and underlying mechanisms of chronic cell death, which clearly occurs after collagenase-induced ICH (Auriat, et al., 2012, MacLellan, et al., 2008, Nguyen, et al., 2008) (present data).

Our findings show that the number of degenerating peri-hematoma neurons, which we had not previously evaluated (Auriat and Colbourne, 2009, DeBow, et al., 2003), was significantly reduced after one week of ER treatment (two weeks post-ICH), but this did not lead to a significant reduction in lesion volume at that time. While these findings appear contradictory, we suspect that it simply takes more time and perhaps more therapy (e.g., the second week of our standard ER treatment) before there is a discernable reduction in the volume of tissue loss. Specifically, in Controls there would be ongoing injury (lesion expansion) beyond one or two weeks, as we have repeatedly found after collagenase-induced ICH (Auriat, et al., 2012, MacLellan, et al., 2008, Nguyen, et al., 2008). At the same time ER treatment would attenuate this progressive

injury thereby culminating in a long-term reduction in the volume of injury (Auriat and Colbourne, 2009, DeBow, et al., 2003). Thus, it would take weeks to notice the cumulative effects of reducing peri-hematoma damage. Of course other effects might also impact the volume of tissue loss (e.g., ER promotes dendritic growth). Further work should evaluate whether rehabilitation also prevents cell death beyond the end of treatment, and if not, whether extending the duration of treatment would provide greater protection. An additional unresolved issue concerns the contribution of these salvaged neurons to functional recovery. Unfortunately, there is no obvious way to causally link neuroprotection from ER to improved behavioural recovery after ICH. The fact that ER also improves recovery in the whole blood model of ICH (MacLellan, et al., 2011), which does not result in as much chronic cell death (MacLellan, et al., 2008), suggests that behavioural benefit is not solely dependent on neuroprotection. Regardless, we have observed an ~30% reduction in tissue loss after collagenase-induced ICH from rehabilitation at long survival times (Auriat and Colbourne, 2009, DeBow, et al., 2003), and it is difficult to argue that this would not be of behavioural significance. One must consider too that neuroprotective treatments rarely achieve this in any ICH model, let alone when the drug treatment is delayed for a week following ICH.

Non-heme iron levels were substantially higher in the injured hemisphere at 1 month following collagenase infusion. This is in line with our previous findings in this model that used both a Ferrozine-based method and x-ray fluorescence imaging (Auriat, et al., 2012), and with studies using the whole blood model (Wu, et al., 2003). Our findings also parallel the whole blood model with regard to the accumulation of iron-positive cells (Perls' stain), known to be mostly macrophages and microglia, and

increases in ferritin and transferrin proteins (Wu, et al., 2003). In total, such findings point to a chronic degenerative / repair process. Rehabilitation had no discernable effect on any of these processes thereby suggesting that ER is not mitigating iron load or the removal of degenerating erythrocytes and damaged brain tissue. However, several additional experiments are needed to confirm this. First, rehabilitation may influence oxidative stress by altering free radical defenses (Boveris and Navarro, 2008, Briones, et al., 2011). Second, whereas ER did not influence the number or morphology of microglia, or reactive astrogliosis, it is possible that ER influenced certain biochemical responses of these cells. Third, we did not examine multiple survival times following ICH; without this it is possible that some effects were missed. Thus, ER may alter the rate of hematoma clearance or iron detoxification, but we expect this is more likely following earlier interventions. We are currently examining these hypotheses. Along with this, ER likely has numerous other beneficial effects (e.g., changes in growth factors) that aid in recovery / neuroprotection.

The collagenase and whole blood models have a number of similarities as well as important differences (Kirkman, et al., 2011, MacLellan, et al., 2012). While it appears that key mechanisms of injury, such as iron toxicity and inflammation, are at play in each model, it is quite likely that their contribution varies by model and the fact that lesions are larger with collagenase than with a whole blood infusion of comparable hematoma size (MacLellan, et al., 2008). Neuroprotective treatments, therefore, may not be equally effective. Indeed, iron chelators, such as deferoxamine, appear to be more effective against whole blood (Chiu, et al., 2010, Huang, et al., 2002, Nakamura, et al., 2003) than collagenase induced ICH (Warkentin, et al., 2010, Wu, et al., 2011).

The same argument regarding differential mechanistic and efficacy effects may be made for rehabilitation. Thus, until additional data are available to better validate these models, it makes sense to test treatments in both models as we have been doing. Additional studies are also needed to vary insult location and size along with other important variables, such as age, that are known to influence outcome (Wasserman and Schlichter, 2008, Wasserman, et al., 2008).

In summary, ER substantially improved functional recovery after striatal ICH. The fact that chronic cell death was observed and that ER attenuated this, suggests that clinical rehabilitation therapies, at least when properly timed and at an appropriate dose, may be neuroprotective in humans suffering from ICH. Our study shows that rehabilitation is at least not markedly changing two key mechanisms of injury – iron toxicity and inflammation. This suggests that co-treating against these injurious processes coupled with rehabilitation might be additively or synergistically advantageous. Lastly, additional studies are needed to identify how rehabilitation improves recovery and reduces peri-hematoma injury after ICH. This information is needed to rationally devise more effective treatments that are especially needed in more challenging cases (e.g., older individuals, greater intervention delays, more severe stroke).

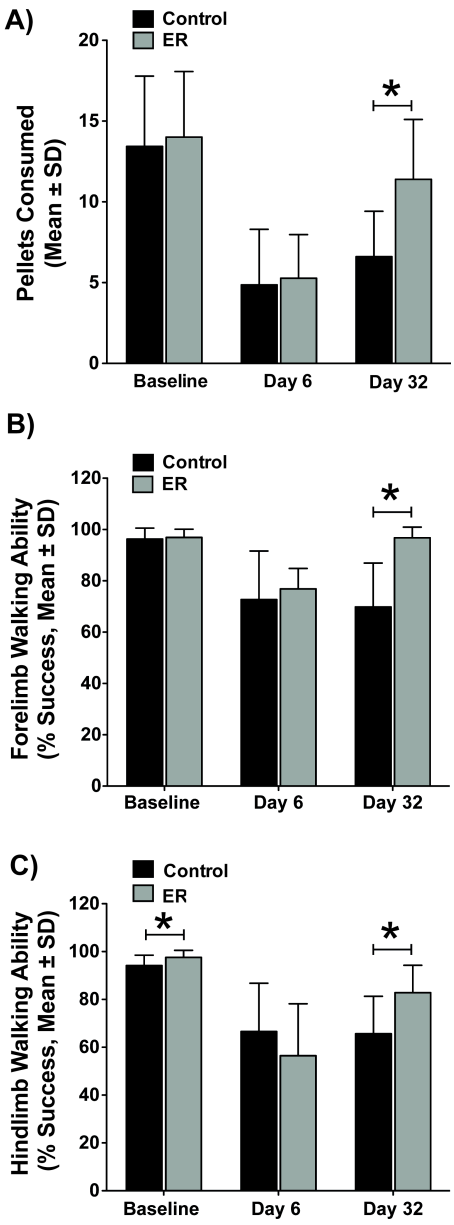

Figure 1. A striatal ICH caused significant skilled reaching (A) and walking impairments (B, C), which were significantly ameliorated by ER treatment. An * denotes $P < 0.05$. There was a small difference in baseline scores for the hind limb, but this did not meaningfully affect our findings as determined by an analysis on % baseline scores (not shown).

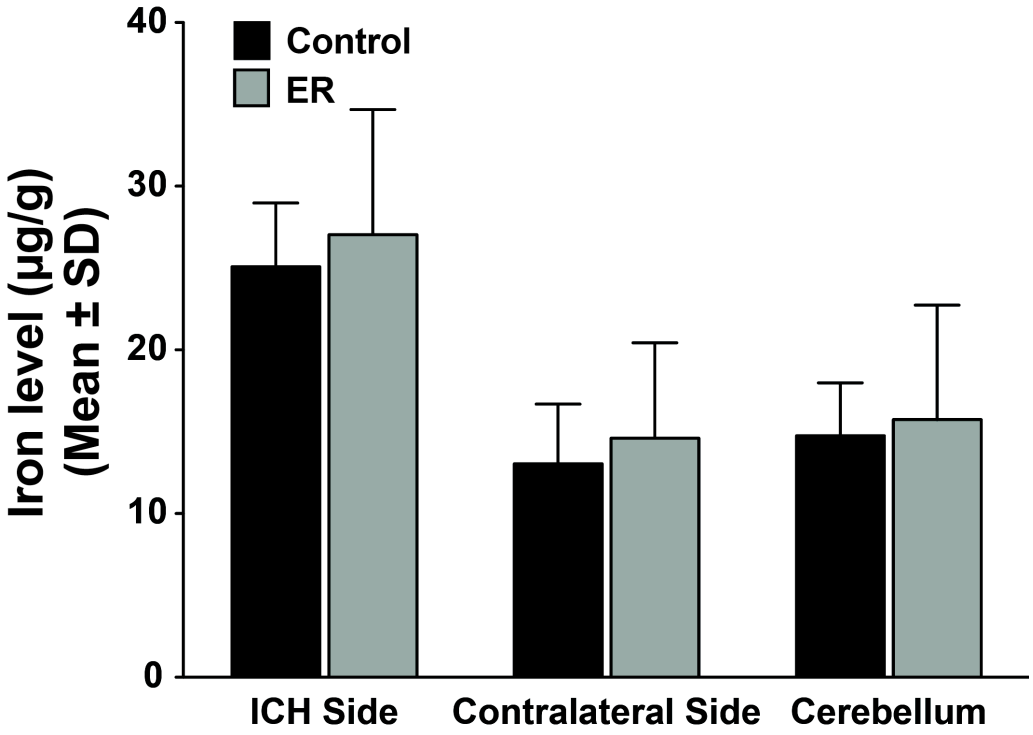

Figure 2. Non-heme iron levels were significantly higher in the injured hemisphere at 32 days after ICH (vs. contralateral side, $P < 0.001$). The ER treatment had no impact on any region sampled.

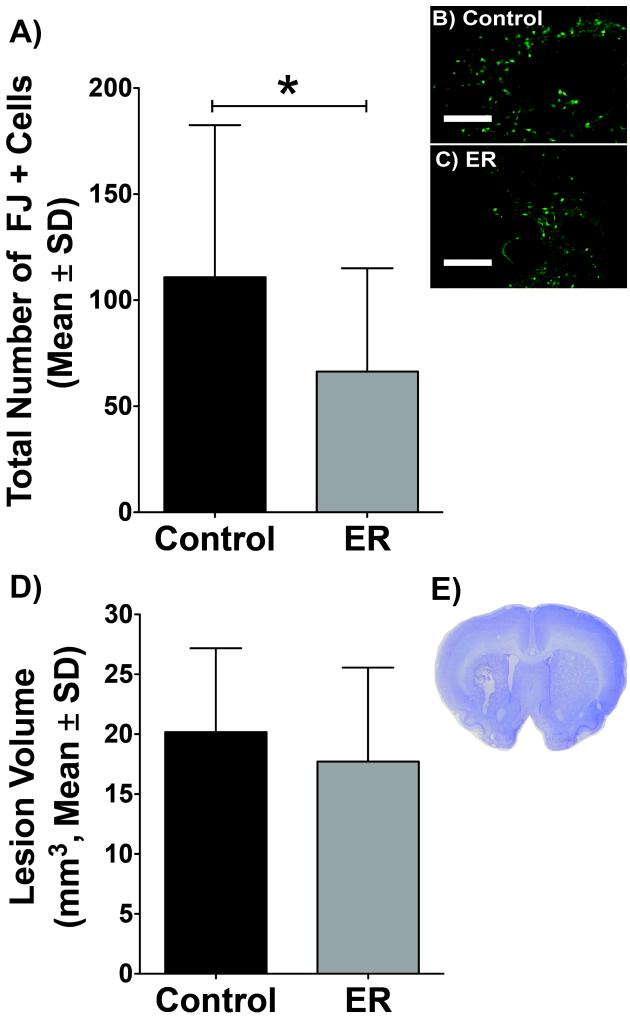

Figure 3. The ER group had significantly fewer degenerating neurons (Fluoro-jade positive – FJ+) in the peri-hematoma region than controls at 14 days post-ICH (A, **P* = 0.040). Representative photomicrographs are shown in B and C (scale bar = 500 μm). The volume of tissue loss was not different (D). A photomicrograph of the typical injury (E) illustrates the residual cavity and ventriculomegaly caused by the ICH 14 days earlier.

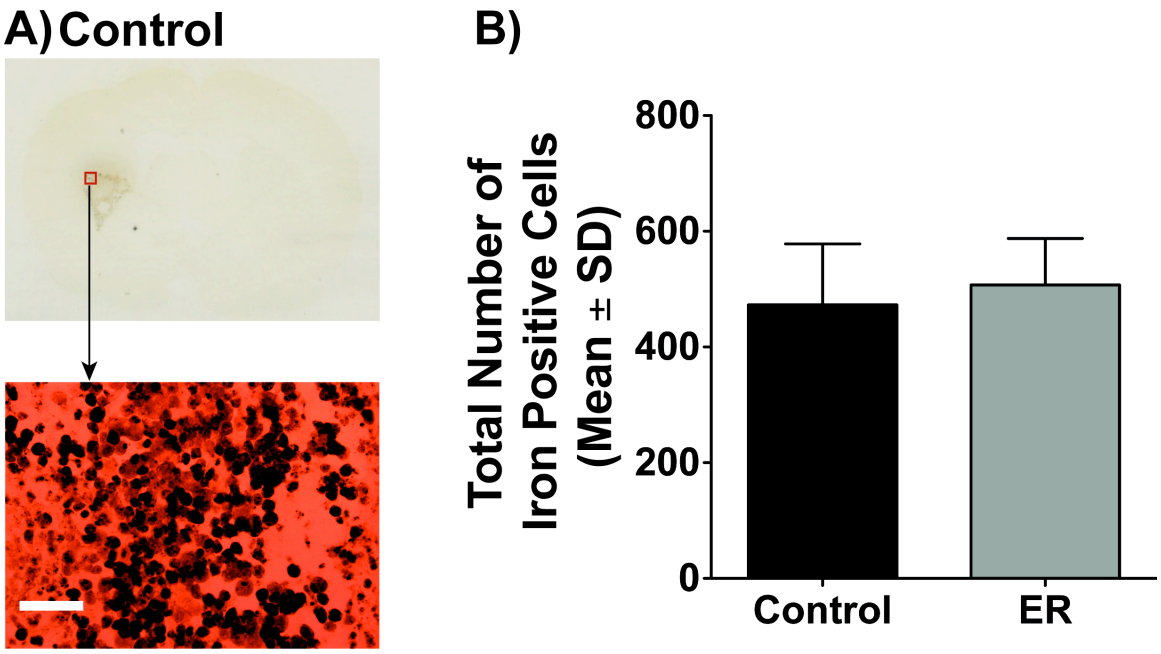

Figure 4. Representative photomicrographs of a control rat showing Perls' staining, which largely identifies ferric iron loaded in macrophages and microglia (A, scale bar = 50 μm). Quantification showed no significant difference between groups (B).

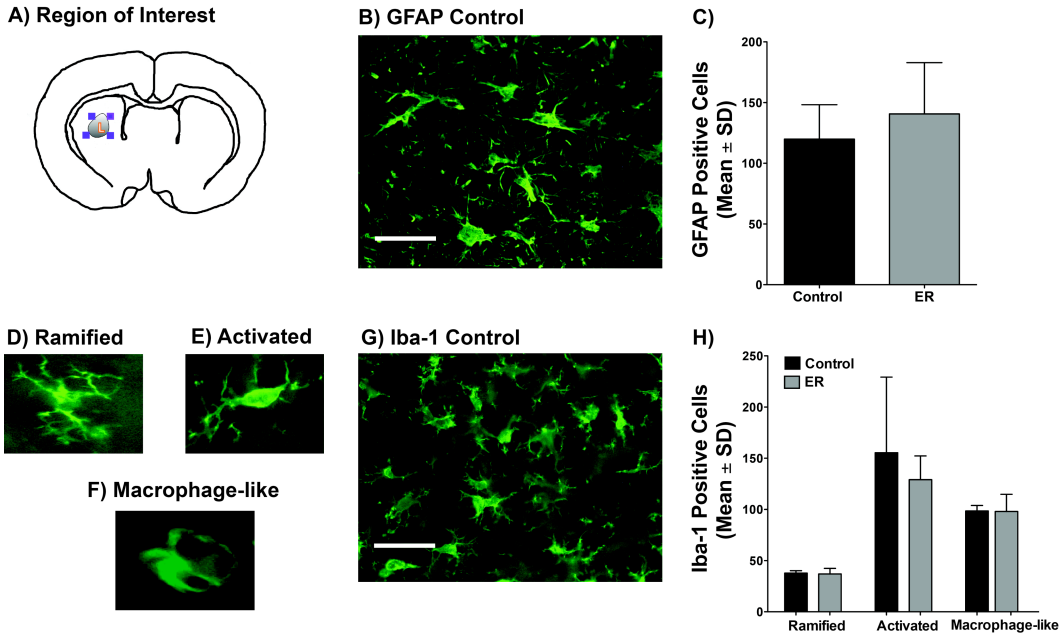

Figure 5. We counted GFAP and Iba-1 labeled cells in the peri-hematoma region (A). Labeled astrocytes from the control group are shown in B (scale bar = 50 μm). Quantification showed no group difference (C). The different stages of macrophages are illustrated in D, E and F along with a representative photomicrograph of a control animal in G (scale bar = 50 μm). There were no group differences in the number of these cells (H).

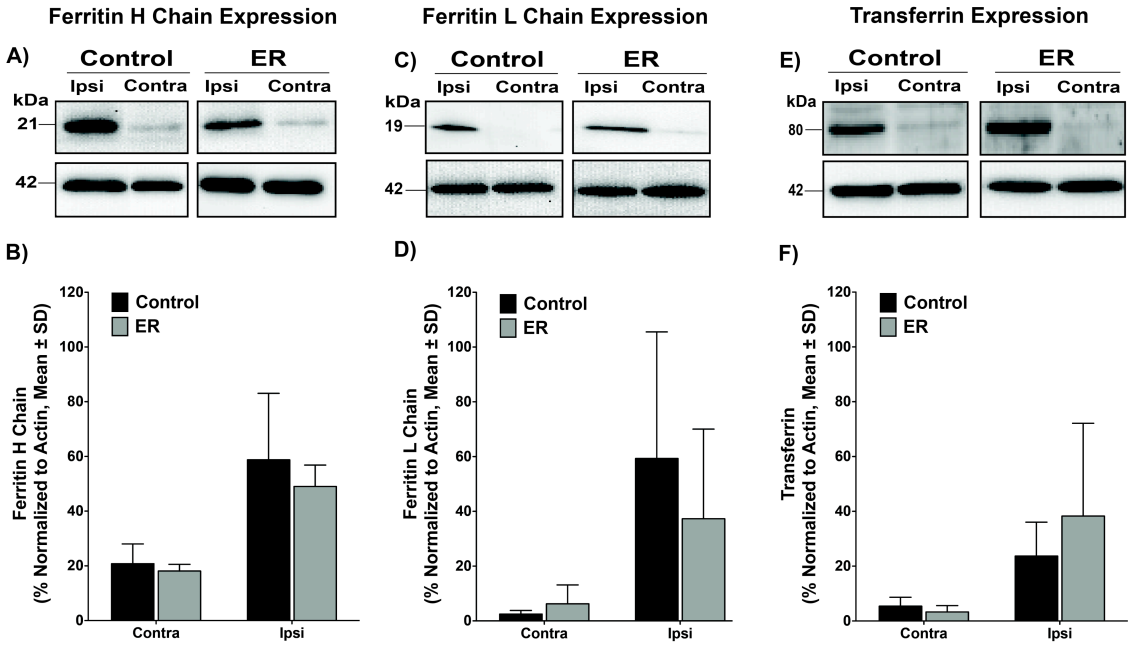

Figure 6. Representative Western blots showing the expression of iron handling proteins: ferritin H-chain (A), ferritin L-chain (C) and transferrin (E) at 14 days post-ICH. Data were normalized to actin (B, D and F) showing significant differences between injured and contralateral hemispheres $P < 0.001$), but no significant effect of ER treatment.

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Chapter 6

General Discussion

1. Summary of Findings

The focus of this thesis was to evaluate the role of thrombin and iron in causing brain damage associated with intracerebral hemorrhage. Understanding the time-period in which thrombin and iron are detrimental is crucial for developing therapies to target their deleterious effects. Another goal of this thesis was to understand the underlying mechanisms of recovery. The work done on the effects of rehabilitation after ICH reveals the mechanistic roles played by iron and inflammation in post-stroke recovery. The data presented in this thesis are novel, and contributes further to the understanding of the pathophysiology of ICH.

Cumulatively, the findings reported in the preceding chapters indicate that thrombin causes behavioural dysfunction, as well as the atrophy of surviving neurons and acute tissue loss. Interestingly, our findings indicate that thrombin is not responsible for chronic injury after ICH. Similar experiments with iron showed that iron causes behavioural dysfunction and neuronal atrophy, as well as short- and long-term tissue loss, neurodegeneration, and edema. Knowing the long-term detrimental effect of iron, we specifically targeted it using an iron chelator bipyridine. However, bipyridine did not reduce edema, al dysfunction, tissue loss or neurodegeneration.

As iron causes secondary neuronal degeneration for about 60 days (Caliaperumal, et al., 2012), we hypothesized that rehabilitation may be neuroprotective by reducing the ongoing cell death caused by iron. The data presented in chapter 6 show that rehabilitation, started at 7 days post-ICH and continued for about 2 weeks, improves the behavioural impairments. Importantly, we found a neuroprotective role of rehabilitation in significantly reducing the ongoing cell death. To elucidate the putative mechanism of

action we measured the inflammatory cells such as microglia and astrocytes, as well as iron-positive cells, iron handling proteins (ferritin and transferrin) and total iron levels. We did not find any changes in inflammation or iron toxicity post-ICH. This is the first study to investigate the influence of rehabilitation treatment on iron toxicity.

Even though the studies examined important mechanisms of injury and recovery, additional work is needed to understand the underlying mechanism behind the neuroprotective effect of rehab. The limitations of each experiment are already mentioned in the respective chapters (2-5). Hence this last chapter will add some extra points about the limitations and future directions.

2. Limitations and Future Studies for Thrombin and Iron Experiments

In chapter 2, the study showed that thrombin causes acute injury, along with behavioural impairments. Previous studies have shown that thrombin infusions into rat striatum can cause neurological deficits (Gong, et al., 2005, Hua, et al., 2002). For this reason we did only corner turn test to make sure we get behaviour deficits after thrombin injection. We administered thrombin and iron infusions in striatum by bolus injection in chapters 2 and 3. The bolus injection of thrombin and iron does not exactly mimic what happens in ICH patients: in patients, the release of iron from rupturing erythrocytes starts after 24 hours, and proceeds for many days (Wu, et al., 2010). Hence, using an osmotic pump to achieve the slow release of iron and thrombin would have been more physiologically accurate. However the infusion pumps are better than bolus injection but this method also results in greater concentration of iron release than found in ICH.

Our work has established that thrombin is deleterious after ICH, the mechanism through which it is harmful is still not clear. As previously mentioned, there are 3 types of thrombin receptors, i.e. PAR-1, PAR-2, PAR-3. The activation of PAR-1 leads to apoptosis and BBB damage (Noorbakhsh, et al., 2003, Xi, et al., 2001, Xi, et al., 2006, Xi, et al., 2003, Xi, et al., 2003). Experimental studies have shown that after ICH there is expression of PAR-1 receptor surrounding the hematoma (Guan, et al., 2004). Hence, it would be reasonable to evaluate the expression pattern of the PAR-1 receptor after ICH. Previously after collagenase ICH it has been showed that PAR-1 is activated up to 14 days (Zheng, et al., 2009). Studies are needed to evaluate long-term contribution of PAR-1 to thrombin induced injury. Further studies focusing on intracellular signaling cascades would help to determine whether the activation of PAR-1 has a beneficial, or a detrimental, role after ICH. For example, by combining with different G-proteins, PAR-1 may affect the morphology, proliferation, survival and transcriptional responses of neurons, as well as induce apoptotic cell death if coupled to independent tyrosine-kinase pathways. Hence, studying the temporal profile of the PAR receptor activity on different G-proteins will give us an idea of how long these receptors are active. Previous studies have shown that intracerebral injection of argatroban (thrombin inhibitor) three hours after ICH significantly reduced edema at 48 hours. (Nagatsuna, et al., 2005). Likewise hirudin was also shown to reduce edema and improve neurological deficits (Hua, et al., 2002, Lee, et al., 1996). Using thrombin antagonist drugs (e.g. argatroban, hirudin) during up-regulation of PAR-1 could be a potential therapeutic approach to reduce thrombin-induced cell death.

Other studies have also shown that thrombin can exacerbate iron-induced brain damage (Nakamura, et al., 2005). Further studies are needed to evaluate the combined role of thrombin and iron and find whether blocking the thrombin activation decreases iron-induced toxicity. Therefore, future studies are needed to study the combined role of thrombin, iron, heme and to see if these components interact to cause detrimental effects after ICH.

3. Limitations and Future Directions for Iron Chelator Experiments

Iron contributes to injury after ICH and hence in chapter 4 we tried an iron chelator (bipyridine) to attenuate the injury. However, the treatment failed to lessen the injury and behavioural dysfunction. First and foremost, there always remains controversy in some of the neuroprotective treatments. For example, deferoxamine has been shown to provide beneficial effect by reducing the lesion size, edema and neurological deficits (Gu, et al., 2009, Hatakeyama, et al., Hua, et al., 2008, Nakamura, et al., 2003, Nakamura, et al., 2004, Wan, et al., 2006) but another study showed no effect on multiple end points such as on edema, behaviour, lesion size etc. (Warkentin, et al., 2010)

There are several reasons for the variability of results seen in drug studies like bipyridine after ICH. The success and failure rate of the drug may be dependent on matching various factors such as infarct size, location of insult, experimental design variations, end points used, duration of treatment, survival time, group size and behaviour tests (Diguet, et al., 2004, Kirkman, et al., 2011, MacLellan, et al., 2012, MacLellan, et al., 2009).

4. Enhanced Rehabilitation (ER) Treatment and ICH

4.1 ER treatment and Oxidative Stress:

In chapter 5, we found out that rehab treatment decreased the ongoing cell death; therefore a follow-up experiment was performed in which oxidative stress was measured. Souza M et al (unpublished data from our lab) evaluated whether ER treatment affects oxidative stress. We evaluated oxidative stress after one week of rehab therapy, which was at 14 days post-ICH. We measured the levels of GSH (glutathione), GSSH (glutathione disulfide), GST (glutathione S transferase), GPx (glutathione peroxidase) and TBARS (lipid peroxidation). At 14 days post-ICH most of the oxidative stress measurement on the ipsilateral (ICH side), was same as the contralateral (normal side). However we found a significant effect of ER treatment in cytosolic levels of ratio of reduced and oxidized glutathione (GSH/GSSH). Therefore, ER increased the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSH), and prevented the cell from redox reactions or cellular toxicity. This may be a possible mechanism by which ER treatment showed a neuroprotective affect by reducing the ongoing cell death in the peri infarct region after ICH. Hence, future studies can aim to add antioxidant therapies with ER treatments, to get a synergistic affect of the treatment. Some limitations of the oxidative stress study include using smaller samples limited to peri-hematoma region. We should have also used other techniques such as synchrotron rapid scanning X- ray fluorescence (RS-XRF) imaging method, which helps to map and quantify the oxidative stress elements in the tissue slides.

4.2. Limitations and Future Studies of ER treatment

Firstly, the main limitation in chapter 5 as well as in other chapters 2, 3 and 4 is the use of young healthy animals to induce stroke, despite the fact that ICH commonly occurs in aged population. So future studies are needed to evaluate whether the same level of functional benefit occurs in aged animals. The reason for not using aged animals is mainly because we wanted to study the mechanism of recovery in young animals and then translate our results to old animals. One important consideration when performing rehab studies is the large amount of time required executing an experiment of this nature.

Secondly, we did not evaluate whether the neuroprotective effect was due to true recovery or by compensation. We used the Montoya staircase test, which measures the independent forelimb skilled reaching ability (Montoya, et al., 1991). The main disadvantage of using staircase test is that the detailed observations of animal behaviour are more difficult. For example, the precise motor organization of reaching cannot be performed. Thus, if we did assess different foot placement scores such as deep slip, slight slip, replacement, correction, partial placement and correct placement (Metz and Whishaw, 2002, Metz and Whishaw, 2009), we would have been able to determine whether there was true recovery versus compensation. Nonetheless, we do not think this would alter our conclusions that rehab improved functional performance.

There are several other issues to consider when selecting a sensitive behaviour test for efficacy studies like ER. We chose behaviour tests based on the location of injury, for example damage to dorsomedial striatum disrupts locomotor activity whereas lateral striatal lesions affect skilled motor control (Kirik, et al., 1998, Pisa and Schranz, 1988).

After ICH, often damage occurs to corpus callosum, internal capsule and striatum, which cause a broad range of deficits. Hence batteries of tests are needed rather than one or two test, for neuroprotection studies (MacLellan, et al., 2009).

Thirdly, we showed that delayed rehabilitation (combination of reach training and enriched environment), has shown to significantly reduce the neurodegenerating cells. The collagenase ICH model produces delayed atrophy and progressive tissue damage for 60 days (Nguyen, et al., 2008). Similar lesion growth occurs in stroke patients (Skriver and Olsen, 1986). Delayed rehabilitation interventions may be beneficial by recovering the atrophied neurons and decreasing cell death (Auriat, et al., 2010). However we do not know whether we will get the same neuroprotective effect when we reduce the intensity of and duration of treatment. It would be very difficult to investigate all the issues in one study; therefore future studies should study early versus late therapy, intensity, duration and amount of the therapy.

Lastly, we looked at inflammation and iron handling protein expression after one week of ER treatment; in the future it will also be important to look at other glial cells such as oligodendrocytes and ependymal cells and their roles after ER treatment (Wasserman and Schlichter, 2008). Future studies also need to evaluate the level of neurotrophic factor (BDNF, VEGF) expression after ER treatment. Hence the role of angiogenesis, axonal growth proteins (e.g. ephrin A5) can also be altered by ER treatment to provide a neuroprotective effect. Figure 1 illustrates the future possible studies to evaluate the mechanisms of recovery after ER treatment.

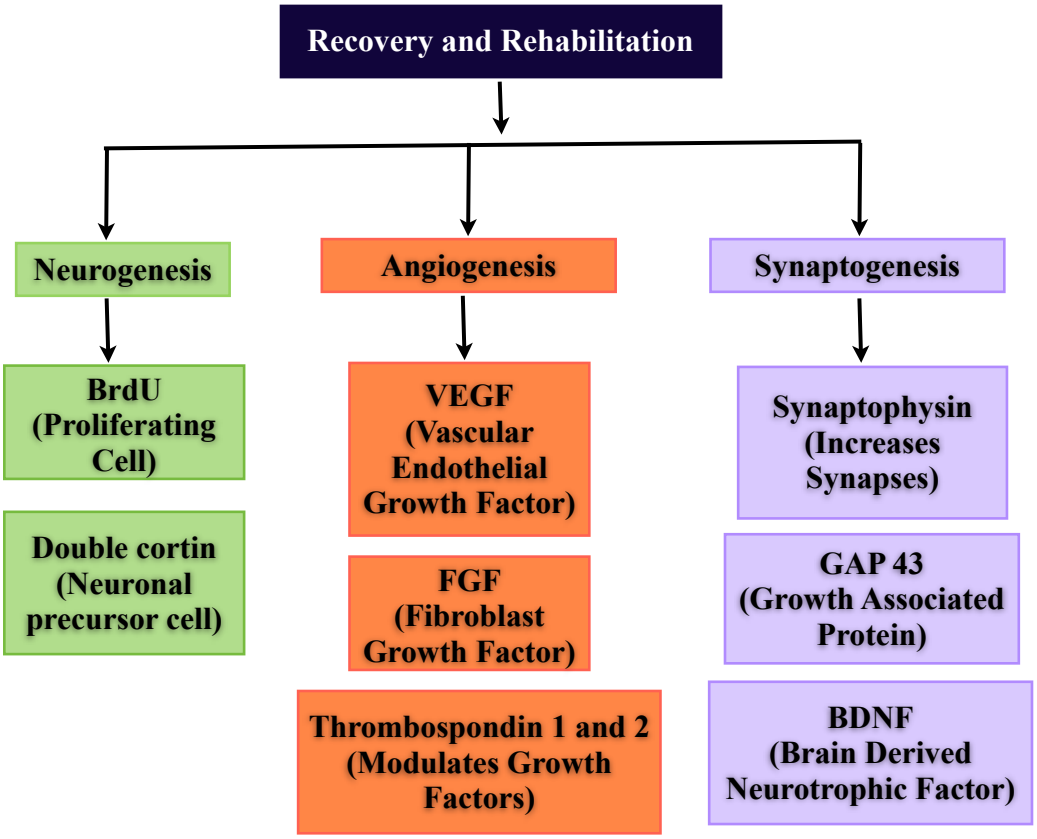

Figure 1. Future directions can evaluate the role of ER treatment on the above mentioned factors listed in the table. The green coloured tables illustrate markers that can be used to identify neurogenesis (newly formed cells). Likewise orange and purple colour denotes the proteins and growth factors that can be measured to study the role of angiogenesis and synaptogenesis respectively.

In summary, we believe that rehabilitation is an effective therapy for striatal ICH. Rehabilitation is effective in reducing neurodegeneration by lessening the oxidative stress. However ER did not lower the total iron levels or iron handling protein expression (ferritin H and L chain, transferrin) or inflammation (microglia and astrocytes) after ICH. Hence, future studies should consider using this neuroprotective therapy and study ways to further improve the recovery mechanisms after ICH.

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