University of Alberta

USE OF ESTROGEN AND REHABILITATION TO IMPROVE RECOVERY IN THE COLLAGENASE MODEL OF INTRACEREBRAL HEMORRHAGE IN RATS

By

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ABSTRACT

Intracerebral hemorrhage (ICH) is a devastating injury which produces chronic and debilitating neurological deficits. While the histologic features of ICH have been well established in the acute period (seven days) following injury, longer term data are lacking. I chose to fill this gap by inducing ICH in rats using the collagenase model and examined the histological development of the injury up to eight weeks. Lesion volume measurements, white matter quantification, and Golgi staining to quantify the injury to both white and grey matter were used. Briefly, I found an ongoing loss of white matter extending to eight weeks, whereas grey matter not lost during the late period. This confirmed previous similar work using serial magnetic resonance imaging. Possible mechanisms are discussed.

The second portion of my thesis concerns the behavioural deficits associated with ICH. Intensive rehabilitation improves functional outcome after ICH in rats. Environmental enrichment (EE) promotes recovery in several other stroke models. Estrogen treatment prior to ICH has also been shown to be neuroprotective. We hypothesized that either estrogen and/or EE might modify the natural history of ongoing injury following ICH when applied in the late period. Moreover, we questioned whether estrogen and EE might act synergistically when given together. I therefore examined the effect of both interventions on functional and histologic measures of ICH in rats. Briefly, I found that EE can promote recovery after ICH injury; however, estrogen treatment does not add to his effect. The possible interpretations of these results and future directions are discussed.

Dedication

I would like to dedicate this thesis to my mother, who has always been my strength and inspiration.

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I would like to thank my family for their love and support throughout the course of my thesis. I would also like to thank the members of my committee (Drs. Kathryn Todd and Dallas Treit) for their comments and suggestions. I am also grateful to the members of my lab, who have all made grad school enjoyable and memorable. Lastly, I would like to thank Dr. Frederick Colbourne for his guidance and supervision throughout the entire thesis.

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List of Abbreviations

BBB – Blood brain barrier

BDNF – Brain derived neurotrophic factor

CBVA – Cortical blood vessel avulsion

CC – Corpus callosum

CIMT – Constraint-induced movement therapy

Coll – Collagenase infusion

CT – Cortical thickness

E2 – 17β-estradiol

EE – Environmental enrichment

ICH – Intracerebral hemorrhage

ICP – Intracranial pressure

MMP – Matrix metalloproteinase

MRI – Magnetic resonance imaging

OVX - Ovariectomy

rFVIIa – Recombinant factor VIIa

ROS – Reactive oxygen species

SH - Sham

STD – Standard

tPA – Tissue plasminogen activator

TUNEL - Terminal uridine deoxynucleotidyl transferase dUTP nick end labeling

WBI – Whole blood infusion

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

Between 40,000 and 50,000 strokes occur annually, resulting in a 32% mortality rate, making stroke the leading cause of disability and the fourth leading cause of death in Canada. Approximately 300,000 Canadians currently live with the effects of stroke, and more than half of stroke survivors are left dependent on others for daily living (Rothwell et al., 2004). The financial burden of stroke on the Canadian economy was estimated to be $2.7 billion in 2002; with a growing elderly population, the cost of stroke is expected to increase. Approximately 15% of strokes result from primary intracerebral hemorrhage (ICH) (Thrift et al., 1995; Brown et al., 1996; Mayo et al., 1996; Broderick et al., 1999; Qureshi et al., 2001; Lee et al., 2007), and about 30% of thrombo-embolic strokes undergo hemorrhagic transformation (Lyden and Zivin, 1993), particularly after following treatment with anti-coagulants (Larrue et al., 1997; Larrue et al., 2001; Castellanos et al., 2004; Foerch et al., 2007).

Intracerebral hemorrhage occurs when a damaged blood vessel spontaneously ruptures. The mortality rate of ICH is about 35 - 52% within 30 days of stroke, and about half of these patients die within two days (Broderick et al., 1993; Broderick et al., 1999). Level of consciousness strongly predicts adverse outcome; age and heart disease also correlate with poor outcome (Nilsson et al., 2002). Early mortality is caused by the primary mechanical injury due to the space occupying effects of a hematoma (Fujii et al., 1994; Kazui et al., 1996; Brott et al., 1997), and brain herniation resulting from increased levels of intracranial pressure (Fewel et al., 2003). Few treatments improve functional outcome clinically (i.e., rehabilitation (Paolucci et al., 2003) or experimentally (i.e.,

CIMT, (DeBow et al., 2003); hypothermia, (MacLellan et al., 2004); free-radical scavengers, (Peeling et al., 2001)). Three main goals in treating ICH are (1) prevention of early death from herniation; (2) limiting the initial injury; and (3) promoting recovery after ICH (Ferro, 2006). Surgical (Juvela et al., 1989; Zuccarello et al., 1999; Skidmore and Andrefsky, 2002; Xi et al., 2002; Mendelow et al., 2005) and hypotensive (Jauch et al., 2006; Sorimachi et al., 2007) treatments have been used previously in an attempt to control intracranial pressure, with little clear beneficial evidence (Aguilar and Demaerschalk, 2007), though patients with more superficial hematomas do show benefit from early surgical intervention (Lee et al., 2003; Teernstra et al., 2003; Mendelow et al., 2005). There has been more progress with treatments that minimize the initial injury, such as using the hemostatic agent recombinant factor VIIa (rFVIIa) (Flaherty et al., 2005; Mayer et al., 2005b; Mayer et al., 2005a; Ferro, 2006; Aguilar and Demaerschalk, 2007; Marietta et al., 2007).

Despite these advances, stroke survivors and their families are left to deal with the devastating disabilities (Taylor et al., 1996; Broderick et al., 1999; Gebel and Broderick, 2000). Rehabilitation can sometimes dramatically reduce disabilities in stroke survivors (Chae et al., 1996; Kaste et al., 2000; Paolucci et al., 2003). Rehabilitative therapies rely on promoting the spontaneous neurological recovery, by maintaining and strengthening the remaining connections, as well as teaching the patient to learn new compensatory techniques which could improve activities of daily living. Current research in rehabilitation is focused on understanding the underlying mechanisms of recovery, such as synaptogenesis and dendritic arbourisation to maximize the recovery process. A better understanding of these processes can contribute to developing better therapies or

combinations of therapies that will give stroke patients and their families a higher quality of daily living.

1.2 Risk factors

Several risk factors predict the development of stroke. Age is the single most important risk factor for both ischemic and hemorrhagic stroke. The rate of ischemic stroke doubles for every successive decade after the age of 55 in both men and women (Wolf et al., 1992; Brown et al., 1996). Similarly, the rate of incidence for hemorrhagic stroke increases exponentially with advancing age (Skidmore and Andrefsky, 2002; Woo and Broderick, 2002). Sex, race, and heredity have also been identified as non-modifiable risk factors for stroke. Alternatively, several modifiable risk factors for stroke have been identified, hypertension being the most powerful modifiable risk for both ischemic and hemorrhagic strokes. Antihypertensive treatment is highly effective in preventing both ischemic (SHEP Cooperative Research Group, 1991; MacMahon and Rodgers, 1994) and hemorrhagic stroke (SHEP Cooperative Research Group, 1991). Several important lifestyle factors widely regarded as unhealthy have also been identified as modifiable risk factors for ischemic stroke. These risk factors include cardiac disease (Wolf et al., 1991a; Wolf et al., 1991b; Bikkina et al., 1994), physical inactivity (Kiely et al., 1994), excessive alcohol consumption (Hillbom and Kaste, 1990; Regan, 1990), and cigarette smoking (Shinton and Beevers, 1989). Lifestyle risk factors which specifically apply ICH include anticoagulant, antithrombothic therapies and cerebral amyloid angiopathy characterized by ß-amyloid protein build-up on vessel walls (Mandybur, 1986), and anticoagulation and antithrombotic therapy (Radberg et al., 1991; Hart et al., 1995). Management of

modifiable risk factors and attending to lifestyle factors may aid in stroke prevention or improved outcome after stroke.

1.3 PATHOLOGY

Intracerebral bleeding has historically been considered a monophasic event, whereby bleeding from a ruptured blood vessel was completed within minutes. Recent studies show that hemorrhagic stroke is a much more complex and dynamic process with effects that last weeks beyond the first several minutes. The complex pathophysiology of hemorrhagic stroke can be divided into two general categories of primary and secondary injury. The primary injury and initial cell death are due to the space occupying effects of the hematoma (Fujii et al., 1994; Brott et al., 1997). ICH can occur as a single large bleed, but many patients have ongoing bleeding, which contributes to hematoma expansion (Fujii et al., 1994; Kazui et al., 1996; Qureshi et al., 2001). These shearing forces and elevated intracranial pressure cause direct and immediate tissue destruction. Hematoma expansion, when severe enough can cause a midline shift, associated with seizures after ICH (Broderick et al., 1999). The immediate nature of primary injury limits treatment efficacy, particularly as half of the early mortality cases occur within the first two days (Broderick et al., 1993). Further, the initial primary injury is complicated by secondary events.

Secondary events occur downstream from the initial hemorrhage (Xue and Del Bigio, 2000; Gong et al., 2001). The effects of ICH are not only localized to the area of hematoma itself, but can be observed in distant brain regions. These effects include edema (Yang et al., 1994; Wagner et al., 1998; Song et al., 2003), inflammation (Gong et

al., 2000; Xue and Del Bigio, 2000), and disruption of the blood brain barrier (BBB) (Nakashima et al., 1999; Matsushita et al., 2000; Qureshi et al., 2001; Qureshi et al., 2003). Early vasogenic edema following ICH is due to an increase in extracellular fluid that accumulates in the brain as the blood brain barrier breaks down (Butcher et al., 2004). This edema occurs immediately after the bleeding and peaks about four to five days after the initial injury in humans (Butcher et al., 2004) and at three days post-injury in rats (Fingas et al., 2007). The delayed edema that occurs during the first two days after ICH in rats is both vasogenic and cytotoxic, and is induced by the components of the coagulation cascade and thrombin production (Xi et al., 1998b), blood degradation products (Xi et al., 1998a; Huang et al., 2002), and matrix metalloproteinases (MMPS) (Alvarez-Sabin et al., 2004).

Inflammation plays a highly complex role in mediating ICH injury, and occurs soon after ICH when the blood components and plasma proteins enter the brain; the inflammatory response peaks several days later (Gong et al., 2000; Wang and Dore, 2007). The cellular components of inflammation involve movement of leukocytes, macrophages, and microglia into the affected brain tissue (Del Bigio et al., 1996; Gong et al., 2000). This inflammatory response is necessary as it limits the injury by clearing the hematoma. These cellular components release molecular signals such as cytokines, proteases, MMPs, and reactive oxygen species (ROS) to develop and maintain the inflammatory response (Xue and Del Bigio, 2000, 2003). However, these molecular signals can further contribute to the injury and neurotoxicity (Wang and Dore, 2007; Wasserman et al., 2007). Studies in both the bacterial collagenase and whole-blood models of ICH show the presence of neutrophils in and around the hematoma, with

similar temporal patterns, beginning as early as four hours after ICH, peaking two to three days after, and disappearing three to seven days after ICH (Xue and Del Bigio, 2000, 2003; Wasserman and Schlichter, 2007; Wasserman et al., 2007).

As such, the mechanisms of damage that occur following ICH are expected to affect fibre tracts as well as cell bodies. It was previously thought that ICH injury occurs quickly; however recent data suggests a slow maturation of brain injury that evolves over days, and even weeks after the initial insult (Xue and Del Bigio, 2000; Gong et al., 2001; Felberg et al., 2002).

1.4 Rodent models of intracerebral hemorrhage

Experimental stroke therapies are generally tested in the laboratory setting with *in vitro* and *in vivo* models of stroke. Although *in vitro* models can mimic some aspects of stroke such as oxygen or glucose deprivation, it is difficult to approximate the combination of events of stroke *in vitro*. This is particularly true of ICH where there is a cascade of complicated events such as the hematoma growth and elevated intracranial pressure (ICP), inflammation, and disrupted BBB. Researchers have modeled strokes in non-human primates, pigs, and dogs; however, rodent models of stroke are most often studied due to ethical and financial concerns. Common rodent models of stroke can be categorized according to subtype of stroke: global ischemia, focal ischemia, and hemorrhage. Species of rodents used are dependent on several factors such as vasculature and type of experimental endpoint. For example, mouse models of stroke are utilized when investigating genomics; however, rats are most often used because of the relatively larger brains and access to a wider battery of behavioural tests. To study the basic

processes and possible treatments for stroke in the laboratory, it is essential to have animal models of stroke that are clinically relevant, whereby the pathophysiological parameters are similar to those that occur in the human condition. The appropriateness of animal models of stroke has come into question as many experimentally neuroprotective treatments have failed in the clinical setting (STAIR, 1999; Gladstone et al., 2002; Dirnagl, 2006; O'Collins et al., 2006). Additionally, it is essential to consider not only the model, but also the experimental endpoints. It is important to consider the behavioural endpoints and their relation to histological measures. There are many cases whereby stroke therapies have failed in the clinical setting despite having shown some histological benefits in the experimental setting because these histological benefits do not persist (Corbett and Nurse, 1998). Furthermore, some studies have found functional improvements, but these are generally limited to simple tests and disappear in the face of more demanding tasks. It is most likely that many of the treatments for a devastating stroke type such as ICH generally target only one mechanism of injury and do not assess the long-term effects of ICH.

The majority of studies examining hemorrhagic stroke use rodent models of intracerebral hemorrhage. The most common and widely accepted ICH models are the bacterial collagenase and autologous whole-blood injections into the striatum (Bullock et al., 1984; Andaluz et al., 2002). Infusion of bacterial collagenase into the striatum will cause digestion of the Type IV collagen of the basal lamina in surrounding blood vessels, causing vessels to rupture and resulting in hemorrhage (Rosenberg et al., 1993). Although widespread degradation of the basement membrane does not reflect the human condition, this bacterial collagenase model of ICH is a consistent model with persistent and well-

characterized behavioural and histological deficits (MacLellan et al., 2006). In the whole-blood model, autologous whole blood is withdrawn from the rat and injected into the striatum (Bullock et al., 1984; Maclellan et al., 2007). This model is commonly used to study the pathophysiology, biochemistry, and behavioural deficits associated with ICH. Although this model is more similar to the monophasic single large bleed seen in the clinical setting, the long-term histological effects are less detrimental than those observed clinically. Accordingly, the resulting behavioural deficits are generally resolved three to four weeks following ICH. A recent MRI study comparing the progression of injury and functional recovery in the bacterial collagenase and whole-blood models of ICH has shown major differences between the models (Maclellan et al., 2007). In general, injury is greater and functional deficits persist for longer in the collagenase model, and the differences in injury may be related to how the hematoma is formed in each model (Maclellan et al., 2007). Other ICH models examine specific components of ICH damage. For example, implantation of an inflatable micro-balloon in the striatum mimics the space-occupying effects of a large bleed. Alternatively, injections of blood components such as thrombin are often used to characterize pathophysiology of ICH.

1.5 BEHAVIOURAL DEFICITS

Behavioural endpoints and the relation to histological measures are important for the validity of an experimental stroke study. Several behavioural tests exist to measure cognitive and motor impairments following stroke. Measures of cognitive impairment include the widely used Morris water maze, radial arm maze, and Barnes maze and their several permutations to examine spatial learning and memory deficits. There are also

several tests for locomotion, skilled movements, and sensory and sensorimotor deficits following stroke, such as the horizontal ladder walking (Metz and Whishaw, 2002), single pellet (Whishaw, 2000), and limb-use asymmetry tests (Schallert et al., 2000; Schallert, 2006; Shanina et al., 2006). When examining a variety of brain deficits resulting from injury and stroke, a battery of sensitive behavioural tests should be used to detect dysfunction and measure neuroprotective effects and/or recovery. Comprehensive histological and functional evaluation at long survival times has been highly recommended for experimental stroke therapies.

1.6 Objective of the Present Thesis

The purpose of this thesis was to examine the time course of ICH injury to define an optimal therapeutic window for appropriate pharmacological and rehabilitative interventions. In the present thesis, I sought to evaluate the time course of injury after ICH and to determine whether delayed degeneration could be ameliorated with a combination of putative cytoprotective and rehabilitation treatments. Existence of delayed cell death would indicate a possible therapeutic target for recovery following ICH injury. Clinical studies of stroke recovery suggest that there is functional reorganization around the area of infarct (Cramer et al., 1997; Cuadrado et al., 1999). Although high mortality rates are associated with hemorrhagic insults, studies have shown a better functional outcome following a hemorrhagic than a non-hemorrhagic injury when matched for initial severity, as initial symptoms such as edema resolve (Chae et al., 1996; Paolucci et al., 2003). However, patients are nonetheless left with permanent functional impairment. It is therefore important to address the issue of recovery following

ICH experimentally to maximize the functional recovery after ICH. Furthermore, behavioural and histological benefits are seen following rehabilitation treatment after experimental ICH (DeBow et al, 2003). Therefore, I also examined a novel rehabilitation treatment using a combination therapy to promote survival and recovery of cells following ICH injury in a second study. I chose to use delayed estrogen (E2) treatment because previous studies have shown that its pre-treatment reduces cell death from global (Sudo et al., 1997; Jover et al., 2002; Shughrue and Merchenthaler, 2003), and focal (Simpkins et al., 1997; Dubal et al., 1998; Carswell et al., 1999; McCullough et al., 2001) ischemia, as well as after ICH (Auriat et al., 2005; Nakamura et al., 2005). Furthermore, E2 promotes dendritic arbourisation and synaptogenesis in the hippocampus, which is correlated with hippocampal-dependent improvements in memory (McEwen and Woolley, 1994). Estrogen may facilitate spontaneous recovery, as well as promote neuronal survival and plasticity that is associated with rehabilitative treatment after stroke (Biernaskie and Corbett, 2001). As such, I am proposing using a multimodal approach to investigate maturation of injury after ICH, which may be a target for pharmacological or rehabilitation interventions.

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CHAPTER 2

PROGRESSIVE INJURY AFTER COLLAGENASE-INDUCED INTRACEREBRAL HEMORRHAGE IN RATS

2.1 INTRODUCTION

Intracerebral hemorrhage (ICH) accounts for ~15% of all strokes (Broderick, 1994; Mayo et al., 1996; Qureshi et al., 2001). It is one of the most lethal and functionally devastating strokes with few treatment options. Primary ICH results from the spontaneous rupture of a blood vessel(s) damaged from chronic hypertension and/or amyloid angiopathy whereas secondary ICH originates from bleeding vascular abnormalities (e.g., aneurysm), coagulation defects or tumors. Hemorrhagic transformation sometimes follows occlusive stroke, occurring spontaneously (Lyden and Zivin, 1993) or from use of tissue plasminogen activator (tPA) (The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group, 1995).

Tissue destruction results from the immediate mechanical damage caused by infiltrating blood (e.g., shearing forces, space occupying effects) followed by secondary degenerative events (e.g., edema, disrupted blood-brain barrier, inflammation). An ICH was initially thought of as a monophasic event wherein bleeding is rapidly terminated by clotting and the tamponade effect. However, in many patients the hematoma expands, as noted with successive imaging, leading to poor outcome (Fujii et al., 1994; Kazui et al., 1996; Brott et al., 1997; Fujii et al., 1998). Continued bleeding in this acute period probably occurs from the initial site of bleed as well as from injury to other vessels caused by the initial bleed.

For many reasons, the substantial amount of tissue destruction caused by the rapid, primary injury will likely continue to go untreated. However, it is expected that benefit would result from limiting ongoing bleeding such as with rFVIIa (Mayer et al., 2006); although, a recent clinical trial found no improvement in functional outcome

despite a reduction in hematoma size (Mayer et al., 2005). Beyond this time, secondary effects, which take place over weeks, can be targeted as these clearly exacerbate the initial insult (Xue and Del Bigio, 2000; Gong et al., 2001; MacLellan et al., 2007). Thus, an understanding of such delayed injury is of high therapeutic importance especially given the difficulty with attenuating the primary insult.

The loss of neural tissue in the hematoma along with eventual resolution of inflammation leads to cavitation and commonly ventriculomegaly (e.g., following basal ganglia injury). Several groups (Gong et al., 2001; Wasserman et al., 2007), using rodent models, have shown that cell death occurs in the peri-hematoma region over a few weeks, and this likely contributes to the increase in total tissue lost seen in a recent study that used magnetic resonance imaging (MRI) out to four weeks post-ICH in rats (MacLellan et al., 2007). The loss of neurons in the peri-hematoma region, however, seems too small and it does not occur late enough to fully account for the substantial and delayed increase in tissue loss observed. Given the tendency of blood to dissect along white matter tracts during the bleed, it makes sense that ongoing white matter injury and atrophy probably contributes to delayed tissue loss. Unfortunately, most ICH studies do not assess white matter injury. Indeed, clinical trials of cytoprotective agents may be less successful because the effect of white matter injury is usually overlooked (Stys, 1998; Dewar et al., 1999).

In a series of three experiments, we examined the maturation of long-term tissue loss following collagenase-induced striatal ICH in rats. This widely-used model, developed by Rosenberg and colleagues (Rosenberg et al., 1993), consistently injures the striatum and results in a well-characterized profile of behavioural deficits that make it

well suited to evaluating treatments (Peeling et al., 2001; DeBow et al., 2003; MacLellan et al., 2006). In Experiment 1, we quantified the volume of tissue lost at 7, 14 and 60 days after ICH using standardized histological procedures. We hypothesized that there would be continuing tissue loss over time as suggested by our recent MRI work (MacLellan et al., 2007). In Experiment 2, we used gold chloride staining to assess white matter loss from 7 to 60 days post-ICH. Here we measured the areas of the anterior and hippocampal commissures, and the corpus callosum – all at the mid-sagittal level. We hypothesized that there would be a continuing loss of white matter tracts over time. In the final experiment, we used the Golgi-Cox stain for a detailed examination of the dendritic arbourisation of medium-sized spiny neurons in the striatum. We hypothesized that neuronal atrophy in the peri-hematoma region would contribute to the total loss of tissue expected.

2.2 MATERIALS AND METHODS

2.2.1 Animals

All procedures are in accordance with the Canadian Council on Animal Care guidelines and were approved by the Biological Sciences Animal Care and Use Committee. Seventy male Sprague-Dawley rats were obtained from the biosciences animal colony at the University of Alberta, weighing between ~200 and 250 g and at ~ 16 weeks of age at the time of ICH. All animals had ad libitum access to food and water, and were individually housed on a 12 hr light cycle for the duration of the experiment.

In experiment 1, animals were randomly assigned to survive for 7, 14, or 60 days (n = 10 each) following ICH and the volume of tissue lost was calculated. In experiment

2, white matter loss was studied in animals that survived 7 or 60 days (n = 8 each) following ICH. In the third experiment, we used Golgi-Cox staining to investigate neuronal dendritic structure in animals that survived for 7 or 60 days following ICH (n = 8 each), and these were compared to a naïve control group (n = 8).

2.2.2 Intracerebral Hemorrhage

Surgical procedures were performed using aseptic technique (e.g., sterilized instruments, drapes, etc.). We used the bacterial collagenase model of ICH based on Rosenberg and colleagues with minor modifications (Rosenberg et al., 1993; MacLellan et al., 2007). Rats were anesthetized with isoflurane in 70 % N_2O and 30 % O_2 (4 % induction; 1.5 – 2 % maintenance) and placed in a stereotaxic frame. Core body temperature was measured with a rectal thermocouple probe and maintained at normothermia (36.5 – 37.5°C) with a heating blanket throughout surgery. A midline scalp incision was made and the skull was leveled between Bregma and Lambda. A small burr hole was drilled in the skull 3.5 mm lateral and anterior-posterior level of Bregma. A 26 gauge needle (Hamilton syringe, Hamilton, Reno, NV, USA) was lowered 6.5 mm below the surface of the skull and 0.6 μL sterile saline containing 0.12 U bacterial collagenase (Type IV – S; Sigma, Oakville, ON, Canada) was infused into the striatum over five minutes to create an ICH. The needle remained in place for an additional ten minutes following the infusion in order to prevent reflux. The burr hole was sealed with a metal screw (model MX – 080 – 2; Small Parts, Miami Lakes, FL, USA). The scalp wound was infiltrated with Marcaine (Sanofi Canada, Markham, ON, Canada) and stapled closed.

2.2.3 Histopathology

All rats were anesthetized with sodium pentobarbital (80 mg / kg, i.p; Somnotol, MTC Pharmaceuticals, Cambridge, ON, Canada) and then transcardially perfused with 0.9 % saline. In experiments 1 and 2, this was followed by 10 % neutral buffered formalin (Fisher Scientific, Ottawa, ON, Canada). Brains in experiment 1 were extracted from the skull and placed in 10 % neutral buffered formalin until sectioning whereas those in experiment 2 were split mid-sagittally prior to reveal the cross-sectional area of the commissures. The hemispheres were stored in 10 % neutral buffered formalin until staining. In experiment 3, the brains were removed and processed with a modified Golgi-Cox staining procedure (Gibb and Kolb, 1998). All histological analyses were performed in a blinded fashion.

Cresyl violet stain

Brains from experiment 1 were removed from 10 % neutral buffered formalin and placed in 20 % sucrose formalin solution for cryoprotection. Frozen 40 μm coronal sections were taken every 200 μm throughout the lesion and stained with cresyl violet. Lesion volume was quantified using Scion Image J 4.0 (Scion Corporation, Frederick, MD, USA) according to the following method we have routinely used (MacLellan et al., 2007):

- Volume of Lesion = remaining volume of tissue in the normal hemisphere – remaining volume of tissue in the injured hemisphere.

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

Whole mount gold chloride staining

The mid-sagittal areas of white matter tracts were visualized via whole-mount staining of the lesion hemisphere with gold chloride (Wahlsten et al., 2003). Each hemisphere was removed from the 10% neutral buffered formalin, quickly blotted dry, and incubated in about 20 mL 0.2% gold chloride in phosphate buffered myelin stain for about 40 minutes, or until the commissures appeared purple brown. Each hemisphere was then immersed in 2.5 % sodium thiosulfate anhydrous for five minutes at room temperature. An image of the hemisphere was obtained at 1200 DPI with a flatbed scanner as the brain was mounted on a glass slide. The maximum cross-sectional areas for the anterior commissure, hippocampal commissure, and corpus callosum were subsequently quantified using Scion Image.

Golgi-Cox staining

Brains were immersed in 20 mL of Golgi-Cox solution in an opaque container for two days. The brains were then immersed in 30% sucrose solution for another two days before sectioning. Coronal brain sections were cut using a vibratome at 200 μm and developed (Gibb and Kolb, 1998).

Medium-sized spiny neurons in the striatum were traced onto paper using camera lucida with a 20× objective by a researcher blinded to treatment conditions. Five neurons

per hemisphere were drawn for each animal and only those completely impregnated with Golgi-Cox solution were drawn. Segments of the dendritic branches were counted and classified according to branch order, that is: dendrites originating from the cell body were classified as first order whereas successive branch orders were based on subsequent dendritic bifurcations. As well, a Scholl analysis of ring intersections was used to estimate dendritic length. In this analysis dendritic length was estimated by placing a transparent grid of concentric rings, equivalent to 20-μm spacing, over the dendritic drawing, and the number of branches intersecting each ring was counted (Gonzalez and Kolb, 2003; Monfils et al., 2005).

2.2.4 Statistics

Data were analyzed with SPSS (v. 15, SPSS Inc, Chicago, IL) using one-way ANOVAs and Scheffé post-hoc tests. Results are considered statistically significant at the level of $p \leq 0.05$ and data are presented as mean ± S.D.

2.3 RESULTS

2.3.1 Lesion volume

The volume of tissue lost at 7, 14, and 60 days following collagenase-induced ICH injury is show in Fig. 2.1A, whereas representative photomicrographs are illustrated in Fig. 2.1B – D. Tissue loss was primarily in the striatum, but, to a lesser extent, surrounding areas were affected including the globus pallidus, thalamus, and corpus callosum. Ventricular enlargement was also prominent. An ANOVA revealed significantly greater tissue loss with increasing survival times such that the 60-day

survival group had more injury than both the 7 ($p < 0.001$) and 14-day ($p = 0.003$) groups.

2.3.2 White matter tracts

The mid-sagittal area of white matter was visualized via gold chloride staining (Fig. 2.2A). There was a significant decline (22%) in the area of corpus callosum from 7 to 60 days ($p = 0.025$; Fig. 2.2B). However, the anterior commissure was not significantly ($p = 0.134$) different between the 7 (0.34 ± 0.02) and 60-day survival times (0.29 ± 0.08). Similarly, the hippocampal commissure had similar ($p = 0.779$) measurements at 7 (0.62 ± 0.11) and 60 days (0.60 ± 0.14).

2.3.3 Golgi-Cox staining

Average dendritic length in the peri-hematoma region (Fig. 2.3A) was significantly different among groups ($p = 0.006$) as there was an initial atrophy at day 7 ($p = 0.032$ vs. naïve controls) that fully recovered by 60 days post-ICH ($p = 0.855$ vs. naïve controls; $p = 0.010$ vs. day 7 post-ICH group). The average dendritic length in the contralateral-to-ICH striatum (Fig. 2.3B) was also significantly different among groups ($p = 0.002$). Specifically, it was significantly longer at 7 and 60 days ($p \leq 0.013$) compared to naïve controls, but not different between the 7 and 60 day post-ICH groups ($p = 0.842$).

An ANOVA on branch order frequency (sum scores) revealed significant group effects for both the injured and non-injured sides ($p \leq 0.026$). For the injured side the 7-day post-ICH survival group had significant fewer branches than naïve animals ($p =$

0.046) whereas the 60-day survival ICH group and naïve animals were not different ($p = 0.968$) as seen in Fig. 2.4A. In contrast, there were significantly more branches on the contralateral-to-stroke side at 7 days versus naïve animals ($p = 0.034$). The 60-day animals were not significantly different than either the 7-day ICH rats or the naïve animals ($p \geq 0.113$). Order complexity was considered by taking the ratio of lower order branches (average of 1, 2 and 3) compared to higher order branches (average of 4, 5 and 6). These ANOVAs showed that there was a significant effect on the lesioned side ($p = 0.034$), but not on the non-lesioned side ($p = 0.653$). On the non-lesioned side the average ratio was 2.1 indicating that there were almost twice as many lower order branches than higher order branches. For the injured side, this increased to a ratio of 4.2 ± 2.4 and 2.9 ± 1.4 for the 7 and 60-day ICH groups, respectively (ca. 1.9 ± 0.3 for naïve animals). Only the 7-day group was significantly different than naïve animals ($p = 0.034$). Thus, it was branches of higher orders that were initially lost as can be also seen in Fig. 2.4B.

Representative Golgi-Cox stained neurons are shown in Fig. 2.5, which shows peri-hematoma dendritic atrophy at 7 day post-ICH 5A, compared to naïve animals 5B, and recovery at 60 days post-ICH in the peri-hematoma region 5C.

2.4 Discussion

This study used well-established histological techniques to confirm our recent magnetic resonance imaging (MRI) findings (MacLellan et al., 2007) wherein we show that injury continues for weeks following collagenase-induced ICH in rats. Further, we extend these findings by demonstrating that at least part of this quite delayed tissue loss is due to a thinning corpus callosum. We also show important changes in dendritic

arbourisation, which were measured in Golgi-Cox stained neurons. Notably, we report that there is an initial dendritic atrophy in the peri-hematoma region that eventually recovers to normal levels, whereas in the contralateral striatum there is an early and sustained enhancement in dendritic arbourisation following ICH. Accordingly, the continuing loss of hemispheric volume after striatal ICH is not due to ongoing dendritic atrophy. Overall, these findings have important implications for assessing outcome in this model of ICH. Specifically, white matter damage should be evaluated and long-term survival studies are required, as short survival times may not accurately predict final outcome. Furthermore, our findings give some insight into how spontaneous recovery occurs in this model, and that may result from both ipsi- and contralateral dendritic alterations.

The primary implication of finding protracted tissue loss after ICH is that treatments may mitigate it to improve outcome. Interestingly, a rehabilitation treatment has already been shown to mitigate the striatal injury when given from one to two weeks after collagenase-induced ICH (DeBow et al., 2003). That study used constraint-induced movement therapy (CIMT) to force the rats to use their impaired forelimb during rehabilitation exercises and in their home cage. Animals given this treatment had significantly smaller lesions at 60 days post-ICH. Thus, it is possible that this protective effect stemmed from attenuating white matter loss, or enlarging the dendritic arbor of peri-hematoma neurons, among other possibilities. For instance, forced running in rats soon after ICH appears to be neuroprotective (Lee et al., 2003) and it can promote cellular proliferation (Lee et al., 2005). Thus, more delayed, clinically relevant rehabilitation paradigms may also have these effects.

A MRI study (MacLellan et al., 2007) compared the collagenase and whole blood models to show that the volume of tissue lost increased from one to four weeks after collagenase infusion, but not after whole blood injection. At this time there is no clear explanation for the difference, and given the lack of comparable time-course data in humans it would be premature to say which rat model better predicts the clinical course of ICH. Thus, we recommend that both models be used and that a similar histological study is completed using the whole blood model. Such as comparative study should take into account insult severity, as this is likely to influence the maturation of injury.

Dendritic atrophy in the peri-hematoma region is an expected consequence of an ICH. Likely it stems, in part, from the direct mechanical insult to dendrites at a level that was insufficient to kill the neuron. As well, atrophy may stem from regional edema and blood brain barrier breakdown, inflammatory cell activity, loss of contact from and with destroyed neurons, and / or from toxicity caused by erythrocyte breakdown (e.g., iron catalyzed free radical injury). These events, which continue for days after ICH in rats (Xi et al., 2006; MacLellan et al., 2007), can be treated resulting in improved functional recovery [e.g., deferoxamine - (Wan et al., 2006)]. Commonly, these treatments do not affect the volume of injury, at least at relatively short survival times (e.g., seven days), but it is possible that long-term protective effects might be observed had they been carefully investigated. Similarly, various treatments may limit dendritic atrophy, which may not be evident with standard volumetric analyses. An increase in peri-hematoma dendritic arbourisation above that seen in naïve rats might also occur either spontaneously with time or through a neuroprotective intervention. Thus, it seems prudent to evaluate dendritic arbourisation in neuroprotection studies.

The finding of increased dendritic arbourisation above control levels in the contralateral striatum suggests that this region mediates at least some of the recovery seen after ICH. Indeed, increased dendritic complexity and spine density has been repeatedly associated with recovery after brain injury (Johansson, 2000; Kleim et al., 2003; Kolb, 2003). Furthermore, the contralateral hemisphere has been implicated in recovery, especially in cases of severe injury (Calautti and Baron, 2003; Teasell et al., 2006; Noskin et al., 2007). Thus, rehabilitation treatments, such as CIMT, may facilitate recovery by acting on both peri-hematoma and contralateral striatal circuits. Further research is needed to test this hypothesis and to determine if dendritic effects are observed elsewhere (e.g., motor cortex).

There are several limitations with our study that must be considered. First, we did not examine behaviour in this study, and thus cannot directly relate histological changes observed to recovery. We have measured behaviour following collagenase-induced ICH in other studies and have repeatedly found marked functional recovery especially in the first week following ICH (MacLellan et al., 2006; MacLellan et al., 2007) as others also note (Hua et al., 2002). Further study is clearly needed to determine the contribution of many factors (e.g., resolution of edema, synaptogensis) to recovery post ICH, and it should not be assumed that mechanisms found to be important after ischemia will contributed similarly after ICH.

A second limitation is that, by necessity, we used a between subjects design to evaluate dendritic structure over time as there is no method that allows one to examine the same striatal neurons repeatedly over a long period. Accordingly, selection bias may have influenced our results. Specifically, some of the atrophied peri-hematoma neurons,

which we sampled at a 7-day survival, may have died off leaving only normal neurons to sample from at a 60-day survival time. Thus, it is possible that atrophied neurons did not recover, but instead they died off. Arguing against this are those studies, using the collagenase model, that show only a few neurons dying beyond seven days post-ICH (Felberg et al., 2002; Xue and Del Bigio, 2003; Wasserman and Schlichter, 2007). However, it is possible that this relates to the size of the initial lesion. In our study, the injury may have reached a critical mass, whereby the lesion continues to expand, in comparison to the previously mentioned studies that use small lesions to examine neuronal death beyond seven days.

A third limitation is that while we have shown the area of the corpus callosum to diminish with time, we did not established whether other axonal tracts, such as the internal capsule, undergo delayed degeneration and whether this is due to loss of axonal bundles, demyelization, or both. Furthermore, similar white matter loss was not seen in the anterior or hippocampal commissures. It is also impossible to determine the exact contribution of delayed white matter loss to the measured volume of tissue lost over this protracted time. Nonetheless, our findings do emphasize that delayed white matter loss occurs and this should be considered in cytoprotection studies.

Fourth, many other factors could have contributed to the delayed loss of tissue. While it has been shown that only a few neurons die beyond seven days post-ICH in studies using smaller lesion volumes (Felberg et al., 2002; Xue and Del Bigio, 2003; Wasserman and Schlichter, 2007), a loss of neuropil, axons, myelin and other cells (e.g., inflammatory cells) could also contribute to our observed effects. For instance, while the bulk of the inflammatory response normally subsides by 14 days (Gong et al., 2000;

Wasserman et al., 2007), it remains possible that the presence of some inflammatory cells in the peri-hematoma zone contributes in a small way to our estimate of "healthy" tissue and their eventual removal makes it appear as if additional neural tissue is lost. Likewise, regional edema may contribute to an overestimation of "healthy" tissue at 7 days post-ICH. Arguing against this possibility are rat ICH studies showing that edema resolves by 7 days (Fingas et al., 2007). Furthermore, we found a substantial loss of tissue occurred after 14 days when it is very unlikely that there is any residual edema. Regardless of the relative contributions of each of these possibilities, our findings illustrate that long-term survival studies are needed to truly gauge the level of brain injury.

In summary, we report that a substantial amount of injury occurs long after collagenase-induced ICH in rats, thus indicating that long-term endpoints must be used in this model, including white matter assessment. Unfortunately, most neuroprotection studies do not examine the significance of white matter injury (Coleman and Perry, 2002), which likely has contributed to the failure of clinical trials (Stys, 1998; Dewar et al., 1999). Furthermore, we report that neuronal dendritic structure is markedly affected in both the peri-hematoma and contralateral striatum, which likely influences behavioural outcome. Few studies have examined recovery mechanisms or rehabilitation treatments after ICH, and of the latter it is common to find only relatively small effects (Nguyen et al., 2007; Auriat and Colbourne, 2008). Thus, further rehabilitation-ICH studies are needed as findings in ischemia models may not necessarily apply, and those surviving ICH are often left with significant disability necessitating rehabilitation.

2.5 FIGURES

Figure 2.1

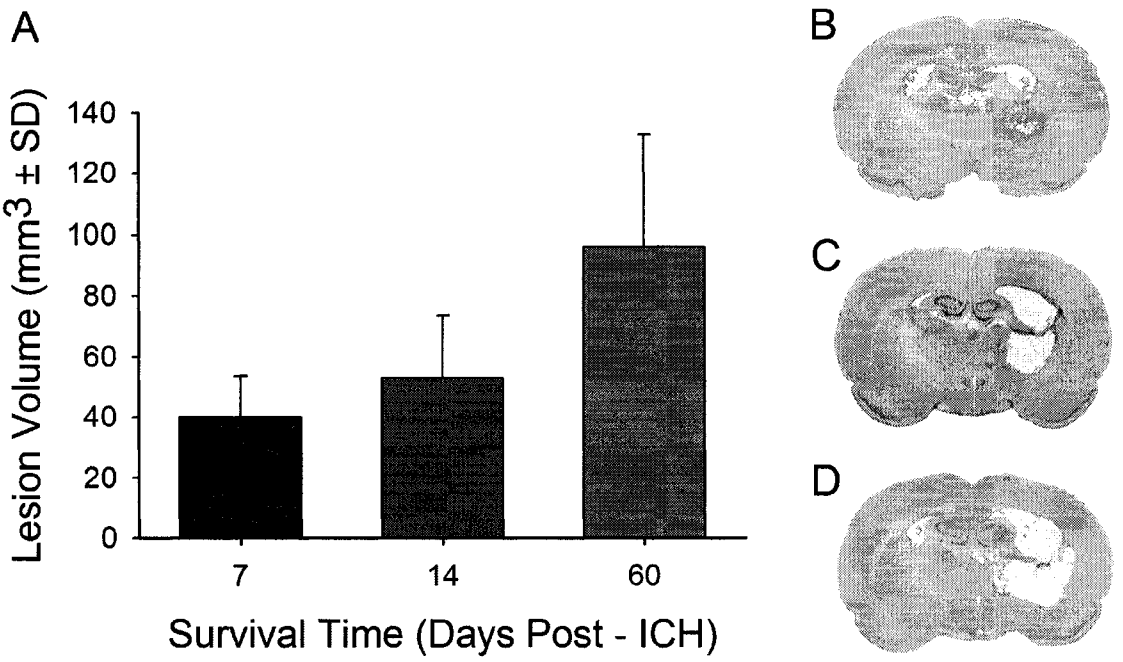

Figure 2.1. (A) The volume of tissue lost at 7, 14, and 60 days after ICH. Lesion volume significantly increased with time. See results for statistics. Representative coronal sections are shown at the level of maximal injury in animals at 7 (B), 14 (C), and 60 (D) days post-ICH.

Figure 2.2

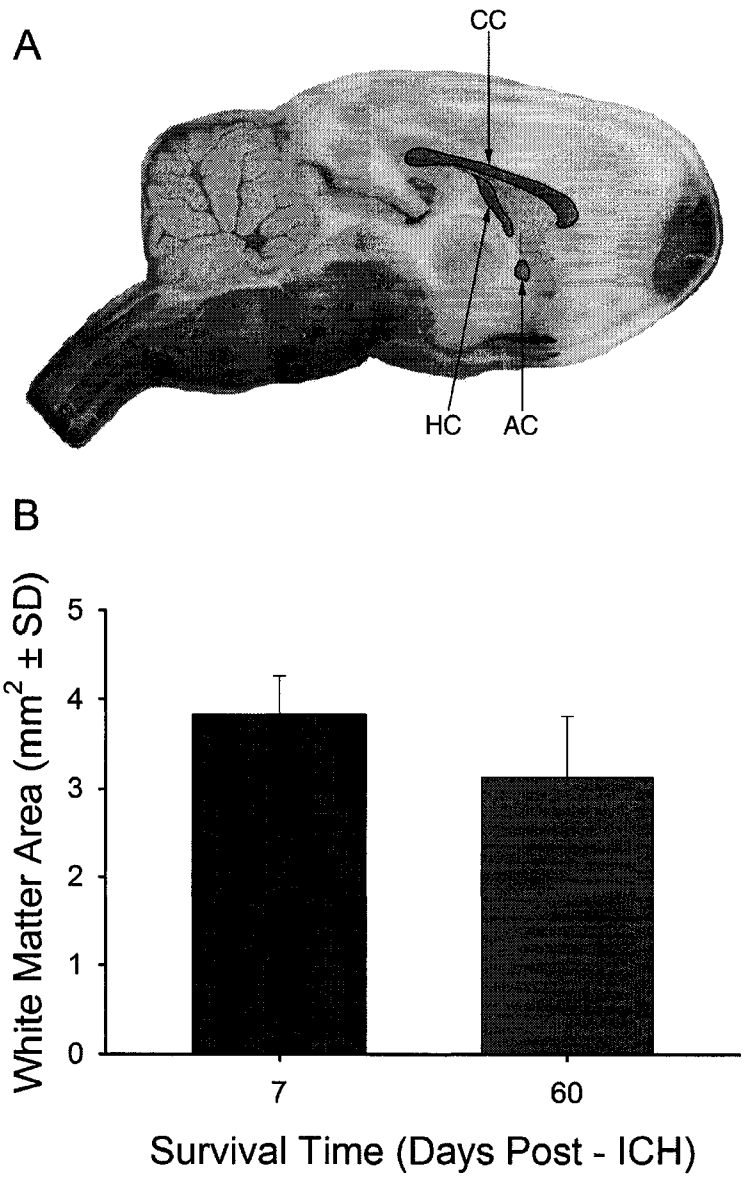

Figure 2.2. Gold chloride staining of white matter. Representative photo of the mid-sagittal area of white matter visualized via gold chloride staining (A). The area of white matter was quantified as the area of the anterior commissure (AC), the hippocampal commissure (HC), and the corpus callosum (CC). The area of white matter was quantified at 7 and 60 days after ICH. The CC area (B) was significantly smaller at 60 days, whereas no significant effects were found for the AC and HC regions. See results for statistics.

Figure 2.3

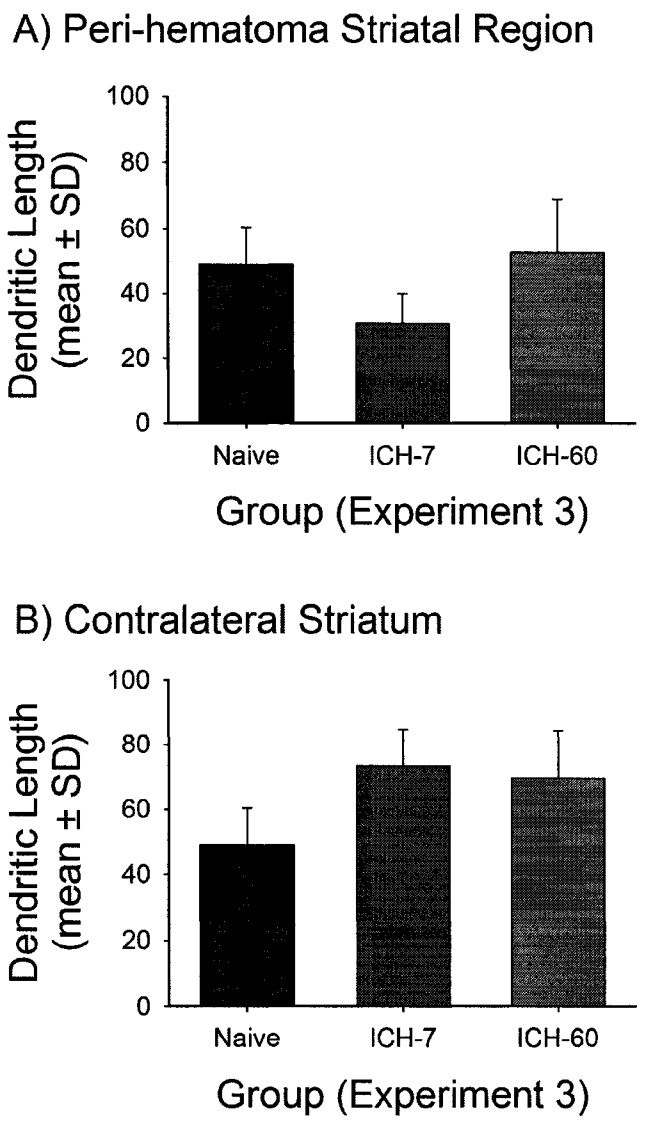

Figure 2.3. Dendritic length as measured by the Scholl analysis for the peri-hematoma striatal region (A) and contralateral to stroke striatum (B) for the control (naïve rats), 7 and 60 days post-ICH survival groups. In the peri-hematoma striatal region, dendritic length is significantly shorter for the 7-day survival group than the control and 60-day survival groups. In the non-lesion hemisphere, dendritic length was significantly shorter in the control group than the 7 and 60-day survival groups. See results for statistics.

Figure 2.4

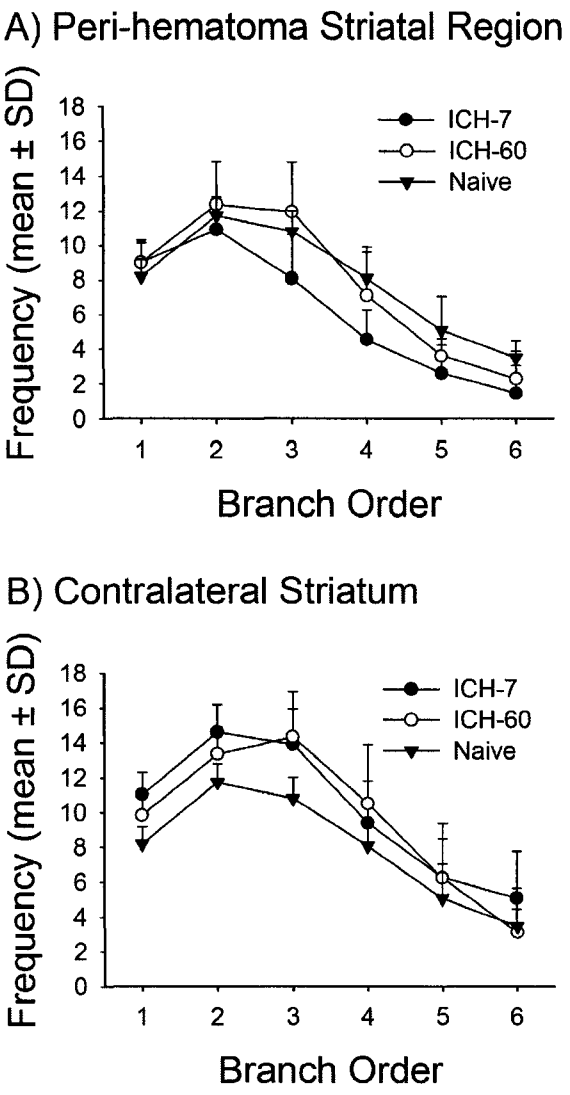

Figure 2.4. Dendritic complexity as measured by branch order analysis for control, 7 and 60 days survival groups in the peri-hematoma striatal region (A) and contralateral to stroke hemisphere (B). The 7-day survival group had significantly fewer branches than naïve animals (A) in the lesioned hemisphere. In the non-lesion hemisphere (B), there were significantly more dendritic branches as compared to the naïve and 60-day survival groups. See results for statistics.

Figure 2.5

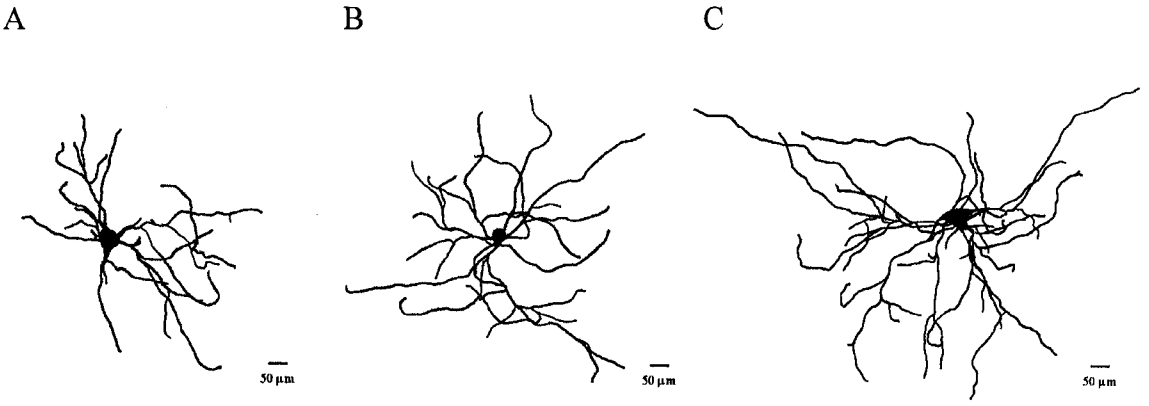

Figure 2.5. Representative tracings of Golgi-Cox stained neurons are shown. (A) shows peri-hematoma dendritic atrophy at 7 day post-ICH, compared to naïve animals (B), and increased arbourisation beyond control levels at 7 days post-ICH in the contralateral striatum (C).

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CHAPTER 3

FAILURE OF ESTRADIOL TO IMPROVE SPONTANEOUS OR REHABILITATION-FACILITATED RECOVERY AFTER HEMORRHAGIC STROKE IN RATS

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3.1 INTRODUCTION

The incidence of stroke is lower in pre-menopausal women than in men, which has been largely attributed to the high levels of estrogen, predominantly 17β-estradiol (E2), circulating in women (Ayala et al., 2002; Wise et al., 2005; Bushnell et al., 2006; Prentice, 2007). Unexpectedly, the Women's Health Initiative (WHI) trial reported that estrogen replacement therapy actually increased stroke incidence in postmenopausal women (Wassertheil-Smoller et al., 2003), although it has been argued that this trial has serious design flaws (Klaiber et al., 2005; Wise et al., 2005; Bushnell et al., 2006) Regardless, the potential of using E2 to protect the brain (neuroprotection) in both women and men following stroke is of great interest. Indeed, in experimental models of global cerebral ischemia E2 treatment has been repeatedly shown to attenuate hippocampal CA1 sector injury (Sudo et al., 1997; Jover et al., 2002; Shughrue and Merchenthaler, 2003). Similarly, E2 reduces infarct size following focal ischemia (Simpkins et al., 1997; Dubal et al., 1998; McCullough et al., 2001). It is not surprising then that E2 pretreatment reduces injury in models of intracerebral hemorrhage (ICH) that target the striatum (Auriat et al., 2005; Nakamura et al., 2005). Estrogen is thought to exert its neuroprotective actions against ischemia via numerous mechanisms (Gibson et al., 2006). Direct mechanisms include preserving cerebral blood flow and enhancing post-ischemic reperfusion (Hurn and Brass, 2003) as well as suppression of pro-apoptotic signals (Dubal et al., 1999; Harms et al., 2001). While similar mechanisms likely contribute to E2's neuroprotective effects in ICH models, estrogen may further affect ICH by promoting hemostasis and lessening the hematoma size following vessel rupture (Auriat et al., 2005). While ischemia and ICH share many mechanisms of injury,

fundamental differences remain (Xi et al., 2006). Thus, it is important to test putative treatments in ICH models rather than rely on findings in ischemia models.

While the ability of E2 to reduce injury, such as CA1 sector cell loss after global ischemia, has translated into improved functional recovery (e.g., memory tasks) (Li et al., 2004a; Li et al., 2004b; Auriat et al., 2005; Gulinello et al., 2006), it is also well known that E2 affects brain function in ways that should independently improve functional recovery. For example, E2 promotes the formation of new dendrites and excitatory synapses in the hippocampus (Woolley and McEwen, 1992), and this effect is correlated with improvements in hippocampal-dependent memory (McEwen and Woolley, 1994; Packard and Teather, 1997). As well, E2 affects the expression of brain-derived neurotrophic factor (BDNF) (Sohrabji et al., 1995; Berchtold et al., 2001), which not only promotes neuronal survival but also regulates plasticity (Lindsay, 1988; Alderson et al., 1990). Interestingly, E2 receptors in the forebrain are co-localized with BDNF (Miranda et al., 1993), and E2 replacement in ovariectomized (OVX) female rats increases BDNF expression in the forebrain (Jezierski and Sohrabji, 2000; Allen and McCarson, 2005). Accordingly, E2 may affect recovery by enhancing dendritic arbourisation and spine density (Berchtold et al., 2001; McEwen, 2001; Jezierski and Sohrabji, 2003), among other mechanisms, and these may be used to improve spontaneous recovery after stroke. Effective rehabilitation therapies also promote increases in dendritic arbourisation and spine density following stroke (Biernaskie and Corbett, 2001). Thus, it is possible that E2 treatment may further enhance rehabilitation efforts.

In this study, we assessed whether delayed E2 treatment affects spontaneous and rehabilitation-facilitated recovery after ICH in rats (Fig. 3.1 – timeline of events). The

collagenase model of ICH (Rosenberg et al., 1993) was used because it produces relatively consistent bleeding within the striatum along with well-characterized behavioural deficits (MacLellan et al., 2006; Auriat and Colbourne, in press). Beginning 1 week after ICH, rats were group housed in either standard (STD) or EE cages, and received either E2 pellets or they underwent sham procedures (SH). We used environmental enrichment (EE) as a rehabilitation treatment as it improves functional recovery following several types of brain injury (for a review, see (Will et al., 2004)), including ICH in rats (Auriat and Colbourne, in press). Rats were group housed in the EE cages, which contained ramps, tunnels, beams and various objects to explore. Estrogen was administered continuously, via an implanted pellet, until the end of the study to mimic estrogen replacement therapy. Furthermore, the onset of E2 treatment was delayed to one week post ICH to isolate E2's ability to independently promote functional recovery from its direct effects on cell death and hematoma size found with pre-treatment (Auriat et al., 2005). Comprehensive histological and functional evaluation at long survival times has been highly recommended in the assessment of putative stroke therapies (STAIR, 1999; MacLellan et al., 2006). Thus, we gauged recovery at 2, 5 and 8 weeks after ICH with several well-characterized behavioural tests that included: the tray task to assess skilled reaching (Whishaw et al., 1986), the cylinder test of forelimb-use asymmetry (MacLellan et al., 2006; Shanina et al., 2006), the horizontal ladder walking test (Metz and Whishaw, 2002; MacLellan et al., 2006) and the elevated beam task to assess balance and locomotion (Feeney et al., 1982; MacLellan et al., 2006). We predicted that delayed EE would promote functional recovery, and that delayed E2 would

facilitate recovery independently of and in combination with EE. Neither treatment was expected to alter lesion size.

3.2 Materials and methods

3.2.1 Animals

Seventy-two female Sprague-Dawley rats were entered into this study. They were obtained from the Biosciences breeding colony at the University of Alberta and were cared for in accordance with the Canadian Council on Animal Care guidelines. Procedures were also approved by the Biosciences Animal Care and Use Committee at the University of Alberta.

Rats were housed in groups of three to four in standard polycarbonate cages (38 cm width × 49 cm length × 20 cm height) unless otherwise stated, and on a 12 hr light cycle. They had free access to food and water, except during behavioural testing when they were kept at 90% of their free-feeding weight taking into account natural gains with age. They were handled for 15 minutes per day for five days prior to the start of the experiment. Rats were randomly assigned to treatment conditions: STD-SH (n = 17), STD-E2 (n = 12), EE-SH (n = 17), and EE-E2 (n = 19). Seven other rats died during surgery prior to treatment assignment. Figure 3.1 illustrates the time line of experimental procedures.

3.2.2 Body weight measurements

Body weight (g) was measured at the time of OVX and ICH surgeries and at 1, 5 and 8 weeks post ICH.

3.2.3 Behavioural training and testing

We used a battery of tests to assess skilled reaching (tray task), spontaneous forelimb usage (cylinder task) and walking ability (elevated beam, horizontal ladder). Tests were chosen because they have been previously shown to be sensitive to motor system injury including that caused with the collagenase model of striatal ICH (MacLellan et al., 2006).

Tray reaching task

The tray reaching box (Whishaw, 2000) measures 26 cm high × 28 cm deep × 19 cm wide and is made of Plexiglas with 2 mm vertical steel bars interspaced 9 mm in the front. Rats learn to reach through the bars to retrieve food pellets (17% Layer Prostock Feed; Masterfeeds, Edmonton, Alberta) placed in a shallow tray (4 cm wide × 0.5 cm deep) just outside the bars. Following food deprivation to 90% of baseline weight, rats were trained over 10 consecutive days (1 hr per day). On days -1, 14, 35, and 56 (relative to ICH), rats were video recorded for 10 minutes, which was subsequently analyzed as % successful reaches ((successful reaches / total reaches) × 100). A successful reach was one in which the rat successfully reached through the bars with its initially preferred (dominant) forelimb and retrieved and ate the food.

Forelimb use asymmetry (cylinder) test

Rats were placed in a transparent cylinder (20 cm diameter, 45 cm high) for 10 minutes while being videotaped from below. Spontaneous movements to explore the

walls were categorized as an independent wall contact with either the ipsilateral (to ICH lesion) or contralateral paw or co-usage (Schallert et al., 2000; Schallert, 2006; Shanina et al., 2006). At least 5 independent wall touches was needed to be considered a reliable measure of forelimb use and rats that did not reach this cutoff were excluded from this analysis. From videotape analysis we calculated an asymmetry score defined as the (number of contacts with contralateral forelimb + ½ both) / (ipsilateral forelimb use + contralateral forelimb use + both) × 100 (MacLellan et al., 2006; Shanina et al., 2006). With this measure normal animals score near 50% whereas those with motor system damage, such as a striatal ICH (Auriat et al., 2005; MacLellan et al., 2006; Shanina et al., 2006), have smaller scores indicating diminished usage of independent contralateral paw movements relative to the ipsilateral paw and co-usage. Rats were assessed the day prior to ICH and on days 14, 35 and 56 following ICH.

Beam task

On the last day of tray reaching task training, rats were trained to cross an elevated horizontal beam (1.10 m long; 3.20 cm wide). This was achieved by initially placing them on the beam at increasing distances from the goal box, located at the end of the beam, until they easily crossed the entire beam. Baseline performance was measured on the day prior to ICH, whereas testing occurred on days 14, 35 and 56 post-ICH. We used a modified version of Feeney's (Feeney et al., 1982) rating scale to score videotaped beam task sessions (MacLellan et al., 2006; Auriat and Colbourne, in press). Briefly, a rating scale (0 to 7) was used to assess each cross (5 per test day) on the beam walking test with 0 being the worst case and 7 being the best performance (MacLellan et al., 2006;

Auriat and Colbourne, in press). The median score of the 5 sessions was analyzed as was the summed score of the first two sessions.

Horizontal ladder walking task

On the last day of the tray reaching task training, rats were trained to cross a 1 m long horizontal ladder with variably spaced rungs (3 – 5 cm; 4 crosses). On behavioural testing days (14, 35 and 56 days post-ICH), rats were videotaped crossing the middle 0.5 m segment of the horizontal ladder (Metz and Whishaw, 2002). The total number of steps and slips made with each limb was recorded for 4 crosses per behavioural test day. A slip was counted when the limb slips completely through the bars. The success rate for each limb was calculated as follows: (number of successful steps / total number of steps) × 100. Performance on this task is affected by striatal ICH, at least for the contralateral forelimb, which was our primary endpoint (DeBow et al., 2003; MacLellan et al., 2006).

3.2.4 Major surgical procedures

Aseptic surgical technique was used (e.g., autoclaved or hot-bead sterilized instruments, autoclaved surgical drapes). The OVX surgery was done two weeks prior to ICH and three weeks prior to E2 treatment. All rats were subjected to OVX and ICH surgeries.

OVX surgery

Rats were anesthetized with isoflurane (4% induction, 2% maintenance in 70% N_2O and 30% O_2). An approximately 1 cm long incision was made in the skin and muscle

on each side demarcated by the caudal end of the ribs. Both ovaries were identified, tied off with suture, and excised. The incision was infiltrated with Marcaine (Sanofi Canada, Markham, ON, Canada) and sutured closed.

Surgery

Rats were anesthetized under isoflurane as for the OVX surgery. They were then placed in a stereotaxic frame while body temperature was measured with a rectal thermocouple probe and maintained at normothermia (36.5 – 37.5°C) with a heating pad. A midline scalp incision was made and the skull was leveled between Bregma and Lambda. A small burr hole was made 3 mm lateral to Bregma, on the side contralateral to the preferred limb (as determined by the tray reaching task). A 26 gauge needle (Hamilton syringe part # 80308, Hamilton, Reno, NV, USA) was lowered 6.0 mm below the surface of the skull and 0.7 μL of sterile saline containing 0.14 U bacterial collagenase (Type IV – S; Sigma, Oakville, ON, Canada) was infused into the striatum over 5 minutes to create an ICH (Rosenberg et al., 1993; DeBow et al., 2003; MacLellan et al., 2006). The needle remained in place for another 10 minutes to prevent reflux. The burr hole was sealed with a metal screw (model MX–080–2; Small Parts, Miami Lakes, FL, USA) then the incision was infiltrated with Marcaine and stapled closed.

3.2.5 Treatment conditions

Groups of 3 – 4 rats were randomly assigned to Housing and Hormone treatment conditions at one week after ICH. Groups of animals were assigned to either the same standard housing (STD) or environmental enrichment (EE) as changing cage mates would

have caused undue stress. Rats were randomly assigned to receive either estrogen pellet implantation (E2) or sham procedure (SH).

Estrogen treatment

One week following ICH, rats were quickly anesthetized with isoflurane anesthesia for a small incision on the back of the neck. A 17 β-estradiol (E2) pellet (0.36 mg; 60 day release; Innovative Research of America Inc., Sarasota, FL, USA), which continuously releases estrogen, was then implanted subcutaneously (E2 treatment) or no pellet was given (SH treatment). The wound was sutured closed.

Housing treatment

Immediately following E2 or SH treatment the rats were either returned to STD housing or placed in an EE wire cage that measured 77 cm long × 77 cm high × 37 cm wide. These cages, which had 3 levels connected by ramps, allowed rats to access a 30 cm diameter running wheel adjacent to the EE cage. Each week new "toys" (e.g., beams, plastic children's toys, etc.) were introduced to replace existing ones. As well, the location of the food and water inside the cages was also changed weekly to encourage exploration. Animals remained in their assigned housing until the end of the experiment.

3.2.6 Histopathology

Rats were euthanized the day following the last test session (~ two months post-ICH) with an overdose with sodium pentobarbital (80 mg / kg, i.p; Somnotol, MTC Pharmaceuticals, Cambridge, ON, Canada). Following clamping of the descending aorta,

the rat's upper body was transcardially perfused with 0.9% saline followed by 10% neutral buffered formalin. Uteri, which were not formalin fixed, were dissected from adhering fat and mesentery to measure uterine weight. The formalin-fixed brain was extracted and immersed in a 20% sucrose-formalin solution until sectioning. Frozen coronal sections taken every 200 μm throughout the lesion were stained with cresyl violet. The volume of tissue lost was quantified using Scion ImageJ for Windows (v. 4.0, Scion Corporation, Frederick, MD). The volume of injury plus atrophy (e.g., ventricular enlargement) was quantified and expressed as follows:

- Volume of Lesion = remaining volume of tissue in the normal hemisphere – remaining volume of tissue in the injured hemisphere.
- Volume of tissue in a hemisphere = average (area of the complete coronal section of the hemisphere – area of ventricle – area of tissue damage) × interval between sections × number of sections.

The cortical thickness for each hemisphere was measured at maximal lesion site (Fig. 3.7A) using Scion Image as previously done (MacLellan et al., 2007). Similarly, corpus callosum injury was quantified by measuring its area in each hemisphere at the maximal lesion site (MacLellan et al., 2007).

3.2.7 Statistics

All behavioural and histological analyses were performed in a blinded fashion. Data were analyzed with SPSS (v. 15, SPSS Inc, Chicago, IL) using ANOVA for parametric data (e.g., cylinder scores) and non-parametric statistics for beam test scores. A p value of < 0.05 was considered statistically significant.

3.3 RESULTS

3.3.1 Protocol violations and mortality

Thirteen rats were excluded entirely from this study. Of these, 7 died during surgery of unknown causes, likely due to anesthetic complications. Six others were excluded due to surgical errors (e.g., no lesion likely due to a blocked injection needle). The remaining group sizes were as follows: STD-SH (n = 16), STD-E2 (n = 12), EE-SH (n = 14), and EE-E2 (n = 17). Some data for several other rats were excluded from one or more behavioural tests for failing to meet criteria stated in the Methods.

3.3.2 Body weight

Rat body weight (Fig. 3. 2) was initially analyzed with a 3-factor ANOVA (Housing and Hormone factors; Time factor: OVX, ICH, and weeks 1, 5 and 8 post-ICH); owing, however, to significant Day interactions in this analysis ($p \leq 0.003$), the data were analyzed with 2 between factor ANOVAs at each time. There were no significant Housing or Hormone main effects or interactions ($p \geq 0.196$) on the days of OVX, ICH and 1 week post-ICH. However, both main effects were significant ($p < 0.001$) at 5 and 8 weeks post-ICH as E2 and EE treatments caused rats to have a lower body weight.

3.3.3 Uterine weight

Uterine weights at euthanasia were 0.035 g ± 0.048 (mean ± SD), 0.1849 ± 0.247, 0.055 ± 0.071 and 0.165 ± 0.126 in the STD-SH, STD-E2, EE-SH and EE-E2 groups, respectively. With a 2-way ANOVA we found that the Hormone main effect was

significant ($p = 0.001$), but the Housing main effect ($p = 0.997$) and the interaction ($p = 0.583$) were not. Thus, E2 treatment promoted excessive uterine growth.

3.3.4 Behavioural outcome

Tray task

Most rats showed a clear limb preference for grasping food in the tray task. For instance, during the last training session (a ten minute baseline session) rats reached an average of 83.0% of the time with one limb (Fig. 3.3A). A 3-way ANOVA (Housing and Hormone factors; Time factor: baseline, weeks 2, 5 and 8 post-ICH), for which the interactions were non-significant ($p \geq 0.070$), showed that asymmetry scores were similar among groups (Housing main effect: $p = 0.593$; Hormone main effect: $p = 0.594$) and significantly lower at 2, 5 and 8 weeks post-ICH (simple within-subjects contrasts: $p < 0.001$ vs. baseline). Thus, following ICH rats altered their limb preference to more frequently use their ipsilateral-to-stroke limb instead of their contralateral, "preferred" limb, but neither EE nor E2 affected this change.

Unfortunately, many rats used their contralateral-to-stroke limb too infrequently (< 5 reaches) after ICH to adequately gauge their reaching success at obtaining food. While the loss of data was approximately equal over the weeks and among groups, it amounted to an averaged loss of ~42% of data points, and, therefore, a loss of statistical power in this analysis. Furthermore, the loss of data was inconsistent, as some animals did not attain our criterion on only one or two test times. Therefore, the reaching success data were analyzed with 2-way ANOVAs (Housing and Hormone factors) at the four measurement times. Reaching success with the preferred limb during baseline was similar

among groups and averaged 61.9% (Housing main effect: $p = 0.733$; Hormone main effect: $p = 0.389$; interaction: $p = 0.456$; Fig. 3.3B). Following ICH there was a Housing main effect favoring EE treatment on week 2 ($p = 0.030$), but not on weeks 5 ($p = 0.333$) or 8 ($p = 0.313$). The Hormone main effects ($p \geq 0.577$) and interactions ($p \geq 0.324$) were not significant on weeks 2, 5 or 8. Despite the loss of data, a 1-way ANOVA (Time: 4 levels) showed a clearly significant main effect ($p < 0.001$) and specific contrasts shown that animals had significantly lower reaching success at 2, 5 and 8 weeks post-ICH ($p < 0.001$ vs. baseline). Thus, ICH significantly impaired reaching success, but this was only improved by EE treatment when tested at 2 weeks post-ICH.

Cylinder task

As expected asymmetry scores were close to 50 during baseline testing in the cylinder task and these scores decreased following ICH (Fig. 3.4). A 2-between (Housing and Hormone factors) 1-within (Time factor: baseline, weeks 2, 5 and 8 post-ICH) ANOVA revealed a significant Time effect ($p < 0.001$), with significant forelimb use asymmetry favoring the ipsilateral-to-stroke limb following ICH (each post-ICH week vs. baseline; $p < 0.001$). The Housing ($p = 0.275$) and Hormone ($p = 0.315$) main effects were not significant, nor were any of the interactions ($p \geq 0.321$). Thus, neither E2 nor EE treatment affected asymmetry scores, which were significantly affected by ICH.

Beam task

Beam traversing scores were normal (i.e., 7) at baseline for all animals. The median score of 5 traverses was significantly worse (lower score) at 2 weeks post-ICH (p

= 0.001 vs. baseline, Wilcoxon Signed Ranks Test; Fig. 3.5); although many animals performed normally. The lower median scores observed at 5 ($p = 0.066$) and 8 ($p = 0.109$) weeks post-ICH were not significantly worse than baseline scores (data not shown). Thus, with this analysis, ICH seemed to only transiently impair performance on this test. Furthermore, there were no group effects at 2 ($p = 0.851$; Kruskal Wallis Test), 5 ($p = 0.630$) or 8 weeks ($p \geq 0.298$). The sum score of the first 2 beam sessions was also analyzed as performance would be worse on these trials (v. median score) and this might help reveal group effects. With this analysis there were significant impairments after ICH (v. baseline) on weeks 2 ($p < 0.001$), 5 ($p = 0.027$), and 8 ($p = 0.007$). Nonetheless, there were no group differences at 2 ($p = 0.832$), 5 ($p = 0.953$) or 8 weeks post-ICH ($p = 0.060$). Thus, neither E2 nor EE treatment statistically lessened impairment following ICH.

Horizontal ladder task

A 3-way ANOVA (Housing and Hormone factors; Time factor: 4 levels) on stepping success with the contralateral forelimb (Fig. 3.6A) revealed a significant Time effect ($p < 0.001$) with significant impairment evident at 2, 5 and 8 weeks following ICH (within-subjects contrasts: $p < 0.001$ vs. baseline). All interactions in this 3-way ANOVA were non-significant ($p \geq 0.091$) as was the Hormone main effect ($p = 0.808$). However, the Housing main effect was significant ($p < 0.001$). Thus, ICH caused persistent impairment in walking with the contralateral forelimb that was attenuated by EE, but not E2, treatment. As stepping success with the contralateral forelimb is a primary endpoint in this study, and given the variability, the data were also analyzed with 2-way ANOVAs

at each time. These analyses revealed that the Housing main effect (favoring EE treatment) was significant at 5 ($p = 0.005$) and 8 weeks ($p = 0.003$), but not at week 2 ($p = 0.118$). The interactions and the Hormone main effect were non-significant ($p \geq 0.102$).

Owing to significant interactions (e.g., Time by Housing by Hormone: $p = 0.008$) in the 3-way ANOVA on the contralateral hind limb data (Fig. 3.6B) we conducted simpler analyses at each time. First, a 2-way ANOVA on baseline scores with the contralateral hind limb showed no significant differences among groups (Housing main effect: $p = 0.565$; Hormone main effect: $p = 0.389$; interaction: $p = 0.115$). Second, following a significant interaction in the 2-way ANOVA ($p < 0.001$) for week 2 data we conducted a 1-way ANOVA ($p < 0.001$) with post-hoc Scheffé tests that showed only the STD-E2 group to be significantly impaired versus the STD-SH ($p = 0.005$) and EE-E2 groups ($p = 0.001$). Third, a 2-way ANOVA showed that there was a significant effect of Housing, favoring EE treatment, at the 5 week test time ($p = 0.014$), whereas the Hormone main effect ($p = 0.369$) and interaction were not significant ($p = 0.510$). Fourth, a 2-way ANOVA on the week 8 data shown that the Housing ($p = 0.051$) and Hormone ($p = 0.594$) main effects, and interaction ($p = 0.380$) were not significant.

Owing to a significant Time by Housing interaction ($p = 0.002$) in the 3-way ANOVA, the ipsilateral forelimb data (Fig. 3.6C) were analyzed with 2-way ANOVAs at each time. Analysis of the baseline scores showed no significant differences among groups (Housing main effect: $p = 0.282$; Hormone main effect: $p = 0.492$; interaction: $p = 0.710$). There was a significant Housing main effect ($p < 0.001$) at week 2 where the Hormone main effect ($p = 0.916$) and interaction ($p = 0.425$) were not significant. At week 5, however, both Housing ($p < 0.001$) and Hormone ($p = 0.020$) main effects were

significant; the interaction was not ($p = 0.663$). Only the Housing main effect was significant ($p = 0.004$) at week 8; the Hormone main effect ($p = 0.451$) and interaction ($p = 0.343$) were non-significant. Thus, EE treatment consistently improved performance, whereas E2 only improved performance at one time.

Owing to a significant Housing by Hormone interaction ($p = 0.044$) in the 3-way ANOVA on the ipsilateral hind limb data (Fig. 3.6D) it was analyzed with 2-way ANOVAs. For the baseline data (2-way ANOVA) the Housing ($p = 0.552$) and Hormone main effects ($p = 0.317$) were not significant, nor was the interaction ($p = 0.293$). However, the Housing main effect, favoring EE treatment, was significant ($p \leq 0.006$) at each post-ICH week, whereas the Hormone main effects ($p \geq 0.065$) and interactions ($p \geq 0.053$) were not. Thus, the ICH did not cause ipsilateral hind limb impairments and EE treatment further improved performance.

3.3.5 Histological outcome

The collagenase-induced ICH caused injury primarily to the striatum, and to a lesser extent to surrounding structures such as globus pallidus, thalamus, and corpus callosum. Ventricular enlargement was prominent (Fig. 3.7A). The total volume of tissue lost (Fig. 3.7B), which was analyzed by a 2-way ANOVA, was not significantly affected by either Hormone ($p = 0.774$) or Housing treatments ($p = 0.724$) and the interaction was not significant ($p = 0.148$). Two-way ANOVAs showed a significantly smaller cortical thickness (CT; $p < 0.001$; Fig. 3.8B) and area of the corpus callosum (CC; $p < 0.001$; Fig. 3.8C) ipsilateral to the ICH. There was no significant effect on CT of Housing ($p = 0.272$) or Hormone treatment ($p = 0.369$) and the interactions were not significant ($p \geq$

0.484). Similarly, the CC area data showed no effect of Housing ($p = 0.850$) or Hormone ($p = 0.092$) treatment and this interaction was not significant ($p = 0.068$).

3.4 Discussion

This is the first study to assess whether delayed and chronic E2 treatment influences functional recovery after striatal ICH in rats. A sustained E2 treatment beginning 1 week following collagenase-induced ICH did not notably influence functional recovery or brain injury. Environmental enrichment did facilitate recovery on some tests, but this was mostly unaltered by E2 treatment. These findings, in conjunction with previous work (Li et al., 2004b; Auriat et al., 2005), suggest that E2 treatment improves functional recovery only when it limits injury size (e.g., attenuates bleeding and/or directly reduces cell death), and this effect occurs when E2 is on-board around the time of ICH (Auriat et al., 2005; Nakamura et al., 2005).

Our E2 findings mirror those of Farr et al (Farr et al., 2006) who found that delayed E2 treatment did not promote spontaneous functional recovery (a rehabilitation condition was not used) nor increase synaptogenesis in a rat model of focal ischemic stroke. They used a daily dose (1.07 mg pellet, 90 day release; 11.8 μg / day) that was approximately double that we used (6 μg / day) thus arguing against the possibility of finding benefit with a larger E2 dose after ICH. Furthermore, dosages calculated to be between 2 and 24 μg per day lessen injury when given prior to ICH (Auriat et al., 2005). Thus, it would appear that E2 simply does not affect recovery within a natural dose range and with doses that provide considerable neuroprotection in rats when administered prior to ICH. Finally, although we did not measure serum E2 levels, which would have

strengthened this study, it was clear that the dose used had a significant effect on body and uterine weights, which are well known to occur, and we had used the same delivery system as in other stroke studies (Auriat et al., 2005; Farr et al., 2006). These E2 pellets, however, may release a large amount of E2 initially (Auriat et al., 2005) and this may be counterproductive. Conversely, it is possible that such a high dose would provide benefit had it been sustained. Thus, alternative dosing regimens (e.g., intermittent), delivery methods, or pharmacologically high doses might influence recovery and warrant further study, especially if they can be administered for a relatively short time and provide benefit while avoiding side effects.

The EE treatment significantly facilitated recovery after ICH, which is in line with studies examining traumatic and ischemic brain injury (Biernaskie and Corbett, 2001; Johansson, 2003; Will et al., 2004). However, improvements were not found on all four behavioural tests and they were not large. For example, compared to STD-treated animals, who obtained an average success of ~74% with the contralateral forelimb in the ladder test, EE improved post-ICH performance to ~82%, but this was still substantially below baseline performance (93% success). Moreover, the only significant effect in the tray test occurred at two weeks post ICH, and no effect was found in the cylinder and beam tasks. In order to explain the failure to broadly and more completely improve performance, one might argue that striatal ICH is simply more difficult to treat than motor cortex injury where EE treatment appears to be more efficacious. This appears to be the case with forced running exercise, which often facilitates recovery after ischemic injury (Wang et al., 2001), but not after ICH-induced striatal damage (Auriat et al., 2006). Similarly, amphetamine has been repeatedly, but not always, shown to improve recovery

after ischemic injury whereas it provides no benefit for striatal ICH (Auriat and Colbourne, in press). In contrast, the comparable EE treatment protocol used in that study partially facilitated recovery after ICH as we presently observed. Another possibility is that some EE studies may overestimate the effects of enrichment by comparing those animals with ones singly housed. In this and our previous work we group housed our control rats, which should then diminish the 'effect size' with EE treatment.

It is also possible that the behavioural tests we used were unable to detect small treatment effects. Although, given that EE improved performance, it is hard to use such an explanation to explain the failure of E2 to improve recovery. Furthermore, we used four behavioural tests, which have all been previously shown to detect ICH-induced striatal injury to varying extents (MacLellan et al., 2006), which we presently showed. Nonetheless, while many behavioural tests are effective lesion detectors, they are sometimes unable to effectively distinguish among treatment groups including those with markedly different ICH-induced lesion volumes (Auriat et al., 2005; MacLellan et al., 2006). This potential weakness (test insensitivity) may be especially concerning with the beam task in which only a subset of animals showed impairment. The analysis of reaching success in the tray task was also compromised by a loss of statistical power. Here many rats switched limbs from the initially-preferred forelimb to the ipsilateral-to-stroke forelimb following ICH. Thus, significant benefit may have been observed with EE at 5 and 8 weeks had many rats not switched preference; although it is also possible that no effects would have been observed with greater group sizes (i.e., transient benefit). Interestingly, the substantial change in limb preference observed in the tray task was persistent and unaffected by EE or E2 treatment, which concurs with the data from the

cylinder task. Cumulatively, these data suggest that a rat's ability to use a limb may be improved by rehabilitation (EE) while its preference to use it, if given the choice, may not be altered. Perhaps this effect stems from the failure of conventional therapies to effectively deal with learned non-use (Taub and Uswatte, 2003). Indeed, rats are free to use whichever limb they choose in EE cages; thus, we did not specifically target the impaired limb with this treatment. Therefore, EE treatment may be improved by adding task-specific training (Biernaskie and Corbett, 2001), such as skilled reaching with the impaired limb, in order to counteract any over-reliance upon the ipsilateral-to-stroke limb that may be promoted by EE treatment. Indeed, constraint-induced movement therapy, focusing on skilled reaching, effectively promotes recovery after striatal ICH in rats (DeBow et al., 2003). This is why we did not force rats to use their impaired limb (e.g., restricting use of the normal limb) in the tray task as it might have rehabilitated all groups.

Our study has several practical implications for assessing outcome after collagenase-induced striatal ICH in rats. First, while the beam task is able to detect long-term deficits, it seems relatively insensitive to treatment effects because many animals show little to no impairment. Of course, this will depend upon the insult severity and location. Second, while the cylinder task was sensitive to ICH, it appears to assess a behaviour (spontaneous limb usage) that is resistant to therapy. Thus, the cylinder should not be the only behavioural test used, but we do recommend its use with other tests, especially considering the brief time required to conduct testing. Third, the tray task, which is designed to assess skilled reaching, is also persistently sensitive to ICH-induced striatal damage, but the possibility of rats switching limb preference must be considered.

MacLellan et al. also reported that animals switch limb preference in the single-pellet reaching task and this depended upon the ICH lesion size (MacLellan et al., 2006). Thus, these tests can determine whether limb preference is affected by therapy. In either case, we do not recommend forcing rats to use their contralateral-to-stroke limb, such as by bandaging their ipsilateral limb, as it may rehabilitate the rat, thereby confounding or masking treatment effects. A similar problem would occur with providing extensive testing (to increase sample size). Either way, this also adds to the time required to complete testing, which is considerable with such tests. Fourth, of the tests used, the horizontal ladder test seemed most sensitive to ICH and treatment effects, and it is quick to conduct and easy to analyze. Thus, we recommend its use in future studies with the caveat that there may be no ipsilateral limb impairment, as is commonly seen (DeBow et al., 2003; MacLellan et al., 2006), and that the contralateral hind limb data are generally more variable.

The EE treatment did not lessen the total volume of brain tissue lost at 8 weeks after ICH. This contrasts with a study using constraint-induced movement therapy after ICH wherein rehabilitation lessened tissue loss when administered after a one-week delay (DeBow et al., 2003). In addition to rapid tissue destruction at the time of ICH, the collagenase model of ICH also causes some loss of tissue over weeks, likely due to continuing atrophy such as of white matter tracts (MacLellan et al., 2007). Thus, it is possible for delayed rehabilitation treatments to reduce injury volume. The E2 treatment also did not reduce injury, but this is not surprising given that it was administered after a delay of 1 week and previous studies found benefit with pre-ICH treatment. Nonetheless, it remains possible that the EE and E2 treatments had a transient effect on cell loss or

atrophy that dissipated over the lengthy survival time used in this study. Indeed, weak neuroprotectants have a history of providing only transient benefit whereas more potent therapies provide lasting benefit (STAIR, 1999). This may be the case with rehabilitation treatments as well.

In summary, E2 treatment failed to notably influence either spontaneous or rehabilitation (EE) facilitated recovery in the collagenase rat model of ICH. In contrast, EE treatment provided significant benefits, albeit not on all tests nor to a great extent. Accordingly, E2 treatment appears to improve sensory / motor recovery from striatal injury only when administered around the time of ICH. The same situation appears to be the case with ischemic injury to the motor system. While these findings indicate that E2 will not be an effective therapy when given later after a stroke, they do show that E2 will not harm recovery from motor system injury. Thus, studies that administer E2 early to limit stroke damage should not have to worry about subsequently impeding recovery by continuing the treatment over an extended time. This is an important clinical issue as stroke patients may conceivably receive chronic hormone therapy.

3.5 Figures

Figure 3.1

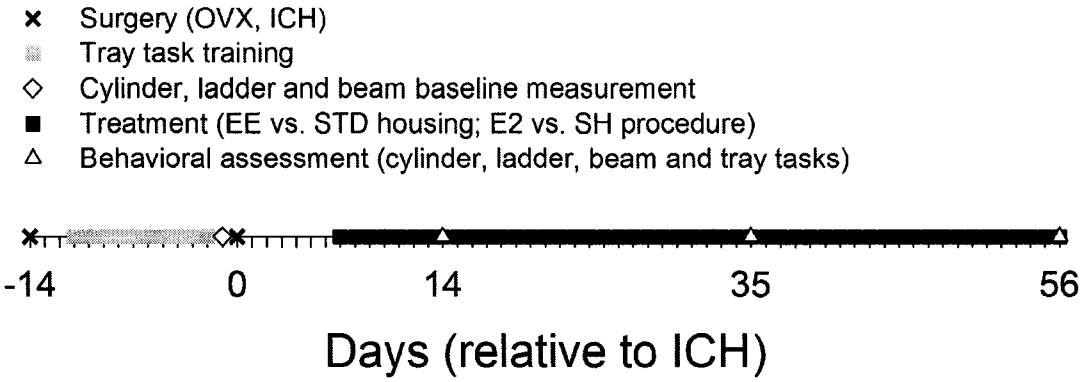

Figure 3.1. Timeline of behavioural training and testing, and the surgical procedures. Rats were euthanized at 8 weeks post-ICH. Animals were housed in environmental enrichment (EE) or standard (STD) cages and received an estradiol (E2) pellet implant or they underwent a sham (SH) procedure. Thus, there were four groups: STD-SH, STD-E2, EE-SH, and EE-E2.

Figure 3.2

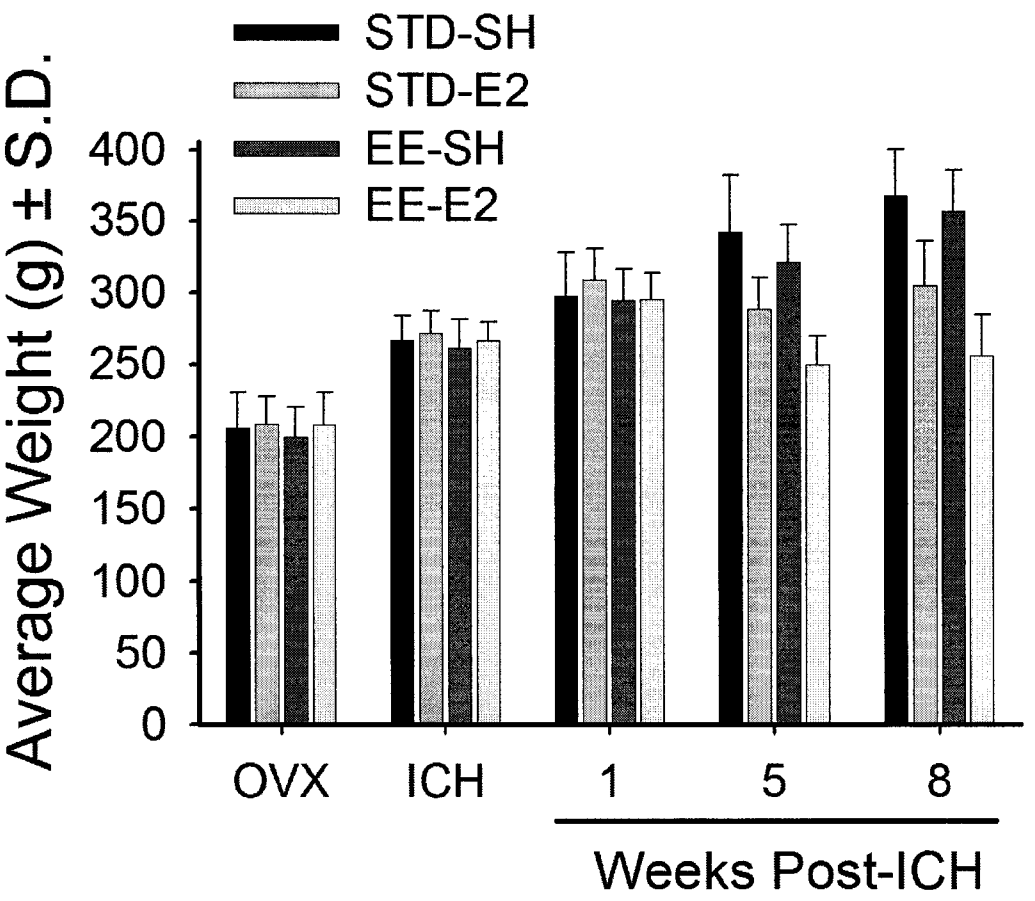

Figure 3.2. Body weight measured at the time of OVX and ICH surgeries and at 1, 5 and 8 weeks post-ICH. Both E2 and, to a lesser extent, EE lessened body weight at 5 and 8 weeks post-ICH. See Results for statistics.

Figure 3.3

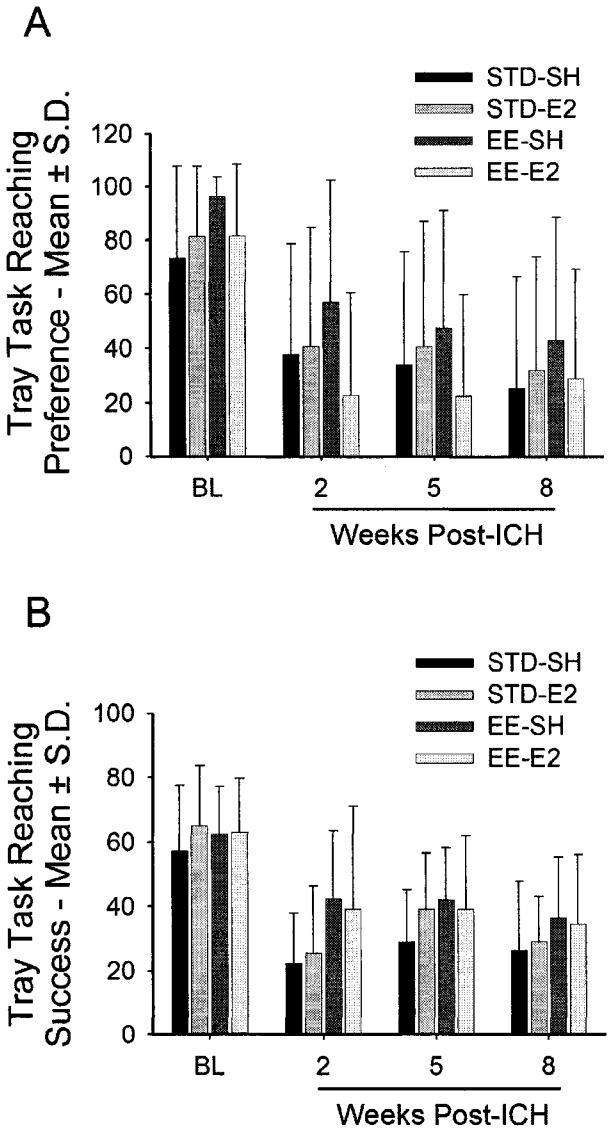

Figure 3.3. Reaching preference (A) and success (B) in the tray task during baseline (BL) training and at 2, 5 and 8 weeks post-ICH. All groups showed a significant reduction in reaching after ICH with the initially dominant limb. Reaching accuracy was also impaired. Neither EE nor E2 treatment affected limb preference while EE transiently lessened the skilled reaching impairment (week 2 post-ICH). See Results for statistics.

Figure 3.4

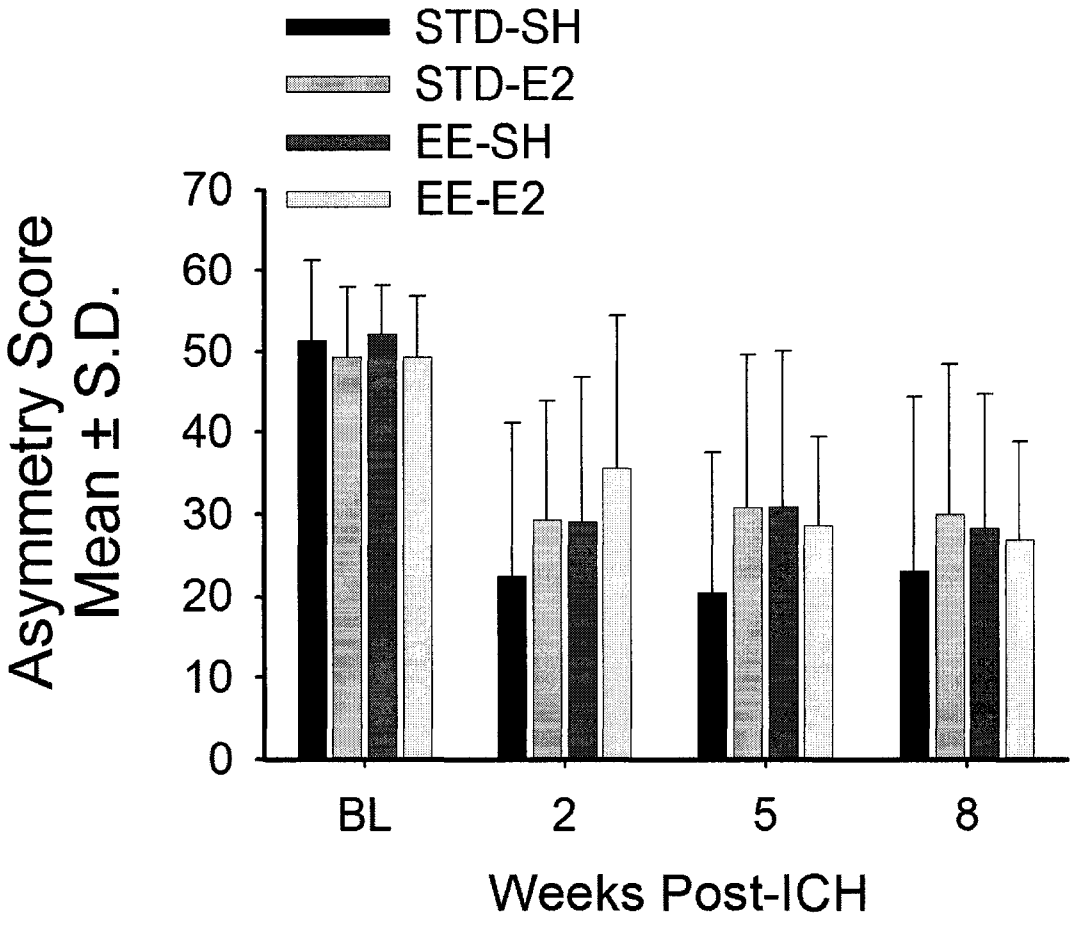

Figure 3.4. Contralateral forelimb usage for wall contacts in the cylinder task during baseline (BL) assessment and at 2, 5, and 8 weeks after ICH. All groups showed asymmetrical limb usage after ICH, but there were no significant differences among the groups. See Results for statistics.

Figure 3.5

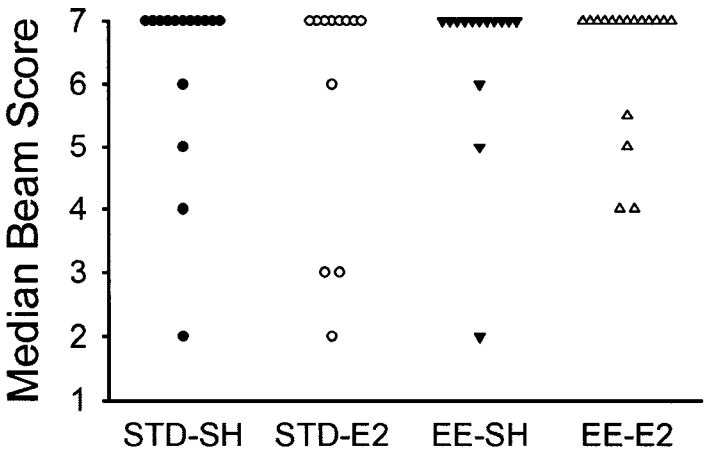

Figure 3.5. Beam traversing performance at 2 weeks after ICH. Each symbol represents the median beam traversing score of 5 trials. All groups has significantly lower scores (versus baseline), but there were not significant differences among groups. See Results for statistics.

Figure 3.6

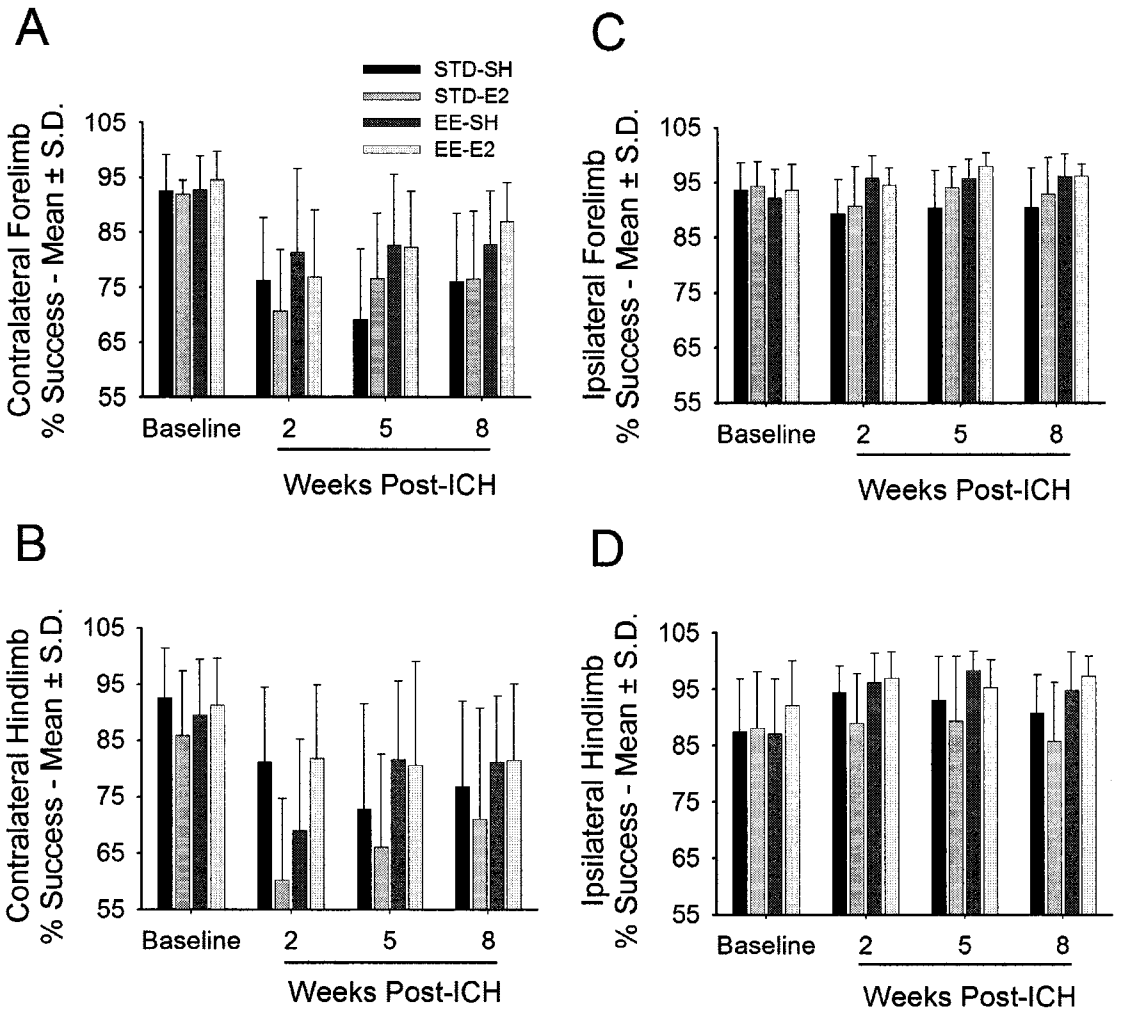

Figure 3.6. The % success on the horizontal ladder walking task for the contralateral-to-stroke forelimb (A), contralateral hind limb (B), ipsilateral forelimb (C) and ipsilateral hind limb (D) during baseline training (BL) and on weeks 2, 5 and 8 post-ICH. All groups had significant impairments with their contralateral forelimbs, which was significantly improved with EE, but not E2 treatment. Use of EE also improved performance with the ipsilateral limbs. See Results for statistics.

Figure 3.7

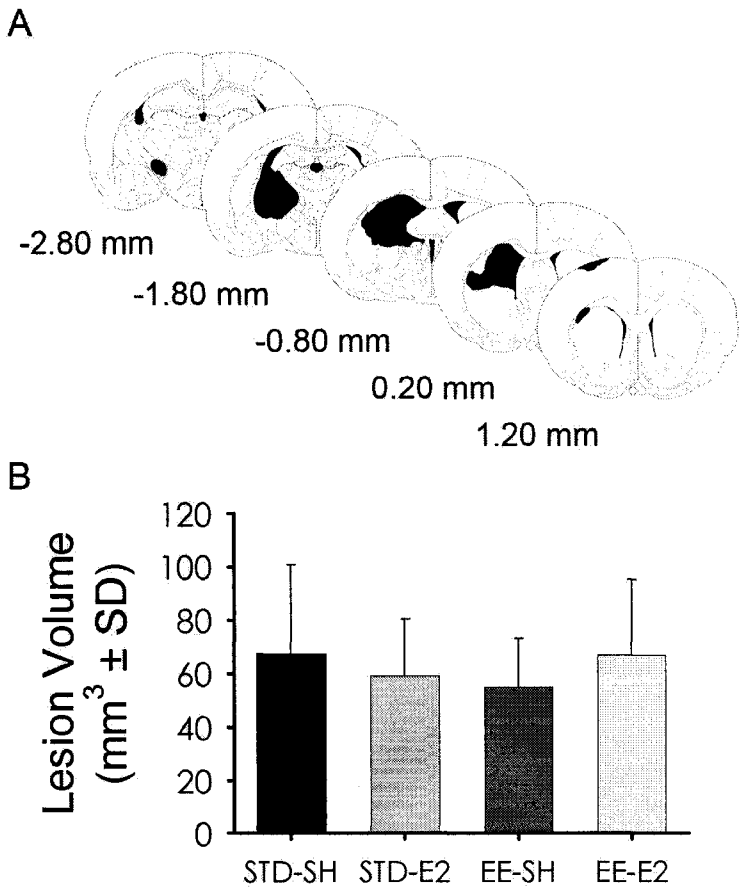

Figure 3.7. A diagram of an ICH-induced lesion from a representative animal (A). The black region represents ventricular space (e.g., ventriculomegaly on the side of the ICH) or dead tissue. The volume of tissue lost at 8 weeks after ICH surgery (B) was not significantly different among groups. See Results for statistics.

Figure 3.8

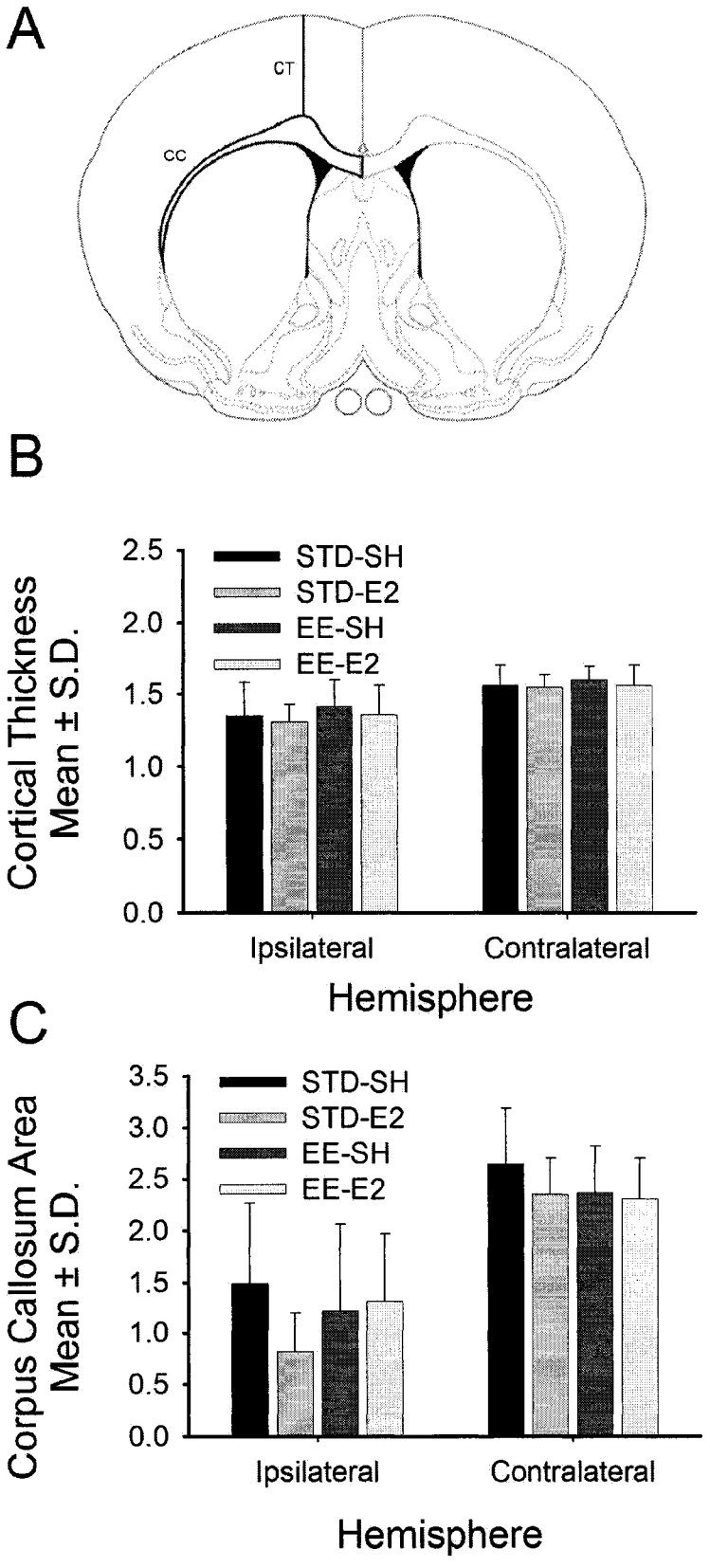
A
CT
CC
B
Cortical Thickness
Mean ± S.D.
2.5
2.0
1.5
1.0
0.5
0.0
STD-SH
STD-E2
EE-SH
EE-E2
Ipsilateral
Contralateral
Hemisphere
C
Corpus Callosum Area
Mean ± S.D.
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
STD-SH
STD-E2
EE-SH
EE-E2
Ipsilateral
Contralateral
Hemisphere

Figure 3.8. An illustration (A) of our method to measure cortical thickness (CT) and corpus callosum (CC) area, which was assessed in one coronal section of each rat that contained the largest area of injury. These measurements were done on both the ipsilateral and contralateral-to-ICH hemispheres. Cortical thickness (B) and CC area (C) were significantly smaller in the ICH side, but there were no other treatment effects. See Results for statistics.

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CHAPTER 4

DISCUSSION

4.1 MAJOR FINDINGS

Intracerebral hemorrhage (ICH) is a devastating neurological injury that occurs in up to 15% of strokes (Brott et al, 2003). For patients who survive ICH, functional deficits are chronic, as is the rehabilitation process. Despite the fact that ICH might be expected to be a long-term process with a gradually developing natural history, the large majority of studies of ICH-related tissue changes have focused on the acute window of the first seven days post-injury (Gong et al., 2000; Huang et al., 2002; Belayev et al., 2007). A minority of studies have examined histological and behavioural findings at longer survival times; selected landmark studies are summarized in Table 1 (Del Bigio et al., 2001; Felberg et al., 2002; MacLellan et al., 2007). However, none of these studies have explicitly examined histologic evolution of ICH over time. Because we know little of the longer-term changes in the brain following ICH, we hypothesized that tissue loss may evolve after day seven on the basis of atrophy and/or ongoing cell death. We therefore chose to address this question in a well-established experimental model of ICH in rats.

Ongoing tissue loss

The experiments detailed in chapter 2 quantified the ongoing injury post-ICH by examining histological sections of the rat brain at up to 60 days post-procedure. Firstly, we analyzed the total volume of tissue lost at 7, 14, and 60 days post-ICH. Importantly, we found that there was a significantly larger lesion volume at 60 days than at 7 days post-injury, verifying our hypothesis. To further characterize the ongoing tissue destruction after ICH, we examined the loss of white matter at the midline at 7 and 60 days. Here we found that indeed there was further thinning of the corpus callosum at the

midline 60 days after ICH, confirming ongoing white matter involvement. Next we examined the grey matter surrounding the hematoma at 7 and 60 days after ICH, measuring dendritic length and arbourisation. In contrast to our findings with white matter, dendritic length and arbourisation initially decline at 7 days and recover by 60 days post-injury in the grey matter surrounding the hematoma. An unexpected finding was that the dendritic length and arbourisation actually increased in the contralateral striatum, potentially indicating compensation by the contralateral hemisphere. Although it is possible that the neurons sampled from the perihematoma region at day seven may have died off as the lesion expanded leaving only healthy cells to sample from. Cumulatively, these studies indicate that white matter continues to degenerate up to 60 days post-ICH, whereas grey matter begins to recover earlier. This is a novel result that indicates a need for attention to the longer-term injury related to ICH.

In a previous study of the long-term effects of ICH in rats, Felberg and colleagues found that neuronal *density* remained normal in the previously lesioned striatum, and that neuronal loss was proportional to the amount of gross tissue atrophy observed. This would appear to corroborate our findings in that grey matter is relatively spared by the ongoing injury (Felberg et al., 2002).

Possible mechanisms of lesional expansion

While the process causing ongoing loss of tissue following ICH remains unclear, any or all of several processes may be involved. One potentially important mechanism could be Wallerian degeneration, which would be consistent with an ongoing (late) loss of primarily white matter. Given the abundance of white matter in the striatum,

destruction of axons due to the mechanical injury of ICH is expected to contribute to the ongoing tissue loss. Following destruction of cell bodies adjacent to the hemorrhage, axons degenerate within one week (Stovring and Fernando, 1983; Kuhn et al., 1988). However, other tissue components of white matter may similarly be affected by damage to the grey matter. Following Wallerian degeneration of the optic nerves in rats, degeneration of the myelin sheath and oligodendrocytes is very slow and can take up to 22 months to clear (Ludwin, 1990). Furthermore, demyelination has been observed to continue for up to six months in brain injured patients (Kuhn et al., 1988). The gradual demise of these cells may contribute to the ongoing loss of white matter following ICH. We can consider further studies that could be used to test this hypothesis. Degenerating oligodendrocytes, for instance, are detectable by staining for myelin-oligodendrocyte glycoprotein. Electron microscopy could also be used to study changes in myelin following ICH. Furthermore, Wallerian degeneration following long-term ICH injury can be examined in human patients with magnetic resonance imaging (MRI) (Kuhn et al., 1988).

A second mechanism that may be responsible is cell death. The cell death which occurs following ICH is generally necrotic due to the mechanical injury and toxicity of blood products released from the hematoma (Gong et al., 2001). Apoptotic cell death is observed in and around the hematoma, but is most extensive 24 hours after ICH, nearly absent at seven days, and not detectable at 100 days (Felberg et al., 2002). Cell death is not seen by Fluoro-Jade staining after the first week following ICH (Wasserman and Schlichter, 2007), suggesting that neither apoptosis nor necrosis is occurring within the perilesional grey matter at this time. However, in contrast, Wilson et al have detected

apoptosis in the white matter (pons) following traumatic brain injury by TUNEL at late time points from four weeks to more than one year (Wilson et al., 2004). This is, however, limited in extent and occurs mainly in microglia and macrophages. Furthermore, the difference between injury models is likely to be important. Similar (late TUNEL) studies in human or animal ICH are lacking. It may be interesting to repeat these studies in an ICH model to determine whether apoptosis is indeed occurring in the white matter at late time points.

Inflammation is a third process which has been the subject of much interest in ICH; however, the inflammatory reaction to cerebral hemorrhage appears to subside by day 14, prior to the time-points we studied (Xue and Del Bigio, 2003; Wang and Dore, 2007; Wasserman and Schlichter, 2007). The inflammatory response is observed with leukocytes infiltrating as early as four hours after the initial insult, peaking at two to three days, and generally subsiding by day 14 (Xue and Del Bigio, 2000). Microglia are also involved in the inflammatory response after ICH and are responsible for clearing the hematoma, and have been found to contribute to early injury (Hickenbottom et al., 1999). The hematoma volume can remain unchanged for the first few days, but is nearly absent at one week in rodent models of ICH, suggesting clearance, which is likely accomplished by activated microglia (Xue and Del Bigio, 2000; Peeling et al., 2001; Wang et al., 2003). Indeed, time-course studies show that activation of microglia begins as early as one to two hours after ICH, peaks at seven days, and can persist for up to three to four weeks (Hickenbottom et al., 1999; Xue and Del Bigio, 2000; Wang et al., 2003). It may be that late inflammation may cause ongoing cell death. However, it is possible that the apparently increasing size of the lesion following ICH could be a function of *declining*

inflammation, if we presume that the inflammatory cells/reaction contribute some volume to the brain substance itself. For instance, inflammatory cells (such as microglia) in the peri-hematoma region could contribute to a greater volume of remaining tissue early after injury. Resolving edema of the peri-hematoma tissue could similarly produce this effect, although this would be unlikely, as edema resolves by day 7 after ICH (Yang et al., 1994; MacLellan et al., 2006b; Fingas et al., 2007).

Methodologic limitations

While our experimental design was successful in addressing long-term tissue loss after ICH, there were some potential limitations of our approach. Firstly, microscopic examination of the brains did not allow for longitudinal observations of each lesion as it developed over time. This is best achieved with other methods such as MRI; similar studies using this technique have been performed in parallel by another student in our laboratory (MacLellan et al., 2007). These latter experiments verified the above findings by demonstrating a gradually increasing lesion size in the collagenase model of ICH in rats. One potential advantage of the method presented here over MRI is that I was able to directly observe dendritic morphology; it might however be ideal to use both methods in combination.

A second limitation of our study is that of the two models of ICH, we evaluated long-term injury in only one (the bacterial collagenase model). A second, the whole-blood model, may provide a more physiological perspective; however, it behaves less comparably to clinical ICH because rats do not show functional deficits in the long term post-injection (MacLellan et al., 2006a; MacLellan et al., 2007). Furthermore, injection of

bacterial collagenase produces a persistent tissue defect, as is seen in human ICH, whereas the tissue defect caused by injection of whole blood is only transient (MacLellan et al., 2007). For our purposes, then, the collagenase model is the more relevant model when examining long-term injury.

A third limitation of the above work is that behavioural outcomes were not tested. However, functional deficits and recovery post-ICH have been extensively studied in multiple experiments including the MRI studies mentioned above (MacLellan et al., 2007).

Effect of late intervention

The findings outlined in chapter two showed that ICH in the collagenase model in rats is an ongoing injury that continues to evolve beyond one week. This may indicate that the window of opportunity for treatment in human ICH may be longer than previously presumed. In chapter three, I investigated whether ongoing functional deficits after ICH could be mitigated by a delayed intervention applied one week following the initial injury. Two treatments, estrogen supplementation and environmental enrichment, were applied singly or in combination one week following ICH. Briefly, estrogen treatment did not produce significant histological or functional improvements. In contrast, environmental enrichment did produce statistically significant improvement on one functional test, the horizontal ladder test, although this effect was not observed in the tray reaching, forelimb asymmetry and beam tests. There are several possible reasons that an effect of treatment was only observed in the ladder test. It may be that this test is more sensitive to ICH injury and functional improvements over time than the other tests.

However, because the floor of the environmental enrichment cage consists of a grid, which is similar to the horizontal ladder, it is possible that the improvement on this test simply reflects a practice effect. We could distinguish between these two possibilities by using a more sensitive behavioural testing method. MacLellan et al 2005 examined several functional tasks after ICH and showed that the most sensitive measure is a composite of several behavioural tests, some of which we did not use in this experiment (single pellet, Montoya staircase reaching, neurological deficit score). If the effect of environmental enrichment is robust, we may observe it on one or more of these other tests. We could also try to increase the size of our effect by using a more intensive rehabilitation regime, such as constraint-induced movement therapy which has already been proven beneficial in the period immediately following injury (DeBow et al., 2003). Another potentially interesting question would be whether the addition of one or more growth factors (eg. BDNF) could augment the effects seen with rehabilitation when used in combination.

Our study did not definitively answer the question of whether estrogen can facilitate spontaneous recovery after ICH. To address this more directly, we could use a larger, supraphysiological dose of estrogen, and employ a battery of functional tests as described above. We could also re-examine the effect of estrogen on histologic changes following ICH by studying dendritic morphology using the same methods outlined in chapter two.

4.2 Conclusions and clinical significance

In summary, my thesis looked at long-term injury and recovery in a rat model of ICH. I found that maturation of ICH injury does indeed occur over a two-month period following the event, and, moreover, that white matter appears to be primarily affected during this late phase. I also demonstrated that at least one intervention, environmental enrichment, can be used to facilitate spontaneous recovery when applied in the late post-injury period. These results may have some significance for clinical ICH in humans. ICH is a devastating subtype of stroke for which there are no currently effective treatments. Moreover, most treatments tried to date focus on the acute setting immediately post-injury. My own work suggests that the window of opportunity for ICH treatment may be wider than previously thought, and moreover that rehabilitative strategies may be effective in helping achieve recovery weeks or months after the event. It remains to be seen whether the promising results presented here will eventually hold true in human patients, however, these findings warrant further study, and may ultimately lead to new approaches to the treatment of hemorrhagic stroke.

Authors	Model	Time Points	Major Measure	Key Findings
(Felberg et al., 2002)	WBI	20min	MRI	The majority of damage after ICH is in the peri-hematoma region and the destruction of neurons occurs over at least three days as they come into proximity of the hematoma.
		1, 3, 7, 100d	Cytochrome c release	
		100d	Neuronal density Cell count Brain atrophy White matter characteristics Neuronal density in substantia nigra pars reticulata	
(Xue and Del Bigio, 2003)	Coll.	1, 4h 1, 2, 3, 7, 21d	Hematoylin & eosin Lymphocyte infiltrates Reactive microglia and macrophages TUNEL Fluoro-Jade	The temporal pattern of cell death and inflammation are similar in all models, although the magnitudes of injury differ. Cell death peaked at one to three days after injury, but continues, along with inflammatory cell infiltration for several weeks after injury.
	WBI	1, 4h 1, 2, 3, 4, 28d		
	CBVA	1, 8h 1, 2, 3, 7, 28d		
(MacLellan et al., 2007)	Coll. WBI	1, 2, 4h	Bleeding profile	Injury in the collagenase model is greater and resolves more slowly than in the whole blood infusion model. Functional deficits are more persistent in the collagenase model than in the whole blood infusion model.
		6, 12h 2, 4, 7, 14, 28, 42d	MRI	
		1, 28d	Functional assessment	
		42d	Histopathology	

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