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Recovery after intracerebral hemorrhage

by

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This thesis is dedicated to my family, Mary, Terry, Andrew and Tyler.

Abstract

There are two types of stroke: ischemic and hemorrhagic. Intracerebral hemorrhage (ICH) accounts for about 15% of all strokes and is often severe. Currently no treatments are available to reduce injury, but rehabilitation may improve recovery. Most studies focus on ischemia, putting little emphasis on understanding recovery after hemorrhage.

In chapter 2, we evaluated exercise prior to and/or following ICH. Similar protocols improve recovery after ischemic stroke, and we hypothesized that the treatment would also reduce deficits after hemorrhagic injury. However, exercise was not beneficial for ICH and increased intensity of treatment worsened functional outcome. In chapter 3 we assessed amphetamine and/or rehabilitation after ICH, an intervention also shown to improve recovery after ischemia. The rehabilitation consisted of environmental enrichment (EE) with modest amounts of training on beam and skilled reaching. Rehabilitation but not amphetamine partially improved recovery. Skilled reaching was not improved by rehabilitation so we decided to combine EE with more reach training. In chapter 4, we found that two weeks of rehabilitation (EE and skilled reaching), started one week after ICH significantly reduced lesion volume, and improved recovery on walking and skilled reaching tests. We were particularly interested in the mechanisms contributing to the reduction in lesion volume after ICH, and attempted to identify these.

In chapter 5, we used the same rehabilitation intervention as in chapter 4 to determine if treatment alters dendritic complexity, spine density, or cell proliferation. Unfortunately, the reduction in lesion volume from chapter 4 was not replicated. But we were able to identify several plastic changes. Dendritic complexity was increased in neurons of the forelimb motor cortex ipsilateral to injury. Dendritic complexity of neurons in the peri-hematoma region and corresponding area in the uninjured hemisphere were also increased. In contrast, rehabilitation did not alter spine density or cell proliferation.

In summary, we found that treatments that work for ischemic stroke do not necessarily work for hemorrhagic injury. Some methods of rehabilitation are able to reduce functional deficits and in some cases lesion volume after ICH. These rehabilitation effects are likely due to enhanced plasticity and not cell genesis.

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Table of Contents

List of Tables:

List of Figures:

List of Abbreviations:

AMP	d-amphetamine
ANOVA	analysis of variance
BDNF	brain derived neurotrophic factor
BrdU	5-bromo-2-deoxyuridine
CIMT	constraint-induced movement therapy
DAPI	4',6-diamidino-2-phenylindole
EE	environmental enrichment
eNOS	endothelial nitric oxide synthase
EX	exercise
GFAP	glial fibrillary acidic protein
Hr	hour
ICH	intracerebral hemorrhage
Iba1	ionized calcium binding adaptor molecule 1
i.p.	intraperotineal
MABP	mean arterial blood pressure
MCAO	middle cerebral artery occlusion
m/min	meters per minute
NDS	neurological deficit score
NeuN	neuronal nuclei
PBS	phosphate buffered saline
REHAB	rehabilitation
rFVIIa	recombinant factor VIIa
ROI	regions of interest
rt-PA	recombinant tissue plasminogen activator
SAL	sterile saline
SEM	standard error of the mean
TUNEL	transferase dUTP nick end labeling

Chapter 1:

Introduction

1-1 Introduction to stroke

Stroke is the third leading cause of death in Canada and the leading cause of disability. The demand on rehabilitation services is increasing, because as the population ages more people are suffering a stroke (1). In addition, due to improvements in acute care the percentage of patients surviving has also been steadily increasing (2).

There are two forms of stroke, ischemic and hemorrhagic, but their acute presentation is often indistinguishable. Symptoms for both include a sudden onset of any one or a combination of signs such as weakness, speech difficulties, vision problems, headache and dizziness. However, ICH patients more often present with impaired consciousness and deteriorate rapidly. The underling mechanisms of injury differ, as does the time course of damage and recovery (3-5). Therefore, different stroke types may respond differently to rehabilitation.

1-1.1 Ischemia

Ischemia, which is the interruption of blood flow to a region of the brain, accounts for about 80% of all strokes. Focal ischemia is caused by a blockage in a blood vessel within the cranium, whereas global ischemia occurs when blood flow to the entire brain is compromised, such as during cardiac arrest. Acute treatment for focal ischemia is now available in the form of intravenous recombinant tissue plasminogen activator (IV rt-PA), an enzyme that can degrade the stroke-causing clots within vessels.

Acute treatment with IV rt-PA is highly beneficial, reducing death and dependency (6). However, to avoid the risk of hemorrhagic transformation this therapy must be provided within 4.5 hours of stroke onset, which limits the number of patients who can benefit (7). With increasing awareness and a larger number of institutions developing dedicated stroke units the number of patients receiving IV rt-PA has been steadily increasing, but many still do not meet the criteria for treatment. Other treatment options include intra-arterial thrombectomy, which is a surgical intervention to mechanically remove the clot. This treatment has a slightly longer time window of effectiveness (6 hours compared to 4.5 hours for IV t-PA), but few centers are equipped to perform the procedure (8). Antithrombotic agents such as aspirin are commonly used to reduce the risk of recurrent stroke (9).

1-1.2 Hemorrhage

Hemorrhage accounts for about 20% of all strokes and is often severe (10). There is a high mortality rate of 34-51%, with the majority of deaths occurring within the first two days of the insult (11). ICH presents with a rapid onset of neurological symptoms, similar to ischemia with focal deficits depending on the brain region involved (e.g. blurred vision, weakness), and often a severe headache and altered consciousness. Those patients who survive are frequently left with severe disability; an estimated 66% never regain functional independence (12). Improving

stroke outcome is an important goal, which can have significant benefits for the patient, while reducing the economic burden of long-term care.

A. Pathophysiology

ICH results from the rupture of a blood vessel in the brain, which can be further classified as primary or secondary. Primary hemorrhages are the most common, accounting for about 80% hemorrhages, and result from the spontaneous rupture of blood vessels. Hemorrhages resulting from hypertension or amyloid angiopathy are included in this class. Secondary hemorrhages result from any of several underlying conditions or events, including vascular malformations, trauma, drug use or coagulopathies (10).

Hypertension is a major cause of primary ICH. Hemorrhaging often occurs in subcortical structures (basal ganglia, thalamus, cerebellum, and pons) where small vessels branch off the circle of Willis. These locations are particularly sensitive to the occurrence of ICH because of their vascular connections. Normally pulse pressure is decreased at each branch point in the vasculature. However, in subcortical structures pulse pressure is still high due to the proximity to their large supplying vessels, making hypertension induced hemorrhaging common in these locations.

The other major cause of primary ICH is amyloid angiopathy (13). White matter and cortical hemorrhages are more likely due to amyloid angiopathy than hypertension. The buildup of amyloid beta-protein in the

walls of cerebral vessels makes them brittle and susceptible to rupture (14). There is a high rate of recurrent hemorrhage (~10%) associated with cerebral amyloid angiopathy (15). In fact, this might be a gross underestimation as the rate of non-symptomatic hemorrhaging is high. Accumulation of small microhemorrhages often results in dementia, focal deficits, and seizures. Cerebral amyloid angiopathy increases with age, and is found in about 5-8% of the population in their seventies but in 58% of the population over the age of 90 (16, 17).

Secondary ICH accounts for a minority of cases (~20%), and results from a variety of vascular abnormalities, trauma, coagulation disorders, or tumors (18). These forms of injury are more likely to account for hemorrhage in younger patients, as primary ICH is associated with risk factors more frequent in the elderly (i.e., cerebral amyloid angiopathy and hypertension) (19). Vascular malformations are the most common cause of hemorrhage in young normotensive patients. Aneurysms frequently occur at the branch point of major arteries, where the high rate of blood flow can produce quite large and devastating strokes. Hemorrhages due to trauma often occur with multiple locations of bleeding on the surface of the brain. Primary hypertensive and secondary ICH can affect a young population, emphasizing the need for improved rehabilitation to decrease the long-term dependency of these patients.

B. Risk Factors and Prevention

Age, sex (males have a greater risk), diabetes and race (African American and Asian) are significant risk factors (20). Hypertension is the primary modifiable risk factor for hemorrhage. Even a slight elevation in systolic hypertension (140 - 160 mm Hg) increases the risk for hemorrhage (20). Other modifiable risk factors include heavy alcohol or caffeine consumption, smoking and low serum cholesterol (19). The best method to reduce the impact of stroke is by preventing it. However, convincing people to make lifestyle changes and stick to them is not an easy feat.

C. Current treatment options

Current treatment options are limited and not entirely clear. Dedicated stroke units are beneficial, reducing mortality and rate of institutionalization (21). However, many other aspects of treatment remain controversial. Early hematoma growth in ICH occurs in 18 – 38% of patients and is associated with clinical deterioration and mortality (22). Blood pressure, hemostatic therapy and surgical intervention have the potential to alter hematoma growth and outcome. Unfortunately, it is difficult to reduce injury after ICH; therefore research on how to improve recovery is necessary.

Blood pressure optimization

Proper blood pressure management in acute hemorrhagic patients is critically important. Hypertension is associated with hematoma expansion (23), but there is worry that lowering blood pressure too much may contribute to ischemia in the area surrounding the hematoma (24). Many clinical trials are currently assessing the ideal strategy for blood pressure management. Currently the recommendations are to treat systolic blood pressure exceeding 180 mmHg or mean arterial blood pressure (MABP) exceeding 130 mmHg (25).

Hemostatic therapy

Great optimism for ICH treatment was generated from the use of the early hemostatic therapy recombinant factor VII (rFVIIa). Use of rFVIIa had already been approved for treatment of bleeding disorders, and preliminary clinical findings suggested early use would improve functional outcome by reducing ICH growth and mortality (26). However, a larger phase III trial failed to find significant reduction in mortality or disability in spite of confirming reductions in hematoma expansion (27). Based on current findings use of rFVIIa is not recommended, but ongoing research is addressing its potential benefits for specific subgroups of patients (28).

Surgical Management

Surgery has the potential to provide benefit for ICH patients in several ways. For example, removing the potential toxic breakdown products of the blood may decrease cell death. In addition, reducing the space occupying effects of the clot could reduce intracranial pressure. However, there are also surgical risks such as damaging overlying tissue, or causing re-bleeding by disrupting the clot. The International Surgical Trial in Intracerebral Hemorrhage (STICH) trial found that outcome and survival was not improved by hematoma removal (29). Although, it appears that for those with superficial hemorrhages (within 1 cm of the cortical surface) surgery may be beneficial (30). Patients with a cerebellar hemorrhage that is 3 cm in diameter or larger often require surgery, as rapid deterioration and coma frequently occurs without intervention. Future studies may be able to combine rFVIIa with surgical intervention, thus reducing the risk of recurrent bleeding following surgical intervention.

1-2 Recovery

1-2.1 Ischemia vs. ICH

Although initial functional disability is often substantial, significant spontaneous recovery often occurs (31), partially due to the resolution of initial injury processes (e.g., edema). Ischemic and hemorrhagic injuries have different mechanisms of injury, suggesting that there could be differences in recovery. Several studies have compared recovery in these

two groups of patients with no clear answer. Patient comparisons often vary depending on whether population (32-34) or rehabilitation (35-38) based assessments are made. Population based studies assess all patients who have a stroke whereas rehabilitation studies only look at individuals selected for therapy.

Hemorrhagic stroke has a high acute mortality rate. About 22% die within the first day, 42% are dead within the first week, and only 49% survive the first month (33). The overall fatality rate for ischemic stroke is low in comparison, 12% at 28 days (39). Predictors of early death following an ICH include age, altered consciousness (often assessed as a low score on the Glasgow coma scale), hematoma volume, hemorrhages that extend into the ventricular system, lateral shift, elevated MABP or blood glucose, and being on an anticoagulation treatment at the time of ICH (33, 40-48).

There is some evidence that the difference in fatality rates between stroke subtypes is a result of stroke severity rather than type. For example, The Copenhagen Stroke Study compared outcomes in unselected stroke patients (32). When stroke severity was controlled for, stroke type had no influence on mortality, functional outcome or time course of recovery, suggesting that initial severity determines outcome. However, the rate of hemorrhage increased with stroke severity, meaning that the typical ICH patient will have substantial injury. Barber and colleagues have provided additional support for this hypothesis (34); they

found that outcome is worse in ICH patients (greater deficits and mortality), but after controlling for stroke severity, there is no longer an effect of stroke type. Hemorrhage patients have more severe strokes, which if not fatal leaves them with substantial functional deficits.

The most severe stroke patients are often placed directly into long-term care and the mildest patients go directly home. This means that only those patients with moderate impairments tend to receive inpatient rehabilitation. Several studies have compared how stroke type affects outcome following inpatient rehabilitation. Comparisons between stroke types are often difficult, as hemorrhagic patients tend to be younger, are often admitted later, have greater deficits on admission and stay longer in rehabilitation. When these differences are controlled for, efficacy of therapy (the amount of improvement corrected for the length of stay) is often higher in hemorrhagic patients (35, 37, 38, 49). One reason for this difference may be the delayed reduction of neurological symptoms after ICH, as the hematoma and edema resolves so to does the compression of brain areas that may be able to regain function, thus reducing deficits (37). However, not all studies find greater recovery in ICH patients: some find no difference (50) or greater improvement in ischemic patients (51, 52). Differences in the type and time of assessment as well as the initial severity of patients likely contribute to the conflicting findings.

1-2.2 Factors Predictive of Recovery

Stroke patients recover over time but the majority have persistent deficits (5). Age is a negative predictor of outcome (36, 53), which may be related to increasing balance difficulties and the higher occurrence of comorbidities (e.g., arthritis) (54). The most important factor contributing to the rate and degree of recovery is stroke severity (5). Severe strokes recover gradually and although a few improve considerably most are left with substantial impairment. Patients who have had mild strokes recover quickly and have the least amount of residual deficits. Patients who suffer a moderate stroke patients benefit most from rehabilitation, making significant gains in function, although few recover completely. Lesion location also has a large role, as minor damage to some regions (e.g., internal capsule or brainstem) can result in substantial deficits and poor recovery (55). Admittance to a stroke unit improves outcome after stroke (56, 57). Interdisciplinary stroke rehabilitation improves functional outcome, reduces mortality, decreases length of stay and sends more patients home as opposed to institutional care.

1-3 Rehabilitation Treatments

1-3.1 Critical Factors

Delay

Recovery processes in the brain are transiently engaged following an insult (58). Therefore, it makes sense that early rehabilitation would

best take advantage of this temporary peak in plasticity. Functional recovery and plasticity are maximized when rehabilitation is started early (59). In a study by Biernaskie and colleagues, rehabilitation was started 5, 14, or 30 days following an ischemic stroke in rats. Recovery was best with the brief delay; benefits were diminished after a 14-day delay; and no benefit was achieved with 30-day delay. Only the early intervention enhanced dendritic growth. Clinical studies have shown that earlier rehabilitation is also associated with better functional outcomes (60-63). Clinical and animal research suggests that long delays between stroke and rehabilitation may decrease the impact of rehabilitation. However, there is no consensus on the optimal time for rehabilitation. Several studies support beginning rehab as soon as the patient is stable (62, 64), but both clinical and animal data indicate that early intensive therapy can be detrimental (65, 66).

Intensity

High intensity of rehabilitation is associated with improved outcome (67). Kalra (68) assessed the difference in functional outcome between patients receiving care in a stroke rehabilitation unit versus those in a general unit. Both units provided the same amount of therapy overall, but the stroke unit therapy was more intensive and specialized. Recovery was faster and more complete in the stroke unit as reflected in the functional assessment at discharge and the reduced length of stay. Therapy 7 days

a week compared to the standard 5 days per week also improves recovery and shortens length of stay (69). Unfortunately, the standard of care is far from optimal. There is evidence for poor patient participation (70), which decreases functional improvement and lengthens the time spent in care. Improvements in stroke unit care are necessary because the majority of patient time is spent inactive and alone (60%), with only 13% of the day spent in therapy (71). The structure of rehabilitation units must be altered to enhance the environment and optimize patient treatment.

Task-Specific

The benefits of rehabilitation are task-specific, there is very little generalization (72). For instance providing feedback about weight distribution during standing improved balance during stance but did not improve limb movement or balance during locomotion (73). Constraint induced movement therapy and body-weight supported treadmill training both combine the forced use of an impaired limb with task relevant training to achieve functional improvement (74-76).

1-3.2 Types

Exercise

Exercise is important to healthy aging, reducing the risk of developing dementia (77, 78). Physical activity also decreases the risk of stroke (79, 80), in part through lowering blood pressure and helping to

maintain a healthy weight (81). Pre-stroke exercise reduces stroke severity in rodents (82, 83); similarly early post stroke running is neuroprotective (84, 85). There are insufficient clinical findings to support any conclusions. Stroud and colleagues reported that moderate to high levels of pre-stroke physical activity decrease functional deficits following stroke (86); It is unclear if physical fitness in this case reduced lesion severity, or if pre-stroke activity had some other positive carryover effects on outcome (e.g., higher level of post stroke exercise). Exercise post stroke as a method of improving outcome in patients has many conflicting findings (87). Recently, a meta-analysis indicated that exercise has task specific benefits. Exercise programs using treadmill training improved walking ability. However, programs, which used a combination of resistance training or circuit training, produced unclear results. Although several mechanisms of action have been suggested more research is needed to clarify the role of exercise in brain health.

Environmental Enrichment (EE)

In comparison to healthy young adults, older individuals and especially those with brain injury often live in an impoverished environment. For instance, during hospitalization most patients lie in bed for over 60% of the day (71). Based upon extensive animal research showing the importance of environmental enrichment, novel activities, and social engagement, many stroke units are now attempting to enrich the

lives of their patients (88, 89). Many units are now incorporating more social spaces and encouraging activities among patients. Virtual reality therapies, which utilize video games, are an affordable way to increase activity (90). The proper balance of focused therapy and stimulating activities will provide enhanced and more cost effective rehabilitation.

Constraint-Induced Movement Therapy (CIMT)

Taub developed the idea that stroke patients may acquire learned non-use of their impaired limb (91). Initial neurological injury will create a maximal level of impairment, which will resolve over time, allowing some recovery. However, patients learn during the initial period to decrease dependence on the impaired limb, and will even continue to underuse this limb once more function has returned (92). Restraint of the non-impaired limb is combined with task-specific training, forcing the patient to use the impaired limb. Many patients show a long-term improvement after CIMT (74, 93). This therapy is believed to work through two processes, first through extinction of the learned non-use (measured as an increase in limb use), and second by improving the function of the impaired limb. There are some contradictory findings regarding whether CIMT can improve the quality of limb use, in some cases the therapy only increases the amount of limb use (94, 95). Constraint therapy is an effective tool for improving long-term recovery after stroke.

1-4 Animal Models

1-4.1 Models of Brain Injury

Many models of injury are used to identify cell death and repair mechanisms. However, it is necessary to use a model that closely mimics the pathology you are trying to treat as several aspects of injury, such as type of cell death and its time course, will alter efficacy of neuroprotection and rehabilitation interventions. Many lesions such as aspiration and middle cerebral artery occlusion can be used to study recovery, but the resulting changes in plasticity, which likely contributes to recovery, varies across models (96). When studying stroke recovery it is important to use injuries that model the human condition in as many ways as possible (97). Several aspects of stroke, which affect recovery such as the progression of injury over time, type of cell death and location and size of injury, can be studied in stroke models (97).

Several experimental models of ICH are currently used to study mechanisms of cell death and plasticity and to test novel treatments. Injection of blood components, such as iron and thrombin or implanting an inflatable balloon, can model specific components of injury such as the toxicity of blood and mass effect (98-100). Injury often targets the striatum as this structure is frequently the site of ICH in humans (32, 101), but some models do target other structures such as the pons or cerebellum (102, 103). Two common models of ICH are whole blood and collagenase injections (104).

The whole blood model involves injecting blood into the brain (105). This mimics a single large bleed. Once the hematoma resolves a small lesion remains, therefore in many studies an early measure of edema is the primary outcome measure that is quantified (106). The amount of blood injected can be modified to change lesion size. However, large lesions tend to result in blood coming up the needle track, limiting the size of the lesion. The resulting functional deficits are small and often recover by three weeks, indicating that long-term functional assessment with this model requires sensitive behavioural tests (106, 107).

Collagenase is an enzyme that breaks down collagen, a major component of blood vessels (101). Injecting collagenase into the striatum induces active intracerebral bleeding, that can model hematoma enlargement, which occurs in patients (22, 108, 109). Some interventions may alter the size of the hematoma, which must be assessed in a model with active bleeding (110, 111). Two disadvantages of the collagenase model are that it produces an elevated inflammatory reaction as compared to the whole blood model (105, 112), and it damages many blood vessels as it diffuses around the site of injection. The collagenase model produces consistent lesions (106), and also results in delayed atrophy, which often occurs in patients (113-115). Persistent functional deficits occur and can be evaluated at late time points (116). The collagenase model is well suited for the long-term study of functional outcomes and lesion

assessment, which is why it is often favored for rehabilitation studies (84, 117, 118).

1-4.2 Measuring Recovery

Researchers often use functional recovery as a primary outcome measure. Several factors contribute to difficulties in behavioural testing, such as differences in lesion placement. For example ICH lesions that target the dorsolateral striatum affect reaching ability, whereas those in the dorsomedial striatum disturb locomotion (119, 120). Behavioural tests may not be sensitive enough to differentiate treatment effects if there is variable lesion location, requiring larger group sizes or the use of lesion models with greater consistency. Injury models such as whole blood injection, in addition to producing variable lesion location, also result in small behavioural deficits, which can recover with time (106, 116). In these cases, more difficult tasks such as skilled reaching, which can differentiate compensatory movements from true recovery, are required to assess long-term recovery (121). These problems also suggest the need for using multiple tests that can indicate recovery mediated by more than one brain area, and at multiple times from injury.

Several tests have been developed to assess sensorimotor recovery following ICH (107, 116). Basic neurological deficits are frequently assessed with a battery of tests such as forelimb grasping, hindlimb retraction, and spontaneous circling. Many of these components

rely on subjective judgments of behaviour, and because of the high degree of spontaneous recovery late assessment often does not detect impairments (122). Walking tests including horizontal beam and ladder tests can measure balance and coordination. The number and/or degree of foot slips can be assessed as an estimate of walking ability (116, 123). The degree of recovery can be quite high and rats often make a near complete recovery, in these cases more difficult versions of the test may be called for (e.g. narrower beam or greater distance between bars). Spontaneous forelimb usage can be measured in a cylinder (124). When naive animals explore the walls of the cylinder they use both forelimbs approximately equally, but after injury they reduce the use of the contralateral limb, resulting in a forelimb use asymmetry. Caution must be used when assessing animals with large lesions as some bilateral brain damage may occur, resulting in an asymmetry score that underestimates the true deficits. Skilled reaching tests are sensitive behavioural assessments that measure the ability to grasp food, but they require pre-injury training and mild food deprivation (116, 125-127). The single pellet test can be broken down into individual reach components, which can differentiate between reacquiring normal movements and compensation (126). In the tray test, rats reach through vertical bars to obtain food pellets placed just outside the box. This task allows for reaching success to be measured with each forelimb. However, after injury rats can refuse to reach with their impaired limb (128). Staircase testing, where a rat is

placed into a box with a raised central platform with stairs on either side, allows for reaching success to be measured independently for both forelimbs (125). The types and timing of the testing must be carefully considered, as some treatments may increase the rate of recovery and/or the absolute recovery achieved. Although, too much testing must be avoided as it can act as rehabilitation, preference should be given to tests that can detect a long-lasting change in performance.

1-4.3 Recommendations for Translational Research

Basic research findings often fail to translate to the clinic (129). Although most translational studies focus on neuroprotection and acute interventions, many of the same principles apply to treatments aimed at enhancing rehabilitation (130). For example, amphetamines enhance rehabilitation based recovery in several species (123) but do not provide clear benefit for patients (131). Several aspects of basic research could be improved to increase the probability of success at the clinic. In the case of amphetamine, although several doses have been used in animal studies, no one study has directly assessed this factor (123, 132). Clear dose response curves are beneficial in guiding clinical trials. Likewise, many durations of treatment were tested but only one study made direct comparisons (133). Improved information from basic research may have reduced the number of unsuccessful clinical trials for amphetamine.

There are also several other aspects of basic research that can be improved. Delays between injury and treatment may be extended for drugs promoting recovery as opposed to neuroprotection, but the time frame is still limited (59) and should be identified in animal models prior to assessment in humans. Few studies use animals with comorbidities that are common in the patient population such as diabetes, hypertension and obesity. Most data is collected in young male rats, ignoring the importance of age and sex. Species differences are also critical, in humans stroke affects both white and gray matter, but rats, which are frequently used in basic research, have relatively little white matter. Pre-clinical testing is also done in pigs and primates, both of which have more white matter, and can better model aspects of white matter injury (134). Multiple species should be assessed prior to translation. Also, no one model can account for all aspects of patient stroke, which is why multiple models should be used. The use of guidelines for pre-clinical testing can increase the quality of studies and ensure that treatments going to clinical trial have a greater likelihood of success.

1-5 Mechanisms of Recovery and Rehabilitation

There are several periods during which recovery can be targeted: 1) prior to injury, 2) the acute phase and 3) the post-acute phase. Interventions have been used at each of these stages to augment recovery. Understanding the processes through which outcome is

enhanced at each stage will lead to new and superior strategies for stroke rehabilitation.

Rehabilitation is technically a post-injury intervention, but much can be learned from studying treatments that reduce stroke severity. In rats, exercise prior to temporary and permanent models of middle cerebral artery occlusion reduces lesion volume (82, 135). These benefits are thought to be due to enhanced levels of neurotrophic factors such as brain derived neurotrophic factor (BDNF), and insulin-like growth factor-1 (136, 137). Exercise also induces angiogenesis (138, 139), and can have antithrombotic actions, reducing blood viscosity (140), fibrinogen levels (141), platelet aggregability (142) and enhancing fibrinolysis (143). Although these circulatory changes are beneficial for ischemic injury they have the potential to worsen ICH.

Environmental enrichment, prior to or following ischemia, also reduces injury (83, 135). Environmental enrichment typically refers to social housing in a large cage with a variety of objects, which are frequently changed, and may often include access to a running wheel. It is likely a combination of factors that produces EE's effects as no single variable (socialization or general activity) can account for all benefits (144, 145). Enhanced neurotrophin expression (83, 146), which plays an important role in plasticity and neuroprotection (147), is thought to be a major contributor. Understanding why some treatments reduce brain injury

provides a picture of how to achieve a healthy brain and will allow us to target recovery in novel ways.

It is difficult to prevent or decrease the amount of brain injury, as demonstrated by the fact that most neuroprotectants have failed in the clinic (129). It is harder to use rehabilitation to produce neuroprotection, because no physical therapy can start before the patient is medically stable and able to participate. It is more likely that understanding the mechanisms underlying neuroprotection could lead to the development of pharmacological treatments, which can be given early. Rehabilitation is often started as soon as patients are stable, and is increased gradually as ability improves; but many of the interventions that provide neuroprotection in animal models of stroke (84, 148, 149) involve an intense level of early activity that is unobtainable by the majority of patients.

Rehabilitation is most beneficial during the early post acute period (62, 64). The mechanisms responsible for recovery during this period likely play a critical role in determining post-stroke outcome. Spontaneous recovery, which is thought to be due to the resolution edema and return of circulation to the penumbra (150), accounts for some of the behavioural improvements that occur during this period. Dramatic spontaneous recovery occurs after ICH where the resolution of the clot and edema reduces compression of uninjured brain areas (37). However, spontaneous recovery does not account for all the functional changes that

occur during this period. Animal and human studies have indicated that changes in the brain's functional organization occur (151-153).

Reorganization of the affected hemisphere following stroke contributes substantially to recovery. The brain regions connected to the injury have altered output to and input from the damaged area (154), which will result in reorganization; these changes are associated with increased dendritic arborisation and spine density (155). For example, damage to forelimb motor cortex will cause rearrangement of the peri-injury cortex, hand representations will move into areas that previously controlled the elbow and shoulder (153). The greater the damage to similar intracortical pathways the more secondary areas will be recruited (156). Large cortical lesions, which demonstrate a much slower rate of recovery, cause reorganization in more distant structures including the contralateral hemisphere. This type of brain reorganization has been confirmed in humans.

After damage to primary motor cortex patients have increased activity in the premotor cortex (157). Cramer and colleagues demonstrated that the degree of motor recovery is related to the level of activity in the primary and premotor cortex of the injury hemisphere. Patients with the poorest outcome had large injuries with no intact premotor cortex, which resulted in activation of the uninjured hemisphere (158). Similar reorganization occurs following subcortical strokes (159), where damage to the corticospinal system results in the recruitment of associated cortical

areas, if these connections remain intact. These findings indicate the importance of post-stroke cortical plasticity in stroke recovery, and also confirm that the size of lesion will determine the degree and location of change. Small lesions affect related brain areas and show the best recovery, whereas large lesions affect distant less connected brain areas and have poor outcome.

There are several mechanisms contributing to post-stroke plasticity. However, determining causation is difficult as the many mechanisms involved interact in a complex manner and can be altered by several factors such as lesion size, age, previous experience and time after injury (88). Neurotrophins, such as BDNF, are often upregulated in cases of enhanced plasticity, and likely contribute to recovery (147). Developing a greater understanding of the pathways and molecules involved in brain plasticity will provide a new understanding of therapeutic strategies used to promote post stroke recovery.

1-6 Rehabilitation for ICH: Potential Therapies and Mechanisms

Many therapies are used to promote stroke recovery (160). However, the efficacy of these therapies for hemorrhagic patients is rarely considered. Most clinical trials either focus entirely on ischemic stroke excluding hemorrhage or they include a small subset of patients but fail to independently analyze their outcomes. Given the differences in pathophysiology between ischemic and hemorrhagic injury, for example

location and mechanisms of cell death (3, 4), it is important to test treatments in both, as it is possible that a therapy used for one will not work in the other. For example, exercise has been shown to increase antithrombotic factors in blood (86), which may help ischemic injury (85) but may be detrimental for ICH. Location and size of injury are important, but most pre-clinical work uses models of small cortical ischemic stroke. It is unlikely that large striatal injuries will respond in the same way. Identifying treatments and the processes that reduce injury after striatal injury will help a large number of stroke patients. Little is know about the efficacy of rehabilitation for ICH. Rehabilitation may not work as well or by the same mechanism as it does in ischemia, and it is possible that we may be able to uniquely target ICH.

The goals of my thesis are to identify the potential benefits of exercise and amphetamine treatments for hemorrhagic stroke. These treatments have been frequently assessed after ischemia but little is known about their effects on ICH. Secondly, after identifying a therapy that improves outcome after ICH, I want to determine the role of peri-lesion and cortical plasticity on functional recovery. These studies use the collagenase model of ICH (101), which causes active bleeding into the brain, making it an ideal model for assessing therapies, such as exercise, which may alter the bleeding profile (86). Collagenase induced ICH results in long-term functional deficits, which can be assessed at multiple time points to confirm treatment efficacy (116). Multiple behavioural tests were

used to maximize the probability of detecting treatment effects (116). In all cases skilled reaching was assessed with the Montoya staircase test and in some instances also with the tray-reaching task. These tests are sensitive measures of improvement although they don't detect differences in true recovery vs. compensation as does the single pellet test (161). However, single pellet testing takes significantly longer to train animals, test them, and then analyze the collected data. These studies focus on the importance of understanding rehabilitation and plasticity after ICH. Hemorrhagic stroke can be severe and functionally devastating (33). With better knowledge of the mechanisms involved in the hemorrhagic recovery it will be possible to develop strategies for improving outcome in some of the most impaired and previously neglected stroke patients.

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Chapter 2:

Forced exercise does not improve recovery after hemorrhagic stroke in rats.

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2-1 Introduction

Stroke is one of the leading causes of death and disability in North America. Cerebral bleeding, including intracerebral hemorrhage (ICH), accounts for approximately 15% of all strokes and frequently causes severe disability or death (1). Most experimental and clinical stroke studies focus on finding effective hemostatic or cytoprotective (neuroprotective) therapies. Furthermore, most studies target cerebral ischemia and not hemorrhagic stroke. Given the differences in pathophysiology (2, 3), such as the nature of cell death and the extent and location of injury, it is important to test therapies in ICH models as it is possible that treatments that work in ischemia may fail in ICH. This is not only an issue with cytoprotectants, but rehabilitation therapies may also differ in efficacy between ischemic and hemorrhagic events.

Various rehabilitation interventions promote recovery after ischemic stroke in rats (e.g., environmental enrichment). Interestingly, even simple exercise (EX) treatments such as forced running promote recovery after ischemic stroke, and they also reduce cell death in some situations. For example, Ding and colleagues (4) found that forced EX prior to temporary middle cerebral artery occlusion (MCAO) reduced infarct volume and promoted neurological recovery, which was associated with increased expression of brain derived neurotrophic factor (BDNF) and nerve growth factor. In another study, pre-stroke forced EX reduced edema and lesion volume measured at 24 hr after temporary MCAO (5). Reductions in lesion

volume and improved behavioural recovery also occur when EX is started following ischemia. For instance, forced EX initiated within 24 hr of temporary MCAO reduced functional deficits and lesion volume (6).

Rehabilitation also benefits rats suffering an ICH. DeBow and colleagues (7) showed that constraint-induced movement therapy (CIMT) initiated one week after ICH improved behavioural recovery. A reduction in the total volume of tissue lost also occurred. The CIMT, which lasted 7 days, included a combination of ipsilateral limb restraint (8 hr / day) combined with 1 hr per day of EX (e.g., skilled reaching training, walking). The modest EX treatment alone did not improve outcome. However, others have shown that greater amounts of forced EX (running) starting 1 day following ICH reduces lesion volume, caspase-3 expression, and the number of degenerating cells (8, 9). This raises the possibility that greater amounts of EX may improve recovery and reduce injury when given after a more clinically realistic treatment delay (e.g., 2 weeks).

In this study, we examined the effects of forced EX on outcome following ICH in rats. The collagenase model of ICH, developed by Rosenberg and colleagues (10), was used because it results in consistent hemorrhaging within the striatum and a well-characterized behavioural deficits (11). Forced EX, via motorized running wheels, was used because of its demonstrated efficacy in ischemia models and because an exact amount of EX can be easily administered. Effective pre-clinical testing requires a comprehensive assessment of long-term recovery and not just

use of short-term endpoints (12). Thus, we used a 7-week survival time and gauged skilled reaching, spontaneous paw usage, and walking ability, the staircase, forelimb asymmetry, and horizontal ladder tests, respectively, which are all sensitive to striatal ICH (11, 13). In our first experiment we examined the effects of EX given before and/or after ICH. We hypothesized that EX either before or following ICH would be beneficial, and expected the combination to be superior. The second experiment doubled the amount of daily EX given both before and following ICH. We hypothesized that this EX regimen would lead to greater benefit. In experiment 3 we reduced the lesion size to determine the effects of EX given both before and after ICH as in Experiment 1. We anticipated somewhat greater effects in treating this smaller lesion than that found in Experiment 1. Lastly, in Experiment 4 we quantified hemorrhage volume at 12 hours after ICH to determine if forced EX prior to ICH aggravated bleeding. Given the known angiogenic effects of EX (14-16), we predicted that pre-ICH EX treatment might aggravate bleeding and thereby counteract beneficial effects of EX therapy.

2-2 Methods

2-2.1 Animals

One hundred and eighty-five male, young adult Long-Evans rats (Charles River, Montreal, Quebec, Canada) weighing between 250 and 350 g at the start of the experiment were entered into the experiments.

Ten of these animals were excluded due to technical errors (e.g., spectrophotometric assay processing mistake). The animals were individually housed with food and water available ad libitum. All procedures were in accordance with the Canadian Council on Animal Care guidelines and were approved by the Biological Sciences Animal Policy and Welfare Committee at the University of Alberta.

In the first experiment (N = 79; Figure 1) animals successfully meeting the criterion for staircase training (see below) were randomly placed into one of four conditions: EX prior to and following ICH (PRE&POST-1; n = 20), EX only prior to ICH (PRE-1; n = 16), EX only following ICH (POST-1; n = 17), and a group receiving no EX (CONT-1; n = 18). Eight additional rats were excluded based on poor staircase training performance. The second experiment (N = 36) used a greater amount of EX each day and compared rats receiving EX prior to and following ICH (PRE&POST-2; n = 12) with those not receiving EX (CONT-2; n = 10). Fourteen additional rats failed to meet criterion for staircase training and did not continue in the study. In the third experiment (N = 39) rats received a milder ICH insult. We compared rats given EX, which was identical to Experiment 1 (PRE&POST-3; n = 19), to those that received no EX (CONT-3; n = 20). In an attempt to reduce the number of animals used, rats failing to meet exclusion criterion for staircase continued on in the study and had their staircase data excluded from analysis. In the fourth experiment (N = 21) we assessed hemoglobin content 12 hr after ICH in

three groups of rats. One group received an ICH without any behavioural training or EX therapy (ICH-4, n = 7). The second group were trained behaviourally (e.g., staircase test) prior to ICH but did not get EX therapy (CONT-4, n = 8). The last group were trained behaviourally and subjected to EX (PRE-4, n = 6) identical to the Experiment 1.

2-2.2 Behaviour Training

Montoya Staircase Skilled Reach Training

Three days prior to the beginning of staircase training all animals were food deprived to 90% of their free feeding weight adjusted for natural increases in weight with age. This task measures the skilled reaching ability of each forelimb (17). The rats were trained to reach for food reward pellets (45 mg each; Bio-Serv, Frenchtown, NJ, USA). Each rat received two 15 minute trials a day separated by 3 - 5 hr, five days a week, for three weeks. To successfully complete training an average of 9 of the 21 pellets for each limb must be obtained for 6 consecutive trials.

Horizontal Ladder Walking Test

On the last two days of staircase training (i.e., days 3 and 4 prior to EX), each rat was given three consecutive trials on a 1 m long horizontal ladder with randomly spaced bars (1 – 3 cm apart). The trials occurring on the second day of training were videotaped and the number of errors (limb

slips through the ladder) made with each limb while traversing the middle 0.5 m section were determined (11, 18).

Spontaneous Forelimb Use Asymmetry (Cylinder) Test

On the last two days of staircase training, each rat was placed in a transparent cylinder (height: 45 cm, diameter: 33 cm) and allowed to explore spontaneously for 5 minutes on each training day. The second cylinder session was video recorded and analyzed for the number of forelimb placements on the wall made with each paw. The percent contralateral paw use was calculated as (contralateral forelimb touches / (ipsilateral forelimb touches + contralateral forelimb touches)) × 100, according to established methods (11, 19).

2-2.3 Pre-ICH EX

On days 3 and 4 before EX training, all animals were habituated to the motorized wheel (35 cm in diameter; 10 cm wide, bars 1 cm apart) for 15-minutes at a speed of 2.2 m/min.

Training consisted of a 1 hr daily session conducted 5 days a week for 2 weeks. The speed was 5.5 m/min for the first 5 minutes and then 11 m/min for the remaining 55 minutes. Control animals were placed in a stationary wheel in otherwise identical conditions (e.g., noise, location, etc.). In the second experiment EX-treated rats ran at the same intensity,

but for two 1-hour EX sessions per day. These sessions were separated by 3 – 5 hr. All animals were weighed daily during the EX training.

2-2.4 ICH Surgery

Rats were subjected to an ICH two days after finishing the last EX session or at the equivalent time in non-EX treated groups. The rats were anesthetized with isoflurane (4% induction; 1.5% - 2% maintenance in 70% N_2O, 30% O_2) and placed in a stereotaxic frame. A rectal temperature probe and electric heating pad were used throughout anesthesia to maintain body temperature at ~37°C. Using aseptic technique a midline scalp incision was made and a small hole was drilled 3.5 mm lateral to Bregma, contralateral to the preferred paw (as determined by staircase testing) and at the anteroposterior level of Bregma. One microliter of sterile saline containing 0.2 U of collagenase (Type IV-S; Sigma, Oakville, ON, Canada) was injected into the striatum creating a severe ICH (11). The needle was first lowered 7 mm below the skull and 0.5 µL of saline was injected over 5 minutes. The needle was left undisturbed for 5 minutes and was then raised to a depth of 5.5 mm (i.e., up 1.5 mm), where a second 0.5 µL injection was made over 5 minutes. Five minutes following this infusion the needle was removed and a metal screw (model MX-080-2; Small Parts, Miami Lakes, FL, USA) was used to seal the hole. The scalp was treated with a local anesthetic (Marcaine; Sanofi Canada, Markham, Ontario, Canada) and the wound was closed

with staples and covered with antibiotic ointment. The third and fourth experiments used a single 0.7 µl (0.14 U) injection of collagenase to create a less severe ICH (10, 20). The injection was given at then anteroposterior level of Bregma, 3.5 mm lateral and to a depth of 5.5 mm.

2-2.5 Post-ICH EX

Two weeks after ICH surgery half of the rats began post surgery EX training. Due to the severe motor impairment caused by the ICH insult, the intensity of this EX treatment was reduced compared to pre-ICH EX treatment. The duration of training was the same as the pre-ICH EX, with animals being placed in the motorized wheel for a single 1-hr training session per day five days a week for two weeks (Experiment 2 used two 1-hr sessions). For the first five days the intensity was maintained at a pace of 5.5 m/min for the entire EX session, for the remaining five days the pace was increased to 11 m/min after the initial 5-minutes at 5.5 m/min.

2-2.6 Behavioural Testing

Rats were food deprived to 90% of their free feeding weight 4 days prior to staircase testing. The staircase test was used to assess skilled reaching on days 42 – 46 post ICH (two 15 minute trials per day separated by 3 – 5 hr). The results are expressed as a percent of baseline (average of last 3 training days). Each animal was assessed on the horizontal ladder (3 crosses) and forelimb asymmetry tests (one 5 minute session) at

46 days after ICH. Rats in Experiment 3 were also tested on the forelimb asymmetry and horizontal ladder tests 11 days after ICH.

2-2.7 Histology

Rats in Experiments 1 - 3 were euthanized 7 weeks following ICH surgery with an overdose of sodium pentobarbital (80 mg/kg i.p.; Somnotol; MTC Pharmaceuticals, Cambridge, ON, Canada). They were perfused with saline followed by 10% formalin. Forty-micrometer coronal brain sections were taken every 600 μm with a cryostat and then stained with cresyl violet. The volume of tissue lost was assessed using Scion Image J 4.0 (Scion Corporation, Frederick, MD, U.S.A.) and was calculated by subtracting the remaining volume of injured hemisphere from the remaining volume of normal hemisphere. The volume of each hemisphere was calculated as: (average area of complete coronal section of the hemisphere – area of damage – area of ventricle) × interval between sections × number of sections (11).

2-2.8 Intracerebral Blood Volume Analysis (Experiment 4)

The volume of blood released into the brain 12 hr after collagenase infusion was assessed using a spectrophotometric hemoglobin assay (20, 21). Three groups were compared: CONT-4, PRE-4 and ICH-4. Twelve hours following the induction of ICH rats were deeply anesthetized with 4% isoflurane and decapitated. The brain was extracted and the olfactory

bulbs and cerebellum were discarded. The brain was homogenized (Model 398; Bio-Spect, Racine, WI, USA) in a test tube containing distilled water (total volume 3 mL). This solution was centrifuged (15800 g for 30 minutes; model OM3590; Thermo Electron Corporation, Waltham, MA, USA) and 4 aliquots of supernatant (100 µL each) were reacted with Drabkin's reagent (400 µL; Sigma, Oakville, ON, Canada) for 15 minutes. The absorbance was measured using a spectrophotometer (model RS232; Thermo Electron Corporation, Waltham, MA, USA). The readings from the four samples were averaged. Blood volumes were determined from a previously calculated curve that used known blood volumes (22).

2-2.9 Statistical Analysis

Results are presented as mean ± standard error of the mean (SEM). Composite behavioural scores were created as an index of overall performance by ranking all animals (best to worst) on each test and taking the average, which was analyzed with ANOVA (11). The rest of the data were analyzed with multiple factor ANOVA in the first study, and single factor ANOVA in the remaining experiments. In all cases Scheffé post hoc test was used if needed. Mortality was analyzed with Chi-Square (SPSS 11.0; SPSS Inc., Chicago, IL, U.S.A.).

2-3 Results

2-3.1 Weight Data

In all experiments body weight was similar among all groups at the beginning of the experiment, on the first and last day of PRE and POST EX treatments, surgery, post surgery, and euthanasia ($p \geq 0.066$).

2-3.2 Experiment 1

No animals in the POST-1 group died, whereas 1 in the CONT-1 group, 4 animals in the PRE&POST-1 group, and 1 animal from the PRE-1 group died following surgery ($p = 0.095$). The cause of death was presumed to be due to insult severity; however, this was not verified.

The lesion volumes at 7 weeks after ICH are given in Figure 2a. Injection of collagenase caused significant tissue loss in the striatum as well as damage to thalamus, globus pallidus and the corpus callosum (Figure 2d). The main effects (PRE: $p = 0.412$; POST: $p = 0.212$) and interaction ($p = 0.419$) were not significant.

All groups performed similarly during staircase training (data not shown). There was a significant Day effect ($p = 0.001$; Figure 3a) because all groups improved over time (contralateral limb reaching success). However, the PRE ($p = 0.820$) and POST ($p = 0.440$) main effects and interaction ($p = 0.959$) were not significant. Likewise, the main effects (PRE: $p = 0.586$; POST: $p = 0.391$) and interaction ($p = 0.136$) were not significant for the contralateral forelimb slip rate in the ladder test. Analysis

of contralateral limb use in the asymmetry test revealed a significant POST main effect ($p = 0.039$; Figure 5a) and a significant PRE×POST interaction ($p = 0.033$), but the PRE factor was not significant ($p = 0.075$). However, Scheffé post-hoc tests revealed no significant differences between any of the groups ($p \geq 0.065$) in a one factor ANOVA. The composite score revealed that neither the main effects (PRE: $p = 0.811$; POST: $p = 0.178$) nor interaction ($p = 0.868$) were significant. The CONT-1, PRE-1, POST-1 and PRE&POST-1 groups had mean composite scores (lower reflects better performance) of 35.1 ± 12.7, 34.9 ± 11.7, 31.6 ± 12.6, and 30.5 ± 8.6, respectively.

2-3.3 Experiment 2

Two animals in the PRE&POST-2 group died after ICH whereas no animal died in the CONT-2 group ($p = 0.176$). Mortality was assumed to be due to the ICH.

Histological data for one animal in the CONT-2 group was lost before analysis. The one factor ANOVA revealed no significant difference in lesion size between the PRE&POST-2 and CONT-2 groups ($p = 0.465$; Figure 2b).

There was no significant difference in contralateral reaching ability between the two groups ($p = 0.052$), and Day effect was not significant ($p = 0.090$; Figure 3b). As shown in Figure 4b, the PRE&POST-2 group made significantly more foot slips with their contralateral forelimb than the

CONT-2 group ($p = 0.032$). Analysis of limb use asymmetry revealed no significant difference between the groups ($p = 0.395$; Figure 5b). There was a significant difference in the composite behavioural scores between the PRE&POST-2 and CONT-2 groups ($p = 0.010$), with mean rank scores of 12.6 ± 1.0 and 8.4 ± 1.0, respectively. Thus, the PRE&POST-2 group preformed worse on average.

2-3.4 Experiment 3

One animal in the CONT-3 group did not survive ICH surgery; no other animals died ($p = 0.356$). This death was probably due to anesthesia.

The lesion volumes at 7 weeks post ICH were not different between the PRE&POST-3 and CONT-3 groups ($p = 0.179$; Figure 2c).

In an attempt to reduce the number of animals used, rats that failed to reach criterion performance were excluded from the staircase analysis and remained in the rest of the study. Ten animals were excluded from the CONT-3 and 6 were excluded from the PRE&POST-3 leaving group sizes for skilled reaching of 9 and 13 animals, respectively. There was no significant difference between the groups in contralateral forelimb reaching ability ($p = 0.700$; Figure 3c). Reaching improved over trials as revealed in the significant Day effect ($p < 0.001$). The horizontal ladder test showed no difference between the two groups in contralateral forelimb slip rate ($p = 0.076$; Figure 4c). The Day effect for slip rate in the ladder was significant

($p = 0.021$). In the forelimb asymmetry test animals performed significantly better at week 7 as compared to week 2 ($p = 0.008$). However, there was no difference between the PRE&POST-3 and CONT-3 groups ($p = 0.722$; Figure 5c). The composite score was based upon the mean rank from the final test point in limb-use asymmetry and horizontal ladder and average staircase performance for all 5 testing days. The CONT-3 group had an overall mean rank of 18.8 ± 1.6 and the PRE&POST-3 group had a mean rank of 16.7 ± 1.7 ($p = 0.363$).

2-3.5 Experiment 4

The volumes of cerebral blood at 12 hr after ICH were 40.9 ± 5.5 µl in the ICH-4 group, 46.1 µl ± 4.7 in the CONT-4 group, and 47.7 µl ± 3.9 in the PRE-4 group ($p = 0.606$). Thus, giving behavioural training and/or EX prior to ICH does not apparently affect bleeding after collagenase infusion.

2-4 Discussion

Our primary finding is that EX (running) does not improve outcome after ICH in rats. The first experiment showed that a moderate amount of forced EX did not improve recovery when administered either before and/or after ICH. The second experiment showed that doubling the EX not only failed to improve recovery, but actually impaired it. The third experiment suggests that this failure was not simply due to insult severity because forced EX did not improve recovery after a somewhat milder

insult. Finally, the last experiment showed that a moderate amount of pre-stroke EX did not affect bleeding volume. Accordingly, and in contrast to ischemia studies that report improvements, our forced EX regimen does not improve recovery nor affect lesion size after ICH.

The timing, duration, intensity and type of EX (e.g., voluntary vs. forced) are all critical factors modulating the efficacy of EX rehabilitation (23). Thus, while our results show that our EX regimen does not benefit ICH, alternative forced EX regimens may be of therapeutic value. For instance, an earlier intervention may have improved recovery in our model as others have shown that EX starting 24 hours after ICH reduced lesion volume (8, 9). In the current study, the injury may simply have matured by the time we intervened with the 2-week delayed EX treatment. Comparably, EX started within 24 hours following MCAO reduced lesion volume, whereas the same intervention started one week later did not (6). We delayed treatment for two weeks because rats receiving a severe ICH are usually too impaired to run much earlier (unpublished observation), plus the delay better reflects the clinical situation. Interestingly, other therapies such as CIMT (7, 24) and skilled reach training (25) can improve recovery when delayed a week or more after a stroke in rats. Modifying the duration or intensity of EX training may also have altered the outcome. For instance, two weeks of pre-stroke EX benefits a temporary MCAO insult (5), whereas a 12 week treatment, and not shorter durations, was required prior to permanent MCAO to reduce lesion volume (26). Similarly,

one may argue that extending the length of EX training following ICH may have improved outcome. However, several studies show that one week of EX is effective in reducing lesion and functional deficits following ischemia (6), and two studies show decreased lesion volume with 10 days of EX following ICH (8, 9). It is difficult to compare intensities of EX treatments across studies because of differences in duration, running rate and method of running (e.g., wheel vs. treadmill). We used one or two 60-minute EX sessions per day at a maximum speed of 11 m/min. While several studies have used much higher amounts (5, 6, 26) the rate we used is in line with other studies (8, 9) and was appropriate for the ICH rats; that is, it could be maintained for 1 hr sessions, and did not appear overly stressful.

Besides differences in EX regimens, it should be noted that there are key differences in pathophysiology between ischemic and hemorrhagic insults (e.g., amount of inflammation (2)) that may contribute to the ineffectiveness of forced EX, or any therapy, after ICH. Location and severity of damage also confound comparisons among studies. These issues along with potential differences in EX-induced recovery mechanisms highlight the need to investigate therapies specifically in ICH models. Notably, EX increases blood vessel density in motor cortex, cerebellum, and striatum (4, 14-16). Exercise also up-regulates endothelial nitric oxide synthase (eNOS) (27), and affects the levels of eicosanoids, reducing pro-coagulation factors (i.e., thromboxane) as well as increasing

anti-coagulation factors (i.e., prostacyclin) (28). Such effects may work to lessen the impact of ischemia by improving reperfusion whereas they could conceivably aggravate hemorrhagic insults. Presently, we found that EX did not aggravate bleeding at 12 hr after ICH surgery. The fact that lesion size was not significantly different among groups in each experiment supports this finding as exaggerated bleeding should aggravate lesion size. Likewise, higher mortality might occur in EX groups; however, mortality was not significantly different among groups in any of the 4 experiments. Nonetheless, greater amounts of EX prior to collagenase-induced ICH may worsen outcome through such a mechanism. Experiment 2 shows that greater amounts of forced EX can worsen functional outcome. In this case the volume of tissue lost was not affected; however, this does not exclude the possibility that some cell death or the rate of cell death was affected.

Undoubtedly, several factors contribute to the beneficial effects of EX on recovery after ischemia. One leading candidate is BDNF, which promotes synaptic plasticity and neuronal survival (29), and is elevated following EX (4, 30, 31). Elevations in BDNF after ICH and EX have not been studied, so it is possible that our treatment did not sufficiently affect BDNF levels. However, EX regimens less strenuous than ours have been shown to elevate BDNF levels (31). Another possibility is that the beneficial effects of forced EX were counteracted by deleterious factors (e.g., corticosterone) that might have a greater influence on ICH than

ischemia. Both voluntary and forced EX increases serum corticosterone levels (31), which on its own reduces the amount of BDNF mRNA in the hippocampus and dentate gyrus (32) and aggravates ischemic stroke (33, 34). More extensive forced EX, as used in our second experiment, may thus worsen outcome through such a mechanism. Clearly, further study is needed to understand the relationship, if any, between ICH, forced EX, corticosterone, and recovery.

Our study cannot exclude the possibility that cell death was affected by the EX treatments. A reduction in cell death, while possible, was either insufficient to influence the total volume of tissue lost at 7 weeks post-ICH or occurred transiently and went undetected. We did not assess cell death or lesion size at earlier times as long-term outcome is the more important clinical endpoint. Neuronal death around the 7 week survival time was not assessed because injury is expected to mature well in advance of this euthanasia time (35). Likewise, one could argue that behavioural improvements (treatment effects) occurred and went undetected in these experiments. We used three tests previously shown to be sensitive to ICH-induced striatal injury (11) and treatment effects (7, 22). Nonetheless, other behavioural tests might have detected improvements. Furthermore, earlier testing (e.g., week 4 post-ICH) may have detected transient benefit. The use of more tests and repeated testing was avoided as it can influence recovery by acting as rehabilitation and/or interacting with the EX treatment.

In summary, we assessed the effects of forced EX on long-term outcome after ICH. Exercise before and/or starting two weeks following ICH did not improve outcome or reduce lesion size. Furthermore, 2 h/day of EX before and after ICH worsened functional outcome, indicating that one should not assume more is better. The present findings, and that of our previous studies with CIMT (7, 24), clearly indicate that rehabilitation efforts for ICH are not only far from perfect, but they are inferior to those for cerebral ischemia. Further efficacy and mechanistic studies targeting rehabilitation for ICH are needed.

Figure 2-1: Timeline of procedures for Experiment 1 (days are given relative to ICH surgery). Similar training and testing procedures were used in Experiments 2 and 3 (see Methods for details).

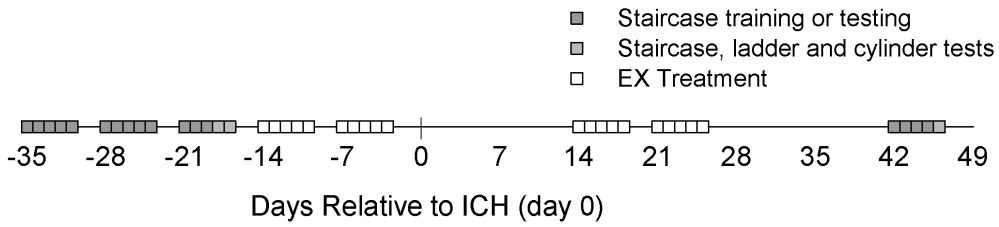


Figure 2-2: Volume of tissue lost ($mm^3 \pm$ SEM) 7 weeks after ICH. There are no significant differences between any of the treatments in Experiments 1 (A), 2 (B), or 3 (C). The photomicrograph (D) is representative of the lesion for Experiment 1 and 2.

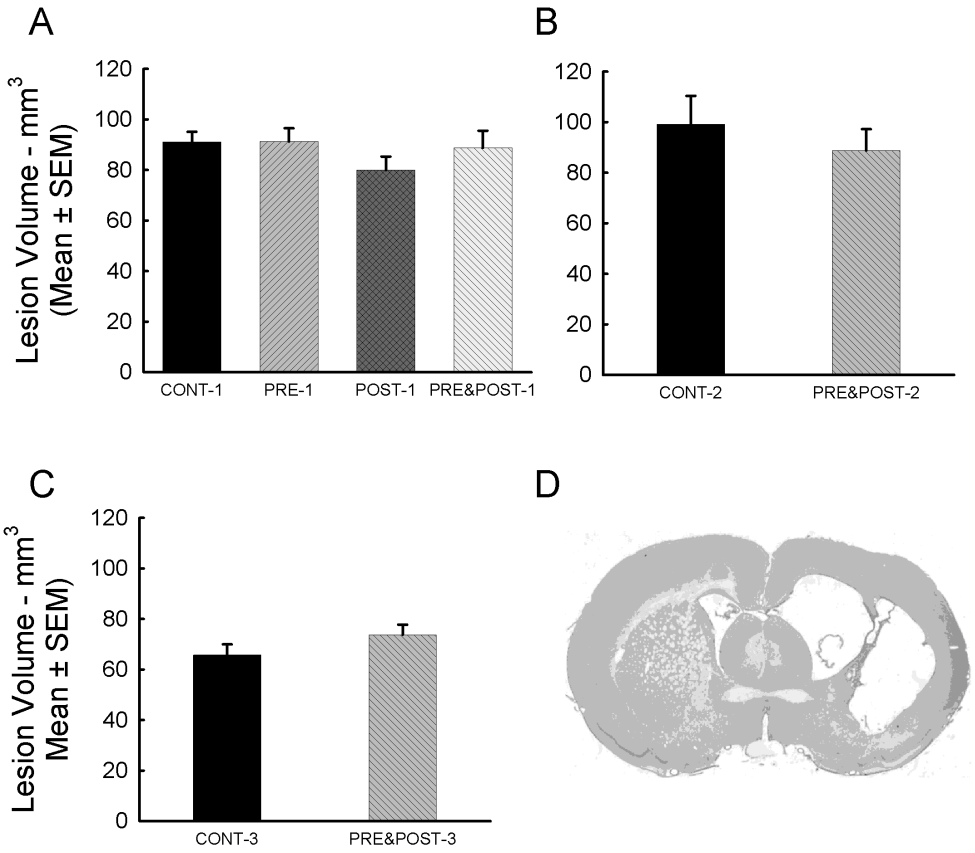


Figure 2-3: Successful skilled reaching on days 42 - 46 after ICH as a % baseline for the contralateral to stroke limb. Rats in Experiment 1 and 3 showed an improvement in contralateral reaching ability over days. There were no significant group differences in any experiment.

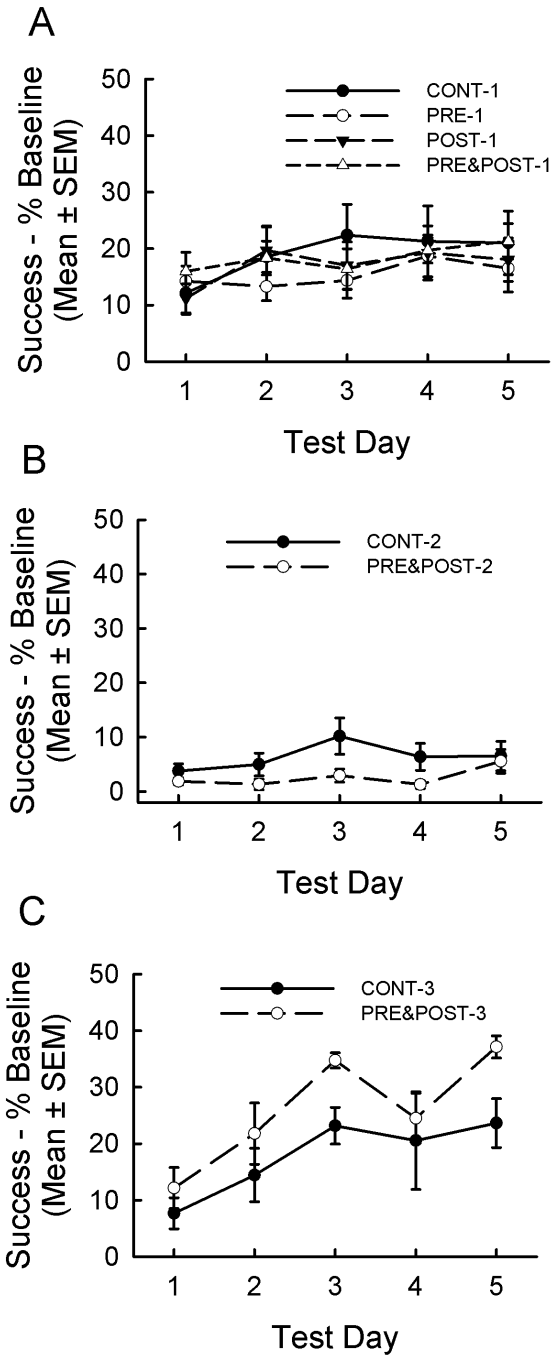


Figure 2-4: Contralateral forelimb slip rate (% slips through bars; mean ± SEM) on the horizontal ladder. There were no significant group differences in Experiment 1 (A) at 46 days after ICH. In Experiment 2 (day 46 after ICH), EX significantly increased slip rate (B). At 2 and 7 weeks, there were no group differences in Experiment 3 (C).

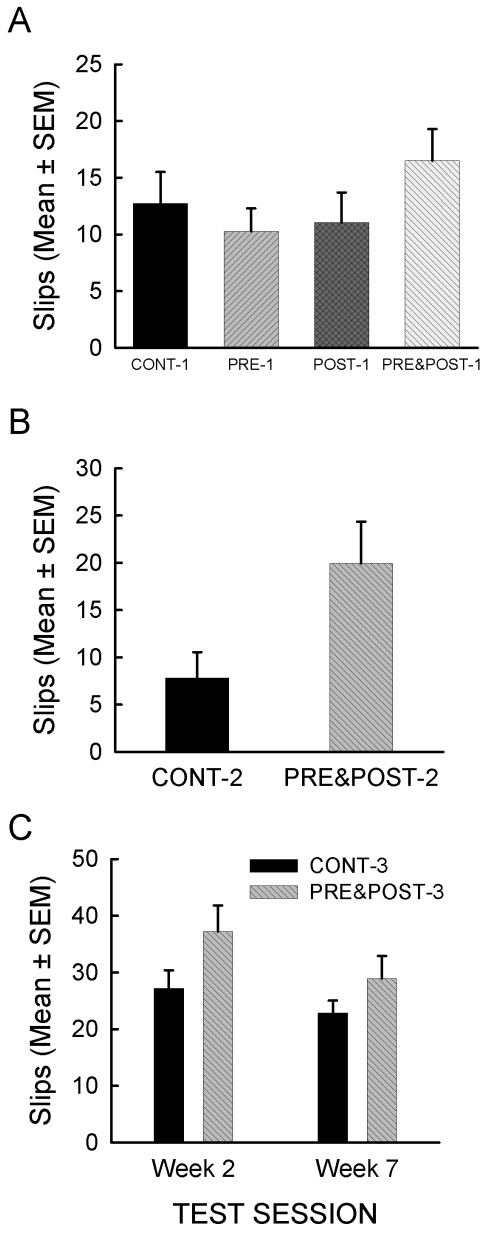
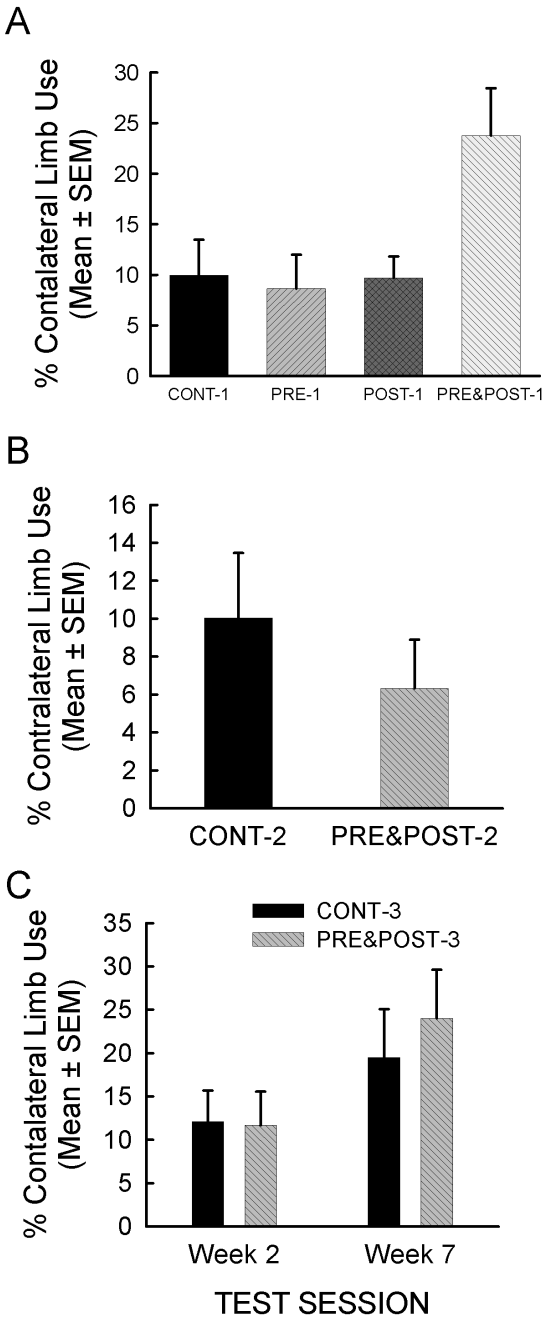


Figure 2-5: Forelimb asymmetry expressed as a % contralateral limb use (mean ± SEM). There were no group differences at 46 days after ICH in Experiment 1 (A) or 2 (B). There was also no difference in Experiment 3 (C) at 2 and 7 weeks after ICH.



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Chapter 3:

Influence of amphetamine on recovery after hemorrhagic stroke in rat.

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3-1 Introduction

d-Amphetamine (AMP) coupled with physical activity promotes sensorimotor recovery after brain injury in rodents (1-4), whereas AMP alone is not considered an effective treatment. Feeney and colleagues have shown that AMP treatment paired with training for traversing a narrow elevated beam significantly improves beam-walking skill after a mechanical insult to the motor cortex. Conversely, a single AMP injection without concomitant training on the beam provided no benefit (1). Furthermore, recent studies using clinically-relevant ischemic insults to the motor cortex show improvements with AMP treatment (3, 4). Nonetheless, clinical trials evaluating AMP have yielded mixed results; some report significant improvements (5, 6), whereas others find none (7, 8). Perhaps the discrepancies are due to patient selection and the fact that clinical studies have been small. Accordingly, further animal experimentation should identify those factors that influence AMP's efficacy (e.g., type and location of stroke) to increase the likelihood of finding benefit in defined clinical stroke populations.

Approximately 15% of all strokes are hemorrhagic and with these significant basal ganglia damage commonly occurs (9). Survivors are often left with long-term disabilities due to permanent functional impairment (e.g., hemiplegia) (10). It is possible that AMP might facilitate recovery in these patients. Given that the pathophysiology of intracerebral hemorrhage (ICH) differs substantially from ischemic and traumatic brain

injury (11), it is likely that the mechanisms of recovery and the response to rehabilitative treatments, such as AMP, will also differ. To date, most animal and clinical studies have focused on ischemic stroke. Studies that included hemorrhagic stroke had very few hemorrhage patients, making it difficult to determine the efficacy of treatment for these patients (6). Thus, neither pre-clinical nor clinical studies have determined whether AMP might promote recovery after ICH.

In this study we assessed whether AMP with or without rehabilitation improves performance on several behavioural tests after a striatal ICH in rats. The collagenase model of ICH, developed by Rosenberg and colleagues (12), was used because it results in consistent hemorrhaging, and a well-characterized pattern of behavioural deficits (13). Environmental enrichment (EE; multi level cages with ramps, toys, and a running wheel) with additional training on reaching and walking (tray and beam tests respectively) was used as our rehabilitation intervention. Enrichment was used because it enhances behavioural recovery in many models of brain injury (14, 15), although the effects of EE on striatal ICH are not known. We compared four groups: rats housed in EE with AMP injections (EE+AMP), those housed in EE with saline injections (EE+SAL), group housed in standard cages with AMP injections (GH+AMP), and group housed in standard cages with saline injections (GH+SAL). Rats in EE also received experience for both walking and skilled reaching (beam and tray tasks, respectively) 30 minutes after each injection of SAL or

AMP. We predicted that EE with additional rehabilitation training, henceforth simply referred to as EE, would improve recovery and that AMP treatment would provide additional benefit. Amphetamine alone (GH+AMP) was not expected to improve recovery.

3-2 Methods

3-2.1 Animals

Sixty, 7-week-old, male, Sprague-Dawley rats, obtained locally (Biological Sciences Animal Services; University of Alberta, Edmonton, Alberta, Canada), weighing approximately 250 g were entered into this study. Rats were group housed, 4 per cage, in standard plastic cages (width: 38 cm; length: 49 cm; height: 20 cm) with wood chip bedding. After being acclimatized to our animal room for 3 days they were handled daily for 4 days (5 - 10 min / day) before starting the experiment. To minimize stress that is likely to result from changing cage mates each group of 4 rats were randomly assigned to one of 2 housing groups (GH or EE). This ensured that the same rats remained housed together for the duration of the study. Rats were then randomly assigned to 1 of 2 injection groups (SAL or AMP), resulting in 4 treatment groups: GH+SAL (n = 17), GH+AMP (n = 15), EE+SAL (n = 14), EE+AMP (n = 14). Animals were given free access to food and water, except when food deprivation was needed for behavioural training and testing. All procedures were approved by the Biological Sciences Animal Policy and Welfare Committee of the

University of Alberta and were in accordance with the Canadian Council on Animal Care guidelines.

3-2.2 Behaviour Training

Montoya Staircase Skilled Reaching Test

Three days before training rats were food-deprived to 90% of their free feeding weight adjusted for natural gains in body weight over this period (13). Prior to surgery, rats were trained to reach for food reward pellets (45 mg each; Bio-Serv, Frenchtown, NJ, USA) in the staircase test (length: 30 cm; width: 6.8 cm; height: 12 cm) twice daily (15 min trials separated by 3 – 4 hr) for 5 days a week over 3 weeks (Fig 1). The test was baited with 3 pellets per stair for a total of 21 pellets per limb. This test measures forelimb reaching ability and is sensitive to striatal ICH (13, 16). Successful completion of training required that animals consume an average of at least 8 pellets per limb during the final 6 trials.

Tray Task

Rats were trained in the tray task over 14 consecutive days (30 min per day) beginning the final week of staircase training. In the tray task (Plexiglas box; width: 19 cm; height: 25 cm; length: 27 cm) the rats must reach through vertical bars (1 cm separation) to obtain food pellets (17% Layer Prostock feed; Masterfeeds, Edmonton, Alberta) placed in a shallow tray located just outside of the box (17). We recorded the first 10 minutes

of the final training day for subsequent video analysis. The numbers of successful and unsuccessful reaches were determined and the % success was calculated as: (successful / total reaches) * 100. In order for the rat to score a reach, the paw had to be inserted through the bars of the cage, and in order for the reach to be considered successful the animal had to consume some food from its paw. Rats were given free access to their regular food after completing this training.

Horizontal Ladder Walking Test

Rats were given one day of training on the ladder (4 crosses) prior to recording baseline performance. On the last day of tray task training rats were videotaped crossing the middle 0.5 m section of a 1 m long horizontal ladder with variably spaced rungs (1 – 3 cm). Several variations of rung spacing are sensitive to brain injury, including ICH (13, 18). Thus, this test, with the parameters used in this study, can detect significant error rates at up to 6 weeks following a moderate-sized ICH (19). The number of errors (complete limb slips through the rungs) made for each limb was determined over four trials. The performance on this test is expressed as the % success = # successful footsteps / (# successful footsteps + # foot slips).

Beam Walking Test

On the final day of tray task training the rats were also trained to cross an elevated beam (length: 1.1 m; width: 3.2 cm) by placing them on the beam at increasing distances from the goal box until they successfully crossed the entire beam. An additional 3 crosses were then videotaped and analyzed on an 8 point scale (1, 19). Briefly, each cross was scored as a 0 (rat fell off the beam within 10 seconds), 1 (rat remained on the beam for more than 10 seconds but could not cross), 2 (rat could not cross but was able to place affected limb on beam), 3 (rat crossed but was unable to place affected limb on beam), 4 (rat crossed beam and placed affected limb on beam at least once), 5 (rat crossed with more than 50% foot slips with the affected limb), 6 (rat crossed with fewer than 50% foot slips with affected limb), or 7 (rat crossed with 2 or less foot slips). This test is sensitive to striatal ICH (13).

Neurological Deficit Scale (NDS)

Neurological deficit scores were assessed at multiple times, once prior to surgery, and 3, 5, 14, 21 and 28 days following ICH (13, 20). The score was compiled from five components: 1) spontaneous circling rated from 0 for no circling to 3 for continuous circling when placed in a plexi glass cylinder (diameter: 33 cm); 2) bilateral forelimb grasp rated from 0 for normal to 3 for unable to grasp at all on a elevated bar (diameter: 3 mm); 3) hind limb retraction rated from 0 for immediate replacement to 3

for no retraction after limb was displaced laterally; 4) contralateral forelimb flexion rated from 0 for uniform extension of forelimbs to 2 for full wrist flexion and shoulder adduction when the rat was lifted by the base of the tail; 5) beam walking ability rated from 0 for a rat that crosses easily to a 3 for a rat unable to stay on the beam for more than 10 s (length: 1.1 m; width: 3.2 cm). Scores for each test were added for a maximum score of 14 indicating greatest impairment. This NDS is sensitive to striatal ICH (13).

3-2.3 Surgery

Rats were food deprived for 12 hours prior to surgery, which was performed aseptically under isoflurane anesthesia (4% induction; 2% maintenance in 70% N_2O and 30% O_2). Rectal temperature was maintained at ~37.0 ºC during surgery with a heating pad. Rats were anesthetized and a catheter placed in the tail artery to measure mean arterial blood pressure (MABP) and to take blood samples for measurement of blood gases, pH, glucose, and hemoglobin concentrations. Two blood samples were taken – one at the start of surgery and one following the infusion of collagenase. Measurements of MABP were recorded every 5 minutes. Heparinized saline was used to prevent clotting in the tail artery and catheter. An identical amount of heparinized saline (1 mL; 10.0 U) was infused into all rats. Rats were placed in a stereotaxic frame. A midline incision was made and a hole was

drilled at 3.5 mm lateral at the level of Bregma contralateral to the preferred paw (limb with highest average number of pellets retrieved over the last week of staircase training). A 26-gauge needle (Hamilton syringe; Hamilton, Reno, NV, U.S.A.) was lowered 5.5 mm below the surface of the skull. After waiting 5 minutes with the needle in place 0.5 µL of sterile saline containing 0.10 U of bacterial collagenase (Type IV-S; Sigma, Oakville, ON, Canada) was manually infused over 5 min into the striatum (21). Previous studies indicate that the this dose of collagenase produces moderate lesion sizes (12, 13) whereas saline injections cause only minimal injury (needle tract) (22). The needle remained undisturbed for 5 min after the injection. A metal screw (model MX-080-2; Small Parts, Miami Lakes, FL, U.S.A.) sealed the hole and Marcaine (Sanofi Canada, Markham, Ontario, Canada) was placed into the wound, which was then closed with staples.

We opted to affect the preferred paw, by lesioning the contralateral hemisphere, to allow for a more consistent level of performance and to avoid floor effects (i.e., not being able to worsen the performance of the non-preferred forelimb). Furthermore, the preferred limb in the staircase test is usually the limb that performs better on other tasks such as the ladder and tray tasks (C. MacLellan and F. Colbourne, unpublished data).

3-2.4 Amphetamine and Rehabilitation

All rats were food-deprived to 90% of their free feeding weight adjusted for normal increases in body weight over days 3 - 14 post-ICH. Starting 5 days after ICH, and lasting until euthanasia, rats in the EE conditions were placed into a cage (width: 35 cm; length: 75 cm; height: 75 cm) with 3 levels, tunnels, ramps, various toys, and a running wheel. Cages were cleaned and changed weekly, with new objects introduced. Animals in the GH conditions remained in standard group housing. Injections of 2 mg/kg d-amphetamine sulphate (i.p.; 2 mg/mL; US Pharmacopia, Rockville, Maryland, USA) or an equivalent volume of sterile saline were given on days 7, 9 and 11 following ICH. Thirty min after the injections of AMP or SAL rats in the EE conditions were given rehabilitation training consisting of 5 consecutive crosses on the elevated beam and 30 min in tray task. Following this training rats were placed back into their EE cages.

3-2.5 Behavioural Testing

Rats were videotaped on the beam (3 crosses) and ladder (4 crosses) tasks on days 5, 14, 21 and 28. Tray testing (10 min) was conducted on days 5, 14 and 24. Two days before staircase testing animals were food deprived to 90% of their free feeding weight. Rats were tested on days 21 – 23 in staircase, 2 times a day for 15 min each. On day 24 rats were tested on tray task and then returned to ad lib feeding.

Animals were only tested on skilled reaching tasks for a limited number of days in order to reduce the need for food deprivation. Additional testing was also not thought necessary in order to accurately gauge recovery. NDS testing was conducted on days 3, 5, 14, 21 and 28.

3-2.6 Histology

Rats were euthanized 4 weeks following ICH surgery with an overdose of sodium pentobarbital (80 mg/kg i.p.; Somnotol; MTC Pharmaceuticals, Cambridge, ON, Canada). They were perfused with saline followed by 10% formalin. Brains were cut into 40 μm coronal sections using a cryostat. Every fifth section was saved starting anterior to and extending to well after the end of the lesion ensuring that we analyzed locations where atrophy may occur. The tissue was stained with cresyl violet and every 600 μm a section was assessed for the extent of lesion. Volume of tissue lost was assessed using Scion Image J 4.0 (Scion Corporation, Frederick, MD, U.S.A.) and was calculated by subtracting the remaining volume of injured hemisphere from the remaining volume of normal hemisphere. The volume of each hemisphere was calculated as: (average area of complete coronal section of the hemisphere – area of damage – area of ventricle) × interval between sections × number of sections (13).

3-2.7 Statistical Analysis

Results are presented as mean ± standard error of the mean (SEM). The behavioural scores and lesion volume were analyzed as multiple factor ANOVA (SPSS 11.0; SPSS Inc., Chicago, IL, U.S.A.). In cases of a significant Levene's test for equality of error variances we used the Kruskal-Wallis test. For nonparametric data (beam and NDS scores) we used Wilcoxon Signed Ranks, Kruskal-Wallis and Mann-Whitney tests. The sum of the scores for the 3 daily trials was analyzed for the beam data. The χ^2 test was used to assess dropout rate (number of rats failing to reach with contralateral limb) in tray task. The level of significance was set at $p < 0.05$.

3-3 Results

One rat (GH+SAL) died during surgery, presumably due to anesthesia. Three other animals, one from each of the GH+SAL, GH+AMP, and EE+AMP groups, were excluded at the time of histological analysis due to surgical error. The remaining group sizes were 15, 14, 14 and 13 in GH+SAL, GH+AMP, EE+SAL, and EE+AMP groups, respectively.

3-3.1 Weight Data and Physiological Parameters

Weight data was recorded for all groups during food deprivation and the period following surgery to ensure the health of all animals. There

were no significant differences in weight among the groups at any time (data not shown).

Physiological parameters (e.g., PCO_2) were not significantly different among groups either prior to or following collagenase infusion (e.g., PCO_2: Drug main effect – $p = 0.659$; Housing main effect – $p = 0.849$; Interaction – $p = 0.529$). These parameters did not change significantly over time (e.g., PCO_2: $p = 0.617$; Table 1).

3-3.2 Lesion Volume

Inspection of coronal sections at a 30 day survival showed that ICH-induced brain injury primarily occurred within the striatum but occasionally affected other structures (e.g., globus pallidus) along with usually causing marked enlargement of the ipsilateral ventricle. This pattern was similar among group as was the lesion volume ($p = 0.474$; Figure 2a), which was analyzed by the Kruskal Wallis test owing to a significant Levene's test ($p = 0.002$). A representative lesion is illustrated in Figure 2b.

3-3.3 Behavioural Testing

All animals received a normal score (i.e., 7 on each of the 3 trials) on the elevated beam task prior to ICH. All groups were significantly impaired on day 5 post-ICH ($p < 0.001$, Figure 3) and the groups were not significantly different at this time ($p = 0.826$), which was prior to

rehabilitation treatment. However, significant group differences emerged on days 14, 21 and 28 ($p \leq 0.020$). Pairwise comparisons indicate that EE+SAL rats performed significantly better than GH+SAL rats on days 14, 21 and 28 ($p \leq 0.014$). EE+SAL rats were also significantly better than GH+AMP rats on days 14, 21 and 28 ($p \leq 0.004$). EE+AMP rats were significantly better than the GH+SAL on days 14 and 21 ($p \leq 0.020$). EE+AMP rats were also significantly better than GH+AMP rats on days 14 and 21 ($p \leq 0.003$). However, there was no significant effect of AMP as there were no significant difference between the GH+SAL vs. GH+AMP on days 14, 21 or 28 ($p \geq 0.693$) or between EE+AMP vs. EE+SAL on days 14, 21 or 28 ($p \geq 0.107$). Therefore, EE treatment improved beam walking ability, but AMP did not.

The NDS scores were not significantly different among groups at baseline (prior to ICH) as all rats received the minimum score of 0 (i.e., no impairment). Three days following ICH all groups were significantly impaired compared to baseline ($p < 0.001$). However, there were no significant group differences prior to rehabilitation treatment on day 3 or day 5 ($p \geq 0.058$) post-ICH. Significant group differences emerged on days 14 and 21 ($p \leq 0.022$), but not on day 28 ($p = 0.119$) post-ICH (Figure 4). On day 14, each EE-treated group was significantly less impaired then their respective GH control group: EE+SAL vs. GH+SAL ($p = 0.009$), and EE+AMP vs. GH+AMP ($p = 0.003$). On day 21, the EE+SAL ($p = 0.019$) and EE+AMP ($p = 0.011$) groups were significantly better than GH+SAL

rats. As it is possible that the beam sub-scale of the NDS contributes considerably to these effects, we also analyzed the NDS without the beam sub-scale. There were no significant differences between groups on days 14, 21 and 28 ($p \geq 0.070$), indicating that EE only improved NDS scores through enhancing beam performance.

Owing to significant Levene's test ($p = 0.001$) for baseline ladder data, indicating heterogeneity, we used a nonparametric test and found no significant baseline differences among groups ($p = 0.144$). The overall baseline performance for all groups was 95.2 ± 5.3 % success. Analysis of day 5 data with ANOVA revealed a significant Drug main effect ($p = 0.010$). Given the presence of a main effect prior to treatment, the following test days were expressed as % improvement (% success on treatment day - % success on day 5). This analysis of day 14, 21 and 24 data revealed a significant Day effect ($p < 0.001$; Figure 5) with all groups improving performance on the later test days. Nonetheless, performance remained significantly below baseline at all these times (Day effect: $p \leq 0.001$, Tukey post hoc each day vs. Baseline: $p < 0.001$). There was a significant main effect of Housing ($p = 0.007$), but not of Drug ($p = 0.121$), and there was no interaction ($p = 0.483$). Therefore, EE, but not AMP, improved performance on the ladder test.

Rats failing to reach baseline staircase exclusion criteria were excluded from the analysis, leaving group sizes of: 10, 10, 12 and 10 in the GH+SAL, GH+AMP, EE+SAL, and EE+AMP groups, respectively.

Performance improved over the test days for all groups (Day effect: $p = 0.006$; Figure 6), but there were no significant Drug ($p = 0.784$) or Housing ($p = 0.735$) main effects and no interaction ($p = 0.085$).

Analysis of the tray task data indicated that there were no baseline differences among the groups (Drug: $p = 0.745$; Housing: $p = 0.468$; Interaction, $p = 0.565$). Following the ICH several animals would not reach with their impaired arm so they were excluded from the tray task analysis. The dropout rates for each group were compared for each test day. The Day 5 dropout rates were 20, 36, 43 and 31 % in the GH+SAL, GH+AMP, EE+SAL and EE+AMP groups, respectively; which is not significantly different ($p = 0.449$). However, on days 14 and 24 ($p \leq 0.015$) significantly more rats in the EE groups stopped reaching with their contralateral limbs. The dropout rates were 40, 36, 71 and 69 % on day 14 and 33, 50, 71 and 77 % on day 24 in the GH+SAL, GH+AMP, EE+SAL and EE+AMP groups, respectively. Given the differences in dropout rates tray data was not analyzed further.

3-4 Discussion

This is the first study to test the separate and combined effects of EE and AMP after ICH. Environmental enrichment, which included rehabilitation exercises, improved recovery after ICH, and this was not facilitated by AMP. Furthermore, EE-facilitated recovery was incomplete and did not occur on all behavioural tests. Notably, EE enhanced walking

ability on beam and ladder tests. The improvement found with the NDS was apparently due to the beam sub-scale. Neither treatment facilitated skilled reaching in the staircase test. These results indicate that AMP does not provide significant functional benefit following striatal ICH. Accordingly, further research is required before AMP should be considered for evaluation in striatal ICH patients.

Beam training during AMP exposure was no better than training (EE) alone. However, the EE+SAL and EE+AMP groups approached a full recovery on the beam test suggesting that it may not have been sensitive enough to detect any further benefit of AMP beyond that provided by EE (i.e., ceiling effect). Accordingly, our beam data are not necessarily at odds with reports that the combination of AMP and beam training improves performance (1, 23). Interestingly, Goldstein and Davis (24) found that if non-AMP treated rats were forced to cross a beam they recovered at a rate similar to AMP treated rats. It follows then that our EE cage, which contained a beam that we frequently observed our rats using, both with and without AMP, was a sufficient treatment to allow recovery on subsequent beam tests. Thus, when sufficient training and recovery time is given the beam test will likely be insensitive to treatment effects (13). This highlights a limitation of studies using this tests as the sole indicator of functional outcome following AMP treatment (1). Although, it is possible that the beam test may be more sensitive under other conditions, such as cases were there is limited exposure to the test, or in other models of brain

injury. In contrast to the beam test, persistent deficits occurred in the ladder test indicating that this is the more sensitive measure of recovery in this study. We found that significant improvement occurred in the ladder test when rats were housed in EE, but AMP provided no benefit either alone or combined with rehabilitation. Recovery on this test may have generalized from training on other tasks, such as beam, or from the experiences the rats had in the EE cages (e.g., climbing and walking).

Amphetamine treatment for skilled reaching has produced equivocal results perhaps owing to study differences such as variations in lesion size, location and insult type, along with the dose of AMP. Two studies found benefit with AMP (≤ 2 mg/kg) and skilled reach training following cortical ischemic injury (3, 4). In contrast, Rasmussen et al. (25) using an embolic stroke model found that reach training improves performance in the staircase test, but this effect disappears with AMP treatment (3.5 mg/kg). This effect was attributed to AMP suppressing reaching indicating the importance of proper dosing and training following AMP treatment. We selected 2 mg/kg as a dose because it did not block reaching and is a commonly used dose in rehabilitation studies in rats. Presently, neither EE nor AMP improved recovery after ICH in the staircase test. We counted the number of pellets retrieved in this test; thus, there may have been performance differences among groups (e.g., % successful reaches). Unfortunately, rats often stopped reaching with their impaired limb in tray task following ICH and thus we were unable to

accurately judge reaching success. Furthermore, the switch in limb preference in tray task was significantly different among groups thereby confounding this test data.

Enrichment has been found to improve recovery in several brain injury models (14, 15, 26), including ICH as presently shown (i.e., walking). We used EE as a way to enhance rehabilitation during AMP exposure, providing abundant opportunity for exploration and exercise. Rats without AMP were repeatedly observed exploring the multiple objects in the cage. This behaviour increased with AMP treatment and some stereotypical behaviours (e.g., head bobbing) emerged that did not appear to notably impact mobility nor did they block performance on the other rehabilitation exercises. Unfortunately, behaviour was not formally quantified in the EE cages or other exercises, and thus it is not possible to determine whether the stereotypical behaviours somehow impeded rehabilitation. Additionally, it is possible that the altered activity of AMP treated rats influenced co-housed animals. However, we did not observe any abnormal interactions between animals following drug treatment. Johansson et al. (27) combined AMP treatment, at the same dose we used (2 mg/kg), with EE following middle cerebral artery occlusion in rats and found that AMP did not provide any additional benefit to EE. Our findings suggest that the inability of AMP to provide benefit to animals housed in EE may be related to the demands of the function being assessed. Finally, AMP was unable to provide additional benefit on beam

because EE animals achieved a near maximum score. However, this was not a limitation of the ladder test where AMP also failed to improve recovery.

Beneficial effects of EE are usually limited to tasks that involve body coordination and balance, such as beam, ladder and rotarod (15, 26, 28) whereas improvements on skilled reaching come from additional forced reach training of the impaired limb (29). Perhaps this is because EE naturally encourages rats to use their non-injured limb for reaching, whereas all limbs must be used for effective locomotion. This learning may be most effective when the impaired limb is used early after injury as during EE exposure. Indeed, more animals in the EE than in the GH groups stopped reaching with their contralateral limb in the tray task on test days 14 and 24. Future studies should consider whether AMP combined with more intensive and forced reach training would facilitate recovery of skilled reaching ability. For instance, constraint-induced movement therapy (CIMT), which involves restraint of the unimpaired limb to force the use of the impaired limb during exercises (e.g., tray training), improved staircase performance following striatal ICH (30). Further improvement, especially needed for severe ICH (31), may be achieved with the addition of AMP to CIMT.

The present findings do not exclude the possible use of AMP for striatal ICH; however, neither do they support the use of AMP for this type of stroke, as there was no benefit of AMP. Accordingly, alternative AMP

treatments should be tested as they may better promote recovery. First, AMP treatment was not started until 7 days after ICH and an earlier start may have improved outcome to a greater extent. However, one must question whether very early treatments have clinical relevance and there is concern about early behavioural interventions aggravating injury as found following ischemia (32). Second, the dosage of AMP may need to be varied to suit the particular rehabilitation goal (e.g., walking vs. skilled reaching). Third, more frequent treatment may provide greater benefit. Unfortunately, the number of treatment days in clinical and experimental AMP studies varies considerably with several studies reporting benefit from a single dose of AMP (1, 4, 25). Only one study, using a model of cortical injury in cats, directly compared single and multiple dosing. They found that multiple doses increase the rate of recovery. However, the single dose eventually reaches the same level of improvement (33). We chose three treatment days because this would likely provide an adequate indication of AMP's effectiveness while avoiding the problem of rats becoming habituated and refusing to complete testing trials. Finally, it is possible that additional behavioural tests might have revealed further group effects. However, not all aspects of recovery can be assessed in a single study and we used several tests thought to be sensitive to a striatal ICH lesion.

Location, size and type of injury critically modulate the extent of recovery following stroke thereby necessitating a thorough evaluation of

putative treatment in multiple stroke types. Here we report that with our selected treatment protocol AMP did not improve function after ICH in rats. Additionally, our results suggest that although EE with additional training improves performance on some tasks, while its use early after injury may further inhibit limb use for other tasks such as reaching. Robust functional benefits in animal models are likely needed if treatments are to be effective clinically. Therefore, our current findings indicate that AMP treatment may not provide substantial benefit to ICH patients.

Table 3-1: Physiological variables measured before (top) and after ICH (bottom). The hemoglobin and glucose values are from the first reading, whereas the mean arterial blood pressure (MABP) is averaged throughout surgery. The values are within normal ranges and are similar among groups and across time points.

	GH+SAL	GH+AMP	EE+SAL	EE+AMP
pH	7.41 ± 0.01	7.41 ± 0.01	7.42 ± 0.01	7.41 ± 0.01
	7.41 ± 0.01	7.41 ± 0.01	7.41 ± 0.01	7.41 ± 0.01
pCO_2, mm Hg	41.6 ± 1.3	40.3 ± 1.3	39.7 ± 1.3	40.3 ± 1.3
	40.8 ± 1.3	39.8 ± 1.5	39.7 ± 1.6	40.4 ± 1.5
pO_2, mm Hg	122.4 ± 3.94	114.9 ± 4.26	115.3 ± 3.94	115.2 ± 4.09
	120.9 ± 4.07	123.2 ± 4.40	118.3 ± 4.07	122.8 ± 4.23
Hemoglobin, g/dL	15.4 ± 0.20	15.9 ± 0.22	15.1 ± 0.20	15.5 ± 0.21
Glucose, mmol/L	8.8 ± 0.65	8.3 ± 0.70	8.7 ± 0.65	8.9 ± 0.67
MABP, mm Hg	105.3 ± 2.3	100.7 ± 2.5	96.8 ± 2.3	100.3 ± 2.4

Figure 3-1: Days of behavioural training and testing given relative to ICH. Rats in EE+SAL and EE+AMP groups were housed in enrichment starting 5 days following ICH. Only animals in EE+SAL and EE+AMP groups were given training on injection days.

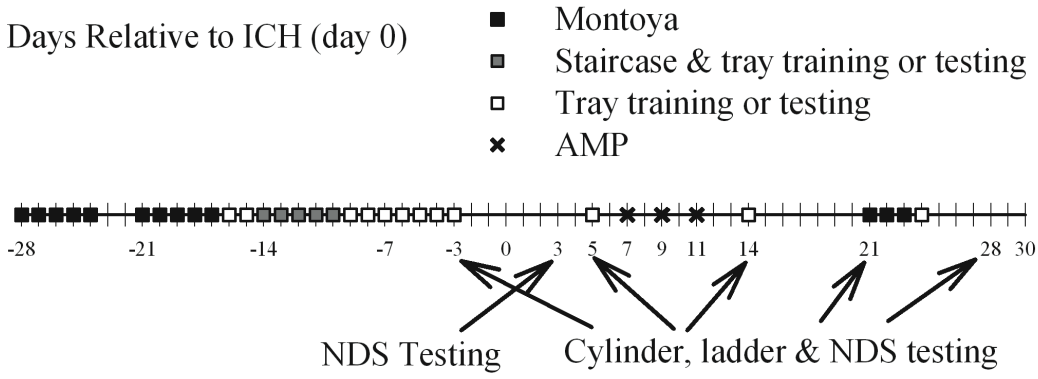


Figure 3-2: A) Volume of tissue lost (mean ± SEM) at 30 days after ICH surgery. The groups were not significantly different from each other. B) Lesion profile of a representative animal. Black indicates the ventricle or cavity and grey indicates dead tissue.

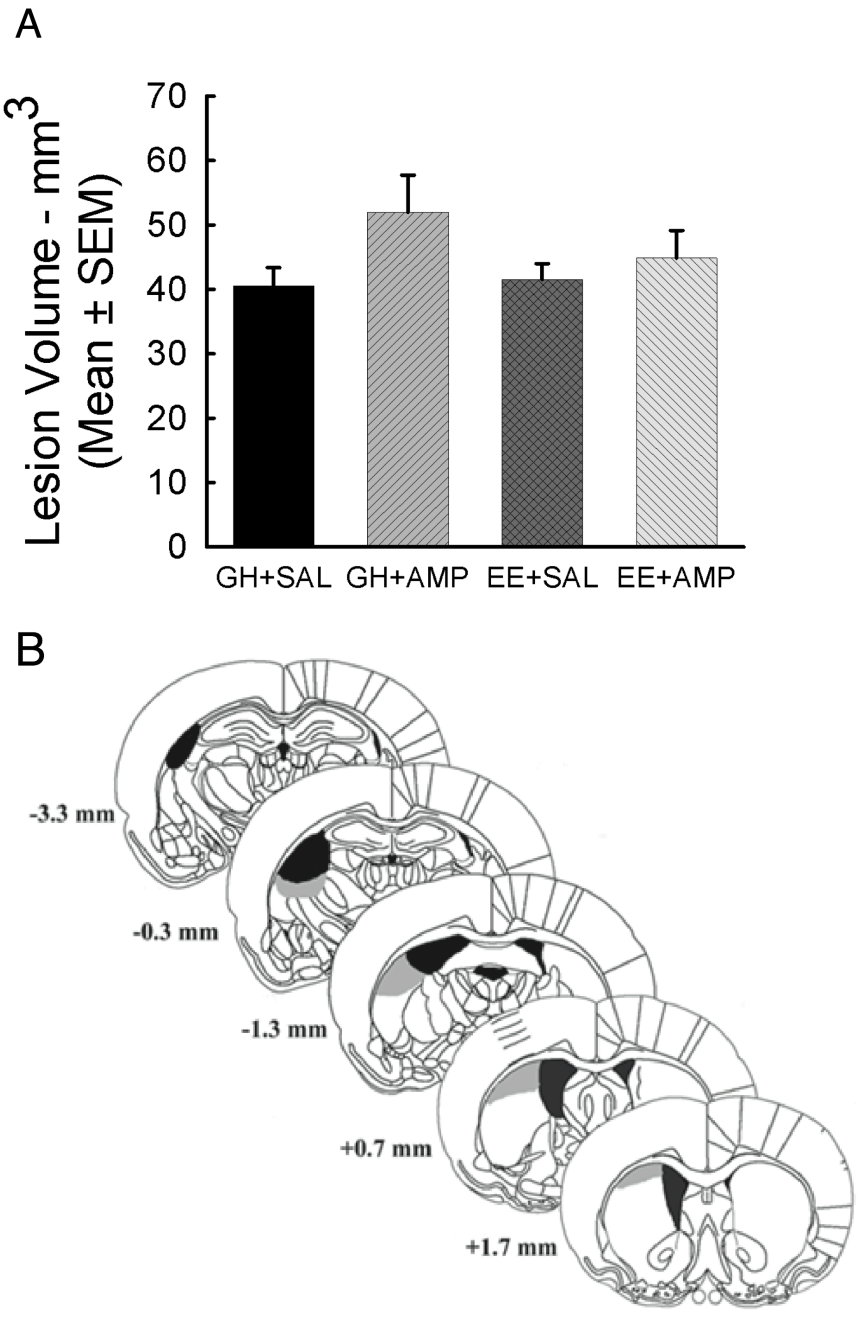


Figure 3-3: Each symbol represents the sum of three scored trials for each rat in the beam-walking test on days 5, 14, 21 and 28 († indicates a significant difference from GH+AMP and * indicates a significant difference from GH+SAL; see results for statistics).

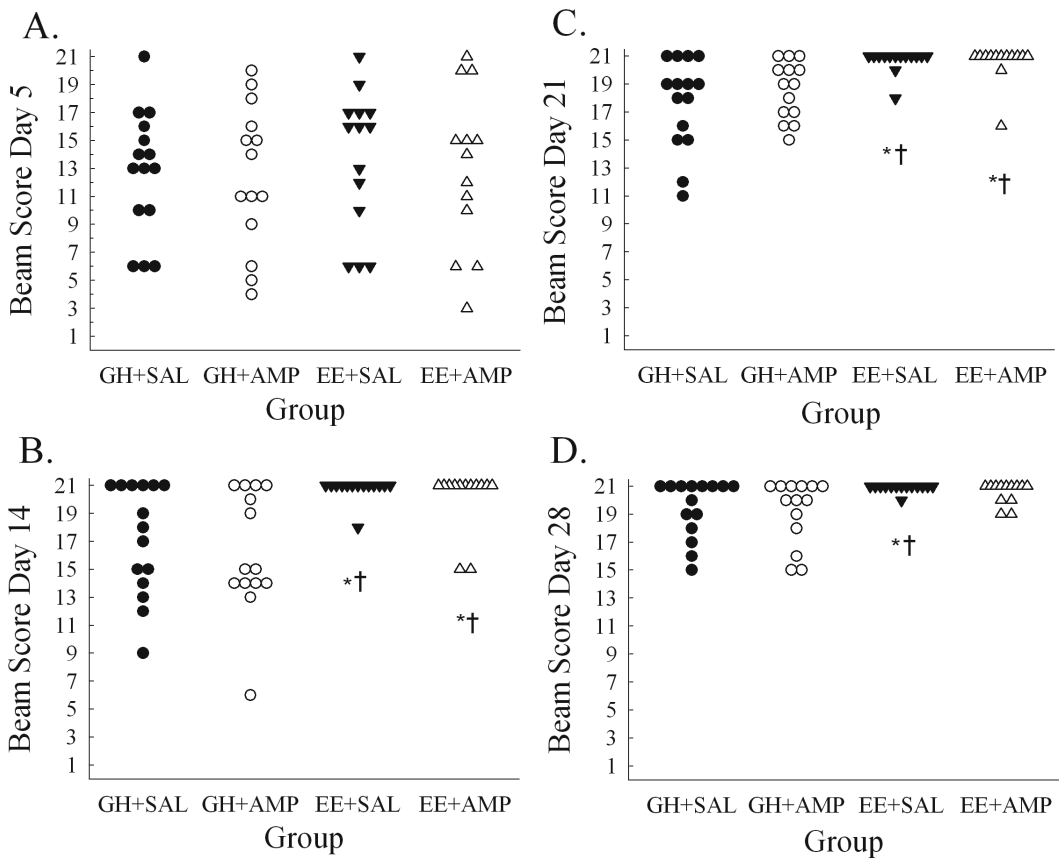


Figure 3-4: Neurologic impairments for days 3, 5, 14, 21 and 28. Each symbol represents the score for one rat († indicates a significant difference from GH+AMP and * indicates a significant difference from GH+SAL).

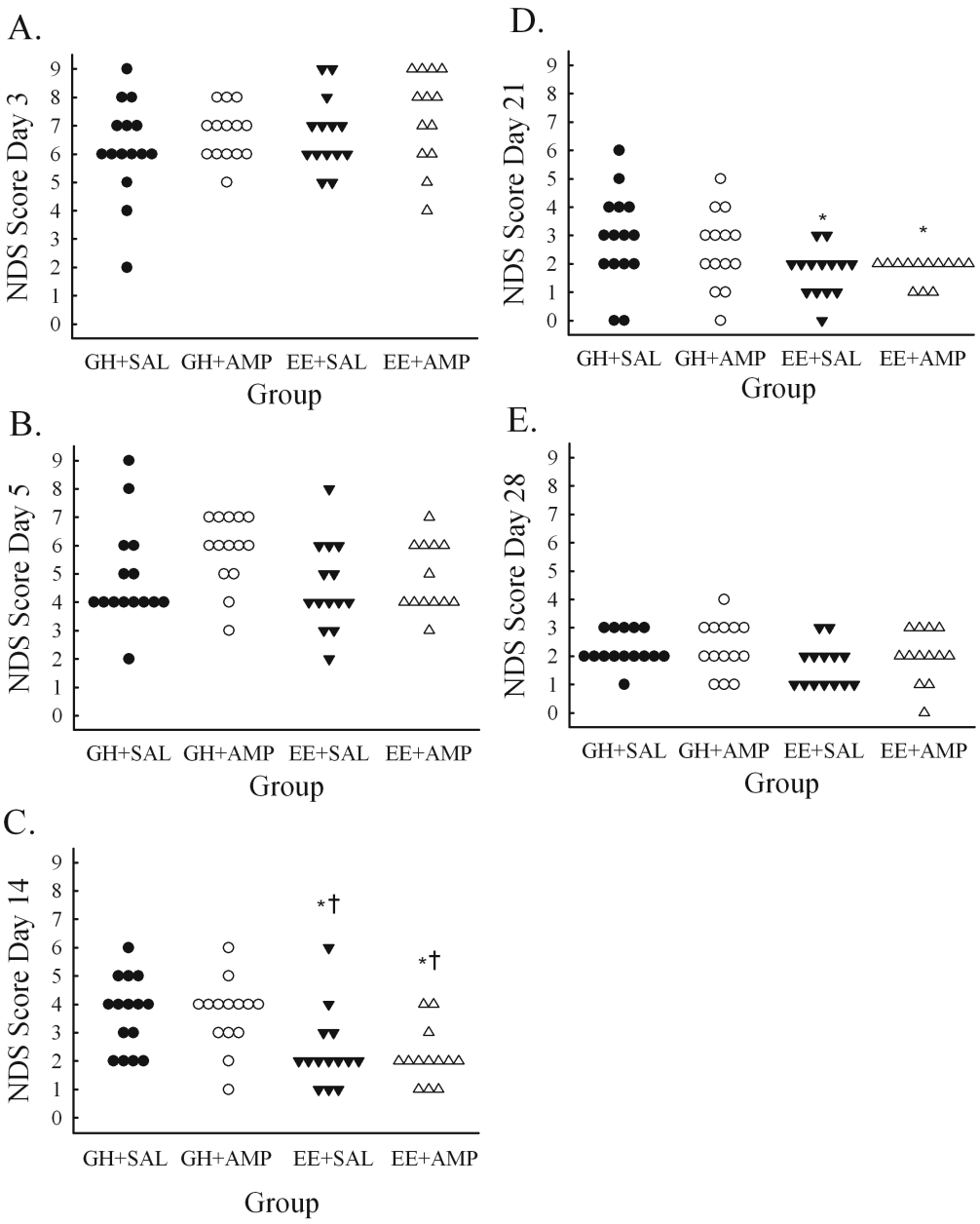


Fig 3-5: Contralateral forelimb slip rate in the horizontal ladder test expressed as an improvement score (% success - % success on day 5 pre-treatment) for test days 14, 21 and 28. There was a significant main effect of Housing.

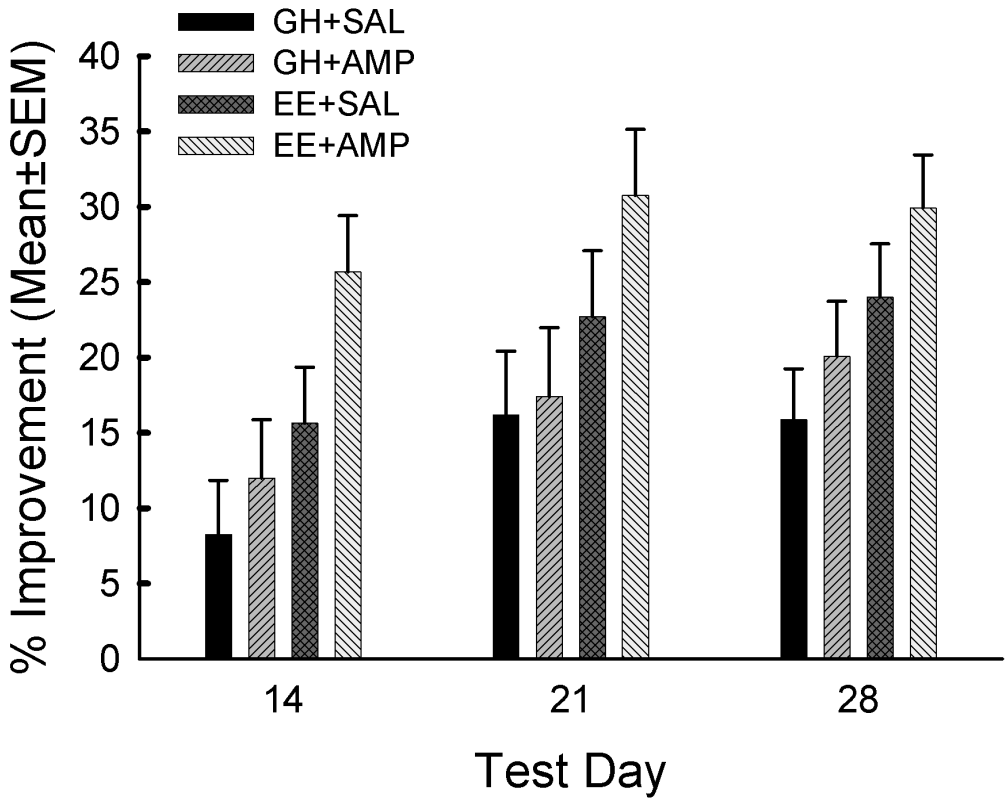
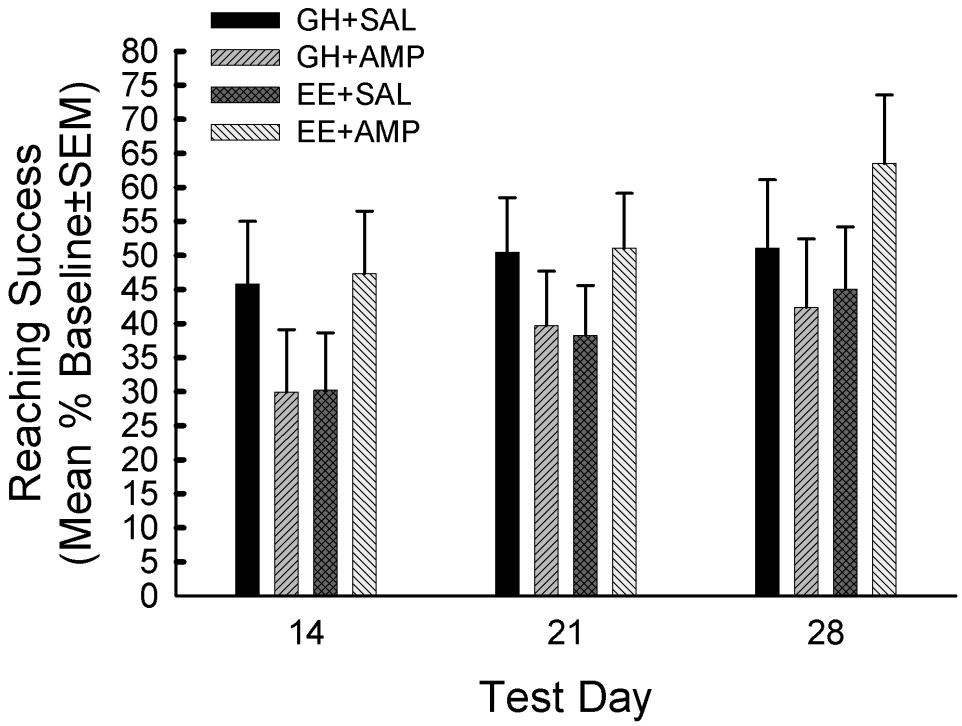


Figure 3-6: The number of pellets retrieved with the contralateral forelimb in the staircase test expressed as a mean % of baseline on days 21, 22 and 23 after ICH. There were no significant group differences.



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Chapter 4:

Neuroprotection with delayed rehabilitation therapy after intracerebral hemorrhage in rats.

A version of this chapter has been published. Auriat and Colbourne 2009. *Brain Research*. 1251: 626-268.

4-1 Introduction

Approximately 10% of all strokes result from an intracerebral hemorrhage (ICH). This stroke causes high mortality and morbidity (1); although, significant recovery can occur (2). Ischemic and hemorrhagic stroke patients often differ in initial clinical presentation, and in the rate and degree of recovery (2, 3). This stems from differences in the pattern and extent of brain injury and its complications (e.g., edema). Furthermore, there are clear differences in pathophysiology (4), thus necessitating unique treatment approaches to mitigate each insult (e.g., rt-PA vs. rFVIIa), and perhaps the use of different neuroprotective approaches. The latter is widely assumed and evident in the failure of many anti-ischemia treatments to markedly alter outcome after ICH in rodent models.

Similarly, differences in pathophysiology between ischemic and hemorrhagic stroke and in the extent and timing of injury and its complications (e.g., edema) suggest that rehabilitation treatments may differentially affect recovery even when patients with comparable brain injury are compared. However, clinical rehabilitation studies usually target ischemic patients, and either exclude hemorrhagic patients or include only a small subset from which no or limited conclusions can be drawn. As well animal studies focus almost exclusively on ischemic injury or they use mixed brain injury models (e.g., pial devascularization, suction lesions)

that do not allow one to determine treatment efficacy and mechanisms in clinically-realistic models of ICH.

There are several rodent ICH models currently in use, and these usually target the striatum. In the whole blood model, autologous blood is injected directly into the striatum over a few minutes thereby mimicking a single large bleed (Bullock et al., 1984). Other models inject blood components, such as thrombin (Xi et al., 1999 Stroke), to help elucidate mechanisms of injury. Finally, ICH can be produced by injecting collagenase into the striatum to cause a hemorrhage that develops over several hours (5). Several studies have directly compared models (6, 7) and while there are advantages and disadvantages of each, it is not clear which better mimics spontaneous ICH in humans or predicts treatment efficacy. The collagenase model results in greater deficits because it is easier to produce larger and more uniform lesions than with the whole blood model (6). Both the collagenase and whole blood (7, 8) models result in delayed cell death, but lesion expansion appears to be greater in the collagenase model. Notably, MacLellan et al. compared these models that were matched for bleeding volume (6). The collagenase model produced a greater lesion volume, which also continued to mature from 1 week to 1 month post-ICH as observed with successive MR images. This has been confirmed with histological techniques (9). Given the remarkable delay in injury, the collagenase model seems to provide an ideal therapeutic target.

Numerous rehabilitation techniques have proven beneficial in rodent models of brain injury (10), with almost all rodent stroke studies targeting cerebral ischemia. For example, use of environmental enrichment (EE), which includes social housing of animals in a large cage with tunnels, ramps, etc., has been repeatedly shown to improve outcome after ischemic injury in rodents (11). After ICH, however, EE appears to be of more limited benefit (12, 13). Forced running improves outcome after ischemic stroke, and when given soon after ischemia the treatment can reduce infarct size (14). Early forced running also appears to be neuroprotective after collagenase-induced ICH (15, 16), but there is no benefit with a more clinically-realistic intervention delay of 2 weeks (17). Overall, treatments that work in ischemia models generally appear less effective for ICH. However, constraint-induced movement therapy (CIMT), which improves outcome in ischemic stroke patients (18), facilitates recovery and lessens injury after a moderate-sized ICH in rats (19). In that study, rats were forced to use their impaired limb during rehabilitation exercises (e.g., skilled reach training) that started 1 week after ICH. In summary, these findings suggest that striatal ICH is more resistant to rehabilitation therapies, but it is possible that focused therapies (i.e., CIMT) can be effective, and they can reduce the delayed injury that occurs after collagenase-induced ICH.

The current study assessed whether rehabilitation (REHAB) started one week following a moderate-sized striatal ICH improves functional

recovery and decreases brain injury compared to standard, group-housed rats (CONT). We used the collagenase model of ICH because it results in consistent hemorrhaging and functional impairment (20). As well, this model of ICH results in progressive brain injury with a significant increase (e.g., 25%) in lesion volume over approximately 4 weeks post-ICH (6). We used a modified version of the enhanced EE therapy that reduces impairment and enhances neuroplasticity following focal ischemia (21) because EE alone provides only slight functional benefit and does not lessen injury after ICH (12, 13). We predicted that EE and reach training following ICH would improve outcome on multiple tasks (e.g., skilled reaching) and reduce lesion volume.

4-2 Methods

4-2.1 Animals

Thirty-six male Sprague-Dawley rats were used in this study. They were obtained locally at a weight of 200 - 250 g and an age of approximately 8 weeks. Rats were housed in groups of four in standard polycarbonate cages (width: 38 cm; length: 49 cm; height: 20 cm) with wood chip bedding. They were housed on a 12 hr light dark cycle with lights on at 7 am. Rats were given free access to water and food except for the periods of food deprivation described below. Rats were habituated to the animal housing room for one week prior to the start of the behavioural training and they were gently handled for 5 minutes a day for

the last 4 of these days. All procedures were in accordance with the Canadian Council on Animal Care guidelines and were approved by the Biological Sciences Animal Care and Use Committee at the University of Alberta.

Rats were trained on several behavioural tests, then subjected to an ICH followed by either rehabilitation (*n*=16; REHAB) or a control treatment (*n*=16; CONT), then testing, and euthanasia at 46 days post-ICH. Animals were randomly assigned to treatment conditions and assessed in a blinded manner. Four other rats were excluded from the study (see Results).

4-2.2 Behaviour Training and Testing

Montoya Staircase Skilled Reaching Test

Rats were first food-deprived (daily rationing) to bring their weight to 90% of their free feeding size, taking into account the natural gain in body weight with time. After three days of food restriction, training began in the staircase test where they learned to reach for 45 mg reward pellets (Bio-Serv, Frenchtown, NJ, USA) in the staircase apparatus. This apparatus (length: 30 cm; width: 6.8 cm; height: 12cm) has a central platform with descending staircases on each side into which 3 reward pellets are placed on each step (22). Training continued for 5 consecutive days per week over 3 weeks with two 15-min trials per day separated by 3 – 4 hours. Rats were excluded from the study if they did not obtain an average of 8

pellets with at least one limb over the final 3 days of training. Ad lib feeding followed completion of the training phase.

Testing (and food deprivation) was conducted on days 28 – 34 and days 42 – 46 following ICH (two 15 minute trials / day). Previous work has established that this test, which measures forelimb reaching ability, is sensitive to motor system injury (22), including striatal ICH (20).

Horizontal Ladder Walking Test

During the final three days of staircase training rats were trained (4 times / day) to cross a 1 m long horizontal ladder situated 23 cm above a table, and consisting of a series of parallel steel rungs (3.0 mm diameter) interspaced 1 – 3 cm. Using video analysis the number of errors (complete foot slips through the rungs) were counted for each limb while traversing the middle 0.5 m section of the ladder on the third day of training. This test is persistently sensitive to motor system damage, including ICH (17, 20). Performance on this test was expressed as % success = successful steps / total steps × 100. Thus, testing for long-term walking ability was conducted on days 32 and 46 post-ICH.

Spontaneous Forelimb Use Asymmetry (Cylinder) Test

During the final day of staircase testing rats were placed in a plexi-glas cylinder (diameter: 20 cm; height: 45 cm) and videotaped from below for 10 minutes. Spontaneous forelimb use during wall movements was

analyzed. Each initial forelimb placement as well as lateral movements was scored as being made by the contralateral or ipsilateral-to-stroke limb or both. Independent forelimb use was expressed as % contralateral use = (contralateral contacts / contralateral contacts + ipsilateral contacts) × 100 (17, 20). The cylinder test measures spontaneous forelimb usage, which is persistently altered by damage to the motor system (23) including ICH (20). Testing was conducted on days 32 and 46 post-ICH.

4-2.3 Surgery

Surgical procedures were done aseptically. After anesthetization with isoflurane (4% induction, 2% maintenance in 60% N_2O and 40% O_2) the scalp was shaved and cleaned with alcohol and betadine, and then 0.2 ml of Marcaine (Sanofi Canada, Markham, OT, Canada) was injected subcutaneously. The rat was placed in a stereotaxic frame and body temperature was maintained at normothermia (36.5 – 37.5 ºC) with a rectal thermocouple probe and heating blanket. The thermocouple probe was previously shown to be accurate by comparing it to a calibration grade glass thermometer. After a midline scalp incision a hole was drilled at 3.5 mm lateral at the level of Bregma. This was done on the side contralateral to the preferred paw (paw with the highest average number of pellets retrieved over the last week of staircase training). A beveled 26-gauge needle (Hamilton syringe; Hamilton, Reno, NV, USA) was lowered 7.0 mm below the surface of the skull, and 0.8 µL of sterile saline containing 0.16

U bacterial collagenase (Type IV – S; Sigma, Oakville, ON, Canada) was infused into the striatum over 5 minutes. The needle was left in place for 5 minutes and then slowly removed. The hole was sealed with a metal screw (model MX – 080 – 2; Small parts, Miami Lakes, FL, USA), and the scalp was stapled closed. We have found this model of ICH, developed by Rosenberg and colleagues, results in consistent hemorrhaging within the striatum (5, 20).

4-2.4 Rehabilitation

Co-housed rats were randomly assigned to either REHAB or CONT treatment conditions. This assured that animals remained housed with the same cage mates for the duration of the study, reducing the stress that likely results from changing housing partners. One week following ICH the REHAB and CONT groups began treatment that lasted 5 days a week for 2 weeks. The REHAB animals received 15 hours of EE a day (5:30 pm – 8:30 am, including the entire dark cycle). When the REHAB group was removed from EE they were temporarily housed in a cage without food. Each REHAB rat received four 15-minute reaching sessions in a skilled reaching task that were separated by at least 1 hour. The reaching task was a staircase box with a modified tray that allowed unlimited access to sugar pellets (Bio-Serv, Frenchtown, NJ, USA) with the impaired paw only. An equivalent amount of sugar pellets was given to the CONT group in their home cage. The EE cage had three levels (width: 35 cm; length: 75

cm; height: 75 cm), which included tunnels, ramps, children's toys and a running wheel (13). Objects were exchanged biweekly. The CONT group remained group housed for the duration of the experiment. The CONT rats were placed in a new polycarbonate cage without food during the period when the REHAB rats were removed from EE (9 hours a day). This was to control for food deprivation and handling effects.

4-2.5 Histology

Rats were euthanized 46 days after ICH with an overdose of sodium pentobarbital (80 mg / kg, i.p.; Somnotol, MTC Pharmaceuticals, Cambridge, ON, Canada). They were transcardially perfused with 0.9% saline followed by 10% formalin. Forty μm coronal sections were taken with a cryostat 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), and calculated by subtracting the remaining volume of injured hemisphere from the remaining volume of normal hemisphere. The volume of each hemisphere was calculated as: (average area of complete coronal section of the hemisphere – area of damage – area of ventricle) × interval between sections × number of sections (20). The volume of corpus callosum was assessed for each hemisphere by using a similar formula: volume = average area of corpus callosum × section interval × number of sections. In order to determine cortical thickness the distance from the cingulum to layer II of the cortex

was measured for sections every 200 μm for the extent of the corpus callosum (6).

4-2.6 Statistical Analysis

Results are presented as mean ± standard error of the mean (SEM). The behavioural scores for forelimb asymmetry and ladder were analyzed as repeated measures ANOVA (SPSS 16.0; SPSS Inc., Chicago, IL, USA), followed by one-way ANOVAs in the case of significant interactions. Staircase was analyzed by repeated measures for period (2 weeks) and day (5 days) followed by an additional repeated measures ANOVA for day. One-way ANOVAs were conducted for the lesion volume, cortical thickness and corpus callosum volume.

4-3 Results

4-3.1 Exclusions

One rat died during surgery, presumably due to anesthesia. Three other rats failed to meet inclusion criteria for staircase test and were not included in the study. However, they were subjected to an ICH and kept in their original groups to minimize disruptions to cage mates. The remaining group sizes were 16 each.

4-3.2 Behavioural Testing

Initial analysis of the staircase data for the contralateral forelimb (Figure 1) revealed that there was no Day × Period × Group interaction (p

= 0.860), and no Period × Group interaction (p = 0.524). However, there were significant Day × Group (p = 0.001) and Day × Period (p < 0.001) interactions. There was a significant effect of Day (p < 0.001) and Period (p < 0.001; Figure 3), with performance being worse than baseline levels at week 4 (p = 0.002) and week 6 (p < 0.001). Thus, ICH impaired skilled reaching ability with the contralateral-to-stroke forelimb. There was also a significant effect of Group (p = 0.006). Further analysis of the effects occurring at each period revealed a significant day effect at baseline (p < 0.001) and week 4 (p < 0.001), but not at week 6 (p = 0.063). The groups were not significantly different at baseline (p = 0.767), but were at week 4 (p = 0.001) and week 6 (p = 0.002). Thus, there was a persistent improvement in skilled reaching ability in the REHAB group.

Horizontal ladder data analysis of the contralateral forelimb revealed a significant Period effect (p < 0.001), and a Period × Group interaction (p = 0.010). The Group effect was not significant (p = 0.109). Planned contrasts showed that stepping success on both post-ICH tests times were significantly lower than baseline performance (p < 0.001), and there was a significant interaction for the week 4 versus baseline contrast (p = 0.005). Analysis of the individual time points revealed no Group difference at baseline (p = 0.309) or week 6 (p = 0.274), but there was a significant Group effect at week 4 (p = 0.021; Figure 2). Thus, the REHAB treatment transiently improved contralateral forelimb walking ability, which was impaired by the ICH.

Analysis of the cylinder data showed significant Period ($p < 0.001$; Figure 3) and Group effects ($p = 0.031$), but no significant Period × Group interaction ($p = 0.052$). Planned contrasts showed that scores were lower at both post-stroke test times compared to baseline testing ($p < 0.001$). However, the Period × Group interactions for these contrasts were significant ($p \leq 0.038$) and thus further analysis was done for each time. At baseline, group differences were not significant ($p = 0.663$), whereas the CONT group made a greater percentage of limb placements with their impaired paw than the REHAB group at week 4 ($p = 0.045$) and week 6 ($p = 0.041$). Overall, this indicates that ICH significantly altered limb use bias such that the contralateral-to-stroke forelimb was used less relative to the ipsilateral forelimb. Furthermore, REHAB treatment exacerbated this effect at both test times.

4-3.3 Lesion Volume

Collagenase infusion caused significant striatal injury with some ventriculomegaly at a 46 day survival as illustrated in other publications (20). Lesion size at this time was significantly less (by 28%) in the REHAB group compared to the CONT group ($p = 0.019$; Figure 4a). However, the groups were not different in corpus callosum volume (lesion side: $p = 0.405$; contralateral: $p = 0.264$; Figure 4b) or cortical thickness (lesion side: $p = 0.300$; contralateral: $p = 0.904$; Figure 4c).

4-4 Discussion

Intracerebral hemorrhage occurs with a sudden outpouring of blood into the brain, resulting in both early and delayed injury. The immediate damage results from the space-occupying effects of the hematoma and shearing forces, which probably will only be mitigated by limiting the hematoma size. Peri-hematoma tissue suffers a more delayed insult owing to increasing blood brain barrier disruption, edema, excessive inflammation and neurotoxicity (e.g., iron-mediated free radicals) (4). Indeed, grey and white matter loss progresses over weeks, at least in the striatum (6, 7, 9). Importantly, this study shows that the delayed loss of striatal tissue can be mitigated, and recovery partly improved, by a REHAB treatment starting 1 week post-ICH. In contrast, most putative neuroprotectants, such as FK-506 (24), fail to lessen long-term lesion volume even when administered at the time of ICH. Our results suggest that patients suffering from an ICH affecting the basal ganglia, and perhaps other structures, may benefit from early rehabilitation interventions.

In this study we used an easily-administered REHAB intervention that persistently reduced tissue loss after collagenase induced ICH. Several studies show that cell death and loss of striatal volume continues over weeks in this model of ICH (6, 7, 9, 25). As well, there is a small loss of corpus callosum volume with time (6, 9); however, our results show that the protective effects of REHAB were not due to influencing this structure

nor cortical thickness. Thus further experiments are needed to determine whether the reduction in tissue volume results from reducing cell death within the striatum, including to white matter tracts within it. Others have shown that forced running beginning on the day after ICH is neuroprotective via suppressing caspase activation (15). Thus, it is possible that our more delayed REHAB treatment was similarly neuroprotective, but this would need to be assessed at several survival times to ensure that the cell death was not simply delayed. Alternatively, REHAB might increase striatal volume by stimulating cellular proliferation (e.g., neurogenesis) and migration into the striatum from the subventricular zone. Cellular proliferation occurs following ICH in rats (26) and humans (27), and one report shows that forcing rats to run soon after ICH increases proliferation in the dentate gyrus (16). Additionally, REHAB may have enhanced dendritic arborization within the peri-hematoma region thereby counteracting the dendritic atrophy that occurs here (9). Such an effect of rehabilitation is commonly found in ischemia models (21). Regardless, neither enhanced dendritic arborization nor cellular proliferation would likely fully account for a 28% reduction in tissue loss observed.

Impairments in skilled reaching often follow stroke in humans (28) as also occurs in rodent stroke models (29), including ICH (20, 30). The REHAB intervention persistently improved reaching success in the staircase test as was also found with the use of CIMT (19), but not with

forced exercise (17), or EE with or without concomitant drug treatment (12, 13). Thus, from comparing these studies it appears that task specific training, in this case on a reaching task, is needed to improve recovery. Similar findings have been reported after focal ischemia insults. For instance, training in a tray reaching task improved skilled reaching ability in the single pellet task whereas voluntary exercise alone or in combination with skill training had no effect (31). Further studies are needed to determine whether improvements in the staircase test (success at obtaining pellets) result from improved reaching accuracy or simply greater persistence. This can be determined with the single pellet test, which would also allow one to determine if true recovery or compensation occurred, via detailed video analysis of reaching movements (32). Finally, further studies are needed to determine why task specific training provides greater benefit, histologically and behaviourally, than EE (e.g., comparing neurotrophin levels with these treatments).

The REHAB treatment lessened error rate in the horizontal ladder test at week 4, but the effect was not significant at week 6. Perhaps this resulted from spontaneous recovery in the CONT group which lessened the effect size. Given that the final deficit was relatively mild, it might have been better to use a more difficult version of the ladder test (e.g., wide spacing between bars) or another more sensitive test in order to detect the treatment effect. Alternatively, it is possible that our REHAB treatment did not provide enough walking practice to persistently improve outcome.

Indeed, EE treatment alone when given all day for a longer period does diminish stepping errors after ICH (12, 13).

Use of EE alone does not lessen limb use asymmetry in the cylinder task after striatal ICH (12). We had hoped that REHAB, which included skilled training and EE, would have lessened asymmetry given that this combination lessens limb use bias after focal ischemia in rats (21). However, REHAB treatment actually increased forelimb use asymmetry scores as those rats preferred to use their “normal” forelimb when exploring the walls of the cylinder. Thus, at least for striatal ICH, extensive reach training with EE may favor the development of learned non-use for the impaired (contralateral-to-stroke) limb, which develops from successfully using the non-impaired (ipsilateral) limb in daily tasks (e.g., grooming, climbing, eating ect.), whereas use of the impaired limb is unsuccessful or more arduous. Thus, despite improving the success rate with the contralateral-to-stroke limb in the staircase test, rats prefer to use it less in a free choice situation (cylinder task).

Our findings suggest that there is a significant therapeutic window in which rehabilitation treatments might improve functional recovery after ICH, more so than comparable studies after moderate to severe focal ischemia which show that rehabilitation programs lessen injury only if begun before or very soon after injury (14). The much delayed maturation of injury in the collagenase model (versus other types of brain injury) likely explains this finding (6, 9). Similarly, it is reasonable to expect that delayed

rehabilitation programs might reduce infarction after milder ischemic insults, which result in more delayed injury (33). Thus, further study is needed to determine whether the tissue-salvaging effects of rehabilitation vary with the type, location, and severity of stroke, all of which influence the maturation of injury and recovery processes. Further variations in the rehabilitation protocol (e.g., longer treatment) may also further improve outcome. As well, experiments are needed to identify the mechanisms of action (e.g., improvement in cerebral blood flow, release of neurotrophins). Finally, the applicability of these findings to stroke in humans will rest upon whether slow cell death occurs. This has not been clearly determined in ICH, but there is some evidence for lesion expansion (e.g., delayed atrophy) from finding of increased ventricular size over time (34).

One limitation of our study was that we did not confirm that lesion size or functional impairment was equivalent between groups in the post-stroke, pre-rehabilitation period. Baseline, pre-stroke performance was the same, and given that rats were randomly assigned after the ICH to receive the REHAB or CONT condition, one should assume that these groups were similar following ICH. Nonetheless, future studies could confirm this by using imaging to determine the size and location of injury prior to treatment. Similarly, functional impairment could be measured prior to treatment. Here some might argue that groups should then be matched on the basis of functional impairment. However, this is difficult to achieve

when multiple tests are used, which often do not inter-correlate well. It also means that animals would not be randomized to treatment conditions.

In summary, our main finding of reduced tissue loss with a 1-week delayed rehabilitation program after collagenase-induced ICH suggests that similar rehabilitation programs might mitigate cell death / atrophy after ICH in patients while improving functional recovery. Further study using our REHAB protocol or similar treatments is needed to identify the nature and mechanisms of this protective effect and optimal treatment parameters. For instance, earlier (e.g., starting on day 1) and aggressive rehabilitation programs can aggravate brain injury (35, 36), so aggressive rehabilitation is not recommended at this time.

Figure 4-1: Number of pellets consumed with the contralateral limb over 5 days prior to ICH, and on days 28 – 32 and 42 – 46 following ICH. The groups were significantly different at 4 and 6 weeks, but not at baseline.

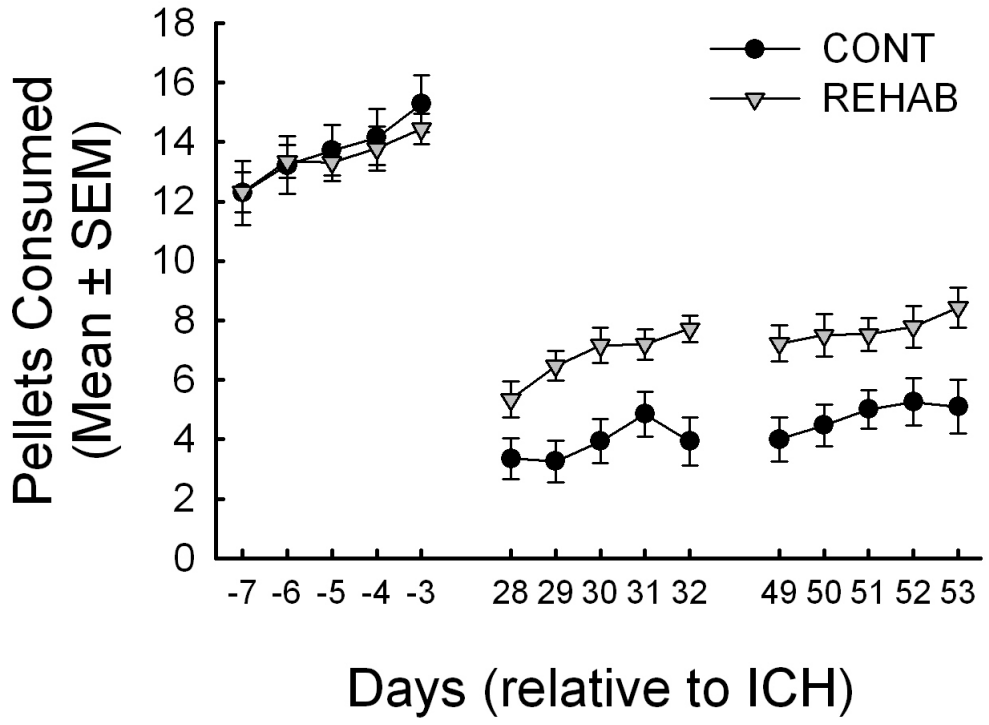


Figure 4-2: Contralateral forelimb slip rate in the horizontal ladder test expressed as a percent success at baseline and days 32 and 46 post-ICH. *p<0.05.

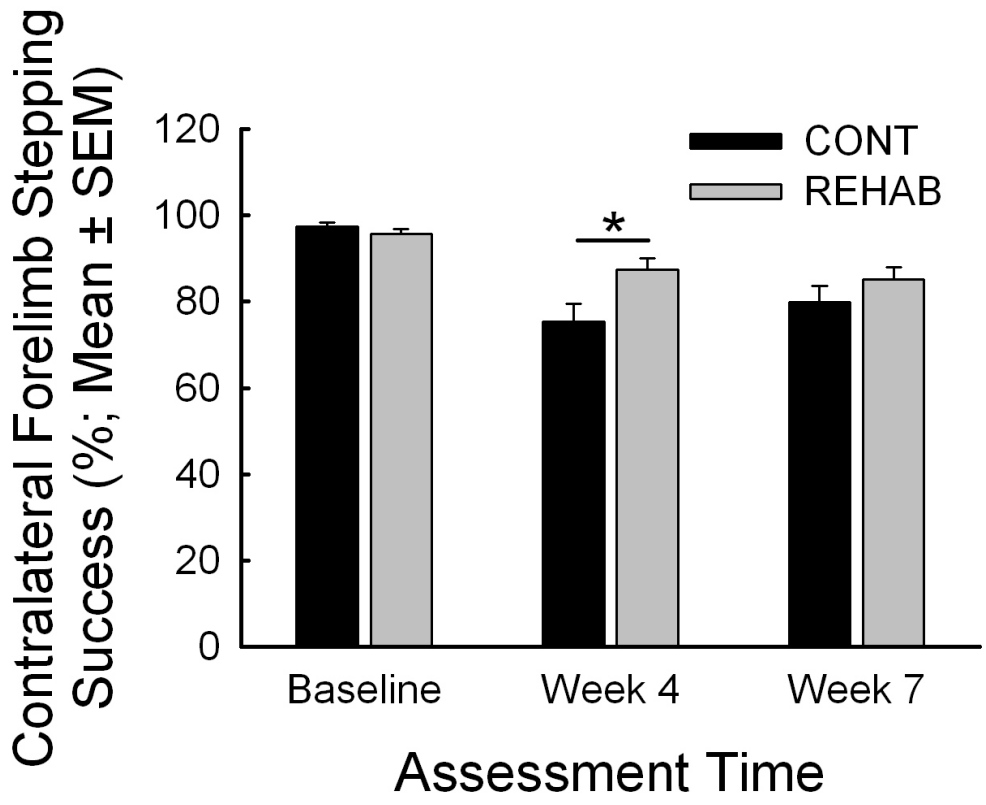


Figure 4-3: Forelimb asymmetry shown as the percentage of wall contacts made with the contralateral-to-stroke forelimb. *p<0.05.

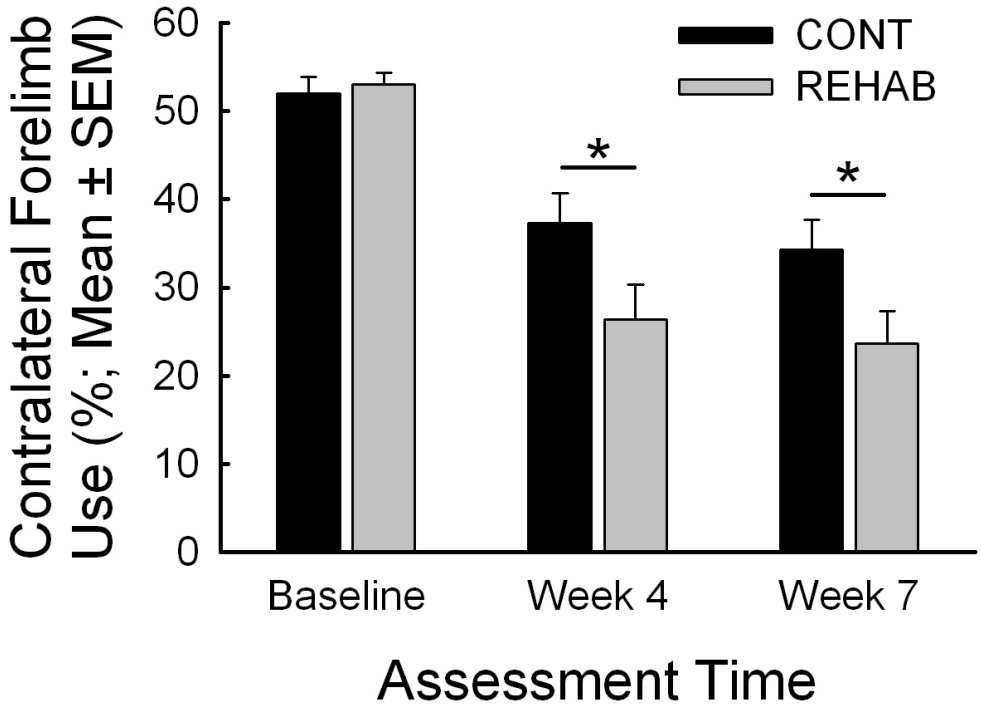
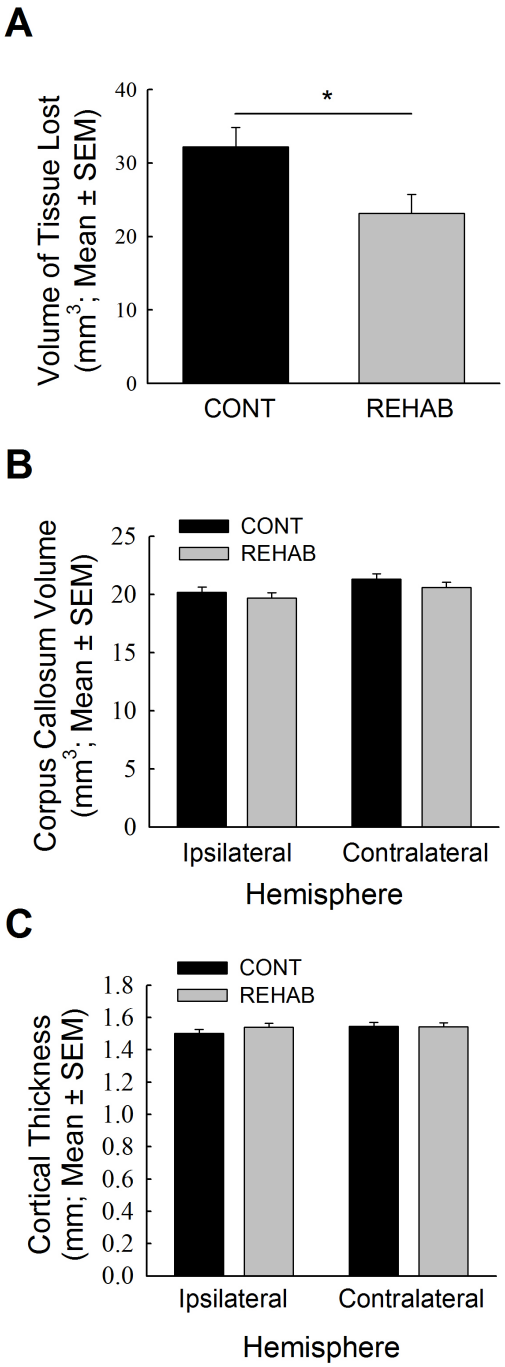


Figure 4-4: (A) Volume of tissue lost at 46 days after ICH (*p<0.05). The corpus callosum volume (B) and cortical thickness measurements (C) did not differ between groups.



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Chapter 5:

Rehabilitation after intracerebral hemorrhage in rats improves recovery with enhanced dendritic complexity but no effect on cell proliferation.

A version of this chapter has been published. Auriat et al. 2010. *Behavioral Brain Research*.

5-1 Introduction

An intracerebral hemorrhage (ICH) is a devastating stroke causing significant mortality and disability (1). While substantial improvement occurs (1, 2), a better mechanistic understanding is needed to further lessen residual deficits. Animal stroke models have been used to identify factors that influence recovery and to test rehabilitation strategies (3-6). Unfortunately, most ICH studies focus on pathophysiology and neuroprotection, ignoring mechanisms of recovery. Thus, there is a paucity of animal data available for ICH.

Nonetheless, rodent experiments show that striatal ICH causes an initial atrophy of peri-hematoma neurons that subsequently appears to recover (7). In contrast, the contralateral striatum shows a sustained increase in dendritic complexity. Greater dendritic branching, indicating enhanced synaptogenesis, has been routinely linked with recovery after brain injury, and is a likely contributor to behavioural improvement after ICH (4-6). Similarly, animal studies show modestly-enhanced neurogenesis after ICH (8, 9), which also appears to occur in humans (10). The contribution of these mechanisms, among others (e.g., inflammatory response), to spontaneous and rehabilitation-facilitated recovery after ICH is not well known.

A variety of rehabilitation strategies have been tested in rodent ICH models with limited success. These putative treatments include: environmental enrichment (EE) (11, 12), simple exercises (e.g., treadmill)

(13-15), and forced-use therapies (e.g., “constraint-induced movement therapy”) (12, 14). Interestingly, in some cases, rehabilitation lessened brain injury after ICH (12, 14, 15). This has also been observed in some ischemia models. Delayed cell death occurs in rodent models of ICH, and is especially prominent in the collagenase model (7, 16). Thus, rehabilitation may also improve recovery through neuroprotection.

In this study, we evaluated these potential mechanisms through which rehabilitation improves recovery after collagenase-induced ICH in rat (17). Rehabilitation (REHAB), which consisted of environmental enrichment and skilled reach training (18), started 1 week after the lesion and lasted for 2 weeks. Control rats (CONT) were group housed. In experiment 1, we assessed whether dendritic complexity was increased at 3 weeks following treatment. In experiment 2, we tested whether rehabilitation influenced cell proliferation and the inflammatory response at 2 weeks following treatment. We assessed cell proliferation and inflammation/gliosis at an earlier time point than dendritic plasticity because these factors were likely to decrease with time from injury. Multiple behavioural endpoints were used in both experiments, but the staircase test (19) of skilled reaching was, *a priori*, the primary endpoint due to its sensitivity to striatal ICH (20) and the effects of rehabilitation (12, 14).

5-2 Methods

5-2.1 Animals

We used 64 male Sprague-Dawley rats obtained from the Bioscience's colony at the University of Alberta. All procedures were approved by the Bioscience Animal Care and Use Committee at the University of Alberta and were in accordance with the Canadian Council on Animal Care guidelines. Rats were kept 4 per cage with free access to water and food, except as noted below, under 12-hour light cycles (on from 7 am to 7 pm). Cages of 4 rats were randomly assigned to either REHAB or CONT treatment (n=16 each per experiment), thereby avoiding stress associated with changing cage mates.

5-2.2 Behavioural Training and Testing

Montoya Staircase Skilled Reaching Test

All rats were food deprived to ≈ 90% of their free feeding weight and maintained at this level adjusting for normal growth. Rats were trained twice daily 5 days a week over 3 weeks. During each 15-min training session the rat was placed in a staircase box (length: 30 cm; width: 6.8 cm; height: 12 cm) containing a central platform and descending stairs on each side (19). On each of 7 steps per side there were 3 food pellets (45 mg, Bio-Serv, Frenchtown, NJ), which the rat grasped with its forepaw. Rats were excluded if they did not obtain an average of ≥8 pellets per session with at least one paw over the final 3 training days. Afterwards,

rats were returned to ad lib feeding until they were food deprived for testing at 6 days, and 5 weeks (experiment 2) or 6 weeks (experiment 1) post-ICH.

Horizontal Ladder Walking Test

Rats walked across a 1 m long horizontal ladder (21) with randomly spaced rungs (1-4 cm apart; diameter: 3 mm). Rats were trained for 3 days (4 crosses per day) and video recorded on the final day. The contralateral to ICH forelimb ratio was analyzed to determine walking ability (% error = (number of slips / total steps) × 100). Walking ability was assessed 3 days prior to injury and at 6 days and 32 (experiment 2) or 39 days (experiment 1) following ICH.

Spontaneous Forelimb Limb Use Asymmetry (Cylinder) Test

Rats were placed in a Plexiglas® cylinder (diameter: 20 cm; height: 45 cm) and video recorded for 10 minutes. Spontaneous forelimb use was measured for each initial contact and movement on the cylinder wall, scored as being made by the ipsilateral or contralateral (to ICH) limb or as both. Contralateral forelimb use was expressed as: ((contralateral forelimb contacts + ½ bilateral contacts) / total contacts)) × 100 (22). Animals were tested 3 days before and at 6 and 32 (experiment 2) or 39 days (experiment 1) following ICH.

Tray Task

Rats in experiment 2 were also trained and tested on a tray task where they reach through vertical bars at the front of a cage (length: 27 cm; width: 19 cm; height: 25 cm) to retrieve food placed on an external tray (17% Layer Prostock feed; Master-feeds, Edmonton, AB). Rats were given a daily 30-minute training session following the completion of staircase training. Reaching was video recorded 3 days before ICH, and at 6 and 32 days post-ICH. The % success was determined as: (successful/total reaches) × 100 (23).

5-2.3 Surgery

Rats weighed ~350 g on the day of surgery. Procedures were similar to those used previously (12). Briefly, they were anesthetized with 4% isoflurane (2% maintenance; 60% N_2O and remainder O_2) for aseptic surgery and placed in a stereotaxic frame. Body temperature was maintained between 36.5 – 37.5 ºC. A hole was drilled at 3.5 mm lateral and 1.0 mm anterior to Bregma on the side contralateral to the preferred paw (one with the better success in the staircase test). A 0.8 µL solution of sterile saline containing 0.16 U of bacterial collagenase (Type IV–S. Sigma-Aldrich, Oakville, ON) was infused through a 26-gauge needle (Hamilton syringe; Hamilton, Reno, NV) at a depth of 7.0 mm below the skull.

5-2.4 Rehabilitation

The REHAB group received EE and skilled reach training starting 7 days after ICH. Treatment lasted 2 weeks for 5 consecutive days per week. On these days, rats were placed in EE cages (width: 35 cm; length: 75 cm; height: 75cm) from 5:30 pm – 8:30 am with free access to food and water. The EE cages contained three levels with multiple 'toys' (exchanged biweekly), tunnels, ramps and a running wheel. During the day, REHAB rats were returned to their standard polycarbonate cages (width: 38 cm; length: 49 cm; height: 20 cm), without food, and they received four 15-minute reaching sessions in a modified staircase-reaching box. Essentially, rats were allowed unlimited access to sugar pellets located in one large well on the side contralateral to the ICH. Weight was monitored daily to ensure no animal dropped below 90% of its free feeding weight. The CONT rats remained group housed in standard cages for the duration of the experiment with the same feeding schedule and cage change cycles. Furthermore, they were given an equivalent amount of sugar pellets scattered on the floor of their home cage as obtained by the REHAB group.

5-2.5 BrdU Injections

All rats in experiment 2 received daily injections of BrdU (100 mg/kg i.p., Sigma, St. Louis, Missouri, USA, B5002) on days 14 through 18 after ICH.

5-2.6 Histology

In experiment 1, rats were euthanized at 39 days post-ICH via an overdose of pentobarbital (80 mg/kg, i.p.). They were then perfused with 0.9 % saline. The brains were removed and placed in Golgi-Cox solution for 14 days. Brains were then immersed in 30% sucrose in distilled water for two days. Tissue was Vibratome-sectioned at 200 μm and stained according to established methods (7, 24). Four brain areas were assessed: 1) medium spiny neurons in the peri-hematoma region, 2) the corresponding area in the contralateral striatum, 3) basilar dendrites of layer 5 pyramidal neurons in the ipsilateral, and 4) contralateral forelimb motor cortex. For each region of interest (ROI) 5 cells were drawn per animal. Neurons were traced via Camera Lucida using a 20× objective by a researcher blinded to treatment conditions. Only those neurons that were fully impregnated with Golgi-Cox solution and unobscured were drawn. Sholl analysis of ring intersections was used to estimate dendritic length and complexity (24). Briefly, a transparent grid of concentric rings was used to assess the number of cell branches that intersected each ring. Finally, spines on the terminal tips of neurons in the same ROIs were drawn at 1000× (oil immersion). The number of spines and length of each segment were determined and the spine density was expressed as the number of spines per unit length.

In experiment 2, rats were euthanized 32 days after ICH via pentobarbital. They were perfused with 0.1 M phosphate-buffered saline

(PBS) followed by fixation with 4% paraformaldehyde. Brains were further fixed overnight and then transferred to 30% sucrose in PBS. Brains were cryostat sectioned at 50 μm. Every fourth section was placed on slides for cresyl violet staining and lesion volume assessment. The volume of each hemisphere was calculated as: (average area of complete coronal section of the hemisphere – area of damage – area of ventricle) × interval between sections × number of sections. Lesion volume was then calculated by subtracting the remaining volume of injured hemisphere from the volume of the normal hemisphere (12, 20).

Sections from a random subset of animals (n = 8 per group) were collected at the level of maximal lesion and placed in centrifuge tubes containing PBS for immunohistochemistry. Three sections per brain were saved; one was double labeled for BrdU and a mature neuronal marker (NeuN), one for BrdU and a macrophage/microglia marker (Iba1), and one for BrdU and an astrocyte marker (GFAP). Prior to BrdU labeling, tissue was exposed to 2 M HCl for 30 minutes at room temperature. Free-floating sections were incubated in blocking solution (10% donkey serum and 0.3% Triton-X 100 in PBS) for 1 hour and then incubated with primary antibodies in 2% blocking solution for 24 hours. Sections were then placed in secondary antibodies (1:500) with DAPI (1:500; nuclear label, Pierce, Rockford, IL, USA, 46190) for 1 hour.

The following primary antibodies were used in this study: rat anti-BrdU (1:1000; Serotec, Raleigh, NC, OBT0030), mouse anti NeuN (1:500;

Millipore, Temecula, CA, MAB377(CH)), mouse anti GFAP (1:400; Sigma, St. Louis, Missouri, G3893), and rabbit anti Iba1 (1:1000, Wako, Richmond, VA, 019-19741) . The secondary antibodies used in this study, all from Jackson ImmunoResearch (West Grove, PA, USA) are: donkey and rat Cy3 (712-165-153), donkey anti mouse 488 (715-485-151) and donkey anti rabbit 488 (711-485-152). Control sections were incubated without primary antibodies, and no nonspecific labeling was observed in these sections.

Six regions (450 μm × 350 μm) per brain were identified for quantification of GFAP+ and Iba1+ cells at 20× magnification (Olympus BX51). BrdU+ cells in the ipsilateral and contralateral ventricles, as well as in the striatum of a single section per brain were counted. All BrdU+ cells in each section were examined for double labeling.

5-2.7 Statistical Analysis

Data are expressed as mean ± SEM and were analyzed by repeated-measures and/or 1-way analysis of variance (ANOVA) using SPSS (v. 17.0, SPSS Inc, Chicago, IL).

5-3 Results

5-3.1 Exclusions

Five rats in experiment 1 did not reach baseline criteria for staircase training and were excluded. Nonetheless, they received surgery and

remained housed with their cage mates to reduce the stress of changing the housing arrangement. One rat in the CONT group was euthanized early due to excessive weight loss following ICH. These factors left group sizes of 13 in each group. There were no exclusions from experiment 2.

5-3.2 Behavioural Testing

In experiment 1, there were significant Day ($p < 0.001$) and Group ($p = 0.018$) main effect, and a Day × Group interaction ($p = 0.001$; Figure 1a) in the staircase test. The groups were not different prior to ICH, or after ICH but prior to intervention ($p \geq 0.714$). However, rehabilitation improved skilled reaching on all test days ($p \leq 0.019$) by ~22% (vs. CONT). Similar results were observed in experiment 2 (Figure 2a) where there was a significant Day main effect ($p < 0.001$) and a Day × Group interaction ($p = 0.001$), but no Group main effect ($p = 0.803$). Rehabilitation significantly reduced impairment by ~29% on the test days ($p \leq 0.044$), but, as expected, there were no group differences prior to treatment ($p \geq 0.244$). Rehabilitation successfully reduced skilled reaching impairments in both experiments.

In experiment 2, reaching ability was also assessed in the tray task (Figure 2b). Those rats that did not make more than 10 reach attempts with their impaired limb were excluded from analysis leaving CONT and REHAB groups with the following group sizes: baseline (16, 15), day 6 (12, 9) and day 32 (12,10). There was an effect of Day ($p < 0.001$), but not

of Group ($p = 0.762$), and no interaction ($p = 0.099$). Owing to the data loss in the repeated measures design, we proceeded with a planned comparison that revealed a significant effect of rehabilitation on the test day ($p = 0.039$). Adding further support to the staircase finding that also found improved reaching ability in REHAB treated rats.

In experiment 1, analyzing walking ability for the contralateral forelimb revealed a significant Day ($p < 0.001$) and Day × Group effect ($p = 0.016$), but there was no Group main effect ($p = 0.222$). Additional one-way comparisons indicated that although the performance between groups did not differ at baseline or day 6 ($p \geq 0.568$), the REHAB group was significantly better at day 39 ($p = 0.009$; Figure 1b). In experiment 2 (Figure 2c), there were significant Day ($p < 0.001$) and Group ($p = 0.016$) main effects and a Day × Group interaction ($p < 0.001$). There were no pre-treatment differences ($p \geq 0.365$), but the REHAB group was less impaired post-treatment ($p < 0.001$). Thus, rehabilitation improved ladder-walking ability with the impaired forelimb in both experiments.

In both experiments (Figure 1c; Figure 2d), cylinder test analysis indicated that there was a Day effect ($p \leq 0.005$), but no Group ($p \geq 0.573$) or interaction effects ($p \geq 0.447$). Rehabilitation did not significantly alter the spontaneous usage of the impaired forelimb.

5-3.3 Golgi-Cox Analysis

Poor staining and technical errors resulted in the loss of some tissue from analysis. Thus, cortical tissue analysis was completed on 12 rats per group whereas the striatal analysis was completed on 11-12 rats per group. Rehabilitation increased the overall dendritic length compared to the CONT group in ipsilateral cortex, peri-lesion striatum and contralateral striatum ($p \leq 0.012$), but not in the contralateral cortex ($p = 0.217$). Dendritic length in the contralateral hemisphere was greater than in the ipsilateral side in both the cortex and striatum ($p \leq 0.023$; Figure 3).

Due to uneven staining several brains were excluded from spine density analysis, leaving the following group sizes for CONT and REHAB hemispheres for each location: cortical contralateral (8, 11) ipsilateral (9, 11), and striatal ipsilateral (6, 6) and contralateral (6, 5). There were no significant differences in spine density between groups for any ROI ($p \geq 0.732$; data not shown). Nor did spine density differ across hemispheres in either cortex or striatum ($p \geq 0.362$).

5-3.4 Lesion Volume and Cell Counts

There was significant striatal injury at 32 days following ICH (experiment 2), as illustrated in our previous work (7, 14, 16). Injury volume did not differ between CONT (27.79 ± 4.50) and REHAB (24.46 ± 3.40) groups ($p = 0.435$). There were more BrdU+ cells near the ventricle of the injured than uninjured hemisphere ($p < 0.001$). However,

rehabilitation did not significantly affect these numbers or those in the striatum ($p \geq 0.147$; Figure 4 and 5a). None of the BrdU+ cells found in the striatum were co-labeled. Analysis of GFAP labeled cells revealed only one group difference, which was in the cortex on the lesion side ($p = 0.030$; Figure 5b); otherwise the group differences were not significant ($p \geq 0.203$). There were no differences between groups for the number of Iba1+ cells in any location ($p \geq 0.247$; Figure 5c). However, there were more Iba1+ and GFAP+ cells in the injured than the contralateral hemisphere ($p \leq 0.007$).

5-4 Discussion

Our rehabilitation intervention, which started one week after ICH, significantly reduced functional impairment (skilled reaching, walking) similar to that we previously reported (12), although benefit was neither complete nor obtained on all tests. Such findings were associated with significantly increased dendritic length in peri-hematoma striatal neurons, the ipsilateral motor cortex and the contralateral striatum. Rehabilitation did not appear to alter neurogenesis or cell proliferation, suggesting that, in this model, a more important contributor to behavioural recovery is synaptogenesis, not replacement of lost cells or alterations in the response of astrocytes and microglia/macrophages.

Our dendritic structure findings with rehabilitation concur with previous work in ischemia and related models indicating that ipsilateral

and contralateral structures are altered following injury and rehabilitation (3-6). However, not all studies show a total increase in dendritic branching in peri-infarct areas. Indeed, Brown and colleagues, using two-photon imaging, report that there are no net changes in apical dendrites bordering an ischemic infarct; although individual dendrites remodel by shortening tips near the infarct and lengthening branches extending away from the infarct (25). Unlike this imaging technique, Golgi-Cox labeling cannot be used to longitudinally monitor individual dendritic branches, but it has the advantage of allowing one to assess deeper cortical layers as well as subcortical structures. Thus, differences among studies may relate to the region evaluated (e.g., cortex vs. striatum; apical vs. basilar dendrites), as well as to the type of insult, animal model, etc.. Regardless, such studies, including our findings, cannot causally link increased dendritic branching, indicative of enhanced synaptogenesis, with behavioural recovery. Furthermore, our study does not sort out the contribution of ipsilateral versus contralateral structures to recovery and compensation, which has been widely studied after ischemia with contradictory findings (e.g., (26, 27)). It is likely that issues, such as dependence upon initial lesion volume (20), are also relevant to recovery after ICH as after ischemic stroke.

We observed BrdU-labeled cells migrating toward the injured striatum, which supports previous observations (8, 9). However, there are contradictory finding with regard to the effects of rehabilitation after injury. Some report increased proliferation and neurogenesis (28) while others

report a decrease (29). Many factors such as type, location and extent of injury as well as the timing, duration, intensity and method of rehabilitation likely influence neurogenesis. The current rehabilitation intervention did not alter cell proliferation, and we found no co-labeling to indicate that new cells become neurons, astrocytes or microglia. However, one cannot exclude the possibility that other rehabilitation treatments would influence these measures after ICH. Furthermore, it should be noted that we administered BrdU relatively late after ICH, and we did not assess multiple survival times. Thus, while our results suggest that rehabilitation does not work through enhancing cellular proliferation and differentiation, the findings certainly do not exclude a possible role, especially in spontaneous recovery. Indeed, this is indicated by the significantly increased number of GFAP+ and Iba1+ cells found in the injured hemisphere. Finally, while there were significantly fewer GFAP+ cells in the ipsilateral cortex of REHAB rats, it seems unlikely that this lone difference accounts for the behavioural effects, but further study is warranted.

We did not observe a protective effect of rehabilitation on lesion volume (experiment 2), which we had previously found (12, 14). Given the general similarity in methods, it is difficult to reconcile these findings, but it might relate to differences in initial insult severity and/or the greater demands of testing (tray task) used in the second experiment. We also cannot exclude the possibility that rehabilitation reduced tissue loss in experiment 1. This was not measured due to processing the tissue with

Golgi-Cox stain. Nonetheless, the present findings and our previous work (14) suggest that the neuroprotective effect of rehabilitation, which sometimes occurs, does not solely account for the functional benefit of rehabilitation.

In summary, rehabilitation improves functional recovery after ICH in rodents and this effect has at least some overlapping mechanisms to those engaged after ischemic and traumatic brain injury. Further study is needed to identify factors unique to ICH so that the efficacy of rehabilitation can be maximally enhanced thereby minimizing disability in survivors of ICH.

Figure 5-1: Skilled reaching (A) and walking ability (B) data in experiment 1 show that rehabilitation lessened impairment (*p < 0.05). There was no effect in the cylinder test (C; 50% is normal).

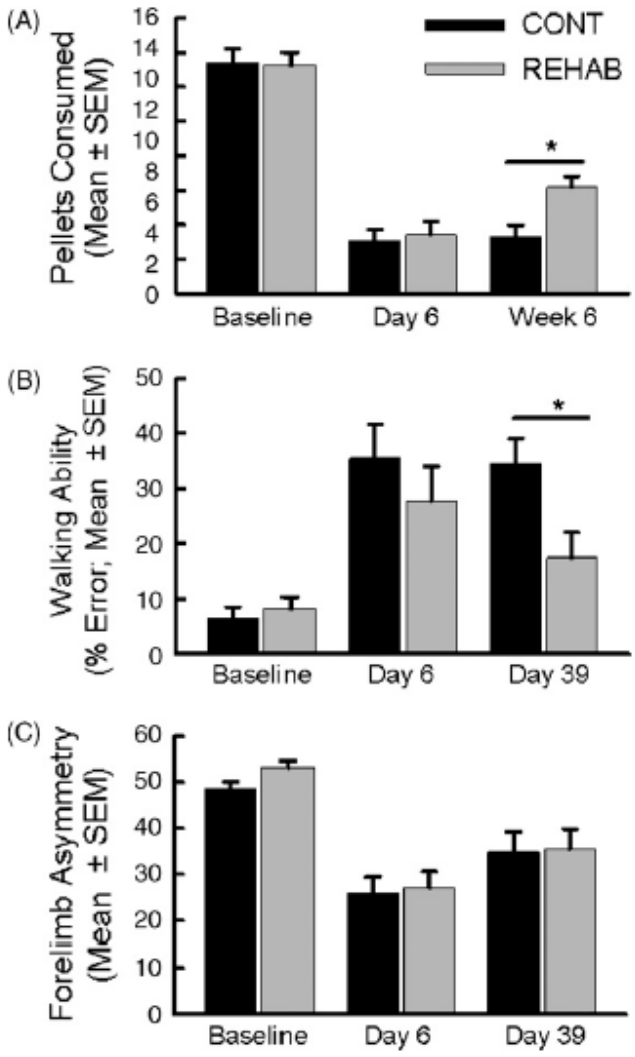


Figure 5-2: Data in experiment 2 for the staircase test (A), tray task (B), horizontal ladder (C) and cylinder task (D). Rehabilitation improved outcome on several tests (*p<0.05), but not on the cylinder task (50% = normal performance).

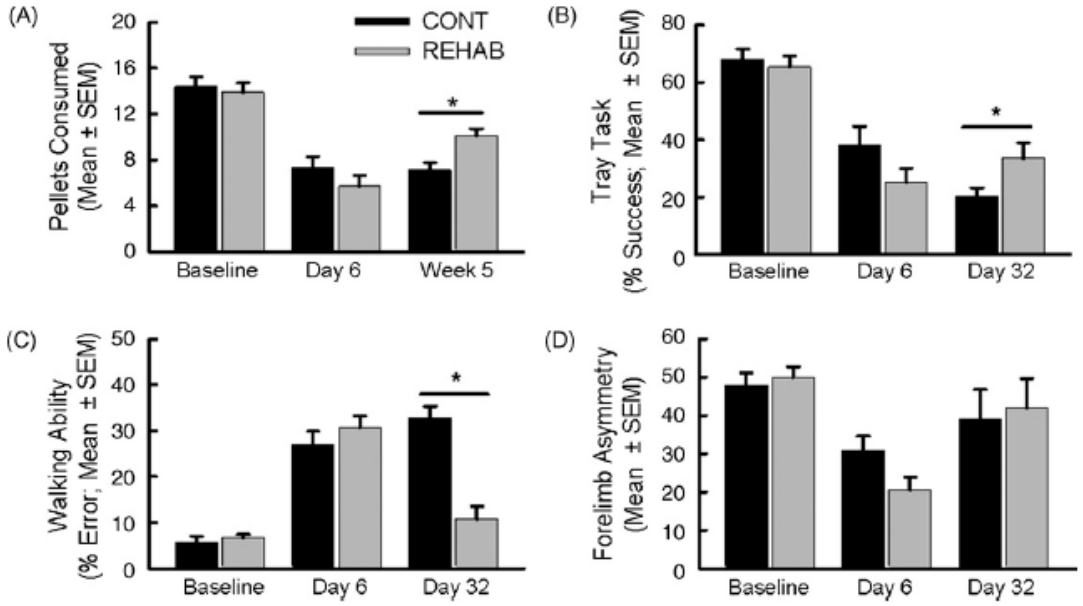


Fig 5-3: (A) An example of Sholl analysis where the number of ring intersections is assessed for each neuron, this is a standard measure of dendritic length for Golgi-Cox stained neurons. (B) The mean number of circle crossings in each group were taken in experiment 1 (*p < 0.05 for group comparisons). The ipsi- and contralateral hemispheres were significantly different. (C) Representative examples of camera lucida drawings of neurons from the ipsilateral primary motor cortex (C) and striatum (D).

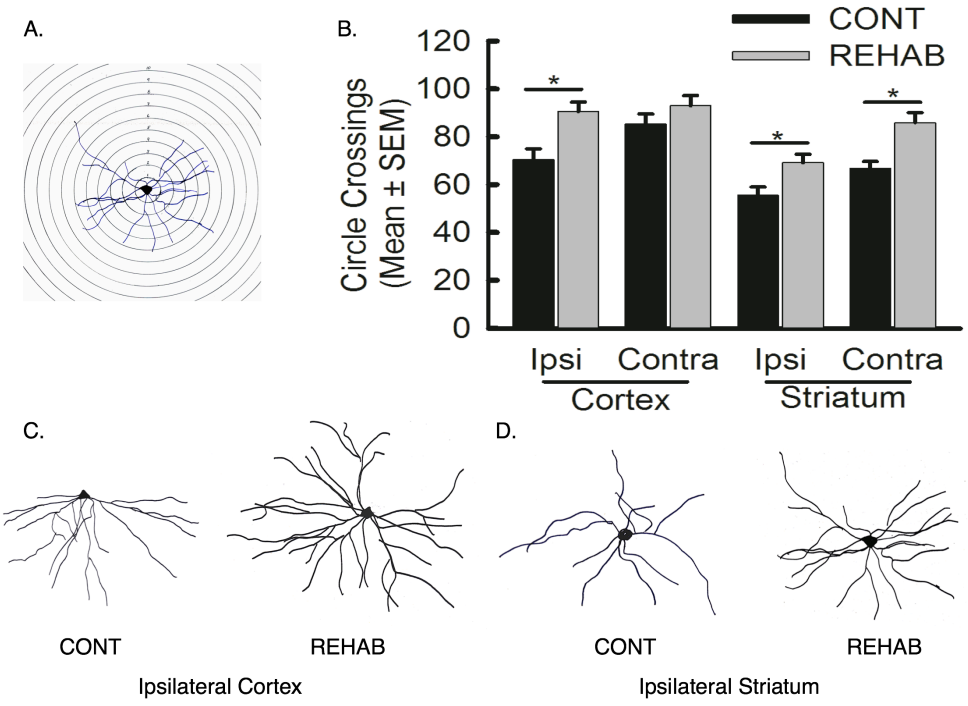


Figure 5-4: Diagram illustrating the location of cell counts (A), with examples of BrdU (B), GFAP (C), and Iba1 (D) positive cells taken at 40× magnification.

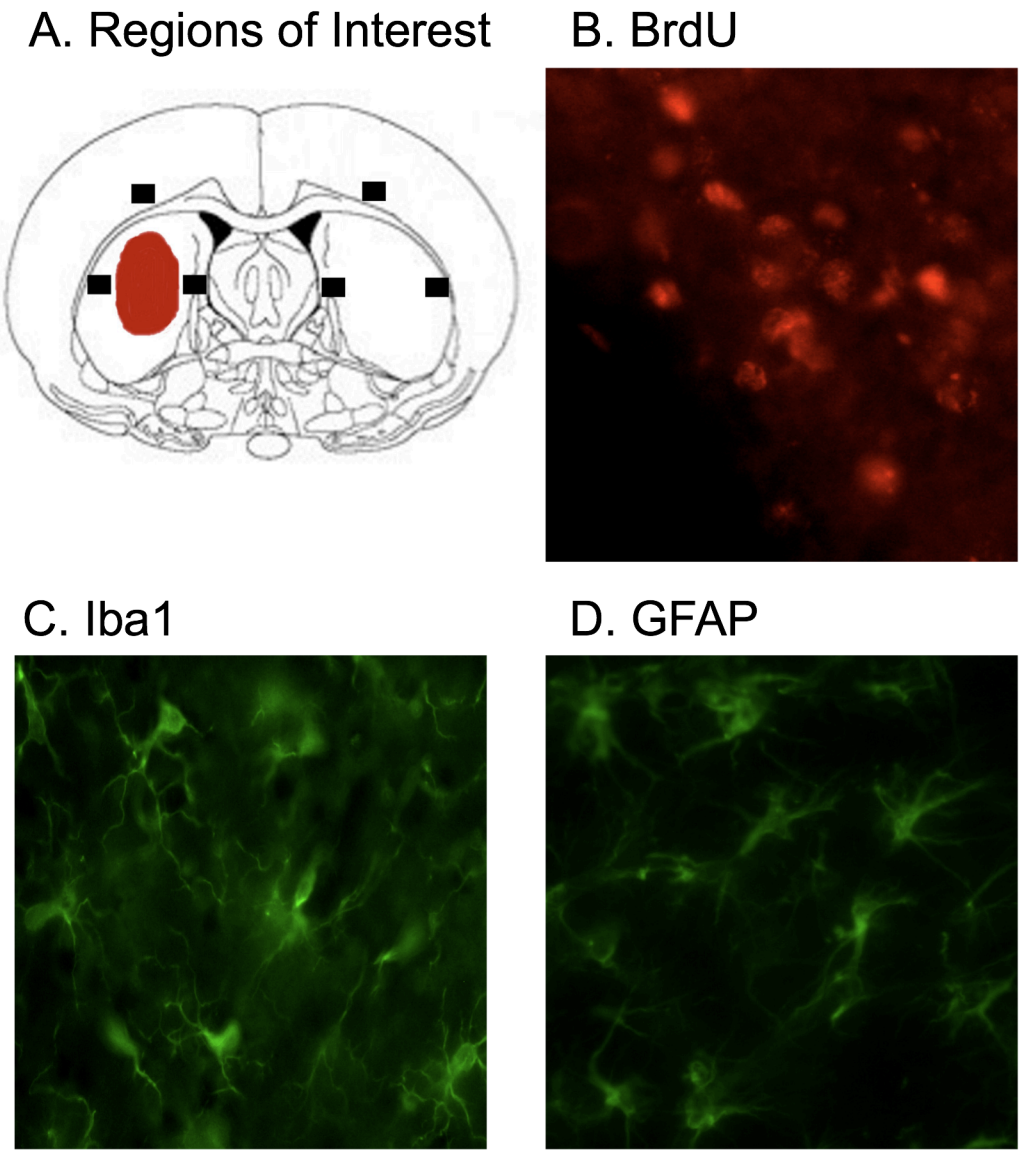
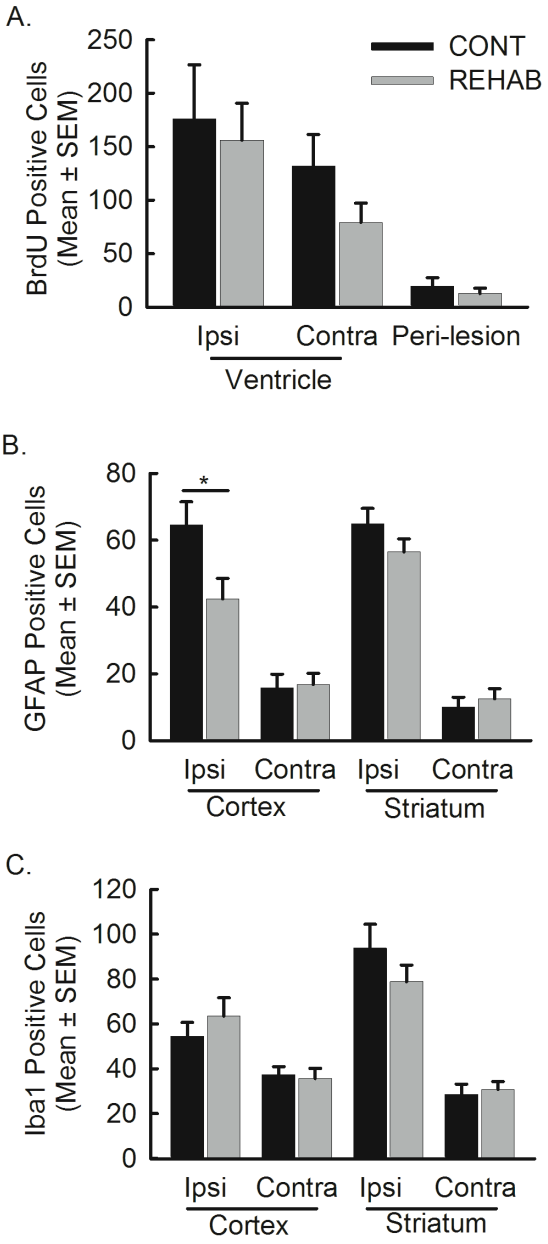


Figure 5-5: The numbers of BrdU+ (A), GFAP+ (B) and Iba1+ (C) cells counted in experiment 2 (see Fig. 3a for ROIs). There were significantly more of these cells in the hemisphere subjected to ICH, but there were no group differences except for GFAP+ cells in the ipsilateral cortex (*p < 0.05).



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Chapter 6:

Discussion

6-1 Introduction

The vast majority of basic and clinical research neglects hemorrhagic stroke, focusing instead on ischemia. The goals of my thesis were to identify treatments that improve recovery after ICH, and to elucidate the mechanisms through which they affect outcome. I assessed three treatment protocols, which improve outcome after ischemia (1-3), and found that only the combination of environmental enrichment (EE) and reach training improved recovery after ICH (4-6). We also confirmed that rehabilitation can reduce lesion volume after ICH (5, 7). Measures of brain plasticity (dendritic length and spine density assessed with Golgi-Cox staining), and cell proliferation (via BRDU injections), indicated that there are both similarities as well as differences between ischemic and hemorrhagic recovery mechanisms. Specifically, enhanced plasticity occurs in both cases but the specific pattern of changes differs (1, 8). Together these studies indicate that stroke type contributes significantly to recovery and should be taken into consideration when treating stroke.

6-2 Rehabilitation

6-2.1 Exercise

In chapter 2, exercise prior to and/or following ICH did not alter outcome (4). This is in contrast to several studies that find a preconditioning effect when exercise is given prior to ischemia (9, 10). Exercise increases angiogenesis and several antithrombotic factors, which

may reduce ischemic insult by enhancing collateral blood flow, but may also enhance bleeding during ICH (11). However, exercise pre-treatment did not alter cerebral blood volumes at 12 hours after collagenase injection (4). Therefore, the lack of benefit in the Pre&Post running groups can not be explained by greater bleeding. Additionally, similar lesion sizes were observed in all groups, this would be unlikely had there been differences in bleeding. It is possible that greater amounts of exercise prior to ICH could aggravate bleeding, this possibility should be assessed in future studies.

Doubling the amount of running prior to and following ICH worsened walking ability in the ladder test. This finding cautions against assuming that more therapy is always better. Exercise is physically demanding and increasing its intensity may result in elevated stress. Running can increase circulating corticosterone levels (12), which aggravates ischemic stroke (13, 14). The increased intensity may have been detrimental for a number of reasons such as difficulty of the task, size of lesion, location of injury, or type of injury. To avoid the risk of aggravating outcome, future studies should assess which factors can cause a detrimental effect when combined with intense therapy.

We hypothesized that if the ICH injury was too large, exercise would not be beneficial. However, when lesion severity was reduced exercise remained ineffective. It appears that lesion severity was not the primary reason for the failure of exercise. Although, the reduced lesion size was moderate in comparison to other ICH studies, it still remained

large in comparison to the typical ischemic injuries used to assess exercise (10, 15). Given that lesion size and location normally vary between ischemic and hemorrhagic injuries it is difficult to control for this factor. Future studies matching for these factors are needed to directly compare the effects of running on recovery after ischemia and hemorrhage.

6-2.2 Amphetamine and Environmental Enrichment

Amphetamine (AMP) is a therapy of interest primarily because it is frequently assessed in stroke patients, despite contradictory results in animal models of stroke (16). Many basic research studies, using multiple species and models of injury, have found dramatic improvement from pairing rehabilitation with amphetamine treatment (2, 17-21). However, little was know about the effects of AMP on ICH. We found that AMP alone or in combination with EE and rehabilitation training did not improve recovery after ICH (6).

Amphetamine acts through stimulating the release of norepinephrine and dopamine, which modulate neuronal function, enhancing cortical excitation (22). However, the exact mechanisms behind AMPs facilitation of recovery remain unclear. Data from rodents have indicated the role of neuronal plasticity (23) and reversal of widespread metabolic depression (24). It is unclear if these mechanisms of recovery

are less important after ICH, or if differences in lesion location and size resulted in AMP's decreased efficacy.

Previous findings indicate that it is necessary to combine AMP with rehabilitation training (19). We used a combination of task specific training (beam walking and reach training) and EE after AMP or control treatment. We selected EE as a part of the therapy because of its effects on naïve and brain injured rats, resulting in increased, cortical thickness, and dendritic spines and length (25-28).

Environmental enrichment improved recovery after ICH, but only on walking tests (beam and ladder). Although reach training was given on days 7, 9 and 11, after saline or AMP injections, it did not improve skilled reaching. More task specific training may have improved recovery; this was assessed in chapter 4.

6-2.3 Reach Training and Environmental Enrichment

In chapter 4, delayed rehabilitation, the combination of EE and reach training, was shown to improve skilled reaching ability and significantly reduce lesion volume (5). Recovery from brain injury is enhanced by EE, likely utilizing enhanced neuronal plasticity in uninjured brain areas (29). On its own, EE does not improve skilled reaching ability after focal ischemic injury or ICH in rats (28, 30). Biernaskie and Corbett (1) found that following middle cerebral artery occlusion the combination of EE and reach training improved skilled reaching ability after middle

cerebral artery occlusion in rats. We used a modified version of this therapy to improve recovery and decrease lesion volume after ICH.

The collagenase model of ICH produces delayed atrophy, as lesion size more than doubles between 7 and 60 days (34); similar lesion growth also occurs in stroke patients (31, 32). Delayed rehabilitation interventions may decrease this delayed atrophy, or perhaps diminish lesion volume by enhancing cell proliferation. Chapter 5 of this thesis attempted to clarify the cause of the observed reduction in lesion volume.

6-3 Mechanisms of Recovery and Rehabilitation

The reduction in lesion volume observed in chapter 4 was not replicated in chapter 5. Given the similar interventions used in the two chapters there is no apparent reason for the conflicting findings. Lesion sizes were similar in the two studies; the control group in chapter 4 had a lesion volume of 32.23 ± 2.47 mm^3 versus 27.79 ± 2.50 mm^3 for the control group in chapter 5. The second study used both the staircase test and tray test to assess reaching ability, therefore it is possible that this increased volume of training prior to injury or extra testing following ICH affected lesion volume. Future studies are required to identify why in some cases rehabilitation after ICH can reduce lesion volume (5, 7), but fails in others (8). Regardless of this unexpected finding the studies in chapter 5 provided substantial knowledge about plasticity changes and cell genesis that occurs after ICH.

6-3.1 Plasticity

Dendritic complexity is increased in the primary motor cortex of the injured hemisphere following ICH (8). We assessed layer V pyramidal neurons because they are the major output neurons controlling skilled reaching. It seems reasonable that the reach training contributed significantly to the enhanced dendritic complexity in the cortex ipsilateral to injury, although EE may have also contributed. There are no changes associated with rehabilitation in the primary forelimb motor cortex in the uninjured hemisphere; this differs from ischemic findings, which may be a result of different stroke type or lesion location (1, 33). In these cortical ischemia studies it was impossible to assess the primary forelimb motor cortex in the injured hemisphere as this area was in the core of the infarct. Clinically, large lesions to the primary motor cortex are frequently associated with the involvement of the unimpaired hemisphere in recovery (34).

Rehabilitation increased the complexity of medium spiny neurons in the striatum in both the peri-lesion region as well as in the corresponding region of the contralateral hemisphere. Similar plastic changes may be responsible for supporting recovery in patients. Enzinger et al. studied how body-weight supported treadmill training affected brain activity in subcortical stroke patients. Functional improvements after treadmill training were associated with changes in activation of cortical areas in the

injured hemisphere, and in bilateral activation of subcortical structures (35).

6-3.2 Cell Proliferation

Cell proliferation is not enhanced by rehabilitation following ICH (8). Only two other studies have looked at the effects of rehabilitation on cell proliferation after ICH. The first found that forced exercise, after ICH increases cell proliferation in the dentate gyrus in hyperglycemic rats (36). The second concluded that voluntary exercise for at least 3 weeks following ICH increases proliferation in the subventricular zone, and migration toward the injured area (37). More extensive research has been completed looking at the effects of rehabilitation on cell proliferation after ischemic injury. Some groups find that rehabilitation increases cell proliferation and neurogenesis whereas others fail to find such effects (38, 39). Several factors contribute to the conflicting results, (i.e. degree and location of injury vary), as does the time and number of BrdU injections. More detailed and controlled comparisons of ICH and ischemic injury must be made to determine if injury type affects cell genesis and survival. Stroke type could affect cell genesis by altering the amount of proliferation, the location of cell migration, the time-course of cell proliferation, or altering cell survival. Given the differences in pathophysiology of injury it seems likely that the microenvironment affecting cell proliferation and survival will differ. Greater understanding of the role of stroke type on

stem cell response following injury and rehabilitation could provide great insight into how to most efficiently enhance recovery.

6-4 Limitations

Although the work within this thesis addresses several important issues relating to recovery after ICH there are several limitations to the findings, and areas that need further study. Several of the criticisms can be applied to the majority of work done in the stroke recovery field, and must be taken into consideration when interpreting findings. None of the studies contained in this thesis can establish causation. For instance when reviewing the findings from chapter 5 we can not conclude that the changes in dendritic complexity in the rehabilitation group resulted directly from rehabilitation or that these changes accounted for enhanced recovery. When assessing mechanisms involved in recovery it is very difficult to parse out one individual piece of this process. Many changes in the brain can contribute to improved function, and it is impossible to address all processes in a single study.

The staircase and tray tests do not differentiate between compensation and true recovery, regaining the same function with unimpaired movements. Each component of skilled reaching can be analyzed in the single pellet test, to determine if any compensatory movements are being made (40). In all studies within this thesis functional impairments on the reaching tests remained suggesting that none of the

groups showed complete recovery. Single pellet analysis may have identified specific components of the reaching that were making the biggest contribution to the remaining deficits as well as indicating the areas that were improved in the rehabilitation group. Although the increased training and testing required for the single pellet test may have influenced our findings, acting as rehabilitation for both groups.

Durations, delays and intensity should all be studied prior to excluding the benefits of a therapy. The protocols selected in chapters 2 and 3 were used to parallel treatments that worked for ischemia. Each aspect of the therapy either on its own or by interacting with the other factors could affect the efficacy. It would be very difficult to investigate all these issues in one study; therefore we had to select the protocol we believed would have the greatest probability of success.

We used Golgi-Cox staining to assess plasticity. It is possible that looking in different areas or with different techniques may have indicated a different pattern. For instance in vivo two-photon imaging has been used to assess changes in dendritic complexity and spine numbers after ischemia but these techniques are limited to cortical structures (41, 42). The same argument can be made for the selection of behavioural tests. Although, no benefits were seen at specific times with select tests, we can not preclude the possibility that functional performance was not enhanced at different times or on different tests. It is impossible to test all

combinations, and we avoided excessive testing as it may act as rehabilitation (15).

Finally, my work emphasized the need to study recovery after ICH as it may differ from ischemic injury. However, I did not directly compare the two types of stroke. These comparisons, matching lesion size and location, are important to determining differences in the recovery profiles, and response to treatment, although they do ignore the normal differences in lesion size and placement. Comparisons of stroke type would be influenced by which stroke models and injury locations are selected. My preliminary work comparing endothelin-1 (a vasoconstrictant used to model ischemia) and collagenase injections found that because of backflow of liquid out of the injection site, the placement of cortical injury was too variable. Perhaps the use of subcortical injuries would reduce the variability and allow for direct comparisons between these models.

6-5 Future Directions

There are many exciting areas of research that have yet to be fully explored. Blood breakdown components produce an extended period of toxicity following hemorrhage (43). Would decreasing the delayed toxicity of blood components in combination with early rehabilitation enhance efficacy? Mitigating some of the early neurological symptoms that resolve over time, such as edema and elevated intracranial pressure, could allow ICH patients to participate in rehabilitation earlier. Further studies would

have to confirm that earlier intervention is better in these cases and that the brain is primed at this time for plastic changes that contribute to recovery (44).

Growth factors are important to plasticity, and likely contribute to ICH recovery. Artificially enhancing plasticity by injecting growth factors such as brain-derived neurotrophic factor (BDNF) may improve recovery. BDNF is important to neuroplasticity in both the intact and damaged brain (45). When given soon after ischemia BDNF decreases cell death and lesion volume (46, 47), and can also improve long-term functional recovery (48-50); whereas interfering with normal BDNF function impairs recovery after ischemia (51). Future work would be needed to determine if administration of BDNF with a relevant behavioural therapy could augment recovery after ICH.

6-6 Summary

The finding of this thesis support the idea that there are important differences between ischemic and hemorrhagic stroke patients, and that rehabilitation may need to be customized for each type of stroke. Both Exercise and amphetamine failed to improve outcome after ICH, conflicting with findings in ischemia (2-4, 6, 10). Injury processes interact with recovery mechanisms to alter outcome. Developing a greater understanding of these factors will improve stroke rehabilitation. We used intervention delays and intensities similar to those used in ischemia; it may

simply be a case of adjusting these factors to make the treatments beneficial for hemorrhage. Identifying the ideal treatment parameters for ischemic and hemorrhagic patients will increase recovery.

Rehabilitation improves outcome after ICH. Similar to clinical findings task specific training is required to improve reaching ability (52). In some cases lesion volume can be reduced by rehabilitation (5, 7), perhaps due to an increase in dendritic complexity (8). Cell proliferation does not appear to be elevated after rehabilitation for ICH, although it is necessary to assess more times to make any certain conclusions. Rehabilitation enhances dendritic complexity in several brain regions and likely contributes to improved recovery. Treatments that can augment rehabilitation-induced plasticity may be able to provide substantial functional benefit for stroke patients. Researching the mechanisms of recovery after stroke is a promising area of investigation but it is important to be mindful of different types of injury, and how this may interact with rehabilitation.

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