EFFECTS OF ENVIRONMENTAL ENRICHMENT ON ISCHEMIC TOLERANCE

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Effects of Environmental Enrichment on Ischemic Tolerance

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A thesis submitted to the
School of Graduate Studies
in partial fulfillment of the
requirements for the degree of
Master of Science

Faculty of Medicine
Memorial University of Newfoundland
December 2001

St. John’s Newfoundland
Abstract

In this study, we sought to determine whether environmental enrichment could alter the pattern of delayed CA1 cell death and functional impairment in a gerbil model of ischemic tolerance. Gerbils received either ischemic preconditioning, 5 minutes of ischemia or sham surgery. Three days after ischemia, gerbils were placed in either an enriched environment or standard laboratory housing. Animals were tested in an open field arena followed by two versions of a T-maze task. Following behavioural testing, extracellular CA1 field potential amplitudes and CA1 cell counts were determined. In open field testing (day 60), enriched, ischemic preconditioned and ischemic gerbils were not different than shams whereas, non-enriched, ischemic preconditioned and ischemic gerbils continued to have higher activity scores. Preconditioned and enriched ischemic animals learned the win-shift T-maze problem as quickly as shams whereas, only the enriched preconditioned group acquired the win-stay paradigm. Surprisingly, CA1 cell counts were significantly lower in enriched versus non-enriched ischemic preconditioned animals.

These data demonstrate that early, intensive intervention after ischemia can improve functional outcome but this is accompanied by increased brain damage.
Acknowledgements

I would like to thank Dr. Dale Corbett, my supervisor, for his guidance and encouragement.

I thank Sue Evans for her excellent ideas and incredible technical support.

I thank Drs. Carolyn Harley and Penny Moody-Corbett, members of my supervisory committee, for their invaluable advice, as well as all of the members of the Corbett lab for their unique contributions to the project.

I must also thank Tina Giles for her friendship and good humour.

Finally, I thank my husband Ian and my parents for their exceptional and unconditional love and support during my graduate program.
PUBLICATIONS

Portions of this thesis have been published or submitted in the following:

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Introduction

Stroke

Stroke (cerebral ischemia) is one of the leading causes of death and the leading cause of permanent disability in Canada. To date there are very few options for the treatment of patients after an ischemic episode. The paucity of viable treatment options makes research in ischemia critical to the survival and quality of life of stroke victims.

According to the Canadian Heart and Stroke Foundation (1995), for each death due to a stroke, 3 victims survive but suffer from neurological after-effects, which necessitate extended hospitalization and rehabilitation therapy.

The types of stroke seen in humans are predominantly either hemorrhagic or ischemic with ischemic episodes being the most prevalent (90%) (Hunter, Green et al., 1995). Ischemic stroke is either focal, a local decrease in cerebral blood flow via a blockage in a major artery, or global, the loss of blood to a large area of the brain as seen in cardiac arrest/hypoxia. The types of deficits experienced by victims vary with the type of stroke. Depending on the area of ischemia, victims of focal ischemia may exhibit difficulties in speaking, moving an arm or a leg, or have problems with coordination. Global ischemia victims usually experience a profound anterograde amnesia leaving them with an inability to form new memories (i.e. loss of declarative memory) (Zola-Morgan, Squire, et al. 1986; Petito, Feldman, et al. 1987).

Research on stroke has focused both on preventing the occurrence of stroke and on improving the outcome for stroke victims. Many risk factors have been identified as being predictive of a stroke including smoking, obesity, high blood pressure and high
cholesterol. Thus, one of the main lines of attack against the disease has been to reduce the prevalence of these risk factors in the general population. Some success has been achieved in the prevention arena through increases in physical activity, use of aspirin (inhibits platelet aggregation) and antihypertensive medications (McMahon et al., 1991; Gubitz et al., 2000).

Another approach is to shorten the ischemic episode by restoring blood flow to the affected area. Indeed, the only effective treatment currently employed during an ischemic attack is tissue plasminogen activator (t-PA) (Caplan, 1999; Tanne et al., 1999; Buchan et al., 2000; Wang et al., 2000). This thrombolytic therapy works by dissolving blood clots obstructing arteries and must be given within 3 hours of the onset of stroke. Obstacles in applying this treatment stem from victims not recognizing the symptoms of a stroke and medical personnel not treating stroke as an urgent medical emergency, subsequently delaying medical attention. Thus, t-PA therapy benefits only a small group of patients (<5%) (Buchan et al., 2000).

Another strategy has been to rescue neurons after ischemia using neuroprotective drugs. Many of these drugs appeared protective in animal models so these compounds were advanced to clinical trials. None of these drugs have been effective in stroke patients (De Keyser, Sulter et al., 1999; Dirmagl, Iadecola, et al., 1999). Therefore, for the majority of stroke patients the best hope for long-term functional improvements is through rehabilitation.

The focus of this thesis is on the rehabilitation and promotion of recovery of function in the gerbil model of global ischemia.
**Neuroprotection**

Brief (3-5 min) occlusions in the gerbil model of global ischemia produce severe but selective damage to the hippocampus (Kirino, 1982). The gerbil is a valuable species in which to study global ischemia due to gerbils incomplete Circle of Willis (lacks posterior communicating arteries). As such, clamping of the carotid arteries produces an effective and complete blockage of blood flow to the forebrain (Levine and Payan, 1966).

Neurons of the hippocampus are selectively vulnerable to global ischemia with the most susceptible dying within hours of the occlusion (e.g. dentate hilar, CA2 and subicular neurons). CA1 neurons undergo a process of delayed neuronal death (Kirino, 1982) wherein they recover from the occlusion and die 2-4 days later. Other regions of the hippocampus are much more resistant to global ischemia episodes (e.g. CA3 and dentate gyrus) and will tolerate 15-30 min of ischemia without appreciable cell loss.

Animals with loss of CA1 neurons show impairment in working memory and habituation (Colbourne & Corbett, 1995). A similar pattern of cell loss seen in humans after cardiac arrest/hypoxia, results in profound cellular loss in the hippocampus (specifically CA1) producing anterograde amnesia (Zola-Morgan et al., 1986).

In global cerebral ischemia, “delayed neuronal death” affords a therapeutic window in which many potential neuroprotective compounds have been administered in the hopes of preventing cellular demise. Intuitively, an attempted rescue of dying cells would either counteract the deleterious cellular events which occur following an ischemic episode, (e.g., increased glutamate release, increased intra-cellular Ca²⁺) or enhance the events that are down-regulated and potentially beneficial after an ischemic episode (e.g. gamma-
aminobutyric acid (GABA) input). To that end, many different potential neuroprotective interventions have been investigated.

One prominent theory of ischemic cell death is glutamate excitotoxicity (Rothman & Olney 1986; Choi 1988). The brain has an extremely high metabolic rate consuming high levels of oxygen and glucose for production of energy (Dirnagl. Iadecola, et al., 1999). Ischemia results in a rapid depletion of brain ATP-stores. Thus the ionic gradients cannot be maintained allowing a large influx of Na⁺ and efflux of K⁺ triggering the depolarization of neurons and glia. Subsequently, there is a massive release of glutamate (increases 15 fold post-ischemia: ~ 28 pmol/µL) into the extracellular space (Mitani & Kataoka, 1991). The rise in extracellular glutamate causes excessive stimulation of glutamate receptors (AMPA, NMDA and kainate sub-type). This altered state allows for an influx of calcium into neurons via NMDA receptor-gated ion channels accompanied by other ions (e.g. Cl⁻) and water. Moreover, ischemia further increases the cytosolic levels of free Ca²⁺. These abnormally high levels of Ca²⁺ are thought to trigger cellular death cascades (Choi, 1988; Siesjo & Agardh et al., 1989).

Consequently, the use of glutamate antagonists was proposed to effectively limit the amount of glutamate active in the cellular environment. Initially, the glutamate antagonist (NMDA sub-type) MK-801 was reported to be effective in preventing neuronal loss after a global ischemia attack (Gill, Foster, et al. 1987). However, Corbett et al. (1990) revealed that the neuroprotection seen with MK-801 stemmed from the protracted hypothermia induced by this drug. When these hypothermic actions were controlled, the neuroprotective action was lost. Furthermore, Valtysson and colleagues
(1994) reported that MK-801 does not have a beneficial effect on infarct size in focal ischemia. Valtysen et al. measured infarct size at 3 and 28 days after focal ischemia. At 3 days there was a 40% decrease in infarct size compared to placebo treated animals. However, at 28 days there was no significant difference between the MK-801 treated animals and controls indicating that there was no lasting protective effect (Valtysson, Hillered, et al., 1994).

A second glutamate antagonist NBQX (AMPA antagonist) was reported to be effective in preventing delayed neuronal death (Sheardown, Nielsen, et al., 1990; Frank, Bruhn, et al., 1993). However, these data were assessed at relatively early survival times (e.g. 4 - 7 days post-ischemia). Moreover, when survival times were extended, protection declined (Nurse & Corbett, 1996) and by 28 days was lost altogether (Colbourne, Li, et al., 1999).

Further efforts to arrest delayed neuronal death have focused on blocking the entry of Ca\(^{2+}\) into the post-synaptic cell. Ca\(^{2+}\) entry blockers like nimodipine (Germano et al., 1987) and Verapamil (Hunter, 1997) were proposed to halt the lethal influx of Ca\(^{2+}\) due to the overactivation of glutamate receptors coupled to ion channels (Choi, 1988). Unfortunately, each was proven ineffective at preventing damage in focal ischemia models (Harris et al., 1982; Reedy et al., 1983). Nimodipine was also shown to possibly increase the susceptibility of brain tissue to ischemic damage (Harris et al., 1982).

Colbourne et al. (1999) demonstrated that the Ca\(^{2+}\) channel antagonist SNX-111 simply delayed but in no way prevented CA1 cell death when assessed 28 days post ischemia.

An alternative approach is to increase the inhibitory GABA-ergic input in the hippocampus and counteract the post-ischemic excitotoxic input. Specifically the use of
the drug diazepam, a compound which enhances GABA\(_A\) transmission, has been reported to be neuroprotective in the gerbil model of global ischemia (Schwartz, Huff. et al., 1994). In the experiment conducted by Schwartz et al. (1994) gerbils were subjected to 5 minutes of global ischemia then given diazepam either once (30 minutes post-ischemia) or twice (30 and 90 minutes post-ischemia). When assessed at day 7, 62% of one-dose diazepam gerbils had virtually all CA1 neurons remaining and 67% of the two-dose diazepam gerbils had completely protected CA1 pyramidal neurons. More recently a study by Dowden. Reid. Dooley and Corbett (1999) demonstrated that, while somewhat efficacious in preventing neuronal death, the neuroprotective effect of diazepam arises from its hypothermia-inducing effects (e.g. lowering temperature to 28° - 32° C). The basis for this hypothermic action is unknown, however it may be due to the well-known sedative effects and metabolic depression of this and related drugs particularly at the doses employed. This study revealed that when brain temperature was not regulated during and after a 5 minute episode of global ischemia there was significant cellular and behavioral protection at 10 days with diazepam administration. However, when animals were maintained at a normothermic brain temperature, there was a reduction in the cellular preservation and a complete loss of the behavioral protection in diazepam treated animals (Dowden. Reid. et al., 1999).

Thus the drugs that “appeared neuroprotective” in animal models were never truly neuroprotective. The drugs either provided protection by inducing hypothermia or in other cases delayed rather than prevented cell death. This latter effect became apparent when survival times were extended beyond one month (Corbett & Nurse, 1998). As
pointed out in this and other studies (e.g. Buchan et al., 1990; Corbett et al., 1990) when hypothermic effects are eliminated there is no significant protection obtained. Moreover, these drugs (e.g. diazepam) do not produce hypothermic effects in humans, due to the large difference in body size in humans versus rodents. Therefore it is imperative that confounding hypothermic effects in rodent stroke models be eliminated.

**Ischemic Preconditioning**

Delayed or postponed cell death is not unique to purportedly neuroprotective drugs, it is also seen with ischemic preconditioning. Ischemic preconditioning (ischemic tolerance) is the acquisition of tolerance to a severe ischemic insult by the prior administration of brief episodes of ischemia which alone do not produce any appreciable cell death or loss. Initially, this phenomenon was discovered in the heart (Kuzuya et al., 1993: Lawson & Downey, 1993; Baxter, Yellon et al., 1994) and was first demonstrated in brain by Kitigawa and colleagues (1990) in both rat and gerbil models of global and focal cerebral ischemia (Kitagawa, Matsumoto et al., 1990; Corbett & Crooks, 1997; Toyoda et al., 1997; Dooley & Corbett, 1998; Stagliano et al., 1999). Focal ischemic models have demonstrated similar protection in several species when shorter episodes of ischemia precede a more severe episode. For example, focal ischemic preconditioning has been shown to produce "rapid tolerance" to a middle cerebral artery (MCA) occlusion in mice (Stagliano et al., 1999). Similarly, Toyoda et al. (1997) showed that Wistar rats subjected to a 20 minute focal ischemia (MCA-occlusion) followed 24 hours later by a 1 hour focal episode had a significant reduction in infarct volume.
The protection conveyed by preconditioning episodes depends on their duration and the length of time between the preconditioning episodes and the final more severe ischemic insult. In global ischemia, effective preconditioning paradigms include 2 minutes of ischemia or 2 episodes of 1.5 minutes followed by a severe (e.g. 5 min) insult (Kitigawa, Matsumoto et al., 1990). The repeated ischemic preconditioning episodes must be separated sufficiently (>24 hours) so as not to act cumulatively and inflict cellular damage (Kitigawa, Matsumoto et al., 1990). Also, the severe ischemia must be administered at least 24 hours after but within a week of the ischemic preconditioning in order for the cells to be protected (Tomida et al., 1987). The preconditioning durations used in the present experiment consisted of two 1.5-minute occlusions 24 hours apart followed 72 hours later by a 5 minute occlusion.

In the gerbil model of ischemic preconditioning substantial neuroprotection is seen at day 10 (i.e. preconditioned animals CA1 cell counts are ~80% of sham). However, at 30 days, cell survival dropped to 50% of sham (Corbett & Crooks, 1997). Further studies have demonstrated that death of CA1 neurons continues in a preconditioning model at least to post-ischemic day 90 (Dooley & Corbett, 1998).

As a clinical treatment, ischemic preconditioning is obviously not practical. However, if the protective mechanisms underlying ischemic preconditioning can be identified this may lead to the development of effective pharmacological interventions, which could protect the brain from ischemia.
Functional Outcome Measures

Many studies have drawn conclusions on the effectiveness of post-ischemic interventions based solely on the quantification of cellular loss and/or infarct volume. This approach assumes erroneously, that the histological evaluation is directly indicative of treatment success or failure (Colbourne & Corbett, 1995). To effectively evaluate a treatment paradigm one must use more than one experimental endpoint. Assessing the functionality and viability of remaining cells with behavioral testing and electrophysiological investigation combined with the power of histological analysis gives a more complete picture of the true state of the remaining cells (Nurse & Corbett, 1994; Corbett & Nurse, 1998). Case in point: a paradox exists between the histological and behavioral data obtained with ischemic preconditioning, especially at 10 days. At this point there is close to total histological preservation but the amplitudes of CA1 dendritic field potentials are reduced and animals exhibit high levels of open field activity (behavioral assessment). Typically, these electrophysiological and behavioural abnormalities are observed in animals with near total CA1 cell loss (Dooley & Corbett, 1998). Thus it appears that many of the "protected" neurons are functionally abnormal.

The degree of damage seen with the global ischemia model can be assessed using several behavioral tests that include the Morris water maze, T-maze and open field. In the present experiment both the T-maze and open field were used to assess behavioral outcome. Introducing the animal to a novel open field (10 minute trial) starting on day 3 post-ischemia has been shown to be a reliable means of distinguishing a normal animal from an ischemic animal (Corbett & Nurse, 1998). Typically, an ischemic animal differs
from a normal in two ways. Firstly, the level of activity of an ischemic animal is much higher than a sham. Secondly, during the 10 minute session a minute by minute analysis reveals that ischemic animals habituate slowly to the novel open field whereas normal animals show a marked habituation even during the first trial (Corbett & Nurse, 1998). When gerbils are exposed to a novel open field environment after ischemia they demonstrate increased locomotor activity which continues over many test days. In a sense, the ischemic gerbils continue to treat the open field as novel and persist in exploring the environment. When gerbils were exposed to the open field prior to ischemia (5 days 10 minute sessions) and then tested for 5 days post-ischemia the increased open field activity was eliminated (Wang & Corbett, 1990) indicating that the increase in activity was not simple hyperactivity.

A second behavioral test used to assess hippocampal damage is the T-maze. The T-maze has been shown to be sensitive to hippocampal injury and assesses working memory (Volpe et al., 1988; Hagan and Beauchard, 1990) because the stimulus information that the animal receives in each trial is useful only for one trial. Previous work illustrates the ability of this test to distinguish between normal and ischemic animals. For example, in a study by Colbourne and Corbett (1995) ischemic animals showed a significant performance deficit at 1 month as compared to shams and hypothermic treated animals in a win/shift T-maze test. When trained in the win/stay version of the T-maze test the ischemic animals did not learn after 250 trials whereas all of the sham animals and half the hypothermic animals learned the new task. Other studies have also shown a correlation between the amount of CA1 cellular loss and the
ability to choose the correct arm in a delayed alternation T-maze task (Andersen et al., 1998).

Since functional outcome is the most important clinical endpoint after stroke it is important to include functional (e.g. behaviour and electrophysiology) measures in animal models. Another important reason for employing functional tests is that histological preservation does not necessarily guarantee functional preservation (Corbett and Crooks, 1997; Corbett and Nurse, 1998). Therefore, this thesis utilized behavioural, electrophysiological and histological measures that were obtained from the same animals (as much as possible).
Rehabilitation and Neuronal Plasticity

Due to the lack of effective neuroprotective treatments for stroke, interest has turned towards rehabilitation. Rehabilitation can include consistent, intensive physiotherapy, speech and/or occupational therapy or it can be poorly structured and sporadic. Indeed, there are a number of fundamental questions concerning the optimal rehabilitation treatment for stroke patients. For example, what is the best time to enter treatment? Is early intervention after stroke best or should treatment be delayed for several weeks? What specific rehabilitative exercises are helpful to a victim of stroke? Some of these questions are now being addressed by basic medical research.

Nudo, Milliken et al. (1996) showed that considerable functional reorganization occurs in the primary motor cortex of the squirrel monkey after focal ischemic infarct. A severe functional deficit was seen in the contralateral hand when tested in a reaching task coincident with loss of cortical hand movement representation in areas adjacent to the motor cortex lesion. In a subsequent experiment, post-stroke training in a skilled reaching task was shown to provide substantial recovery of function following a focal ischemic infarct in primary motor cortex (Nudo, Wise et al., 1996). The skilled rehabilitative training halted the loss of adjacent digit representation of the cortex and in some animals increased the area of the cortex representing the digits into areas occupied previously by the movements of the elbow and shoulder (Nudo, Wise et al., 1996).

Skilled rehabilitative training may be necessary to regain the fine motor skills lost after focal ischemia (Kleim et al., 1998; Biernaskie and Corbett, 2001). Rats trained in a skilled motor learning task showed an increase of the wrist and digit representations in
the motor cortex while those trained in an unskilled task did not achieve these same benefits. This illustrates the need for fine motor rehabilitation after ischemia. In fact, stroke patients rarely fully regain the ability to use their digits in fine motor tasks (Nudo, Wise et al., 1996). A recent study by Biemaskie and Corbett (2001) further highlighted the importance of skilled reach training after focal ischemia. In this study rats were given either endothelin-1 induced focal ischemia or sham surgery followed 15 days later by environmental enrichment or standard housing coupled with a daily skilled reaching task (staircase test). Ischemic enriched animals improved 30% on the skilled reach task whereas non-enriched ischemic animals showed no improvement when assessed at 4 and 9 weeks post-ischemia. Moreover, ischemic enriched animals experienced enhanced dendritic complexity and length in layer V of the undamaged motor cortex. This research reveals that enrichment must be combined with intensive skill training for animals to regain fine motor skills after focal ischemia. This body of research suggests that humans may benefit from intensive skill training after stroke perhaps increasing their chances of functional independence.

Recently, human studies have started to focus on the reorganizational abilities of the brain after stroke by using a rehabilitation method referred to as constraint-induced movement therapy (Taub et al., 1993; Kunkel et al., 1999; Liepert et al., 2000). Victims of motor stroke (focal) have their unaffected arm placed in a sling preventing its involvement in tasks, forcing use of the affected limb. Patients who have received this therapy have shown enhanced motor ability with the affected limb and have increased the cortical representation of the affected hand (Liepert et al., 2000). Constraint-induced
Movement therapy was developed to aid patients to attain a level of competence in using
the affected limb in the tasks of daily life and to help them lose their dependence upon
the unaffected extremity. Patient's reliance on their unaffected limb is a phenomenon
referred to as learned non-use (Taub et al., 1993). Learned non-use occurs when stroke
patients attempt to use their impaired limb to perform simple tasks and fail. These
patients soon stop trying to use the impaired limb and learn that it is more efficient for
them to use the unaffected limb. Constraint-induced therapy, initiated months or years
after stroke, has provided benefit in patients whose recovery has reached a plateau based
on traditional rehabilitation methods. The unaffected extremity is placed in a sling for 14
days, 10 of which are training days (6hrs/day) (Taub et al., 1993; Kunkel et al., 1999).
Constraint-induced therapy increases quality and speed of movement as well as the
amount of time the patients use the affected limb in the real world environment up to 3
months post-therapy (Kunkel et al., 1999).

Animal studies appear to be instructive in suggesting effective rehabilitative strategies
after stroke. Intensive rehabilitation may be approximated in animal models through the
use of the enriched environment paradigm. Human rehabilitation involves sensori-motor
stimulation, social interaction and exercise, as does an enriched environment in animal
studies. Environmental enrichment in the laboratory includes greater voluntary physical
activity and social interaction and provides a variety of objects for the animals to explore.
Environmental enrichment induces a host of structural and functional changes in the
brains of both normal and injured animals (e.g. Johansson, 1996; van Praag et al., 1999).
Much of what has been learned about environmental enrichment effects involves animals with brain lesions and focal ischemia. Enrichment increases behavioral abilities in animals after aspiration lesions of the frontal cortex. Kolb et al. (1991) placed rats in enriched environments (90 days) after unilateral or bilateral frontal lesions which counteracted the lesion-induced deficits when compared to lesioned, standardly housed control rats. In a study by Mattson and colleagues (1997) rats were subjected to a focal ischemic infarct and one week later given fetal neocortical grafts. Animals were housed either in enriched or standard cages. The enriched animals performed significantly better on behavioral tasks when compared to grafted standard housed animals. Therefore, the effects of the neocortical grafts enhanced functional outcome only when combined with enrichment (Mattsson, Sorenson, et al., 1997). Several experimental investigations have demonstrated improved motor function in animals housed in an enriched environment: (Ohlsson & Johansson, 1995; Johansson, 1996; Johansson, Mattsson, et al., 1997).

The effects of enrichment on global ischemia have been less thoroughly investigated. In a study by Puuronen and colleagues (1997) rodents were given global ischemia (20 minutes, four vessel occlusion (4-VO) method) and were placed in an enriched environment 3 days after the ischemia. The rats were tested behaviourally in the open field test and water maze learning task. It was found that rats housed in enriched housing showed better acquisition of the water maze learning set task after 1 week of housing. In the open field task the ischemic animals were slightly hyperactive however the ischemic enriched animals were not significantly different from shams. This study demonstrates
that enriching the post-ischemic environment appears to facilitate habituation and spatial memory formation in the rat model of global ischemia.

While environmental enrichment has been successful in enhancing the behavioral outcomes for brain injured animals, there appears to be no effect on cerebral infarct volume or hippocampal cell death (Johansson, 1996; Puurunen, Sirvio, et al., 1997). Thus, improved outcome occurs in the absence of any neuroprotective effect.

Environmental enrichment in the present experiment refers to a complex environment where animals are housed in social groups with the opportunity for voluntary physical activity. Are the effects of exercise or social interaction alone as beneficial to animals as a complex environment combining the two? As was previously mentioned exercise and social interaction provide some of the same benefits as environmental enrichment. However, the theory had not been tested in a single experiment. A study by Johansson and Ohlsson (1996) placed animals, 2 weeks after permanent MCA occlusion, in three experimental groups: (1) enriched environment; (2) social housing (standard laboratory cage); and (3) isolated standard housing with a running wheel. The results showed that while there was no difference in infarct size among the groups the greatest behavioral benefit was achieved by the animals in enriched environment. Animals exposed only to social interaction performed significantly better than those housed alone with the running wheel but not as well as enriched animals. Therefore, exposure to an enriched environment led to a significantly better functional outcome than either social interaction or isolated housing + physical activity alone.
What causes the ameliorative effects of enrichment? Many changes are observed in the brains of enriched animals when compared with animals in standard housing. Changes include increased cortical thickness, increased brain mass, enhanced dendritic branching, upregulation of growth factors and enhanced neurogenesis (Katz et al., 1984; Carughi et al., 1989; Kolb & Gibb, 1991; Ickes et al., 2000). Many growth factors are upregulated through enriched housing (Ickes et al., 2000).

Upregulation of growth factors may mediate the functional and morphological changes described above that are seen in enriched animals. Basic fibroblast growth factor (bFGF) decreases thalamic degeneration following cortical infarction (Yamada et al., 1991).

Brain derived neurotrophic factor is upregulated in cerebral cortex, hippocampus, basal forebrain and hindbrain following environmental enrichment (Falkenberg et al., 1992; Rowntree & Kolb, 1997; Ickes et al., 2000).

Nerve growth factor (NGF) is associated with cognitive functions and shown to improve the performance of rats in spatial learning tasks (Pham, Ickes et al., 1999). Pham and colleagues (1999) examined the effects of placing adult rats in long-term enriched housing on the levels of NGF in the brain as compared with non-enriched controls. NGF levels were increased in the hippocampus, visual and entorhinal cortices of enriched animals. Increased NGF levels have also been correlated with enhanced learning and memory in enriched animals (Kolb, Cote et al., 1997; Pham, Ickes et al., 1999).

The increase in growth factor levels in enriched animals may be the biochemical trigger for the morphological and functional improvements of enrichment.
The discovery of use-induced plasticity by Hebb (1947) introduced the notion that experience can lead to changes in the organization of the nervous system. Clayton and Krebs (1995) discovered that birds that store food have larger hippocampi than similar non-storing species. Jones and colleagues (1996) gave rats electrolytic lesions to the forelimb representation area of the sensorimotor cortex (FL-SMC). These rats used their unaffected forelimb preferentially and this was correlated with increases in dendritic growth, number of synapses per neuron and membrane surface area of dendritic processes in the sensorimotor cortex opposite the affected limb. It is suggested that these plasticity changes were induced via use-dependent mechanisms.

Morphological changes are also induced by exposure to an enriched environment. Increased dendritic branching and increased spine density have been documented in the neocortical and hippocampal neurons of enriched animals (Kolb & Gibb. 1991; Moser, Trommald. et al., 1997). Moser et al. (1994) showed that training in a complex environment increased the number of excitatory spines on basal dendrites of CA1 neurons and training increased the number of excitatory synapses per neuron. Kolb and Gibb (1991) examined the effects of enrichment on both normal and unilateral or bilateral frontal cortex lesioned animals. Enrichment attenuated many of the behavioural effects due to the lesion and increased the dendritic branching in the visual cortex of enriched lesioned and normal animals. Enrichment also increases the dendritic branching on basilar dendrites of pyramidal neurons in the occipital cortex when animals are placed in enrichment pre-weaning (Venable. 1989) and prevents the normal decrease in synaptic density seen in senescent animals (Nakamura et al.. 1999).
The birth of new neurons (neurogenesis) in the adult brain may have functional significance, although this has yet to be determined. This phenomenon naturally occurs in the adult brain of rodents, primates and humans alike. Both the olfactory bulb and the dentate gyrus of the hippocampus undergo mature neurogenesis (Kuhn et al., 1996; Eriksson et al., 1998; Kempermann, Kuhn et al., 1998; Gould et al., 1999). Exercise (wheel running) also increases neurogenesis in the dentate gyrus of adult rats when compared to normal rodents (van Praag et al., 1999). The benefits of exercise following ischemia are seen functionally by increased ability in behavioral tests (e.g. limb placement, beam walking) (Johansson, 1996). Environmental enrichment significantly enhances neurogenesis over the level of neurogenesis seen in wheel-running animals (Kempermann et al., 1998; Kempermann et al., 1999; van Praag et al., 1999). Recently, an experiment by van Praag, Kempermann and Gage (1999) compared neurogenesis in the dentate gyrus of normal adult mice in various conditions: water maze learning; swim-time-yoke control; voluntary wheel running; enriched housing; and standard housing. Enrichment doubled the number of surviving newborn cells compared to control animals. Of the experimental groups only the running group achieved similar numbers of surviving newborn cells as the enriched group. Nilsson et al. (1999) found that enrichment not only enhanced neurogenesis but also improved learning and memory in adult rats.

It is unknown which of the above mechanisms are critical to the production of the beneficial effects of enrichment. However, it is likely that many factors are responsible for the benefits provided by enriched environments.
This thesis employed ischemic preconditioning, a well-documented model of delayed neuronal death, such as that seen with many putative neuroprotective drugs. The protracted cell death seen in ischemic tolerance provided a unique opportunity to evaluate the neuroprotective efficacy of environmental enrichment. In addition, the effects of enrichment on previously documented functional impairments seen in the tolerance paradigm were investigated in this thesis.

Experimental Overview

At the present time, other than t-PA, there appears to be no way to prevent cell loss after ischemia. This thesis employed a post-ischemic treatment of environmental enrichment for animals after ischemia. This intervention may help identify a potential treatment or suite of treatments that could promote the function of remaining cells after an ischemic attack. In this thesis it was hypothesized that environmental enrichment might attenuate the delayed cell loss that appears in ischemic preconditioned animals and potentially eliminate the behavioral deficits that characterize this model.

All gerbils received either 5 minutes of ischemia (I), preconditioning prior to 5 minutes of ischemia (IP) or sham surgery (S). Animals entered either enriched housing (5 gerbils/cage) or standard housing (1 gerbil/cage) 3 days after ischemia. The gerbils remained in enrichment for the duration of the experiment. Activity in the open field was recorded on days 3, 7, 10, 30 and 60. At 60 days the gerbils were habituated to a T-maze for 3 days. At this point training in a win-shift t-maze paradigm began and lasted for 10 days, 10 trials per day. After 10 days the animals had a 6 day break followed
immediately by training in the win-stay t-maze paradigm for 25 days. 10 trials per day. The animals survived 105-110 days from the last episode of ischemia. CA1 cell counts were performed on all animals while assessments of the CA1 stratum radiatum tEPSPs were conducted in a subset of these animals due to the extreme time consuming nature of these studies.
Experimental Methods

Subjects, surgery and housing

This experiment used 84, 4-5 month old female Mongolian gerbils (Meriones unguiculatus) weighing 50-76 g (High Oak Ranch, Baden, Ontario, Canada). Animals were housed in groups of 5 in standard plastic laboratory cages on a 12/12 h light/dark cycle. All experimental manipulations were performed during the light phase. Food and water were freely available. Three days after surgery/sham occlusion (see below) animals were housed either 5 per cage (enriched condition) or singly (non-enriched condition). All procedures were approved by the Animal Care Committee of Memorial University of Newfoundland in accordance with the guidelines set out by the Canadian Council on Animal Care. Every attempt was made to minimize animal suffering and use as few animals as possible.

A 20 gauge stainless steel guide cannula was positioned above the dural surface overlaying the dorso-medial striatum under sodium pentobarbital anesthesia (65 mg/kg, i.p.). The cannula was attached to the skull with dental cement and kept patent with a 27 gauge stainless steel stylet. Two days prior to ischemia/sham occlusion the animals were anesthetized with 1.5–2.0% Halothane in 30 % O₂ and 70 % N₂O and an 8 mm brain temperature probe (model XM-FH, Mini-Mitter, Bend, Oregon, USA) was inserted to measure striatal temperature. Animals were then placed in individual plexiglas cages (20 x 14 x 17 cm) resting upon AM receivers (RA-1010, Data Sciences, St. Paul, Minnesota, USA) that sampled brain temperature in freely moving animals every 30 sec over a 3 hour period.
Forty-eight hours later animals were re-anesthetized with 1.5 - 2.0 % Halothane in 30 % O₂ and 70 % N₂O and brain probes inserted to measure temperature during surgery. A ventral midline incision was made and the common carotid arteries were isolated from the surrounding tissue. A piece of 4-0 silk suture was threaded underneath the arteries allowing them to be lifted away from the underlying tissue and occluded for either 1.5 or 5 min using microaneurysm clips. Brain and rectal temperature were maintained at approximately 36.5 °C during occlusion using feedback regulated heating blankets wrapped around the head and body (Nurse & Corbett, 1994). In this experiment, animals in the IP groups received two 1.5 min occlusions given 24 hours apart followed 72 hours later by a 5 min occlusion. Ischemic animals received a single 5 min occlusion, while sham animals had their arteries isolated but not occluded. Following occlusion the arteries were checked for reflow and the wound closed. Animals recovered in individual plexiglas cages and their brain temperature was maintained above 36.5 °C for 30 min with the aid of a heating lamp. Subsequently, they were monitored for either 8 (after 1.5 min occlusion) or 24 hours (after 5 min occlusion).

Animals were placed in enriched or non-enriched housing 3 days after ischemia, immediately after the first open field test session (described below) (see Figure 1). Enriched animals were housed 5 per cage (usually 2 ischemic, 2 IP and 1 sham animal) for the duration of the study. The enriched environment consisted of a stainless steel cage (61 x 46 x 38 cm) containing a wooden log, tubes and shelves for climbing, as well as extra bedding materials and a running wheel. Non-enriched gerbils (ischemic, IP and
sham) were housed singly in standard laboratory housing (16 x 20 cm) for the remainder of the experiment.

**Behavioural Testing**

**Open field**

Animals were not exposed to the open field prior to ischemia. Starting on day 3 (also days 7, 10, 30, 60) animals were placed in the open field apparatus (72 x 76 x 57 cm) housed in a soundproof room with constant environmental cues (e.g. shelves, computer, etc.). The floor of the open field arena was electronically divided into 25 equal squares and the number of squares entered during each 10 min testing session was recorded by a visual tracking system (HVS Systems, Kingston, UK).

**T-Maze**

Starting at 60 days post-ischemia the gerbils were habituated to the T-maze which measured 47 (stem) x 30 (arms) x 10 (width) cm (see Figure 2). Testing room cues remained constant throughout the experiment. On days 60, 61 and 62 animals were habituated (2 x 5 min/day) to the maze. The animals were rewarded with 1/2 a sunflower seed. Initially the seeds were spread throughout the maze but as habituation training progressed the seeds were localized to the reward cups in each arm. On days 63-72 gerbils were trained in a win-shift T-maze paradigm using 10 pairs of forced (FT) and choice (CT) trials per day. The first trial consisted of the animal being forced into either the right or left arm by a partition blocking the opposite arm. On the second trial (CT) the animal was placed back in the start box and was allowed to enter either of the arms.
Gerbils received a reward only if they entered the opposite arm (i.e. win-shift). After a 6-day break animals were then trained for 25 days (10 trials per day, days 79-104) using a win-stay strategy in which the FT is identical to that of the win-shift. However, to receive the reward during the CT the animal must enter the previously entered arm. The criterion for learning both these strategies was an average of 9/10 correct trials over 3 consecutive days.

**Electrophysiology**

The electrophysiological properties of the CA1 region of the hippocampus were evaluated in a slice preparation (Figure 3). After 105-110 days 42 of the 84 animals were lightly anesthetized with 2% Halothane in 30% O₂ and 70% N₂O and cooled to 32°C. Animals were then decapitated, brains quickly removed, cut in half and the left hippocampus dissected out. The left hippocampus was placed in cold, oxygenated, modified (Nurse & Corbett 1994; Kombian et al., 2000) sucrose Krebs solution containing (mMol/L): sucrose 215.8, KCl 3.5, CaCl₂ 2.0, NaHCO₃ 25.0, MgCl₂ 1.3, glucose 11.0, NaH₂PO₄ 1.2, bubbled with 95% O₂/5% CO₂. The right hemisphere was placed in 10% phosphate-buffered formalin (4°C) and subsequently processed for histology. The left hippocampus was immediately sliced in 500µm transverse sections with a tissue chopper (Model # 51425, Stoelting, Wooddale, IL, USA) and again placed in modified sucrose Krebs solution bubbled with 95% O₂/5% CO₂ for 10-15 min. After this incubation slices were submerged in Krebs solution (NaCl replaces sucrose) containing (mMol/L): NaCl 126.0, KCl 3.5, CaCl₂ 2.0, NaHCO₃ 25.0, MgCl₂ 1.3, glucose...
11.0. NaH₂PO₄ 1.2, where they were further incubated for 60 min. The individual slices were placed in an interface chamber (Fine Science Tools, Vancouver, BC, Canada) for ~45 min before experimentation. Slices were perfused with oxygenated Krebs solution at a flow rate of 2 ml/min and the temperature of the bath was maintained at 33°C.

The Schaeffer collaterals were stimulated (20 μs pulses, 0.5 Hz) using a concentric bipolar stimulating electrode (100μm, Frederick Haer, Brunswick, ME, USA) and CA1 field excitatory postsynaptic potentials (fEPSPs) were recorded from the stratum radiatum (Figure 3). Population spikes in dentate granule cells were evoked by perforant path stimulation (100μs pulses, 0.5 Hz). The slice was discarded if the population spike could not be generated.

**Histology**

Immersion fixed hemispheres (i.e. from animals used for electrophysiology) were placed in 10% formalin for at least 4-5 days prior to paraffin embedding. Animals not used for electrophysiology (n = 42) were transcardially perfused using a heparinized saline flush followed by fixation with 10 % phosphate-buffered formalin. The brains remained in formalin for at least 48 h before being embedded. Brains were cut on a microtome into 6 μm sections and stained with hematoxylin and eosin.

The quantification of ischemic brain damage was done by counting the basophilic cells seen in medial, middle and lateral sectors of CA1 in hematoxylin and eosin stained 6 μm sections at two different levels of the hippocampus (1.7 mm and 2.2 mm posterior to bregma). At the caudal pole of the hippocampus (2.7 mm posterior to bregma) only the
middle region was counted. Counting was performed using a light microscope at a
magnification of 400x with a counting grid measuring 200 x 200 μm superimposed over
the 3 different sectors of the CA1 region as described previously (Colbourne & Corbett,
1995). Only neurons within the counting grid with a well-defined nucleus and intact cell
membrane were counted. Eosinophilic neurons were not present at the long survival
times used in this study. All counting was done blind with respect to treatment condition
and one person (RF) performed counting. Previous studies (Corbett and Crooks, 1997;
Dooley & Corbett, 1998) have demonstrated that the pattern of CA1 cell loss is not
different between hemispheres in the same animal. Therefore, in the case of animals in
the present study with just one hemisphere available for CA1 counts, the total was
doubled.

Statistical analysis

All of the data were analyzed using ANOVA followed by Newman-Keuls post-hoc
tests whenever a significant F value was obtained. The choice of the particular post hoc
test was based on the fact that some tests, though valid do not correct for multiple
comparisons (e.g. Fishers test) whereas others such as Tukey and Scheffé are most
appropriate when every conceivable comparison is of interest. With these latter tests it is
often the case that significant results go unreported. Therefore, a Newman-Keuls test,
which is moderately corrective, was selected as done previously (Corbett and Crooks,
1997; Dooley and Corbett, 1998; Dowden and Corbett, 1999).
The number of gerbils attaining criterion in the win-shift and win-stay versions of the T-maze were analyzed using Chi-square tests. The level of significance was set at $P < 0.05$. It is necessary to use the chi square test since in each version of the t-maze some animals did not learn the task. As a result, frequency, not trials to criterion, was used to ascertain significant differences between groups.

Pearson correlation coefficient was calculated for each group to determine the relationship between the CA1 neuron counts (level A) and open field activity levels and EPSP amplitudes. T-maze correlation data could not be accurately ascertained due to the need to assign false values to the animals that did not learn the tasks thus not providing accurate data.

On all outcome measures (open-field, T-Maze, field potential amplitude and CA1 cell counts) it was found that sham enriched and sham non-enriched animals did not differ. Thus, these 2 groups were combined into a single sham group in order to simplify statistical analysis and data presentation.
Results

Brain temperature

Brain temperature was recorded 2 days before either preconditioning or the 5 min ischemia to ensure cannula patency. During the 5 min occlusion brain temperature ranged between 36.4±36.7°C for all experimental groups. Brain temperature did not differ between IP and ischemic groups during the 24 hour post-ischemic temperature monitoring period (F = 0.557, P > 0.05). However, as noted previously in this model (Corbett & Crooks, 1997), all ischemic animals exhibited a period of mild hyperthermia (~0.8°C) that persisted for 8 hours after occlusion (Figure 4). Consequently, their 24 hour post-ischemic brain temperature differed significantly from shams (P < 0.0001).

During surgeries and temperature recording the animals had not yet been placed into their respective experimental conditions. However, the data is presented with animals in their experimental groups to illustrate that the animals' brain temperature did not differ.

Open field activity

The first day of open field testing, day 3, was performed on the gerbils prior to their entry into enriched or non-enriched conditions. The data is shown for day 3 (Figure 5) testing with animals divided into their experimental groups to demonstrate there was no difference in the activity levels of IP and ischemic groups (P > 0.05) prior to entry into enriched or non-enriched conditions (mean±S.E.M.: IP enriched, 856.47 ± 32.3: IP non-enriched, 800.0 ± 34.7: I enriched, 840.8 ± 39.0: I non-enriched, 811.1 ± 46.8: S. 570.9 ± 26.13). However, as shown in Figure 5 these groups exhibited a heightened
level of activity in the open field relative to the activity levels of sham animals during the 10 min session (enriched ischemic and IP, \( P < 0.0001 \); non-enriched ischemic and IP, \( P < 0.001 \)). By day 7, there was no change in the relative activity levels. Shams continued to exhibit significantly lower activity scores than each of the other groups (enriched ischemic and IP, \( P < 0.0001 \); non-enriched ischemic and IP, \( P < 0.001 \)) (mean±S.E.M.: IP enriched, 693.9 ± 44.7; IP non-enriched, 684.5 ± 38.6; I enriched, 727.56 ± 49.4; I non-enriched, 675.1 ± 38.5; S, 413.1 ± 22.6). This general pattern was maintained on day 10 (enriched ischemic, enriched IP and non-enriched IP, \( P < 0.05 \); non-enriched ischemic, \( P < 0.01 \)) (mean±S.E.M.: IP enriched, 486.5 ± 40.2; IP non-enriched, 531.4 ± 34.8; I enriched, 547.3 ± 46.4; I non-enriched, 598.8 ± 35.1; S, 383.8 ± 24.6). Exposure to the open field on day 30 revealed the activity level of both enriched ischemic and enriched IP groups were reduced to near sham levels (\( P > 0.05 \)) while non-enriched ischemic and non-enriched IP groups remained significantly more active than shams (\( P < 0.05 \)) (mean±S.E.M.: IP enriched, 419.7 ± 29.7; IP non-enriched, 465.8 ± 22.0; I enriched, 444.3 ± 32.7; I non-enriched, 472.6 ± 26.5; S, 356.1 ± 24.5). The final exposure to the open field on day 60 showed that the non-enriched ischemic and non-enriched IP groups continued to have significantly higher activity levels of than shams (\( P < 0.05 \)) (mean±S.E.M.: IP enriched, 373.5 ± 18.4; IP non-enriched, 422.4 ± 19.2; I enriched, 382.1 ± 30.6; I non-enriched, 438.4 ± 34.2; S, 316.8 ± 17.5).

Correlation data

The Pearson correlation coefficient was calculated for all animals to determine the relationship between the CA1 cell counts (Level A) and open field activity (Table 1).
Ischemic animals' (enriched and non-enriched) day 3 open field data were significantly negatively correlated with CA1 cell counts ($r = -0.59, P < 0.01; r = -0.45, P < 0.05$). That is, the animals with low CA1 cell counts had high open field activity scores. IP animals showed no significant correlation between day 3 open field scores and CA1 cell counts ($P > 0.05$). By day 60 none of the groups' CA1 cell counts were significantly correlated to the open field activity since all groups had habituated over the five sessions in the open field.

*T-Maze acquisition*

T-maze testing commenced with habituation on days 60-62 followed by training in the win-shift t-maze task from days 63 - 72 and win-stay training from day 73-104. Most animals learned the win-shift (Table 2 and Figure 6) task within 100 trials with the notable exception of the ischemic non-enriched group where only 25% of animals met criterion ($\chi^2 = 22.5, P < 0.0001$ versus shams). Non-enriched IP animals learned the task and were somewhat, but not significantly, slower in learning than other groups. Enriched IP and ischemic animals learned the task as quickly as sham animals.

In contrast, all animals, including shams had more difficulty learning the win-stay version of the T-maze (Table 3 and Figure 7). Consequently, enriched ischemic animals that readily learned the win-shift task, did not learn the win-stay task. Indeed, only 1/14 animals met the 250 trial criterion ($\chi^2 = 28.2, P < 0.0001$ versus shams). None of the non-enriched ischemic animals learned the task ($\chi^2 = 32.0, P < 0.0001$ versus shams) and a majority (70%) of the IP non-enriched animals failed to meet criterion by the end of the
training period ($\chi^2 = 17.6$, $P = 0.0001$ versus shams). IP enriched gerbils learned the win-stay task almost as readily as sham animals ($\chi^2 = 3.7$, $P = 0.05$).

When using this and other learning tasks acquisition criteria must be set. Based on advice (Dr. G. Martin, personal communication) the stringent criterion of an average of 9/10 trials correct over 3 consecutive days was adopted. Within each group some variability is present as in all behavioural studies with animals or humans, in that some subjects fail to acquire the task or do so very slowly.

**Electrophysiology**

Extracellular field potentials were recorded from the CA1 region of the hippocampus following Schaeffer collateral stimulation. Recordings were obtained from 73 slices derived from 42 animals (shams = 11; ischemic enriched = 7; ischemic non-enriched = 8; IP enriched = 8; IP non-enriched = 8). Slice recordings were restricted to rostral and middle portions of the hippocampus so as to avoid the caudal portion which is less susceptible to ischemic damage and therefore may yield false positive data (i.e. appearing to be protected by preconditioning) (Ashton, Reempts, et al., 1989).

All animals were assessed 105-110 days post-ischemia (i.e. after 102-107 days in enrichment). The maximum fEPSP amplitudes recorded from enriched and non-enriched IP gerbils were not significantly different from each other (Table 4) or from sham values ($P > 0.05$). However enriched and non-enriched ischemic values were significantly reduced compared to shams ($P < 0.0001$).
Ischemic enriched and ischemic non-enriched animals' CA1 cell counts were positively correlated with the EPSP amplitude ($r = 0.72, P<0.01; r = 0.69, P<0.05$). i.e. animals with low cell counts have smaller EPSP amplitude. IP enriched animals' CA1 counts did not significantly correlate with the EPSP amplitude however there was a trend toward a positive correlation indicating that those with higher CA1 cell counts had higher EPSP amplitude and vice versa. Interestingly, there was a significant negative correlation between IP non-enriched CA1 cell counts and EPSP amplitude ($r = -0.73, P<0.01$). It appears that IP non-enriched animals with higher cell counts have lower EPSPs and vice versa.

**CA1 cell counts**

The mean number of CA1 neurons in the rostral, middle and caudal regions of the hippocampus are depicted in Table 5 and Figure 8. In rostral and mid-hippocampus (levels A and B) the 5 min occlusion produced a striking loss of CA1 neurons in both enriched and non-enriched gerbils ($P < 0.0001$ versus shams). The cell loss in these ischemic groups was less severe in the caudal (level C) hippocampus ($P < 0.01$ versus shams). The CA1 cell counts of IP enriched animals were significantly lower than the IP non-enriched group ($P <0.01$) at rostral and mid-hippocampus ($P < 0.01$) but not caudal hippocampus (level C). At these levels IP non-enriched animals had cell counts similar to sham values ($P > 0.05$).
All cell counts were performed as described in the methods. These counts were performed at the end of the experiment (survival time 105-110 days post-ischemia) once sections were stained with hematoxylin and eosin (see Figure 9).
Figure 1: Experimental Time Line - Animals entered environmental enrichment or standard housing 3 days after the 5 min ischemia or sham surgery. Open field testing was conducted on days 3 (prior to enriched or standard housing), 7, 10, 30 and 60. T-maze habituation occurred on days 60-62 followed by training on win-shift (d 63-72) and win-stay (d 79-104) T-maze tasks. Gerbils were sacrificed between days 105-110 post-ischemia by either transcardial perfusion or decapitation for electrophysiology.
**Figure 2:** T-Maze testing was conducted from day 60-104 post-ischemia. First gerbils were habituated to the maze and trained to eat the food reward from the food cups located at the end of each arm. Gerbils were then trained in win-shift T-maze task consisting of a forced trial: animals are forced in one arm to receive reward followed by a choice trial – animals must choose the opposite arm to receive reward. Animals were then trained in the win-stay T-maze consisting of a forced trial, which is the same as win-shift, and a choice trial - animals must return to the same (i.e. stay not shift response strategy) arm to receive reward.
Procedure
Habituation

Food Cup
(1/2 Seed)

Training (Win-Shift)

6 day break

Training (Win-Stay)

Sliding Doors

Start Box
Figure 3: Schematic diagram of hippocampal slice preparation showing location of stimulation and recording electrodes. SC refers to Schaeffer collateral fibres arising from CA3 neurons.
Figure 4: Mean brain temperature (°C) patterns recorded for 24 hours post-ischemia (5 minute) or sham surgery for all treatment conditions: sham (S; n=20), enriched 5 minute ischemic (I+; n=16), non-enriched 5 minute ischemic (I-; n=16), enriched ischemic preconditioned (IP+; n=17), non-enriched ischemic preconditioned (IP-; n=15). Time 0 corresponds to reperfusion.

Note: At the time of the 5 min ischemia or sham surgery, gerbils were not in enriched or non-enriched housing. Data is presented in this way to illustrate that prior to enrichment animals that had been assigned to the experimental groups did not differ in brain temperature.
Figure 5: Open field activity scores (mean ± S.E.M.) measured on days 3, 7, 10, 30 and 60 post-ischemia. By day 30 and continuing on day 60 the activity scores of both enriched IP (IP+: n = 17) and enriched ischemic (I+: n = 16) groups had declined to sham (S) levels (P > 0.05: n = 20), whereas, activity scores of non-enriched IP (IP-: n = 15) and non-enriched ischemic animals (I-: n = 16) continued to be significantly higher than shams (P < 0.05). (* = P < 0.0001; ♦ = P < 0.001; ◊ = P < 0.01; ∆ = P < 0.05 with respect to sham animals.)
Table 1: Correlation Data - Pearson Correlation Coefficient (r)
CA1 cell counts (Level A) versus Open field activity and EPSP amplitude

<table>
<thead>
<tr>
<th></th>
<th>Day 3</th>
<th>Day 7</th>
<th>Day 10</th>
<th>Day 30</th>
<th>Day 60</th>
<th>EPSP amplitude</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ischemic enriched n=16</td>
<td>-0.59**</td>
<td>-0.34</td>
<td>-0.29</td>
<td>-0.29</td>
<td>0.05</td>
<td>0.72**</td>
</tr>
<tr>
<td>Ischemic non-enriched n=16</td>
<td>-0.46*</td>
<td>-0.63**</td>
<td>-0.66**</td>
<td>-0.39</td>
<td>-0.21</td>
<td>0.69*</td>
</tr>
<tr>
<td>IP enriched n=17</td>
<td>0.24</td>
<td>0.1</td>
<td>-0.11</td>
<td>-0.15</td>
<td>-0.13</td>
<td>0.34</td>
</tr>
<tr>
<td>IP non-enriched n=15</td>
<td>-0.07</td>
<td>-0.03</td>
<td>-0.29</td>
<td>-0.25</td>
<td>-0.17</td>
<td>-0.73**</td>
</tr>
</tbody>
</table>

* = p < 0.05; ** = p < 0.01

Ischemic animals’ day 3 open field data was significantly negatively correlated with CA1 cell counts (r = -0.59, P < 0.01; r = -0.45, P < 0.05). IP animals showed no significant correlation between day 3 open field scores and CA1 cell counts (P > 0.05). By day 60 none of the groups CA1 cell counts were significantly correlated to the open field activity.

Ischemic enriched and ischemic non-enriched animals’ CA1 cell counts were positively correlated with the EPSP amplitude (r = 0.72, P < 0.01; r = 0.69, P < 0.05). IP enriched animals’ CA1 counts did not significantly correlate with the EPSP amplitude.

Interestingly, there was a significant negative correlation between IP non-enriched CA1 cell counts and EPSP amplitude (r = -0.73, P < 0.01).
Table 2: T-maze acquisition (win-shift rule)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Trials to criterion</th>
<th># Animals meeting criterion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>46 ± 4.2</td>
<td>20/20</td>
</tr>
<tr>
<td>IP enriched</td>
<td>35.9 ± 2.4</td>
<td>17/17</td>
</tr>
<tr>
<td>IP non-enriched</td>
<td>88.9 ± 4.1</td>
<td>13/15</td>
</tr>
<tr>
<td>Ischemic enriched</td>
<td>48.1 ± 5.0</td>
<td>16/16</td>
</tr>
<tr>
<td>Ischemic non-enriched</td>
<td>98.1 ± 1.9</td>
<td>4/16*</td>
</tr>
</tbody>
</table>

Acquisition of win-shift T-maze task conducted 63-72 days after ischemia or sham surgery. Criterion values expressed as mean (± S.E.M.). * χ² tests showed that the non-enriched ischemic group was markedly impaired compared to all other groups (P < 0.001). Animals failing to meet criterion after 100 trials were assigned a score of 100.
Figure 6: T-maze win-shift trials to criterion scores for enriched ischemic (I+, n = 16), non-enriched ischemic (I-, n = 16), enriched ischemic preconditioned (IP+, n = 17), non-enriched ischemic preconditioned (IP-, n = 15) and sham (S, n = 20) at 63-73 days post-ischemia. Note that non-enriched ischemic (I-) and ischemic preconditioned (IP-) were significantly impaired in learning the task ($P < 0.001$) when compared to sham groups. Conversely, enrichment prevented the learning impairments of ischemic (I+) and ischemic preconditioned (IP+) groups. Each symbol represents an individual animal.

Experimental groups are represented by the following symbols:

$I+ = \bullet; I- = \bigcirc; IP+ = \Delta; IP- = \triangle; S = \bigtriangleup$
Table 3: T-maze acquisition (win-stay rule)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Trials to criterion</th>
<th># Animals meeting criterion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>212.2 ± 6.5</td>
<td>18/18</td>
</tr>
<tr>
<td>IP enriched</td>
<td>225.6 ± 7.0</td>
<td>13/16*</td>
</tr>
<tr>
<td>IP non-enriched</td>
<td>245.7 ± 2.7</td>
<td>4/13**</td>
</tr>
<tr>
<td>Ischemic enriched</td>
<td>249.3</td>
<td>1/14**</td>
</tr>
<tr>
<td>Ischemic non-enriched</td>
<td>250.0</td>
<td>0/14**</td>
</tr>
</tbody>
</table>

Acquisition of win-stay T-maze task conducted 79-104 days after ischemia or sham surgery. Non-enriched IP and both enriched and non-enriched ischemic groups were markedly impaired compared to shams ** (P < 0.0001). Enriched IP animals learned the task slower than shams * (P = 0.05). Animals failing to meet criterion after 250 trials were assigned a score of 250.
Figure 7: T-maze win-stay trials to criterion scores for enriched ischemic (I+, n = 14), non-enriched ischemic (I-, n = 14), enriched ischemic preconditioned (IP+, n = 16), non-enriched ischemic preconditioned (IP-, n = 13) and sham (S, n = 18) at 79 to 104 days post-ischemia. Enriched IP animals learned the task as quickly as shams. However, enriched ischemic and both non-enriched ischemic and IP groups failed to meet criterion by 250 trials. Experimental groups are represented by the following symbols:

I+ = ●; I- = ○; IP+ = △; IP- = ▲; S = ◇
Table 4: CA1 fEPSP amplitudes

<table>
<thead>
<tr>
<th>Groups (n = 42)</th>
<th>CA1 fEPSP amplitudes</th>
<th>% of sham.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham (n=11)</td>
<td>2.76 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>IP enriched (n=8)</td>
<td>2.30 ± 0.1</td>
<td>83.4</td>
</tr>
<tr>
<td>IP non-enriched (n=8)</td>
<td>2.19 ± 0.1</td>
<td>79.2</td>
</tr>
<tr>
<td>Ischemic enriched (n=7)</td>
<td>0.62 ± 0.3</td>
<td>22.5</td>
</tr>
<tr>
<td>Ischemic non-enriched (n=8)</td>
<td>0.68 ± 0.3</td>
<td>24.5</td>
</tr>
</tbody>
</table>

CA1 fEPSP amplitudes are reported as the mean (mV±S.E.M) and as the mean per cent of the sham group. Both ischemic groups had significantly lower fEPSP amplitudes compared to sham (P < 0.0001). IP enriched and non-enriched were not significantly different from each other or sham (P > 0.05).
Table 5: Mean CA1 cell counts (105-110 days post-ischemia)

<table>
<thead>
<tr>
<th>Level</th>
<th>Sham (n=20)</th>
<th>Ischemic enriched (n=16)</th>
<th>Ischemic non-enriched (n=16)</th>
<th>IP enriched (n=17)</th>
<th>IP non-enriched (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>265.7 ± 4.0</td>
<td>51.3 ± 16.6</td>
<td>41.9 ± 13.3</td>
<td>126.4 ± 19.6*</td>
<td>193.9 ± 14.7</td>
</tr>
<tr>
<td>Level A</td>
<td>269.4 ± 7.5</td>
<td>75.3 ± 17.0</td>
<td>72.1 ± 18.3</td>
<td>149.1 ± 18.6*</td>
<td>219.0 ± 17.4</td>
</tr>
<tr>
<td>Level B</td>
<td>87.1 ± 3.2</td>
<td>55.7 ± 6.7</td>
<td>54.2 ± 6.4</td>
<td>60.0 ± 5.6</td>
<td>73.5 ± 4.2</td>
</tr>
<tr>
<td>Level C</td>
<td></td>
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</tbody>
</table>

Mean CA1 cell counts (± S.E.M.) were taken across the anterior-posterior extent of the hippocampus (level A, -1.7 mm; level B, -2.2 mm bregma; level C, -2.7 mm relative to bregma). * Indicates the IP enriched group had significantly fewer cells than the IP non-enriched group at levels A (P < 0.01) and B (P < 0.01) measured 105-110 days post-ischemia (see results for other details). All animals were sacrificed 105-110 days post-ischemia and cell counts were performed following histology.
Figure 8: CA1 cell counts were taken across the anterior-posterior extent of the hippocampus (level A, 1.7 mm posterior to bregma; level B, 2.2 mm posterior to bregma; level C, 2.7 mm posterior to bregma). Each symbol denotes an individual animal while the horizontal bars represent group means. The enriched ischemic preconditioned (IP+, n = 17) group had significantly fewer cells than the ischemic preconditioned non-enriched (IP-, n = 15) group at levels A and B (P < 0.01) measured 105-110 days post-ischemia.
Figure 9: Histology pictures of CA1 region of the hippocampus (Level A) at 20X magnification. Sections chosen represent the mean cell counts for each group (mean ± S.E.M.). A. Sham enriched. (265.7 ± 4.0); B. Sham non-enriched. (265.7 ± 4.0); C. Ischemic enriched. (51.3 ± 16.6); D. Ischemic non-enriched. (41.9 ± 13.3); E. IP enriched. (126.4 ± 19.6); F. IP non-enriched. (193.9 ± 14.7).
Discussion

The main findings of this study were that environmental enrichment attenuated the behavioural abnormalities associated with IP but paradoxically enrichment increased the loss of CA1 neurons. Indeed, IP enriched animals habituated to the open field and learned the win-shift and win-stay t-maze at a similar rate as sham animals. Furthermore, the IP enriched group showed no deficit in fEPSP amplitude while exhibiting reduced CA1 neuron counts when compared to IP non-enriched animals.

Enriched and non-enriched sham groups did not differ from each other in any of the endpoints. Therefore, the two were combined for ease of reporting. Previous studies have revealed inconsistencies in the effects of enrichment on normal animals. Some studies have not seen significant behavioural improvement after environmental enrichment (Kolb & Gibb, 1991; Biernaskie & Corbett, 2001). Others indicate an improvement in intact animals after exposure to enrichment (Moser et al., 1994; Kempermann et al., 1997). In the current experiment the lack of distinction between enriched and non-enriched sham animals may have been due to the gerbils being housed in groups prior to the experiment. Also, this experiment involved a large amount of behavioural testing during which sham animals were handled repeatedly which may have blunted the effects of enrichment by increasing the activity and stimulation of these standardly housed animals.

There was no difference between the CA1 cell counts of ischemic enriched and non-enriched animals. This was anticipated as 5 min of global ischemia in gerbil produces
delayed neuronal death in which CA1 neurons die 2-4 days post-ischemia (Kirino, 1982). As animals were not placed in enriched housing until day 3 post-ischemia virtually all CA1 neurons would have died prior to enrichment.

Enrichment (large cages with ropes for climbing, various toys and social interaction) enhances the performance of animals with various types of brain injury (electrolytic lesions, chemical lesions, focal and global ischemia) as well as normal animals (e.g. Kolb & Elliot, 1987; Kolb & Gibb, 1991; Johansson, 1996; Puurunen et al., 1997). Spatial training in a complex environment for several hours per day has also been shown to improve animals' performance on both spatial and non-spatial learning tasks (Moser et al., 1994; Rampon, Tang et al., 2000). Rampon and colleagues tested the effects of enrichment training on hippocampal CA1 NMDAR1-knockout mice. Due to the mutation these mice experienced severe learning and memory deficits in object recognition, olfactory discrimination and contextual fear memory. Nevertheless, after training in an enriched environment (specially designed box with running wheel, toys and little houses) (3 hours/day for 2 months) there was a significant reduction in the memory deficits in all three tests for the mutant animals.

Enriched animals exhibited a reduction in both habituation and working memory deficits in the present study. Preconditioned animals ordinarily show levels of heightened open-field activity comparable to those of ischemic animals in the first 10 days after ischemia. This is surprising because in previous studies at day 10 post-ischemia these animals exhibit relatively high numbers of surviving CA1 cells (~70-90%) (Corbett & Crooks, 1997; Dooley & Corbett, 1998; Dowden & Corbett, 1999). However,
these "protected" CA1 neurons display reduced fEPSPs (Dooley & Corbett, 1998; Dowden & Corbett, 1999), which along with the open field data suggest that these cells, though viable, are functionally abnormal. In the present experiment enriched animals reduced exploratory activity to a level similar to sham animals by 30 days, a pattern that continued at 60 days post ischemia. Thus enrichment largely blunted the behavioural deficits typically exhibited by IP animals when compared to sham. These data are in agreement with results from another study that used a rat model of global ischemia in which enriched ischemic rats exhibited reduced activity in an open field task after 5 weeks post-ischemic enrichment (Puurunen, Sirvio, et al., 1997).

Deficits in working memory with the win-shift T-maze have been demonstrated in both male and female gerbils 1 week and 1 month after ischemia (Colbourne & Corbett, 1995; Andersen & Sams-Dodd, 1998). In the present study, enrichment dramatically abated the effects of ischemia on working memory as reflected by the ischemic animals learning the win-shift T-maze as quickly as normal animals. In contrast, the non-enriched ischemic group was significantly impaired in learning this task. Both the enriched and non-enriched IP animals acquired the win-shift task as quickly as sham animals.

When the T-maze paradigm shifted to the win-stay rule all animals had much more difficulty learning this task compared to the win-shift version as evidenced by the substantial increase in the number of trials to meet the learning criterion. Unlike the win-shift task, all groups were significantly slower than sham. However, the majority of IP enriched animals learned the task within the 250 trials (13/16 or 81.3%) whereas just 30% (4/13) of the non-enriched IP animals acquired the win-stay task. The win-stay task
is difficult to learn because the animals are required to revisit the previously baited arm to receive a reward. This response strategy is the reverse of a gerbil’s normal foraging behavior (Babcock & Graham-Goodwin, 1997). It is interesting to note that the deficits in the win-stay task were apparent 3 months after ischemia in animals with mild (~ 30%) CA1 (i.e. IP non-enriched) and severe (~ 98%) CA1 cell loss (i.e. both ischemic groups) (see Table 3 and Figure 8). Other behavioural tests such as the Morris water maze are far less sensitive to ischemic injury with "apparent" recovery being evident within days or weeks (Auer. Jensen. et al., 1989; Corbett. Evans. et al., 1992; Corbett & Nurse. 1998). Obviously task selection is an important consideration in assessing recovery or neuroprotection after brain injury, since improved performance on some tests does not guarantee recovery on other more demanding tasks (Corbett & Nurse. 1998; Hunter. Hatcher. et al., 2000).

Unlike previous results from this laboratory (Dooley & Corbett. 1998; Dowden & Corbett. 1999) the IP gerbils did not show a reduction in CA1 tEPSP amplitude. The most likely explanation for this apparent discrepancy is the much longer survival time used in the present experiments (3 months versus 10, 30 and 60 days). This study began recording tEPSPs beyond the time in previous studies when field potentials had largely normalized. In earlier studies where enrichment was not used, tEPSP amplitudes largely recovered by 60-90 days post-ischemia perhaps as a result of endogenous recovery processes. Enrichment may have enhanced dendritic growth and spine formation as well as other processes that could have normalized tEPSP responses within the first few days or weeks after ischemia (Moser. Trommald. et al., 1994: Moser. Trommald. et al. 1997).
However, studies incorporating both short (e.g. 1-2 weeks) and long (2-3 months) survival times are required to address this issue.

Post-ischemic exposure to an enriched environment in this and other studies results in enhanced functional recovery (Ohlsson & Johansson, 1995; Johansson, 1996; Johansson, Mattsson, et al., 1997; Biernaskie and Corbett, 2001) in both sensori-motor and cognitive based tasks. It is generally assumed that this improvement is due to biochemical and/or structural changes in the brain (Kolb, Forgie, et al., 1998). For example, increased dendritic growth and spine formation, expression of neurotrophic factors, gene expression, synaptogenesis and neurogenesis have all been observed in animals placed in enrichment or complex learning environments (Kolb & Gibb, 1991; Moser, Trommald, et al., 1997; Kempermann & Gage, 1999; Rampon, Jiang, et al., 2000; Rampon, Tang, et al. 2000; Biernaskie and Corbett, 2001). Similar changes in plasticity are triggered by the brain injury itself making the post-ischemic milieu an environment that should promote neuronal rewiring and functional reorganization (Jones & Schallert, 1994; Jones, 1999; Cramer & Chopp, 2000; Schallert, Leasure, et al., 2000).

It is probable that a number of the above mechanisms contributed to the improved behavioural performance we observed in the open-field and T-maze tasks and possibly the normalization of fEPSP responses in IP animals.

It has been previously reported that structural changes such as increased dendritic branching and increased spine formation occur as a result of spatial training in a complex environment (Moser & Trommald 1994; Moser et al., 1997). Training increases the number of excitatory hippocampal CA1 spine synapses and spine density on basal
dendrites as compared with either isolated or standardly housed animals (Moser et al., 1994; Moser et al., 1997). Dendritic spine density can be used as a reflection of the quantity of excitatory hippocampal CA1 synapses. It is possible that spine density increased on the remaining CA1 neuron dendrites of IP animals and consequently the increased number of excitatory synapses strengthened processing in the remaining neurons to permit the superior learning abilities exhibited by the enriched animals.

Dendritic growth may also be a theoretical explanation for the decreased CA1 cell counts. Motor learning in rats results in changes in dendritic morphology of the stellate cells in the cerebellum (Kleim et al., 1997; Klintsova et al., 2000). The reduction in cell density did not change the density of synapses or unit volume (Kleim et al., 1997; Klintsova et al., 2000). Perhaps the acquisition of a learning task and increased motor activity by the enriched IP animals caused increased dendritic arborization of CA1 neurons. Instead of neuronal death the reduced numbers may be due to the neuronal density decreasing due to dendritic hypertrophy (Kleim et al., 1997; Klintsova et al., 2000). This may account for the paradoxical maintenance of the EPSP amplitudes of enriched IP animals even though cell counts are decreased.

Neurotrophic factor levels have been reported to both increase and decrease in various brain areas in response to environmental enrichment (e.g. Dahlqvist et al., 1999; Ickes et al. 2000). Ickes and colleagues (2000) placed normal rats in enrichment at 2 months of age and at 14 months various neurotrophic levels were measured. Levels of nerve growth factor (NGF) and brain-derived growth factor (BDNF) were increased in the hippocampus, cerebral cortex, basal forebrain and hindbrain when compared to isolated
control animals. Aged rats placed in an enriched environment were found to have higher levels of NGF mRNA in the visual cortex and hippocampus (Torasdotter et al., 1998). A higher level of this growth factor has been previously implicated in cognitive functions and was shown to improve the performance of aged rats in spatial learning and memory tasks. The study by Torasdotter et al. (1998) revealed that exposing aged rats to an enriched environment increased the level of NGF mRNA in those who were housed in groups in the stimulating environment for 30 days as compared with those housed singly in non-enriched standard laboratory cages. Hence, a higher level of NGF may contribute to the increased spatial and memory abilities of enriched animals. Furthermore, environmental enrichment reversed the deleterious behavioural effects of deprivation (no handling post-weaning) and concurrently increased NGF levels in the hippocampus (Pham, Soderstrom et al., 1999).

Dahlqvist and colleagues (1999) reported a decrease in the levels of NGF-induced gene A and glucocorticoid receptor mRNA after ischemia. In standard housed animals this decrease lasted for just 2 – 3 days while the decrease continued for 20 days in enriched animals. Interestingly, after 30 days, enriched animals showed an increase in NGF-induced gene A expression when compared to animals in standard housing. Therefore, in this case, enrichment after focal ischemia provided a dynamic alteration in the expression of NGF-induced gene A over a 30 day period.

Zhao and colleagues (2000) investigated the effects of environmental enrichment on the levels of BDNF mRNA, after focal ischemia. BDNF mRNA levels were higher in standard housed rats compared to enriched rats up to 12 days after ischemia and both
groups had below baseline levels at 20 and 30 days. This study provides further evidence that growth factors are not necessarily increased due to environmental enrichment.

What can be the explanation for these conflicting data? Perhaps growth factors are not chronically elevated due to enrichment. Instead specific growth factors may be elevated for a short period of time early in enrichment and return to pre-enrichment levels as time progresses or perhaps some are decreased for the entirety of the animal's enrichment. Conceivably, a decrease in growth factor levels may be beneficial as it may reduce the post-ischemic hyperexcitability and prevent further deterioration due to ischemia (Zhao et al., 2000). It must not be overlooked that increases in growth factors may be an indicator of cellular damage. Several growth factors are seen to increase after brain injury (e.g. NGF, BDNF, NT-3) (e.g. Hashimoto et al., 1992; Kokaia et al., 1995). Therefore, the increase in growth factors may also be a result of the neurotoxic environment after injury not a beneficial environment.

It is likely that many growth factors are involved in the functional improvements seen in enriched animals. Further studies are needed to identify the time at which the growth factor levels increase and/or decrease after ischemia and the duration of the alteration in levels.

Other explanations, especially of the open field data, are possible. For example, it is known that the open field must initially be "novel" in order to elicit postischemic locomotor hyperactivity. If the animals are exposed to the open-field several days prior to ischemia then the typical postischemic profile of heightened locomotor activity is abolished (Wang & Corbett, 1990; Babcock, Baker, et al., 1993).
enrichment is that the animals are continuously exposed to a varied environment. During enrichment housing, objects were moved into different locations in the cage and the presence of other animals ensured changing sensori-motor stimulation. All of this may have blunted any perceived novelty effects of the open field particularly after the initial open field test exposure. This explanation probably does not account or contribute importantly to our T-maze results which do not depend on novelty since the animals were habituated to the T-maze prior to training on win-shift and win-stay tasks.

Environmental enrichment did not attenuate the delayed loss of CA1 neurons after ischemic preconditioning. In fact, 105-110 days after ischemia, enriched IP animals had significantly fewer CA1 cells than their non-enriched counterparts. These data are similar to results from an earlier study reporting use-dependent injury after electrolytic lesions of the motor cortex in rats (Kozlowski, James, et al., 1996). In that study the unaffected limb was placed in a plaster cast immediately after the lesion thereby forcing use of the impaired limb. This intervention increased the extent of cortical injury and worsened behavioural outcome. A related experiment using a focal ischemia model demonstrated an increase in infarct volume when rats were put into an enriched environment plus training on several motor tasks beginning 24 hours after ischemia.

Delaying enrichment and motor task training until 7 days after ischemia had no effect on infarct volume. Interestingly, both early and late enrichment improved functional outcome even though early enrichment increased use-dependent cortical damage (Risedal, Zeng, et al., 1999). These findings parallel the present results where increased
injury in IP enriched versus non-enriched IP gerbils was associated with better functional recovery.

An alternative explanation for the difference between the enriched and non-enriched IP cell counts exists. It is possible that the increased CA1 loss of the IP enriched animals reflects a type of “cellular pruning”. Enrichment may have inadvertently removed abnormal neurons (i.e. those with sub-lethal injury). A study by Mumby et al. (1996) illustrated that animals with complete hippocampal ablation performed significantly better on an object recognition delayed non-matching-to-sample (DNMS) task than animals with global ischemic damage that only partially damages the hippocampus. In the present experiment non-enriched IP animals had significantly more cells than enriched IP animals. Consequently, these viable but abnormal cells may have contributed additionally to the deficit seen in non-enriched IP animals with both habituation (open field) and working memory (T-maze).

When is the appropriate time for intensive rehabilitative interventions after stroke? Many focal ischemia studies indicate delayed entry into enrichment may be preferable. Humm and colleagues (1999) determined that casting an animal’s non-impaired forelimb within 7 days of ischemia caused increased infarct size and interfered with restoration of function. However, if casting occurred during the next 7 days (8-15) there was no increase in infarct size but interference with restoration of function continued (Humm et al., 1999).

Previous investigations have not found evidence of use-dependent injury in the hippocampus. Colbourne examined the effects of early behavioral testing in a gerbil
model of global ischemia (Colbourne, Auer et al., 1998) by subjecting the animals to either 5 min ischemia with or without hypothermia. Four days after ischemia the animals were exposed to a battery of behavioral tests (novel mazes and an open field) but no exacerbation of CA1 cell loss in either the normothermic ischemic or the hypothermic ischemic groups was found. However, the hypothermia produced almost complete protection of CA1 neurons so a worsening effect of training may have been difficult to detect. Likewise, in untreated ischemic animals CA1 cell loss was so extensive that there was little latitude for injury exacerbation. To avoid these potential problems Dowden, Evans et al. (1999) used IP to assess whether early open field testing (days 3, 7, 10 and 30) would increase CA1 cell loss. These animals were assessed 30 days after ischemia but behavioural testing did not affect cell survival. Therefore, while the focal ischemia studies suggested waiting for longer than 3 days after ischemia to enter enrichment, the gerbil model of global ischemia did not seem as susceptible to damage through early intervention.

It is possible that the increased CA1 cell loss in the enriched IP group of the present study is due to the combined effects of post-ischemic behavioural testing (open field, T-maze) and the continuous, early (i.e. 3 days) exposure to an enriched environment. In other words, use-dependent injury in hippocampus (present results) and cortex (Kozlowski, James et al., 1996; Risedal, Zeng et al., 1999) may require both intensive behavioural interventions, such as enrichment plus behavioural testing or limb casting, and early initiation of these interventions (e.g. < 3 days). A recent study suggests that the size of injury may also influence use-dependent injury since use-dependent worsening of
cortical infarction appears most readily demonstrated after mild ischemic insults (Bland, Schallert, et al., 2000). In this regard, the use of global ischemia models may be particularly helpful for elucidating the mechanisms of use-dependent damage because the amount of cell death can be easily varied and the degree of necrosis readily quantified.

A question arises as to the mechanisms of the use-dependent injury exacerbation. Several factors may be contributing to this phenomenon. First, we know that during and after ischemia there is a massive release of glutamate from hippocampal and other neurons into the extracellular space which reach excitotoxic levels (Choi, 1988). Second, behavioural testing and spatial learning further enhance glutamate release as part of the learning process (Richter-Levin, Canevari, et al., 1998). Thus, the combination of enrichment and open field testing in the IP enriched gerbils starting 3 days after ischemia may have been sufficient to cause a secondary increase of glutamate to neurotoxic levels. In contrast, less intense stimulation (Colbourne, Auer, et al., 1998; Dowden, Evans, et al., 1999) such as open-field testing may not provoke such a sustained elevation of glutamate. This idea was originally proposed by Schallert and colleagues who found that glutamate antagonists block use-dependent cortical injury following limb casting procedures (Humm, Kozlowski, et al., 1999). Therefore, early intensive rehabilitation or behavioural testing given in the first few days after stroke or other forms of brain injury may be neurochemically equivalent to delivering a second ischemic or traumatic insult.

This study is the first to assess the effects of environmental enrichment on ischemic preconditioning and the first to demonstrate a possible use-dependent exacerbation of hippocampal injury. In the present study one survival time was used to assess the effects
of enrichment. Future investigation using multiple survival times (early to late) needs to be performed to determine when the CA1 cell counts of enriched IP animals were reduced to a level significantly lower than non-enriched IP and to determine when plasticity changes are occurring (e.g. alteration of dendrites).

Another limitation is the use of isolated animals as the control. Isolated control gerbils were used to determine whether any differences existed between enriched and isolated animals since there is just one previous study using global ischemia and enrichment. Isolated rats have fewer spines on dendrites compared to paired rats (Moser et al., 1997). Gerbils in paired housing should be used as controls for any future investigations.

The present study incorporated behaviour (i.e. open field, 2 types of T-maze tests) histology and electrophysiology. Given the intensive nature of repeated behavioural testing and electrophysiological recording involved it was impossible to include further investigations of cellular integrity. However, it would be of interest to attempt various immunoassays to determine the effects of enrichment on these levels. One possible investigation would be to stain for microtubule associated protein 2 (MAP-2). Previous studies have shown MAP-2 levels (ROD) of IP animals in the apical and basal dendrites of CA1 were not different from sham (Dooley and Corbett, 1998). But perhaps enriched IP animals, due to the loss of cells, may have increased MAP-2 levels when compared to non-enriched IP. A more sensitive method to assess dendritic changes in enriched animals is Golgi staining. Focal ischemia studies have revealed enhanced dendritic arborization following enrichment (e.g. Biernaskie and Corbett, 2001). It is necessary to assess the state of dendrites in the gerbil model of ischemic preconditioning with and
without enrichment. In this thesis Golgi staining may have revealed dendritic hypertrophy and/or increased spine densities in the CA1 region, perhaps explaining the disparate findings of reduced cell counts and normalized fEPSP amplitudes. Further immunocytochemical staining for markers of synaptogenesis (e.g. synaptophysin) and various growth factors may reveal further information as to the effects of environmental enrichment in the gerbil model of ischemic preconditioning. Previous work has illustrated a correlation between enhanced dendritic branching, synaptogenesis, neurotrophic levels and improved functional outcome after environmental enrichment (Moser et al., 1994; Moser et al., 1997; Ickes et al., 2000).

As previously mentioned, early behavioural testing did not exacerbate CA1 neuronal death in IP animals (Colbourne, Auer et al., 1998; Dowden, Evans et al., 1999). In contrast, this study which used a much more intensive behavioural experience (e.g. early open field plus environmental enrichment) revealed a use dependent injury to the CA1 neuron population of IP gerbils with the combination of enrichment and behavioral testing. The question arises as to whether the degree of CA1 protection obtained in the IP animals would have been greater if the animals’ entry into enrichment had been further delayed or instituted more gradually. Environmental enrichment instituted 3 days after global cerebral ischemia attenuated the behavioural deficits in both habituation and working memory. Enriched IP animals performed better in all behavioural tests but experienced a more severe loss of cells than non-enriched IP animals. Enrichment is effective in improving functional outcome when initiated even 15 days after focal ischemia (Johansson, 1996; Biernaskie & Corbett, 2001) however, the window for global
ischemia has not yet been determined. The present study as well as the Puurunen et al. (1997) study placed animals in enriched housing 3 days post-ischemia. Further experiments to determine the optimal time point for entry into enrichment are needed.

Previous focal ischemia studies have revealed use dependant cortical injury (Risedal et al., 1999). In this thesis for the first time early behavioural testing plus enrichment was associated with increased hippocampal neuronal loss. The fact that use-dependent injury has now been demonstrated in several different animal models including global and focal ischemia suggests that rehabilitation be initiated gradually especially during this critical period of enhanced susceptibility to brain injury exacerbation.

In summary, the original hypothesis that environmental enrichment might attenuate CA1 cell death and increase functional recovery was not entirely supported. Interestingly, increased CA1 cell death was seen in enriched ischemic preconditioned animals however these animals displayed improved functional outcome relative to their non-enriched counterparts. The timing of entry into enrichment/rehabilitation must be carefully investigated. However, it should be noted that the prevention of neuronal loss may not always be desirable. For example, preservation of potentially abnormal neurons (e.g. ones that are hyperexcitable) may impede recovery of learning and memory or predispose a patient towards another debilitating condition (e.g. epilepsy). These complex issues must be addressed experimentally and are of considerable importance for future rehabilitation of stroke patients.
References


