CHARACTERIZING THE NEUROPROTECTIVE EFFICACY OF ISCHEMIC PRECONDITIONING (ISCHEMIC TOLERANCE): IS AGE AN IMPORTANT FACTOR?

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Characterizing the Neuroprotective Efficacy of Ischemic Preconditioning (Ischemic Tolerance): Is Age an Important Factor?

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ABSTRACT

In this dissertation, the phenomenon of ischemic preconditioning was investigated in a gerbil model of global ischemia. Specifically, the neuroprotective efficacy of ischemic preconditioning was evaluated in aged and young gerbils to determine if age is an important factor influencing outcome. Outcome was assessed using histological, behavioural and electrophysiological endpoints in order to ensure an accurate measure of the extent of neuroprotection.

In the first experiment, ischemic preconditioning was assessed in 18-20 month old gerbils. Ischemic preconditioning conveyed robust and long-lasting functional and histological protection. Furthermore, it appeared to be more effective than what had been previously reported in young animals (3-5 months).

In the second experiment, a direct comparison of ischemic preconditioning protection was made in young (3-5 months old) and aged gerbils (18 months old). It was determined that aged animals had a higher level of protection compared to young animals. In addition, the results of this experiment suggest that one mechanism by which ischemic preconditioning produces protection may involve activation of astrocytes in the CA1 region.

Finally, in the last experiment, the partial protection provided by ischemic preconditioning was used as a model to determine if early postischemic behavioural testing affected CA1 cell survival. In this study, there were no differences in cell counts between animals that had been exposed to a novel environment (open field apparatus) and those that were not.

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LIST OF ABBREVIATIONS

ACSF..... artificial cerebrospinal fluid

AMPA..... α-amino-3-hydroxy-5-methyl-4-isoxazole propionate

BBB..... blood brain barrier

CNS..... central nervous system

CC..... corpus callosum

DAB..... diaminobenzidine

EPSP..... excitatory postsynaptic potential

ET-1..... endothelin-1

fEPSP..... field excitatory postsynaptic potential

GABA..... gamma-amino-butyric acid

GFAP..... glial fibrillary acidic protein

H&E..... haematoxylin and eosin

HSP..... heat shock protein

I..... ischemic

IP..... ischemic preconditioned

LTP..... long term potentiation

MAP2..... microtubule associated protein 2

MCA..... middle cerebral artery

NBQX..... 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo (F) quinoxaline

NMDA..... N-methyl-D-aspartate

PO..... preconditioned only

ROD..... relative optical density

S..... sham

TIA..... transient ischemic attack

2-VO..... 2 vessel occlusion

4-VO..... 4 vessel occlusion

PUBLICATIONS

The majority of the data presented in Chapter 3 of this thesis has been previously published; "Ischemic preconditioning in 18- to 20- month old gerbils: Long term survival with functional outcome measures." J. Dowden and D. Corbett, Stroke, Volume 30, pages 1240-1246. The data from Chapter 5 has been included in a manuscript submitted for publication; "Effects of early behavioural testing on histological outcome following global ischemia." D. Corbett, J. Dowden, S. Evans and S. Nurse, Brain Research, submitted.

The following is a list of publications and abstracts that formed part of my graduate program.

PUBLICATIONS

Papers

Dowden, J. and Corbett, D. Ischemic preconditioning in 18-20 month old gerbils: Long term survival with functional outcome measures, *Stroke*, 30 (1999) 1240-1246.

Dowden, J., Reid, C., Dooley, P. and Corbett, D. Diazepam-induced neuroprotection: Dissociating the effects of hypothermia following global ischemia, *Brain Res.*, 829 (1999) 1 - 6.

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Corbett, D., Dowden, J., Evans, S. and Nurse, S. Effects of early behavioural testing on histological outcome after global ischemia, *Brain Res.*, Submitted.

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Abstracts

Dowden, J., Evans, S., Nurse, S. and Corbett, D. Effects of early behavioural testing on histological outcome after global ischemia, *Neurosci. Abst.*, 1999.

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CHAPTER 1: INTRODUCTION

1.1 Stroke

Stroke or cerebral ischemia occurs when blood flow to the brain is reduced or blocked depriving the brain tissue of essential stores of oxygen and glucose. Cerebral ischemia is the third leading cause of death in North America and the leading cause of permanent disability (Hademenos, 1999; Bonita, 1992). Typically cerebral ischemia is classified into two categories. Global ischemia commonly occurs following cardiac arrest when blood flow to the entire brain is interrupted. Focal ischemia, on the other hand, occurs when blood flow to a specific region of the brain is blocked due to the occlusion of a major artery such as the middle cerebral artery (MCA).

A tremendous amount of progress has been made in identifying the risk factors for stroke (e.g. age, hypertension, smoking, hypercholesterolaemia,) thereby reducing the incidence of stroke (Gorelick, 1995). In addition, recent breakthroughs have made it possible to restore cerebral blood flow during a stroke by administering thrombolytics such as tissue plasminogen activator (tPA) within 3 hr of stroke onset (Barinag, 1996). However, this narrow therapeutic window coupled with an increased risk of hemorrhage limits the use

of thrombolytic therapy. Despite these advances, there is currently no effective neuroprotective drug treatment for minimizing damage once a stroke has occurred.

1.2 Animal Models

Increasing interest in the pathology of stroke has given rise to several animal models that have aided in the understanding of the mechanisms of injury. MCA occlusion is the most common model of focal ischemia. The most popular technique is the method developed by Zea Longa where a suture is introduced into the lumen of the internal carotid artery and advanced forward to block the MCA at its origin (Longa et al., 1989). Other methods include a surgical craniotomy whereby a piece of skull is excised to expose a section of the MCA. The artery is then occluded either with a clip (Buchan et al., 1992) in the case of the reversible ischemia model or with cauterization or ligation (Chen et al., 1986) in the permanent model of focal ischemia.

Although these models have been useful they have several disadvantages. Most notable is the impact that such invasive techniques have on the health of the animals. Typically, vasculature supplying the facial muscles and teeth is disrupted during these surgeries leading to eating difficulties, poor

weight gain and tooth overgrowth (Sharkey and Butcher, 1995). These complications make it difficult to employ behavioural measures especially over long survival times. In addition, these models are often inconsistent producing infarcts of variable size. Finally, these models are associated with a high incidence of mortality.

An alternative method of MCA occlusion involves the application of endothelin-1 (ET-1), a potent vasoconstrictor, onto the MCA via intracerebral injection (Sharkey et al., 1993; Marston et al., 1996). This method is less invasive and avoids the complications associated with disrupting the musculature and blood supply of the face.

All of these models mimic the clinical situation and result in the death of all tissue elements within the core of the ischemic region where blood flow is less than 15 ml/100g tissue/minute. In addition, there is delayed and selective death of vulnerable cells in the penumbra, the region surrounding the core infarct, where blood flow is compromised (Hunter et al., 1995).

As previously mentioned, global ischemia occurs following cardiac arrest/hypoxia. In the rat models, global ischemia is produced in one of two ways. In the first method, known as the 4 vessel occlusion (4-VO) model, the vertebral arteries are isolated and cauterized for 10-30 min followed 24 hr later by

bilateral carotid artery occlusion (Pulsinelli and Brierly, 1979). In the 2 vessel occlusion (2-VO) model only the carotid arteries are occluded with concomitant systemic hypotension (Eklof and Siesjo, 1972; Smith et al., 1984). Hypotension (45-50 mm/Hg) is required in this model as it has been shown that in the rat bilateral carotid artery occlusion alone does not result in a reduction in blood flow sufficient to produce histological damage (Schmidt-Kastner and Freund, 1991). Typically an occlusion duration of 10-20 min is necessary to produce substantial loss of vulnerable hippocampal CA1 neurons. An alternative method of global ischemia involves the gerbil (*Meriones unguiculatus*) (Levine and Payan, 1966; Kirino, 1982) which lacks a complete circle of Willis leaving the anterior and posterior cerebral circulations distinct (Berry et al., 1975; Kahn, 1972). Therefore, bilateral occlusion of the carotid arteries alone is sufficient to produce deep forebrain ischemia. With good temperature control, this model is highly reproducible and 5 min of ischemia produces highly consistent CA1 damage with a low incidence of mortality (Colbourne and Corbett, 1994; Nurse and Corbett, 1994).

1.3 Pathophysiology of Cerebral Ischemia

The brain's high metabolic rate and low energy stores make it particularly vulnerable to an interruption of blood flow even for a limited time (Siesjo, 1978). During ischemia a number of events occur in rapid succession. Within seconds of ischemic onset the brain's energy stores are rapidly depleted resulting in extensive energy failure (Siesjo, 1984 and 1988). This is followed by the failure of the Na^+ / K^+ pump, which is ATP dependent, leading to major ionic shifts where K^+ accumulates extracellularly while Ca^{2+}, Na^+ and Cl^- are internalized with the obligatory accumulation of water (Hansen, 1985; Somjen et al., 1993). The result is cellular swelling, destabilization of the membrane potential and depolarization of the neurons. In addition, there is a rapid accumulation of metabolic waste including lactic acid and a subsequent decrease in pH (Combs et al., 1990; Munekata and Hossman, 1987). All of these factors contribute to the disruption of various cellular processes including the inhibition of protein synthesis (Bodsch et al., 1985). If blood flow is restored in a timely manner (e.g. < 2 min in global ischemia and < 1 hr in focal ischemia) and reoxygenation occurs then many of the above mentioned events are reversible and normal neuronal function can be expected (Hossman, 1985; Schmidt-Kastner and Freund, 1991). ATP levels recover in minutes and ion gradients are quickly reestablished. The

normalization of pH typically takes a bit longer and protein synthesis can take hours to recover if at all, especially in vulnerable regions such as CA1 (Thilmann et al., 1986).

While the characteristics of focal and global ischemia are very similar during the ischemic period there are a number of differences in the pathology that follows. As previously mentioned focal ischemia produces an area of core infarct where all tissue elements (neurons, glia and vasculature) are destroyed and a surrounding region or penumbral zone where only specific cell types are affected. The time course for the development of the infarct is typically 24 hr (Garcia et al., 1995) with additional maturation of injury taking place over several days or perhaps up to 1-2 weeks after ischemia.

Global cerebral ischemia, on the other hand, results in the neuronal death of selectively vulnerable cells, most notably the CA1 cells of the hippocampus (Kirino and Sano, 1984). In addition to CA1, other regions vulnerable to global ischemia include CA2 neurons, hilar neurons of the dentate gyrus and medium sized striatal neurons (Hsu and Buzaki, 1993; Matsuyama et al., 1993; Akai and Yanagihara, 1993). In contrast, some neuronal populations (e.g. CA3 and dentate granule cells) are particularly resistant to ischemic episodes (Petito and Pulsinelli, 1984).

The selective vulnerability of certain neuronal populations was first noted by Sommer over 100 years ago (Sommer, 1880) and since then investigators have sought to determine the reason behind differences in susceptibility. Over time, two main theories have emerged: one argues for differences in the vasculature supplying the various regions (Spielmeyer, 1927; Coyle, 1978; Imdahl and Hossman, 1986) while the other argues that differences in the physiology and biochemistry of the cells themselves (Vogt, 1936/37) are responsible. While there is evidence to support and refute both theories, the prevailing view is that intrinsic non-vascular properties are responsible for regional differences in vulnerability.

The selectively vulnerable neuronal populations can be further classified based on the time course of cell death following ischemia. Striatal and hilar neurons are thought to undergo rapid cell death by necrosis with irreversible cellular changes occurring within minutes to hours after ischemia (Akai and Yanagihara, 1993; Kirino, 1982; Kirino and Sano, 1984; Pulsinelli et al., 1982; Crain et al., 1988). In contrast, CA1 cell death takes 2-4 days to develop; a process that has been termed delayed neuronal death (Kirino, 1982; Pulsinelli et al., 1982). While light microscopy indicates that these neurons appear morphologically normal for up to two days postischemia (Kirino, 1982), electron microscopy has

revealed early distinctive changes. Soon after ischemia CA1 neurons show transient mitochondrial swelling and disintegration of cristae, cytoplasmic vacuoles, disaggregation of polyribosomes, a decrease in rough endoplasmic reticulum and loss of Golgi apparatus cisterns and vesicles. On the second day a massive proliferation of membranous cytoplasmic organelles is seen with overt cellular disintegration occurring only after a survival period of four days (Petito and Pulsinelli, 1984; Yamamoto, 1986; Siesjo, 1993). The delayed nature of CA1 cell death provides a unique window of opportunity for intervention and thus a number of strategies have evolved that are aimed at attenuating the loss of these selectively vulnerable neurons.

1.4 Current Theories of Ischemic Cell Death

The mechanisms by which cells die following ischemia have been and continue to be intensely studied. Depolarization of the neurons during ischemia results in the release of various neurotransmitters including glutamate, aspartate, dopamine and gamma-amino-butyric acid (GABA) into the extracellular space (Benveniste et al., 1984; Globus et al., 1988). The excitatory neurotransmitter glutamate has been prominently implicated in the neurobiology of ischemic brain injury (Choi, 1990; Rothman and Olney, 1986). Ischemia followed by reperfusion

is associated with an increase in the release of glutamate and a simultaneous decrease in reuptake leading to a prolonged activation of N-methyl-D-aspartate (NMDA) receptors and influx of Ca^{2+} and other ions into the postsynaptic cells (Benveniste et al., 1984; Globus,1991). In addition, the energy failure associated with ischemia results in an increase in free cytosolic Ca^{2+} concentration and also affects the ATP-dependent extrusion of Ca^{2+} and its intracellular sequestration (Siesjo and Bengtsson, 1989; Siesjo, 1991; Hansen, 1985). These abnormally high levels of intracellular Ca^{2+} are then thought to activate a myriad of enzymes such as proteases, caspases, kinases, phospholipases and endonucleases initiating cascades that culminate in cell death (Choi, 1988; Siesjo, 1988; Siesjo and Bengtsson, 1989; Korshetz et al., 1996). While prompt restoration of blood flow can attenuate neuronal death it may also contribute to the disease process. Reperfusion injury has been implicated in neuronal death following ischemia primarily because reoxygenation of the tissue leads to an increase in free radical production that results in lipid peroxidation and destruction of cell membranes.

1.5 Neuroprotective Strategies

As alluded to in the preceding section a pervasive theory of ischemic cell death is the theory of glutamate excitotoxicity. The main tenant of this theory is

that the massive increase in extracellular glutamate following reperfusion is responsible for initiating a number of events (e.g. increased levels of intracellular Ca^{2+}) that ultimately lead to the selective death of vulnerable cells. Early evidence supported a role for glutamate in ischemic cell death. In conjunction with the fact that glutamate is accumulated extracellularly following ischemia (Benveniste et al., 1984; Benveniste et al., 1989; Hagberg et al., 1985) there is a high concentration of NMDA receptors in the vulnerable CA1 region (Monaghan and Cotman, 1985). In addition, in 1984, Simon and colleagues demonstrated that when the glutamate antagonist – aminophosphonheptanoate was injected into the hippocampus there was a dramatic reduction in the neuronal damage after ischemia (Simon et al., 1984). A year later Pulsinelli showed that removing the glutamatergic input into hippocampus reduced ischemic damage (Pulsinelli, 1985). Therefore one of the first strategies developed to protect neurons following ischemia was to block the effects of excess glutamate. A number of drugs including the NMDA antagonist MK-801 and the AMPA antagonist NBQX initially appeared promising in that a number of investigators reported substantial neuronal protection in both global and focal models of ischemia (Gill et al., 1987; Park et al., 1988; Diemer et al., 1992; Nellgard and Weiloch, 1992; Buchan et al., 1991; Sheardown et al., 1993). However, more

careful analyses revealed that these compounds produced hypothermia that when prevented abolished any protection (Buchan and Pulsinelli, 1990; Corbett et al., 1990; Nurse and Corbett, 1996).

Despite the fact that there has been little success with the glutamate antagonists researchers continue to investigate new drugs that block glutamate excitotoxicity. This is surprising since by the time these drugs would be administered to a patient (i.e. likely several hours after stroke) the effects of the glutamate would have already taken place. Thus, the rise in intracellular calcium would have already occurred along with activation of calcium mediated caspase and protease activity and free radical formation. Unfortunately, when strategies have been employed to counteract these latter events (i.e., Ca^{2+} channel blockers, Ca^{2+} chelators and free radical scavengers) they have been largely unsuccessful at preventing neuronal death. Again, the explanation could be that because there are so many events taking place, each with the capability of destroying the cells, blocking one of them provides very little benefit. Therefore, it is likely that a more broad pharmacological approach is required. Perhaps a cocktail of agents each targeting a specific element of the ischemic cascade would provide significant neuroprotection.

While the above would imply that finding an effective treatment for stroke is a daunting task there have been some success stories. As previously mentioned many of the pharmacological agents that appeared to prevent neuronal loss did so through a hypothermic effect. Both intraischemic (Nurse and Corbett, 1994) and postischemic (Colbourne and Corbett, 1995; Colbourne and Corbett, 1994; Busto et al., 1987; Minamisawa et al., 1990) hypothermia have been proven to provide significant and long lasting neuroprotection. In fact, it is now considered to be the gold standard of neuroprotection (Colbourne and Corbett, 1994; Colbourne and Corbett, 1995; Green et al., 1992; Nurse and Corbett, 1994). Although hypothermia has been used during repair of heart defects in children to prevent ischemic brain damage (Bigelow et al., 1950; Lewis et al., 1954; Clifton et al., 1989) its potential use in stroke has met with both skepticism and resistance. One of the reasons for this includes the logistics from moving from rodent models of ischemia to treating human stroke. What depth and duration of hypothermia would be necessary to provide protection? These parameters need to be worked out and side effects of hypothermia (e.g. shivering) controlled. In addition, the patient would have to be anesthetized for the entire time. This fact alone poses two additional difficulties. First, the fact that the patient is immobilized and unresponsive means that frequent

assessments cannot be made; something that is currently an important aspect of stroke management. Second, prolonged anesthetization would require constant care at the ICU level, which necessarily puts more demands on space and personnel. While these concerns are real, they are not insurmountable and, considering the potential benefits, hypothermia should be investigated as a clinical option.

1.6 Ischemic Preconditioning

Ischemic preconditioning (ischemic tolerance) was first demonstrated in the heart where it was shown that a brief non-injurious episode of ischemia was able to protect heart tissue against a subsequent more severe insult (Murray et al., 1986; Li et al., 1990). The brain can also be made tolerant to ischemia. For example, in the gerbil model of global ischemia four days following a 5 min occlusion of both carotid arteries 80-90% of the CA1 cells are destroyed. However, if the gerbil is subjected to one or two brief episodes (e.g. 2 min) of ischemia prior to a more severe insult, then approximately 60-90% protection of CA1 neurons is achieved (Kitagawa et al., 1991; Kitigawa et al., 1990).

The effectiveness of ischemic preconditioning in the brain depends on both the intensity of the preconditioning episode and the time between insults

(Perez-Pinzon et al., 1997). Repeated ischemia, if given after short intervals, is known to have a cumulative deleterious effect on neuronal survival. Tomida and colleagues have previously shown in the gerbil that three episodes of 5 minutes of ischemia each separated by 1 hour produced more damage than one 15 minute episode (Tomida et al., 1987). Similarly, repeated episodes of non-lethal ischemia can produce neuronal damage if given close together (Kato et al., 1990). Studies designed to determine the parameters of ischemic preconditioning seem to indicate that at least 1 day following the preconditioning episode is required to confer protection and that the positive effects of the preconditioning episode can last up to one week (Kato et al., 1991; Kirino et al., 1991; Kitagawa et al., 1990).

Another interesting feature of ischemic preconditioning is that it appears to work in both global and focal ischemia models (Kato et al., 1991; Kirino et al., 1991; Kitagawa et al., 1990; Corbett and Crooks, 1997; Dooley and Corbett, 1998; Chen et al., 1996). Further, it has been demonstrated in the rat that an episode of focal ischemia (20 min) given prior to a global ischemic episode was protective (Glazier et al., 1994). Similarly, Simon and colleagues observed that when global ischemia preceded focal ischemia a decrease in infarct size was observed (Simon et al., 1993). The fact that preconditioning with focal ischemia is protective in a

global model and vice versa suggests that the mechanisms of cell death in these two models are similar. Finally, it has been demonstrated that transient focal ischemia can protect against subsequent lethal focal ischemia in terms of decreased infarct size (Chen et al., 1996). This last example of the focal-focal model of ischemic preconditioning is quite interesting in light of the fact that it may relate to the clinical situation of transient ischemic attacks (TIA's) preceding full blown stroke.

This phenomenon of ischemic preconditioning has generated widespread interest since it may provide insight into endogenous protective mechanisms that could be harnessed so as to attenuate ischemic cell death.

1.7 Assessment of Neuroprotection

Historically, neuroprotection has been assessed using histological endpoints. In general traditional histological staining methods (e.g. haematoxylin and eosin, H&E) are not very sensitive indicators of *early* neuronal injury since cell death may not become obvious until several days after ischemia. However, as long as the histological assessments are made after this time, quantification can be relatively simple especially within the stratified CA1 region of the hippocampus. The laminar distribution and large size of CA1 neurons makes

performing cell counts an easy task. The problem arises when neuroprotective treatments are being investigated. A major drawback of many ischemia studies is that survival times of only a few days are used. There is now substantial evidence that cell loss may continue for weeks and months postischemia and that many neuroprotective therapies are only delaying cell death (Valtysson, 1994; Colbourne and Corbett, 1995; Colbourne and Corbett, 1994; Corbett and Nurse, 1998; Dietrich et al., 1993; Corbett and Crooks, 1997; Dooley and Corbett, 1998). Therefore it is essential that long survival times of one or more months be employed to determine the true extent of histological protection.

In addition to the widespread use of relatively short survival times, another problem with the majority of ischemic studies is that only histological endpoints are being used. This evaluative method is unable to determine if the remaining, apparently normal, cells are functioning properly. In fact, there is evidence that this may not be the case. Hori and Carpenter (1994) revealed that following global ischemia in rats there was a reduction in the amplitude of the population excitatory postsynaptic potential (EPSP) recorded from healthy looking CA1 neurons with normal membrane potentials and resistances. Furthermore, long term potentiation (LTP) could not be produced in these cells. Upon closer examination, the CA1 dendrites had an abnormal, beaded

appearance which is an early sign of neuronal injury (Hori and Carpenter, 1994; Kitagawa et al., 1989; Matesic and Lin, 1994). Similarly, behavioural impairments (as assessed by acquisition of operant behaviour) following unilateral forebrain ischemia have been noted despite hippocampal pyramidal neuron preservation (Bothe et al., 1986). In another study, it was found that while pentobarbital administration following global ischemia protected CA1 pyramidal cells, it was unable to preserve hippocampal cholinergic function (Ishimaru et al., 1995). Finally, a study involving ischemic preconditioning recently reported a dissociation between histological protection provided by ischemic preconditioning and behavioural protection as assessed by an open field test of habituation (Corbett and Crooks, 1997). Considering that functional outcome is of paramount importance following clinical stroke, it is essential that functional endpoints also be used as an indicator of neuronal protection in experimental studies.

1.8 Overview of Experiments

1.8.1 Ischemic Preconditioning in Aged Gerbils (Chapter 3)

Stroke is most prevalent in individuals 65 years and older yet most research is done using young subjects. It is possible that vulnerability to ischemic injury is dependent upon age. Therefore, the aim of this experiment was to evaluate the neuroprotective efficacy of ischemic preconditioning in aged (18 month old) gerbils. Experimental animals were assessed at three time points over a two month period using a multifaceted approach. First, hippocampal function was determined using both behavioural and electrophysiological measures. Starting three days postischemia and throughout the survival period animals were repeatedly exposed to a novel open field meant to assess spatial memory formation. At the end of the survival period, measurement of evoked CA1 field potentials provided a direct indication of hippocampal function. These results were then compared to the histological outcome of the same animals to yield an overall picture of neuroprotection. Finally, an immunocytochemical stain for MAP2 was used to assess the dendritic morphology of CA1 that might explain the previously reported early dissociation between functional impairment and

apparent histological preservation following ischemic preconditioning (Corbett and Crooks, 1997; Dooley and Corbett, 1998).

1.8.2 A Comparison of Young and Aged Animals (Chapter 4)

Based on the results from the experiment in Chapter 3 and other experiments performed in our lab (Corbett and Crooks, 1997; Dooley and Corbett, 1998), it appeared as though ischemic preconditioning was more effective in aged animals than young ones. However a number of factors (e.g. different surgeon) unrelated to age could account for this apparent difference. Therefore, it was necessary to do a direct comparison of aged and young animals to confirm this finding. Young and old animals were assessed simultaneously. Again, a multifaceted approach was used determine the extent of neuroprotection provided by ischemic preconditioning. In addition, an immunocytochemical stain for glial fibrillary acidic protein (GFAP) was used to determine if a change in the astrocytic response could be a contributing factor to protection provided by ischemic preconditioning as previously reported (Kato et al., 1994).

1.8.3 Early Behavioural Testing and Ischemic Outcome (Chapter 5)

It is widely accepted that rehabilitation following stroke is beneficial (Dobkin, 1989; Ernst, 1990). However, little has been done in the way of clearly defining the parameters of rehabilitative therapy. Recent studies have indicated that when rehabilitation is initiated early after the ischemic episode it can exacerbate injury and inhibit recovery. In the previous experiments (Chapters 3 and 4), open field testing was initiated three days postischemia. This may be problematic as the testing may tax the recovering, but compromised, neurons to an extent that they eventually die. In this experiment, ischemic preconditioning was used as a model to determine whether or not open field testing alters histological outcome. Following ischemic preconditioning half of the animals in the study were exposed to a novel open field on days 3, 7, 10 and 30. The other animals were handled in the same manner but not exposed to the open field environment. Following sacrifice on day 35, CA1 cell counts were compared between groups.

CHAPTER 2: GENERAL METHODS

2.1 Animals

All experimental procedures were carried out in accordance with the animal care guidelines of Memorial University of Newfoundland and the Canadian Council on Animal Care. All experiments involved the use of female Mongolian gerbils (*Meriones unguiculatus*) purchased from High Oak Ranch Limited (Baden, ON, Canada). Females were used because males scent mark regularly and this would interfere with open field testing. The gerbils were group-housed (four per cage) for a minimum of two weeks before being included in any experiment. In addition, it was necessary to treat all the animals with piperazine citrate (0.34% w/v in the drinking water), a drug for treating pinworm infections that are common in gerbils and other rodents. Drug administration typically lasted 3 days.

The lights in the animal room were kept on a 12 hr light/dark cycle and the housing conditions of the animals varied depending on the experiment. For the experiments involving young animals (aged 3 months), the animals were housed in groups of four prior to the start of the experiment and then housed individually thereafter. For the experiments involving old animals (aged 18 months), the animals were housed in groups of two and kept in the animal care facility for 16

months until they reached 18 months of age and were housed singly when included in an experiment. All the cages were changed bi-weekly and the gerbils were given fresh water daily. The gerbils' diet consisted principally of guinea pig pellet food. In addition the animals were given a seed mixture containing sunflower seeds and corn kernals twice weekly and fresh vegetables either carrots, apples or broccoli once a week. Finally, shredded paper and cardboard tubes were placed in the cages for the gerbils to shred and build nests.

2.2 Cannula Implantation

Four days prior to the induction of ischemia the gerbils were anesthetized using either sodium pentobarbital (65 mg/kg, i.p) or halothane (1.5%, 70% N_2O / 30% O_2) and implanted with a guide cannula overlying the dorsal medial striatum. This permitted the later placement of a brain temperature probe (model XM-FH, Mini Mitter, Sunriver, OR, USA). The cannula was a modified needle (20 gauge) that was 5 mm in length and held in place using dental cement (Colbourne and Corbett, 1994; Nurse and Corbett, 1994). Briefly, under anesthesia, the animal's head was immobilized and an incision was made in the scalp. The skin was retracted and the skull was gently rubbed using a cotton swab to remove the overlying periosteum. A small hole was then drilled in the skull, approximately 1

mm in front of Bregma and 2 mm lateral from the midline suture. Two plastic screws (#MN-080-2, nylon machine screw, pan head #0-80 x 1/8", Small Parts Inc., Miami Lakes, FL, USA) were fixed to the skull, head down, using Lepage accu-flo™ super glue. The glue was given five minutes to set. The cannula, held in place with a stereotaxic arm, was then placed in the hole and rested on the dura. A cap made of dental cement was carefully built around the plastic screws and the cannula to keep it all in place. After the cap had hardened, the skin was sutured and the cap was marked with the last two digits of the animal's identification number. Finally, a 27 gauge stylet was placed in the cannula to keep it patent, the anesthesia was discontinued and the animal was placed back into it's home cage and allowed to recover.

2.3 Normal Temperature Measurement

Two days after implantation of the cannulae, the gerbils were anesthetized with 1.5% halothane in a mixture of 30% O_2 and 70% N_2O and the brain probes were inserted through the guide cannula into the striatum to a depth approximating that of the hippocampus. The probes were kept in place using surgical tape. Anesthesia was discontinued and the animals were placed in plexiglass cages atop AM receivers (model # RA-1010, Data Sciences Inc., St.

Paul, MN, USA). Brain temperatures were monitored and recorded every 20 sec (Experiment 1) or 30 sec (Experiment 2) for a period of 3 hr to establish baseline records of temperature. All the brain probes were sterilized by soaking them in Cidex for 20-30 min prior to use. This was done in order to avoid potential brain infections.

The brain temperature probes are battery operated and transmit signals based on frequencies. In order to ensure the accuracy of the signal being transmitted, the probes were frequently calibrated. Calibration consisted of using a precision thermometer to first calibrate a thermocouple probe (HYP1-30-1/2-T-G-60-SMP-M) with a microprocessor thermometer (model HH23, both from Omega Technologies Co., Stanford, CT, USA). The latter two were then used to determine the frequency emitted by the probes at particular temperatures. Specifically, the frequency of the signal emitted from two temperatures was determined. Briefly, both the probe and the thermocouple were floated in a beaker containing hot water. The water was then allowed to stand and slowly cool. When the temperature reading on the thermocouple reached 38.0°C a frequency trace was started and run until temperature reached 37.9°C. This same procedure was followed using a temperature change from 33.0°C to 32.9°C. The frequency values received from this calibration were then entered into the

configuration file of the Datasciences program. The accuracy of the probe was then tested again using the thermocouple probe and had to be within 0.1 °C in the temperature range of 36-37°C in order to be considered useable. The signal being transmitted is completely dependent on the viability of the battery. Therefore, probes were only used for 150-200 hr per battery before a new battery was inserted into the probe and the probe was recalibrated.

2.4 Induction of Ischemia

Prior to surgery the animals were divided into treatment groups. Under halothane anesthesia (2%, 30% O_2 / 70% N_2O), brain probes were again inserted into the guide cannula for direct measurement of brain temperature during the surgery. The animals' necks were shaved and a ventral midline incision was made. The carotid arteries were then carefully isolated so as to free the arteries from the surrounding tissue. Care was taken to ensure that the vagal nerve was separated from the artery and that the artery was as clean as possible. Silk suture was then looped under each artery so that they could easily be picked up for occlusion.

When brain temperature was stable (36.5°C) the arteries were occluded with miniature aneurysm clips for various lengths of time depending on the

experiment. Brain and rectal temperature were monitored throughout surgery and during the occlusion period. Brain temperature was maintained as close to 36.5°C as possible using a flexible blanket (Gaymar heat therapy Mul-T-Pad™ model TP-3E, 31/2" x 23", Gaymar Industries Inc., Orchard Park, NY) perfused with circulating water at 47°C. Core temperature was maintained between 36.0°C and 37.0°C using an overhead lamp and a homeothermic heating blanket wrapped around the animal's body (Harvard Apparatus, South Natick, MA, USA). Following ischemia the clips were removed and reflow was visually confirmed. The incision was closed, anesthesia was discontinued and the animals were again allowed to recover in the plexiglass cages and brain temperature was monitored continuously for either 8 or 24 hr. Sham animals underwent the same procedures in which the carotid arteries were exposed but not occluded. The brain temperature of all animals was maintained at 36.5°C, with a heating lamp, for thirty minutes following occlusion. This approximates the length of time for a 5 min ischemic animal (most severe) to recover from surgery and begin to self regulate temperature. While being monitored, the animals were given free access to food and water. In addition, a piece of paper towel was placed in the bottom of each cage. Gerbils will shred this paper in expressing their nest building behaviour. However, ischemic animals have been shown to lose this behaviour

for a few days following the ischemic episode. Therefore even the observation of whether or not the paper is shred is a reliable early indicator of the severity of the ischemic insult (Antonawich et al., 1997).

At the end of the monitoring period the animals were reanesthetized and the brain probes were removed. The stylets were replaced and the animals were placed back in their home cages and returned to the animal care facility.

2.5 Behavioural Testing

All animals were tested in an open field apparatus with the exception of one group in experiment 3 (see Chapter 5). Behaviour in the open field was assessed on days 3, 7, 10, 30 and 60 postischemia (depending on survival time) in a soundproof room (2.1 m x 3.4 m). The position of various environmental stimuli in the room (e.g., location of shelves) remained constant throughout the experiment. The floor of the open field apparatus (72 x 76 x 57 cm^3) was electronically divided into 25 equal squares and the number of squares entered per minute over a 10 min test session was recorded by a visual tracking system (HVS Systems Kingston, UK). The total number of squares entered throughout the 10 min test session was then used for analysis.

Various factors such as excessive handling, loud noises and transport can confound behavioural assessments. Therefore, the animals were not tested within 30 minutes of being moved from the animal care facility to the laboratory. In addition, every effort was made not to disturb the animals during this rest period.

Immediately prior to testing, the animal's cage was placed in the open field room, the animal was gently removed from the cage and placed in the corner of the open field apparatus. The computer tracking system was initiated and the experimenter left the room. At the end of the test session, the experimenter re-entered the room, removed the gerbil from the open field and placed it back in it's home cage. After each session, the open field was washed with mild soapy water and allowed to dry. This was done in order to minimize odor that may influence the next animal's behaviour. Following testing, each gerbil was weighed and returned to the animal care facility.

2.6 Electrophysiology

Electrophysiological recordings were taken from the majority of animals in experiments 1 and 2 (see Chapters 3 and 4, respectively). After the last day of behavioural testing, animals were anesthetized with 2% halothane (30% O_2/70%

N_2O) and cooled to a brain temperature of 30°C with the use of a water blanket circulating ice cold water. During cooling the level of halothane was gradually reduced to 0.5%. The gerbils were then decapitated and their brains quickly removed and placed in ice-cold oxygenated modified sucrose Krebs solution (Aghajanian and Rasmussen, 1989; Nurse and Corbett, 1994; Payne and Neuman, 1997) containing (mmol/L) sucrose 215.8 (in place of NaCl), KCl 3.5, $CaCl_2$ 2.0, $NaHCO_3$ 25.0, $MgCl_2$ 1.3, glucose 11.0, NaH_2PO_4 1.2 that was bubbled with 95% O_2/ 5% CO_2. The brains were hemisected; one hemisphere was immersed in 10% phosphate-buffered formalin (4°C) for histology, and the other hemisphere was used for electrophysiology. The hippocampus was isolated and cut in 500 µm transverse sections with a tissue chopper (Model # 51425, Stoelting, Wood Dale, IL, USA). Slices were again placed in the above modified artificial cerebrospinal fluid (ACSF) solution at room temperature for 10-15 min, as done previously (Nurse and Corbett, 1994). Slices were then transferred into oxygenated (95% O_2/ 5% CO_2) KREBS solution containing (mmol/L) NaCl 126.0, KCl 3.5, $CaCl_2$ 2.0, $NaHCO_3$ 25.0, $MgCl_2$ 1.3, glucose 11.0 and NaH_2PO_4 1.2 and incubated for at least 1 hr before recordings.

Individual slices were placed in a fluid interface chamber (Fine Science Tools Inc) perfused with oxygenated ACSF at a flow rate of 2 ml/min. The

temperature of the bath was maintained at 33°C. Orthodromic stimulation (0.02-msec constant-current pulses delivered at a rate of 0.05 Hz) of the Schaeffer collaterals was achieved using Ultrasmall concentric bipolar stimulating electrodes (100 μm; Frederick Haer Company). Glass micropipettes with a tip diameter of ~ 20 μm and filled with 2 mol/L NaCl were used for recording EPSPs in stratum radiatum. Responses were amplified and displayed on an oscilloscope, digitized and stored on a computer for later analysis using ASYST software. The viability of the slice was tested first by orthodromic stimulation of the perforant path and recording a population spike in dentate granule cells (uninjured after ischemia). In slices that failed to yield a dentate population spike, no recordings in CA1 were attempted.

2.7 Histology

In the animals from which electrophysiological recordings were taken, the hemisphere reserved for histology was immersion fixed in 10% buffered formalin for a minimum of two days. All other animals were given an overdose of sodium pentobarbital and perfused transcardially with heparanized saline followed by 10% buffered formalin. To avoid the occurrence of dark neuron artifact, the brains were kept in the skulls overnight in a jar of formalin and then carefully

removed the next day. All of the tissue was subsequently embedded in paraffin and 1-2 series of 6 μm thick coronal sections were cut on a microtome. The first series was stained with haematoxylin and eosin (H&E) and the number of neurons exhibiting distinct cell membranes and nuclei (not eosinophilic) remaining in a 200 x 200 μm grid were counted in medial, middle and lateral sectors of dorsal CA1 at 1.7 mm (level A) and 2.2 mm (level B) posterior to Bregma. Cells were also counted from the middle sector of CA1 2.8 mm posterior to Bregma (level C).

The second series of sections were immunostained with either anti-microtubule associated protein 2 (MAP2) in experiment 1, or anti-GFAP in experiment 2 (see Chapters 3 and 4, respectively for details).

CHAPTER 3:

ISCHEMIC PRECONDITIONING IN 18-20 MONTH OLD GERBILS: LONG TERM SURVIVAL WITH FUNCTIONAL OUTCOME MEASURES

3.1 Introduction

Approximately three-quarters of all stroke cases affect those aged 65 years or older (Millikan, 1992). Despite this fact most stroke research utilizes young animals. This may be problematic since there are numerous age related differences in brain biochemistry, morphology and electrophysiology (Tamaru et al., 1991; Luine et al., 1990; Gonzales et al., 1991; West, 1993; Barnes, 1993). Previous studies examining the influence of age on ischemic outcome have produced conflicting results. In experiments involving focal ischemia, infarct volume was consistently increased in aged versus young rats (Davis et al., 1995; Sutherland et al., 1996). Global ischemia studies, however, have yielded more variable results. In one study there was increased neuronal loss in both the hippocampal CA1 region and striatum of aged (18-22 month) versus young (5-6 month) rats (Yao et al., 1991). In contrast, another study reported an age dependent regional vulnerability following global ischemia whereby there was less CA1 neuronal loss in old (26-28 month) than young (2-3 month) rats, but increased striatal and neocortical damage (Sutherland et al., 1996). Finally, Corbett and colleagues reported a similar degree of neuroprotection following global ischemia in old and young gerbils treated with intraischemic but not with

postischemic hypothermia. These authors cautioned that the efficacy of a particular treatment should be confirmed in aged animals (Corbett et al., 1997).

As previously mentioned, Corbett and Crooks (1997) recently reported a dissociation between histological protection provided by ischemic preconditioning and behavioural protection as assessed by an open field test of habituation. Increased locomotor activity in an open field is thought to reflect an animal's inability to habituate to a novel environment. Increased open field activity has been linked to loss of CA1 neurons (Wang and Corbett, 1990; Gerhardt and Boast, 1988) and not to loss of striatal or cortical neurons (Mileson and Schwartz, 1991). However, the open field task is not specific to hippocampal function and even animals with extensive CA1 loss eventually recover, most likely due to compensation by other brain regions (Corbett et al., 1992). Thus, a valuable additional functional endpoint is to record CA1 field potentials which have been found particularly effective when combined with behavioural and histological measures (Nurse and Corbett, 1994).

It has been suggested (Kitagawa et al., 1989; Matesic and Lin, 1994) that a sensitive indicator of early neuronal injury is loss of MAP2 which is primarily localized to dendrites (Caceres et al., 1984). A reduction in this structural protein could conceivably alter neuronal function in the absence of conspicuous

changes in Nissl staining of CA1 somata. If so, this could account for our previous observation that ischemic preconditioning preserves CA1 neurons (i.e., using Nissl stains) even though functionally, these neurons appear compromised (Corbett and Crooks, 1997; Dooley and Corbett, 1998).

In view of the above considerations, the purpose of this study was to examine ischemic preconditioning in a population of aged gerbils (18-20 months) and assess its efficacy at preserving hippocampal CA1 neurons. To this end behavioural, electrophysiological, immunocytochemical (MAP2) and standard histological endpoints were employed as well as long survival times (10, 30 and 60 days). The use of longer survival times ensures that treatments are providing true neuroprotection rather than merely delaying cell death (Colbourne and Corbett, 1995; Corbett and Nurse, 1998).

3.2 Materials and Methods

3.2.1 Subjects

A total of 78, female, Mongolian gerbils weighing 55-114g and aged 18-20 months were used for this study.

3.2.2 Temperature

Brain temperature was closely monitored in all animals prior to, during and following the ischemic episode as previously described (Corbett and Crooks, 1997; Nurse and Corbett, 1994; Colbourne and Corbett, 1994). Under 1.5% halothane anesthesia (30% 0_2/ 70% N_20) all animals were implanted with a 5mm/20 gauge guide cannula overlying the dorsomedial striatum as described in the general methods in section 2.2. Forty-eight hr later, direct measurements of brain temperature were recorded every 20 sec for a period of 3 hr to establish a record of normal brain temperature.

3.2.3 Induction of Ischemia

Animals were allocated to groups prior to the experiment: Sham (S); preconditioned only (PO) consisting of two 1.5 min occlusions separated by 24 hr; ischemic preconditioned (IP) - two 1.5 min occlusions separated by 24 hr followed three days later by a 5 min occlusion and a 5 min ischemic group without prior preconditioning (I).

Surgical procedures and postischemia temperature monitoring and regulation are described in section 2.4 of the general methods.

3.2.4 Behavioural Testing

Behaviour in the open field was conducted as described in Chapter 2 (section 2.5).

3.2.5 Electrophysiology

Following the last day of behavioural testing, animals were sacrificed and brain slices were prepared for electrophysiological recordings. Field EPSPs (fEPSPs) were recorded from the apical dendrites of CA1 following Schaeffer collateral stimulation (see section 2.6 for details).

3.2.6 Histology

Following immersion fixation, the hemisphere reserved for histology was embedded in paraffin and two series of 6 μm thick coronal sections were cut. One series of slides was stained with H&E and CA1 cell counts determined as described in section 2.7.

The second series of sections was immunostained with anti-MAP2 diluted 1:500 (Sigma Chemical Co., St. Louis, MO) and developed using avidin-biotin-peroxidase complex (Vectastain ABC) followed by incubation with diaminobenzidine (DAB) - nickel chloride as previously described (Dooley and

Corbett, 1998). Negative controls were run repeatedly by omitting the primary antibody. There was no staining in the control sections. Semi-quantitative analysis of the stained sections at level A was done using NIH Image software running on a Macintosh 7600 computer to determine relative optical densities (ROD). Briefly, the ROD for the apical dendritic fields of CA1 were measured and the data normalized to the unstained corpus callosum (CC) using the following formula: (ROD CA1 - ROD CC)/ ROD CA1.

3.2.7 Statistics

With the exception of day 3, 7 and 10 open field data, all other data were analyzed using one way ANOVA. The open field data for days 3, 7 and 10 were analyzed using repeated measures analysis of variance. Individual post-hoc comparisons were performed, when necessary, using the Neuman-Keuls test to compare group means.

Temperature profiles, behaviour in open field and cell counts were all obtained from the same animals while electrophysiological and MAP2 immunocytochemical data were obtained from subsets of the animals in each group.

Correlational analyses (simple regression) were also conducted in order to determine the relationship between each outcome measure.

3.3 Results

There were no group differences in weight at the start of the experiment. Eight animals died throughout the course of the experiment. Six died as a result of respiratory failure (during or just after ischemia), one from a severed artery and one animal was sacrificed due to weight loss resulting from a brain infection. One animal in the PO group was excluded from the study. In this animal, there was complete loss of CA1 cells on histology, most likely due to reflow impairments following clip removal.

The behavioural, electrophysiological and histological data of the PO animals were not influenced by survival time and so the day 10 and day 30 data were pooled. This was also done for the ischemic groups.

3.3.1 Temperature

The mean baseline temperature for the groups ranged from 36.8°C to 36.9°C. Similarly, there were no significant differences in the intraischemic (5 min) brain temperatures between groups ($F_{2,51} = 2.991$, $p = 0.06$). The postischemic

brain temperature profiles of each group are presented in Fig. 1. The mean postischemic brain temperatures recorded over 24 hr were 36.66 ± 0.23, 36.96 ± 0.34 and 37.14 ± 0.29°C for the S, IP and I groups, respectively. The postischemic temperatures of the IP and I groups were significantly different from sham ($p < 0.05$), reflecting the brief period of hyperthermia that is common following global ischemia in this model (Colbourne and Corbett, 1994).

3.3.2 Behavioural Testing

Data from the open field are presented in Fig. 2. The scores of the animals within the IP group did not differ with survival time and so were pooled for analysis. Repeated measures analysis of variance indicated a significant treatment effect ($F_{3,65} = 35.908$, $p < 0.01$), day effect ($F_{2,6} = 80.514$, $p < 0.01$) as well as a significant treatment by day interaction ($F_{6,130} = 5.504$, $p < 0.01$). On the first test day, sham animals exhibited normal levels of exploration and showed habituation both within the test session (data not shown) and on subsequent test days. The PO group followed the same behaviour pattern as shams and although levels of recorded activity were slightly higher on all test days they were not significantly different from sham levels. In contrast, the I group displayed heightened levels of activity during all test sessions and their scores

were significantly different from the sham group on days 3, 7, 10 and 30 ($p < 0.01$). In addition, these ischemic animals showed very little within session habituation (data not shown). Interestingly, similar levels of increased activity were seen in the IP group on all test days and although overall activity levels did decline with repeated exposure, the behaviour of these gerbils in open field remained significantly different from the sham group on days 3, 7 and 10 ($p < 0.01$) as well as on days 30 and 60 ($p < 0.05$). A comparison of the IP and I animals revealed significant differences on days 7 ($p < 0.05$) and 10 ($p < 0.01$) in open field activity indicating a more rapid recovery in the IP group.

3.3.3 Electrophysiology

CA1 field potentials were recorded from approximately 100 slices taken from 35 animals (sham : n = 5; PO : n = 5; I : n = 6; IP 10 : n = 6; IP 30 : n = 6 and IP 60 : n = 7). Three recordings of maximal fEPSP amplitude were averaged for each rostral, middle and caudal section from every animal. These means were then averaged to yield an overall maximum amplitude representative of the whole hippocampus. Following stimulation of the Schaeffer collaterals the mean maximum amplitude of the field excitatory postsynaptic potentials recorded from the stratum radiatum of gerbils in sham and PO groups was not significantly

different (-4.04 ± 0.19 mV versus -3.514 ± 0.72 mV). The mean amplitude of the fEPSP of the I group was -1.07 ± 0.45 mV ($p < 0.01$ versus S). In keeping with the deficits in behaviour in open field, EPSPs measured in the IP group at 10 days survival were significantly lower than those of the sham and PO groups ($p < 0.05$). However, with longer survival times (Fig. 3) there was recovery of the evoked responses from -2.487 ± 0.55 mV at 10 days to -2.975 ± 1.34 mV and -3.173 ± 1.16 mV at 30 and 60 days, respectively. The potentials at 30 and 60 days were not significantly different from the sham animals.

3.3.4 Histology

The results of the histological assessment are presented in Figure 4. There was extensive loss of CA1 pyramidal cells at all rostral-caudal levels in the ischemic group ($p < 0.01$ versus S). PO animals were not significantly different from S. Ischemic preconditioning with 10 and 30 days survival provided robust protection of CA1 neurons at all levels measured. However, the percent savings at levels A and B did decline with continued survival such that by 60 days (IP 60) 75% of CA1 neurons were remaining at level A ($p < 0.05$ versus S).

There was a significant difference in the ROD of MAP2 immunostaining between the S and I groups (0.71 ± 0.007 vs 0.031 ± 0.186, $p < 0.01$). There was a

near absence of MAP2 staining in the apical dendritic region of CA1 from ischemic animals while the area under CA3 remained normal (Fig 5). IP gerbils, irrespective of survival time, showed no significant changes in MAP2 compared to S animals. However, the mean ROD of the IP group was slightly lower than that of the S group (0.65 ± 0.13, $n = 23$ vs. 0.71 ± 0.07, $n = 9$, respectively).

3.3.5 Correlations Among Outcome Measures

In S and I animals, each of the endpoints used to assess neuroprotection was highly correlated with the other outcome measures. Histological outcome was predicted by both behaviour in open field and fEPSP amplitudes elicited from CA1 (see Table 3.1). This is consistent with previous reports (Nurse and Corbett, 1994). In contrast, in IP animals open field behaviour was not correlated with fEPSP amplitude and neither functional assessment was able to predict histological outcome at any survival time (see Table 3.2).

Figure 3.1: Postischemic Brain Temperature

Brain temperature profiles of S, I and IP animals followed for 24 hr after surgery. Note that both I and IP animals exhibit a hyperthermic period postischemically. The mean postischemic brain temperatures of the I and IP groups are significantly different from S ($P<0.05$).

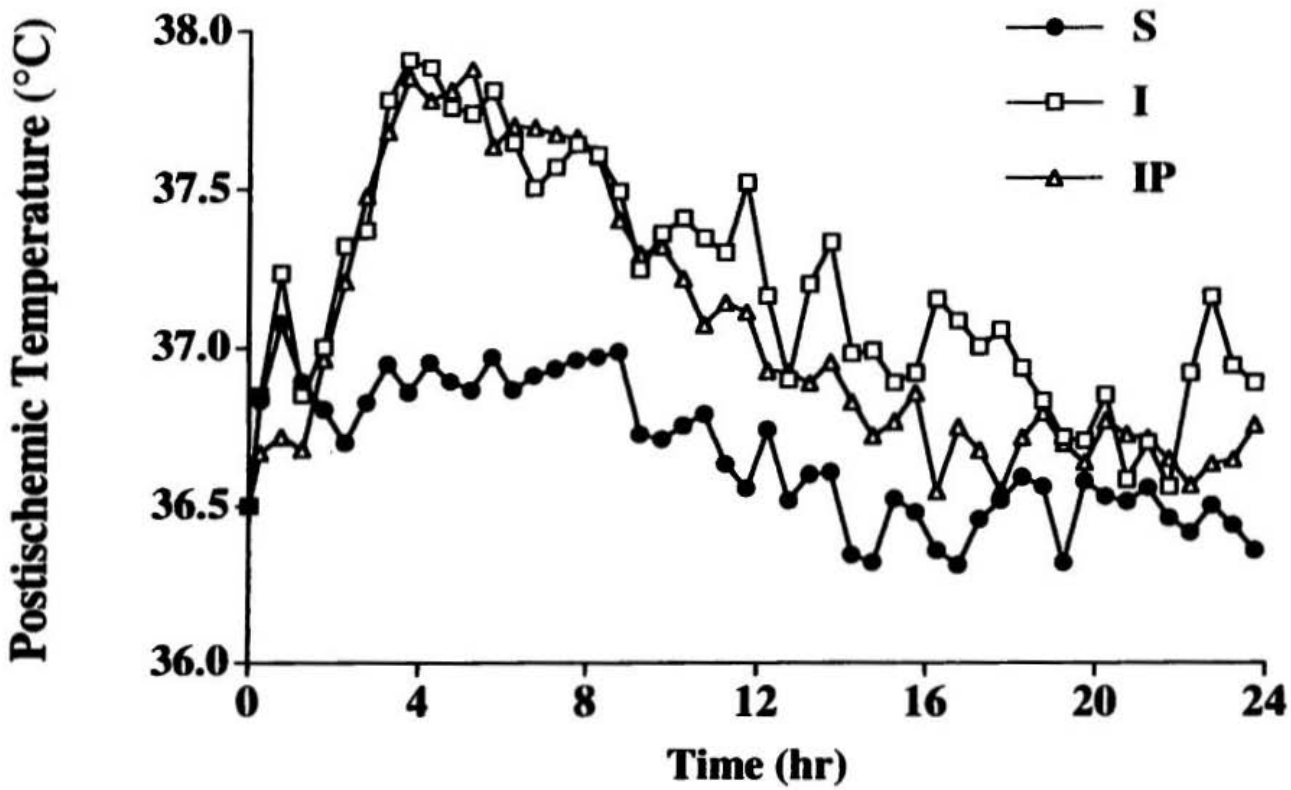

Postischemic Temperature (°C)
38.0
37.5
37.0
36.5
36.0
0
4
8
12
16
20
24
Time (hr)
S
I
IP

Figure 3.2: Total Open Field Score

Open field activity score (mean ± SD of number of squares entered in a 10 min test session) for sham (S), preconditioned only (PO), ischemic (I) and ischemic preconditioned (IP) groups on days 3, 7, 10 ,30 and 60 after ischemia/sham surgery. The number of animals in each group on test days 3, 7 and 10 was as follows: group S, n=9; group PO, n=15; group I, n=13 and group IP, n=32. Animals tested on day 30 included group S, n=9; group PO, n=6; group I, n=7 and group IP, n=23; and for day 60, group S, n=6 and group IP, n=8. Behaviour in open field is impaired in ischemic and ischemic preconditioned animals, although there is a trend for recovery in the latter group with repeated exposure and longer survival time.

*$P<0.01$, †$P<0.05$ with respect to group S; ‡$P<0.05$ comparing group IP with I.

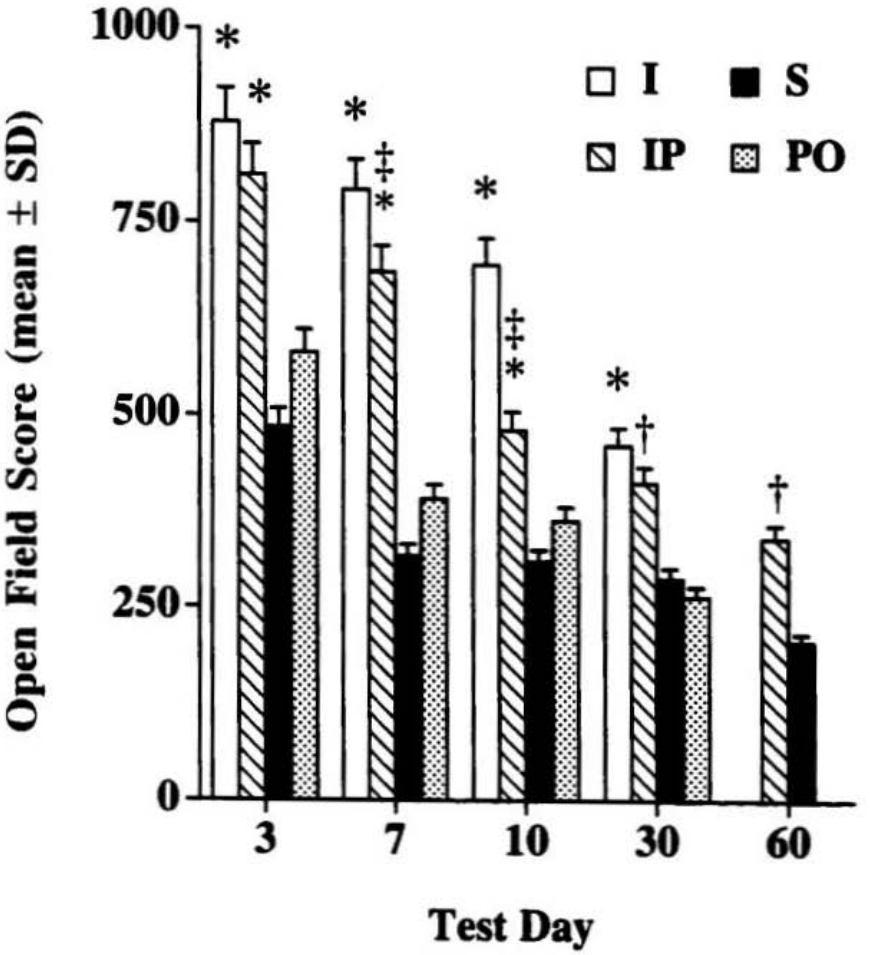

1000
750
500
250
0
Open Field Score (mean ± SD)
I
S
IP
PO
3
7
10
30
60
Test Day

Figure 3.3: CA1 Field Potentials

Maximum amplitude of CA1 fEPSPs (mean ± SD) recorded in stratum radiatum from group S (n=5), PO (n=5), I (n=6), IP-10 (n=6), IP-30 (n=6) and IP-60 (n=8) animals. Note the recovery of CA1 fEPSPs in IP animals with increasing survival time. Ischemic preconditioning provided significant protection at all survival times ($P<0.01$, versus group I). Recordings from S, PO and I animals were performed after 60-, 10- and 10-day survival, respectively, because survival time (i.e., 10 versus 30 versus 60 days) would not affect fEPSP amplitude in these groups (see "Results").

*$P<0.05$ with respect to S, †$P<0.05$ with respect to I.

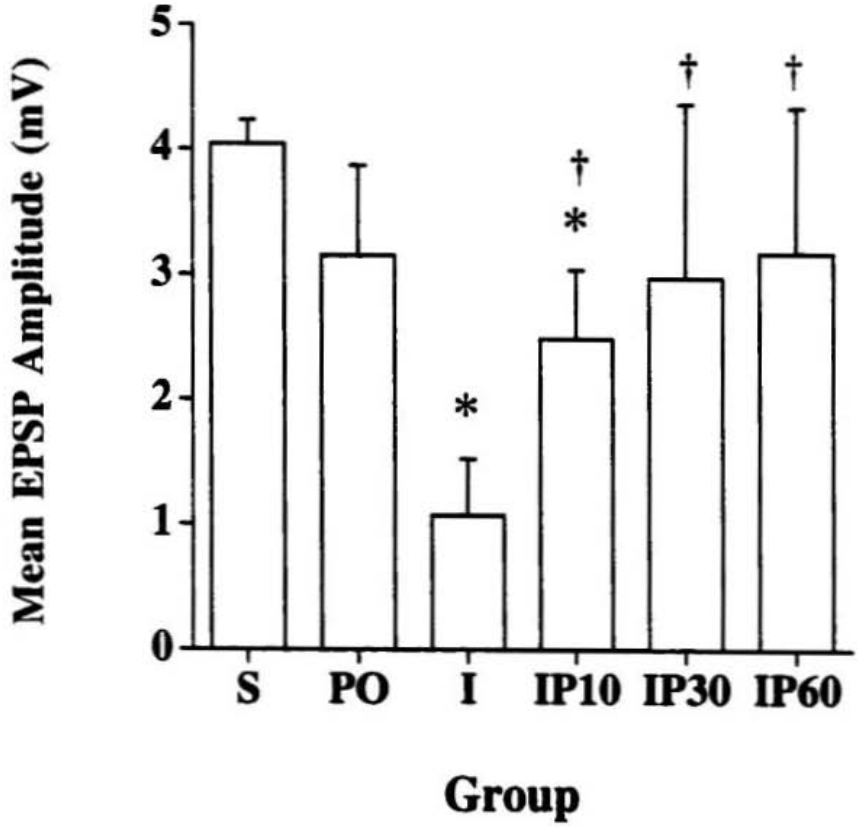

Mean EPSP Amplitude (mV)
5
4
3
2
1
0
S
PO
I
IP10
IP30
IP60
*
*
†
†
†
Group

Figure 3.4: Histological Assessment at Levels A, B and C

Total CA1 cell counts from 1 hemisphere 1.7 mm (level A), 2.2 mm (level B) and 2.8 mm (level C) posterior to Bregma. Groups as in Figure 1. Each symbol represents an individual animal while horizontal bars indicate group means. There were no significant differences in cell counts between S and PO animals. Five minutes of ischemia (I) resulted in significant neuronal loss at all levels ($P<0.01$). Ischemic preconditioning provided robust histological protection at 10 and 30 days. CA1 cell death continued with increasing survival time such that there was significant loss from levels A and B at 60 days ($P<0.05$, versus S). Cell counts from S, PO and I groups have been pooled since they did not differ with survival time (see "Results").

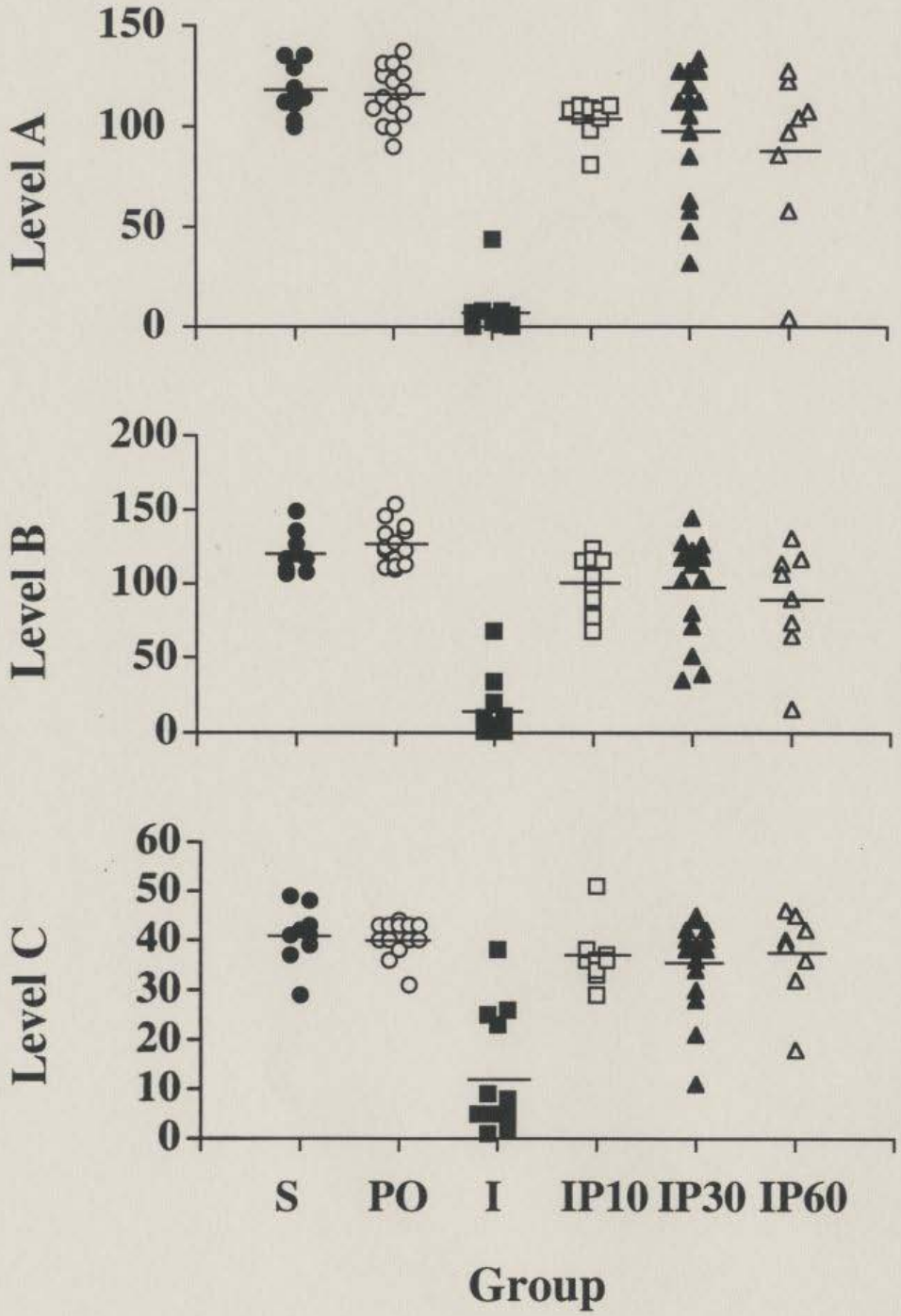

Level A
150
100
50
0
Level B
200
150
100
50
0
Level C
60
50
40
30
20
10
0
S
PO
I
IP10
IP30
IP60
Group

Figure 3.5: Representative Photographs of MAP2 Staining in the Hippocampus

Representative photographs of MAP2 immunoctyochemistry from groups S (top left), I (top right), and IP after 10- and 60-day survival (bottom left and right, respectively). Note the complete absence of MAP2 staining in the apical dendritic region of CA1 in group I compared with group S. Ischemic preconditioning did not significantly alter the pattern or intensity of MAP2 immunocytochemistry assessed at 10, 30 and 60 days after ischemia. Scale bar = 0.5 mm.

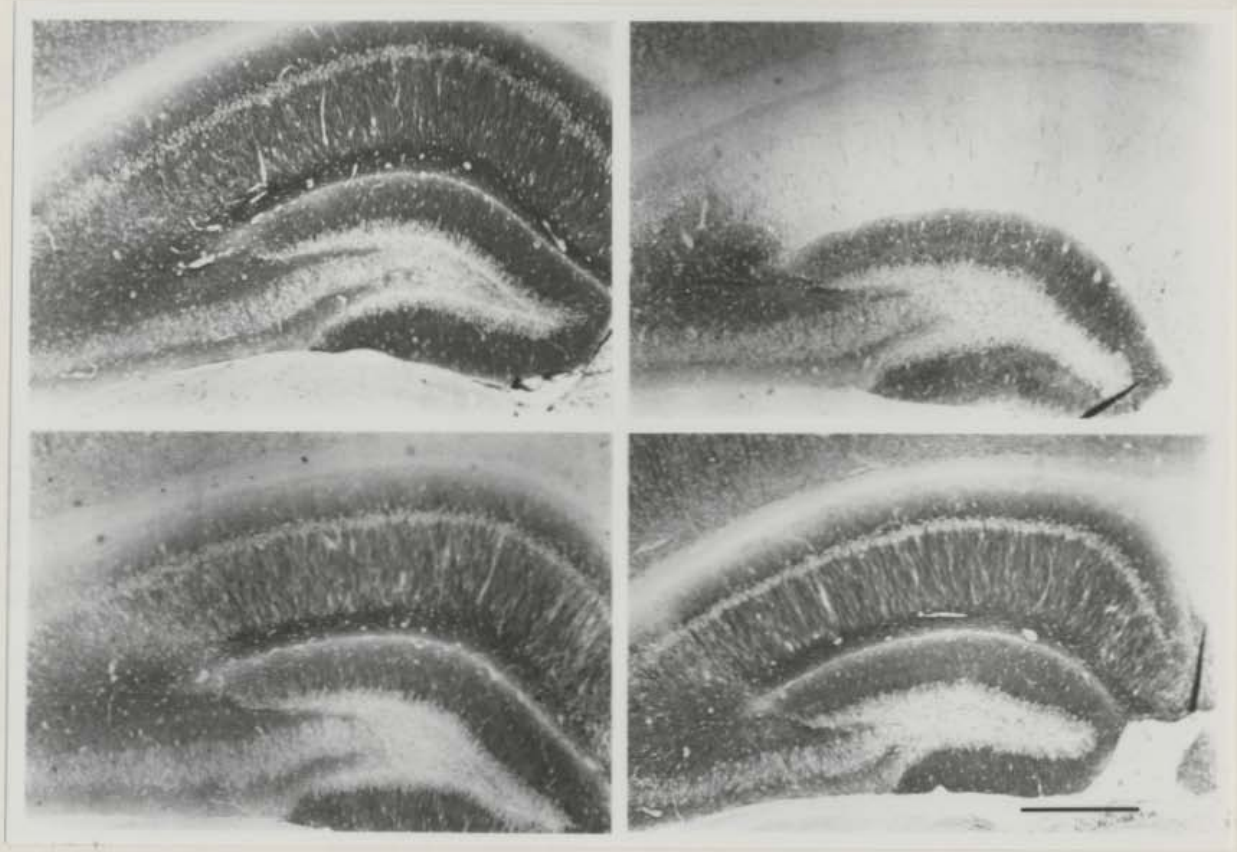


Table 3.1 Correlation Matrix for Sham and Ischemic Groups

A regression analysis was performed to determine the correlation among each of the outcome measures: behaviour in open field, extracellular field potentials and cell counts from Level A of CA1. CA1 cell counts were highly correlated with fEPSP amplitudes and behaviour in open field on days 3, 7 and 10 post ischemia or sham surgery.

*$p < 0.01$

	Open Field Behaviour					fEPSPs
	Day 3	Day 7	Day 10	Day 30	Day 60	
fEPSPs	0.852 *	0.929 *	0.859 *	0.439	0.456	
Cell Counts Level A	0.681 *	0.842 *	0.701 *	0.482	0.424	0.965 *

Table 3.2 Correlation Matrix for Ischemic Preconditioned Animals

A regression analysis was performed to determine the correlation among each of the outcome measures: behaviour in open field, extracellular field potentials and cell counts from Level A of CA1. There were no significant correlations between any of the outcome measures in the ischemic preconditioned animals.

	Open Field Behaviour					fEPSPs		
	Day 3	Day 7	Day 10	Day 30	Day 60	IP 10	IP 30	IP 60
fEPSPs								
IP 10	0.126	0.167	0.451					
IP 30	0.304	0.417	0.210	0.025				
IP 60	0.424	0.351	0.733	0.437	0.403			
Cell Counts Level A								
IP 10	0.237	0.047	0.474			0.213		
IP 30	0.057	0.051	0.366	0.471			0.242	
IP 60	0.121	0.195	0.037	0.043	0.099			0.480

3.4 Discussion

Numerous studies have confirmed the neuroprotective efficacy of ischemic preconditioning in animal models (Kitigawa et al., 1990; Corbett and Crooks, 1997; Dooley and Corbett, 1998; Kirino et al., 1991). However, these studies have typically used young subjects and therefore it was unknown whether similar levels of protection would be obtained in older animals.

In the present study there was significant histological protection of CA1 neurons across three anterior-posterior levels of the hippocampus (88.2%, 83.5% and 89.4% of sham controls) at 10 days in preconditioned animals. The rostral CA1 region is known to be highly vulnerable to cerebral ischemia (Kirino, 1982; Pulsinelli et al., 1982). In this study, the degree of neuronal protection found at this level was similar to that seen in similar studies from our laboratory using young (three month old) animals (Corbett and Crooks, 1997; Dooley and Corbett, 1998). Interestingly, there was not as rapid nor as great a decline in CA1 cell numbers in the aged animals with longer survival times. Thirty days following ischemic preconditioning there was ~53% (Corbett and Crooks, 1997) and ~60% (Dooley and Corbett, 1998) savings of rostral CA1 in the young animals. This is in contrast to the 75% savings found 60 days following a 5 min ischemic episode in aged ischemic preconditioned gerbils. This suggests that ischemic

preconditioning is at least as, if not more, effective in aged versus young animals. Nonetheless, as in other long term survival studies using both hypothermia and neuroprotective compounds (Nurse and Corbett, 1994; Colbourne and Corbett, 1995; Colbourne and Corbett, 1994; Li and Buchan, 1995) neuronal loss continued with increasing survival time (88.2% of S at day 10 compared to 75% at day 60, level A).

As previously mentioned several studies have reported a dissociation between histological and functional protection following both ischemia and ischemic preconditioning (Hori and Carpenter, 1994; Bothe et al., 1986; Corbett and Crooks, 1997; Dooley and Corbett, 1998). In this study animals subjected to ischemic preconditioning displayed heightened levels of locomotor activity in the open field similar to those of ischemic animals without any preconditioning. This was in spite of the fact that cell counts at this time in preconditioned gerbils were near normal (e.g., 88% of S at Day 10). With repeated exposure to the open field the animals in all groups showed habituation, however testing on Day 60 still revealed significant differences between S and IP animals ($p < 0.05$). These results are consistent with previous studies in that ischemic animals tend to recover with repeated exposure to a novel environment especially when testing is conducted at short intervals (Wang and Corbett, 1990; Colbourne and Corbett,

1994; Colbourne and Corbett, 1992; Babcock et al., 1993). However, persistent increases in locomotor activity are observed when test sessions are spaced out over weeks or months (Colbourne and Corbett, 1995; Mileson and Schwartz, 1991; Babcock et al., 1993). Moreover, when apparently recovered animals are exposed to a semi-novel environment they again show elevated levels of activity (Wang and Corbett, 1990; Babcock et al., 1993).

Increased open field activity has consistently been a reliable indicator of ischemic damage (Wang and Corbett, 1990; Gerhardt and Boast, 1988; Karasawa et al., 1994) however, it is not a specific measure of CA1 or even hippocampal function. Therefore, measurement of fEPSPs provides a more direct assessment of hippocampal CA1 function. Recordings from gerbils that had received ischemic preconditioning were characterized initially by a decreased amplitude in CA1 evoked potentials. This coincides with the deficits seen in the open field and occurs at a time when cell counts are virtually normal. In conjunction with the improvement in behaviour, there was recovery of the fEPSP amplitudes in ischemic preconditioned animals with increasing survival time such that by day 60 they were not significantly different from sham values. Although there was a trend for increased cell death with longer survival times, it is not known if cell death would continue past day 60 and if it did how this would ultimately affect

field potentials. It is possible that through alteration of postsynaptic receptor density or increased transmitter release from Schaeffer collaterals that fEPSPs would remain essentially normal. Alternatively, if CA1 cell death continues, these compensatory mechanisms may not be able to sustain normal fEPSP amplitudes.

MAP2 has been reported to be an early indicator of ischemic injury (Kitagawa et al., 1989; Matesic and Lin, 1994). The results of our analysis revealed complete absence of MAP2 in the ischemic animals. However, there were no differences in MAP2 staining in IP and S animals and therefore changes in the dendritic morphology of CA1, as assessed with MAP2 immunocytochemistry, cannot account for the early functional impairments in the IP animals.

The mechanisms responsible for the protection provided by ischemic preconditioning remain speculative. For example, it has been shown that (Heurteaux et al.. 1995) there is increased release of adenosine following preconditioning in the rat (global model). Adenosine is thought to open K_{ATP} channels via the A_1 adenosine receptor and subsequently decrease glutamate release following severe ischemia (Heurteaux et al., 1995). This could be beneficial if the decrease in glutamate release is prolonged. Additionally, Ohta

and colleagues (Ohta et al., 1995) found increased Ca^{2+} sequestration by mitochondria and increased activity of Ca^{2+}-ATPase, which transports Ca^{2+} from the cytosol to the extracellular space, in gerbils with preconditioning. There is also evidence for a more rapid rate of recovery from protein synthesis inhibition after severe ischemia in preconditioned gerbils (Kato et al., 1995) as well as new protein synthesis (induction of heat shock proteins) following preconditioning (Chen and Simon, 1997). Finally, differences in the activation of microglia and astroglia may play a role in ischemic preconditioning. Astroglia support neuronal growth and survival whereas microglia release cytotoxic agents which may exacerbate neuronal injury (Giulian, 1993). Kato and co-workers (Kato et al., 1994) found that two days following ischemia there was intense staining of microglia and very little reaction of astroglia whereas in ischemic preconditioned animals there was astroglial activation with mild activation of microglia. This suggests that the ratio of astroglia to microglia may be important in determining the fate of compromised neurons.

Regardless of the mechanism(s), the results of this study clearly show that ischemic preconditioning is quite effective in aged gerbils, possibly more so than in young animals. Although evidence in the literature generally supports the notion that there is increased damage following ischemia in aged animals (Yao et

al., 1991; Corbett et al., 1997) one study (Sutherland et al., 1996) found less damage in CA1 in aged rats following global ischemia. Undoubtedly some of these differences are due to variations in the model used (global vs. focal), the severity of the insult, the survival times employed and the adequacy of temperature control during and following ischemia. Increased efficacy of ischemic preconditioning in older animals may reflect differences in the aged populations vulnerability to ischemia and/or ability to tolerate treatment procedures compared to young subjects. There is evidence for decreased density of NMDA receptor complexes as well as decreased responsivity to glutamate in the aged rodent brain (Tamaru et al., 1991; Gonzales et al., 1991). Prolonged activation of NMDA receptors by glutamate and entry of calcium into the cells has been thought to be a key component of the excitotoxic theory of ischemic cell death (Choi, 1988; Choi et al., 1988). A decrease in the number and sensitivity of NMDA receptors in aged animals could potentially reduce this excitotoxic response and decrease neuronal death.

The results of the current study raise several important issues concerning neuroprotection. First, there may be subtle differences in aged versus young animals ability to tolerate ischemia. If so, it is important that future experiments be done to confirm these findings and investigate the factors that can account

for the influence of age. Second, a functional approach is necessary for determining the extent of neuroprotection. In studies using intraischemic and postischemic hypothermia, deficits in behaviour and fEPSP amplitude were directly related to degree of neuronal loss (Nurse and Corbett, 1994; Corbett et al., 1997). Thus, in the case of hypothermia, any one of several endpoints (e.g. behaviour, CA1 fEPSP amplitude and CA1 cell counts) are valid indices of neuroprotection. A correlational analysis indicated that this was clearly not the case with ischemic preconditioning and may not be the case with other (e.g. drug) protective treatments.

CHAPTER 4:

A DIRECT COMPARISON OF THE EFFICACY OF ISCHEMIC PRECONDITIONING IN OLD AND YOUNG GERBILS.

4.1 Introduction

In the previous chapter, it was shown that ischemic preconditioning is highly effective at providing long lasting protection to CA1 neurons in aged gerbils. It was also suggested that the protection provided by ischemic preconditioning appeared more robust in aged gerbils than in young ones. This observation is potentially important in that it questions the validity of using young animals in ischemia studies. Others have also noted age dependent differences in ischemic outcome (Sutherland et al., 1996; Davis et al., 1995; Yao et al., 1991; Corbett et al., 1997, see section 3.1 for discussion). The fact that stroke is more prevalent in the elderly population suggests that a more accurate model would be one that utilized aged subjects.

The aim of this study was to directly compare the efficacy of ischemic preconditioning in young versus old gerbils. In addition, an attempt was made to uncover potential protective mechanisms of ischemic preconditioning. As alluded to in the previous chapter, there is evidence to suggest that the ratio of astroglia/microglia is important to neuronal survival. Glial cells outnumber neurons nine to one and play an integral role in the physiology and function of the brain (Travis, 1994). Astroglia and microglia are both intimately involved with the maintenance and survival of neurons. As such both glial types are

rapidly activated following brain injury (Gottlieb and Matute, 1999; Petito et al., 1990; Morioka et al., 1991). Astrocytes have long been considered important for neuronal survival. Following injury, they help restore ion homeostasis, reform the tight junctions of the blood brain barrier (BBB) and provide the CNS with neurotrophic factors (Yong, 1998; Giulian, 1993). Microglia, on the other hand, are known to secrete neurotoxic agents (e.g. free radicals) (Giulian and Baker, 1986) and contribute to neuron loss after injury (Giulian, 1993). There is evidence to suggest that the preconditioning episodes result in an early activation of astroglia. This increases the ratio of astroglia/microglia and thereby may contribute to the protection of neurons (Kato et al., 1994). Therefore, in addition to investigating the influence of age on the efficacy of ischemic preconditioning, an attempt was made to determine if there were differences in astroglial activation within the hippocampal CA1 region. To this end the astrocytic marker GFAP was used to compare preconditioned only and ischemic preconditioned animals to ischemic and sham controls.

Finally, the same multifaceted approach (histology, behaviour and electrophysiology) was used to assess the extent of neuroprotection at both shorter and longer survival times to fully compare young and old animals.

4.2 Methods

4.2.1 Subjects

143 female Mongolian gerbils were used in this study. The age of the gerbils ranged from 3-5 months (young) and from 18-19 months (aged). All animals were purchased from High Oak Ranch at 10 weeks of age and kept in the animal care facility until use as described in the general methods, section 2.2.1.

4.2.2 Brain Temperature Measurement

As in the previous experiment, all animals were implanted with a guide cannula for the direct measurement of brain temperature (see sections 2.2 and 2.3 for details). A 3 hr normal temperature profile was measured two days prior to the start of ischemia. Brain temperatures were again monitored during the occlusions and for 8 hr (in the case of 1.5 min occlusions) and 24 hr (following 5 min of ischemia/sham surgery) postischemically. Measurements were taken every 30 sec and averaged every 10 min.

4.2.3 Induction of Ischemia

Young and aged animals were allocated to groups prior to the experiment: Sham (S); preconditioned only (PO), ischemic preconditioned (IP) and an

ischemic group (I). All operations were performed as described in section 2.4 of the general methods.

4.2.4 Behavioural Testing

Animals were repeatedly tested in the open field starting 3 days after the final occlusion and then again on days 7, 10, 30 and 60 (depending on survival time). Refer to section 2.5 for more details.

4.2.5 Electrophysiology

Following the last day of behavioural testing, brains were removed and one hemisphere was prepared for electrophysiological recordings. Field EPSPs were recorded from the apical dendrites of CA1 following Schaeffer collateral stimulation. Stimulus and recording parameters and apparatus were the same as outlined in the general methods section 2.6. Responses were visualized and analyzed using Scope software (MACLAB).

4.2.6 Histology

The hemisphere reserved for histology was immersion fixed in 10% phosphate buffered formalin and subsequently embedded in paraffin. Two series

consisting of 6 μm thick coronal sections were cut from each brain. The first series was stained with H&E and used for cell counts as previously described (Section 2.7). The second series of sections were immunostained with anti-GFAP diluted 1:250 (DAKO Diagnostics Canada Inc, Mississauga, ON) and developed using strept-avidin-biotin-peroxidase complex (streptABC, Dimension laboratories, Mississauga, ON) followed by incubation with diaminobenzidine (DAB). Negative controls were run repeatedly by omitting the primary antibody. There was no staining in these sections.

4.2.7 Statistics

Direct comparisons of young versus aged gerbils cannot be made due to inherent differences between the populations (e.g. weight, activity levels, overall health). Therefore, age appropriate sham and ischemic controls were used to assess ischemic preconditioned animals on all outcome measures.

There were no differences (i.e. cell counts, open field and fEPSP amplitude) between PO 3, PO 10 and PO 60 animals and so these data were pooled for each outcome measure. All data were analyzed using one way ANOVA. Individual post-hoc comparisons were performed using Neuman-Keuls test to compare group means. Significance was set at 0.05.

4.3 Results

Three animals (2 young and 1 old) died during the surgeries, 1 animal (young) died due to injury sustained from the insertion of the rectal probe and 1 animal (old) died in the 2 month survival period after surgery. Four animals were excluded due to suspected seizure activity triggered by exposure to the open field environment. These animals exhibited freezing behaviour in the open field (characteristic of seizures) and did not move for several minutes within the test period.

4.3.1 Temperature

Intraischemic brain temperatures ranged from 36.28°C to 36.57°C and 36.40°C to 36.47°C in young and aged gerbils, respectively. None of the group averages were significantly different from each other. Similarly, there were no significant differences in the postischemic brain temperatures of the groups. However, I and IP animals displayed a mild hyperthermic response after ischemia as noted in other studies (Corbett and Crooks, 1997; Dooley and Corbett, 1998). Fig. 1and 2 present the 24 hr postischemic temperature profiles of the young and old groups, respectively.

4.3.2 Behaviour

The open field data for the young animals are presented in Fig 3. S and PO groups were not significantly different from each other on any test day. Both the IP and I groups were significantly different from S and PO groups ($p < 0.05$) on days 3 and 7, displaying heightened levels of activity typical of ischemic injury. A comparison of the I and IP groups revealed a significant difference in open field score on day 3 ($p < 0.05$), with the IP group having a higher score. This difference was not apparent on days 7 or 10. Ischemic animals were not significantly different from S on day 10 while IP animals were ($p < 0.05$, IP versus S). Finally, on days 30 and 60 all animals appeared to have habituated to the open field environment and none of the groups were significantly different.

An assessment of open field behaviour in the old animals revealed a similar pattern to that of experiment 1 (Chapter 3). That is, there were no significant differences between the S and PO groups at any test day (Fig. 4). In addition while both the I and IP groups had significantly higher scores than the S and PO animals ($p < 0.05$) they were not significantly different from each other on test days 3 and 7. However, by day 10 the IP animals showed significantly lower open field scores than the I animals indicating a faster rate of recovery/habituation and therefore less ischemic damage. By day 30 these

differences were reduced but both IP and I remained significantly different from S ($p < 0.05$). There were no differences between any of the groups by day 60.

4.3.3 Electrophysiology

Field potential amplitudes from S and PO animals were not significantly different. Three days following 5 min of ischemia it was still possible to record field potentials from the young animals. However, the field potentials of the I 3 animals were significantly reduced in amplitude compared to S animals (1.51 ± 1.44 mV, $n = 5$ versus 4.33 ± 1.03 mV, $n = 8$, $p < 0.05$). Ischemic animals surviving for 60 days (I 60) exhibited a further diminution in field potential amplitude to 0.65 ± 0.50mV ($n = 9$, $p < 0.05$ versus S). Ischemic preconditioning followed by 3 days survival (IP 3) also resulted in a decrease in fEPSP amplitude that, although higher, was not significantly different from I 3 or I 60 animals. In addition, there was no recovery with increasing survival time (compare 1.97 ± 0.98 mV, $n = 7$ at day 3 to 2.04 ± 1.42 mV, $n = 7$ at day 60) and none of the IP groups were significantly different from the I groups (Fig. 5).

With respect to the aged gerbils, the mean maximum amplitudes for the I 3 and I 60 groups were 1.18 ± 0.43 mV ($n = 5$) and 0.43 ± 0.02 mV ($n = 8$) respectively. These were significantly different from the S group that had field

potentials of 3.71 ± 1.56 mV ($n = 7$, $p < 0.05$). Three days following ischemic preconditioning the mean EPSP amplitude was 2.17 ± 1.10 mV ($n = 9$) which was significantly different from the I 60 group but not the S group. In animals that survived 10 days fEPSP amplitudes decreased to 1.67 ± 0.75 mV ($n = 7$, $p < 0.05$ versus S). However, this was followed by subsequent recovery to 2.64 ± 0.87 mV ($n = 6$) which was significantly different from I 60 ($p < 0.05$) animals but not S. The data are presented in Fig. 6.

4.3.4 Histology

There was no significant difference between S and PO groups in the young animals. Five minutes of ischemia (I) resulted in very different histological outcomes depending on the survival time assessed. At 3 days, 96.5% of CA1 neurons remained at level A (ns from S). This is in marked contrast to the 60 day survival group (I 60) where only 4.6% of CA1 neurons were viable ($p < 0.05$ versus S). Ischemic preconditioning provided significant protection to CA1 neurons at all survival times when compared to I. However, only partial protection was achieved (78.8% at day 3, ns from S) and cell death appeared to continue with longer survival times (45.3% at day 10 and 36.8% at day 60, $p <$

0.05 versus S). The same patterns emerged at Levels B and C. The data are presented in Figure 7.

Similarly, there was no difference in cell survival between S and PO animals in the aged group. Five minutes of ischemia produced 89% CA1 cell loss in the I 60 group ($p < 0.05$, versus S). Three days after ischemic preconditioning cell counts were normal. However when assessed at 10 days neuronal survival decreased to 62.2% which was significantly better than both I groups ($p < 0.05$) but not as good as S ($p < 0.05$). Cell loss seemed complete since there was no further loss of CA1 neurons at 60 days (61% of S). Cell counts of levels B and C displayed the same patterns (Fig. 8).

The pattern of GFAP immunostaining was very similar between young and old animals and the following description applies to both age groups. In hippocampal sections from S animals immunoreactive for GFAP, there was very little staining (Fig. 9). There was a distinct band of staining in the hippocampal fissure that was common in all groups. In addition there was sparse and diffuse staining of astrocytic processes within the apical region of CA1. Three days following 5 min of ischemia, there was a small increase in GFAP immunoreactivity in the hilar region. In addition, it appeared as though more processes were stained with GFAP than in S animals. In ischemic animals that survived 10 days

there was a dramatic upregulation of GFAP immunoreactivity. This was readily apparent in the hilar region as well as in both apical and basal dendritic regions of CA1. Furthermore, intense GFAP staining extended in a medial-lateral plane from the subiculum to the CA1/CA2 border. The CA3 region was less intensely stained although more so than S. In the I animals, the astrocytes were fuller, the processes appeared thickened and the staining was more dense. In the majority of 3 day PO animals (independent of age) the pattern of GFAP immunostaining was moderately increased in the whole hippocampus compared to S, however, staining was not as pronounced as it was in the I3 animals. Again, there was an upregulation in hilus as well as an increase in apical CA1 staining. A majority of the animals in the PO 10 group had staining that was very similar to the PO 3 animals with the exception that the hilus was weakly stained in the PO 10 gerbils. GFAP staining of astrocytes was more intense in IP 3 and IP 10 animals than in PO and I3. The process of reactive astrocytosis seemed to mature from 3 to 10 days as determined by an increase in the number of visible astrocytes and the thickness of their processes. The distribution and pattern of staining was similar between the IP 10 and I 10 groups. However, the staining of individual astrocytes appeared to be more intense (aggregates of dark brown granules

within the cytoplasm) in the I 10 animals and astrocytes appeared to be infiltrating the CA1 cell band.

Figure 4.1: Postischemic Brain Temperature of Young Gerbils

Brain temperature profiles of young animals during the 24 hr postischemia monitoring period. Groups are sham (S, n = 8); 5 min ischemic (I, n = 13) and ischemic preconditioned (IP, n = 24). There were no significant differences between the groups in terms of mean 24 hr temperature. Note the characteristic period of hyperthermia in the I and IP animals following occlusion.

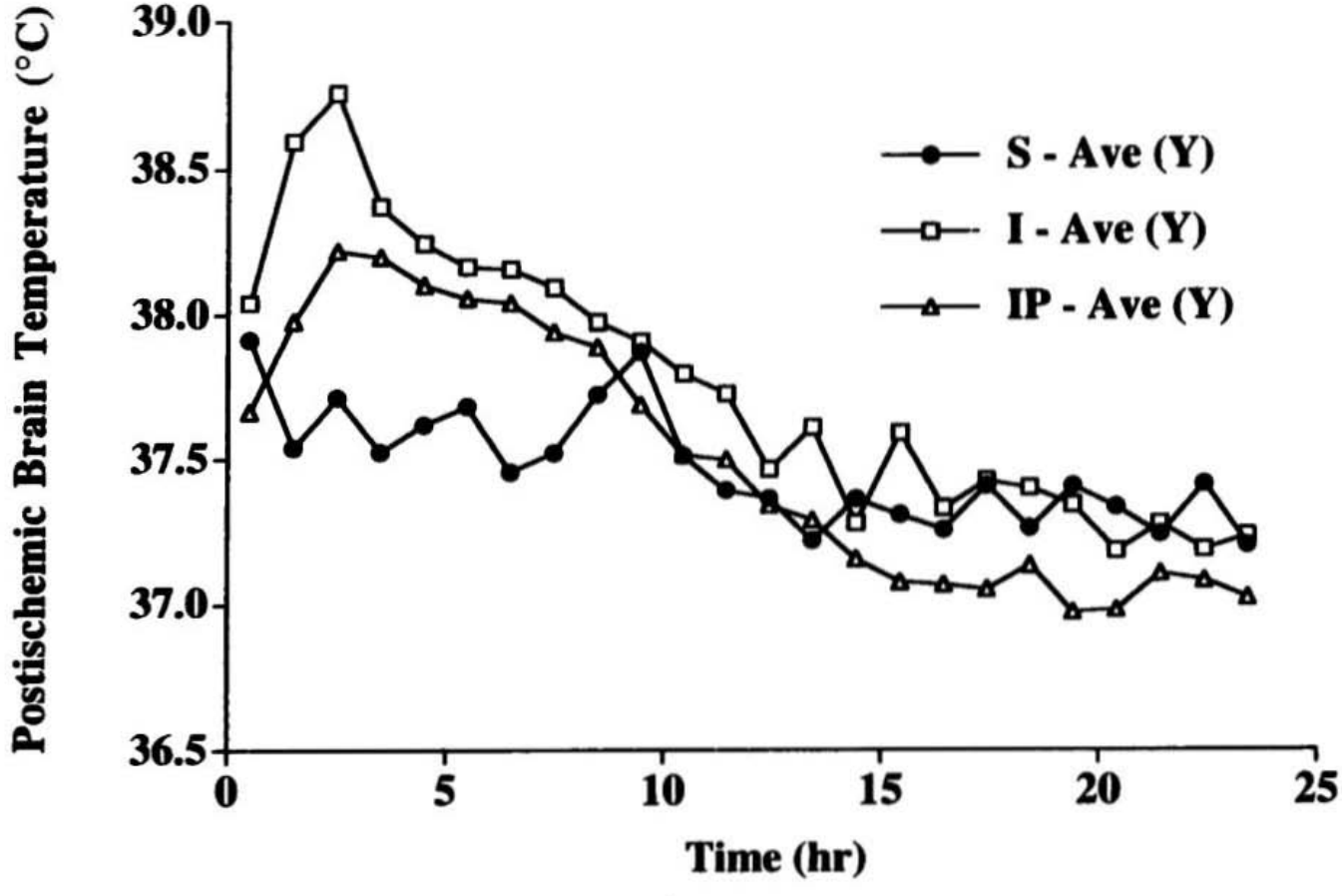

Postischemic Brain Temperature (°C)
39.0
38.5
38.0
37.5
37.0
36.5
0
5
10
15
20
25
Time (hr)
S - Ave (Y)
I - Ave (Y)
IP - Ave (Y)

Figure 4.2: Postischemic Brain Temperature of Aged Gerbils

24 hr postischemic brain temperature profiles of the aged animals. Groups are (S, n = 8); 5 min ischemic (I, n = 13) and ischemic preconditioned (IP, n = 23). Note the period of hyperthermia following occlusion typical of I and IP animals. Mean 24 hr temperatures were not significantly different from each other.

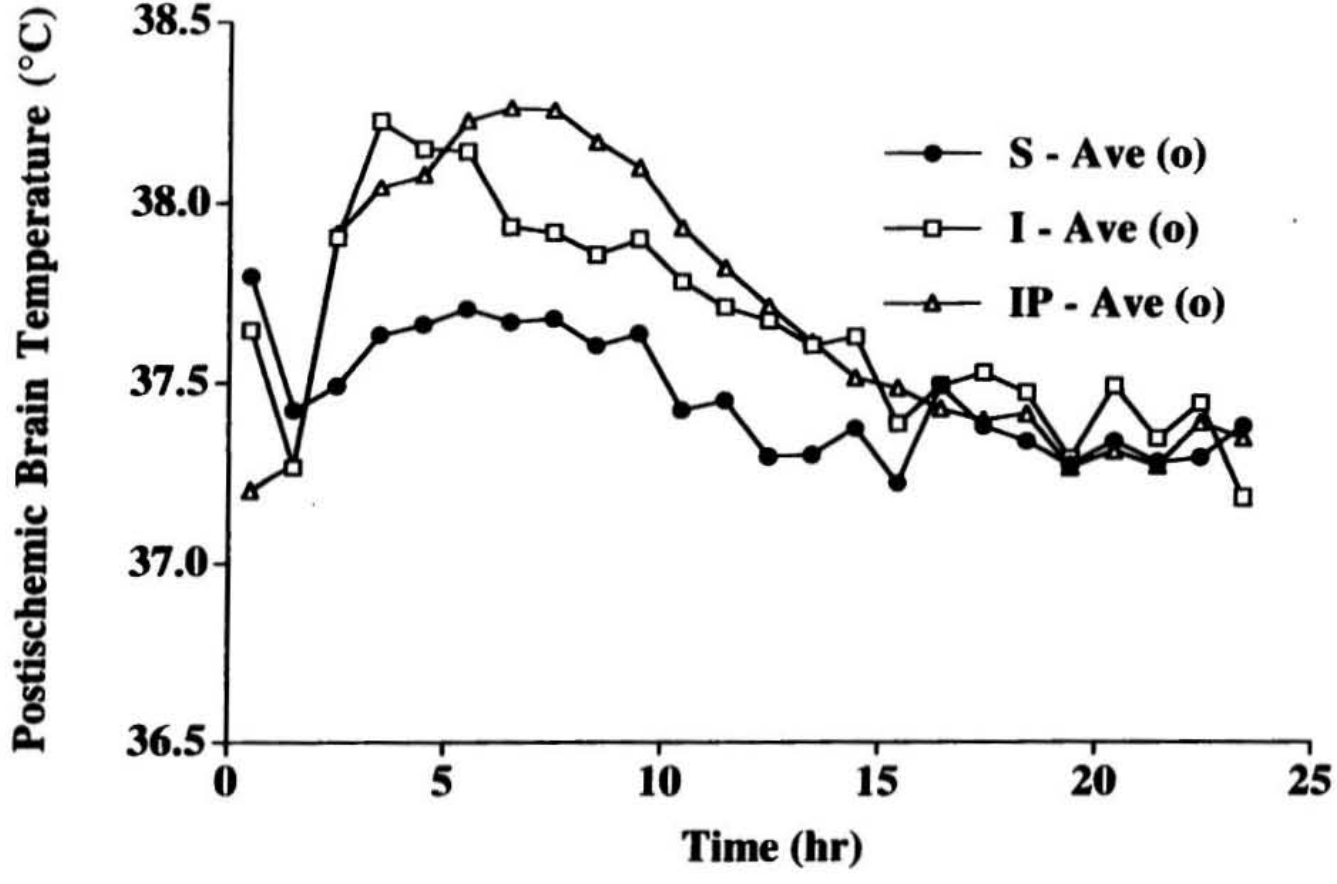

Postischemic Brain Temperature (°C)
38.5
38.0
37.5
37.0
36.5
0
5
10
15
20
25
Time (hr)
S - Ave (o)
I - Ave (o)
IP - Ave (o)

Figure 4.3: Open Field Scores of Young Gerbils

Open field activity (mean ± SD) for young animals tested 3,7,10, 30 and 60 days post ischemia/sham surgery. Groups sizes were as follows: day 3 – S, n = 8; PO, n = 22; I, n = 18; IP, n = 24; days 7 and 10 – S, n = 8; PO, n = 14; I, n = 13; IP, n = 16. On days 30 and 60 all groups had 8 animals. Both the I and IP groups were significantly impaired compared to S on days 3 and 7. While IP animals were significantly different from S animals on day 10, I animals were not. Habituation occurred in all groups with repeated exposure and none of the groups were significantly different on days 30 and 60. Note that the open field scores of the I groups were less than the IP scores on all test days which is not normally the case (see discussion)

* $p < 0.05$ with respect to S.

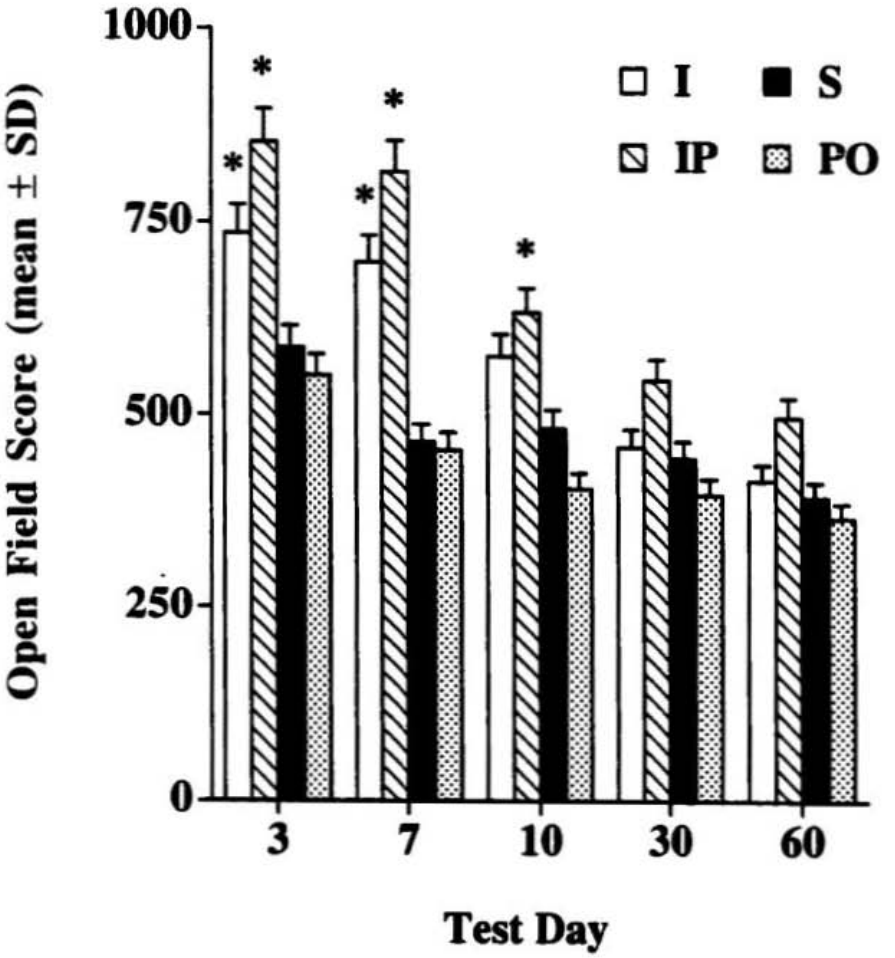

1000
750
500
250
0
Open Field Score (mean ± SD)
I
S
IP
PO
*
3
7
10
30
60
Test Day

Figure 4.4: Open Field Scores of Aged Gerbils

Open field activity (mean ± SD) for aged gerbils tested 3,7,10, 30 and 60 days post ischemia/sham surgery. Groups sizes were as follows: day 3 – S, n = 8; PO, n = 22; I, n = 13; IP, n = 23; days 7 and 10 – S, n = 8; PO, n = 14; I, n = 8; IP, n = 15; days 30 and 60 - S, n = 8; PO, n = 6; I, n = 8; IP, n = 7. Behaviour was impaired in I and IP groups on day 3, 7, 10 and 30 but showed a trend towards recovery. IP animals appeared to improve at a faster rate as they were significantly different from I animals at day 10.

* $p < 0.05$ with respect to S; † $p < 0.05$ comparing IP to I 60.

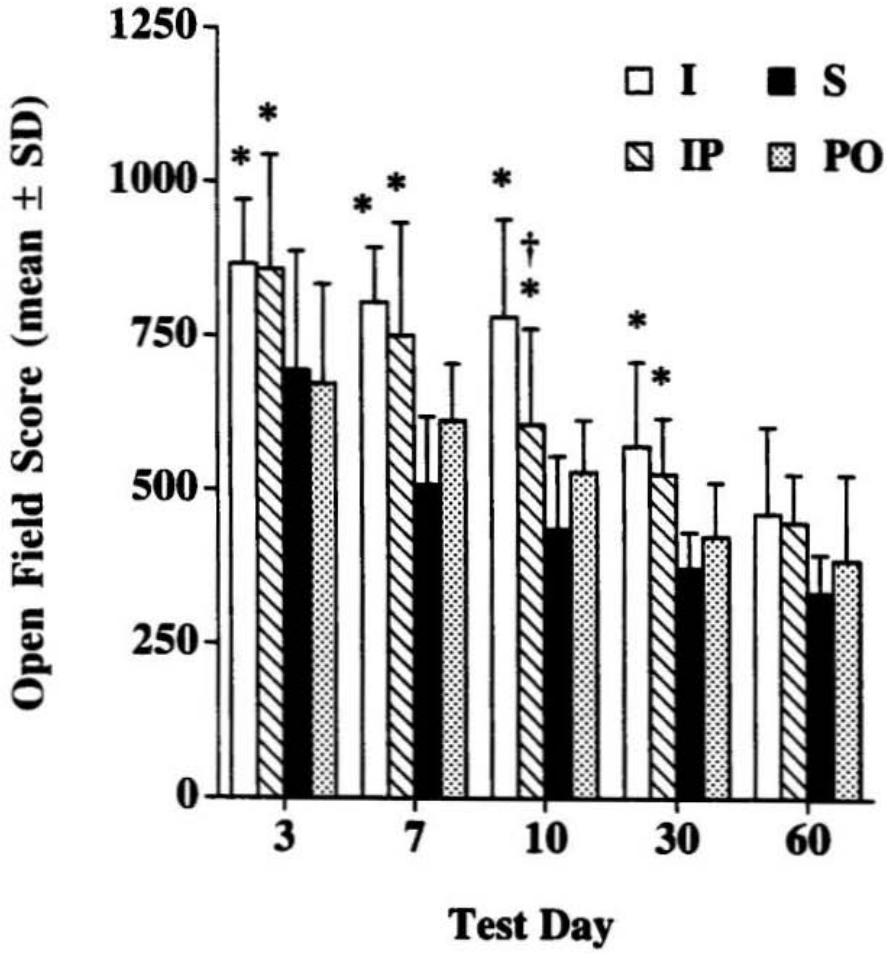

1250
1000
750
500
250
0
Open Field Score (mean ± SD)
I
S
IP
PO
*
†
3
7
10
30
60
Test Day

Figure 4.5: CA1 Field Potentials from Young Gerbils

Maximum amplitude (mean ± SD) of CA1 field potentials recorded from young gerbils. Groups include sham (S, n = 8); preconditioned only (PO, n=22); 5 min ischemic 3 (I 3, n = 5) and 60 (I 60, n = 9) day survival; ischemic preconditioned 3 (IP 3, n = 7), 10 (IP 10, n = 8) and 60 (IP 60, n = 7) days survival. While mean fEPSP amplitudes of the IP groups were higher than those of the I 60 group, they were not significantly different at any survival time. All I and IP animals irrespective of survival time were significantly different from S.

* $p < 0.05$ with respect to S; † $p < 0.05$ comparing IP to I 60.

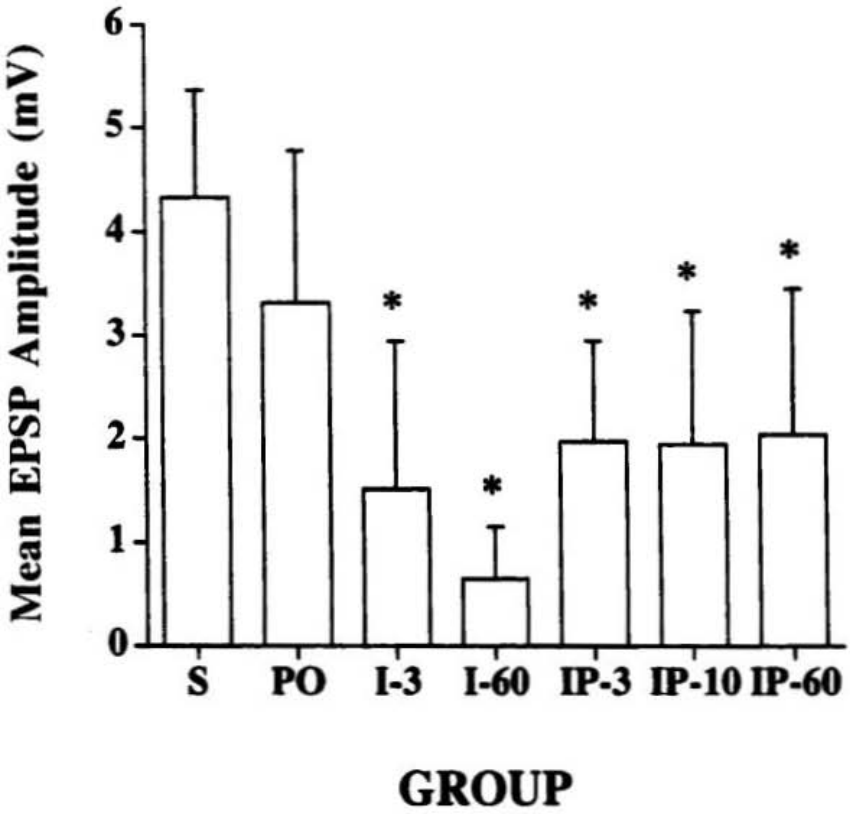

Mean EPSP Amplitude (mV)
6
5
4
3
2
1
0
*
*
*
*
*
S
PO
I-3
I-60
IP-3
IP-10
IP-60
GROUP

Figure 4.6: CA1 Field Potentials from Aged Gerbils

Maximum amplitude (mean ± SD) of CA1 field potentials recorded from aged gerbils. Groups include sham (S, n = 7); preconditioned only (PO, n=20); 5 min ischemic 3 (I 3, n = 5) and 60 (I 60, n = 8) day survival; ischemic preconditioned 3 (IP 3, n = 9), 10 (IP 10, n = 7) and 60 (IP 60, n = 6) days survival. Field potentials were significantly reduced compared to S. Three days after ischemic preconditioning field potentials were not significantly different from S. However at 10 days they were reduced in amplitude only to recover by 60 days.

* $p < 0.05$ with respect to S; † $p < 0.05$ comparing IP to I 60.

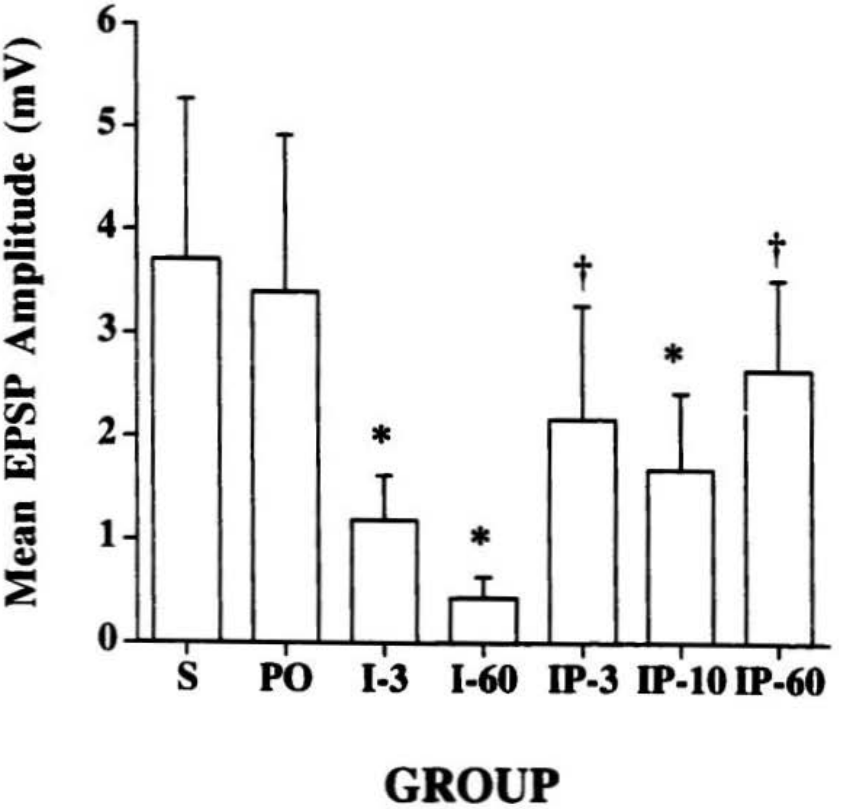

Mean EPSP Amplitude (mV)
6
5
4
3
2
1
0
*
*
†
*
†
S
PO
I-3
I-60
IP-3
IP-10
IP-60
GROUP

Figure 4.7: Histological Outcome in Young Gerbils

Viable CA1 neurons remaining in young hippocampus. Cell counts were taken from three levels: 1.7 mm (level A), 2.2 mm (level B) and 2.8 mm (level C) posterior to Bregma. Each symbol represents an individual animal while horizontal bars indicate group means. There were no significant differences between S, PO, I 3 and IP 3 groups. Five min of ischemia followed by 60 days survival produced substantial loss of CA1 neurons. Ischemic preconditioning provided significant protection; however, cell loss continued with increasing survival time. A significantly greater number of CA1 neurons was evident in IP 10 and IP60 animals compared to I 60 gerbils. However, these values were substantially reduced with respect to shams.

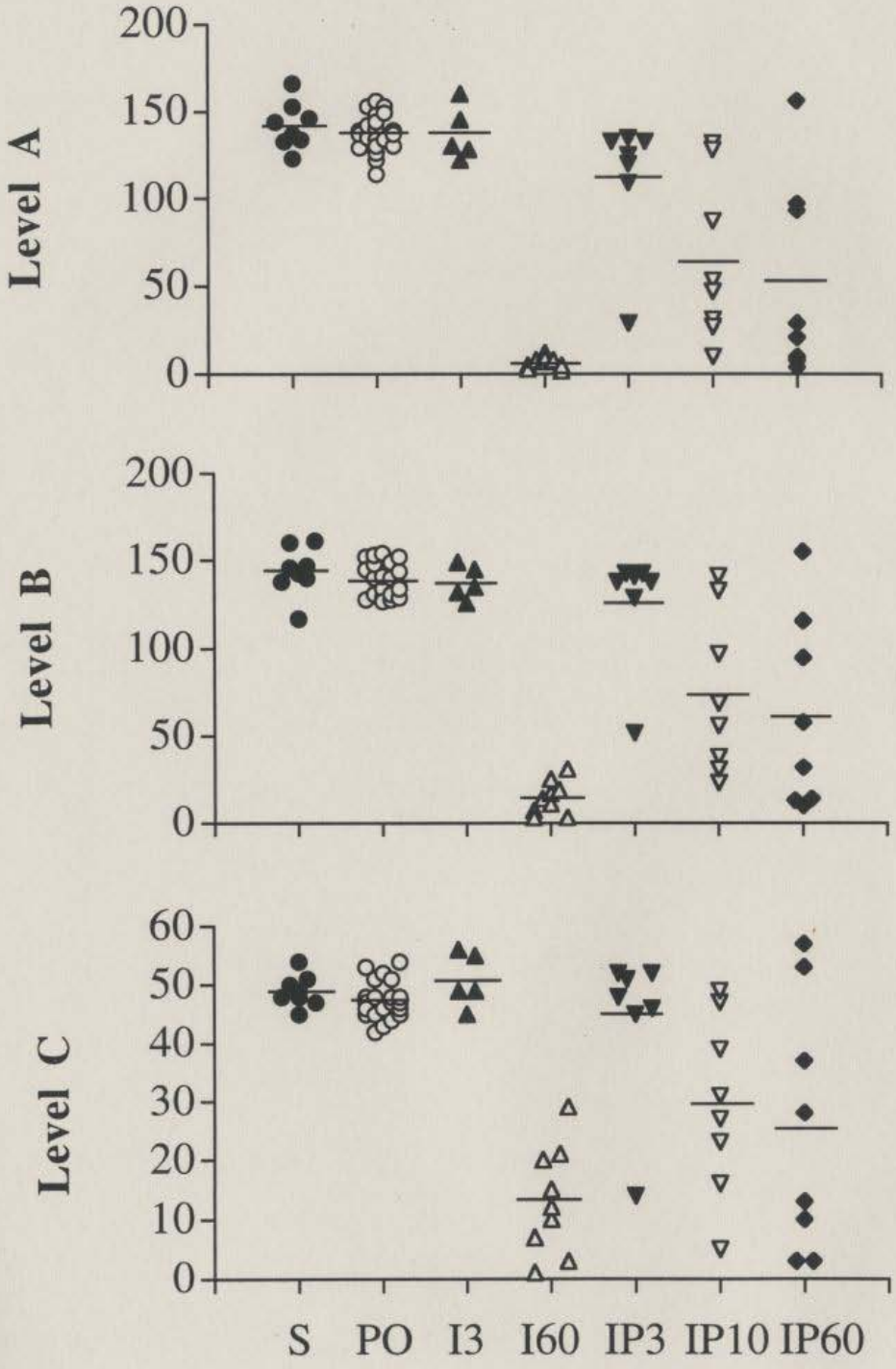

Level A
200
150
100
50
0
Level B
200
150
100
50
0
Level C
60
50
40
30
20
10
0
S
PO
I3
I60
IP3
IP10
IP60

Figure 4.8: Histological Outcome in Aged Gerbils

Total cell counts of the aged gerbils from three levels: 1.7 mm, 2.2 mm and 2.8 posterior to Bregma and corresponding to Levels A, B and C above. There were no significant differences between S, PO, I 3 and IP 3 groups. Five min of ischemia followed by 60 days survival resulted in near-total loss of CA1 neurons. Ischemic preconditioning provided robust histological protection despite increased cell death over longer survival times. Cell counts from the IP 10 and IP 60 groups were significantly lower than counts from the S group but not significantly higher than I 60 values.

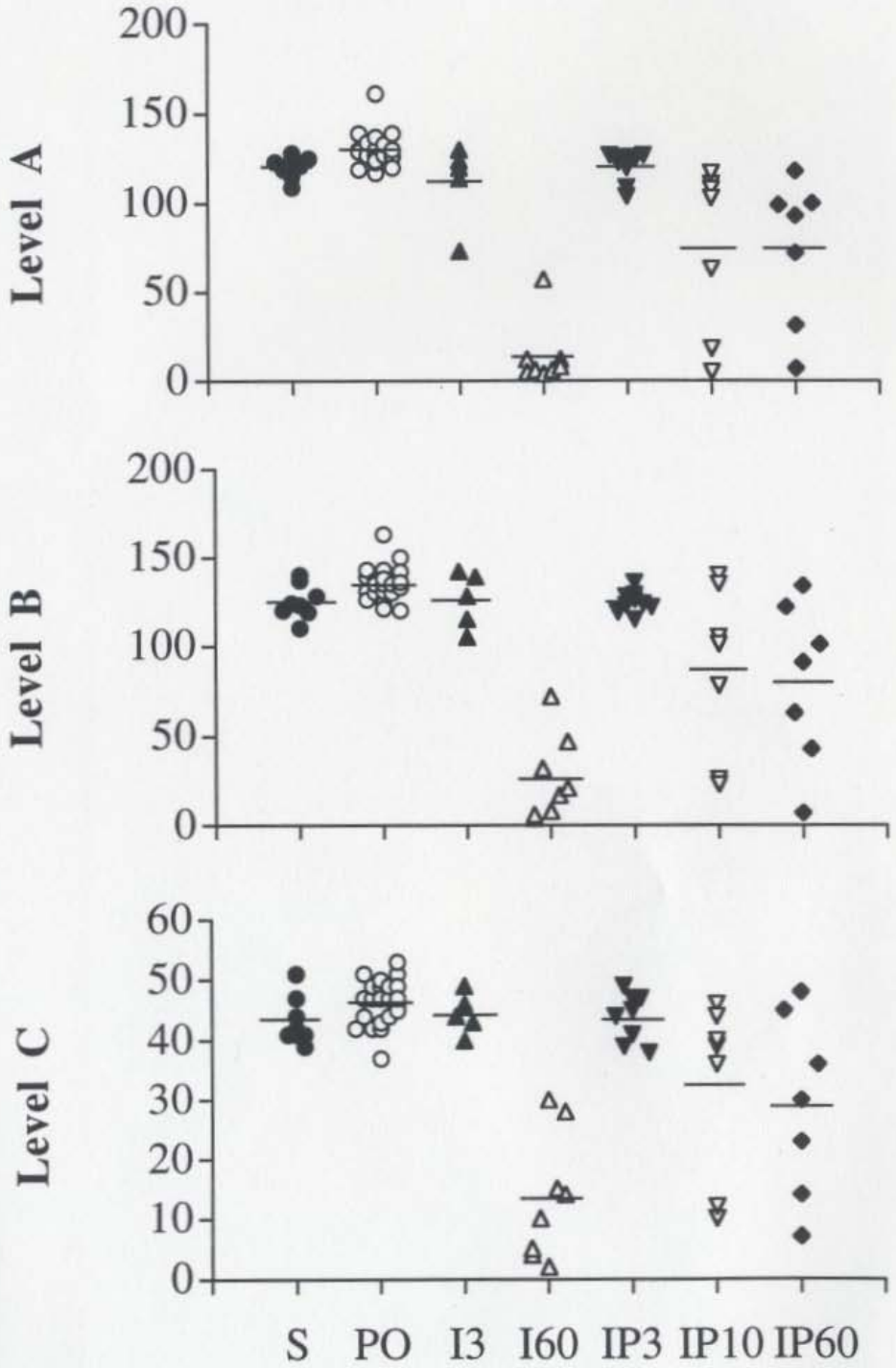
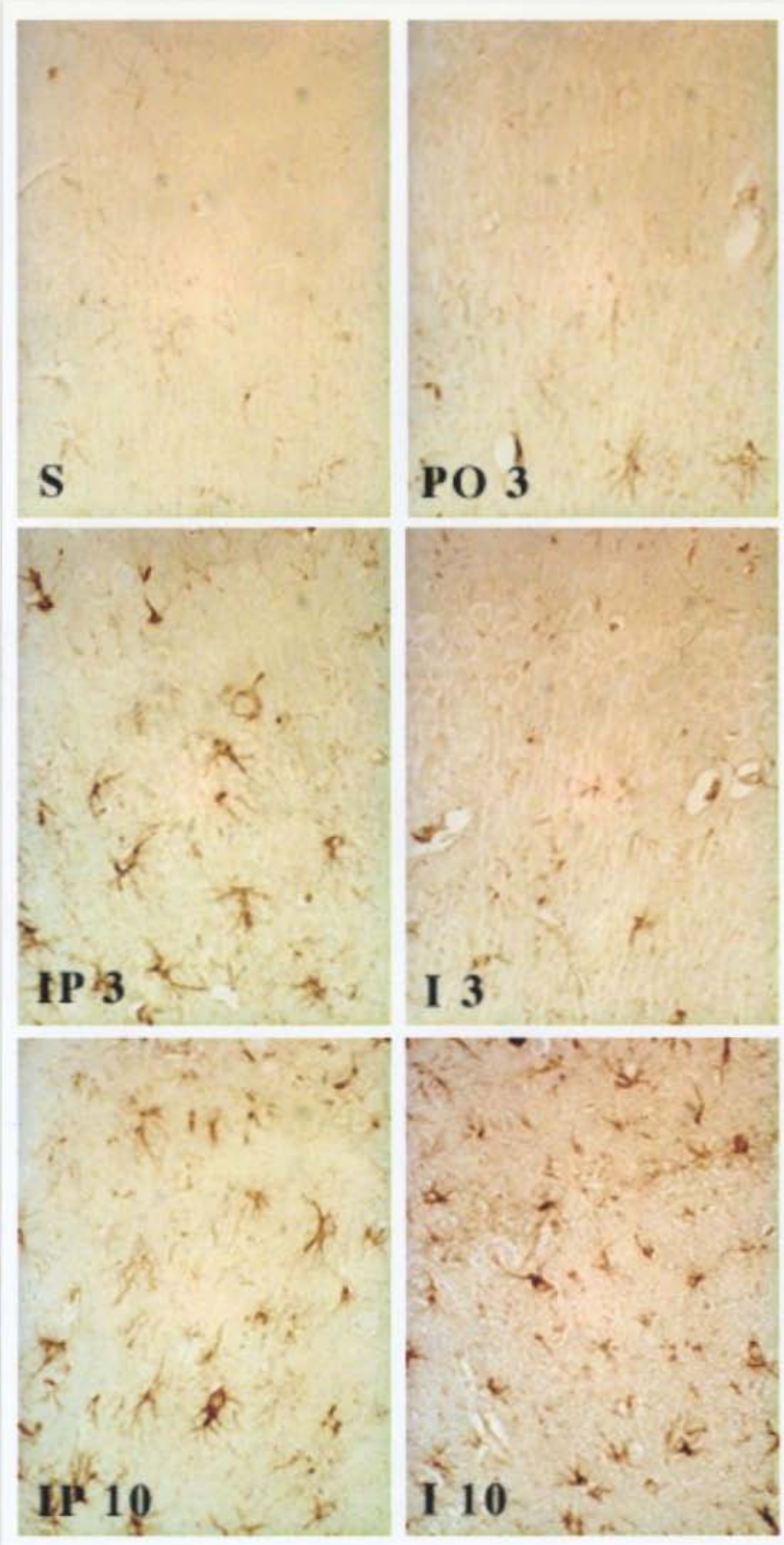

Level A
200
150
100
50
0
Level B
200
150
100
50
0
Level C
60
50
40
30
20
10
0
S
PO
I3
I60
IP3
IP10
IP60

Figure 4.9: Representative Photographs of GFAP Stained Astrocytes in the Hippocampus.

Pictures were taken from sham (S, young), ischemic 3 (I 3, young) and 10 (I 10, young) days survival, preconditioned only 3 days survival (PO 3, old) and ischemic preconditioned animals 3 (IP 3, young) and 10 (IP 10, old) days survival. Note the slight increase in GFAP staining in PO animals compared to S (arrowheads in PO panel are pointing at astrocytes). Three days after the 5 min occlusion, staining of astrocytes and their processes is increased in IP animals compared to I. Ten days after the 5 min occlusion there was an upregulation of staining in astrocytes in I and IP animals. Note the difference in the appearance astrocytes between I 10 and IP 10 (compare arrowheads). Astrocytes of I 10 animals appear to have dense GFAP staining in the cell bodies indicative of hypertrophic reactive astrocytes. (20x magnification of CA1 region)


S
PO 3
IP 3
I 3
IP 10
I 10

4.4 Discussion

The results of this study confirm the hypothesis that ischemic preconditioning is more effective in aged gerbils. Following a long survival time of 60 days there was 61.6% protection of CA1 neurons in aged animals compared to only 36.8% in young animals. Furthermore, this higher level of neuronal preservation was consistent with a more significant recovery of fEPSP amplitude. EPSPs from IP 60 old animals were not significantly different from S while those from the IP 60 young group were.

Once again, the data revealed an early dissociation between histological and functional protection. On day 3, in spite of robust neuronal preservation, there was evidence of impaired open field behaviour and reduced CA1 field potentials. In the old animals as behaviour and field potentials recovered, the process of cell death continued. The patterns of recovery and cell loss observed in the aged gerbils was very similar to that reported in the first experiment (Chapter 3).

There were still CA1 neurons present 3 days after 5 min of ischemia in both age groups. A reassessment of the methodology revealed that it had been less than 72 hr since they were occluded. All the animals were sacrificed early on the third day for electrophysiological recordings. Also notable is the fact that

field potentials could be recorded from I 3 animals of young and old animals. In both groups, fEPSPs were significantly reduced in amplitude compared to S and although not significantly different from I 60 day fEPSPs, their mean maximum amplitudes were higher. This indicates that some CA1 neurons were still viable ~3 days after ischemia.

The open field data for the young animals did not follow the same pattern as reported previously (Corbett and Crooks, 1997; Dooley and Corbett, 1998). That is, in both of those studies IP animals had lower open field scores than I animals on all test days, although this was not significant. In contrast, IP animals from this study had higher open field scores than I animals. It appears as though I animals were not as active in this study as they were in the previous studies (Corbett and Crooks, 1997; Dooley and Corbett, 1998). Normally, the average open field score for ischemic animals tested on day 3 is > 1000. This is compared to the average score of 735 in this study. Furthermore, ischemic animals typically display higher levels of open field activity than S animals (Colbourne and Corbett, 1994; Colbourne and Corbett, 1995; Nurse and Corbett, 1994; Corbett and Crooks, 1997; Dooley and Corbett, 1998). However, in the present study I animals were no different than S animals on day 10. While there is no obvious explanation for the lower activity of the I gerbils, it is possible that the 5 min of

ischemia produced "covert injury" (i.e. scattered necrosis distal to the hippocampus) that went undetected. This injury coupled with severe CA1 cell loss could have resulted in *lower* open field scores.

There was also a difference in the pattern of recovery of field potentials in young IP animals. In the previous study using young animals (Dooley and Corbett, 1998), field potentials recorded from CA1 dendrites of IP animals were initially impaired but recovered over time to a level that was not significantly different from S. In the present study recovery was less evident. The mean maximum amplitudes of all IP groups were higher than I 60 values but were not significantly different from I 60 values, regardless of survival time. CA1 cell counts from the IP 60 group were significantly higher than the counts from the I 60 group. However, because fEPSP is a measure of a population response, it is likely that the CA1 neurons remaining in the IP 60 group were not able to maintain normal output.

The pattern of GFAP immunoreactivity in PO animals indicates that the two preconditioning episodes initiated reactive astrocytosis in the hippocampus, thereby possibly increasing the capacity of CA1 neurons to tolerate the final 5 min insult. Further evidence for this comes from a comparison of the I 3 and IP 3 groups. There was increased staining in the IP 3 animals compared to I 3. This

three day survival time corresponds to the time when CA1 neurons are normally dying following a 5 min period of ischemia (Kirino, 1982; Pulsinelli et al., 1982). It is possible that the reactive astrocytes present in the hippocampus of ischemic preconditioned animals at this time assist in the survival of CA1 neurons (growth factor upregulation). Kato and colleagues reported that microglia are activated early after lethal (i.e. 5 min occlusion) ischemia even before neuronal loss is evident and that the astrocytic response lags behind (Kato et al., 1994). They concluded that preconditioning altered both the pattern and timing of these processes and shifted the ratio of astroglia and microglia in favour of neuronal survival. The results of the present study support these findings.

The level of protection obtained in the young and old animals with ischemic preconditioning in this study was not as great as previously reported (Corbett and Crooks, 1997; Dooley and Corbett, 1998). Unlike the previous studies, several animals in the 10 day survival group exhibited cell loss. This coupled with continued cell death over longer survival times resulted in a decrease in efficacy of ~ 20% (comparing 30 day outcomes of Dooley and Corbett, 1998; Corbett and Crooks, 1997 with 60 days outcome in this study) and ~ 13% (comparing 60 day outcomes of experiment 1 and this study) at Level A in young and aged gerbils, respectively. The reasons for the decrease in efficacy

are unclear. One notable difference between this and the previous studies is that there has been an increase in mean baseline temperature of 0.6°C and 1.0°C in old and young animals, respectively. Correspondingly, there has been an increase in the 24 hr postischemic brain temperatures. It is possible that this shift is responsible for the decrease in efficacy as 24 hr postischemic temperature was correlated with histological outcome at 60 days ($r = 0.67$, $p < 0.01$, $n=15$). The reason for this shift remains unknown but could not be explained by differences in methodology of either probe calibrations or brain temperature measurements.

Finally, it is known that ischemic preconditioning is not protective in some animals. Abe and Nowak, 1995 stated, "protection observed in most studies has been variable and it has not been possible to predict with confidence the degree of tolerance to be expected in a given animal." This may be due to inherent variability in arterial collateralization in the gerbil (Mayevsky and Zipi, 1990). Alternatively, a recent study suggests that differences in vulnerability to hypoxic insult in female mice are dependent upon which phase of the estrus cycle they are in (Kasischke et al., 1999). During proestrus and estrus, when estrogen levels were high and progesterone levels were low, there was a decreased capacity to recover from a hypoxic insult. Conversely, estrogen levels were low and progesterone levels were high during diestrus when maximal recovery was seen.

Furthermore, chemical preconditioning was able to increase the capacity of slices, taken from mice in proestrus but not estrus, to recover from hypoxia (Kasischke et al., 1999). This indicates that ischemic preconditioning is least likely to work in females during the estrus phase of their cycle. Considering that only female gerbils were used in our studies it is possible that differences in efficacy of ischemic preconditioning could be dependent on which phase of the estrus cycle the gerbils were in. Furthermore, this could be an important factor in the difference between aged and young gerbils in that it is possible that some of the 18 month old gerbils have stopped cycling. It has been shown that acyclicity in laboratory rodents begins between 12 and 16 months of age (Finch et al., 1984). Gerbils have been reported to reproduce successfully as late as 24 months; however, such cases are rare and in these animals the frequency of cycling is decreased (Cheal, 1983). Therefore the aged gerbils may not be experiencing the same fluctuations in hormones and so perhaps are less susceptible to ischemic injury (by avoiding high levels of estrogen during estrus and proestrus) and more likely to benefit from ischemic preconditioning (which may be ineffective during estrus).

CHAPTER 5:

DOES EARLY BEHAVIOURAL TESTING INFLUENCE ISCHEMIC OUTCOME FOLLOWING ISCHEMIC PRECONDITIONING

5.1 Introduction

Next to immediate supportive care, rehabilitative therapy is probably the most vigorous clinical intervention following stroke. In fact it is widely accepted that rehabilitative therapy following injury is not only beneficial but is essential for maximum recovery (Dobkin, 1989; Ernst, 1990). For example, it was demonstrated in monkeys that retraining of skilled hand use following motor cortex lesions prevented the loss of hand territory in areas adjacent to the infarct (i.e. penumbral cortex). In addition, the rehabilitative therapy was linked to reorganization of the adjacent intact cortex where hand representation took over areas formerly dedicated to elbow and shoulder. Finally, these structural changes coincided with behavioural recovery of skilled hand function in these animals (Nudo et al., 1996). However, recent experimental evidence suggests that in some circumstances rehabilitation can impair recovery and exacerbate the injury. Koslowski and colleagues, (Koslowski et al., 1996) showed that forced use of the impaired limb (i.e. contralateral limb) immediately following focal ischemia significantly increased infarct size and prevented functional recovery. However, in order to force the use of the impaired limb, it was necessary to immobilize the unaffected limb by casting. No doubt, this procedure was quite stressful for the animals and stress has been shown to exacerbate ischemic injury

(Morse and Davis, 1990; Sapolsky and Pulsinelli, 1985). Therefore, the poorer outcome may be due to heightened stress rather than a "use-dependent" exacerbation of injury. More recently, Risedal and co-workers conducted a similar experiment but avoided the influence of stress (due to inability to use the unaffected forelimb) on outcome. While there was an increase in infarct size as a result of early behavioural testing in the animals there was evidence of improved functional outcome compared to the control group (Risedal et al., 1999).

In keeping with this theme, one concern with our studies is that behavioural tests are routinely conducted soon after global ischemia. If early forced use of a neural system (i.e. use dependency) can have negative effects it could be contributing to the delayed but continued neuronal death following many protective treatments (e.g. hypothermia, neuroprotective drugs) including ischemic preconditioning. Since excessive glutamate may be responsible for initiating the cascades that culminate in ischemic cell death (Choi, 1990; Schurr and Rigor, 1992) even normal synaptic activation of CA1 cells by glutamatergic inputs during the postischemic period may be detrimental. While CA1 neurons would normally be able to handle this activation, neurons recovering from an ischemic episode may not. It has been demonstrated that low frequency stimulation of the input fibers to CA1 following global ischemia leads to

abnormal Ca^{2+} accumulation in the neurons and ultimately cell death (Tsubokawa et al., 1992).

As mentioned above several studies have reported that stress can exacerbate neuronal injury. The open field test, often used as a sensitive indicator of neuronal injury, has been shown to be a stressor and has been used to promote seizure activity in certain strains of gerbils (Harriman, 1980; Thiessen et al., 1968). These two factors - stress and working neurons, may negatively influence cell survival following ischemia. Alternatively, early behavioural testing (like rehabilitation) might convey some benefit thereby prolonging CA1 survival. A recent study has shown that increased locomotor activity (as would occur in the open field) increased hippocampal neurogenesis (Van Pragg et al., 1999). Similarly, prior exposure to a running wheel attenuated damage produced by global ischemia (Strummer et al., 1995). In the following experiment ischemic preconditioning was employed to see whether early exposure to a novel environment following ischemia can alter the pattern of CA1 neuronal loss. Because ischemic preconditioning provides only partial protection of CA1 neurons (Corbett and Crooks, 1997; Dooley and Corbett, 1998) it is sensitive to any influence that can either enhance or impair neuronal survival.

5.2 Methods

5.2.1 Subjects

Twenty-five female mongolian gerbils purchased from High Oak Ranch were used in this study. The gerbils were approximately 12 weeks of age and ranged in weight from 53.1-65.0 g.

5.2.2 Baseline Temperature

See section 2.2 of the general methods for details. The only difference was that gerbils were anesthetized with sodium pentobarbital (65 mg/kg, i.p) and given a subcutaneous injection of atropine (0.03 mg/kg) during cannula implantation and kept under a light in order to keep them warm until the effects of the sodium pentobarbital wore off.

5.2.3 Induction of Ischemia

All animals were subjected to 2 - 1.5 min occlusions followed by a 5 min occlusion as previously described in section 2.4 of the general methods.

5.2.4 Behavioural Testing

Testing in the open field arena was conducted on days 3, 7, 10 and 30 days postischemia in half of the animals. A description of this task was previously detailed in Chapter 2 (section 2.5). The other animals, though not tested, were handled in exactly the same way without being placed in the open field. That is, they were transported to the lab and handled by the experimenter, weighed and then returned to the animal care facility.

5.2.5 Histological Assessment

On day 35 postischemia, all of the gerbils were given an overdose of sodium pentobarbital and perfused transcardially with heparanized saline and 10% phosphate buffered formalin. The heads were removed and the brains remained *in situ* for 24 hr submerged in formalin. After this time, the brains were carefully extracted from the skull. They were subsequently embedded in paraffin and cut in 6 μm sections on a microtome. The sections were then stained with H & E and cell counts were taken from three sectors of rostral and middle sections of CA1 and one sector of caudal CA1 as described in the general methods.

5.2.6 Statistics

Group weights, occlusion temperatures, post-ischemic temperatures and cell counts were analyzed using unpaired t-tests.

5.3 Results

Three animals died during the occlusions leaving 11 animals per group. There were no significant differences between groups in terms of weight and mean occlusion temperature. Similarly, there were no significant differences in the mean postischemic temperatures between the groups. The average postischemic temperature for the animals tested in open field was 37.50 ± 0.25°C versus 37.72 ± 0.23°C for the animals which were not tested. The 24 hr postischemic temperature profiles are presented in Figure 5.1.

Although there was a trend for lower cell counts in the group tested in open field at all the levels assessed there were no significant differences between the groups ($t_{1,20}$ = 0.76-0.92, ns, Fig. 5.2). Similar to the two previous experiments (Chapters 3 & 4) there was variability in the protection provided by ischemic preconditioning. Sham animals have ~250 cells (Corbett and Crooks, 1997) and using this number there was ~ 65% protection of CA1 neurons in animals not exposed in the open field and ~55% protection in those that were exposed.

Figure 5.1: Postischemic Brain Temperatures of Animals with and without Exposure to Open Field

24 hr postischemic temperature profiles for animals subjected to ischemic preconditioning. There were no significant differences in brain temperature between the animals exposed to the novel open field environment and those that were not.

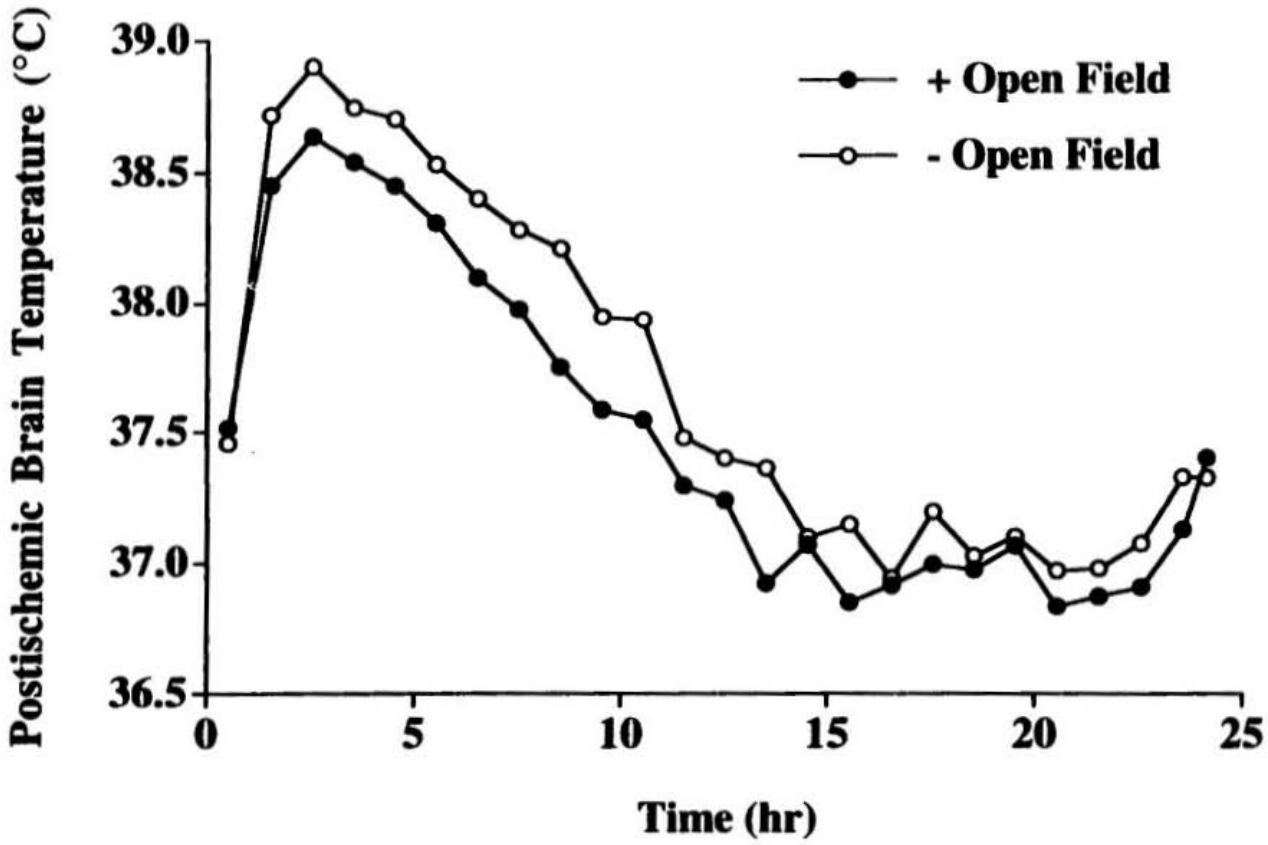
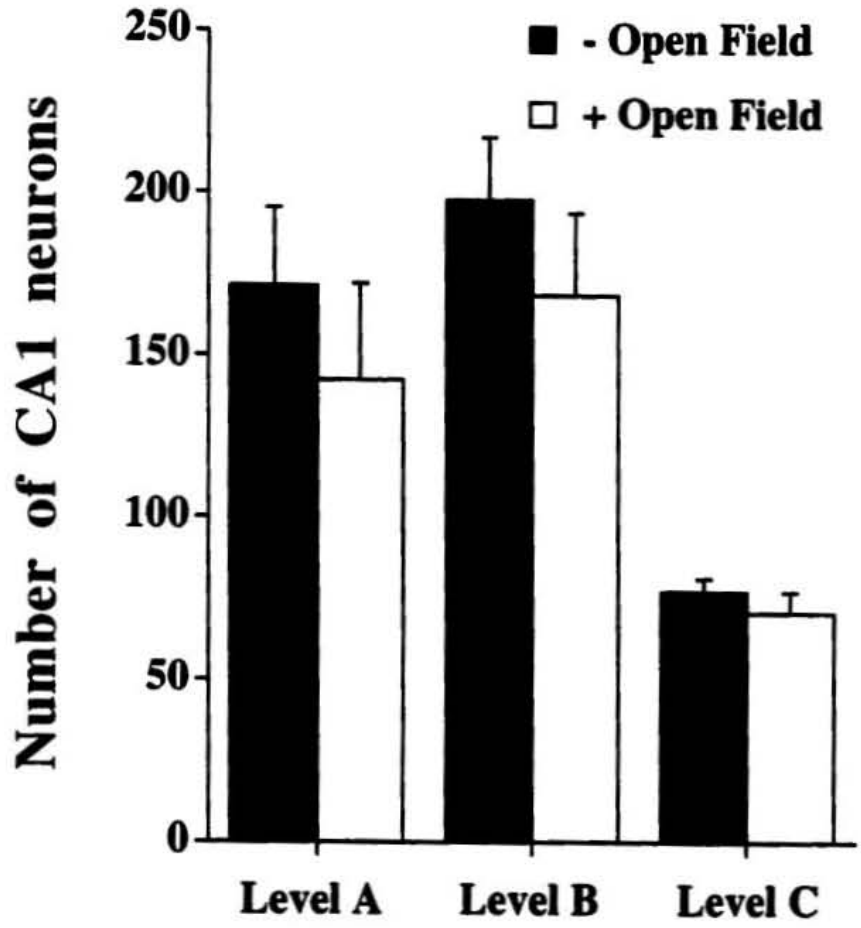

Postischemic Brain Temperature (°C)
39.0
38.5
38.0
37.5
37.0
36.5
0
5
10
15
20
25
Time (hr)
+ Open Field
- Open Field

Figure 5.2: CA1 Cell Counts from Animals with and without Exposure to Open Field

Effects of open field testing on CA1 cell counts (mean ± SEM) following ischemic preconditioning. Repeated exposure to a novel open field environment at 3, 7, 10 and 30 days following the final ischemic episode did not alter histological outcome at 35 days.


Number of CA1 neurons
250
200
150
100
50
0
- Open Field
+ Open Field
Level A
Level B
Level C

5.4 Discussion

Several animal studies have revealed that the CNS can undergo considerable reorganization following injury (Nudo and Millikan, 1996; Castro-Almanacos and Borrel, 1995; Jenkins and Merzenich, 1987) and that spontaneous recovery of function often occurs. It has also been demonstrated that experience (behavioural and environmental) during the recovery period can influence these plasticity processes and significantly improve the level of recovery attained. (Jones and Schallert, 1994; Nudo et al., 1996). However, recent studies have challenged this view by demonstrating a worsening of ischemic injury with early rehabilitation (Koslowski et al., 1996; Risedal et al., 1999).

The theory that early behavioural testing may exacerbate neuronal injury following ischemic preconditioning has not been supported by the results obtained here. It is possible that the amount of exposure to the open field apparatus was not enough to significantly alter neuronal survival. However, a similar study using postischemic hypothermia followed by intense behavioural testing also did not report any significant differences between animals exposed to novel environments and those that were not (Colbourne et al., 1998).

The results of this study do not negate the legitimacy of the studies that have shown exacerbation of injury with early rehabilitative interventions. It is

likely that factors such as the type of injury, where the injury is produced, and the subsequent protocol for therapy contribute to final ischemic outcome. For example, some obvious differences between this study and that of Koslowski's (Koslowski et al., 1996) include the timing and duration of therapy. In their study rehabilitation, in the form of forced use, was initiated immediately after surgery and continued for 10 days. This is in contrast to the 3 day delay and intermittent exposure used here. One of the criticisms of the studies that have shown worsening of injury is that rehabilitation was initiated very soon after injury (within 24 hr) (Koslowski et al., 1996; Risedal et al., 1999). This time frame of ≤ 24 hr is not clinically relevant. In the first few days following a stroke, patients are encouraged to sit up and ambulate only if they can do so safely. This is done to avoid complications such as pneumonia. Intensive rehabilitation, akin to that conducted in the animal studies (Koslowski et al., 1996; Risedal et al., 1999) is typically initiated days or weeks after stroke (Heart and Stroke Foundation of Canada, 1996). Further, in one study of the above studies, when rehabilitation (enrichment and motor training) was delayed by 2 weeks, there was no increase in infarct size (Risedal et al., 1999).

Despite the fact that very few studies have been done to determine the best course of action for rehabilitation, stroke patients are continuing to be

enrolled in programs to promote recovery. While rehabilitation is generally considered to be beneficial, it may not be optimal. It is essential that key issues such as the optimal timing, duration and intensity of rehabilitative therapy be addressed. It is only after we understand the mechanisms underlying functional recovery and how rehabilitative therapy influences this physiology, that we can begin to offer scientifically based and sound treatments to stroke patients.

Chapter 6: Overall Discussion

6.1 Summary of Major Findings

Ischemic preconditioning has consistently been shown to significantly protect CA1 neurons against a severe ischemic episode (Kato et al., 1991; Kirino et al., 1991; Liu et al., 1992; Kato et al., 1994; Corbett and Crooks, 1997; Dooley and Corbett, 1998). One drawback of the early studies is that protection was defined as preservation of neurons as determined by cell counts. Yet, this measure does not determine if the remaining cells are functional. Subsequent studies (Corbett and Crooks, 1997; Dooley and Corbett, 1998) revealed disturbances in behaviour and physiology early after ischemic preconditioning, illustrating the need for the use of functional, in addition to histological, endpoints when investigating neuroprotective treatments.

The objective of this thesis was to further characterize the extent of neuroprotection provided by ischemic preconditioning, particularly in aged animals. This was thought to be important because there are numerous morphological and physiological differences between aged and young animals (Tamaru et al., 1991; Luine et al., 1990; Gonzales et al., 1991; West, 1993; Barnes, 1993). For example, evidence suggests that the density of NMDA receptor

complexes is decreased in aged rodent brain (Tamaru et al., 1991) and that NMDA-mediated responses are attenuated (Gonzales et al., 1991). Therefore, it seemed possible that aged animals might react differently to ischemic preconditioning than young animals. The importance of investigating this phenomenon in aged animals was further underscored by the fact that stroke is a disease that primarily afflicts the elderly (Millikan, 1992).

The results of experiment 1 (Chapter 3) suggested that ischemic preconditioning may be more effective in aged animals than young ones. In this study it was demonstrated that 60 days after ischemic preconditioning animals had ~75% of the CA1 neurons remaining. This level of protection was remarkable since in this model CA1 neuronal loss after 5 min of ischemia is ~ 95% (Colbourne and Corbett, 1995). Furthermore, these cells appeared to be functioning normally as the maximum fEPSP amplitudes elicited from the CA1 dendritic region were not significantly different from sham animals. This level of neuronal preservation was higher than that obtained in young animals from similar experiments, conducted in this laboratory, where 30 days following ischemia there was only 53% (Corbett and Crooks, 1997) and 59% (Dooley and Corbett, 1998) protection. The fact that cell counts were lower at 30 days in young animals versus 60 days in old animals is a strong indication that ischemic

preconditioning is more effective in aged animals. Protracted cell death is known to occur when extended survival times are used (Colbourne and Corbett, 1995; Dooley and Corbett, 1998). Therefore, it is likely that cell counts would be lower if the young animals had survived an additional 30 days. Thus the difference between old and young animals would be more pronounced.

Since historical comparisons of cell counts between experiments are problematic, it became necessary to perform a study whereby a direct comparison of aged and young animals could be made. This was the aim of Chapter 4. In this study, experiments were carried out in young and aged gerbils simultaneously. The results of this study confirmed the hypothesis that ischemic preconditioning was more effective when an aged population of animals were used. Sixty days after the final 5 min occlusion, aged animals had 26% more CA1 neurons remaining in rostral hippocampus than young animals. Furthermore, the level of cellular preservation in the old gerbils was accompanied by relatively normal CA1 field potential amplitudes. This is in contrast to the data derived from young IP animals where field potentials were not significantly different from ischemic values.

These studies were not the first to examine age differences in ischemic vulnerability. A review of the literature describing age-related changes in the

brain reveals an overall view that aged brain is more likely to be vulnerable to ischemic injury than young. For example, morphological studies reveal that with aging there are changes in the cerebral vasculature including thinning of the endothelium (Hajdu et al., 1989; Bar, 1978), thickening of the basal lamina (Topple et al., 1991) and impaired responses to vasodilators (Mayhan et al., 1990; Hongo et al., 1988). There is also evidence of decreased levels of neurotrophins (Whittemore et al., 1985), impaired free radical scavenging systems (Delbarre et al., 1992) and alterations in several neurotransmitter systems (Cortes et al., 1989; Shen and Barnes, 1996). Furthermore, previous studies, examining the influence of age on ischemic outcome, reported more damage in aged animals compared to young (Davis et al., 1995; Sutherland et al., 1996; Yao et al., 1991). However, there is also evidence to support our finding of reduced injury in aged animals. Sutherland and colleagues (1996) found that following global ischemia, there was less CA1 neuronal loss in 26-28 month old compared to 2-3 month old rats. In addition, there is some evidence consistent with decreased vulnerability of the aged brain to ischemic injury. For example, it has been shown that aged brain has a decreased immunological response (Futrell et al., 1991), decreased NMDA receptor numbers and decreased NMDA responsivity (Tamaru et al., 1991; Gonzales et al., 1991). It has also been demonstrated that there is a decrease in

the neuron to glial cell ratio in aged brain (Brizee et al., 1968; Miquel and Johnson, 1983). Finally, as mentioned in section 4.4, it is possible that the fluctuations in hormone levels during the estrus cycle in young gerbils may make them more vulnerable to ischemic injury in this model. Future experiments are required to address the issue of whether or not variations in cycling influence outcome after ischemic preconditioning.

Although there is a difference in the final outcome of aged and young animals, the *early* dissociation (within the first few weeks) between histological and functional protection occurred independent of age and is consistent with previous studies (Corbett and Crooks, 1997; Dooley and Corbett, 1998). Using the dendritic marker MAP2, an attempt was made to account for this dissociation. There were no differences in MAP2 staining in sham and ischemic preconditioned animals 10 days post surgery. MAP2 has been shown to be a sensitive indicator of early neuronal injury (Matesic and Lin, 1994), however this was following lethal ischemia (5 min occlusion) without any treatment intervention. In these animals gross structural changes in the dendrites were apparent and were consistent with ultimate cell death. These types of changes (3 days after ischemia) were also detected by MAP2 immunostaining in the I animals of experiment 1 as well as in a previous study (Dooley and Corbett, 1998).

MAP2 staining was essentially normal in ischemic preconditioned animals indicating that the structural integrity of the dendrites is intact. However, it is possible that the IP animals have structural changes in their dendrites but perhaps the MAP2 antibody was not sensitive enough to detect subtle alterations. Alternatively, it is possible that less obvious changes have occurred at the synaptic level, involving dendritic spines. This idea is supported by the following observations. *In vitro* NMDA application on to hippocampal dendritic spines has been shown to decrease spine length (Segal, 1995). Similarly, it is known that CA1 dendrites and their spines are particularly vulnerable to ischemic injury (Matesic and Lin, 1994). Segal has postulated that dendritic spines have a neuroprotective role, whereby they accumulate Ca^{2+} from extracellular sources and 'compartmentalize' it (Segal, 1995). This would effectively decrease the levels of Ca^{2+} that reach the soma and prevent Ca^{2+} mediated toxicity (e.g. activation of caspases and endonucleases) (Segal, 1995). Changes in spine density have been triggered by a variety of environmental manipulations/conditions (Greenough and Chang, 1988; Kolb et al., 1997; Woolley et al., 1990; Fifkova and Van Harreveld, 1977). For example, increases in spine density have been seen following recurrent epileptiform activity in the rat hippocampus (Bundman and Gall., 1994) and in animals exposed to environmental

enrichment (Moser et al., 1994). Fluctuations in spine density have also been seen in female rats during the estrus cycle with changes in spine density occurring rapidly (within days) (Woolley et al., 1990). It is conceivable that the preconditioning episodes also result in an increase in spine density. This might be protective if the addition of more spines provided the neurons with a greater capacity for dealing with the elevated Ca^{2+} associated with the 5 min ischemic insult. In this scenario many of the spines may succumb to ischemic injury but because the cell body and dendrites have been spared the capability for restoration of dendritic spines is retained. This mechanism may ultimately be responsible for recovery of function. Following the 5 min occlusion, the number of synaptic contacts would be greatly reduced if the dendritic spines are damaged due to Ca^{2+} toxicity. This could result in functional impairments and account for the early behavioural (i.e. heightened open field activity) and electrophysiological (i.e. reduction in fEPSP amplitude) disturbances.

One way to determine if this is the case, would be to examine the dendritic structure of neurons exposed to ischemic preconditioning using Golgi impregnation and/or electron microscopy. Both techniques can be used to quantitatively assess spine morphology (i.e. multiple headed spines) and synaptic density. Specifically, they can be used to count the number of spines

along a dendritic segment (Kolb et al., 1997; Jones et al., 1996). Therefore, either technique would be useful to determine if there are differences in spine density between sham and preconditioned only animals and also if there are differences between sham and ischemic preconditioned animals. Such information could explain the early dissociation between histological protection and functional impairment.

The eventual recovery that occurs in the majority of ischemic preconditioned animals is an extension of this process. Early after the 5 min insult the neurons are in a vulnerable state and it may take several days for cellular activities (e.g. neuronal repair of damaged proteins) to normalize. While many contacts with other cells may remain (through axo-somatic or axo-dendritic synapses) the majority of synapses would have been lost and must be reformed in order to restore normal function. This process may take days or weeks to complete, therefore explaining the impairments in the first 10 days with a trend for recovery over time (i.e. 30 and 60 days). Further, while ischemic preconditioning provides only partial histological protection, at long survival times, field potentials are normal. The reorganization that has been hypothesized to occur may involve compensation by the remaining neurons through increases in synapse formation.

In Chapter 5, it was determined that the early behavioural testing, employed as a functional outcome measure in several ischemia studies (Nurse and Corbett, 1994; Colbourne and Corbett, 1994 and 1995; Dooley and Corbett, 1998; Corbett and Crooks, 1998) including the ones in this thesis, does not alter histological outcome. It was possible that early and repeated exposure to a novel environment could be beneficial and enhance cell survival based on the principle that early functional use is necessary or function may be lost. It was also possible that forced use (e.g. forming spatial maps) would have been detrimental due to overtaxing compromised neurons. Both outcomes have been seen with proposed rehabilitative interventions following brain injury (Koslowski et al., 1996; Nudo et al., 1996; Risedal et al., 1999; Jones and Schallert, 1994).

Finally, a retrospective look at all the IP animals from each of the studies confirms the finding that the protection by ischemic preconditioning is variable (Abe and Nowak, 1995). In addition, the decrease in efficacy seen in Chapter 4 compared to Chapter 3 and previous studies (Corbett and Crooks, 1997; Dooley and Corbett, 1998) was, at first, attributed to an apparent shift upward in brain temperatures. However, the IP animals from Chapter 5 exhibited elevated temperature profiles compared to those from IP animals in Chapter 4 (compare Fig. 4.1 and Fig. 4.2 with Fig. 5.1) and yet had better overall (55-65%) preservation

of CA1 at 35 days. The reasons for these differences in efficacy remain unknown.

6.2 Ischemic Preconditioning as a Model

In the clinical situation it is often not possible to treat stroke patients within 24 hr of onset. Consequently, current treatments such as t-PA, which have to be administered within three hr of stroke onset, cannot be used (Barinag, 1996). Furthermore, in animal studies no pharmacological agents have been proven to be protective when administered more than 2 hr after ischemia. A more useful approach may be to take advantage of increased neuronal plasticity following injury. If it is possible to manipulate plasticity changes in the brain it may be possible to promote recovery of function. In studies such as these treatment is often delayed for several days or weeks. Therefore, a model of untreated global ischemia would not be very useful since near-total loss of CA1 neurons occurs by 4 days postischemia (Kirino, 1982; Pulsinelli et al., 1982). Similarly, in cases of near-total protection of CA1 neurons (i.e. following long duration postischemic hypothermia) it would be difficult to detect any additional benefits of a putative treatment. However, the partial protection provided by ischemic preconditioning makes it a suitable model for assessing delayed

therapeutic interventions. By exploiting the fact that ischemic preconditioning provides only partial protection it is possible to assess how other factors influence outcome. In Chapter 5, this model was used to answer the question of whether or not early behavioural testing influenced histological outcome. In this experiment, it was conceivable that repeated early exposure to a novel environment could be either beneficial or detrimental. Both possibilities would have been easily detected by this model.

Ischemic preconditioning is currently being used in our laboratory to determine the effects of environmental enrichment on outcome. Potentially, it can also be used to assess new pharmacological treatments.

6.3 Ischemic Preconditioning Mechanisms

In the same way that many factors are involved in mediating cell death, many factors are believed to contribute to the phenomenon of ischemic preconditioning. One of the first areas to be intensely studied with respect to ischemic preconditioning was the role of heat shock proteins (HSPs). HSPs are a family of protective proteins that are induced following a period of hyperthermia or other types of stress (Currie and White, 1981). It has previously been demonstrated that HSP induction is upregulated following ischemia (Nowak et

al., 1985). Furthermore, it has been shown that HSP induction occurs even after brief periods of ischemia that are not associated with neuronal injury (Kitigawa et al., 1991). These two factors supported the theory that HSP induction may be an important aspect of ischemic preconditioning protection. However, subsequent studies provided evidence against a role for HSP. First, it was illustrated that the temporal profile of HSP immunostaining did not correlate well with that of induced tolerance (Kirino et al., 1991). Second, when protein synthesis was blocked and prevented the production of HSPs ischemic preconditioning still worked (Kato et al., 1992). Finally, Abe and Nowak (1996) were able to induce tolerance using ischemic durations that were too short to increase HSPs (Abe and Nowak, 1996). The results of these more recent studies have led to diminishing interest in HSPs as mediators of preconditioning protection.

Recently, research has focused on the role of mitochondria. It is thought that Ca^{2+} toxicity arising from glutamate stimulation occurs when mitochondrial capacity to buffer Ca^{2} is exceeded (White and Reynolds, 1995). Excess Ca^{2+} leads to an uncoupling of electron transfer from ATP synthesis and results in the degradation of the mitochondrial membrane potential and increased free radical production that ultimately kills the cells (Schnider et al., 1996). With respect to ischemic preconditioning it has been suggested that the preconditioning

episodes increase the ability of the mitochondria to buffer Ca^{2+}. Ohta and colleagues (1995) demonstrated that the mitochondria of CA1 are able to sequester more Ca^{2+} in animals with preconditioning than those without (Ohta et al., 1995). This is in contrast to a recent report that indicates a decrease in mitochondrial Ca^{2+} levels after preconditioning (Rytter et al., 1999). In this study, it was shown that cellular swelling due to a rise in $[Ca^{2+}]_i$ still occurred. However, through changes brought about by preconditioning, the mitochondria did not sequester the same levels of Ca^{2+} as control animals. By decreasing the Ca^{2+} uptake capabilities of the mitochondria, the authors hypothesized that detrimental cell signaling cascades (mediated by cellular Ca^{2+} homeostasis) are also downregulated, thereby contributing to protection. It seems more likely that preconditioning would decrease mitochondrial exposure to Ca^{2+} rather than increase it. Excess Ca^{2+} is known to destroy mitochondria by the mechanisms discussed above and may, in part, be contributing to the cell death in ischemic animals without preconditioning. Nevertheless, more research in this area is necessary to resolve this controversy.

The potential involvement of glial cells as a mechanism of ischemic preconditioning was addressed in Chapter 4. The results from the GFAP immunostaining indicated changes in the pattern of astroglial reactivity that were

consistent with results from a previous study (Kato et al., 1994). Preconditioned only animals at day 3 exhibited subtle changes in GFAP staining compared to sham animals. Furthermore, GFAP staining in IP 3 animals was more robust than that of I 3 animals. Taken together, these two observations suggest that ischemic preconditioning may promote neuronal survival by initiating reactive astrocytosis after the sub-lethal insults and providing CA1 cells with increased neuronal support at a time when they are vulnerable.

It is clear from the above discussion that endogenous mechanisms are mediating neuroprotection in ischemic preconditioning. It is likely that a number of factors are responsible for conveying protection, each one contributing to the final outcome. With this in mind, it is possible to envision the following scenario. Exposing gerbils to sub-lethal ischemia results in an influx of Ca^{2+} into the cells. Through as yet unidentified mechanisms (possibilities include growth factors, synaptic stimulation by glutamate and/or cell signaling mechanisms initiated by Ca^{2+}) there is an increase in spine density on CA1 neurons over the next few days. Coinciding with this is the alteration in the mitochondrial sequestration of Ca^{2+} followed by the process of astrocyte activation. Several days later, at the time of the final 5 min insult, the spines, to their own demise, effectively protect the cell bodies and dendrites from toxic levels of Ca^{2+} by

compartmentalizing it. The decreased capacity of the mitochondria to take in any excess Ca^{2+} would further protect the cells from detrimental cell signaling processes. In addition, activated astrocytes could help restore ion homeostasis and reform the BBB (Yong, 1998). The loss of synaptic contacts formed through the spines may account for the behavioural and electrophysiological impairments early after ischemic preconditioning. Over time, cell homeostasis is restored and the neurons begin to recover. New spines are formed and synaptic contacts are reestablished, likely mediated, in part, by growth factors (e.g. bFGF) released from astrocytes. Finally, this may be contributing to the observed functional recovery at extended survival times.

Ischemic preconditioning is not the first treatment thought to act through a variety of mechanisms. Both intraischemic (Nurse and Corbett, 1994) and postischemic (Colbourne and Corbett, 1995) hypothermia have been shown to provide robust and long lasting protection to CA1 neurons. The effects of mild hypothermia are widespread and many of its actions are thought to ultimately contribute to neuronal preservation after ischemia (see Colbourne et al., 1997 for a review).

Obviously, ischemic preconditioning would not be used clinically as a treatment for stroke. The interest in studying this phenomenon lies with trying

to uncover the mechanisms through which it works. In doing this it might be possible to develop pharmacological agents that would protect the neurons in the same fashion as ischemic preconditioning. Administered postischemically, they may be able to reduce the deficits associated with severe cerebral ischemia. For example, it may be possible to mimic the function of reactive astrocytes either by increasing growth factor levels in CA1 specifically or by directly antagonizing or inhibiting microglia. However, even if the mechanisms through which ischemic preconditioning conveys protection to CA1 neurons become known, an additional problem exists. Ischemic cell death is known to be mediated through a variety of processes (e.g. loss of Ca^{2+} homeostasis, increased free radicals, proteolysis). In conjunction with this, is the fact that the two most effective neuroprotectants to date (hypothermia and ischemic preconditioning) also act via several mechanisms. Therefore, it is likely that a broad spectrum of pharmacological agents would be necessary to accomplish similar levels of neuronal protection. This would make treatment very difficult due to unknown drug interactions. With respect to ischemic preconditioning, it is possible that some of the protective actions of preconditioning have to be in place *prior* to the lethal insult (i.e. altered mitochondrial capacity for Ca^{2+}) and so pharmacological simulation may be difficult to achieve.

6.4 The Necessity for Functional Assessments and Long Survival Times

Several studies have now demonstrated severe functional impairments in cases where normal histology was preserved. Furthermore, this dissociation has been seen in instances of ischemia with (Ishimaru et al., 1995; Dooley and Corbett, 1998; Corbett and Crooks, 1997) and without (Hori and Carpenter, 1994; Bothe et al., 1986) protective treatments. Repeated examples have been shown in this thesis where ischemic preconditioning results in a dissociation between histological and functional protection early in the recovery period. Despite this knowledge, a majority of researchers are still drawing conclusions based on histological information alone. This is problematic for at least two reasons. First, it is clear from the studies cited above that normal histology does not guarantee functional integrity. Second, with respect to clinical stroke, functional recovery is of paramount importance. It may be catastrophic to save neurons from ischemic cell death if they function abnormally (e.g. rendering the neurons epileptogenic). This last point is particularly important when investigating potentially protective treatments.

In this thesis, behavioural testing and electrophysiological recordings were employed as two measures of functional outcome. The open field test provides a sensitive measure of ischemic injury (Wang and Corbett, 1990; Nurse

and Corbett, 1994; Gerhardt and Boast, 1988; Colbourne and Corbett, 1994). This combined with the fact that it is easy to conduct and requires very little extra time makes it a useful tool in ischemia research. However, as stated previously, the open field task is not specific to hippocampal function and even ischemic animals eventually recover. Therefore, field potentials elicited from the CA1 apical dendritic region were used as a more direct functional measure of CA1 integrity. Without these functional measures it may have been erroneously concluded that ischemic preconditioning provides robust protection early after ischemia but that efficacy decreases as cell death continues over longer survival times. Instead, it was demonstrated that fEPSP amplitudes from ischemic preconditioned animals (aged gerbils) were similar to sham values despite the fact that they had fewer CA1 neurons.

The issue of postponed cell death poses additional problems in ischemia studies. Typically, survival times $\leq$ 7 days have been used in a majority of the published literature assessing neuroprotective treatments. The basis for such short survival times originated from earlier work which showed that cell death following global ischemia is more or less complete by ~72 hr (Kirino, 1982; Pulsinelli et al., 1982). However, more recently studies using extended survival times have revealed that many treatments only postpone cell death (Morse and

Davis, 1990; Li and Buchan, 1995; Nurse and Corbett, 1996; Dietrich et al., 1993; Colbourne and Corbett, 1994; Corbett and Crooks, 1997; Dooley and Corbett, 1998). For example, the AMPA antagonist, NBQX, appeared to provide significant neuronal savings when assessed at early time points (Sheardown et al., 1990; Nurse and Corbett, 1996; Colbourne et al., 1999). However, when survival time was extended to 10 days (Nurse and Corbett, 1996) or 28 days (Colbourne et al., 1999) protection was significantly reduced (Nurse and Corbett, 1996) or lost all together (Colbourne et al., 1999). Even the most effective treatments (i.e. postischemic hypothermia and ischemic preconditioning) exhibit prolonged cell death. In a study that included extended survival times, protection provided by postischemic hypothermia declined from 90% at 30 days to 70% at 6 months Colbourne and Corbett, 1995). However, more recent work indicates that this level of protection (70%) is permanent (Colbourne and Corbett, 1997).

From the above discussion it is reasonable to argue that the failure of most clinical trials resulted from weaknesses in the pre-clinical studies. Obviously assessments made at 7 days may not provide an accurate picture of histological outcome. Additionally, functional endpoints are also essential to determine the true extent of neuroprotection provided by a specific treatment. It

is only when these two criteria are met that researchers can be confident of their assessments. Finally, only when a specific treatment provides long lasting *functional* and histological protection should it be considered for acute stroke therapy.

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