ISCHEMIC PRECONDITIONING PROVIDES HISTOLOGICAL BUT NOT BEHAVIORAL PROTECTION IN THE GERBIL

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ISCHEMIC PRECONDITIONING PROVIDES HISTOLOGICAL BUT NOT BEHAVIORAL PROTECTION IN THE GERBIL

By

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DEDICATION

I would like to make this dedication to my little sister, Gillian, who was diagnosed with multiple sclerosis at the same time this thesis was nearing completion. This symbolizes my faith and hope that medical research will very soon have an impact on her life as well as the lives of the many afflicted by these elusive neurological disorders.

ABSTRACT

It has been demonstrated that brief duration (e.g. 1.5-2 min) ischemia can provide neuroprotection against subsequent insults of longer duration. This phenomenon of ischemic tolerance or ischemic preconditioning was first reported in 1990 and has since received tremendous attention. The purpose of this investigation was to determine what effect ischemic preconditioning has on post-ischemic brain temperature, to assess whether histological preservation translates into functional protection and to observe whether there is a decline in neuroprotection with a longer survival time in a gerbil model of global ischemia.

This study clearly demonstrated that preconditioning episodes of short duration ischemia provided significant histological protection of CA1 pyramidal cells against a subsequent ischemic insult that would typically produce roughly 95% necrosis of these neurons. Interestingly, histological protection in preconditioned ischemic animals did not result in concurrent behavioral protection. Preconditioned ischemic animals displayed a consistent habituation deficit when placed in the open field and were not different from untreated ischemic gerbils. A significant decline in CA1 preservation in preconditioned animals was observed when survival time was extended from 10 (81% protection) to 30 (53% protection) days. The lack of functional protection within the first 10 days postischemia, concomitant with the decline of cellular preservation over time suggests that this paradigm may not provide permanent protection if assessed at 2 or 3 months later.

Consistent damage was observed in sector CA2 of the hippocampus in 21/22 preconditioned gerbils. Tolerance to ischemia failed to be induced in these neurons. In addition, several preconditioned animals displayed damage in the subiculum. Preconditioning ischemia did not affect the degree of hyperthermia observed following the lethal occlusion but did significantly reduce the duration of hyperthermia. However, this effect was small and thus ischemic preconditioning does not appear to be acting via a temperature mediated process.

In light of the present findings, future research must be directed towards determining if the histological protection observed at 30 days is permanent or whether cell death would continue with longer survival times. Electrophysiological recording from CA1 would also help determine if these "preserved" CA1 neurons are fully functional. In the future the mechanisms of ischemic tolerance may be able to be activated pharmacologically thereby providing a novel therapy for the treatment of stroke.

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My parents played a tremendous supportive role in absentia. I cannot express enough my gratitude for all that they have done.

And last but not least, Amanda, where do I begin...!

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1. INTRODUCTION

Stroke is a general term that refers to the sudden appearance of neurological symptoms as a result of interruption of cerebral blood flow. Stroke is the third leading cause of death in Canada and is the first most common cause of chronic functional incapacity. The traditional approach to the treatment of stroke victims has been purely symptomatic. However, increasing the awareness for the identification and control of risk factors such as hypertension, diabetes, nicotine and sodium intake has helped curb the incidence of stroke. As well, new technological advances in brain imaging and the growing number of stroke research laboratories worldwide are creating an optimistic approach for new treatment strategies for stroke patients in the future.

Any perturbation in blood flow to the brain will result in a consequential interruption to the critically dependent supply of oxygen and glucose. Ischemia is defined as a reduction in normal blood flow to a level that is insufficient to meet metabolic demands. Neurons are highly sensitive to situations of reduced or complete ischemia and even brief durations can cause irreversible damage which can potentially result in serious neurological dysfunction (Kirino, 1982; Pulsinelli, Brierly and Plum, 1982).

This thesis is concerned with "ischemic tolerance" first reported by Kitagawa, Matsumoto, Tagaya, Hata, Ueda, Niinobe, Handa, Fukunaga, Kimura, Mikoshiba and Kamada (1990b) who demonstrated that short duration ischemia can provide neuroprotection against subsequent insults of longer

duration. Although there are a number of laboratories currently contributing to the growing body of literature on ischemic tolerance, also referred to as ischemic preconditioning, there are several key issues that require elucidation. In our laboratory the main focus has been the role of brain temperature in determining ischemic outcome as well as assessing functional in addition to histological preservation. Using this approach it was possible to examine what effect ischemic tolerance has on brain temperature, behavior and histological outcome.

1.1 Clinical Features of Ischemia

In the clinical setting, the underlying etiologies of cerebral ischemia are diverse. Ischemia can occur gradually, abruptly, or can be transient due to atherosclerosis, embolism and cardiac failure respectively. In all cases the common denominator is a failure of normal blood supply which leads to brain injury. The extent of damage is dependent on both the degree and duration of the ischemic episode (Kirino, 1982).

Blood flow is reduced to zero during global ischemia, such as the case during cardiac arrest which represents the most frequent clinical cause. The brain can only tolerate very brief periods (e.g. 5-10 min) of complete interruption in blood flow before irreversible cell death ensues. Short periods of global ischemia cause necrosis in discrete areas of the brain that have been found to

be selectively vulnerable, such as the CA1 sector of the hippocampus (Kirino, 1982; Pulsinelli, Brierly and Plum, 1982). With longer durations of global ischemia the damage is evident in additional areas to CA1 including neocortical layers 3,4 and 6, cerebellar Pukinje cells and striatum (Horn and Scholte, 1992). The most pronounced clinical symptom of patients that have suffered some form of global ischemic insult is that of memory dysfunction which has been linked to damage to the hippocampus (Zola-Morgan, Squires and Amaral, 1986).

The inherent lack of control over the majority of the variables contributing to the onset of stroke in human patients has prevented the development of an effective therapy based on human data alone. It is known that the early events following an ischemic occlusion are critical (Kuroiwa, Bonnekoh and Hossman, 1990) and thus can only be studied under experimental conditions in the laboratory. Therefore, animal models of ischemia have become invaluable tools in the search for stroke therapies.

1.2 Gerbil Model of Global Cerebral Ischemia

In the majority of experiments, ischemic injury is produced by the temporary occlusion of large extracranial arteries supplying the brain. Under anesthesia the vessels are surgically exposed, clamped for a predetermined occlusion time and released to initiate reperfusion. Gerbils, unlike other

animals, have a characteristic abnormality in their cerebrovasculature; they possess an incomplete circle of Willis (Levine and Sohn, 1969; Berry, Wisniewski, Suarzbein and Baez, 1975). The absence of posterior communicating arteries which connect the carotids to the vertebrobasilar arterial supply prevents any compensatory residual blood flow to the forebrain when the carotids are occluded bilaterally. Thus bilateral occlusion of the carotids will reduce cerebral blood flow to the forebrain to zero while sparing the vegetative control centers which reside in the brainstem. The relative convenience, ease of induction and consistent damage produced has resulted in widespread use of the gerbil in assessing pathological mechanisms (Ginsberg, Mela,Wrobel-Kuhl and Rievich, 1977; Yoshida, Inoh, Asano, Sano, Kubota, Shimazaki and Ueta, 1980; Suzuki, Yamaguchi, Kirino, Orzi, and Klatzo, 1983; Moskowitz, Kiwak, Hekimian and Levine, 1984; Nurse and Corbett, 1994) and assessing pharmacological efficacy (Alps, Calder, Hass and Wison, 1988; Corbett, Evans, Thomas, Wang and Jonas, 1990; Nurse and Corbett, 1995 in press) following global ischemic insults. Gerbils do have certain limitations in their use when efforts are made to assess physiological variables. For example, due to their very small size, repeated blood sampling cannot be accomplished. It has been reported that gerbils have a genetic predisposition to seizure activity which could potentially create a confounding variable in assessing ischemic outcome (Schmidt-Kastner and Freund, 1991). However, the presence of seizure activity in our laboratory is rare (unpublished observations) which coincides with observations reported by others (Kirino and

Sano, 1984a) that gerbils display seizure activity only after relatively prolonged ischemic episodes (e.g. > 15 min) well exceeding the durations employed in the present study. Armstrong, Neill, Crain and Nadler (1989) recorded EEG continuously for four days from gerbils subjected to 5-10 minutes of ischemia and did not report the presence of ictal activity. Thus, ischemic injury in the gerbil is not the result of epileptiform activity.

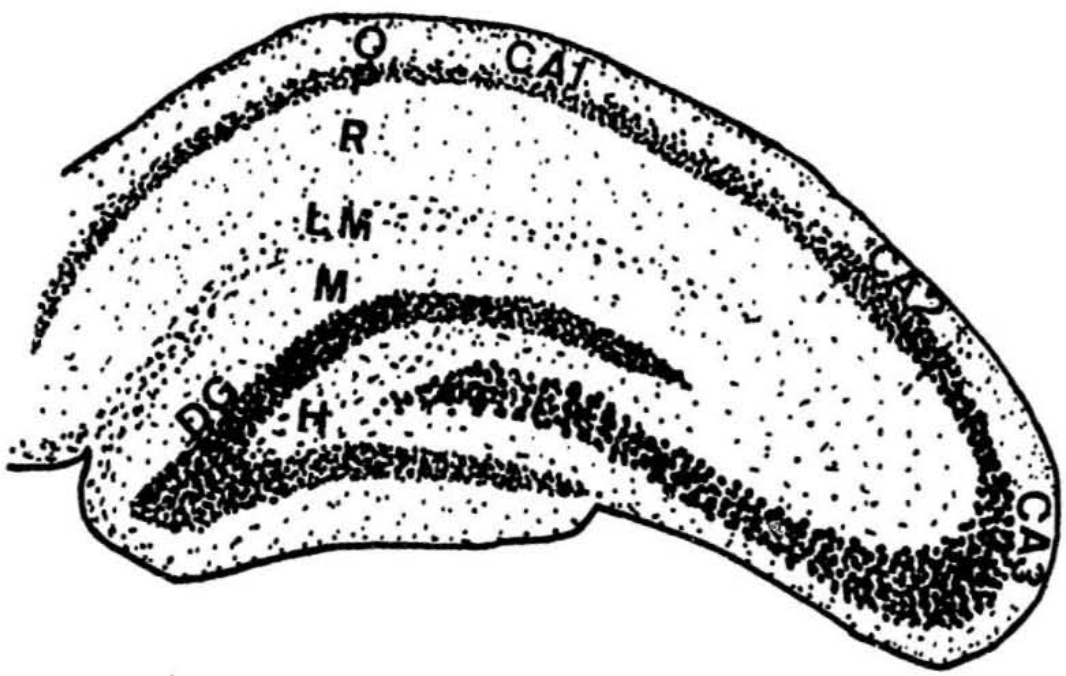
For a comprehensive review of rodent models of ischemia readers are directed to a paper by Ginsberg and Busto (1989).

1.3 Hippocampal Anatomy / Selective Vulnerability

Global ischemic insults produce a histopathological response in which certain brain regions are selectively damaged and this is termed "selective vulnerability". Hippocampal CA1 pyramidal cells display selective neuronal vulnerability to brief episodes of ischemia (e.g. 3-5 min) while the adjacent CA3 sector and dentate gyrus remain largely unaffected (Kirino and Sano, 1984a).

The anatomy of the hippocampus is well-suited for comparing a population of selectively vulnerable neurons to neighboring resistant populations within the same structure. Figure 1 illustrates the major neuronal populations within a coronal section through the rostral hippocampus. Using the "CA" terminology of Lorento de No (1934), the dorsal band of small pyramidal neurons is the CA1 band. Lying at the medial border with CA1 is an area that

Figure 1. Subfields and layers of the hippocampal formation: (O) stratum oriens; (P) stratum pyramidale; (R) stratum radiatum; (LM) stratum lacunosum-moleculare; (M) stratum moleculare of the dentate gyrus; (DG) dentate gyrus and (H) hilar region (modified diagram from Johansen, 1993).


O
CA1
R
LM
M
DG
H
CA2
CA3

has been referred to as the subiculum (Woodhams, Celio, Ulfig and Witter, 1993), subiculum-CA1 (Akai and Yanagihara,1993), or paramedian CA2 (Akai and Yanagihara,1993). There is evidently a lack of consensus as to the proper terminology of this area in different pathological studies, therefore for simplicity it will be referred to here as the subiculum. At the lateral border of CA1 lies CA2. The pyramidal cells of CA2 are larger in comparison to CA1 and make the border between these sectors easy to delineate. However, adjacent CA3 pyramids are equal in size and blend in with CA2, preventing this transition from being discernable with regular light microscopy and conventional stains (Akai and Yanagihara,1993). The large CA3 pyramidal cells form the lateral curvature which makes a ventral turn and runs into the hilar region and dentate gyrus.

The entorhinal cortex provides the major input into the hippocampal formation via the perforant path to the granule cells of the dentate gyrus. Dentate granule cells give rise to mossy fiber projections which send excitatory synaptic transmission to CA3 pyramids. Area CA1 is innervated by a powerful association projection known as the Schaffer collaterals arising from CA3 neurons. CA1 efferents go to the subicular complex which completes the circuit. The subiculum is the source of major output to the entorhinal cortex, cingulate cortex, anterior thalamic nuclei, nucleus accumbens and mamillary bodies (Paxinos, 1985).

1.4 Delayed Neuronal Death

Kirino (1982) described the differential cellular reaction to ischemia in each sector of the hippocampus. It is now well accepted that CA1 pyramidal cells display unique features in manifestation of ischemic injury. The progression of neuronal death in CA1 has been shown to be dependent on the duration of the ischemia (Ito, Spatz, Walker and Klatzo, 1975). Longer duration ischemia will result in a more rapid development of subsequent necrosis, while shorter durations will follow a slower development of cell death.

CA1 neurons are among the most vulnerable neurons to periods of global ischemia in the entire forebrain. The temporal profile of cell death in CA1 is of particular interest. Histological analysis one day after occlusion shows no change in CA1 cellular morphology (Kirino, 1982). Cellular alterations in CA1 are not noticeable with Nissl stains until two days post-occlusion (Kirino and Sano, 1984b), however by 4 days most neurons are extensively damaged. Damage, as reported earlier, is accelerated with extended periods of ischemia (> 5 min in the gerbil). The understanding of the time-course for delayed neuronal death indicated that there may exist a novel mechanism for the manifestation of ischemic damage in CA1.

CA2, as mentioned previously, is difficult to discern at its lateral border with CA3. Thus, it has not received the same degree of attention as neighboring CA1. Delayed neuronal death has not been documented in CA2. This sector does not appear to be affected by very brief durations (e.g. < 2 min)

of ischemia; however, when its threshold for damage has been exceeded, ischemic cell change occurs rapidly and precedes damage in CA1 (Crain, Westerkam, Harrison & Nadler, 1988). Akai and Yanagihara (1993) have demonstrated with a combined immunohistochemical and zinc histochemical procedure that neurons in CA2 display irreversible damage as early as 4-5 minutes following 10 minute of global ischemia in the gerbil. A second area which they term the "subiculum-CA1" which lies at the medial most aspect of the CA1 cell band, displays identical damage with the same time-course and threshold for injury as CA2. The pathophysiological mechanism for the death of CA2 and subicular neurons is not known and it is important not to mistake them for post-ischemic lesions of the CA1 sector.

Certain subpopulations of dentate hilar neurons are more vulnerable to ischemia than CA1 (Kirino, 1982; Kirino & Sano, 1984; Crain et al., 1988). Very brief periods of ischemia that do not cause any histological damage to CA1 (e.g. 1 min in the gerbil) will result in significant loss of somatostatin containing hilar neurons (Mastuyama, Tsuchiyama, Nakamura, Matsumoto & Sugita, 1993). Due to the great diversity of neuronal types in the hilar region (Amaral, 1978), the identification of vulnerable subtypes is technically daunting. Damage to CA1 has not been reported in the absence of hilar lesions which always precede CA1 necrosis.

CA3 pyramidal cells and dentate granule cells are remarkably resistant to ischemic cell death. Prolonged periods of ischemia in the gerbil (5-20 min) and rat (30 min of 4 vessel occlusion) reveal a "reactive change" in these cells;

however, they return to normal in two to three days (Kirino, 1982; Pulsinelli, Brierly and Plum, 1982). The reversible morphological changes noted by Ito et al. (1975) were densely stained cytoplasm, vacuolization and a movement of the nucleus to the periphery. These early morphological alterations are similar to those of CA1, therefore it has been suggested these sectors differ in their capacity to recover from ischemic insults. The reason for this differential vulnerability of CA1 and CA3 and the delayed neuronal death of CA1 remains elusive.

Delayed neuronal death has also been detected in neuropathological analysis of human patients that have suffered from some form of cardiac arrest (Horn & Schlote, 1992). This indicates that it is not a phenomenon limited to experimental animal models but in fact has significant clinical implications. Quantification of the severity of ischemic injury was assessed in the hippocampal CA1 sector, cerebellar Purkinje cells and frontal neocortex by direct counting of stained necrotic neurons. In contrast to the delayed neuronal death defined as lasting roughly 3-4 days in rodents, delayed neuronal death in human CA1 sectors lasted about 7 days following cardiac arrest . This prolonged period of delayed neuronal death in humans emphasizes the importance of this phenomenon in enabling a potential therapeutic window of opportunity to implement some post-ischemic intervention to limit neuronal injury.

1.5 *Neurochemical Alterations*

Restoration of impaired blood flow is essential to halt the cascade of abnormal metabolic processes that results from global insults. Cessation of blood flow causes a depletion of glucose and oxygen necessary for survival. Neurons have comparatively high energy demands but their capacity to store energy is very low. The majority of energy comes from aerobic oxidative phosphorylation in mitochondria and to a lesser degree from anaerobic glycolysis in the cytoplasm. With reduced or no blood flow mitochondrial phosphorylation ceases and the energy-rich metabolites ATP, ADT, AMP and phosphocreatine and the energy stores of glucose and glycogen are quickly utilized. These energy stores can sustain cellular function for only a short period. Anaerobic glycolysis produces biproducts such as lactate and H^+ and the hydrolysis of ATP also produces H^+ which together cause a decrease in pH, this process has been termed tissue acidosis. Tissue acidosis has been suggested to be an important factor contributing to ischemic injury (Siesjo, 1988).

Energy levels are quickly depleted during ischemia which is sufficient to cause a breakdown in metabolism (Pulsinelli and Duffy, 1983). Energy failure triggers a wealth of secondary events. Ion homeostasis is adversely affected, energy-dependent ion pumps can no longer function. ATP energy is required to actively transport Na^+, K^+, Cl^- and Ca^{2+} which regulates ionic potentials across neuronal membranes. With a decrease in ATP-dependent ionic transport there

is a passive efflux of K^+ and influx of Na^+, H^+ and most importantly Ca^{2+} (Hansen, 1985; Hossman and Grosse Ophoff, 1986), Ca^{2+} has been implicated as a key mediator of ischemic cell death (Siesjo and Bengtsson, 1989; Nakamura, Hatakeyama, Furuta and Sakaki, 1993).

Increased cytosolic calcium can arise from a variety of sources: (1) passive leakage from the extracellular space, (2) depolarization and (3) release from internal stores (endoplasmic reticulum and mitochondria). During ischemia there is a massive release of the excitatory amino acids (EAAs) glutamate and aspartate from presynaptic terminals which act on postsynaptic NMDA and metabotropic glutamate receptors. This excessive release of EAAs has been termed "excitotoxicity" and is believed by many to be responsible for cell death in ischemia (Rothman, 1984; Rothman and Olney, 1986; Choi, 1988). Excitatory amino acids will open receptor-gated Ca^{2+} channels and mobilize second messenger systems, inositol triphosphate (IP_3) and diacylglycerol (DAG), respectively. The resultant depolarization activates voltage-sensitive Ca^{2+} channels causing an influx of Ca^{2+}. Calcium entry has been shown to be causally related to delayed neuronal death in CA1 of the hippocampus (Siesjo & Bengtsson, 1989). Increased concentrations of free cytosolic Ca^{2+} activate degradative processes which attack membrane lipids and proteins, nucleic acids and a host of other second messengers that initiate further detrimental effects on cell survival.

During ischemia the activation of second messengers such as DAG, which mobilizes protein kinase C, prevents normal protein functioning and

synthesis. Upon reperfusion there is a persistence in suppression of protein synthesis in CA1 whereas sectors CA3 and the dentate gyrus recover (Bodsch, Takahashi, Barbier, Grosse Ophoff & Hossman, 1985; Araki, Kato, Inoue and Kogure, 1990). In addition, proteases attack and digest critical enzymes required for metabolism. With the restoration of oxygen supply, through hypoxic mitochondria, there is a production of oxygen free radicals. The generation of toxic oxygen metabolites can overwhelm the endogenous antioxidant defense mechanisms and lead to tissue injury. The role of free radicals in cerebral ischemia is extensively reviewed elsewhere (Kirsch, Helfaer, Lange and Traystman, 1992; Siesjo, 1993).

Neuronal survival is dependent on reversing or halting the degradative processes triggered by the ischemic event. Shortly after reperfusion there is a relatively quick recovery of energy supply, intracellular pH and ion homeostasis. Depending on the severity of the insult many of the degradative processes (free radical formation, increased Ca^{2+} concentration, activated proteolytic enzymes and endonucleases) may continue post reperfusion. If protein synthesis, under these conditions, is not restored injured cells cannot repair themselves and resume normal function (Thilman, Xie, Kleihues and Kiessling, 1986; Araki, Kato, Inoue and Kogure, 1990). Many studies have and are currently aimed at further exploring what role each of these respective factors play in neuronal cell death.

1.6 Ischemic Tolerance

The molecular mechanisms of ischemia discussed thus far have come from studies employing single ischemic episodes. The selective vulnerability of hippocampal CA1 neurons suggests that certain nerve cell types respond to and resist ischemic stress, such as the CA3 pyramidal and dentate granule cells. Several studies have shown induced tolerance to subsequent stress in cell types other than CA1 (Gerner & Schneider, 1974; Li & Werb, 1982; Li, Meyer, Mak & Hahn, 1983).

Kitagawa et al. (1990b) investigated the possibility that hippocampal CA1 cells acquire tolerance to subsequent lethal (death of CA1 neurons) ischemic durations by prior exposure to mild ischemic stress. They used the gerbil model of global ischemia which enables easy induction of repeated ischemic episodes. Gerbils were subjected to either 1 or 2 minutes of ischemia at a 12 or 24 hour interval prior to a 5 minute occlusion two days later. The paradigm that afforded the greatest neuroprotective effect was two 2-min episodes separated by 24 hours followed 2 days later by 5 min of ischemia. One minute of preconditioning ischemia was unable to provide any histological protection against a subsequent 5 minute occlusion nor sufficient to perturb cellular metabolism. Two minutes of ischemia in this model is sufficient to transiently alter metabolism, deplete high-energy phosphates and perturb protein synthesis (Nowak, Fried, Lust & Passonneau, 1985). These results suggested that neurons possess an endogenous protective tolerance to ischemia if the

threshold for the induction of this "protectant" is exceeded (≅ 2). This was the first systematic study describing the parameters to obtain histological neuroprotection of CA1 pyramidal cells against ischemic insults by prior ischemic stress.

A second study (Kato, Liu, Araki & Kogure, 1991) further elucidated the parameters of the protective effects of sublethal (causing no discernable damage to CA1 neurons) ischemia. By varying the interval between preconditioning ischemia and the lethal insult it was determined that a 2 minute occlusion in the gerbil given at *short* intervals (up to 6 hours apart) would potentiate damage, whereas if the lethal duration ischemia was administered up to 7 days later, protection would be observed. The cumulative damage of repeated insults at short intervals has been well documented (Tomida, Nowak, Vass, Lohr & Klatzo, 1987; Kato, Kogure & Nakano, 1989; Araki, Kato & Kogure, 1990; Kato and Kogure, 1990 & Yamamoto, Takahashi, Ohyama, Yamaguchi, Saitoh, Yatsugi & Kogure, 1993). Kato et al. (1991) found a maximal protective effect if the lethal insult was delayed between 3-4 days after the initial brief preconditioning episode.

Kitagawa, Matsumoto, Kuwabara, Tagaya, Ohtsuki, Hata, Ueda, Handa, Kimura and Kamada (1991a) investigated whether or not ischemic tolerance was limited to protection against delayed neuronal death in CA1 neurons or was present in other regions as well. In addition, the synthesis of heat shock proteins (HSPs) was investigated for a possible role in the production of ischemic tolerance. HSPs are a family of proteins that are expressed at

increased levels in cells subjected to a variety of environmental stresses (Lindquist, 1986) such as hyperthermia (Currie and White, 1981), trauma (Brown, Rush and Ivy, 1989) and ischemia (Nowak, 1985). Ischemic tolerance was found in widespread areas of the brain and it was also found that 2 minutes of ischemia was sufficient to induce HSPs.

Studies have shown that the survival of hippocampal CA1 neurons can be enhanced by prior exposure to elevated temperature before the induction of the final ischemia in rats (Chopp, Chen, Ho, Dereski, Brown, Hetzel and Welch, 1989) and gerbils (Kitagawa, Matsumoto, Tagaya, Kuwabara, Hata, Handa, Fukunaga, Kimura, and Kamata, 1991b). These results created further interest in the role of HSPs and the induction of ischemic tolerance (Kawagoe, Abe and Kogure, 1992; Aoki, Abe, Kawagoe, Sato, Nakamura and Kogure, 1993 and Liu, Kato, Nakata and Kogure, 1993). It has also been shown that the preconditioned hippocampus displays an accelerated induction of the HSP gene following transient ischemia (Aoki, Abe, Kawagoe, Nakamura and Kogure, 1993). This phenomenon however, cannot be explained exclusively by the induction of HSPs as Kirino, Tsujita and Tamura (1991) have shown that the temporal profile for the induction of tolerance is not the same as the temporal profile for HSP synthesis. In addition, treatment with the protein synthesis inhibitor, anisomycin, which prevents the induction of HSPs, did not inhibit the induction of ischemic tolerance (Kato, Liu, Araki and Kogure, 1992). The exact role of HSPs in ischemia and ischemic tolerance remains elusive; however,

there is little doubt that they play some role in the protection of neurons against subsequent stress.

As described previously the excitatory amino acid glutamate is believed by many to contribute to the pathogenesis of ischemic injury. It was hypothesized that the intra-ischemic release of extracellular glutamate might be blunted by pretreatment with sublethal ischemia. The concentration of extracellular glutamate in hippocampal field CA1 was measured following pretreatment with sublethal ischemia using an intracerebral microdialysis technique (Nakata, Kato, Liu and Kogure, 1992). It was found that preconditioning did not alter the amount of glutamate released. A second investigation into the role of both excitatory and inhibitory amino acids was done with a similar technique (Nakata, Kato and Kogure, 1994). As expected there was a massive release of glutamate, glycine, g-aminobutyric acid and taurine in untreated animals exposed to 3 minutes of ischemia. The levels of amino acids released following 3 minutes of ischemia in animals pretreated with 2 minutes of sublethal ischemia was not different from non preconditioned controls. Ischemic tolerance is thus not due to a reduction in ischemic severity as a result of modifying glutamate or GABA release.

In addition to the unchanged amino acid release there is a concomitant preservation of neurotransmitter receptors and calcium channel binding (Kato, Araki and Kogure, 1992). Gerbils not pretreated with 2 minutes of ischemia had the majority of their CA1 pyramidal cells destroyed and an obvious reduction in calcium channel binding following 3 minutes of ischemia. However,

preconditioned gerbils only show a transient reduction in binding during the early reperfusion period which returned to normal within 1 day. This transient downregulation could possibly play a role in the prevention of histopathological neuronal death and reductions in neurotransmitter and calcium channel binding.

Alterations in second messenger systems may also be important in producing cell death since during pathological conditions Ca^{2+} operates as a second messenger in the brain (Siesjo and Bengtsson, 1989). With preserved receptor and calcium channel binding (Kato et al., 1992) following the induction of ischemic tolerance it is possible that neuroprotection may be the result of alterations in second messenger signalling. Kato, Araki, Murase and Kogure (1992) observed no changes in second messenger function following a single 2 minute preconditioning occlusion in the gerbil. However, using a 3 minute insult as their lethal occlusion, there was a 75% reduction in IP_3 binding and 15-20% reduction in forskolin, cAMP and rolipram. In the induced tolerance animals there was a transient downregulation of the IP_3 system in CA1 while the others remained unchanged coincident with cellular protection. These experiments suggest that preservation of second messenger systems accompanied by the transient reduction in IP_3 receptors may be important for induced neuroprotection. Activation of IP_3 receptors leads to the mobilization of intracellular calcium, believed to be causally linked to ischemic cell death (Siesjo and Bengtsson, 1989).

It has been suggested that oxygen-derived free radicals play a critical role in ischemic cellular degeneration (Watson, 1993). Treating animals with free radical scavengers such as exogenous superoxide dismutase (SOD) can prevent neuronal damage caused by ischemia (Kitagawa, Matsumoto, Oda, Niinobe, Hata, Handa, Fukunaga, Isaka, Kimura, Maeda, Mikoshiba and Kamata, 1990a). The roles of cytosolic copper-zinc SOD (CuZnSOD) and mitochondrial manganese SOD (MnSOD) were investigated in the hippocampus of gerbils with induced tolerance (Kato, Kogure, Araki, Liu, Kato and Itoyama, 1995). Immunostaining of these substances never recovered in non preconditioned 3 minute ischemic animals. Ischemic tolerance caused decreases both in the CuZnSOD and MnSOD immunoreactivities in the early reperfusion period, however, there was eventual recovery. It was also determined that MnSOD staining increased after the initial sublethal insult when normally constitutive CuZnSOD levels are higher than MnSOD in the normal hippocampus. Therefore, recovery of the antioxidant system may play a potential role in the development of ischemic tolerance.

Disturbance of protein synthesis has been shown to persist in hippocampal CA1 neurons until cell death occurs (Bodsch, Takahashi, Barbier, Grosse Ophoff and Hossman, 1985). Despite the temporary restoration of energy supply, electrophysiological activity and pH, CA1 neurons maintain suppressed protein synthesis. In contrast, resistant CA3 neurons recover normal amino acid incorporation and do not undergo delayed neuronal death (Thilmann, Xie, Kleihues and Kiessling, 1986). Recovery of protein synthesis

was compared autoradiographically in gerbils with and without induced tolerance (Nakagomi, Kirino, Kanemitso, Tsujita and Tamura, 1993; Furuta, Ohta, Hatakeyama, Nakamura and Sakaki, 1993). In both the preconditioned and ischemia only groups during early reperfusion there was polyribosomal disaggregation, loss of dendritic microtubules and suppression of radiolabeled amino acid incorporation. Animals without ischemic preconditioning showed no recovery of protein synthesis while gerbils with induced tolerance remained suppressed but returned to normal by 24 hours. This evidence suggests that the recovery of protein synthesis is essential for neuronal recovery. Interestingly CA2 neurons did not regain normal protein synthesis and remained abnormal for four days until being sacrificed.

1.7 Rationale for Experiments

Understanding the mechanism(s) of ischemic preconditioning could provide insights into novel treatments for stroke patients; however, the mechanisms underlying preconditioning remain unclear. In all studies of ischemic tolerance efforts are made to maintain body temperature close to normal during ischemia. However, work completed in our laboratory (Colbourne, Nurse and Corbett, 1993) has shown dissociations between body and brain temperature. In addition, intra- and post-ischemic brain temperature, if not monitored and maintained, can drop and convey considerable

neuroprotection (Colbourne and Corbett, 1994; Nurse and Corbett, 1994). It is not known if ischemic tolerance is acting via some temperature effect. It is conceivable that sublethal preconditioning might lower the post-ischemic temperature of animals that receive subsequent lethal duration insults, thereby indirectly lessening the severity of the ischemia.

Although preconditioning hippocampal neurons may reduce ischemic injury as assessed by histological evaluation it is not known whether there is concurrent *functional protection.* All of the ischemic tolerance studies to date have relied on histology as the sole index of neuroprotective efficacy. Functional outcome has been measured in our laboratory using an open field test of exploratory behavior (Corbett, Evans,Thomas, Wang and Jonas, 1990; Wang and Corbett, 1990; Colbourne and Corbett, 1994; Nurse and Corbett, 1994). An habituation impairment, as measured by heightened locomotor activity on each test day has been shown to be a very sensitive marker of ischemic injury. In our laboratory increased activity in the open field correlates very highly with the degree of histological damage observed in CA1. Furthermore, Mileson and Schwartz (1991) found increases in locomotor activity did not correlate with striatal or cortical damage following 5 minutes of ischemia in the gerbil. They also concluded that locomotor activity in a novel environment can be used as a predictor of CA1 damage. Similar results have been reported by others (Gerhardt and Boast, 1988; Babcock, Baker and Lovec, 1993).

The issue of survival time after ischemia has been raised by recent studies in which histological analysis was performed on animals allowed to survive for weeks or months instead of the usual 4-7 days. Brief durations of (i.e. 3hrs) of post-ischemic hypothermia appear protective when assessed 3-7 days after ischemia but not when survival times are extended to 2 weeks or more (Dietrich, Busto, Alonso, Globus and Ginsberg, 1993). In our laboratory it was found that increasing the duration of post-ischemic hypothermia to 24 hours resulted in near total protection of CA1 neurons 30 days later, however there was further reduction (approximately 20%) in protection when survival was extended to 6 months. Perhaps the most dramatic loss of efficacy has been demonstrated with NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline), a glutamate AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionate) receptor antagonist, that has been reported to have potent neuroprotective properties (Buchan, Li, Cho and Pulsinelli, 1991). The protection observed with this drug is most apparent with survival times between 4-7 days. When survival time is increased to 10 days and beyond the protective effects decrease and eventually disappear (Nurse and Corbett, 1995 in press; Li and Buchan, 1995). All of the ischemic tolerance papers have used short survival times, between 4-7 days, therefore it is important to determine if ischemic preconditioning is producing permanent protection or, like NBQX, is simply delaying death of CA1 neurons.

The purpose of the present investigation is to determine, first of all, if ischemic preconditioning and the induction of tolerance is acting via a

temperature mediated process. Secondly, it is necessary to assess whether histological preservation will translate into functional protection. Lastly, we attempted to determine if there was a decline in the neuroprotection of CA1 pyramidal cells past the conventional 10 day survival time by studying animals 30 days after ischemia.

2. METHODS

2.1 Animals

A total of sixty-four adult female Mongolian gerbils [*(Meriones unguiculatus)*, High Oak Ranch Ltd., Baden, ON, Canada], aged 12-18 weeks and weighing 50-65g, were used in this study. Animals were assigned to one of two survival times, 10 or 30 days, each with five and four groups respectively. The 10 day survival animals were divided into a sham-operated (S_{10}, n=6), a 5 minute ischemic (I_{10}, n=8), a preconditioned ischemic (PI_{10}, n=11), a sham + ischemia (SI_{10}, n=2) and a preconditioned only (P_{10}, n=6) group. The 30 day survival animals comprised of sham-operated (S_{30}, n=6), a 5 minute ischemic (I_{30}, n=8), a preconditioned ischemic (PI_{30}, n=11), and a preconditioned only (P_{30}, n=6) group. When animals of 10 and 30 day survival times are combined they will be represented as follows: e.g. PI_{10} and PI_{30} = PI_{10+30}.

Animals were all housed individually in plastic cages in a climate controlled room with an ambient temperature of 22 °C. They were maintained on a 12/12 hour light/dark cycle and had access to food and water ad libitum.

All experimental procedures were approved by the Animal Care Committee of Memorial University of Newfoundland and were in conjunction with the guidelines outlined by the Canadian Council on Animal Care.

2.2 Brain Temperature Measurement

Four days before either sham or ischemia surgery, gerbils were anaesthetised by intraperitoneal (i.p.) injection of sodium pentobarbital (50 mg/kg). Atropine methyl nitrate (0.02 mg/kg) was administered subcutaneously once the animal lost consciousness. A 5 mm, 20-gauge stainless steel guide cannula was lowered to the dural surface, anterior to bregma and lateral to the midline suture, just overlying the left frontal cortex. Three plastic screws were inverted and glued to the skull using super glue (Lepage's, Brampton, ON, Canada). The cannula was secured in place with dental acrylic and the screws covered. A 27-gauge obturator (≥ 5 mm) was constructed and inserted to prevent infection and any foreign material from obstructing the cannula.

After a minimum of 48 hours post-operative recovery animals were briefly anaesthetised with 2% halothane in a mixture of 30% O_2 and 70% N_2O. An 8 mm wireless temperature probe [model XM-F8 (8 mm), Mini-Mitter Co., Sunriver, OR, USA] was inserted into the guide cannula and secured in place with tape. Animals were then placed in Plexiglas cages (measuring 15 X 21 X 18 cm) that rested on top of AM receivers (Mini-Mitter, Model RA-1010). Three hours of continuous brain temperature and activity levels were monitored to provide a baseline measure of normal ranges and means for comparison. Temperature and activity data were simultaneously collected using Dataquest III software (Data Sciences, Inc., St. Paul, MN, USA)..

2.3 Cerebral Ischemia

Forty-eight hours after monitoring normal brain temperature animals were anaesthetised again, with 2%Halothane in 30% O_2 and 70% N_2O and maintained throughout the surgical procedure with 1.5% Halothane. The anaesthetic mixture was administered through a modified nosecone mask equipped with a Fluovac Halothane Scavenger system (Stoelting Co., Chicago, IL, USA). The brain probe was inserted through a small hole in a heated water flow-through blanket (Mul-T-Pads, model TP-3E, Gaymar Industries Inc., Orchard Park, NY, USA) which was wrapped around the animal's head leaving the thoracic area exposed. A rectal probe was inserted to measure and regulate core body temperature via a homeothermic heating blanket wrapped around the animal's body (Harvard Apparatus, South Natick, MA, USA). The surgery was performed under a fiber optic cold light source in a room with an ambient temperature of 22°C. This set-up was designed to facilitate the control of brain (36.5 °C) and body (37-38 °C) temperature throughout the duration of the surgery and the induction of ischemia.

An anterior midline cervical incision roughly 1.5-2 cm in length was made. Both carotid arteries were carefully isolated from surrounding connective tissue and vagus nerves using small forceps (Fine Science Tools, Inc., North Vancouver, BC, Canada). A silk vascular suture was looped under each artery which enabled them to be gently lifted in order to apply the micro-aneurysm clips (Fine Science Tools, Inc., North Vancouver, BC, Canada) for occlusion.

Visual inspection of the arteries immediately following the application of the clips was done to ensure complete cessation of blood flow. When the clips were removed visual confirmation of reperfusion was noted. Following inspection, the wound was sutured and anaesthesia was discontinued. The length of the surgical procedure was approximately 15-20 minutes.

Animals were then placed in Plexiglas cages resting on top of the AM receivers. Temperature and activity data were collected for 12 and 24 hours for the preconditioning and 5 min ischemic insults respectively. Brain temperature in all animals, as necessary, was prevented from dropping below 36.5 °C for the first hour of the postischemic period. A 60W lamp was placed over the open cage until the animal was up and regulating its own temperature. This prevented the tendency of some animals' temperature to drop spontaneously and remain low for several hours after the occlusion.

For the preconditioned ischemia groups (PI_{10+30}), animals received two 1.5 minute duration occlusions separated by 24 hours of reperfusion, followed 72 hours later by a 5 minute occlusion. The preconditioned only groups (P_{10+30}) were subjected to the two 1.5 min occlusions at a 24 hour interval but did not receive the 5 min occlusion. The 5 min ischemia groups (I_{10+30}) did not receive the two preconditioning episodes and the sham animals (S_{10+30}) were exposed to the surgical procedure (e.g. anesthesia, exposure of carotid arteries etc...) but did not have their arteries occluded.

2.4 Behavioral Testing

All animals were tested in a novel open field with dimensions of 72 x 76 x 57 cm, on days 3,7,10 and 30 after the last ischemic episode/ sham procedure. The floor was subdivided into 25 equal squares (12 x 12 cm) and was illuminated by two overhead 60 W lamps. An HVS image tracking system (HVS Systems Inc., Kingston, U.K.) was used to measure locomotor activity, the number of squares crossed and total distance travelled in each 10 min trial session. All testing was carried out in an insulated sound proof room in which all external cues remained constant for all test days.

2.5 Histological Assessment

Ten or 30 days after the final ischemia animals were deeply anaesthetised with an i.p. injection of sodium pentobarbital (65-70 mg/kg) and perfused transcardially with 20 ml of 0.9% heperanized saline followed by 50 ml of 10% buffered formalin. Animals were decapitated and the heads stored in 10% buffered formalin overnight. The brains were removed from the skulls 12-24 hours later and left in the same fixative before being dehydrated in a graded series of ethanols and embedded in paraffin. Six μm coronal sections were cut through the rostral-caudal extent of the hippocampus, air-dried overnight and stained with hematoxylin-eosin (HE).

Viable looking neurons, those that had a well-defined nucleus and nucleolus, were counted in a grid placed in the medial, middle and lateral sectors of the CA1 pyramidal cell layer. Cell counts were done at a level -1.7 mm relative to bregma according to the atlas of Loskota, Lomax and Verity (1974). Right and left hemisphere counts were added together for analysis. Cell counts were performed blind to the operation procedure.

2.6 Statistical Analysis

Temperature means were plotted and where obvious differences were observed, were analyzed with individual t-tests.

For behavioral analysis both survival time (10 and 30 days) groups were combined. Open field data for days 3,7, and 10 were analyzed with a 2-factor repeated measures ANOVA. Day 30 data were analyzed with a simple ANOVA. Newman-Keuls post-hoc comparisons were performed on significant data for individual test days. Within each open field session repeated measure ANOVAs were performed on 'groups X minutes' for all possible comparisons.

CA1 cell counts were analyzed with an overall ANOVA with Newman-Keuls post hoc comparisons.

3. RESULTS

3.1 Temperature

The measurement of baseline brain temperature recorded two days prior to surgery was 36.3 °C ± 0.46 S.D. (n=64).

The first preconditioning episode (1.5 minutes in duration) caused a mean peak rise in temperature to 37.5 °C that gradually returned within the normal range by 8 hours post-occlusion (Fig. 2). When the same duration ischemia was induced 24 hours later a very similar pattern of post-ischemic hyperthermia was observed but was significantly lower from 0.5 to 8 hours ($p < 0.001$). The mean peak temperature of the second occlusion occurred at the same time but was not as high (37.2 °C). When the second temperature profile was compared to the animals that received a sham operation there were no differences up to 4.5 hours post-ischemia ($p = 0.79$); however, from 4.5 to 10 hours the preconditioned group was significantly lower ($p < 0.001$). Brief exposure to these short duration occlusions had significant effects on subsequent post-ischemic brain temperature by reducing the overall mean and duration of the mild hyperthermia.

The mean brain temperature profiles for the $I_{10\text{-}30}$, $PI_{10\text{-}30}$ and $S_{10\text{-}30}$ groups are presented in Figure 3. The two animals of the SI_{10} group were not different from the ischemic group on any of the dependent measures and were thus

Figure 2. Mean brain temperature profiles measured from Time 0 (end of occlusion) to 12 hours post-ischemia for the two preconditioning episodes of the preconditioning + ischemia (PI; n=22) group, sham (S; n=12) operated and the mean normal temperature of 36.3 °C. Data were pooled for the 10 and 30 Day survival groups for temperature analysis. Temperature data were averaged every 5 minute from the end of occlusion to 12 hours post-ischemia.

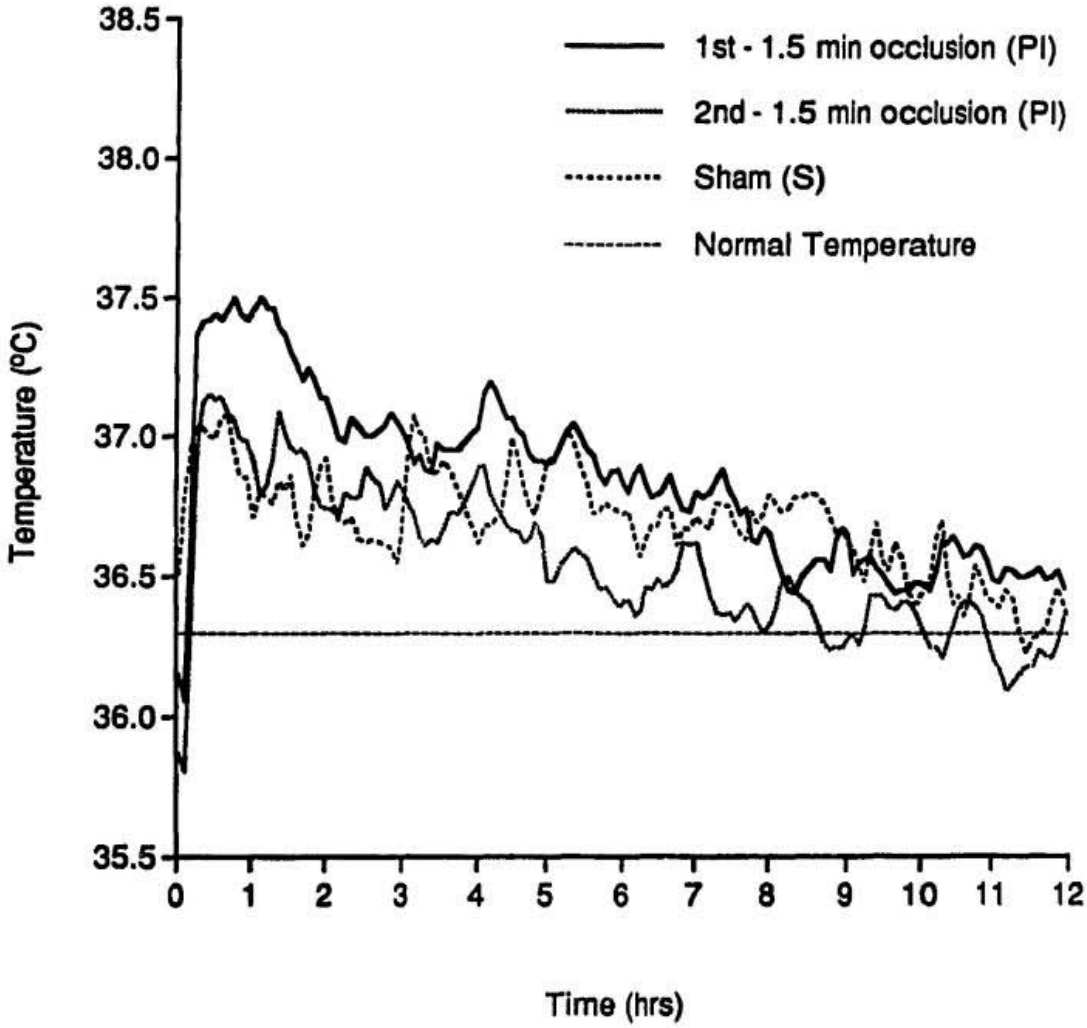
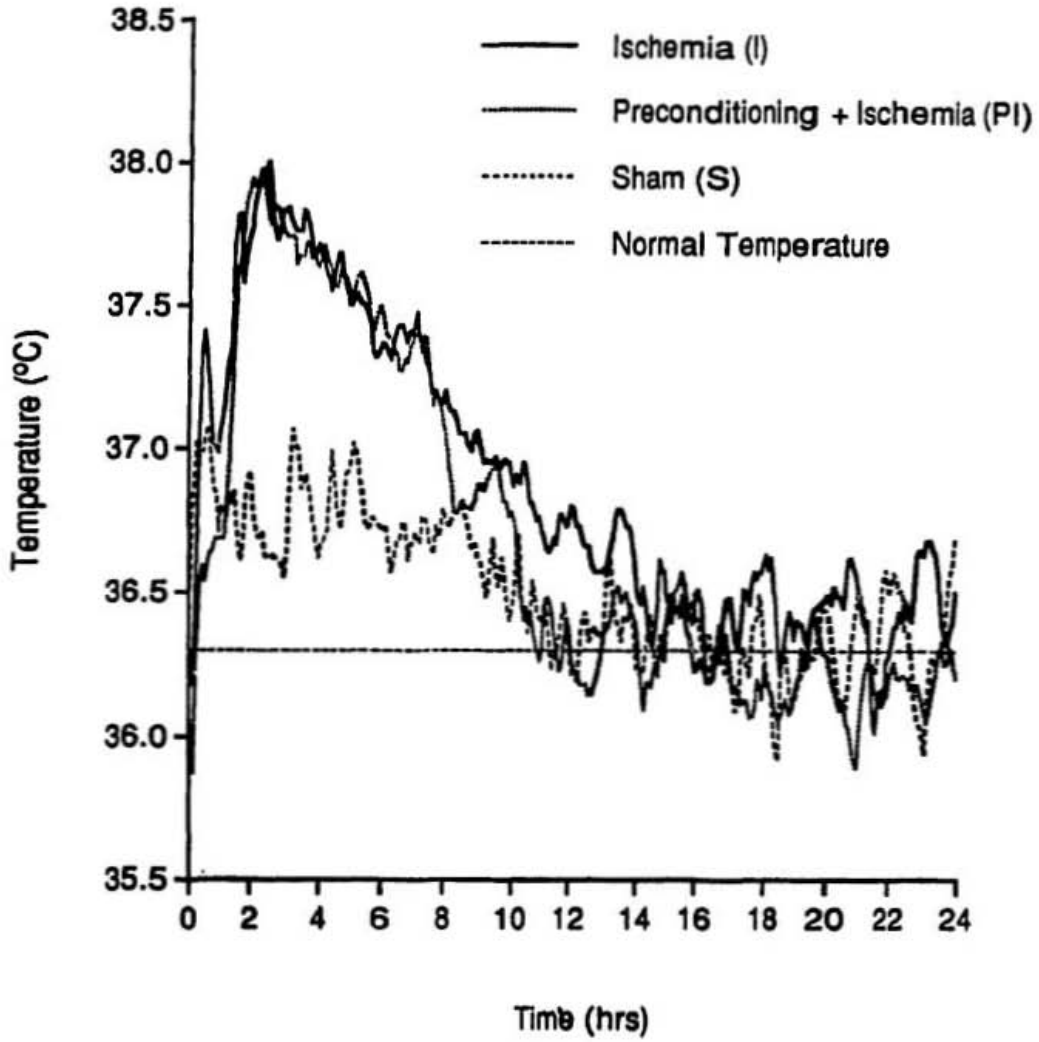

1st - 1.5 min occlusion (PI)
2nd - 1.5 min occlusion (PI)
Sham (S)
Normal Temperature
Temperature (°C)
38.5
38.0
37.5
37.0
36.5
36.0
35.5
0
1
2
3
4
5
6
7
8
9
10
11
12
Time (hrs)

Figure 3. Mean brain temperature profiles measured from Time 0 (end of occlusion to 24 hours post-ischemia for the ischemic (I; n=18), preconditioning + ischemia (PI; n=22) and the sham (S; n=12) operated groups. Data were collapsed for the 10 and 30 Day survival groups for temperature analysis. Temperature data were averaged every 5 minutes from the end of occlusion to 24 hours post-ischemia.


Ischemia (I)
Preconditioning + Ischemia (PI)
Sham (S)
Normal Temperature
Temperature (°C)
38.5
38.0
37.5
37.0
36.5
36.0
35.5
0
2
4
6
8
10
12
14
16
18
20
22
24
Time (hrs)

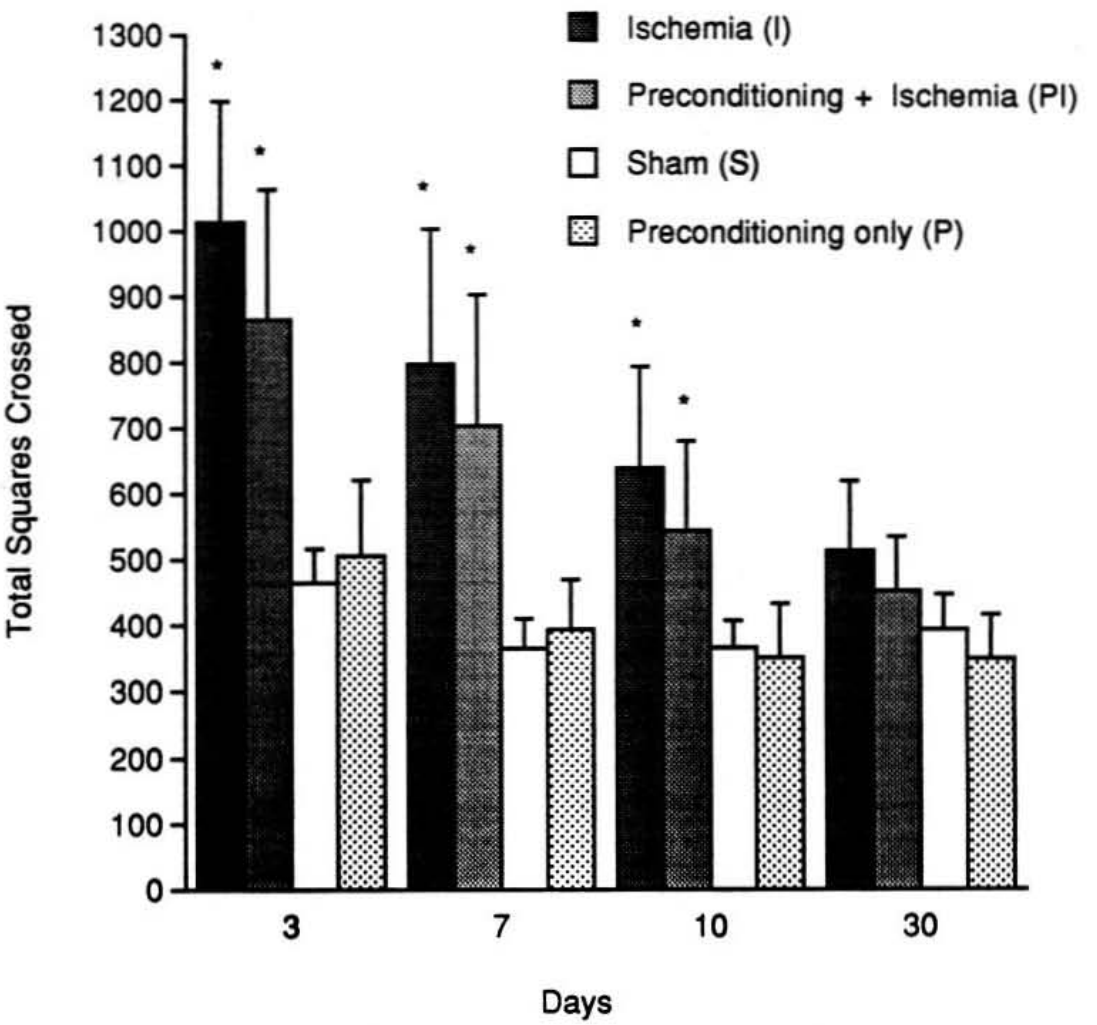
added to the I_{10} group for analysis. Both the I_{10+30} and PI_{10+30} groups had a mean peak of 38 °C at 2.3 hours after occlusion. Their respective profiles remained the same ($p = 0.55$) for the first 8 hours. The group with prior exposure to two preconditioning episodes was significantly lower than the I_{10+30} group from 8-14 hours post-occlusion ($p < 0.001$). Both the S_{10+30} and PI_{10+30} groups returned to their baseline temperature at 11 hours while the I_{10+30} animals remained elevated for 14 hours. Ischemic preconditioning reduced the overall duration of hyperthermia but had no effect on the degree of hyperthermia.

3.2 Behavior

Animals that received the sham operation (S_{10+30}) or the preconditioning only (P_{10+30}) displayed a moderate level of activity on their first exposure to the open field (Fig. 4). This declined slightly by the second exposure on day 7 and did not vary from day 10 to 30. Gerbils in the I_{10+30} and PI_{10+30} groups displayed heightened levels of activity. This resulted in a significant treatment effect (group main effect, $F_{(3,80)} = 38.7$, $p < 0.001$). Habituation over days occurred (day main effect, $F_{(2,80)} = 105.6$, $p < 0.001$), with the degree of habituation differing among groups (significant interaction, $F_{(6,80)} = 6.1$, $p < 0.001$).

Individual comparisons among treatment means revealed no significant differences between the S_{10+30} and P_{10+30} across all test days (Newman-Keuls, $p > 0.05$). The I_{10+30} and PI_{10+30} groups were not different on all test days (Newman-

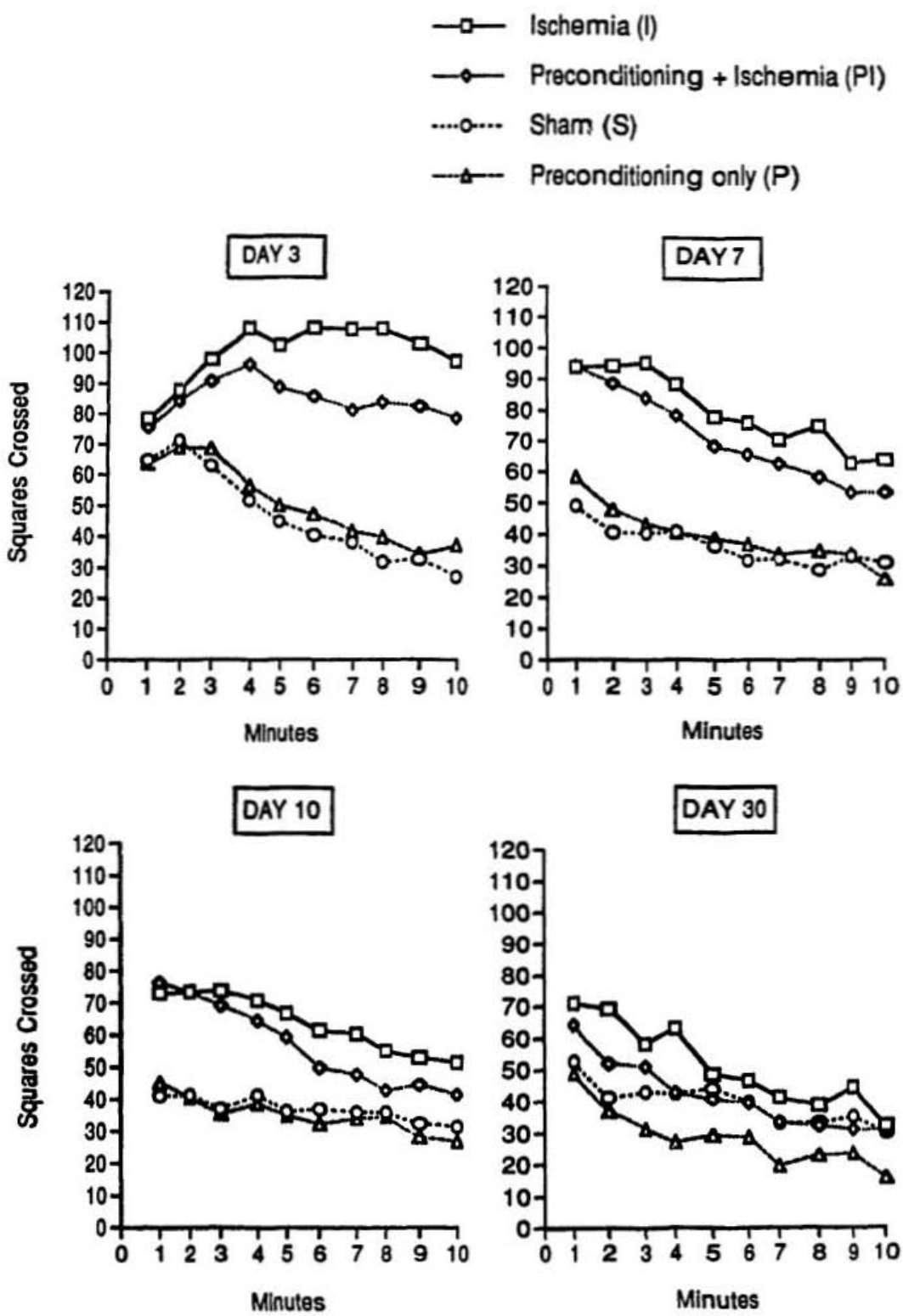
Figure 4. Open field activity scores (mean ± S.D) for the ischemia (I), preconditioning + ischemia (PI), sham (S) and preconditioning only (P) groups on post-ischemic Days 3,7,10 and 30. Ten and 30 day survival animals with the same experimental treatment were not significantly different and were thus added together for analysis. The number of animals in each group on test Days 3,7 and 10 for the I, PI, S and P were 18, 22, 12 and 12 respectively. The number of animals on Day 30 were 8, 11, 6 and 6 for the I, PI, S and P groups respectively. (* denotes significant difference at $p < 0.01$)


Ischemia (I)
Preconditioning + Ischemia (PI)
Sham (S)
Preconditioning only (P)
Total Squares Crossed
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
3
7
10
30
Days

Keuls, $p > 0.05$). Their mean scores were however significantly higher when compared to the S_{10+30} and P_{10+30} groups on test days 3,7 and 10 (Newman-Keuls, $p < 0.01$). By Day 30 the trend was still apparent; however, with smaller numbers the only significant difference remaining (Newman-Keuls, $p < 0.01$) was between the I_{30} and P_{30} groups.

Figure 5 shows the minute by minute mean activity levels for each group on all four test days. The S_{10+30} animals and the P_{10+30} animals display the same pattern of habituation within each 10 minute trial for all test days (Day 3, $F_{(1,22)} = 1.35$, $p = 0.26$; Day 7, $F_{(1,22)} = 1.29$, $p = 0.29$; Day 10, $F_{(1,22)} = 0.49$, $p = 0.49$; Day 30, $F_{(1,10)} = 3.60$, $p = 0.09$). The total number of squares crossed per minute for the S_{10+30} and P_{10+30} groups diminished over time on their first exposure (Day 3; Fig. 5) to the open field. The PI_{10+30} and I_{10+30} groups did not display similar reductions in activity. Habituation is defined as a reduced response to a stimulus over time, the PI_{10+30} animals began at an average of 75.6 squares crossed in the first minute and finished that same test session at 78.3 in the tenth minute, indicating that no habituation occurred. When PI_{10+30} animals were compared across all minutes to the I_{10+30} group a significant difference was found on test Day 3 ($F_{(1,38)} = 6.48$, $p = 0.015$); I_{10+30} animals had greater heightened levels of activity. The PI_{10+30} gerbils did not reach similar levels of habituation as the S_{10+30} on the first three test days (Day 3, $F_{(1,32)} = 37.70$, $p < 0.001$; Day 7, $F_{(1,32)} = 32.44$, $p < 0.001$; Day 10, $F_{(1,32)} = 16.42$, $p < 0.001$), this is apparent by comparing the absolute activity levels obtained at the end of each trial (i.e. the tenth

Figure 5. Mean minute by minute analysis of open field activity for all four post-ischemic Test Days (3,7,10 and 30) for the ischemia (I), preconditioning + ischemia (PI), sham (S) and preconditioning only (P) groups.


Ischemia (I)
Preconditioning + Ischemia (PI)
Sham (S)
Preconditioning only (P)
DAY 3
DAY 7
DAY 10
DAY 30
Squares Crossed
Minutes

minute). By Day 30 the differences among groups had diminished with repeated exposure to the open field.

3.3 Histology

The mean normal CA1 cell count for the S_{10} and S_{30} was 247± 41.7 S.D. and 258 ± 15.1 S.D. respectively (Fig. 6). Animals subjected to the preconditioning only did not differ significantly from the S_{10+30} at either 10 or 30 day survival (Newman-Keuls, $p > 0.05$). Five minutes of ischemia (I) produced near maximal CA1 injury (≅ 93 % cell loss) at 10 days with no significant further loss at 30 day survival. Cell counts in the PI_{10} group were not significantly different from the S_{10+30} with a mean savings of 81%. However, post hoc comparisons revealed a significant loss of CA1 pyramidal cells in the PI_{30} group compared to PI_{10} (Newman-Keuls, $p < 0.05$).The 81% protection observed at 10 days declined to 53% by day 30. This drop in CA1 cell count was significant when compared to S_{10+30} (Newman-Keuls, $p < 0.01$) but remained significantly better than untreated ischemic (I_{10+30}) animals (Newman-Keuls, $p < 0.01$). Representative photomicrographs are presented in Figure 7.

A consistent finding among all animals of the PI_{10} group was the presence of acidophillic neurons in area CA2, indicating that these cells were compromised beyond recovery. Ischemic tolerance was observed in CA1 but not in CA2 (Fig. 7 & 8). Bilateral lesions of CA2 neurons were also observed in

Figure 6. Hippocampal CA1 histology- Animals were sacrificed at 10 and 30 Days following the last ischemic episode, stained with H&E and CA1 pyramidal cell counts were done at a level -1.7mm to bregma. Each symbol represents one gerbil in each of the respective groups. Crosses represent group means.

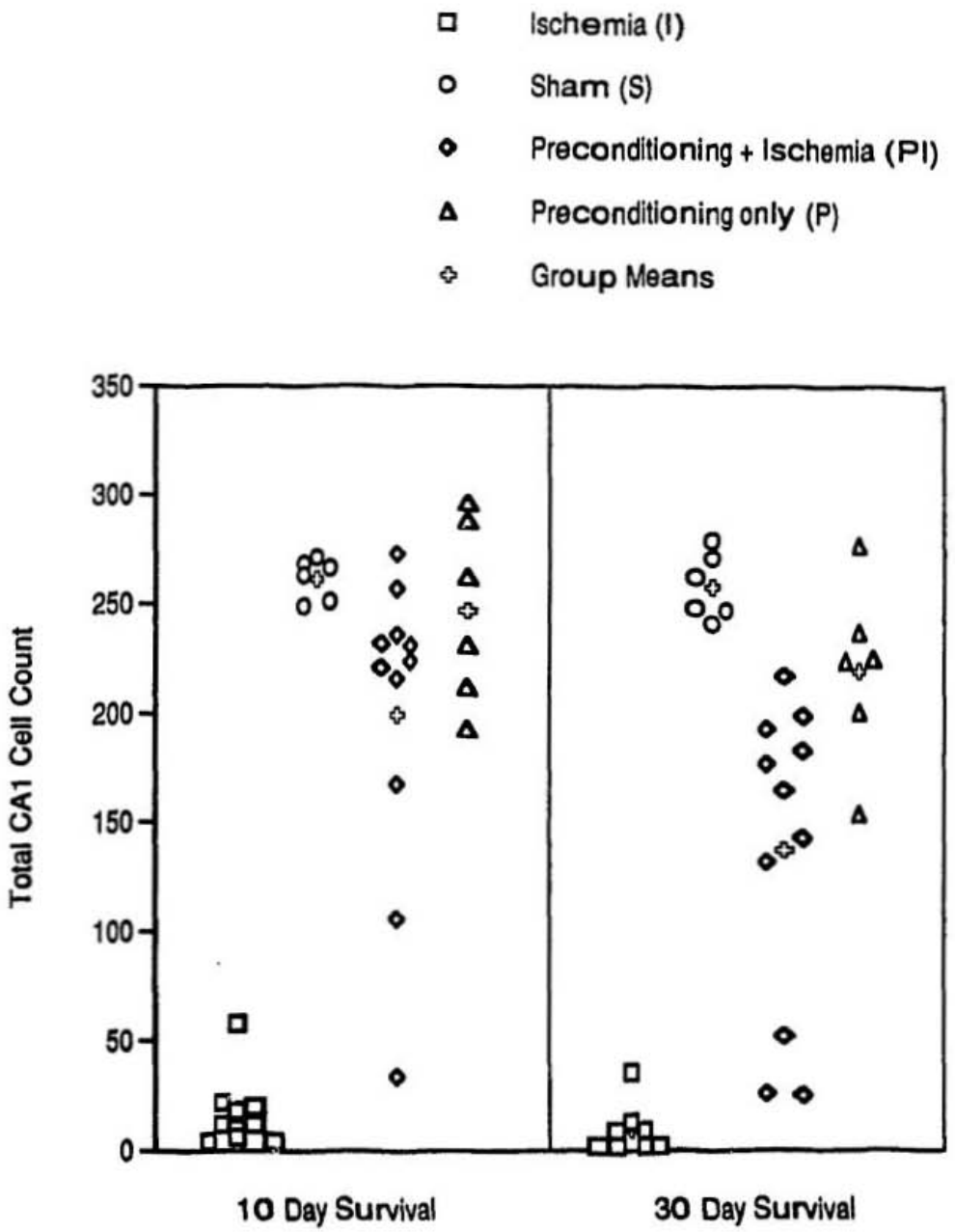
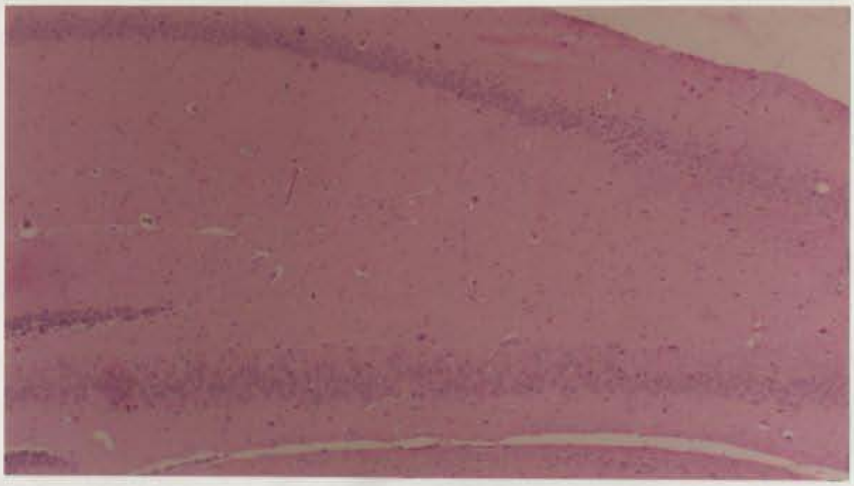
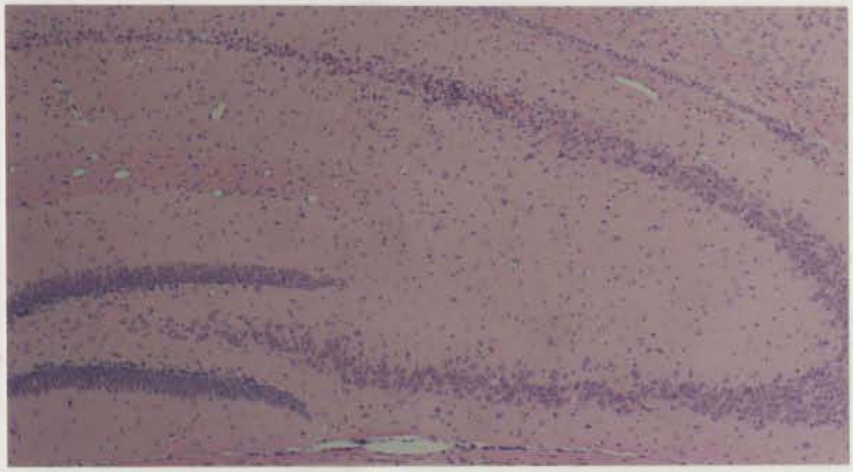

Ischemia (I)
Sham (S)
Preconditioning + Ischemia (PI)
Preconditioning only (P)
Group Means
Total CA1 Cell Count
350
300
250
200
150
100
50
0
10 Day Survival
30 Day Survival

Figure 7. Representative photomicrographs of coronal hippocampal sections taken from normal (S), ischemic (I) and preconditioning + ischemic (PI) animals at 10 Day survival (magnification x10).

Normal (S)



5 min Ischemia (I)



Preconditioning +
5 min Ischemia (PI)

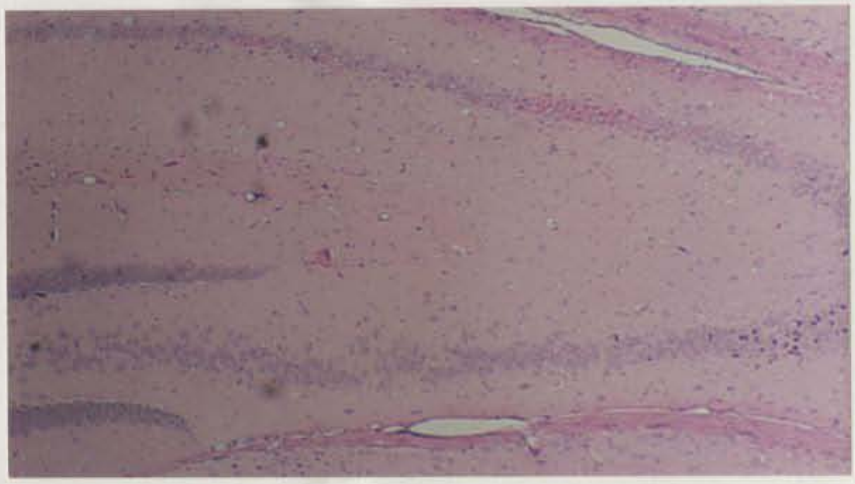
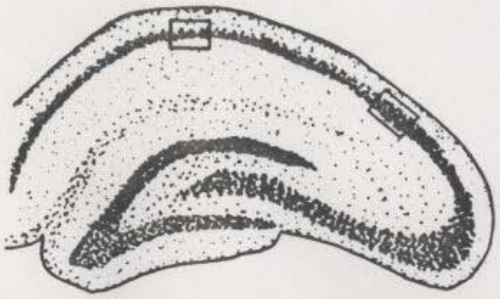
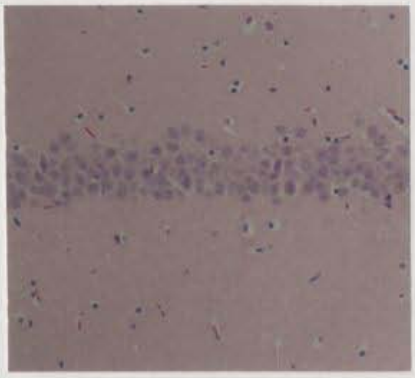


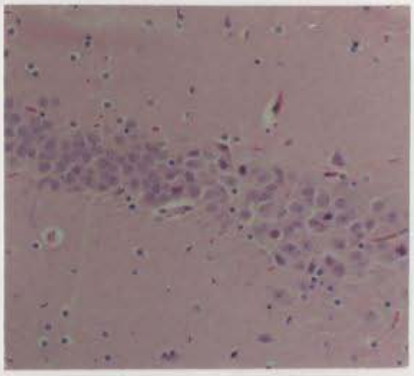
Figure 8. Representative photomicrographs of hippocampal CA1 and CA2 sectors taken from normal (S), ischemic (I) and preconditioning + ischemic (PI) animals at 10 Day survival (magnification x 20).



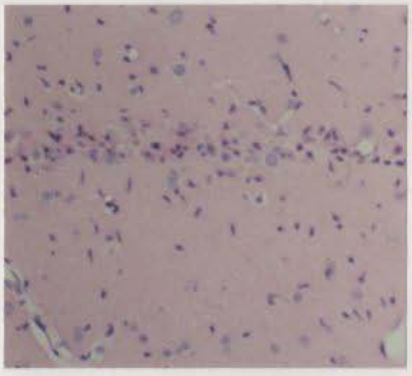
CA1 CA2

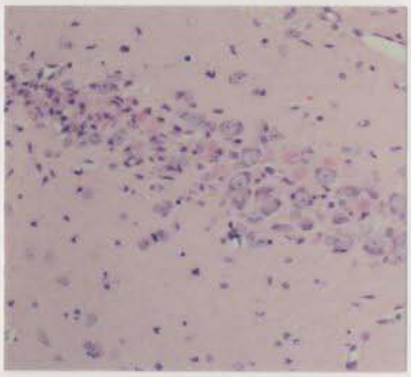
Normal (S)



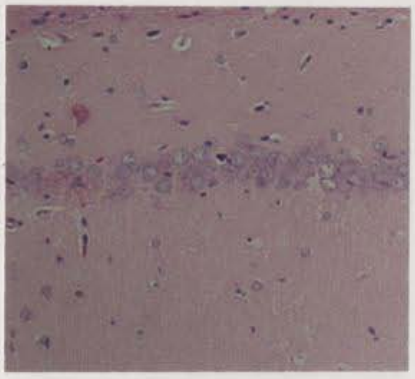


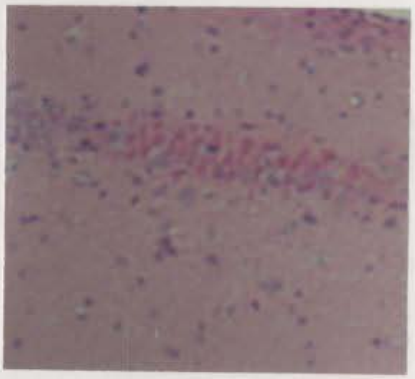
5 min
Ischemia (I)





Preconditioning +
5 min Ischemia (PI)





10/11 PI_{30} animals. The one animal that did not display CA2 damage showed no discernable necrosis anywhere in the hippocampus. CA2 necrosis was more evident but not necessarily greater in the PI_{10+30} animals than the I_{10+30} groups as the preservation of CA1 in the preconditioned ischemic animals provided a medial border not otherwise present in the I_{10+30} animals in which the entire CA1 cell band is absent. Quantification of the damage was not possible due to the difficulty in distinguishing the similar sized CA2 neurons from CA3 neurons in the normal or damaged hippocampus.

4. DISCUSSION

This study has clearly demonstrated that preconditioning episodes of short duration ischemia can provide significant histological protection of CA1 pyramidal cells against a subsequent lethal duration occlusion. However, the most surprising and perhaps the most important finding of this investigation was the dissociation between histological protection and behavioral protection.

Upon initial exposure to the open field, sham animals displayed a moderate level of activity which declined slightly by the second test day and remained at this level for subsequent test days. The lack of difference in open field behavior between the preconditioned and sham gerbils indicates that two 1.5 minute occlusions separated by 24 hours does not have any effect on behavioral outcome. Therefore, the marked habituation impairment of the preconditioned ischemic gerbils results from effects of the lethal occlusion. Ischemic preconditioning must be activating some cellular mechanisms which provide neuroprotection against subsequent lethal insults that by themselves do not affect the functional integrity of hippocampal CA1 neurons. However, the induction of ischemic tolerance followed by the lethal occlusion, while preserving the CA1 neurons morphologically, may not be maintaining functional processes. This is strongly suggested by the persistent elevation in activity levels of the preconditioned ischemic animals across the first three test days following the final occlusion (Fig. 4).

Based on these initial behavioral test results (10 day survival) it was predicted that this paradigm had not provided histological protection. At this point preconditioned ischemic and untreated ischemic animals had similar temperature profiles and similar behavioral deficits. The discovery of robust CA1 protection (81%) 10 days after occlusion was not expected. This was the first time in our laboratory that robust CA1 protection was obtained in the absence of comparable functional protection. The question that immediately arose was whether these cells were compromised beyond repair and destined to die over an extended period (> 10 days) or were simply in a state of "transition" or quiescence until eventual functional recovery.

Neurological dysfunction following various types of central nervous system trauma is usually associated with concurrent and irreversible structural damage. However, several studies have now shown learning and memory deficits in the absence of overt histological damage. Prolonged memory impairments in a rat model of traumatic brain injury have been reported in the absence of hippocampal cell death (Lyeth, Jenkins, Hamm, Dixon, Phillips, Clifton, Young and Hayes, 1990). Persistent memory deficits in rats trained on a radial arm maze task, which has been shown to be a specific marker of hippocampal damage, were observed with no indication of CA1 cell loss or injury to axonal pathways in the hippocampus proper. Similar results have been reported in rats subjected to cerebrovascular insufficiency (de la Torre, Fortin, Park, Butler, Kozlowski, Pappas, Socarraz, Saunders and Richard, 1992) and in rats following transient occlusion of the common carotid arteries (Jaspers,

Block, Heim and Sontag, 1990). Both models create a period of decreased cerebral blood flow that, upon histological analysis, does not produce discernable necrosis in sector CA1. However, animals in both studies reveal spatial memory acquisition deficits as measured by latencies in finding a hidden platform in the Morris water maze.

In light of these findings it is possible that ischemic preconditioning is producing similar results. The morphological appearance of CA1 neurons at 10 day survival is near normal. However, intrinsic functional processes of CA1 neurons may be altered and thus disrupt the overall synaptic transmission within the hippocampus and therefore interfere with learning and memory. Kudo, Tada, Takeda and Nishimura (1990) observed decreases in cytoskeletal proteins (microtubule-associated protein 2, calspectin and clathrin) and impaired learning abilities concomitant with minimal histological damage in gerbils with chronically reduced cerebral blood flow. MAP-2 and the possibility of other vital structural proteins and elements may be compromised with the preconditioning paradigm which could be responsible for the abnormal functioning of hippocampal neurons.

Disturbances in the acquisition of operant behavior have been shown in gerbils subjected to permanent right carotid artery ligation (Bothe, Bosma, Hofer, Hossman and Angermeier, 1986). Gerbils sacrificed 8 days after vascular ligation revealed no change in the number of hippocampal neurons however operant learning behavior was significantly impaired. A second group of gerbils sacrificed 8 weeks later displayed a significant loss of both CA1 and CA2

neurons. They concluded that with appropriate behavioral testing it is possible to detect subtle functional deficits before the manifestation of overt selective injury to the hippocampus. These results coincide with the observed degree of protection at 10 day survival (81%) versus that of the 30 day survival (53%) in preconditioned ischemic animals in this study.

The decline in histological protection between 10 and 30 day survival groups was significant ($p < 0.05$). This emphasizes the importance of late in addition to early histological analysis in the evaluation of neuroprotective paradigms. In the search for pharmacological treatments for experimental stroke it has become apparent that some and perhaps many putative beneficial drugs are not providing long term neuroprotection. Li and Buchan (1995) have shown that the administration of the AMPA receptor antagonist NBQX or the N-channel, Ca^{2+} antagonist, SNX-111, which provide highly significant protection at 7 days, completely lose efficacy at 1 month survival times. Similarly in a rat model of focal ischemia the non-competitive NMDA receptor blocker, MK-801, simply postponed the evolution of infarct when assessed 3 and 28 days after surgery (Valtysson, Hillered, Andine, Hagberg and Persson, 1994). Recently in our laboratory Nurse and Corbett (1995, in press) have shown a significant decline in histological preservation of CA1 cells from 4 to 10 days in gerbils treated with NBQX. In addition, it was shown that the degree of protection was related to the mild hypothermia produced by the drug. This further emphasizes the need to monitor brain temperature both during and after ischemia. Colbourne and Corbett (1994), using mild post-ischemic hypothermia (32 °C for 12 hours), have

shown a significant decline in CA1 neuroprotection from 10 to 30 days. Delayed neuronal death of CA1 neurons in untreated ischemic animals progresses over several days and is virtually complete by 3-4 days (Kirino, 1982). The preconditioning paradigm may be providing transient protection whereby the death of CA1 pyramidal cells has been slowed down. The behavioral deficit observed on the first test session 3 days after the lethal occlusion in preconditioned gerbils may in fact be an early marker of the eventual fate of CA1 pyramidal cells. The addition of an evaluation at > 30 days would provide further insight into the permanence of the neuroprotective effect of ischemic preconditioning.

Alternatively, the number of remaining neurons at 30 day survival may be an accurate reflection of the true neuroprotective potential of ischemic tolerance. Electrophysiological research in our laboratory suggests that functional protection has been achieved. In the gerbil hippocampal slice preparation, CA1 dendritic field excitatory postsynaptic potentials (fEPSPs) were recorded in preconditioned ischemic gerbils 4 days after lethal occlusion. The amplitude of the fEPSPs was approximately 50% of those recorded from sham animals and was roughly 80% larger than untreated ischemic animals. Histological analysis at 4 day survival revealed almost complete (90%) protection of CA1 neurons (Corbett, Crooks, Dooley, Evans and Nurse, 1995). This dissociation between "apparent" histological protection and CA1 function (assessed by fEPSP recordings) is consistent with the poor behavioral outcome. It is interesting to note that the EPSPs' amplitude recorded at 3 days

post-occlusion correlates very highly with the degree of histological protection in the present study at 30 days.

There appears to be some recovery of the CA1 dendritic field EPSP with increased survival time. Preliminary results at 10 days show that the fEPSP has grown to 60% of sham animals and reached 70% by 30 days. There is also some evidence to suggest that remaining neurons may be compensating by becoming hyperexcitable. There is a decrease in latency between the stimulus and the beginning of the slope of the EPSP indicating that neurons have decreased their threshold for excitation. These findings suggest the return of function with increased survival time. A study in which behavioral testing was delayed beyond 30 days might not reveal any behavioral deficits if the remaining CA1 neurons are beginning to resume normal function. It is necessary to determine the exact nature of the depressed electrophysiological activity of tolerance-induced neurons since this could be the mechanism by which protection is obtained. By decreasing synaptic activity in the early post-ischemic phase when increased levels of excitatory amino acids are evident might prevent the selective death of CA1 neurons. Results from whole cell recordings of ischemic hippocampal neurons lend support to this possibility. Input fiber stimulation can lead to abnormal Ca^{2+} accumulation in ischemic neurons resulting in cell death (Tsubokawa, Oguro, Robinson, Masuzawa, Kirino and Kawai, 1992). The suppression of electrophysiological activity may be reducing the amount of free Ca^{2+} inside ischemic neurons.

While ischemic preconditioning appears to convey at least some protection to CA1 neurons Kirino, Tsujita and Tamura (1991) noted that there was consistent neuronal damage in the nearby CA2 sector. The implications of CA2 damage have not been discussed nor reported in other ischemic preconditioning studies. The reason for this is likely due to the difficulty in identifying these cells which are similar in size to neighboring CA3 cells. In the present study 21/22 preconditioned animals had obvious necrotic lesions of CA2. Tolerance to lethal ischemia was not induced in these cells. Therefore, CA2 neurons and also dentate hilar neurons (Sugimoto, Shozuhara, Kogure and Onodera, 1993) do not appear to have the same capability as CA1 neurons for exhibiting ischemic tolerance.

Gerbils that received only the preconditioning treatment did not show any apparent morphological damage in CA2. It therefore seems likely that the cell death occurred as the result of the lethal occlusion. These same gerbils also did not exhibit abnormal habituation when placed in the open field. Thus, it could then be suggested that the loss of CA2 synaptic input might be responsible for the behavioral deficit present in preconditioned ischemic animals. However, comparable examples of CA2 damage accompanied by moderate protection of CA1 pyramidal cells using post-ischemic hypothermia have shown partial behavioral protection (Colbourne and Corbett, 1994, 1995). This is in direct contrast to the present finding in which apparent histological preservation of CA1 did not translate into equivalent behavioral protection.

CA2 cells differ from CA1 and CA3 neurons in the constitutive expression of certain cell-type specific markers such as basic fibroblast growth factor (Gomez-Pinilla, Lee and Cotman, 1992), substance P (Woodhams, Webb, Atkinson and Seeley, 1989), and calcium-binding proteins (Celio, 1990). These neurons also differ in their capacity to produce HSPs (Kirino et al., 1991) and recover protein synthesis and amino acid incorporation following ischemic insults (Nakagomi et al., 1993). Although the mechanism(s) of ischemic tolerance is not well known, the comparative differences of non-inducible (CA2) and inducible (CA1) cell types to tolerance within the hippocampus may provide insights into the underlying physiology of ischemic preconditioning.

This is the first systematic study that has been conducted in which efforts have been made to maintain strict control over brain and body temperature during the induction of ischemia and immediately upon reperfusion. The vast majority of ischemic tolerance studies have used 2 minute occlusions as their preconditioning episode. However, pilot work completed in preparation for this study revealed that 2 minutes of ischemia did not convey protection and in fact resulted in damage even without the administration of the lethal insult. Since we routinely control both brain and body temperatures during ischemia the CA1 damage that we obtain is usually greater and more consistent than that obtained by other investigators who only regulate body temperature. It was for this reason that 1.5 minute durations were used as sublethal preconditioning episodes as they did not cause any discernable damage to CA1. Thus the

threshold for CA1 cell death lies somewhere between 1.5-2 minutes when efforts are made to control brain and body temperature.

It was shown in this study that gerbils with initial exposure to a 1.5 minute occlusion displayed a subsequent rise in brain temperature that persisted for 8 hours post reperfusion. This initial exposure blunted the rise of brain temperature when the second occlusion was administered 24 hours later and only lasted 4.5 hours. Kitagawa et al. (1991) have shown that repetitive hyperthermic (rectal temperature of 42-42.5 °C for 15 min) pretreatments before 5 minutes of ischemia in the gerbil resulted in clear protection against neuronal death in CA1. Their results suggest the involvement of a stress-induced protection in the acquisition of tolerance. Although mild hyperthermia was observed following preconditioning episodes in our study, it was of far less magnitude than that reported by Kitagawa et al. (1991); however, it did persist for much longer and could potentially be acting via the same inducible protective mechanism.

Preconditioned animals displayed the same degree of hyperthermia when given the 5 minute occlusion as that of the untreated ischemic group. The temperature profiles were identical until 8 hours post ischemia when the temperature of the preconditioned animals began to return to normal. Although this difference is significant from 8-14 hours it is unlikely that this slight difference in temperature could account for the amount of histological protection observed in these animals. Colbourne and Corbett (1994) demonstrated a similar degree of CA1 protection in gerbils by lowering brain temperature to

32 °C one hour post ischemia and maintaining this level for 24 hours. The slight reduction in temperature for a few hours observed in the present investigation would, therefore, be unlikely to provide any neuroprotective properties in comparison to the temperature profile necessary for protection reported by Colbourne and Corbett (1994). Thus, ischemic preconditioning does not appear to be mediated by a temperature effect that lessens the severity of the lethal occlusion.

With our current state of knowledge concerning the viability of tolerance-induced neurons and in light of the poor functional protection demonstrated in the present study future research aimed at determining the nature of the "protracted" period of dysfunction is necessary. A long term survival study would determine whether the "histological" protection observed at 30 days in the present study is still present at later times (e.g. 3 months). Electrophysiological assessment at this time would also reveal whether these neurons have regained normal function, as preliminary results from 1 month survival suggest.

Although the mechanisms of this natural protective capacity of neurons to reduce ischemic damage remain elusive, they are of paramount clinical importance. The potential exists to develop pharmacological agents or procedures that mimic ischemic preconditioning that may be exploited therapeutically and utilized as a treatment for human stroke patients.

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