COMPETING PROCESSES OF CELL DEATH AND RECOVERY OF FUNCTION FOLLOWING ISCHEMIC PRECONDITIONING IN THE GERBIL

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By

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A thesis submitted to the School of Graduate Studies

in partial fulfilment of the requirements

for the degree of Master of Science

Faculty of Medicine

Memorial University of Newfoundland

September 1997

St. John's Newfoundland

Abstract

The goal of the present study was to determine the neuroprotective efficacy of ischemic preconditioning using behavioral, electrophysiological and histological endpoints at various time points up to 90 days postischemia. Gerbils were exposed to a brief, non-injurious episode of forebrain ischemia (1.5 min) on each of two consecutive days. Three days following this preconditioning procedure, the animals received a 5 min occlusion. Other animals underwent sham surgery or a 5 min occlusion without preconditioning.

Ischemic preconditioning appeared to provide striking cellular preservation at both rostral (79.7 and 66.9% of sham) and posterior levels of hippocampus (94 and 78% of sham) at 3 and 10 days survival, respectively. However, in spite of the near normal number of CA1 neurons, animals displayed impairments in an open field test of habituation as well as reduced dendritic field potentials in the CA1 area. Additionally, in ischemic animals the basal and apical dendritic regions of CA1 were nearly devoid of the cytoskeletal protein microtubule associated protein 2 (MAP2). Staining levels of MAP2 in sham and ischemic animals were

similar.

With increasing survival time, both open field performance and CA1 dendritic field potential amplitude in the ischemic preconditioined animals recovered. During this time, however, CA1 cell death continued over the 90 day survival period ($p<0.05$, vs. sham).

Ischemic preconditioning provides a significant degree of neuroprotection characterized by a complex interplay of protracted cell death and neuroplasticity (recovery of function). These competing processes are best elucidated using a combination of functional and histological endpoints as well as multiple and extended survival times (i.e. greater than 7-10 days).

ACKNOWLEDGMENTS

Research support was provided by grants awarded to Dr. Dale Corbett from the Medical Research Council of Cananda (MRC). Studentship support was provided to myself from the MRC.

I would like to thank my supervisor, Dr. Dale Corbett for his continued confidence in and support of my thesis work. Were it not for his insight and guidance much of the present work and knowledge gained would not have been possible.

Special thanks also go to past and present members of Dr. Corbett's lab including Sue Evans and Kathy McKay who provided excellent teaching and technical assistance as well as patience early in my program, Fred Colbourne and Suzanne Nurse who offered helpful insight and comments on manuscripts and research directions throughout my thesis work even after they had left the laboratory and Jennifer Dowden and Peter Crooks for support of my efforts and much assistance during preparations of manuscripts and this thesis.

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1. Introduction

Cerebral ischemia refers to a cessation of blood flow to the brain which results in a state of both glucose and oxygen deprivation. Unlike many other tissues, the brain does not possess, to any appreciable extent, the capacity to store energy. This coupled with a high metabolic demand makes the brain particularly susceptible to injury following even relatively brief ischemic episodes (Ito et al.,1975; Kirino, 1982; Kirino and Sano, 1984; Pulsinelli et al.,1982). Furthermore, such brief episodes can result in severe functional impairments depending upon size and location of the injured tissue. It is these features which have made cerebral ischemia/stroke the third leading cause of death and the leading cause of disability in Canada today. These statistics and the related monetary and human costs have resulted in a change in how treatment of acute stroke is approached. It had previously been accepted that little could be done to prevent the pathological developments following a stroke other than to make the patient as comfortable as possible. However, it is becoming increasingly evident through intensive research efforts that measures can be taken to reduce the deficits associated with stroke. Through such strategies, as well as education regarding preventative lifestyle changes, there is much reason for optimism in the prevention and treatment of

stroke.

The present thesis is focused on the phenomenon of ischemic preconditioning or ischemic tolerance which involves the induction of tolerance to a severe ischemic event by prior exposure to one or more brief, non-injurious, ischemic episodes. First discovered in brain in 1990 (Kitagawa et al.,1990), ischemic tolerance has received much attention as delineation of its mechanism of protection could potentially lead to the development of pharmacological agents capable of mimicking this endogenous neuroprotective process. At present however, such mechanisms remain elusive, as do the processes mediating ischemic cell death itself. Furthermore, assessment of the efficacy of ischemic preconditioning has typically been histological in nature (i.e. preservation of cell body integrity) with little attention paid to functional preservation. Finally, it has not been determined whether the neuroprotection afforded by the induction of tolerance to ischemia represents a permanent state or whether it merely extends the interval over which vulnerable neuronal cells die following a severe ischemic insult. Survival times used in the study of ischemic tolerance efficacy have generally been on the order of 1 to 7 days following the final severe insult. The interval over which vulnerable cells

die following ischemia however can be much longer than this short period. Such protracted cell death has been observed following administration of the AMPA antagonist NBQX as well as the Omega conotoxin SNX-111. These drugs were found to provide substantial neuroprotection at relatively short survival times (e.g. 1-7 days) but when assessed over longer postischemic intervals the level of cellular preservation declined significantly (Buchan et al.,1991a; Buchan et al.,1991b; Li and Buchan, 1993; Li and Buchan, 1995; Nurse and Corbett, 1996; Sheardown et al.,1993; Valtysson et al.,1994). Similar results of greatly delayed neuronal death have been found following administration of postischemic hypothermia which exhibited a significant decline in CA1 cell preservation from 30 days to 6 months survival (Colbourne and Corbett, 1995).

In the present thesis, techniques of behavioural assessment and electrophysiological evaluation as well as histological and immunocytochemical procedures were used at both short and long survival times to try and ascertain whether induced ischemic preconditioning is truly neuroprotective.

1.1 Functional Outcomes of Ischemia

Following a cerebral ischemic event there may appear functional abnormalities or deficits in behaviour, the severity of which depends upon duration of the insult as well as location of the ischemic area (Kirino, 1982). During focal ischemia, such as that induced by embolism, the infarcted area may be very well defined and the ensuing functional perturbations are related to those behaviours controlled by those ischemic areas (e.g. movement deficits following injury of motor cortex). In global ischemia, such as may occur during cardiac failure, the duration of the ischemic episode is crucial in determining both the nature and severity of the deficits as brain regions are differentially susceptible to ischemic injury (Kirino, 1982; Pulsinelli et al.,1982). Perhaps the most prevalent functional deficit following global cerebral ischemia in humans is memory impairment and in particular anterograde memory impairment in which the ability to form new memories is deficient while distant recollections remain largely intact. This particular deficit has been extensively studied in humans and found to be associated with injury of the hippocampus and in particular the CA1 subfield (Zola-Morgan et al.,1986). Similarly, tests

designed to assess animals' ability to form new memories (e.g. T-maze, Morris water maze and open field) have shown that selective damage of the hippocampus results in deficiencies in performance (Wang and Corbett, 1990; Corbett et al.,1992; Volpe et al.,1992).

Obviously, the parameters determining the type and severity of an ischemic event are beyond experimental control in the human situation. In the study of cerebral ischemia then the use of animals models has become crucial in attempting to discern both pathological mechanisms and potentially efficacious intervention strategies.

1.2 Gerbil Model of Global Cerebral Ischemia

In the study of cerebral ischemia, many animal models have been employed in an attempt to simulate those processes which occur in the human condition. One such model which has gained popularity is the gerbil bilateral carotid artery occlusion model of forebrain ischemia. In this model the carotid arteries are isolated through a midline incision in the neck and blood flow is temporarily halted by some occluding device for a

predetermined time. This results in a complete forebrain ischemia while allowing continual perfusion of the vegetative control centres of the brain stem via the vertebral arteries. The complete ischemia is produced as a result of the characteristic absence of a posterior communicating artery in the gerbil which results in an incomplete circle of Willis (Levine and Sohn, 1969). This feature prevents compensatory blood flow from the vertebrobasilar arteries when the carotids are occluded.

There are several limitations to this model however. Due to the gerbils small size, blood samples used to assess physiological variables (e.g. blood gas and glucose levels etc.) are difficult to obtain and therefore these animals are typically not regulated during ischemia. Also, it has been reported that gerbils are susceptible to seizure activity (Schmidt-Kastner and Freund, 1991). Such seizures could cause confounded results and complicate assessment of ischemic injury. This has not been found to be problematic in our laboratory as seizure activity is extremely rare (unpublished observations). It has previously been noted that the induction of seizure activity becomes consistent only when excessive durations of ischemia are employed (e.g. > 15 minutes) which far exceed those used in the present study (Kirino and Sano, 1984). A more extensive description

of the gerbil model of forebrain ischemia as well as other rodent models of ischemia has been given by Ginsberg and Busto (1989).

1.3 Regional Disparity in Vulnerability

As mentioned previously, the hippocampus is known to be particulary susceptible to ischemic injury. Within this structure however a nonuniform vulnerability exists such that a single ischemic episode may result in the selective injury of a particular population of cells. For example, a global ischemic insult of relatively short duration(3-5 minutes) results in the degeneration of almost all of the dorsal CA1 pyramidal neurons in gerbils while adjacent CA3 and dentate granule cells maintain normal cellular morphology (Kirino and Sano, 1984). In fact, an ischemic insult of 15 to 30 minutes in duration would be necessary to cause appreciable loss of these relatively stable populations of CA3 cells(Pulsinelli et al.,1982). The same pattern of vulnerability exists in the rat but in general longer durations of ischemia are required to induce cell loss.

Obviously, much interest has been generated regarding why such regional differences in vulnerability exist as this information may yield insight into why neuronal cells die following ischemic insult. There exists very little difference in blood supply between these regions ruling out the possibility that microcirculatory differences are responsible for the disparity (Schmidt-Kastner and Freund, 1991). Therefore there must exist some endogenous differences between these cell populations which account for the observed selective vulnerability.

1.4 Delayed Neuronal Death and the Role of Calcium

Following a global ischemic insult vulnerable cells within the hippocampus exhibit a specific time course of degeneration and death. The rate of progression toward cell death is affected not only by the particular population under investigation but also by the severity or duration of the ischemic insult with more severe insults resulting in a more rapidly occurring neuronal death (Ito et al.,1975). Typically, at 1 day postischemia, CA1 neurons exhibit a near normal appearance (Kirino,

1982) but between 2 and 4 days these cells begin to show distinct abnormalities as judged by conventional Nissl staining procedures (Kirino and Sano, 1984) and by the fourth day CA1 neurons are extensively damaged or entirely gone. Similarly, synaptic responses within the hippocampus are disrupted during and shortly after an ischemic insult but recover again only to be permanently lost as cells begin to die (Urban et al.,1989). As mentioned previously, different neuronal populations die at different times with CA2 and hilar neurons degenerating more rapidly than CA1 neurons, possibly due to differences in the neurodegenerative processes (Crain et al.,1988).

This delayed neuronal death, as it has been referred to, is also observed in the human case following a global ischemic episode such as occurs during cardiac arrest. The time course differs however with the CA1 cells of the hippocampus remaining viable (morphologically at least) for approximately 7 days postischemia (Horn and Schlote, 1992). The importance of this delayed neuronal death is that it offers a window of time during which potentially neuroprotective intervention strategies can be implemented. The discovery of this delay is largely responsible for the changing view that those who have suffered a cerebral ischemic insult are

beyond effective treatment for the prevention of structural and functional impairments. Still more optimism has been gained from studies which have shown that certain drug treatments given postischemically can further delay (but not prevent) the neuronal death of vulnerable cells such as CA1 following an ischemic insult (Buchan et al.,1991a; Buchan et al.,1991b; Li and Buchan, 1993; Sheardown et al.,1993; Valtysson et al.,1994). Such treatments are easily administered and may serve to increase the treatment window for the patient who does not have rapid access to facilities where more complicated and effective procedures can be performed.

As mentioned previously, the specific mechanisms mediating ischemic cell death have yet to be elucidated. However, a number of possible theories have been proposed including prolonged suppression of protein synthesis (Nowak et al.,1985), free radical damage of cell membranes (Watson, 1993) and perhaps the most widely accepted, glutamate excitotoxicity (Rothman and Olney, 1986). Regardless of the particular theory being subscribed to, calcium influx during the insult and altered calcium homeostasis appear to be a common denominator in ischemic cell death. It is known that in response to ischemic insult there occurs activation of various proteases, lipases, and endonucleases each of

which has a deleterious effect on both the structure and function of the cell (Choi, 1988; Siesjo and Bengtsson, 1989). The exact mechanism by which calcium causes neuronal death has not been established and it is likely that a number of calcium mediated events are ultimately responsible for ischemic cell death.

In addition to the adverse effects of elevated intracellular calcium levels within the cell body, calcium influx into dendrites can result in structural changes which lead to abnormal dendritic function. Following overstimulation of NMDA receptors, such as occurs during global ischemia, calcium influx into dendrites has been shown to result in beading of the dendrites and their spines (Matesic and Lin, 1994). If the insult is of sufficient severity the degradation of the dendritic cytoskeletal protein, MAP2, may occur, resulting in the collapse of the dendritic arbour (Aoki et al.,1995; Eguchi et al.,1997). While these changes may not necessarily result in the death of the parent cell they effectively disconnect many of the synaptic connections between neurons which are essential for normal function and are widely believed to be at the level at which processes mediating memory and habituation occur (Bliss and Collingridge, 1993; Edwards, 1995). In the vulnerable hippocampal area for example,

interruption of these connections may result in impairment of memory related behaviours. Thus we see that regardless of the exact mechanisms mediating delayed neuronal death intracellular calcium appears to be an integral component.

Each of the potential mechanisms mentioned thus far may trigger "necrotic" cell death which is a passive form of cell death characterized by the loss of cell membrane integrity and cell swelling. This has been the most accepted theory of ischemic cell death and as such most attempts at rescuing injured neurons have focused on minimizing the deleterious effects of extracellular toxins generated both during and after the ischemic event. An alternative, and increasingly popular view of postischemic cell death is apoptosis or programmed cell death. In apoptosis there is a pre-programmed death process which an injured cell may activate to cause death without cell lysis and the spilling of intracellular toxins which may result in further cell loss. Apoptosis is an accepted process in the development of an organism and necessary for normal development of many bodily systems as well as the maintenance of rejuvenating tissues (Raff et al.,1993). Recently, several studies have indicated that the defining characteristics of apoptosis (cell shrinkage and chromatin condensation) can

be observed in the mature animal following ischemia (MacManus et al.,1993; Linnik et al.,1993; Nitatori et al.,1995; Charriaut-Marlangue et al.,1996). Given the active nature of this form of cell death, therapeutic approaches for the prevention of neurodegeneration would differ markedly from those used when necrosis is suspected. Alternatively, Choi (1995) has proposed that following an ischemic event, the ensuing cell death may contain components of both necrotic and apoptotic cell death and that these two forms of cell death may occur within individual neurons . While providing an interesting alternative, this theory if valid, further complicates the development of effective therapeutic strategies.

1.5 Assessment of Neuroprotection

The ultimate goal of research in the area of cerebral ischemia is to develop pharmacological agents or procedures which effectively reduce or eliminate the deleterious processes which occur during and after a stroke that in turn produce functional deficits. This means that vulnerable neuronal populations (such as dorsal hippocampal CA1 cells) must be

preserved following the ischemic insult. However, it cannot be assumed that maintenance of gross histological integrity is necessarily indicative of functional preservation either of the particular cells themselves or of the neuronal system in which they reside (e.g. CA1 neurons of the hippocampus). Substantial evidence exists that following ischemic insult, standard histological examination of Nissl stained tissue may indicate normal cellular appearance in the presence of disturbed or abnormal functional capacity (Bothe et al.,1986; Hori and Carpenter, 1994; Ishimaru et al.,1995). Despite this evidence, and the acceptance of the importance of functional protection in the clinical setting, researchers have largely continued to use gross histological measures (e.g. counting of Nissl stained cell bodies) as the primary criteria for assessing the efficacy of various intervention strategies.

Using previously validated behavioural (Wang and Corbett, 1990) and electrophysiological measures (Nurse and Corbett, 1994) of functional competence in addition to common histological techniques, the present study extends such a multifaceted approach to the assessment of neuroprotective efficacy. This approach along with the use of multiple and extended postischemic survival times aims to provide a more accurate

assessment of the true extent of neuroprotection.

1.6 Ischemic Preconditioning/Tolerance

It has long been known that tolerance to severe ischemic insults in cardiac muscle can be induced by prior exposure to non-injurious ischemic episodes (Murray et al.,1986; Schott et al.,1990). This ischemic preconditioning or ischemic tolerance initiates some changes which makes the tissue more resistant to the stresses associated with the ischemic environment. The possibility of a similar tolerance in neural tissue and in particular the vulnerable hippocampal CA1 cells was first investigated using the gerbil model of bilateral carotid artery occlusion similar to that used in the present study (Kitagawa et al.,1990). It was discovered that a sub-lethal (i.e. not resulting in the loss of CA1 cells) ischemic insult could confer cellular protection against a subsequent, and otherwise lethal (causing CA1 cell death) 5 minute occlusion when the preconditioning insults (two 2 minute episodes separated by 24 hours) were administered 2 days prior to the severe ischemic event. Both the duration and temporal ordering of the preconditioning episodes is crucial to the induction of

tolerance since it has been shown that ischemic durations too brief to alter cellular metabolism (e.g. 1 minute) are unable to provide subsequent protection against a severe ischemic insult (Kitagawa et al.,1990). Furthermore, damage in response to the severe insult may be potentiated if the interval between insults is too brief (Kato et al.,1991). In fact, it has been shown that ischemic insults of sub-lethal durations (e.g. 2 minutes) do result in substantial neurodegeneration if administered at short intervals (e.g. 1 hour) (Tomida et al.,1987; Kato et al.,1989; Araki et al.,1990; Kato and Kogure, 1990; Yamamoto et al.,1993). It appears that optimal neuroprotection is achieved when preconditioning precedes the severe insult by 3-4 days (Kato and Kogure, 1990).

As previously stated, the significance of ischemic tolerance to stroke research lies in the elucidation of the mechanisms mediating this endogenous neuroprotectant. Such a knowledge could perhaps be used in the development of pharmacological agents which either mimic those events or otherwise activate endogenous cell preserving processes. Unlike the administration of ischemic episodes prior to a severe insult, which may or may not occur, such agents could be administered postischemically to perhaps reduce the deficits associated with severe cerebral ischemia.

Unfortunately these mechanisms, and as a result their exogenous induction, remain elusive. There have however been a number of possible mediators proposed to explain how this neuroprotection is conferred upon vulnerable cells. Perhaps the most active area in the study of potentially mediating mechanisms of the tolerance phenomenon was a study of the role played by heat shock proteins (HSP). First discovered as a family of proteins induced by hyperthermic conditions (Currie and White, 1981), HSPs have since been found to be up regulated following an ischemic insult (Nowak, 1985). The role of these proteins in ischemic tolerance was first considered when it was determined that the levels were increased following a 2 minute ischemic insult which itself was insufficient to induce any neuronal damage but was capable of conferring some cellular protection against subsequent more severe insults (Kitagawa et al.,1991a). Furthermore, exposure to elevated temperatures (known to induce HSPs) prior to a severe ischemic event resulted in a reduced degree of neuronal loss (Kitagawa et al.,1991b). Despite the body of evidence that provides support for the involvement of HSPs in ischemic tolerance, considerable evidence negating its importance also exists. Kirino et al., (1991) assessed the temporal profiles of HSP induction and the induction of tolerance to severe ischemia and found the

two to differ. Perhaps stronger evidence against HSPs involvement in ischemic tolerance comes from studies which have shown that tolerance can be induced in the absence of elevated levels of HSP. For example, protein synthesis inhibition by the drug anisomycin does not prevent the induction of ischemic tolerance (Kato et al.,1992b). More recently, it has been determined that by carefully manipulating the duration of the preconditioning episodes, tolerance can be induced with an ischemic duration that is too short to cause an increase in HSP (Abe and Nowak, 1995). To achieve such finely tuned insults EEGs were used to determine when the ischemic episode had resulted in a loss of normal cortical EEG pattern. By doing this, insults could be of just sufficient duration to induce tolerance to more severe episodes of ischemia without being long enough to cause induction of HSP. As a result of these negative studies, the emphasis on HSP as a potential mediator of ischemic tolerance has deceased considerably in recent years.

While not as popular as HSP, several other mechanisms have been proposed to play a role in ischemic tolerance including the maintenance of second messenger systems following ischemia (Kato et al.,1992a), postischemic recovery of protein synthesis (Nakagomi et al.,1993; Furata et

al.,1993) and the preservation of antioxidant systems which prevent rises in free radical concentrations following the insult (Kato et al.,1995).

More recently, considerable emphasis has been placed on the potential role of mitochondria in the induction of ischemic tolerance. Mitochondria provide the cell with the energy required to maintain normal metabolic processes which are necessary to sustain cellular viability. Recent evidence has indicated that the rise in intracellular calcium concentration following glutamate stimulation is buffered by the mitochondria which serves to prevent calcium activated cell degrading processes discussed earlier (White and Reynolds, 1995). However, if the level of glutamate stimulation becomes excessive (such as occurs during severe ischemia) then the concomitant rise in calcium exceeds the mitochondrial buffering capacity and results in the degradation of the mitochondrial membrane potential (Schnider et al.,1996). This event leaves the mitochondria non functional and the cell without its primary metabolic machinery. Ohta et al., (1995) have provided some evidence that ischemic preconditioning protects cellular integrity by enhancing the mitochondrial buffering capacity thereby increasing the level of glutamate stimulation and the resulting calcium overload which can be sustained

without resulting in failure of this organelle to function. While neither of the above mechanisms have been shown to be exclusively responsible for the phenomenon that is ischemic tolerance, it is possible that each contributes to the overall protection of vulnerable cells against an otherwise lethal ischemic event.

1.7 Rationale for Experiments

Since ischemic tolerance was first discovered in brain (Kitagawa et al.,1990) considerable effort has been expended in attempts to elucidate the mechanisms mediating this neuroprotective procedure since a working knowledge of these would be useful in the development of a potentially effective and usable intervention strategy. There are however several issues which I believe have been overlooked throughout the course of study of this phenomenon and which are the focus of the experiments of the present thesis. The determining factor in assessing efficacy of many possible neuroprotective interventions has been histological preservation of vulnerable neuronal populations such as the hippocampal CA1 cells. While

this is admittedly an important aspect, it should in no way be the sole means of evaluation. It can be argued that in the human case the ultimate goal following ischemic stroke is to preserve, or facilitate the recovery of, functional capacity. The preservation of cellular integrity in its absence would be of little benefit. Given these concerns, the present thesis has incorporated several measures to assess the degree of functional integrity following ischemia. The first of these is the open field test which assesses an animals ability to habituate to a novel environment and has previously been found effective in determining the level of hippocampal function (Wang and Corbett, 1990; Volpe et al.,1992). Unfortunately, with extended survival times, such as are used in the present study, tests such as the open field tend to loose power due to functional compensation by undamaged brain regions (Corbett et al.,1992). Electrophysiological evaluation was therefore used to more directly ascertain the level of functional competence of the hippocampal circuitry, especially at extended survival times. This functional measure was also employed because previous results from this laboratory had indicated that behavioural abnormalities in open field behaviour exist at times when histological analysis is indicative of substantial neuroprotection (Corbett and Crooks,

1997). Furthermore, a substantial amount of evidence exists which supports the possibility of physiological deficiencies in the presence of normal morphological appearance following an ischemic insult (Bothe et al.,1986; Hori and Carpenter, 1994; Ishimaru et al.,1995). The goal, therefore was to attempt to uncover the nature of the behavioural abnormalities exhibited by gerbils which displayed significant neuroprotection as a result of ischemic preconditioning

Evidence from preliminary investigations in this laboratory had indicated that indeed hippocampal electrophysiological function was significantly impaired following induced tolerance at times when histological integrity was near normal (e.g. 3 days postischemia). In an attempt to decipher the origin of this impairment immunohistochemical localization of the cytoskeletal protein MAP2 was carried out. This protein is densely localized in, among other areas, the apical dendritic region of the vulnerable CA1 cells (Caceres et al.,1984). Furthermore, it has been shown that MAP2 can serve as a sensitive indicator of neuronal injury following ischemic insult (Kitagawa et al.,1989; Aoki et al.,1995). These findings lead to the hypothesis that ischemic preconditioning, while providing striking histological protection of hippocampal CA1 cell body

integrity, does not prevent the degradation of dendritic MAP2 thereby possibly impairing normal electrophysiological function of hippocampal circuitry and behaviours mediated by it (e.g. open field performance).

As discussed previously, a number of promising neuroprotective intervention strategies have proven to be of little practical use as the cellular preservation afforded by them declines greatly as the postischemic period is increased in duration. Studies assessing the efficacy of ischemic preconditioning have typically used relatively short survival times (e.g. 4-7 days) and therefore cannot be used to state conclusively its strength as a neuroprotectant (Kirino et al.,1991; Kato et al.,1991). Furthermore, recent evidence from this laboratory has indicated that ischemic preconditioning provides substantial early cellular preservation but this is not maintained as CA1 cell counts declined significantly from 3 to 30 days (Corbett and Crooks, 1997). Since this was the longest survival time used, the possibility remained that cell loss would continue and if so ischemic preconditioning could not be regarded as truly neuroprotective. Therefore the present study incorporated still longer survival times (90 days) in an attempt to determine if this treatment merely delays cell death or if the protracted neuronal loss will cease prior to complete CA1 degeneration.

2. Methods

2.1 Animals

These experiments were carried out in accordance with the guidelines established by the Canadian Council on Animal Care and were also approved by the Memorial University of Newfoundland Animal Care Committee. Fifty-three female, Mongolian gerbils [(Meriones unguiculatus) High Oak Ranch, Baden, ON, Canada] ranging from 12 to 16 weeks old and weighing 50 to 60 gm were used in the study.

2.2 Brain Temperature Measurement

Brain temperature measurement was carried out as previously described (Colbourne and Corbett, 1994). Briefly, four days prior to surgery, gerbils were anesthetized with sodium pentobarbital (65 mg/kg)

and a 5 mm 20 gauge guide cannula was implanted overlying the dorsomedial striatum at the dural surface. Two days later, wireless temperature probes (model XM-FH, Mini-Mitter Co., Inc., Sunriver, OR) were inserted into the striatum at a level approximating the depth of the hippocampus. Animals were placed in plexiglas cages (15x21x18cm) which were positioned on AM receivers (Mini Mitter Model RA-1010) which received frequency signals from the probes relaying information about both temperature and locomotion over the receiver. This temperature and activity data was collected using Dataquest III software (Data Sciences, Inc., St. Paul, MN, USA) and monitored continuously for a 3 hr period to establish a baseline temperature profile.

2.3 Cerebral Ischemia

Anesthesia was induced using a mixture of 2% halothane in 30% oxygen and 70% nitrous oxide and animals were subsequently maintained using 1.5% halothane. Animals were positioned on their backs with probes in place and anesthetic mixture was supplied via a nose cone. Any escaping

anesthetic was retrieved by a Fluovac Halothane Scavenger system (Stoelting Co., Chicago, IL, USA). Brain temperature was measured as previously described and maintained at normothermia. Gerbils were subjected to either 5 min of cerebral ischemia (I), sham surgery (S), or ischemic preconditioning (IP). No animals were subject to preconditioning insults only as previous data from this laboratory have indicated that this procedure does not result in histological or functional deficits (Corbett and Crooks, 1997). Cerebral ischemia was induced by isolation of the common carotid arteries through a ventral midline incision in the neck followed by bilateral occlusion of the arteries using micro aneurysm clips (Fine Science Tools, Inc., North Vancouver, BC, Canada) for the specified duration. Visual Inspection confirmed cessation of blood flow and later reflow upon application and removal of the clips respectively. Sham operated animals underwent exactly the same procedure except that there was no arterial occlusion. Ischemic preconditioning consisted of two brief, non-injurious ischemic insults (1.5 min each) on consecutive days followed three days later by a 5 min occlusion. During all occlusions brain temperature was maintained at 36.0-36.5˚C by wrapping a hot water blanket (model TP-3E, Gaymar Industries Inc., Orchard Park, NY) around the head. Likewise,

rectal temperature was maintained at 37.0-38.0°C by a separate homeothermic blanket system (Harvard Apparatus, South Natick, MA). Following ischemia or sham surgery, all animals were maintained at normothermia using an overhead lamp until they were able to regulate their own temperature (15 to 30 min). Sham and I animals survived for 30 days post surgery. This single survival time was used since it has previously been shown that near complete loss of CA1 neurons (and therefore the associated CA1 dendritic field potentials) has taken place by 3 days postischemia (Kirino, 1982; Pulsinelli et al.,,1982). The 30 day survival was used such that behavioural data, for comparison purposes, could be acquired. IP animals were subdivided into groups surviving for 3 (IP 3), 10 (IP 10), 30 (IP 30) and 90 (IP 90) days after the final (5 min) carotid artery occlusion.

2.4 Behavioural Testing

On days 3, 7, 10 and 30 animals were tested for 10 min in an open field apparatus (72 x 76 x 57 cm) which was divided into 25 equal squares.

A visual tracking system (HVS Systems, Kingston, UK) recorded the number of squares entered per minute. Testing was carried out in a sound proof room in which conditions were maintained constant throughout the experiment. The open field test has been shown to be a sensitive indicator of an animal's ability to habituate to a novel environment (Boast et al.,,1988; Wang and Corbett, 1990; Babcock et al.,,1993).

2.5 Electrophysiological Assessment

Following the last day of behavioural testing, gerbils were re-anesthetized and brain temperature was reduced to 30°C with a cold water blanket. Following decapitation, brains were removed and bisected, with one hemisphere being immersed in cold (4°C) formalin for later histological analysis. The hippocampus was dissected free from the remaining hemisphere and 500 μm transverse sections were cut using a tissue chopper. Slices were placed in a modified artificial cerebrospinal fluid (ACSF) solution containing (in mmol/litre): sucrose, 215.8; KCl, 3.5; $CaCl_2$, 2.0; $NaHCO_3$, 25.0; $MgCl_2$, 1.3; glucose, 11.0; that was bubbled

with 95% O_2/5% CO_2 (pH 7.3-7.4) at room temperature for 10 min. Slices were then transferred to an ACSF solution which differed from the modified ACSF only in that the sucrose was replaced by NaCl (126.0 mmol/litre). Slices were incubated for at least 1 hr prior to experimentation.

Excitatory post-synaptic potentials (EPSPs) were recorded within stratum radiatum in a fluid interface chamber (Fine Science Tools Inc., Vancouver, BC) perfused with oxygenated ACSF at a rate of 2 ml per minute and maintained at 33 °C. Ultrasmall, concentric bipolar stimulating electrodes (100 μm, Frederick Haer Company, Brunswick, ME) were positioned for orthodromic stimulation of the Schaffer collaterals. Stimulation parameters consisted of 0.1 msec constant current pulses delivered at 0.05 Hz. Glass, micropipette recording electrodes filled with 2M NaCl and having a tip diameter of approximately 20 μm and a resistance of 1.0 to 1.5 Mohms were positioned in stratum radiatum. Responses were amplified, digitized and stored for later analysis as well as being displayed on an oscilloscope. A slice was considered viable and recordings used for analysis if a population spike could be elicited from the dentate granule cells in response to stimulation of the perforant path (0.1

msec pulses at 0.05 Hz). Dentate granule cells are not injured by the duration of ischemia employed in the present experiments.

2.6 Histology

The hemisphere that was immersion fixed in cold formalin was paraffin embedded and two series of 6 μm sections were cut. One series was stained with haematoxylin and eosin and neurons exhibiting a distinct nucleus and lack of shrinkage or eosinophilia were counted in medial, middle and lateral regions of CA1 in sections taken from two levels of hippocampus (1.7 [level A] and 2.2 mm [level B] posterior to bregma) by placing a 200 μm long grid over these regions and counting the cells within the grid (Colbourne and Corbett, 1995). Similarly, at 2.7 mm posterior to bregma (level C), cells were counted in the middle region only. The second series of sections was immunostained against MAP2 using the avidin-biotin-peroxidase complex procedure (Vectastain ABC). The specificity of this antibody has previously been established (Yoshimi et al.,1991). The relative optical density (ROD) of MAP2 staining in CA1

was quantified using NIH image analysis software. The ROD of MAP2 in the CA1 dendritic field was normalized to that in corpus callosum (CC) using the formula CA1 ROD - CC ROD/CA1 ROD. This measurement of CA1 ROD was carried out using rostral (Level A) sections only.

2.7 Statistics

Repeated measures analysis of variance was used to analyze the open field data. Based on previous results (Corbett and Crooks, 1997), specific comparisons between treatment means were carried out on individual test days using planned comparison analysis. CA1 fEPSPs and cell counts were analyzed using a simple ANOVA. Individual post-hoc comparisons were evaluated using the Newman-Keuls test.

All behavioural, electrophysiological and histological measurements were carried out in each of the animals tested. Electrophysiological and MAP2 ROD measurements were derived from subsets of these animals.

3. Results

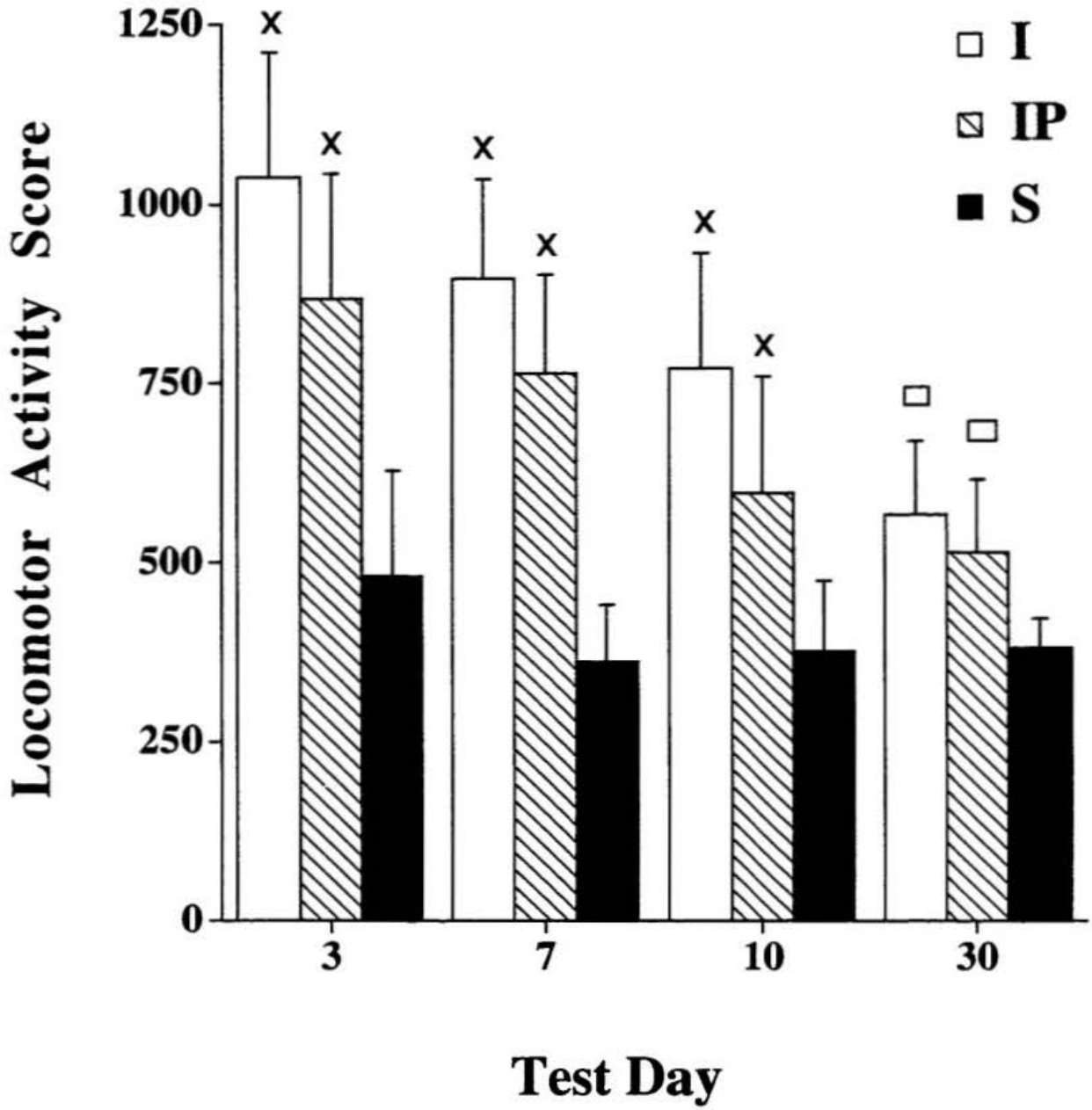
3.1 Temperature

Mean pre-ischemic brain temperature for all animals was 36.4°C during a 3 hr baseline monitoring period. Mean intraischemic brain temperature ranged from 36.2°C to 36.4°C in all groups. Postischemic brain temperature recorded over a 24 hr period following either 5 min occlusion or sham surgery did not differ between groups ($F = 1.680$, $p = 0.1623$). Previous results from this laboratory have indicated that ischemic preconditioning reduces the duration but not the degree of postischemic hyperthermia observed following a severe ischemic insult (Corbett and Crooks, 1997). No evidence of seizure activity was observed in any of the animals following ischemia.

3.2 Behaviour

Mean open field activity levels for each group on post ischemic days 3, 7, 10, and 30 are presented in Figure 1. Repeated measures analysis of

Figure 1: Total open field activity scores (mean ± SD) within each 10 min test session for the ischemia (I), ischemic preconditioned (IP) and sham (S) groups on postischemic test days 3, 7, 10 and 30. Locomotor activity scores for ischemic preconditioned animals did not differ across survival times and were thus pooled for analysis. The number of ischemic preconditioned animals on test days 3, 7, 10 and 30 were 37, 26, 25 and 18 respectively. Sham ($n = 7$) and ischemic ($n = 9$) animals were included on all test days. (x:$p < 0.01$; □:$p < 0.05$ with respect to sham operated animals).


Locomotor Activity Score
1250
1000
750
500
250
0
3
7
10
30
Test Day
I
IP
S
X

variance indicated a significant day effect ($F = 24.574$, $p < 0.01$), likely the result of habituation to the open field as indicated in Figure 1. Both I and IP animals were significantly more active than S on test days 3, 7 ($p < 0.01$) and on day 10 (except for the IP 30 group). This was also the case on day 30 ($p < 0.05$) though the differences between these groups was considerably less at this time. While obvious similarities in open field behaviour exist between the IP and I groups (slow rate of habituation), animals in the I group were significantly more active on all test days ($p < 0.05$).

3.3 Electrophysiology

Following the last day of behavioural testing (dependent on group) orthodromic field EPSPs were recorded from CA1 pyramidal cells in hippocampal slices taken from all animals. As expected, the magnitude (maximum amplitude) of the elicited responses from animals exposed to the 5 min ischemia (no preconditioning) was greatly reduced in comparison with those recorded from sham operated animals (- 1.06 vs. - 4.68 mV

respectively, $p < 0.01$). However, the maximum amplitude of the fEPSPs elicited from ischemic preconditioned animals increased as a function of survival time (Fig. 2) from -2.36mV and -3.06 mV on post-ischemic days 3 (n=6) and 10 (n=4) respectively ($p < 0.05$ versus sham) to -3.98 mV at 30 days survival (n=5) which did not differ significantly from sham. However, this recovery was only transient as responses recorded from ischemic preconditioned animals surviving for 90 days (n=9) were reduced to -3.4 mV which was significantly lower than sham responses ($p < 0.05$).

3.4 Histology

Consistent with functional endpoints, CA1 cell counts confirmed severe neuronal loss at all rostro-caudal levels in the 5 min ischemic group versus sham operated animals ($p < 0.0001$). Cell counts from ischemic preconditioned animals varied as a function of survival time and of the rostro-caudal level being assessed. At Level A (the most rostral), cell counts from ischemic preconditioned animals on day 3 were not significantly different from sham but were significantly lower ($p <0.05$) at 10 and 30 days (Fig 3)

Figure 2: Mean CA1 fEPSP amplitudes (± SD) recorded in hippocampal slices from sham (S; n =8) and ischemic without preconditioning (I; n = 9) animals at 30 days survival and ischemic preconditioned animals at 3 (IP 3; n = 6), 10 (IP 10; n = 4), 30 (IP 30; n = 5) and 90 days (IP 90; n = 9) postischemia. (x:$p < 0.01$; □:$p < 0.05$ with respect to sham operated animals).

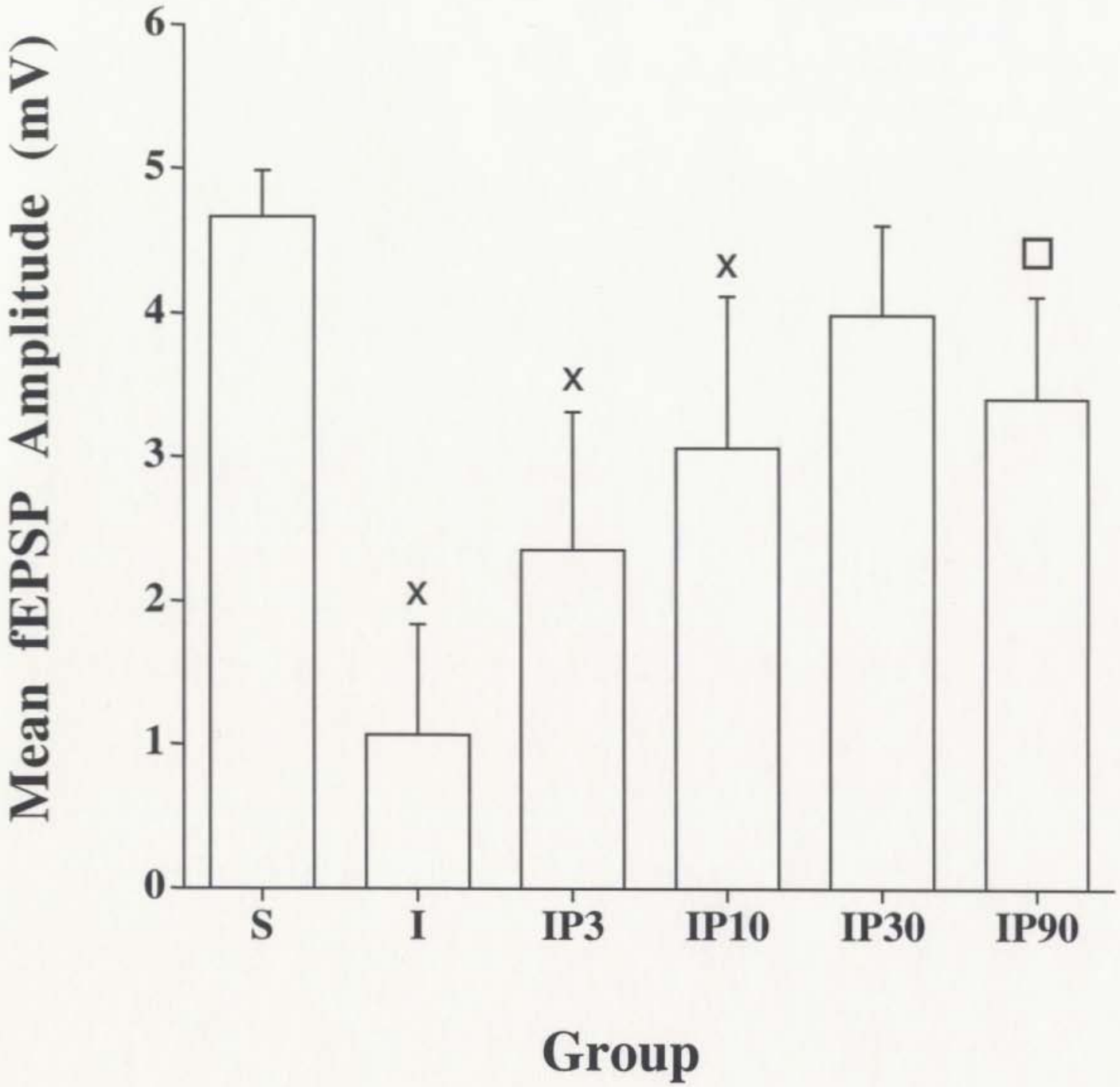
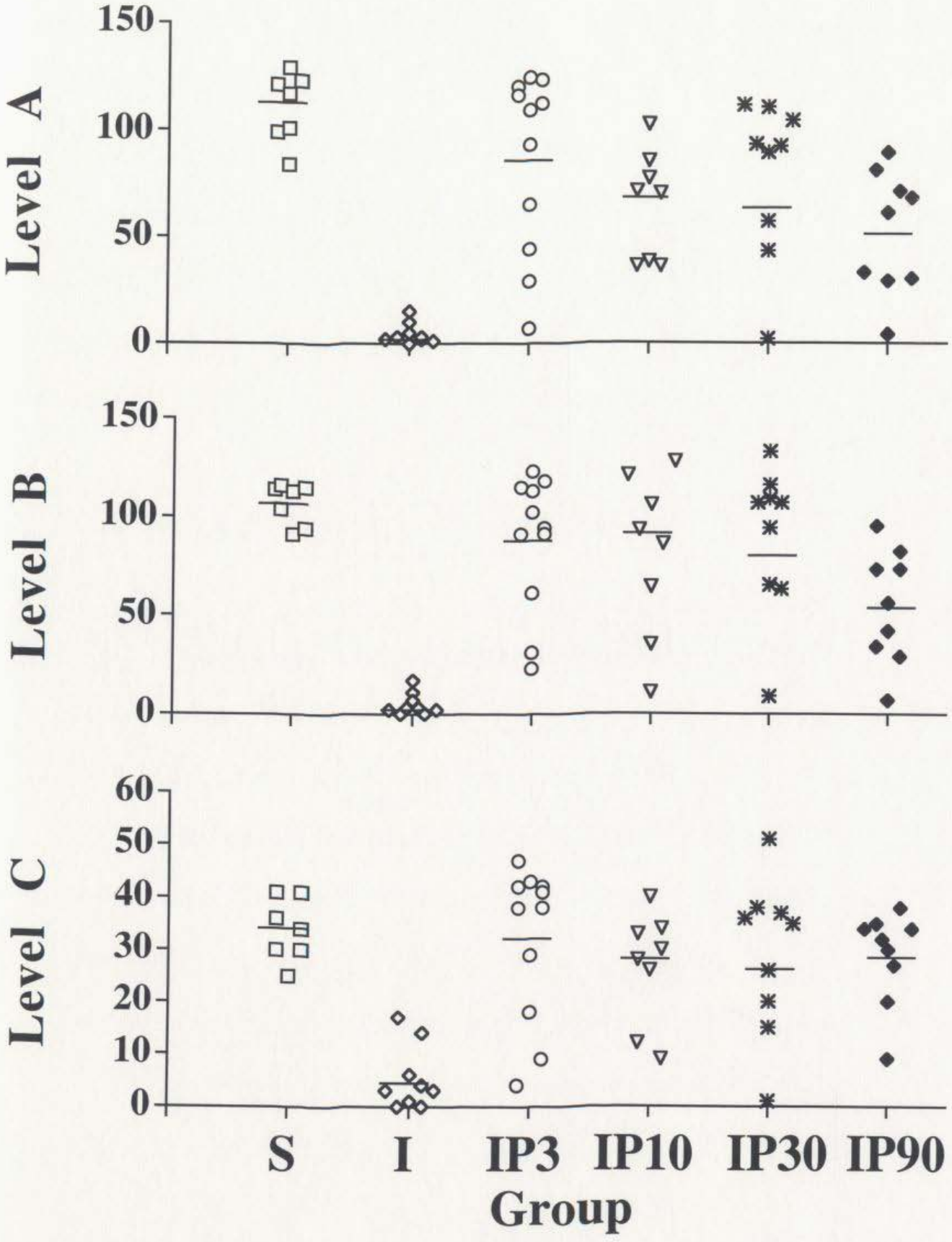

Mean fEPSP Amplitude (mV)
6
5
4
3
2
1
0
X
X
X
□
S
I
IP3
IP10
IP30
IP90
Group

Figure 3: Number of CA1 cells present within 200μm long sections in medial, middle and lateral regions of 6μm H and E stained sections taken from one hemisphere at 1.7 (level A), 2.2 (level B) and 2.7mm (level C) posterior to bregma. Sham (Sham; $n = 8$) and ischemic without preconditioning (I; $n = 9$) animals were sacrificed at 30 days survival. Ischemic preconditioned animals were sacrificed at 3 (IP 3; $n = 11$), 10 (IP 10; $n = 8$), 30 (IP 30; $n = 9$) and 90 days (IP 90; $n = 9$) postischemia. Each symbol represents the number of CA1 cells counted for one gerbil. Horizontal lines indicate the group mean. The I group had significantly ($p< 0.05$) fewer cells than any of the other groups irrespecetive of level (i.e. A,B or C) assessed. The IP90 group had significantly fewer cells than the sham animals at both levels A and B ($p<0.05$) whereas animals allowed to survive for 10 or 30 days (IP10 and IP30) were found to have fewer CA1 cells than shams ($p<0.05$) only ay level A. Cell counts in the IP3 animals were not different from shams.


Level A
150
100
50
0
Level B
150
100
50
0
Level C
60
50
40
30
20
10
0
S
I
IP3
IP10
IP30
IP90
Group

Similar to the decline in fEPSP amplitude at 90 days survival seen in the IP 90 group, these animals showed a significant reduction in the number of viable CA1 neurons ($p < 0.05$, versus sham). At levels B and C (middle and posterior) only CA1 cell counts from level B of the IP 90 animals were significantly less than sham. At all survival times, ischemic preconditioned animals had significantly more intact CA1 cells than 5 min ischemic animals ($p<0.01$).

3.5 Immunocytochemistry

A semi-quantitative assessment of the immunocytochemical localization of MAP2 in rostral hippocampus (level A) indicated that after ischemia there was a near total depletion of MAP2 in the CA1 dendritic region ($p<0.01$, versus sham), while in the ischemia resistant CA3 region it remained well preserved (Table 1). Intensity of MAP2 staining in preconditioned animals, while lower than that in shams, was not significantly different from shams. As was the case with cell counts, MAP2 intensity in the most caudal level assessed (Level C) was not greatly affected by ischemic insult.

Table 1: CA1 MAP2 relative optical density measurements. MAP2 staining of rostral CA1 (Level A) is expressed as the mean ± S.D. ROD of the CA1 apical dendritic field was normalized to the ROD of the corpus callosum (CC) according to the formula: CA1 ROD - CC ROD/CA1 ROD. [a] Indicates $p<0.01$ vs. all other groups.

Group	CA1 MAP2 ROD (mean ±S.D.)
I	0.02 ± 0.04[a]
S	0.69 ± 0.08
IP3	0.44 ± 0.28
IP10	0.42 ± 0.28
IP90	0.47 ± 0.24

4. Discussion

Ischemic preconditioning has repeatedly been shown to confer a substantial degree of histological preservation to the vulnerable CA1 region against an ischemic episode (Kato et al.,1991; Kirino et al.,1991; Liu et al.,1992; Kato et al.,1994). However, it has not been determined whether this protection is permanent or more importantly, whether it extends into the functional domain. The present results demonstrate that cell counts (derived from Nissl stained cell bodies) do not necessarily reflect the degree of functional integrity. At 3 and 10 days survival, IP animals, while exhibiting exceptional cellular preservation (79.7 and 66.9% respectively when rostral hippocampal CA1 levels A and B are combined) were significantly impaired on both behavioural and electrophysiological measures.

The present study also underscores the necessity of using extended survival times. Ischemic preconditioning was found to greatly extend the interval over which CA1 cells die following ischemia especially in rostral hippocampus (i.e. Level A). At 3 days survival, cell counts from ischemic preconditioned animals were not significantly different from shams,

however, by 90 days the number of intact CA1 cells counted in preconditioned animals had declined significantly to 46.8% of sham values from 77.4% in the 3 day survival group ($p<0.05$). Neuroprotection was more persistent at Level B where CA1 cell counts in the ischemic preconditioned animals did not differ significantly from shams except in the 90 day survival group. Indeed several other intervention strategies (e.g. the AMPA antagonist NBQX) have been shown to provide robust neuroprotection initially but with longer survival times it appeared that cell death had merely been delayed (Dietrich et al.,1993; Li and Buchan, 1995; Nurse and Corbett, 1996). The increase in cell loss and attenuated field potential amplitude in the IP 90 animals cannot readily be attributed to differences between it and the other IP groups incurred during induction of ischemia as intraischemic brain temperature did not differ between groups. Furthermore, there were no behavioural differences between the IP 90 and other IP groups that would have indicated variations in the efficacy of the preconditioning insults.

The observed dissociation between histological preservation and function is not unique to ischemic preconditioning since similar results have been reported elsewhere (Bothe et al.,1986; Jaspers et al.,1990; Kudo

et al.,1990; Dirnagl et al.,1990; de la Torre et al.,1992). Furthermore, Hori and Carpenter (1994) have shown that following an ischemic insult, at a time when some functional properties of CA1 cells (membrane potential and input resistance) appear normal, other properties such as the ability to induce LTP are disturbed. These findings were documented at such a time that CA1 cell morphology would have appeared completely normal using standard Nissl staining procedures (24 to 48 hrs postischemia). Interestingly, initial behavioural patterns in the open field seem to predict the final degree of CA1 histological preservation (i.e. high levels of activity are observed in animals which are later found to exhibit substantial CA1 cell loss). Animals receiving 5 minutes of ischemia without preconditioning were significantly more active than sham animals on all test days (3, 7, 10 and 30). Over the course of testing the I group, like IP animals, exhibited a gradual decline in activity scores. At 30 days however, behavioural scores for IP animals approximated those of sham. It is therefore possible that behavioural recovery results from compensation by uninjured structures within (e.g. caudal CA1) or distal to the hippocampus. This "unmasking" of function has previously been suspected to be responsible for behavioural compensations observed

following stroke induced deficiencies (Lee and van Donkelaar, 1995).

Previous studies have used changes in neuronal transmission, assessed electrophysiologically, to evaluate the changes which occur following an ischemic insult (Busaki et al.,1989; Urban et al.,1989; Jensen et al.,1991; Kirino et al.,1992). This approach provides a more direct measure of hippocampal function than behavioural testing. In the present study CA1 fEPSPs recorded in slices from I animals were significantly reduced compared to shams at all survival times. The mean amplitude for the ischemic group was as high as it was (~1 mV) because ventral hippocampus was included in the analysis and this area is resistant to neuronal damage using the occlusion durations of the present study (Crain et al.,1988). Therefore, fEPSPs recorded in this region were often near normal regardless of treatment. Recordings from this level were included however because it is likely that this region contributes to the overall function of the hippocampus. This regional disparity in vulnerability to ischemic injury has also been observed in humans in which the caudal hippocampus remains largely preserved while more rostral areas are severely damaged (Rempel-Clower et al.,1996). It is possible that the reduction in between group behavioural differences is the result of

compensation by ventral CA1 and that this compensation requires time to develop. This would explain why function was impaired at 3 and 10 days postischemia in IP animals that exhibited fEPSPs which were significantly attenuated relative to sham (maximum amplitudes were 51 and 66% of sham respectively, $p < 0.05$). However, by 30 days, fEPSP amplitudes had recovered to near sham levels suggesting true neuroprotection as a result of ischemic preconditioning. Similar evidence of a protracted functional recovery has been observed following induction of epileptiform activity in rat hippocampal slice culture (Muller et al.,1993). However, when the efficacy of ischemic preconditioning was assessed at 90 days, not only had CA1 cell counts continued to decline but the amplitude of fEPSPs were once again found to be significantly attenuated relative to sham values ($p < 0.05$). It would appear that while injured cells recover some degree of functional capacity following ischemic preconditioning, the recovery process is not completely sustained, again illustrating the necessity of assessing neuroprotection at extended survival times. It is possible that the reduction in CA1 dendritic integrity observed in the IP animals, as indicated by the reduction in the ROD scores, removes potentially injurious excitatory inputs to CA1 neurons at a time when they are very vulnerable.

Such enhanced susceptibility of CA1 neurons to excitatory input has previously been observed following an ischemic insult, though at a much shorter survival time (Tsubokawa et al.,,1992).

At present it is unknown whether this trend of continued CA1 neuronal death would continue at still greater survival times (e.g. 6 months or 1 year) or if the loss will cease at some point. For example, long duration postischemic hypothermia has been shown to result in a protection of CA1 neurons which decreases from 90% at 1 month postischemia to 70% at 6 months (Colbourne and Corbett, 1995) but does not continue to decline since the protection observed at 12 months is also approximately 70% (Colbourne et al.,1997). Furthermore it is possible that ischemic preconditioning exhibits a later "aborted recovery". That is at some protracted postischemic time point, processes mediating cellular preservation and recovery of function break down resulting in both histological and functional losses. Similar losses have been noted following removal of neurotrophic support of previously lesioned and regenerating motoneurons 12 weeks after lesion (Novikov et al., 1997). Nonetheless, ischemic preconditioning appears to provide a far greater degree of protection than any currently available drug therapy (Buchan et al.,1991a;

Buchan et al.,1991c; Li and Buchan, 1993) where protection beyond 1 month has not been demonstrated.

In an attempt to identify the origin of the early dissociation between histological and functional preservation, changes in dendritic morphology were assessed since CA1 dendrites and their spines have been shown to be particularly susceptible to ischemic injury (Matesic and Lin, 1994) Furthermore, it is widely accepted that processes mediating memory and habituation occur at the synaptic level (Bliss and Collingridge, 1993; Edwards, 1995). Brain sections were processed for immunocytochemical localization of the structural protein MAP2 which is believed to be involved in the cross linking of microtubules necessary for stabilizing dendritic structure and regulating plasticity (Linnik et al.,1993). This protein is normally present in large quantities in the CA1 dendritic region and is degraded following severe ischemic insult (Matesic and Lin, 1994; Aoki et al.,1995). Thirty days after the 5 min occlusion in the I group when most CA1 neurons have died, we observed a total loss of MAP2 in the apical dendritic region of CA1. In contrast, tissue from sham operated animals exhibited an intense localization of MAP2 in this area. The intensity of MAP2 localization observed in the ischemic preconditioned

animals, while slightly reduced, resembled that of sham animals. Therefore, it seems that dendritic disruption, as revealed using the present technique, cannot account for the behavioral and electrophysiological abnormalities observed in ischemic preconditioned animals. Previous evidence has shown that ischemia-induced dendritic abnormalities result in impaired electrophysiological function within hippocampus, including reduced CA1 field potential amplitude and loss of LTP (Hori and Carpenter, 1994). It may be that a subtle disruption in dendritic morphology (e.g. dendritic beading) undetectable using the present techniques, is responsible for the attenuated fEPSPs in the IP group (at 3 and 10 days postischemia). Furthermore, given the importance of synaptic integrity to the induction of LTP and the postulated relationship of the latter to learning and memory, it is likely that the early dendritic abnormalities in the IP animals are also responsible for the observed open field impairments of the preconditioned group.

While complete recovery of dendritic MAP2 intensity did not occur in ischemic preconditioned animals in the present study, such recovery and repair has been shown to occur in the peripheral and central nervous systems (Muller et al.,1993; Fagan and Gage, 1994; Stroemer et al.,1995).

Again it is possible that more subtle changes in the CA1 dendritic region are responsible for the recovery of fEPSP amplitudes in the IP animals (i.e. at 30 days postischemia).

The mechanisms mediating delayed neuronal death following ischemic insult have yet to be determined. Likewise, processes responsible for the neuroprotective effect of ischemic preconditioning remain elusive. The results of the present study, as well as others (Colbourne and Corbett, 1994; Li and Buchan, 1995), have indicated that neuronal death may occur over a much longer duration than has previously been assumed. It is also possible that the mechanisms responsible for this protracted cell loss may differ from those mediating more rapid neuronal death following ischemic insult. There has been some speculation that dendritic spines may accumulate calcium from extracellular sources following ischemia thereby reducing the levels to which the cell is exposed and decreasing the likelihood that this calcium will reach toxic levels within the soma and destroy the cell (Segal, 1995b). Following a severe insult it is possible that the calcium levels exceed the buffering capacity of the dendrites and it is perhaps this calcium overload which is responsible for the reduction in spine length which has been observed following application of NMDA to

hippocampal dendritic spines in vitro (Segal, 1995a). It may be that this type of subtle morphological alteration is responsible for the reduction in fEPSP amplitude observed in IP animals early after insult in the present study. Thus, the recovery of fEPSP seen with increasing survival times may be the result of a recovery of dendritic spine morphology similar to that observed following epileptic activity induced injury in hippocampal slice culture (Muller et al.,1993). Ischemic preconditioning may therefore enhance the buffering capacity of dendritic spines, reducing levels of calcium to which the cell is exposed and thereby reducing toxicity.

Another possible mechanism mediating the protective effect of ischemic preconditioning involves the role of mitochondria in regulating $[Ca^{2+}]_i$. It has been established that during and following glutamate stimulation of CNS neurons, mitochondria (as well as the Na^+/Ca^{2+} exchanger) serve to buffer the resulting intracellular calcium loads (White and Reynolds, 1995). However, following severe ischemic insult there is a large increase in intracellular calcium that becomes toxic to the mitochondria, resulting in a degradation of the mitochondrial membrane potential (Nathan et al.,1990; Schnider et al.,1996). As a result, calcium sequestration is inhibited and electron transfer is uncoupled from ATP

synthesis (which in turn leads to the generation of free radicals) each of which contributes to processes mediating neurodegeneration. A recent study has demonstrated that the mitochondria of hippocampal CA1 neurons from ischemic preconditioned animals sequester more calcium following a severe ischemic insult than do those from non-preconditioned animals (Ohta et al.,,1995). Perhaps, it is this enhanced calcium buffering capacity which may be partially responsible for the efficacy of ischemic preconditioning. The cell death observed at extended survival times following ischemic preconditioning may therefore be the result of incomplete mitochondrial protection which when coupled with subsequent arousing environmental stimuli (e.g. exploration of the open field) may result in a glutamate-induced increase in intracellular calcium levels that cannot be effectively normalized. One possible scenario is that mitochondrial buffering capacity is enhanced sufficient to protect CA1 cells but not to preserve dendritic morphology. Following loss of these dendrites (and the mitochondria within) CA1 cells are susceptible to the deleterious effects of elevated calcium levels but because of the "functional disconnection" of excitatory synapses, the rate of excitotoxic cell death is reduced. As dendritic morphology recovers and functional connections are

reformed, the cells are once again exposed to elevated levels of calcium as a result of excitatory connections (activated by normal environmental stimuli). The mitochondria may not be able to buffer this excessive calcium load effectively and thus protracted cell loss occurs.

The present study has clearly demonstrated that ischemic preconditioning may not be as efficacious a neuroprotective treatment as has typically been reported. More importantly, it has identified opposing processes of cell death and functional recovery that take place over a much longer time frame than previously thought. These data suggest the intriguing possibility of using interventions long after the initial ischemic insult (i.e. weeks) to rescue cells that would otherwise die months later. Elucidation of the mechanisms mediating the neuroprotective effect of ischemic preconditioning, (which is more effective than current drug therapies) may enable development of pharmacological agents which provide a beneficial and practical treatment strategy. Finally, the present results also illustrate the importance of using both multiple indices of neuroprotection as well as extended survival times in evaluating putative neuroprotective agents. Only when results of these methods of assessment converge can clearcut conclusions be drawn regarding the true efficacy of

a particular neuroprotective intervention.

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