ASSESSMENT OF ISCHEMIC DAMAGE AND NEUROPROTECTION IN AN EXPERIMENTAL MODEL OF FOREBRAIN ISCHEMIA

SUZANNE NURSE

Assessment of Ischemic Damage and Neuroprotection in an Experimental Model of Forebrain Ischemia

by Suzanne Nurse

A thesis submitted to the

School of Graduate Studies

in partial fulfillment of the degree of

Doctor of Philosophy

Faculty of Medicine (Neuroscience Specialization)

Memorial University of Newfoundland

Submitted October 1995

Revised December 1995

St. John's Newfoundland

ABSTRACT

In this dissertation, two treatments, intraischemic hypothermia and glutamate receptor blockade, were evaluated for neuroprotective efficacy against global ischemia. Since the standard assessment of ischemic damage using only histological outcome reveals nothing about function, I used multiple outcome measures including behaviour and electrophysiology, as well as a histological evaluation. In order to further increase the strength of this approach, multiple assessments were made in the *same* animals.

Intraischemic hypothermia induced by selectively cooling the brain conveyed both long-lasting protection against functional loss as well as cell loss. These findings provide a strong argument for the implementation of hypothermia in cases where the occurrence of an ischemic episode can be *predicted* before it occurs (e.g. elective cardiac/neuro- surgery). However, most cases of ischemia, or stroke, occur without warning thereby precluding the use of intraischemic hypothermia.

NBQX, a glutamate receptor antagonist which selectively blocks the AMPA receptor, is reportedly neuroprotective when treatment is delayed several hours postischemia. The same approach developed to evaluate intraischemic hypothermia was used to study NBQX. In the course of these studies it was found that NBQX induced a mild, prolonged, hypothermic action. Due to the ability of hypothermia to mitigate ischemic damage and the confounding effects of hypothermia in previous pharmacological studies of ischemia, it became

important to determine whether the hypothermia induced by NBQX contributed to its mechanism of action. The results strongly suggest that NBQX conveys neuroprotection through a hypothermic action. These data raise important questions concerning current hypotheses of ischemic cell death, which currently focus primarily on involvement of the AMPA subtype of glutamate receptor.

Finally, the behavioural changes that follow an ischemic episode were characterized with reference to variation in ischemic severity. Two types of behavioural alterations were noted: (1) an acute increase in activity following ischemia, and (2) a chronic increase in locomotor activity triggered by novel environments after the acute phase recovers. This second phase of increased locomotor activity was previously thought to reflect an impairment in spatial memory. Instead, it may be due to a form of behavioural sensitization not unlike that induced by stimulant drugs such as amphetamine.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGMENTS

I would like to thank my supervisor, Dr. Dale Corbett, whose strong belief in my abilities often exceeded my own. His encouragement spurred me on through the times when things didn't quite work out, and our mutual enjoyment of scientific research made the rest of the program a pleasure. I doubt whether I'll ever work in a lab with a better atmosphere and working environment than Dale's stroke research lab, which was due in large part to the presence of Suzanne Evans and Kathleen McKay. Even working all night maintaining temperature in a group of animals was enjoyable due to late night chats with Sue and early morning discussions with Dale, at shift changes.

I would like to thank my committee members, Dr. Carolyn Harley and Dr. Richard Neuman, who were always available whether I needed to discuss an academic or technical problem or just scrounge equipment. Not only were they role models for me, but they also acted as surrogate supervisors when Dale was on sabbatical. I would like to acknowledge the contribution of the entire neuroscience group to my growth as a scientist, but especially Dr. Penny Moody-Corbett, Dr. John Evans and Dr. Detlef Bieger. Thanks to my fellow graduate student, Fred Colbourne, with whom I not only shared office space and hotel rooms at meetings, but also interesting talks about new ideas and experiments to test them. I would like to thank Dr. Verna Skanes, Sandra Kelly and Pauline Cole in the Office of Research and Graduate Studies for dealing with

all the administrative details of my graduate programme; and the Faculty of Medicine and Memorial University for studentship support.

Finally, I would like to thank my family, Carmelita and Mike Nurse, Michael Nurse and Kristi Furey, and Mark Hawryluk for continuing to love and support me, especially during the last 6 months. And most importantly to Mark, for putting up with all my late nights and my absence on weekends.

PUBLICATIONS AND PRESENTATIONS

The majority of the data presented in Chapter 3 of this thesis has been previously published; "Direct measurement of brain temperature during and after intraischemic hypothermia: Correlation with behavioural, physiological and histological endpoints." S Nurse and D Corbett (1994), Journal of Neuroscience, Volume 14 (Number 12), pages 7726-7734. The data relating to the hypothermic action of NBQX, Chapter 4, is in press; "Neuroprotection following several days of mild, drug-induced hypothermia." S Nurse and D Corbett, Journal of Cerebral Blood Flow and Metabolism. Some of the data presented in Chapter 5 is being presented at this years Society for Neuroscience meeting in San Diego, California; "Acute and chronic locomotor patterns as predictors of ischemic outcome." S Nurse and D Corbett (1995), Society for Neuroscience Abstracts, Volume 21, 93.16.

Other publications and meeting abstracts which were important for the development and refinement of many techniques used in this dissertation are:

PUBLICATIONS

Corbett D, Evans SJ, Nurse SM (1992) Impaired acquisition of the Morris water maze following ischemic damage in the gerbil. *Neuroreport* **3**:204-206.

Colbourne F, Nurse SM, Corbett D (1993) Temperature changes associated with forebrain ischemia in the gerbil. *Brain Res* **602**:264-267.

Colbourne F, Nurse SM, Corbett D (1993) Spontaneous postishcemic hyperthermia is not required for severe CA1 ischemic damage in gerbils. *Brain Res* **623**:1-5.

ABSTRACTS

Corbett D, Nurse S (1990) Combined treatment with MK-801 and a dopamine (DA) antagonist reduces cerebral ischemic impairments. Presented at the 33rd Annual Meeting of the Canadian Federation of Biological Sciences, Halifax, Canada, June 14-16.

Corbett D, Evans S, Nurse S (1991) A re-examination of the cerebroprotective effects of MK-801 in a gerbil model of global ischemia. Presented at the Third IBRO World Congress of Neuroscience, Montréal, Canada, August 4-9, 50.20.

Nurse S, Evans S, Corbett D (1991) MK-801 blocks the effects of prior learning in an open field test of spatial learning. Presented at the Third IBRO World Congress of Neuroscience, Montréal, Canada, August 4-9, 66.27.

Corbett D, Nurse S, Evans S, McKay D (1991) Impaired water maze acquisition following CA1 ischemic damage. Presented at the Society for Neuroscience Annual Meeting, New Orleans, U.S.A., Volume 17, 426.2.

Nurse SM, Corbett D, Evans SJ (1991) Combined treatment with a D1/D2 dopamine antagonist reduces cerebral ischemic damage. Presented at the Society for Neuroscience Annual Meeting, New Orleans, U.S.A., Volume 17, 501.7.

Nurse SM, Neuman RS, Corbett D (1992) Hypothermic protection against ischemic insult: Assessment using histological, behavioral and electrophysiological measures. Presented at the Society for Neuroscience Annual Meeting, Anaheim, U.S.A., Volume 18, 664.3.

Colbourne F, Nurse SM, Corbett D (1992) Temperature changes associated with forebrain ischemia in the gerbil. Presented at the Society for Neuroscience Annual Meeting, Anaheim, U.S.A., Volume 18, 664.4.

Nurse SM, Corbett D (1993) Does hypothermia contribute to the neuroprotective actions of NBQX? Presented at the Society for Neuroscience Annual Meeting, Washington, D.C., U.S.A., Volume 19, 683.3.

CHAPTER 1: INTRODUCTION

1.1 Stroke

Cerebral ischemia or stroke is a pathophysiological condition in which brain tissue is deprived of oxygen and glucose due to a reduction in blood flow. *Global* cerebral ischemia is commonly observed as a result of cardiac arrest, whereas occlusion of a cerebral artery supplying a specific region of the brain produces an area of *focal* ischemia. While progress has been made in recent years in stroke prevention, primarily by making simple changes in lifestyle (i.e. dietary changes, exercise and quitting smoking), there remains no effective treatment for acute stroke when it does occur. In Canada there are approximately 50,000 new cases of stroke each year, and this disease is currently the leading cause of disability, placing an enormous financial and emotional burden on society. In 1994, the financial cost of stroke due to medical care, rehabilitation and support services, plus the lost revenue from individuals whose disabilities prevented them from returning to work was estimated to be $1.6 billion per year (Sutherland and Peeling, 1994). Obviously, any therapy that would effectively reduce neuronal damage after ischemia would be of tremendous benefit.

1.2 Animal Models of Ischemia

The best way to develop treatments to combat cerebral ischemia is to model the events occurring during human stroke and cardiac arrest in animals

and then test the value of putative protective agents in these well-controlled models. Animal models developed for cerebral ischemia fall into two main categories: (i) focal and (ii) global models (see Ginsberg and Busto, 1989). Focal ischemia can be induced in a number of ways, one common method is to excise the cranium and transiently occlude (i.e. 1 - 2 hours) the underlying middle cerebral artery with a removable clip (Buchan et al., 1992), or permanently occlude the artery by cauterization or ligation (Chen et al., 1986). An alternate method involves introducing a suture into the lumen of a major artery and advancing it up into the brain where it will occlude blood flow at the origin of the middle cerebral artery (Longa et al., 1989; Kawamura et al., 1991). As with the direct application of a vascular clip, focal ischemia induced with the suture model can either be transient or permanent because the suture can be retracted. Animal models of focal ischemia mimic what we typically think of as a stroke in humans and as with human clinical cases, the most common artery occluded in animal models is the middle cerebral artery. The resulting injury is an infarct, death of all tissue elements, in the region where blood flow has been severely reduced (< 10 ml/100g/minute). In the surrounding region, the penumbra, where the reduction in blood flow is not as great due to compensation from the collateral circulation, selective neuronal death may result (Astrup et al., 1981; Siesjö, 1992a,b).

Models of global ischemia mimic the clinical condition of cardiac arrest in that a large region of brain tissue is ischemic. There are several ways of inducing global ischemia in animal models, these include cardiac arrest, inflation of a neck cuff and direct occlusion of the major arteries supplying the brain. In most mammals, occlusion of the pair of vessels supplying the forebrain, the carotid

arteries, will not result in forebrain ischemia because the posterior vertebral arteries can compensate for the lack of blood flow. The carotid and vertebral circulation is linked by communicating arteries which together form the circle of Willis at the base of the brain. Therefore, in order to induce forebrain ischemia, either the vertebral arteries have to be cauterized prior to bilateral occlusion of the carotid arteries, or severe systemic hypotension (45 - 50 mm Hg) has to be induced by exsanguination to prevent the collateral circulation from circumventing the effects of carotid occlusion. Both of these methods are commonly used in rat models of global ischemia and are referred to as the rat 4-vessel occlusion model (4VO, Pulsinelli and Brierley, 1979) and 2-vessel occlusion model (2VO, Smith et al., 1984b), respectively. The gerbil (*Meriones unguiculatus*) is different from most mammals in that it lacks a functional circle of Willis. Therefore, forebrain ischemia can be induced in the gerbil by occlusion of the carotid arteries only (Levine and Payan, 1966). The carotid arteries are isolated through a midline incision in the neck and then transiently occluded with vascular clips. Reproducible brain damage will result in regions such as the CA1 subfield of the hippocampus, after occlusions as brief as 3 minutes (Mitani et al., 1991; Andou et al., 1992), with a very low mortality rate. The advantage of the gerbil model over rat models of global ischemia is that the occlusion is not dependent on the success of the vertebral cauterization or strict maintenance of a blood pressure below 50 mm Hg. The disadvantage of this model is that physiological measurements, such as blood gases and blood glucose, cannot be easily obtained due to the smaller size of the gerbil in comparison to rats, and a correspondingly smaller blood volume. However, changes in many of these

physiological variables results in minimal variation in outcome in the gerbil model, with one exception being temperature which can have a large effect on the degree of ischemic damage (Clifton et al., 1989).

In this thesis, I use a gerbil model of global ischemia in which I have developed a multi-faceted approach for measuring neuroprotection. This approach is used to evaluate the benefit provided by mild hypothermia, "Chapter 3", and that of a selective glutamate α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline (NBQX), "Chapter 4". Finally, I have explored the heuristic value of careful behavioural analysis following ischemia and its use in predicting the severity of ischemic injury, "Chapter 5".

The following sections describe the ionic and metabolic changes that occur during and after an ischemic event (1.3) and the pathology associated with global ischemia (1.4).

1.3 Ionic, Metabolic and Other Pathological Perturbations During and Following Ischemia

Due to the high metabolic rate and limited energy reserves, the brain requires a continual supply of glucose and oxygen. In addition, metabolic waste is normally removed by the circulatory system, preventing a build-up in the local area surrounding neurons and glia. Therefore, an interruption in the blood supply has serious consequences on brain metabolism and hence, brain function. Within ~ 30 seconds following circulatory arrest, the

electroencephalogram (EEG) flattens because ATP levels are not sufficient to maintain synaptic transmission. This is followed about 2 minutes later by an enormous shift in ionic balances (mainly Na^+ and K^+) which require energy-dependent processes for homeostasis (Hansen, 1985) and a massive extracellular accumulation of neurotransmitters including glutamate, aspartate, dopamine and γ-aminobutyric acid (GABA) (Benveniste et al., 1984; Globus et al., 1988a). Within a few minutes of the ischemia, neurons become unresponsive to stimulation and, shortly after this, they depolarize causing a large negative shift in the extracellular potential, otherwise known as anoxic spreading depression (Somjen et al., 1990, 1993). Most neurons will recover from these events, depending on the duration of the ischemia, except the selectively vulnerable cell types (see section 1.4).

In addition to the loss of ionic homeostasis, there is also an accumulation of metabolic waste. Due to the necessity to undergo anaerobic metabolism, lactic acid is one of the products that increases in large amounts (Siesjö et al., 1990) resulting in a pH drop (reports range from 6.2 - 6.9) (Silver and Erecinska, 1992; Kaku et al., 1993). The extent of acidosis is dependent on the glucose level prior to ischemia; hyperglycemia results in an increase in anaerobic respiration and hence a greater fall in pH. Acidosis has been hypothesized to be a major contributing factor to ischemic injury (Ljunggren et al., 1974), but more recent reports have suggested that the pH decline, provided it is relatively mild, may actually be beneficial (Kaku et al., 1993). High-energy phosphates are rapidly depleted during ischemia, but once reflow occurs ATP levels recover relatively quickly (within ~ 5 minutes) with the restoration of oxygen and glucose

(Sutherland et al., 1992). Other processes take longer to recover after ischemia. For example, glycogen stores and protein synthesis can take several hours to normalize (Nowak et al., 1985) and in some brain regions, such as CA1, protein synthesis may be irreversibly inhibited. Although, restoration of blood flow (oxygen, glucose and other constituents delivered by the circulatory system) is necessary to prevent neuronal death, reoxygenation may also contribute to the damaging process. With the restoration of oxygen, free radicals are formed which can lead to the destruction of neuronal membranes through lipid peroxidation reactions (Schmidley, 1990).

1.4 Pathology Following Global Ischemia

Cell injury resulting from focal and global ischemia have different characteristics. Focal ischemia produces an area of dense ischemia, the ischemic core, where an infarct will develop in which all cellular elements are destroyed (i.e. neurons, glia and vascular tissue). Global ischemia, which is the type investigated in this thesis, is characterized by *selective neuronal vulnerability.* Certain classes of neurons display an enhanced susceptibility to global ischemic damage such that occlusion times that do *not* result in irreversible cell injury in most brain regions will kill these vulnerable neurons (e.g. CA1 pyramidal cells). Even within a brain region such as the hippocampal formation, there is a wide variation in the susceptibility of neurons to cell death following global ischemia. Some classes of interneurons in the dentate hilus and stratum lucidum of CA3 are exceedingly vulnerable (Bratz and Grossmann, 1923; Johansen et al., 1985;

Johansen, 1993; Hsu and Buzsáki, 1993), where ischemic durations as brief as 2 minutes will lead to cell death. Similarly, there is a profound loss of dorsal CA1 neurons (> 80 %) following ischemic episodes in excess of 3 minutes (Kirino 1982; Mitani et al., 1991; Andou et al., 1992) in the gerbil and 10 minutes in the rat (Pulsinelli et al., 1982). In contrast, nearby CA3 pyramidal cells and dentate granule cells can tolerate periods as long as 15 - 30 minutes (Pulsinelli et al., 1982). These wide differences in vulnerability within the hippocampal formation are not likely due to differences in blood supply, but probably result from differences in the inherent characteristics of these individual neuronal populations and/or the pattern of innervation they receive (Schmidt-Kastner and Freund, 1991). However, the mechanistic reason for this vulnerability has remained a mystery since Sommer described the selective cell death in CA1, otherwise known as Sommer's sector, over 100 years ago (Sommer, 1880). In addition to the hippocampal formation selective neuronal vulnerability is found in (i) the striatum, where the small- and medium-sized spiny neurons are selectively injured by ischemia, (ii) Purkinje cells of the cerebellum and (iii) pyramidal neurons in layers 3, 5 and 6 of the neocortex (Pulsinelli et al., 1982; Crain et al., 1988).

In addition to local differences in vulnerability, the *type* of cell death also varies. Some neurons (e.g. hilar interneurons and striatal neurons) undergo rapid necrosis soon after the ischemic event, such that within 6 - 24 hours there is histological evidence of irreversible injury (Pulsinelli et al., 1982; Hsu and Buzsáki, 1993). Based on results from cell culture studies, the rapid rate of cell death was originally thought to be due to osmotic injury (Rothman, 1985),

whereas slow cell death appears to be dependent on Ca^{2+} (Choi, 1985). Cellular accumulation of Na^{+} during ischemia leads to an influx of Cl^{-} down its electrochemical gradient. The increased osmolarity of the cell then results in a subsequent accumulation of water. If the osmotically driven influx of water exceeds the capacity of the plasma membrane for expansion, then cell death results. However, it is not clear whether cell lysis occurs *in vivo* (Benveniste, 1991). Therefore osmotic injury may not account for the rapid cell death of striatal neurons.

Other neurons exhibit a different form of cell death which takes days to mature and appears to be mediated by Ca^{2+} (see Siesjö, 1988). This form of cell death has been termed *delayed neuronal death* and is a feature of postischemic necrosis in the CA1 sector of the hippocampus where it was first described (Kirino, 1982). Pyramidal neurons in CA1 appear normal histologically for approximately 48 hours postischemia and will also transiently recover synaptic responses after an ischemic event (Urban et al., 1989). However, the cells progress to necrosis 3 - 4 days after the occlusion. Recently, it has been found that the process of delayed neuronal death in the CA1 region can be postponed by several weeks or months with certain anti-ischemic treatments, as discussed later in section 1.6 and Chapter 4. The mechanism underlying delayed neuronal death is unknown, however, there have been numerous hypotheses developed (e.g. glutamate excitotoxicity, see section 1.5). Most of the current hypotheses, including glutamate excitotoxicity, focus on increased intracellular Ca^{2+} as a common mechanism for the resulting cell injury. The hope offered by necrosis of the delayed type, from a clinical point of view, is that there may be a "therapeutic

window" after an ischemic insult when treatment could be administered which would halt or minimize the processes leading to delayed neuronal death.

1.5 Glutamate Excitotoxicity

Since the mid-1980's, there has been an explosion of knowledge pertaining to cell death induced by cerebral ischemia. For example, at the Society for Neuroscience annual meeting in 1984 there were no sessions specifically related to ischemia, since then the field has grown to such an extent that in 1994 there were 16 ischemia sessions and over 200 ischemia-related presentations. A contributing factor to this rapid growth over the last 10 years has been the interest in glutamate excitotoxicity (Choi, 1985; Rothman and Olney, 1986, 1987) and the hope that glutamate receptor antagonists, in particular N-methyl-D-aspartate (NMDA) antagonists, would be beneficial as a clinical treatment for acute stroke (Gill et al., 1987).

The glutamate excitotoxicity hypothesis is most often used to explain selective neuronal vulnerability to ischemia. The basic premise of the hypothesis is that glutamate rises above normal levels during ischemia and selectively vulnerable neurons are irreversibly injured by exposure to levels which are neurotoxic. The receptor which has been proposed to underlie glutamate excitotoxicity is the NMDA receptor. Since the NMDA receptor-gated channel is permeable to Na^+, K^+ and Ca^{2+}, overstimulation of the NMDA receptor by glutamate could lead to a large accumulation of intracellular calcium (Ca^{2+}_i) thereby triggering a destructive cascade of events (i.e. activation of

Ca^{2+}dependent proteases and lipases) leading to cell death (Choi, 1988; Siesjö and Bengtsson, 1989). The initial evidence that provided support for this hypothesis was that glutamate is neurotoxic *in vitro* and *in vivo* (Rothman and Olney, 1986) and brain regions that are vulnerable to ischemic injury tend to have the highest density of NMDA receptors (Cotman et al., 1987). Moreover, cultured hippocampal cells do *not* die when exposed to hypoxic conditions if they have not developed synaptic connections, but once synaptic contacts form the neurons are susceptible to hypoxia (Rothman, 1983). This requirement of synaptic activity to trigger cell death has also been demonstrated *in vivo* by lesioning the input pathways to the CA1 subfield prior to inducing ischemia (Benveniste et al., 1989; Kaplan et al., 1989). Lesioning afferents to the CA1 region *reduces* the amount of glutamate released during ischemia (Benveniste et al., 1989), which might be a mechanism for the observed protection. Furthermore, intraischemic hypothermia which protects CA1 neurons, decreases glutamate release during ischemia (Globus et al., 1988b; Mitani and Kataoka, 1991), whereas hyperthermia exacerbates both glutamate release as well as the subsequent neuronal injury (Mitani and Kataoka, 1991).

However, the greatest support for the glutamate excitotoxicity hypothesis has come from a large number of studies using *in vivo* ischemia models (both global and focal) that have demonstrated protection against ischemic damage with NMDA antagonists (Gill et al., 1987; Park et al., 1988; Olney et al., 1989). Notwithstanding these results, some of the initial optimism regarding the use of NMDA antagonists to treat cerebral ischemia has been tempered by recent findings. First, glutamate concentrations measured in different brain regions

during ischemia do not predict the subsequent pattern of cellular injury. For example, glutamate concentrations in the CA1 and CA3 regions are similar during ischemia, but the CA1 region is considerably more vulnerable (Mitani et al., 1992). Globus and colleagues have shown that the glutamate levels in the dorsolateral striatum and the anterior thalamus are virtually identical during ischemia, however, only the striatum displays selective vulnerability (Globus et al., 1991). Interventions that alter the glutamate concentration in the hippocampus during ischemia do not consistently alter the extent of cell injury, as do manipulations of intraischemic temperature. For example, theophylline increases glutamate release and kynurenic acid decreases glutamate release. However, neither intervention affects the magnitude of cell injury in the hippocampus following ischemia (Lekieffre et al., 1991, 1992). Second, although the CA1 region has very high levels of NMDA receptors and is quite vulnerable to ischemia, other brain regions have similar NMDA receptor levels and yet are resistant to damage (e.g. dentate granule cells). Third, and most importantly, the protection provided by MK-801, a potent non-competitive NMDA receptor antagonist, has been questioned. Several studies have suggested that MK-801 is neuroprotective because of a hypothermic action, not through a direct pharmacological effect at NMDA receptors on CA1 neurons (Buchan and Pulsinelli, 1990; Corbett et al., 1990; Nellgård et al., 1991; Hara et al., 1992). When MK-801 is administered and the hypothermic effects are prevented, there is little or no neuroprotection found.

The above concerns regarding the excitotoxicity hypothesis, and in particular the questionable involvement of NMDA receptors in ischemic damage,

have caused ischemia research to focus on other subtypes of glutamate receptors. Recently, a selective AMPA receptor antagonist, NBQX has been found to be more protective against global ischemic injury than NMDA antagonists (Sheardown et al., 1990; Buchan et al., 1991a; Nellgård and Wieloch, 1992; Sheardown et al., 1993) even with delayed treatment and there has been a suggestion for clinical trials to be initiated (Buchan et al., 1993).

1.6 Assessment of Neuroprotection

Assessment of ischemic cell damage in cases of *severe* injury is a simple matter of determining how many cells, if any, are remaining in a particular brain region (e.g. dorsal CA1). For example, in the gerbil model of bilateral carotid occlusion, with occlusion times of only 5 minutes, greater than 95 % of CA1 cells are destroyed. In this case, assessment is relatively straightforward using standard histological methods. However, when protection against ischemic injury is evaluated following pharmacological treatment or other anti-ischemic therapies, the issue of defining neuroprotection becomes more complicated. For example, neuroprotection is usually assessed by counting the number of CA1 neurons remaining ~ 3 - 7 days after the ischemic insult, a time when the majority of the cell population would have been destroyed in the absence of any intervention. It has been assumed that the presence of neurons at this stage indicates (i) that neurons whose histological appearance using a light microscope is normal must also be functionally intact and (ii) that the mere presence of cells a few hours to days beyond the time when they normally die

indicates permanent protection. Due to these assumptions, *functional* protection is not typically assessed, instead a histological measure of protection is usually the only endpoint used. However, cellular function can be compromised and behavioural performance can be impaired even when no neuronal injury is detected using conventional (i.e. Nissl stains) histological methods (Bothe et al., 1986; Jaspers et al., 1990; Miyazaki et al., 1992; Sekhon et al., 1994; Hori and Carpenter, 1994). In addition, when a treatment has been shown to provide neuronal protection during the first week, rarely are longer survival times assessed in order to verify that the protection persists. In the few cases where this has been done, decreasing efficacy of the treatment is often found at later survival times (Morse and Davis, 1990; Dietrich et al., 1993; Colbourne and Corbett, 1994a, 1995; Valtysson et al., 1994). Thus, at least in the CA1 region, certain interventions (e.g. brief postischemic hypothermia) simply prolong the time course of delayed neuronal death to weeks or months.

These issues raise the important question of how is neuroprotection best defined? Since functional protection cannot be assumed solely on the appearance of Nissl stained tissue, the assessment of neuroprotection should entail a measure of neuronal function. This is of paramount importance since the aim of ischemia research is to develop treatments for clinical use, in which the ultimate outcome measure is not histological, but functional preservation. Neuroprotection should also provide permanent savings. A treatment that does not preserve normal function and simply postpones cell death will be a disappointment if it eventually reaches clinical trials, not to mention the tremendous cost entailed.

In Chapter 3, I describe a method of functional assessment of ischemic animals that combines *both* behavioural and electrophysiological measures, with a subsequent histological quantification of hippocampal injury, all in the same animals. All measures have been designed to gauge the extent of injury in the CA1 region since this is one of the most vulnerable regions to ischemia. Moreover, this is a brain region that undergoes delayed neuronal death and therefore, is amenable to postischemic treatment.

1.7 Neuroprotective Therapies

One of the most effective therapies in global ischemia to date has been hypothermia initiated during the period of ischemia. In contrast to earlier studies in this field where hypothermia was moderate to severe (i.e. 18 - 28 °C), it has been found that temperature need only be a few degrees below normal in order to provide protection against ischemic injury (Busto et al., 1987). In rodent models of ischemia, intraischemic hypothermia can occur spontaneously during surgery and in particular during ischemia itself. The blood supply is the major heat source to the brain and in small rodents once the blood supply is interupted the brain temperature will drop. Depending on the length of the occlusion, this spontaneous temperature decline is enough to protect neurons. This protective fall in brain temperature is unlikely to be as dramatic in humans during global ischemia due to the larger mass of brain tissue and a much thicker skull that provides insulation.

These declines in brain temperature have been problematic in animal models of ischemia, causing a large variance in outcome, primarily due to

individual differences in intraischemic brain temperature. In view of the confounding influences of uncontrolled intraischemic temperature most investigators now control core and often brain temperature during surgery. However, the ease of cooling the brain during the ischemic period can be taken advantage of in order to study the protective effects of hypothermia. By *deliberately* cooling animals during the ischemic period, it is possible to have control over the magnitude and the rate of cooling. In Chapter 3, I have investigated the protective value of selective brain hypothermia during the ischemic event. Intraischemic hypothermia has been accepted as the gold standard for neuroprotection, but there has been little in the way of functional assessment following this protective strategy. Thus, intraischemic hypothermia was used in order to develop a functional assessment method for global ischemia that could be used in conjunction with a standard histological method of assessment.

In addition to intraischemic hypothermia, there have only been a few pharmacological therapies that have proven to be consistently protective when tested in different research labs. The glutamate AMPA receptor antagonist, NBQX, has received the most attention. Evidence from many studies using different models of global and focal ischemia has been in agreement with respect to the reproducibility of the neuroprotection afforded by this compound (Sheardown et al., 1990; Buchan et al., 1991a,b; Nellgård and Wieloch, 1992) and in contrast to previous studies with MK-801, where the protective action seems to result from a hypothermic effect, NBQX has *not* been reported to induce hypothermia (Sheardown et al., 1990). In addition to the protective effects of

NBQX, other evidence has supported the involvement of the AMPA receptor in ischemic injury. Pelegrini-Giampietro et al. (1992) have found that ischemia causes a change in the mRNA coding for the subunits which make up the AMPA receptor. Following ischemia, there is a decrease in the message for the GluR2 subunit which possesses the Ca^{2+}-impermeability property characteristic of the AMPA receptor (Hollmann et al., 1991). If the reduction in the mRNA for this subunit leads to the formation and expression of AMPA receptors without the GluR2 subunit, then activation of the AMPA receptor would lead to an increased calcium influx during normal glutamatergic stimulation. Activation of the AMPA receptor, even the configuration containing the GluR2 subunit, could be involved in ischemic injury since activation of this receptor depolarizes neurons, thereby opening other channels that allow calcium entry (e.g. voltage-operated calcium channels and the NMDA receptor gated channel). By blocking the AMPA receptor, multiple sites for calcium entry are affected simultaneously, not just a single site as with NMDA antagonists. This effect along with changes in subunit composition, could explain the enhanced efficacy of NBQX compared to MK-801. However, the protection afforded by NBQX has only been subjected to histological verification and the survival times in global ischemia studies have only been ~ 4 - 7 days. Thus, after having developed a combined functional and histological approach to evaluate neuroprotection using intraischemic hypothermia, this assessment method was used to evaluate NBQX (Chapter 4).

1.8 Overview of Experiments

1.8.1 Intraischemic Hypothermia (Chapter 3)

The aim of this study was to critically evaluate the protection afforded by intraischemic hypothermia against ischemic injury to the hippocampus. Hypothermic treatment was applied *selectively* to the brain during a 5 minute carotid artery occlusion. Following a period of recovery, independent functional measures were used to assess hippocampal function- (1) acute activity patterns during the first 3 - 5 hours postischemia, (2) an open field test which has been proposed to assess spatial memory (testing began 3 days postischemia) and (3) measurement of evoked potentials from area CA1 in hippocampal slices (3 weeks after the ischemic episode). The functional outcome portrayed by these measures was compared to a morphological evaluation of CA1 pyramidal cells at three rostro-caudal levels. All evaluations were carried out in the same animals. The outcome from these various assessments should indicate whether intraischemic hypothermia truly is the gold standard of neuroprotective treatments.

This study introduces a novel approach for the evaluation of putative anti-ischemic treatments: combining behavioural, electrophysiological and histological measures. Each method of assessment may provide information relating to hippocampal integrity and when used in combination should yield a more accurate appraisal than any single outcome method.

1.8.2 Evaluation of NBQX (Chapter 4)

The same approach developed with intraischemic hypothermia was to be used to evaluate the protection afforded by postischemic treatment with NBQX, a glutamate AMPA receptor antagonist. NBQX had been repeatedly shown to protect against neuronal cell loss in several ischemia models without the confounding effects of hypothermia which had plagued other agents, such as MK-801. However, when pilot studies with NBQX began we detected that NBQX did produce hypothermia. Therefore, the proposed assessment of NBQX was modified to initially test the hypothesis that hypothermia contributed to the protective action of NBQX.

In initial studies two occlusion durations were used, 3 and 5 mimutes. At *each* occlusion duration a control group received glucose vehicle, and four groups were treated with NBQX (3 x 30 mg/kg, intraperitoneal (i.p.)) starting at either 1 hour or 6 hours postischemia. At each time point the brain temperature in one of the NBQX groups was regulated so that it resembled the pattern in the vehicle controls and in the second NBQX group brain temperature was recorded but *not* regulated. This pilot study (11 treatment groups, n = 90) indicated that in our model, NBQX provided only minimal protection against 5 minutes of ischemia and was much more effective when given at 1 hour postischemia then if treatment was delayed for 6 hours. Therefore, the experiments in Chapter 4 focus on treatment with NBQX 1 hour after a 3 minute occlusion. This study was then followed with a further evaluation of the hypothermic effect of NBQX as well as the functional protection provided by NBQX.

1.8.3 Locomotor Changes Associated with Ischemia (Chapter 5)

In animal models of ischemia, both in the gerbil and rat, a frequently used behavioural hallmark of ischemic injury has been increased locomotor activity. However, reports in the literature have varied with respect to the length of time this behavioural change persists after an ischemic episode, even in the same animal model with identical occlusion durations. I investigated whether these conflicting results were due to differences in the testing environments.

Global ischemia was induced in gerbils for either 1.5, 3.0 or 5.0 minutes. Following ischemia the animal's activity was monitored in cages which the animals had been habituated prior to ischemia. Starting three days after surgery, locomotor activity was monitored for 10 minutes in an open field which was novel to the animals. Repeated open field testing was performed 7 and 10 days postischemia in some cases. All activity measures were compared to results from sham-operated animals.

In the next chapter, Chapter 2, I describe the general methods employed throughout my thesis, some of the specific methodology is repeated when the experiments are described in Chapters 3 - 5.

CHAPTER 2: METHODS

2.1 Animals

All animal research was carried out in accordance with the animal care guidelines of Memorial University of Newfoundland and the Canadian Council on Animal Care. For the majority of the experiments described, female Mongolian gerbils (*Meriones unguiculatus*) were used. Gerbils were purchased from High Oak Ranch Limited (Baden, ON, Canada (previously located in Goodwood, ON)). For the studies on the temperature effects of NBQX, male, Sprague-Dawley rats, bred in the local animal care facility, were used in addition to gerbils.

Gerbils were typically about 2 - 3 months of age upon arrival. They were housed in groups of four and acclimatized to the animal care facility for at least one week prior to experimentation. It was found that housing young animals individually for prolonged periods led to an increase in the number of animals who displayed seizures when handled (some strains of gerbils are known to be very seizure prone and these seizures can be triggered by novelty, Ludvig et al., 1991). Although, with the breeder we use and the above method of housing we seldom observe seizures, perhaps 1 out of 50 animals will display a brief episode of ear and whisker twitching when being weighed at the initiation of an experiment. With the exception of 20 animals used in the intraischemic hypothermia experiments described in Chapter 2, all animals were treated with

piperazine citrate 0.34% w/v in the drinking water for 72 hours when they initially arrived. This antihelmenthic was given to treat pinworm infection which is a common affliction in gerbils and other rodents. Lights in the animal room were on a 12 hour light:dark cycle with lights on at 8 am. All animals were given food and water ad libitum. The diet consisted primarily of guinea pig pellet food, supplemented with a rodent seed mixture which contained sunflower seeds and corn kernels. Gerbils were often provided with shredded paper, papers towels or cardboard tubes which the animals shredded to make nests.

2.2 Brain Temperature and Activity Monitoring

With the exception of 3 normal animals used only for open field behaviour and CA1 cell counts, brain temperature was monitored in all animals. Procedures for brain temperature monitoring have been described in Colbourne et al. (1993) and Nurse and Corbett (1994). A guide cannula was implanted 30 - 48 hours prior to brain temperature monitoring under barbiturate anaesthetic (sodium pentobarbital, 65 - 70 mg/kg i.p.), with atropine (0.02 mg/kg, subcutaneous) given prophylactically. The cannula was made from a 20 G needle and measured 5 mm in length. In some experiments a modified cannula was used, a bend was made at the top to form a 90° arm which acted as a platform for the temperature probe to rest on, tape was fitted under the arm and up and around the probe to secure it in place (modified cannula was designed by Fred Colbourne, unpublished methods). The cannula was positioned in a burr hole approximately 1 mm in front

of bregma and 2 mm to the left of the midline suture. The guide cannula was placed so that it rested on dura, enabling the 8 mm brain probes to sample temperature from the dorsal striatum (~ 3 mm below dura), which was similar to the depth of the dorsal hippocampus. Thus, when the temperature probes were in place the readings obtained should have closely approximated the temperature in dorsal CA1. In experiments involving electrophysiological recording, hippocampal slices were always prepared from the hemisphere opposite the cannula implant. Therefore, in these studies the cannula was placed either to the left or the right of the midline so that hippocampal slices could be sampled from the left or right, not solely from the right hemisphere. Once the guide cannula was positioned, two plastic screws (#MN-080-2, nylon machine screw, pan head #0-80 x 1/8", Small Parts Inc., Miami Lakes, FL, USA) were glued, head down, to the bone plates behind the coronal suture, using Lepage accu-flo™ super glue. Once the super glue had cured (~ 5 - 10 minutes) the guide cannula was cemented in place with dental cement. It was not possible to thread screws into the skull because of the thinness of the skull bone. A 27 G stylet was placed in the guide cannula, to keep the cannula patent. While the dental cement was hardening the skin was sutured and a fine tipped indelible marker was used to write the animal's identification number on the skull cap. All animals were housed individually after cannula implantation, with an identification card on the outside of the cage as well. Any time the animal was removed from its cage for

temperature monitoring, surgery, behavioural testing, etc. the ID on the skull cap was checked against the ID on the cage.

Approximately 2 days after cannula implantation, an amplitude modulation (AM) type temperature probe (model XM-FH (8 mm), Mini-Mitter Co., Sun River, OR, USA) was inserted to sample normal brain temperature. Under halothane anaesthesia, (1.5 % in 30 % O_2/70 % N_2O) the stylet was removed, the temperature probe was lowered into the cannula and secured with adhesive tape. Once the probe was in place, the animal was removed from the anaesthetic and put in a plastic cage resting on an AM-receiver (model # RA-1010, Catalog # 272-0001, Data Sciences Inc., St. Paul, MN, USA) which measured temperature and activity by telemetry.

The temperature readings were recorded on-line every 20 sec by reading the frequency output of the probe and converting it to a temperature using pre-set calibration values. Calibration values at 33 °C and 38 °C were calculated for each probe approximately 48 hours after battery replacement and batteries were usually replaced when the period of temperature sampling reached 150 - 200 hours. A thermocouple probe (HYP1-30-1/2-T-G-60-SMP-M) and a microprocessor thermometer (model HH23, both from Omega Technologies Co., Stamford, CT, USA) were used as the standard to which all brain probes were calibrated. For calibration, the brain probe and Omega thermocouple were floated in a 600 ml beaker containing 300 ml of water at the required temperature. The probes were extended into the water through small holes made in a plastic tissue

culture dish so that the tips of the two probes were at the same depth. Following calibration, the brain temperature probe had to read within 0.1 °C of the thermocouple probe to be considered accurate (accuracy was assessed between 36 - 37 °C).

Activity was scored as events by counting the number of times the automatic gain controller in the receiver had to adjust to maintain relative signal strength during each 20 sec sampling period. Each movement of the probe which caused a change in relative signal strength above the threshold value was scored as one event. Frequency measurements and activity measurements were independent of one another. All data were recorded with Dataquest III software (Data Sciences, Inc., St. Paul, MN, USA).

Brain temperature and activity were typically recorded for a period of 3 hours. Once the session was over the animal was briefly reanaesthetized with halothane as described above, the probe was removed from the guide cannula and replaced with the stylet. Animals were placed in their home cages and returned to the animal room.

2.3 Cerebral Ischemia

Global forebrain ischemia was induced two days after normal brain temperature and activity had been monitored. Under halothane anaesthesia, the animal's head was wrapped in a flexible blanket (Gaymar heat therapy Mul-T-Pad™ model TP-3E, 3 1/2" x 23", Gaymar Industries Inc., Orchard Park, NY) which

could be used to heat or cool the brain by perfusing the blanket with warm or cold water, respectively. A small hole was made in the blanket, in an area that was sealed off from water flow, to allow the brain probe to pass through the blanket into the guide cannula. In some cases a needle thermistor (Model HYP1-30-1/2-T-G-60-SMP-M, Omega Technologies Co., Stamford, CT, USA) was inserted under the skin to measure temperature from the skull, on the side opposite the brain probe. After placing the brain probe (and skull probe), the animal was turned onto its back, a rectal probe was inserted to monitor core temperature and then the body was wrapped in an electrically heated blanket (Harvard homeothermic blanket control unit, small, Model # 50-7503, Harvard Apparatus Ltd., Edenbridge, Kent, UK). Core temperature was controlled with a homeothermic feedback system. Once the temperature probes and blankets were in place the only exposed area was the animal's neck. A midline incision was made and the carotid arteries were gently isolated and then looped with silk suture. A cotton ball moistened with saline was placed over the incision while waiting for the brain temperature to stabilize at normothermia (37.0 °C). Once the target temperature was reached, clamps were placed on both arteries and a stopwatch was started to time the duration of ischemia. Throughout the occlusion, brain temperature was kept as close as possible to 37 °C, except in the case where intraischemic brain hypothermia was deliberately induced. In these animals, following the initiation of ischemia, core temperature was maintained at normothermia as usual, but brain temperature was lowered to ~ 29 - 30 °C (5

minute occlusions) or ~ 31 - 32 °C (3 minute occlusions) during ischemia by switching the water source for the head blanket from a heated water bath to ice cooled water, then rewarming immediately upon reperfusion.

At the end of the ischemic episode, both arterial clamps were removed, reflow was visually confirmed and the incision was sutured. Animals were removed from the anaesthetic setup, however, brain probes were left in place. The animals were placed in plastic cages, resting on the AM receivers, to record brain temperature and activity measurements. During the first hour of reperfusion, brain temperature was kept near normothermic values with a heating lamp if the animal was unable to maintain its own temperature. In some cases, rectal temperature was sampled periodically during the postischemic period. This was easily done by putting the animal on the counter top, grasping and lifting the base of the tail to expose the rectum and then inserting the thermometer parallel to the animal's spine (Digi-Sense Thermometer Model 8523-00, Cole-Parmer, Chicago, IL, USA). In the majority of animals sampled this way (both males and females) a lordosis response was induced and the animals remained motionless while the temperature was being taken, an unusual state for a gerbil!

Behaviour was periodically observed during the first hour post-surgery to determine the length of time it took for animals to right themselves and record whether "ischemic hunch" was apparent. This postural change, resulting from a kyphosis of the spine, was evident during the early period of reperfusion and is a neurological marker of ischemia. Any animal subjected to carotid occlusion for 3

or more minutes at normothermia who did not exhibit this postural change would not be expected to have functional or histological impairments. It is expected that some gerbils will have a circle of Willis (Mayevsky and Zipi, 1990) and therefore may not be affected by a transient clamping of the carotid arteries. However, even in gerbil populations where 10 to 20 % of the animals have some level of posterior communication, forebrain ischemia is still thought to be close to 100 % during bilateral carotid occlusion because the additional vasculature is not sufficient to overcome the secondary vasoconstriction response that occurs during ischemia (Mayevsky and Zipi, 1990). The percentage of animals from the population of gerbils we use that show no evidence of ischemia related changes following occlusion of the carotid arteries is extremely low (< 0.1 %) and none were observed in the experiments described in this dissertation.

Following temperature and activity monitoring, the brain probes were removed as described previously and the animals were returned to the animal room. Body weight was monitored during the postischemic recovery phase. Following ischemic episodes up to 5 minutes in length, an animal may display a transient weight loss but usually regains its pre-ischemic weight by 1 week post-surgery. Animals continuing to lose weight at 1 week post-surgery would be considered unwell and behavioural measures could be confounded, thus not reflecting the true extent of ischemic injury.

2.4 Behavioural Testing

Animals were tested in an open field to which they had *not* been exposed before ischemia/sham operations. The open field was a square wooden box, 72 x 76 x 57 cm, with a white Plexiglas insert that was used as the floor. Testing was carried out in three 10 minute sessions 3, 7 and 10 days post-surgery (in experiments using shorter survival times (4 days), there was only 1 open field test, 3 days post-surgery). A computerized tracking system divided the floor of the maze into 25 squares and counted the total number of squares entered by a gerbil during each trial as well as the total distance traveled (see Wang and Corbett, 1990). The gerbil was monitored by a camera (Panasonic CCTV camera) mounted above the maze and the image was displayed on a video monitor. Image analysis hardware was manufactured by HVS Image Analysing Ltd. (Hampton, England), tracking software was written by Chee Wong. All behavioural testing was carried out in a soundproofed room (2.1 x 3.4 m), distinctive features of the room and lighting conditions were kept constant from trial to trial. The room itself was dimly lit, however, the open field was lit from above by two 60 watt incandescent lights. The animals were kept in an outer room for a *minimum* of 20 minutes before behavioural testing started, care was taken to disturb the animals as little as possible during this waiting period. These precautions were taken in the hope that the activity measurement in the open field would reflect "true" cognitive function and confounding factors such as behavioural arousal due to loud noises, transportation from the animal facility, being handled or weighed,

etc. would be minimized. Each cage was individually moved to the open field test room just before the trial for that animal, the animal was gently lifted out of its cage and placed in the corner of the maze, facing the centre. Once the animal was placed on the floor of the maze, the experimenter initiated computerized data acquisition and then left the room. The animal's behaviour could be viewed through a small window (31 x 31 cm). At the end of the 10 minute session, the experimenter re-entered the test room, removed the animal from the open field and returned it to its home cage. The cage was then taken out of the test room, the floor of the maze was wiped with a sponge using mild, soapy water and dried. A similar procedure was then followed for each additional animal. Following open field testing, all animals were weighed and then returned to the animal care facility.

2.5 Hippocampal Slice Preparation and Electrophysiological Recording

Animals were anaesthetized with 2% halothane in 30% O_2 and 70% N_2O. The head was enclosed in the cranial blanket used previously for ischemic temperature control and a brain probe was reinserted into the dorsal striatum. Brain temperature was *gradually* cooled without regulating core temperature, the percentage of halothane used to maintain anaesthesia was periodically reduced in 0.5% steps, from 2% down to 0.5%. Once brain temperature reached 30 °C (~ 20 minutes), anaesthesia was discontinued for ~ 2 minutes while continuing to keep the head in contact with the cooling blanket. Animals were killed by

decapitation, the brain was quickly removed from the skull, placed on ice-cold filter paper and bisected. The hemisphere used for brain temperature monitoring was reserved for histology (immersion fixed in 10 % buffered formalin) and hippocampal slices were prepared from the other. Briefly, the hippocampus was dissected free, transverse sections (500 μm) from the middle half of the hippocampus (along the septotemporal axis) were obtained with a tissue chopper (Model # 51425, Stoelting, Wood Dale, IL, USA) and transferred to a holding chamber containing modified artificial cerebrospinal fluid (ACSF) consisting of (mmol/L): sucrose 215.8; KCl 3.5; $CaCl_2$ 2.0; $NaHCO_3$ 25.0; $NaHPO_4$ 1.2; $MgCl_2$ 1.3; glucose 11.0, bubbled with 95% O_2/5% CO_2 (pH 7.3 - 7.4). Slices were incubated at room temperature in modified ACSF for 15 minutes and subsequently in normal ACSF which contained (mmol/L): NaCl 126.0; KCl 3.5; $CaCl_2$ 2.0; $NaHCO_3$ 25.0; $NaHPO_4$ 1.2; $MgCl_2$ 1.3; glucose 11.0, saturated with 95% O_2/5% CO_2 (pH 7.3 - 7.4) for at least 1 hr prior to experimentation.

Extracellular recording of evoked potentials was carried out using a submerged slice preparation in a perfusion chamber at 33 - 34 °C. The slice was superfused with warmed, oxygenated ACSF at a rate of 2 ml/minute. Recording electrodes, glass micropipettes were filled with 2M NaCl. Twisted, bipolar tungsten stimulating electrodes (Teflon-coated, tip separation 125 μm) were used for orthodromic stimulation of perforant path and Schaffer-collateral/commissural fibers with constant current pulses (0.1 ms and 20 μs, respectively; stimulus intensities ranged from 50 - 800 μA) delivered at the rate of 0.05 Hz (ST-101

Stimulator, Stimtek, Acton, MA, USA), triggered by a computer. Recordings were amplified and filtered (filter settings were from 0.1 Hz to 10 KHz) (DAM-5 differential preamplifier, World Precision Instruments, Sarasota, FL, USA), displayed on an oscilloscope and digitized for display, storage and subsequent analysis through a Lab Master DMA board (Scientific Solutions Inc., Solon OH, USA, 12-bit A/D converter, A/D conversion rate was 0.02 msec). Software for stimulation triggering (including theta frequency stimulation for induction of long-term potentiation and paired pulse stimulation), data collection, and analysis of excitatory post-synaptic potentials (EPSPs) and population spikes was written in Asyst (Version 4.00, Asyst Software Technologies, Inc., Rochester NY, USA). The software is available from the author.

Slice viability was determined by visual inspection and orthodromic stimulation of the perforant path. If a slice appeared viable and perforant path stimulation could elicit a population spike in the dentate granule cells then it was used for recording in CA1, otherwise it was discarded and no recording in CA1 was attempted. Field potentials for area CA1 were measured in stratum radiatum following stimulation of the Schaffer collaterals, an input/output series was used to determine the maximum EPSP amplitude. Input/output series were performed in a random order, with 3 - 4 stimulations at each intensity in the log series. Once the maximal response was determined all subsequent recordings were made with the stimulus intensity at the setting which produced the half-maximal response.

2.6 Histopathology

All brain tissue to be used for histological assessment was fixed in 10 % buffered formalin. If electrophysiological recording was not carried out then the animal was given a lethal injection of sodium pentobarbital (0.1 ml, approximate dose = 100 - 130 mg/kg), transcardially perfused with heparinized saline (heparin sodium, 2.5 units/ml) for 1.5 minutes (~ 15 ml) followed by 60 ml 10 % phosphate-buffered formalin (SF100-20, Fisher Scientific, Montréal, PQ, Canada). The descending aorta was clamped off during perfusion so that only the upper body and head were fixed. A syringe pump (Model A, Razel Scientific Instruments, Stamford, CT, USA) was used to infuse the perfusate. Following fixation, the animal was decapitated and the brain was left *in situ* overnight, in the same fixative. The following day the brain was extracted from the skull and left in fixative for approximately 3 more days. It was found that taking the brain out of the skull immediately following the perfusion led to dark neuron artifact in some cells due to mechanical compression of the brain tissue during extraction. In order to avoid this artifact, which could be confused with ischemic cell change, the brains were left *in situ* for ~ 24 hours (Cammermeyer, 1961), until the tissue hardened from the fixative. If electrophysiology was carried out, the animal was cooled and decapitated as stated previously, the brain bisected and then the hemisphere used for brain temperature sampling was immersion fixed in 10 % buffered formalin phosphate for at least 4 days prior to embedding or freezing.

For histological assessments of CA1 the brains were blocked (to remove the cerebellum and the region rostral to the nucleus accumbens) and frozen in petroleum ether (cooled with CO_2 in acetone) prior to being sectioned in the coronal plane at 10 μm using a cryostat set at -18 °C. In later studies, the blocks were dehydrated, embedded in paraffin and 6 µm sections were cut on a microtome. Tissue was cut starting at the level of the vertical limb of the diagonal band of Broca through to the level of the red nucleus. Sections were collected on glass slides (slides were gelatin coated for the collection of frozen sections), air dried overnight, then stained with either cresyl violet, cresyl violet plus phloxine, or haematoxylin and eosin. Neurons that have died or undergone irreversible injury, but are still present, prior to fixation are acidophilic and will preferentially take up acidic stains (i.e. phloxine and eosin). In contrast, cells that were alive at the time of fixation preferentially bind basic Nissl stains (i.e. cresyl violet and haematoxylin). This difference in staining when tissue has been double stained with a basic and an acidic dye helps distinguish between cells that appear 'histologically normal' and those that are not at the time of fixation. Acidophilic cells were excluded from the cell counts of the CA1 region. Neurons which are acidophilic correspond to cells that are disintegrating when visualized with electron microscopy. In such cases, there is a breakdown of cellular structures (such as the plasma and nuclear membranes), loss of the endoplasmic reticulum and Golgi apparatus from the cytoplasm and the appearance of mitochondrial flocculent densities (Brown et al., 1979; Auer et al., 1985).

Depending upon the survival time after ischemia, many of the cells that were killed by the ischemic episode may have died and been phagocytosed, thus would not stain at all. Therefore, it is not possible to count the number of cells that have died by counting the acidophilic cells, since this may only represent a subpopulation of the cells that were actually killed by the ischemia. In order to determine the proportion of cells that were killed, the number of basophilic cells remaining must be assessed and then compared to counts/ratings from normal or sham-operated animals.

Hippocampal injury was assessed, while blind to treatment status, in one of two ways. In earlier studies, sectors 400 μm in length of the dorsal and ventral CA1 pyramidal cell band were *rated* at three levels posterior to bregma; 1.7 mm (3 dorsal sectors: medial, middle and lateral), 2.2 mm (4 sectors: medial, middle, lateral and ventral) and 2.8 mm (2 sectors: middle and ventral), see Figure 3-9. The percentage of neurons in each sector which appeared viable (i.e., had well-defined nuclei and were basophilic), relative to a normal gerbil hippocampus, were rated using a five-point scale where: 0 = 0 - 5%, 1 = 6 - 29%, 2 = 30 - 59%, 3 = 60 - 89% and 4 = 90 - 100%. We have examined the precision of this rating scale in relation to actual cell counts and found a high correlation $r = 0.97$, $p = 0.0001$ ($n = 56$, unpublished observations). Assessment of ischemic damage using the above rating scale is also associated with a high degree of inter-rater reliability. However, the literature in the field of cerebral ischemia is filled with a plethora of different rating scales, many of which use only a three-point scale: 1 = no injury

(i.e. < 20 %), 2 = some injury (i.e. 20 - 80 %) and 3 = extensive cell loss (i.e. > 80 %). Since numerous rating scales are essentially all or none and do not distinguish gradations of injury, i.e. 25 % versus 50% versus 75 %, many reviewers are insisting on cell counts and discouraging the use of any rating scales. Therefore, in later studies, assessment of CA1 injury was based on *cell counts* in order to comply with the movement away from rating scales. Unfortunately, the inordinate amount of time involved in doing cell counts limits the amount of tissue that can be assessed. When tissue was assessed by doing cell counts, 3 sectors were counted in a rostral section of dorsal CA1, 1.7 mm posterior to bregma (see Figure 3-9A).

CHAPTER 3:

DIRECT MEASUREMENT OF BRAIN TEMPERATURE DURING AND AFTER INTRAISCHEMIC HYPOTHERMIA

(CORRELATION WITH BEHAVIOURAL, ELECTROPHYSIOLOGICAL AND HISTOLOGICAL ENDPOINTS)

3.1 Introduction

Global cerebral ischemia can result in irreversible brain damage, most noticeably in the CA1 sector of the hippocampus. In both humans and animal models this necrosis is delayed, and using standard histological procedures such as Nissl stains, is undetectable for 2 - 4 days following the ischemic insult and may take at least 7 days to be complete (Kirino, 1982; Pulsinelli et al., 1982; Horn and Schlote, 1992). However, cell death is preceded by a decay in the evoked response of CA1 pyramidal cells to Schaffer collateral stimulation (Urban et al., 1989, 1990). In hippocampal slices taken from animals that have previously undergone an episode of cerebral ischemia, electrophysiological function can be preserved by intracellular injection of Ca^{2+} chelators and deterioration is hastened by IP_3 activation (Kirino et al., 1992; Tsubokawa et al., 1992). This evidence suggests that the irreversible alteration in electrophysiological response following ischemia is Ca^{2+}-dependent. Abnormal Ca^{2+} homeostasis is also thought to be the trigger for the subsequent neuronal death (Choi, 1985; Siesjö and Bengtsson, 1989). Thus, deterioration in the evoked response of CA1 neurons to afferent stimulation could be used as an early marker of cell death.

Putative neuroprotective treatments, such as glutamate receptor antagonists (Warner et al., 1990) or prior lesioning of excitatory amino acid inputs (Kaplan et al., 1989), have been reported to preserve CA1 cells histologically for several weeks. However, if these cells are examined using silver impregnation their appearance is similar to a transition state observed in untreated animals 24 -

48 hours after ischemia, when electrophysiological function has been lost, though neurons are still considered viable (Warner et al., 1990). Thus, it may be that certain anti-ischemic treatments which have appeared neuroprotective simply slow down cell death by several days or weeks, but do not preserve function. Since most researchers tend to quantify ischemic damage using survival times of 3 - 7 days and rely solely on Nissl stained material, a normal or near normal appearance does not necessarily reflect preserved function or long-term protection. Consequently, the electrophysiological responsiveness of CA1 pyramidal cells may be a more sensitive measure of CA1 integrity than histological assessment alone, especially in cases where "protective treatments" have been administered.

Cell death resulting from global ischemia can lead to memory deficits in rodents and primates (Zola-Morgan et al., 1986,1992; Auer et al., 1989; Corbett et al., 1992). Neurobehavioural tests of memory function have been shown to be sensitive indices of hippocampal cell loss resulting from ischemia. Even following mild cases of ischemia, which have not resulted in detectable CA1 cell loss, deficits in spatial memory function were evident several days after occlusion (Jaspers et al., 1990). Following unilateral ischemia in non-sensitive gerbils (i.e. blood flow was only temporarily reduced to ~ 30 % of normal due to compensation by the collateral circulation) there were detectable memory impairments even though the occlusion produced no histological injury (Bothe et al., 1986). However, following a longer survival period, 8 weeks, neuronal cell

death became evident. Thus, the early behavioural test had been more sensitive to the ultimate outcome than early histological assessment. Therefore, it is crucial to combine behavioural testing with histological assessment in order to determine whether putative neuroprotective treatments preserve cognitive functions as well as neurons, since this is the ultimate goal. However, with increasing recovery time memory impairments can be less evident unless more intricate tests are employed. For example, we were able to show a deficit in Morris water maze performance using the simple place learning task starting 3 days, but not 3 weeks, following ischemia (Corbett et al., 1992). Other researchers (Auer et al., 1989; Green et al., 1992) revealed a protracted memory impairment by employing the more difficult place learning-set variation of this task 6 - 8 weeks after ischemia. Recovery of function has been demonstrated in other memory tests as well (Imamura et al., 1991; Katoh et al., 1992) and is presumably due to compensation by undamaged structures within the hippocampal formation itself or other brain regions. The ability of the nervous system to compensate for neuronal loss can complicate the interpretation of behavioural tests if they are used in isolation.

Since behavioural, electrophysiological and morphological evaluations can all be used to assess outcome following cerebral ischemia, combining all three should provide a powerful method for gauging the efficacy of a neuroprotective treatment. If each of these measures are reliable indicators of

CA1 integrity, and if a treatment strategy has been truly beneficial, then converging evidence for neuroprotection should be obtained.

The objective of the present study was to evaluate the protection afforded by intraischemic hypothermia using the above multi-faceted approach. It is well known that mild to moderate intraischemic hypothermia can provide near total protection of CA1 neurons from ischemia (Busto et al., 1987; Chopp et al., 1989; Minamisawa et al., 1990a, 1990b; Welsh et al., 1990; Dietrich et al., 1993; Iwai et al., 1993). Hypothermia has been suggested to be the "gold-standard" to which other therapies should be compared (Buchan, 1992), thus it seemed the best candidate against which to evaluate the potential sensitivity of the above functional and histological measures of ischemic injury. Ischemia was induced by occluding the carotid arteries for 5 minutes in gerbils, while brain temperature was either maintained at normothermia or lowered to ~ 30 °C. These groups were compared to sham-operated controls in a novel open field maze, a test which has previously been shown to reliably reflect the extent of CA1 damage (Gerhardt and Boast, 1988; Wang and Corbett, 1990; Mileson and Schwartz, 1991; Babcock et al., 1993). After a 3 week survival period, hippocampal slices were prepared from one hemisphere in order to measure the responsiveness of CA1 neurons to afferent stimulation, while the other hemisphere was retained for histological analysis. Thus, the behavioural, electrophysiological and histological measures were all derived from the same animals.

3.2 Materials and Methods

3.2.1 Animals

Twenty adult female Mongolian gerbils (High Oak Ranch, Goodwood, ON) were used in this study. At the initiation of the experiment, animals were 3.5 - 6 months of age and ranged in weight from 55 - 90 g.

3.2.2 Preparation for Brain Temperature Measurement

Guide cannule (5 mm) for the brain temperature probes were implanted either above the left or right hemisphere, as described in the general methods section 2.2. Two days prior to ischemia/sham surgery, striatal temperature was measured continuously for three hours to provide baseline values.

3.2.3 Cerebral Ischemia

Anaesthesia was induced with 2% halothane and maintained with 1.5% halothane in 30% O_2 and 70% N_2O. Rectal, skull and brain temperatures were measured as previously described in the general methods section (section 2.3). Rectal temperature was maintained between 37.8 and 38.0 °C throughout the surgical procedure. Brain temperature was maintained near 37.0 °C with a separate blanket system heated by a water bath. This blanket was in direct contact with the top and sides of the head in order to distribute heat to the brain as evenly as possible. The common carotid arteries were isolated through a

midline incision in the neck and looped with silk suture. After carotid isolation animals were divided into three treatment groups: (1) 5 minute ischemia at normothermic brain temperature (NBT; n = 7), (2) 5 minute ischemia with intraischemic brain hypothermia (IBH; n = 7) and (3) a sham-operated control group, treated comparably to (1) except for the induction of ischemia (SHAM; n = 6). Cerebral ischemia was induced by placing vascular clamps on the carotid arteries for five minutes. Following clamp removal reflow was confirmed visually and the incision sutured. Brain temperature was lowered in the IBH group by promptly changing the water source for the head blanket from the heated water bath to an ice-bath just as the arteries were being occluded. Once the brain temperature reached ~ 30.0 °C the water temperature was adjusted to maintain a steady state. Upon reperfusion, normothermia was restored by reverting to the original water source.

Once brain temperatures returned to the preischemic level (5 minutes or less in the NBT group and ~ 10 minutes post-occlusion in the IBH group), the skull thermistor and rectal thermometer were removed and anaesthesia was discontinued. Duration of the entire surgical procedure was similar in all 3 groups, approximately 30 - 35 minutes. Brain temperature monitoring continued after surgery for at least 3 hours in unrestrained animals, rectal temperature was sampled twice, 30 and 60 minutes postischemia. Postischemic brain temperature data for one animal in the sham-operated control group had to be excluded because of irregular readings due to battery failure.

3.2.4 Behavioural Testing

Animals were tested in an open field maze (72 x 76 x 57 cm) to which they had *not* been exposed before ischemia/sham operations. Testing was carried out in three 10 minute sessions 3, 7 and 10 days post-surgery. The floor of the maze was divided into 25 squares and a computerized tracking system counted the total number of squares entered during each trial (see section 2.4). All behavioural testing was carried out in a soundproofed room (2.1 x 3.4 m). Distinctive features of the room and lighting conditions were kept constant for the duration of the experiment.

3.2.5 Hippocampal Slice Preparation and Electrophysiological Recording

Twenty-one to twenty-three days following ischemia/sham surgery animals were anaesthetized with 2% halothane in 30% O_2 and 70% N_2O. The head was again enclosed in the cranial blanket and a brain probe reinserted into the striatum. Brain temperature was gradually lowered as described in section 2.5. Animals were killed by decapitation, the brain was quickly removed from the skull, placed on ice-cold filter paper and bisected. The hemisphere used for brain temperature monitoring was reserved for histology (see below) and hippocampal slices were prepared from the other. Hippocampal slices (500 μm) were incubated at room temperature in modified ACSF (containing sucrose in place of NaCl) for 15 minutes and then transferred to normal ACSF for ~ 2 hours prior to recording.

Extracellular recording of evoked potentials was carried out using a submerged slice preparation in a perfusion chamber. Recording electrodes, glass micropipettes (tip diameter ~ 20 μm, resistance 0.5 - 1.0 MΩ), were filled with 2M NaCl in 0.5% agar. Twisted, bipolar tungsten stimulating electrodes were used for orthodromic stimulation of perforant path and Schaffer-collateral/commissural fibers with constant current pulses (100 and 20 μs, respectively) delivered at the rate of 0.05 Hz. Recordings were digitized for storage and subsequent analysis. The experimenter was blind to treatment status while carrying out the electrophysiological investigation. A slice was considered viable if orthodromic stimulation of the perforant path could elicit a population spike in the dentate granule cells, otherwise it was discarded and no recording in CA1 was attempted.

3.2.5 Histology

The hemisphere retained for histological analysis of CA1 and brain probe placement was fixed by immersion in 10% phosphate-buffered formalin. The brain tissue was frozen in petroleum ether (cooled with CO_2 in acetone), sectioned in the coronal plane at 10 μm and stained with cresyl violet. Sectors of the dorsal and ventral CA1 pyramidal cell band (400 μm in length) were rated blindly at three levels posterior to bregma; 1.7 mm, 2.2 mm and 2.8. The percentage of neurons in each sector which appeared viable (i.e., with well-defined nuclei), relative to a normal gerbil hippocampus, were rated using a five-

point scale where: 0 = 0 - 5%, 1 = 6 - 29%, 2 = 30 - 59%, 3 = 60 - 89% and 4 = 90 - 100%.

3.2.6 Statistics

Brain temperature, open field scores and CA1 evoked potentials were analyzed using ANOVA. Individual post-hoc comparisons were evaluated using Dunnett's *t*-test (two-tailed). Histological results were analyzed with the Kruskal-Wallis test. Significance level was initially set at $p < 0.05$.

3.3 Results

Intraischemic brain hypothermia was achieved rapidly following onset of ischemia (Table 3-1, Figure 3-1; figures and tables are located at the end of the results section). Two minutes into the occlusion, brain temperature in 5 out of 7 IBH animals was below 33 °C and after 3 minutes 4 of 7 animals had reached 30 °C. Skull temperature responded even more rapidly to the induced hypothermia, since it was in closer proximity to the cooling source. This temperature gradient pattern was reversed in the NBT group. In this group, the striatal temperature displayed a slight, non-significant decrease during the occlusion while the temperature at the skull, being closer to the heating source, remained virtually unchanged (Table 3-1). The mean occlusion temperature (brain) for the IBH group was 32.3 ± 2.9 °C SD compared to 36.6 ± 0.6 °C SD, NBT group.

In the early postischemic period following anaesthesia, all groups (including SHAM) exhibited a period of hyperthermia (Figure 3-1). The IBH group displayed the greatest degree of hyperthermia, which peaked at 39.1 ± 0.3 °C thirty-five minutes after ischemia and was significantly higher than both SHAM and NBT groups ($t_{10} = 4.9$ and $t_{12} = 3.8$, respectively, $p < 0.01$). This difference was also reflected in the rectal temperatures measured at 30 minutes postischemia (see Table 3-2). The peak brain temperature during this early postischemic period in the NBT group of 38.5 ± 0.3 °C (also 35 minutes post-occlusion) only just reached significance compared to sham-operated controls ($t_{10} = 2.3$, $p = 0.046$). A secondary rise in brain temperature began approximately 120 minutes post-occlusion in the NBT and IBH groups. At this time sham-operated animals were approaching the range of normal brain temperature recorded two days prior to surgery, 37.4 ± 0.4 °C SD. The secondary temperature rise observed in the ischemic groups appeared to be the result of an *acute* phase of locomotor hyperactivity which was less pronounced and recovered faster in the animals treated with intraischemic hypothermia (Figure 3-2). There was no evidence of seizures during the postischemic observation period and there were no fatalities in this study.

Sham-operated animals displayed normal levels of exploration during the first exposure to the open field, which declined on subsequent test days (Figure 3-3). In contrast, the animals occluded at normothermia exhibited heightened levels of activity during all test sessions, with little or no within session

habituation to the environment. However, in the group subjected to hypothermia no impairment was noted, except for a tendency on Day 3 to have slightly elevated levels of activity relative to controls. When the individual behavioural responses of animals within the IBH group were analyzed two main patterns emerged. Some hypothermic animals had open field scores that were identical to shams, while others closely resembled the normothermic group during the initial part of the session but then began to habituate in the later minutes, Figure 3-4B. When the day 7 open field scores of the hypothermic group were divided (based on day 3 behaviour) the animals with a slight deficit on day 3 started at the same level as the NBT group but quickly dropped down to the same level as the other half of the IBH group, Figure 3-5B. On the third session in the open field (day 10), there was no difference in response patterns between the animals in the IBH group, Figure 3-6B, the entire group responded in the same manner as the sham-operated group as indicated by the total scores in Figure 3-3. All animals appeared healthy on each test day, and there were no group differences in initial body weight or in weight assessed 7, 14 and 21 days after ischemia (Table 3-3).

Evoked responses of CA1 pyramidal cells to orthodromic stimulation were measured in all 20 animals, 3 weeks postischemia. Field excitatory post-synaptic potentials (fEPSPs) from 55 hippocampal slices were recorded in stratum radiatum following stimulation of Schaffer-collateral/commissural fibers. Not surprisingly, in slices taken from animals in the NBT group there was a dramatic reduction of the CA1 response, although a presynaptic fiber volley was often recorded

(Figure 3-7A). The reduction in the amplitude of the maximal EPSP was associated with a decrease in the slope and area of the fEPSPs (Figure 3-8). There was also a significant change in the latency of the response, starting an average of 2.3 ms following stimulation compared to 1.72 ms in sham-operated controls. However, in slices taken from animals treated with intraischemic hypothermia field potentials recorded in CA1 were indistinguishable from shams.

Histological assessment of CA1 confirmed that there was substantial cell loss from dorsal CA1 in the NBT group at all three rostro-caudal levels (Figure 3-9), while intraischemic hypothermia provided remarkable protection against the 5 minute occlusion, as previously suggested by the functional measures. In both ischemic groups greater neuronal injury was observed at the most rostral level assessed (-1.7 mm) and there was no significant cell loss in ventral CA1.

The mean brain temperatures during occlusion, of both ischemic groups combined, accurately predicted histological outcome 3 weeks later (level -1.7 mm; $r = 0.98$, $p = 0.0001$, $n = 14$). Within the IBH group alone this still holds true, animals with higher mean temperatures during occlusion (probably resulting from a slower rate of cooling) had a greater degree of CA1 damage ($r = 0.78$, $p = 0.04$, $n = 7$) but temperatures during occlusion measured at the skull were not predictive of eventual cell loss in this group ($r = 0.07$, $p = 0.88$). Within the NBT group, there was no relationship between temperature during ischemia and histological outcome (most likely as a result of the strict temperature maintenance throughout occlusion). For this group, variability in CA1 damage scores was attributable to

the brain temperature during the first 90 minutes of reperfusion ($r = 0.77$, $p = 0.04$, $n = 7$).

Each of the functional measures used in this study, assessment of open field activity and fEPSPs measured in stratum radiatum, correlated with the extent of CA1 cell loss (see Table 3-4).

Table 3-1. Intraischemic Temperature

Brain, skull and rectal temperature (°C) were recorded at the start of occlusion (S.O.) and during ischemia. Measurements for sham-operated animals were taken at comparable times relative to the period of anaesthesia.

	S.O.	Ischemia (minutes) 1	2	3	4	5
SHAM						
Brain	36.9 ± 0.6	36.9 ± 0.4	36.9 ± 0.3	36.9 ± 0.3	37.0 ± 0.2	37.0 ± 0.2
Skull	37.0 ± 0.6	37.0 ± 0.8	37.1 ± 0.6	37.3 ± 0.5	37.2 ± 0.7	37.1 ± 0.9
Rectal	37.9 ± 0.2	37.8 ± 0.2	37.8 ± 0.2	37.8 ± 0.3	37.8 ± 0.3	37.8 ± 0.3
NBT						
Brain	37.1 ± 0.1	36.8 ± 0.4	36.5 ± 0.6	36.4 ± 0.7	36.3 ± 0.7	36.2 ± 0.5
Skull	37.3 ± 0.5	37.2 ± 0.8	37.2 ± 1.0	37.2 ± 1.0	37.2 ± 0.9	37.2 ± 0.9
Rectal	38.0 ± 0.3	38.0 ± 0.3	38.0 ± 0.3	38.0 ± 0.3	38.0 ± 0.3	38.0 ± 0.2
IBH						
Brain	37.0 ± 0.3	34.7 ± 0.7*	32.5 ± 0.8**	30.7 ± 0.8**	29.6 ± 0.9**	29.4 ± 0.7**
Skull	37.2 ± 0.7	31.4 ± 3.5**	29.8 ± 3.4**	28.7 ± 3.3**	29.6 ± 2.0**	30.2 ± 2.3**
Rectal	37.9 ± 0.3	37.9 ± 0.3	37.9 ± 0.2	37.8 ± 0.2	37.8 ± 0.2	37.7 ± 0.3

Values are the mean ± SD. * $p < 0.05$, ** $p < 0.01$ post-hoc tests (Dunnett *t*) compared the sham-operated group to both ischemic groups.

Figure 3-1. Intra- and postischemic brain temperature

Brain temperature recorded during ischemia/sham operations (-10 minutes to ~ +10 - +15 minutes) and then following surgery in unanesthetized animals. Time 0 indicates the initiation of ischemia, group temperatures at the end of occlusion are indicated 5 minutes after ischemia onset. All values are group means, for clarity the variance has not been indicated but the SD's ranged from 0.05 °C to 0.9 °C. For detailed temperature profiles during ischemia see Table 3-1. Open circles, SHAM group (n=5); solid triangles, NBT group (n=7); solid squares, IBH group (n=7).

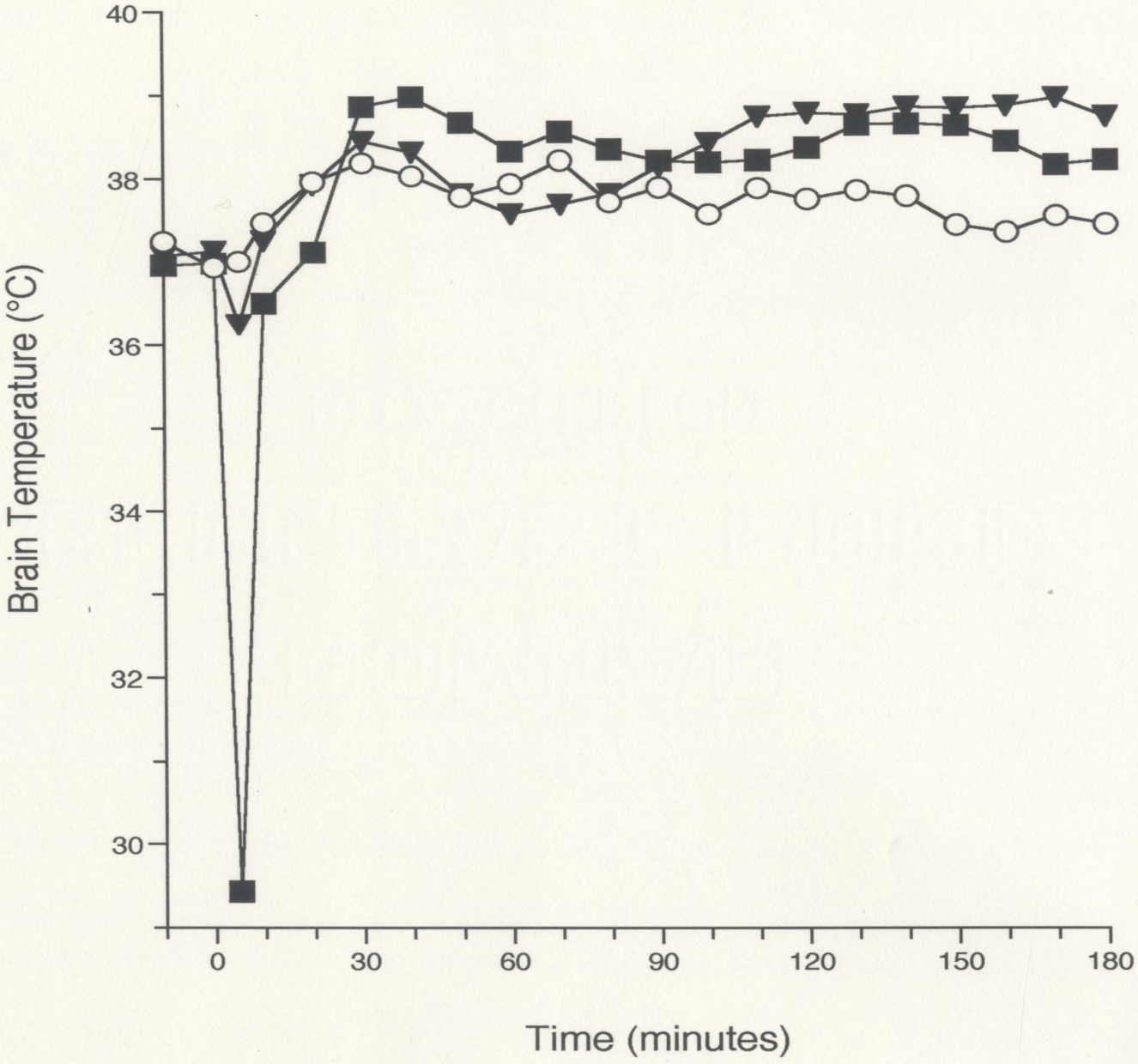

Brain Temperature (°C)
40
38
36
34
32
30
0
30
60
90
120
150
180
Time (minutes)

Table 3-2. Postischemic Temperature

Brain temperature (°C) was recorded on-line every 20 seconds in the postischemic period. Rectal temperature was sampled at 30 and 60 minutes after the end of occlusion. Measurements for sham-operated animals were taken at comparable times relative to the period of anaesthesia.

	Postischemia (minutes)			
	30	60	120	180
SHAM				
Brain	38.3 ± 0.4	37.9 ± 0.2	37.8 ± 0.4	37.4 ± 0.6
Rectal	36.8 ± 0.5	36.5 ± 0.6	------	------
NBT				
Brain	38.4 ± 0.4	37.6 ± 0.5	38.8 ± 0.8	38.7 ± 0.6*
Rectal	37.3 ± 0.7	36.0 ± 0.7	------	------
IBH				
Brain	38.9 ± 0.7	38.3 ± 0.6	38.4 ± 0.6	38.2 ± 0.4
Rectal	38.3 ± 0.9*	37.2 ± 1.2	------	------

Values are the mean ± SD. * $p < 0.05$, post-hoc tests (Dunnett *t*) compared the sham-operated group to both ischemic groups.

Figure 3-2. Postischemic brain temperature and activity counts

Brain temperature and activity were recorded during the postischemic period using Mini-Mitter brain temperature probes which also record activity by telemetry. Brain temperature (°C) is indicated on the left axis and plotted as a line, activity counts are plotted as histograms with the reference scale on the right axis. Time 0 indicates the end of occlusion (and a comparable time relative to anaesthesia in the sham-operated group). All values are group means ± SD, with the number of animals for each group indicated on the graph A, SHAM group; B, IBH group; C, NBT group. Only a subset of animals continued to be monitored after 3 hours therefore a gap is shown on the graph to indicate that the second half of the recordings are not based on the total group. The number of animals after 3 hours varied with increasing time postischemia from the total group at 3 hours, to only 1 animal in the SHAM and NBT groups at 5 hours. Activity and temperature are averaged over 15 minute intervals.

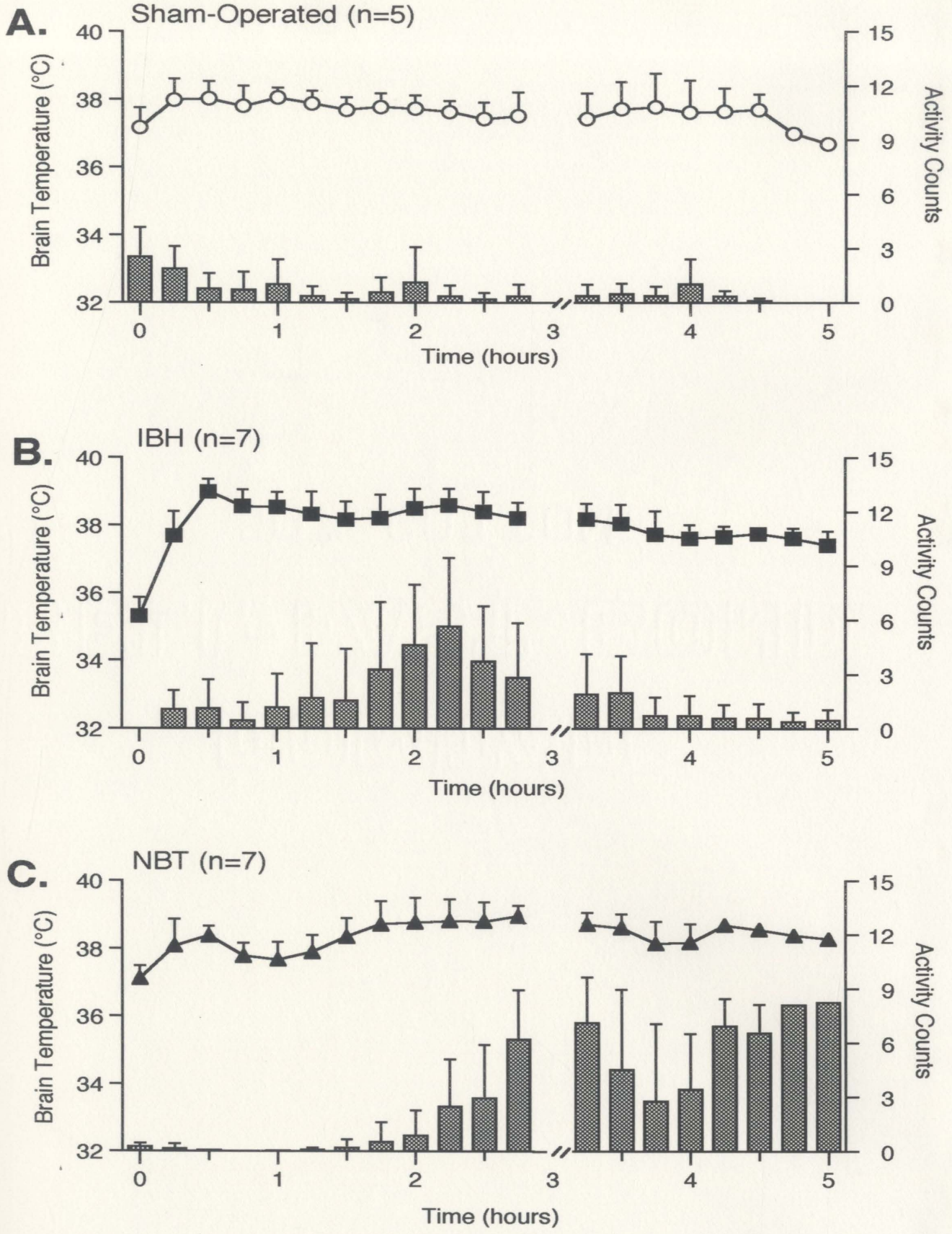

A.
Sham-Operated (n=5)
Brain Temperature (°C)
Activity Counts
Time (hours)
B.
IBH (n=7)
Brain Temperature (°C)
Activity Counts
Time (hours)
C.
NBT (n=7)
Brain Temperature (°C)
Activity Counts
Time (hours)

Figure 3-3. Total open field scores on test days 3, 7 and 10

Sum of the locomotor activity during a 10 minute session in a novel open field, on each of the three postischemic test days. Solid bars, SHAM group (n=6); hatched bars, NBT group (n=7); open bars, IBH group (n=7). All values are the group means ± SEM. There was a significant treatment effect ($F_{2,17} = 37.812$, $p < 0.0001$) as a result of the heightened activity exhibited by the NBT group on all test days, which was significantly different from sham-operated controls (**, $p < 0.01$, Dunnett t).

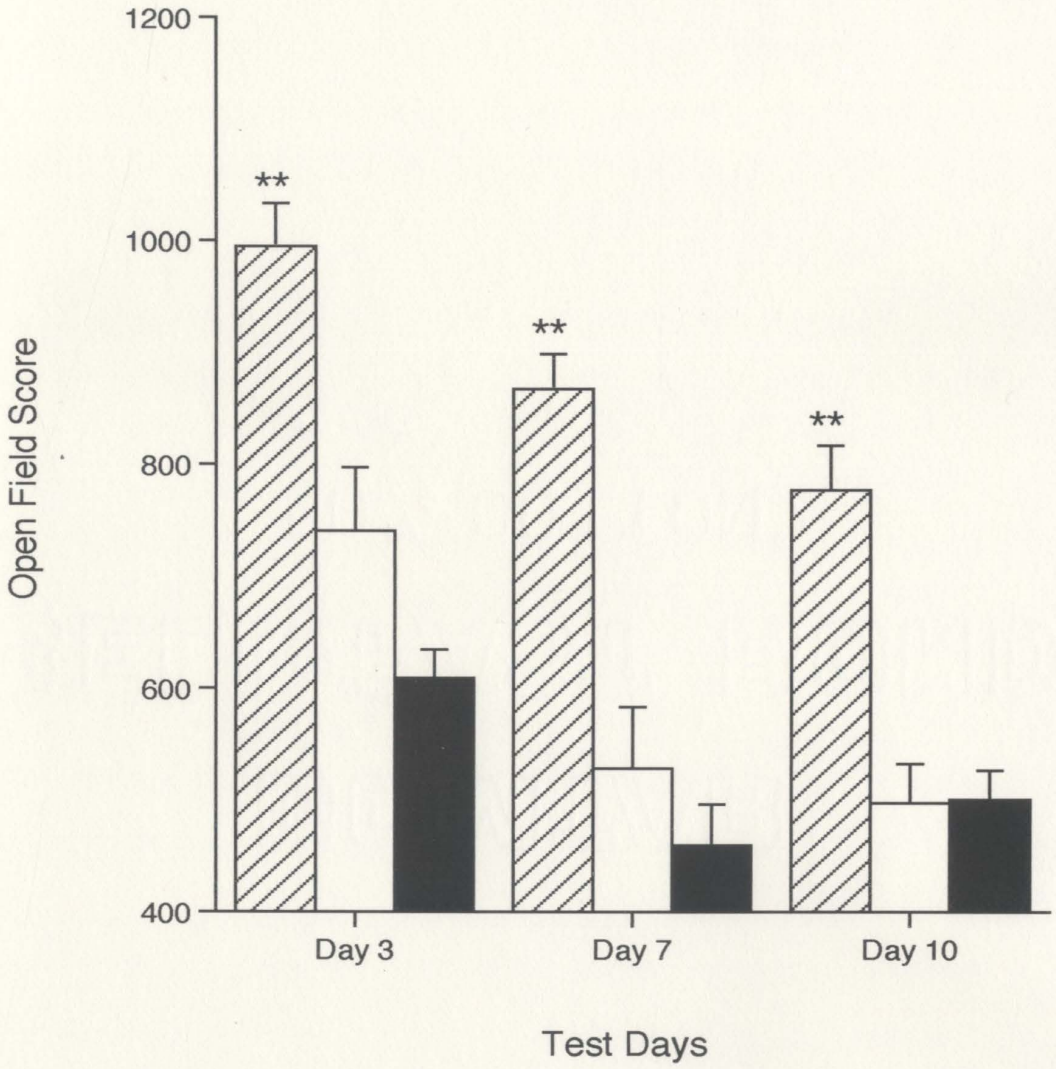
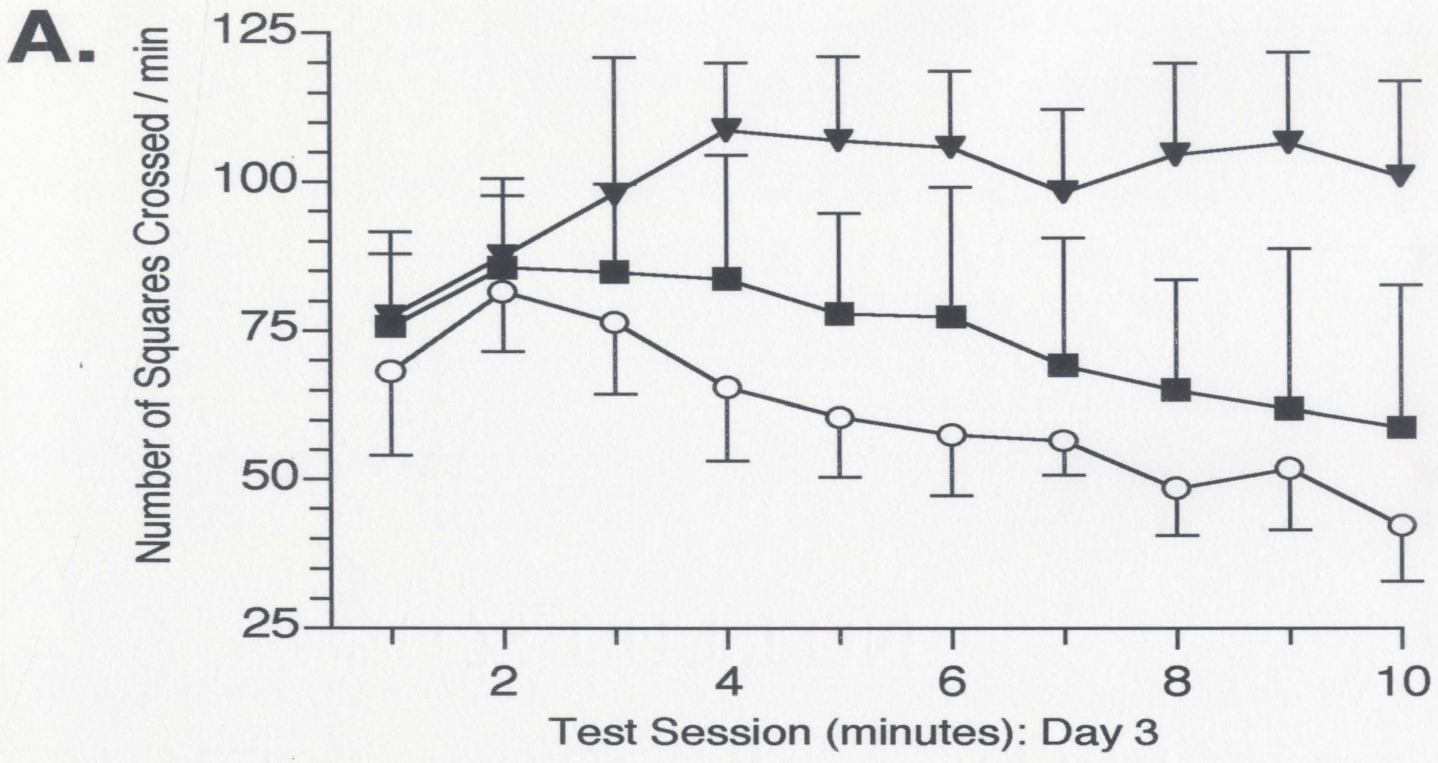

1200
1000
800
600
400
Open Field Score
**
**
**
Day 3
Day 7
Day 10
Test Days

Figure 3-4. Individual group scores, per minute- Open field test day 3

A. This graph is a minute by minute portrayal of the data from Figure 3-3, test day 3. Values are the group means ± SD. Open circles, SHAM group (n=6); filled triangles, NBT group (n=7); filled squares, IBH group (n=7).

B. The IBH group has been divided into two separate groups based on the open field behaviour. Squares with the bottom right corner filled are the IBH-low group (n=4) and these animals behaved in a similar manner as the sham-operated animals; squares with the top left corner filled are the IBH-high group (n=3). The SHAM and NBT groups have been replotted for comparison to the bimodal responses of the IBH group.


A.
Number of Squares Crossed / min
125
100
75
50
25
2
4
6
8
10
Test Session (minutes): Day 3

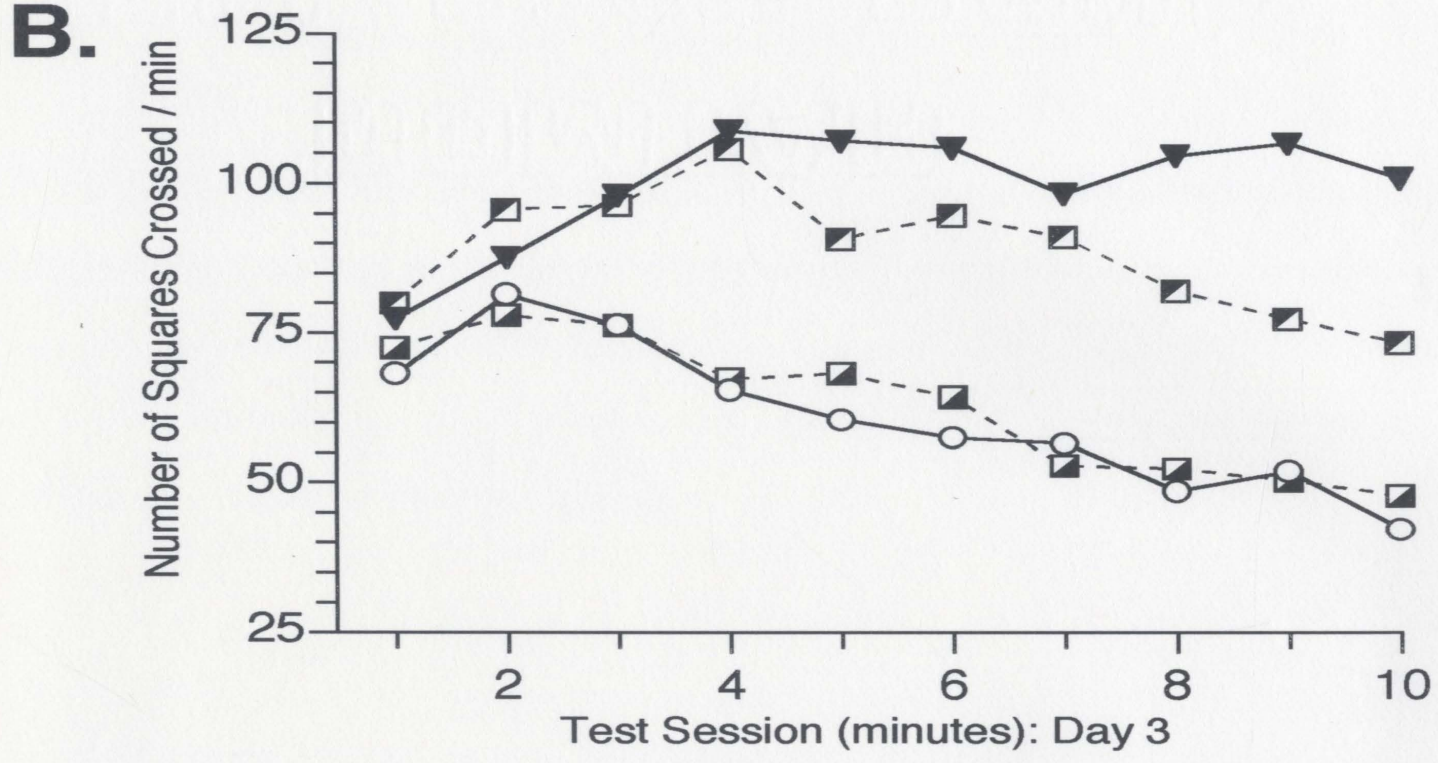
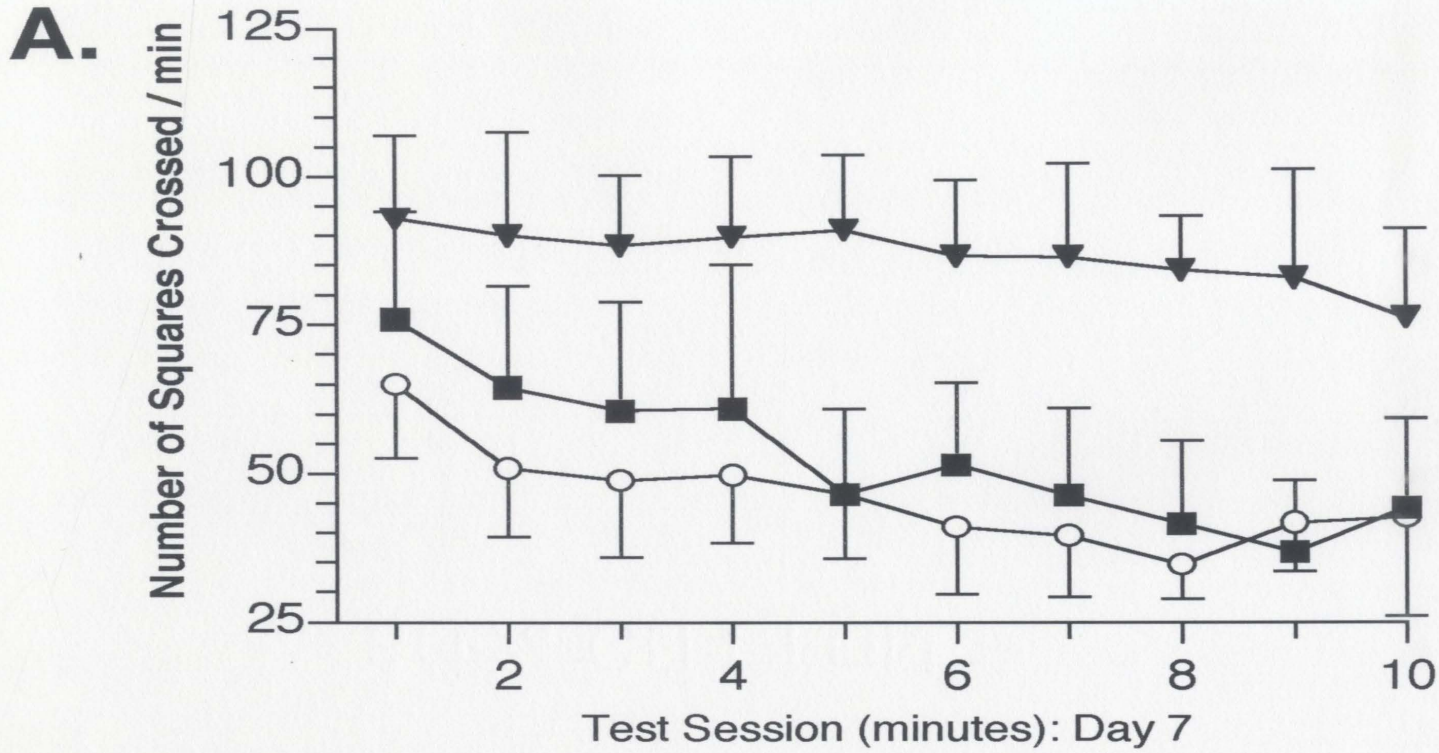

B.
Number of Squares Crossed / min
125
100
75
50
25
2
4
6
8
10
Test Session (minutes): Day 3

Figure 3-5. Individual group scores, per minute- Open field test day 7

A. This graph is a minute by minute portrayal of the data from Figure 3-3, test d 7. Values are the group means ± SD. Open circles, SHAM group (n=6); filled triangles, NBT group (n=7); filled squares, IBH group (n=7).

B. The IBH group has been divided into two separate groups based on the ope field behaviour on day 3. Squares with the bottom right corner filled are the IB low group (n=4); squares with the top left corner filled are the IBH-high group (n=3). The SHAM and NBT groups have been replotted for comparison to the bimodal responses of the IBH group.


A.
Number of Squares Crossed / min
125
100
75
50
25
2
4
6
8
10
Test Session (minutes): Day 7

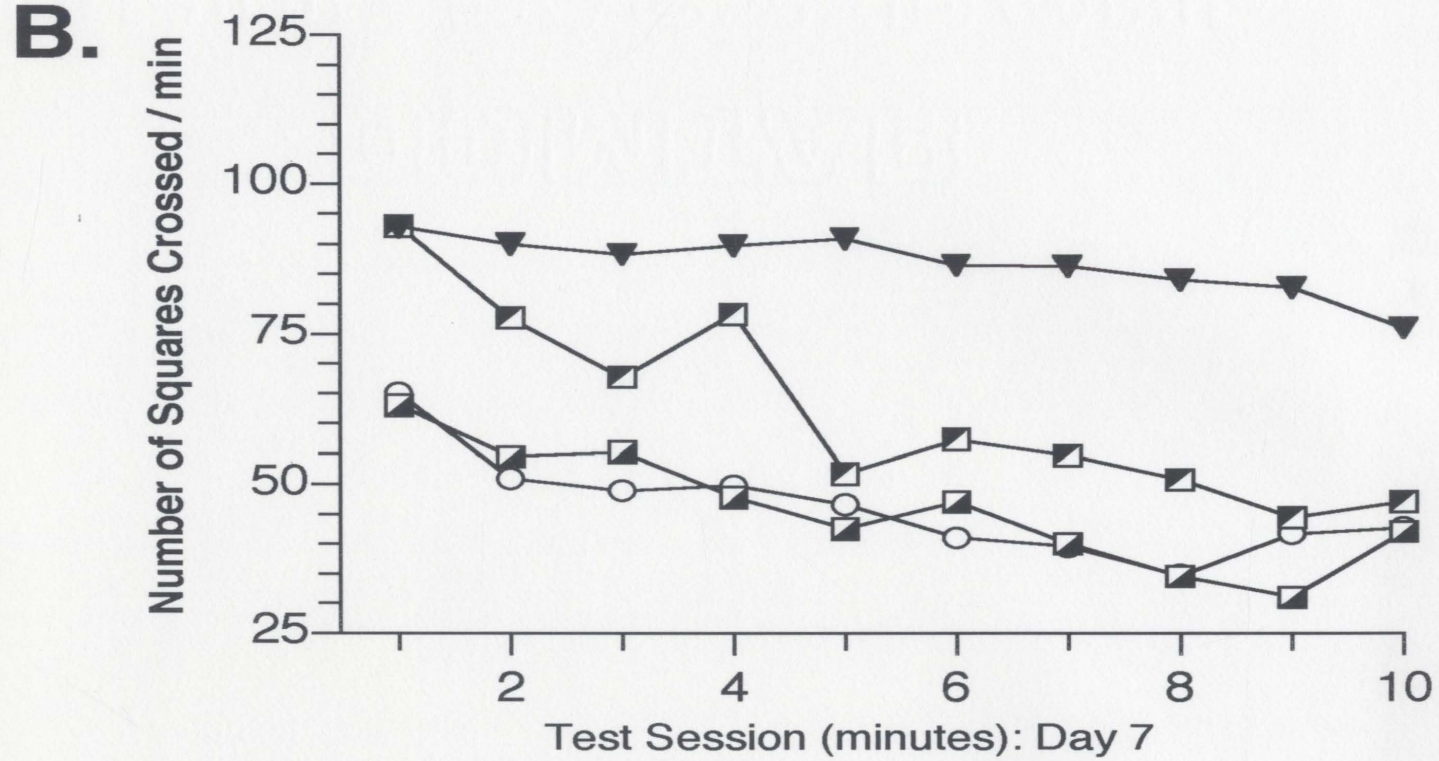
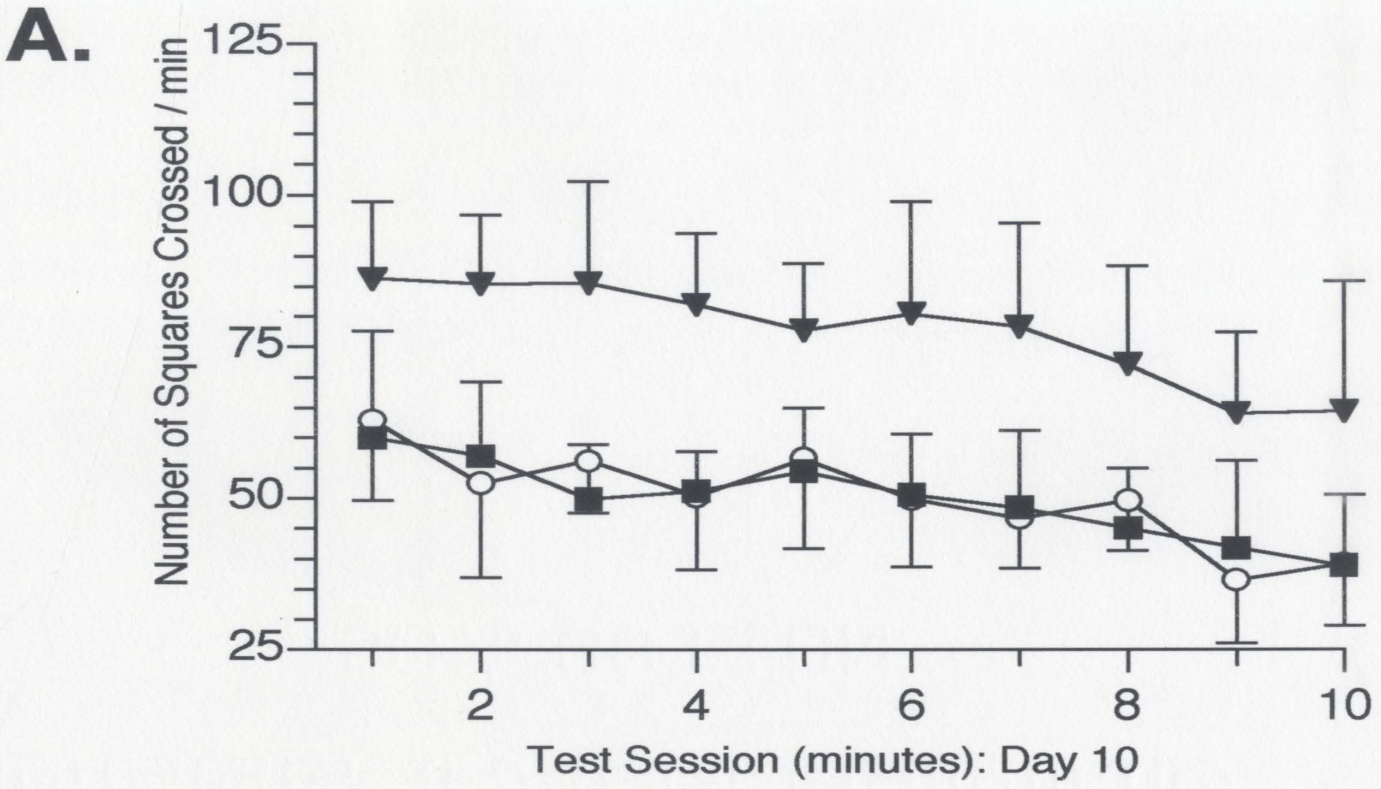

B.
Number of Squares Crossed / min
125
100
75
50
25
2
4
6
8
10
Test Session (minutes): Day 7

Figure 3-6. Individual group scores, per minute- Open field test day 10

A. This graph is a minute by minute portrayal of the data from Figure 3-3, test day 10. Values are the group means ± SD. Open circles, SHAM group (n=6); filled triangles, NBT group (n=7); filled squares, IBH group (n=7).

B. The IBH group has been divided into two separate groups based on the open field behaviour on test day 3. Squares with the bottom right corner filled are the IBH-low group (n=4); squares with the top left corner filled are the IBH-high group (n=3). The SHAM and NBT groups displayed in (A) have been replotted.


A.
125
100
75
50
25
Number of Squares Crossed / min
2
4
6
8
10
Test Session (minutes): Day 10

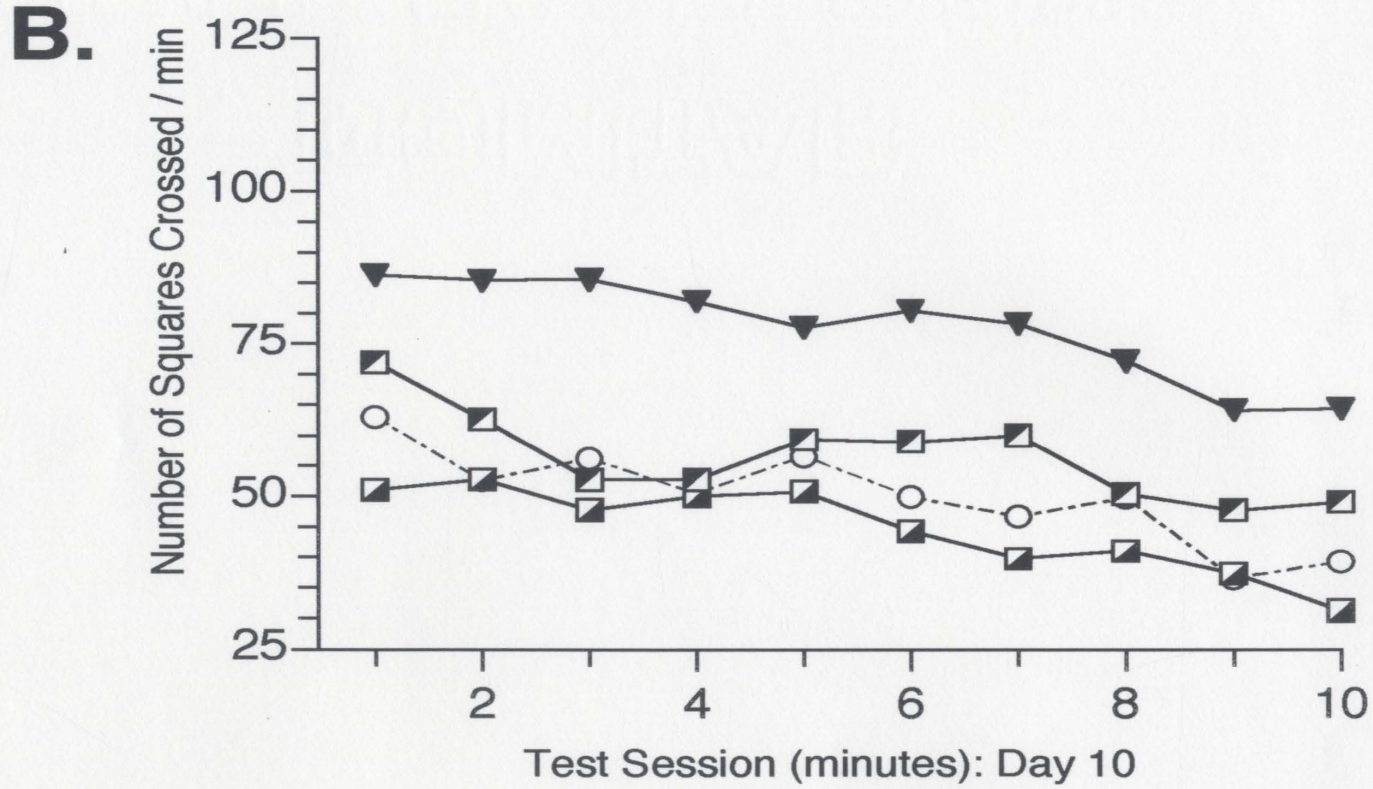

B.
125
100
75
50
25
Number of Squares Crossed / min
2
4
6
8
10
Test Session (minutes): Day 10

Table 3-3. Percentage change in body weight assessed weekly following ischemia.

Treatment Groups	Weight prior to ischemia (g)	Weight change following ischemia (%) 7 days	14 days	21 days
SHAM	69.50 ± 8.33	+ 4.65 ± 8.1	+ 4.57 ± 4.6	+ 8.13 ± 8.5
NBT	71.18 ± 7.24	- 1.04 ± 4.0	+ 1.53 ± 4.5	+ 2.90 ± 5.1
IBH	68.71 ± 7.78	+ 1.13 ± 3.6	+ 2.23 ± 5.8	+ 5.00 ± 5.8

Values are the mean ± SD. There were no significant differences in weight among the three treatment groups.

Figure 3-7. Representative traces of EPSPs

Representative excitatory post-synaptic potentials recorded in the CA1 dendritic field from septal slices in each treatment group: A, NBT; B, IBH; C, SHAM. Field potentials were similar in both the IBH and SHAM groups. However, orthodromic stimulation of CA1 in a hippocampal slice from the NBT group only elicited a fiber volley, which represents multiple action potentials in the presynaptic axons.

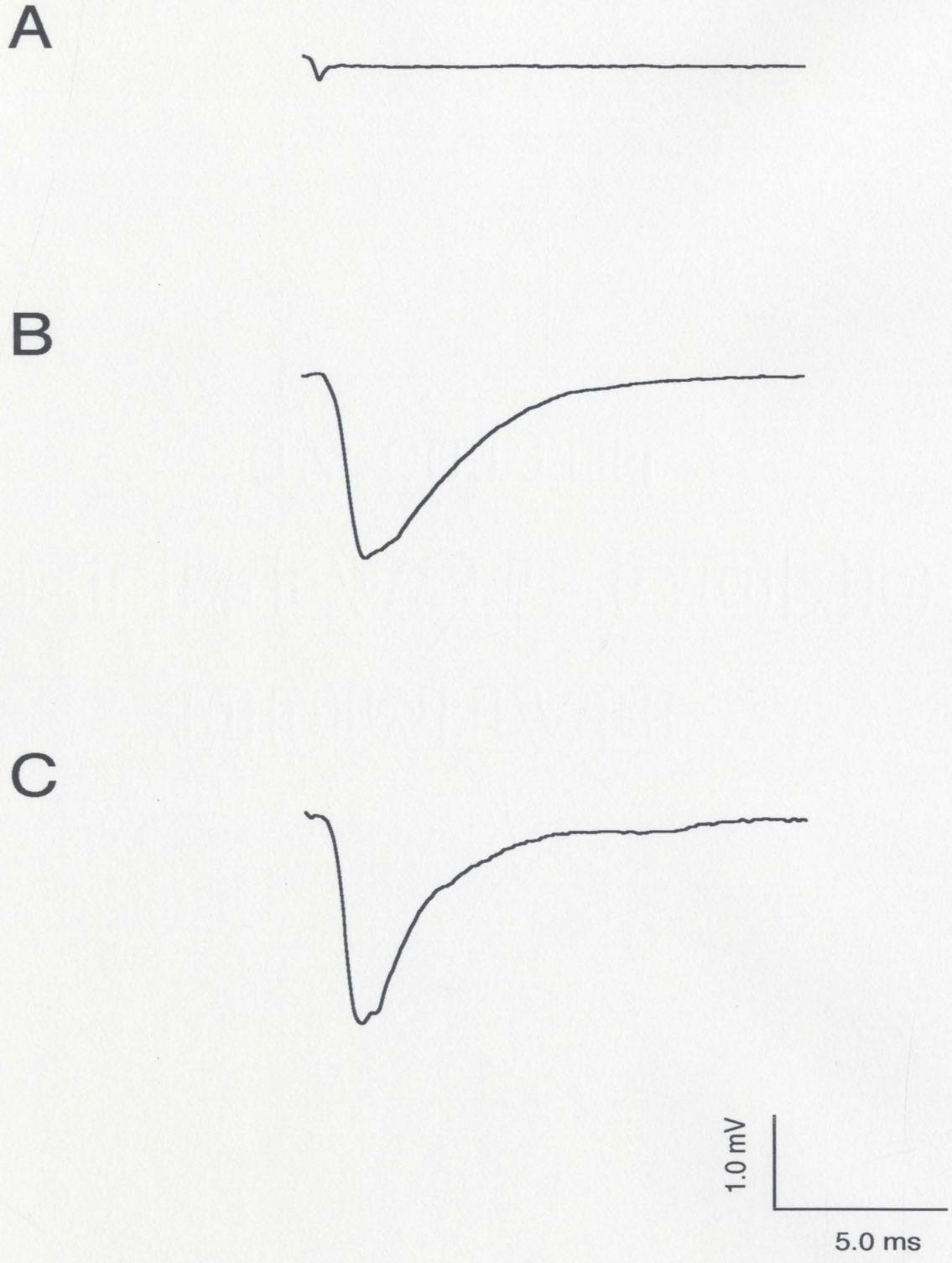

A
B
C
1.0 mV
5.0 ms

Figure 3-8. Summary of amplitude, slope and area measurements

Measurements of CA1 fEPSPs recorded in stratum radiatum from 55 hippocampal slices (SHAM, n=16; NBT, n=19; IBH, n=20) 3 weeks postischemia. Each figure illustrates the group means ± SD. A, fEPSP amplitude; B, fEPSP slope; C, fEPSP area. ANOVA indicated a significant treatment effect for all three measures ($F_{2,52} = 38.97$, 17.701 and 17.198, respectively; $p < 0.0001$) resulting from a reduction in the size of field potentials recorded from the NBT group (**, $p < 0.01$, Dunnett t).

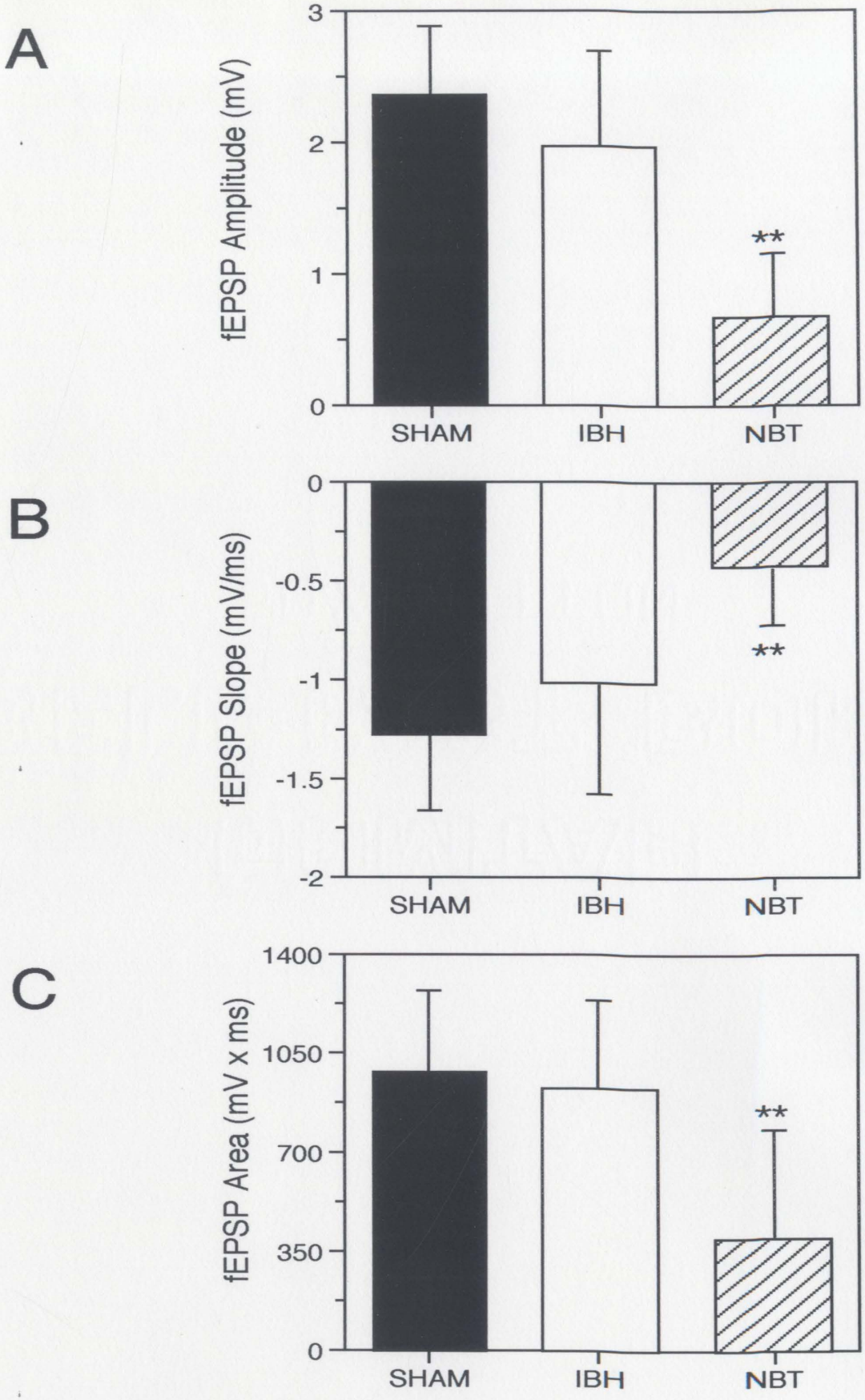

A
fEPSP Amplitude (mV)
3
2
1
0
**
SHAM
IBH
NBT
B
fEPSP Slope (mV/ms)
0
-0.5
-1
-1.5
-2
**
SHAM
IBH
NBT
C
fEPSP Area (mV x ms)
1400
1050
700
350
0
**
SHAM
IBH
NBT

Figure 3-9. Histological assessment of CA1 at three rostro-caudal levels.

CA1 cell counts were made at three levels posterior to bregma (-1.7 mm, -2.2 mm and -2.8 mm), the sectors of CA1 that were rated are indicated by the boxes on the line drawings. Plotted points represent the individual scores for each animal; SHAM group (n=6); NBT group (n=7); IBH group (n=7). Ratings of dorsal CA1 are indicated on the left and ventral CA1 on the right. Evaluation of dorsal CA1 at the most rostral levels (-1.7 and -2.2 mm) is based on the summed ratings from 3 sectors (maximum score = 12). One sector was rated for the assessment of dorsal CA1 at the most caudal level (-2.8 mm) and both ventral levels (maximum score = 4). There was a significant treatment effect at all dorsal levels examined ($H = 14.27$, 13.80 and 12.81 for -1.7, -2.2 and -2.8, respectively; $p < 0.01$, Kruskal-Wallis test) as a result of the extensive cell loss from CA1 observed in animals subjected to ischemia at normothermia (NBT group).

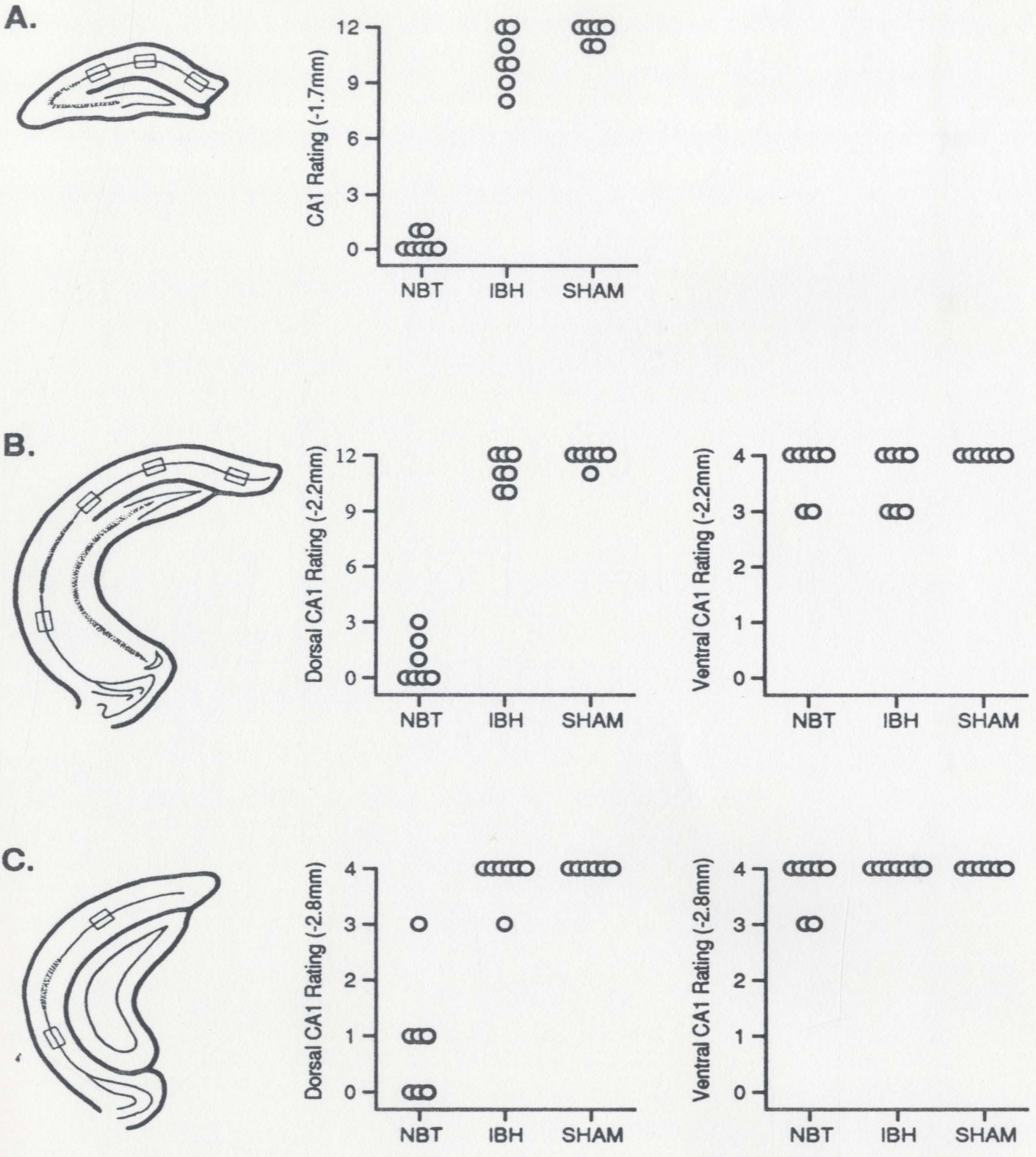

A.
CA1 Rating (-1.7mm)
12
9
6
3
0
NBT
IBH
SHAM
B.
Dorsal CA1 Rating (-2.2mm)
Ventral CA1 Rating (-2.2mm)
4
3
2
1
C.
Dorsal CA1 Rating (-2.8mm)
Ventral CA1 Rating (-2.8mm)

Table 3-4. Correlation matrix.

Variables are derived from the three assessment methods; an open field test, extracellular field recordings obtained from stratum radiatum in hippocampal slices 3 weeks after ischemia and morphological assessment of dorsal CA1 at three rostro-caudal levels (distance from bregma is given in mm).

	Open Field Behaviour			fEPSPs Stratum Radiatum			Histological Assessment Dorsal CA1		
	day 3	day 7	day 10	amplitude	slope	area	-1.7	-2.2	-2.8
Behavior									
day 3									
day 7	0.82								
day 10	0.70	0.90							
fEPSPs									
amplitude	-0.81	-0.76	-0.76						
slope	0.73	0.66	0.66	-0.93					
area	-0.68	-0.70	-0.77	0.87	-0.71				
CA1 Rating									
-1.7 mm	-0.84	-0.90	-0.87	0.88	-0.76	0.82			
-2.2 mm	-0.83	-0.89	-0.88	0.86	-0.76	0.79	0.98		
-2.8 mm	-0.78	-0.85	-0.82	0.76	-0.66	0.72	0.93	0.96	

In all cases, $p < 0.01$, n=20.

Table 3-5. Functional and histological measurements from the low and high sub-divisions of the IBH group.

The IBH group was divided into IBH-low and IBH-high based on the behaviour in the open field on Day 3. The CA1 ratings and fEPSP amplitudes along with the total number of squares crossed on Day 3 are shown below. Values are the mean ± the standard deviation, the small sample size precluded statistical analysis.

Group Sub-divisions	Day 3 Open Field Score	fEPSP Amplitude	Dorsal CA1 Rating		
			-1.7 mm	-2.2 mm	-2.8 mm
IBH-low (n=4)	629.5 ± 68.9	2.13 ± 0.2	11.25 ± 0.96	11.75 ± 0.5	4.0 ± 0
IBH-high (n=3)	886.7 ± 68.3	1.85 ± 0.44	9.0 ± 1.0	10.3 ± 0.58	3.67 ± 0.58

3.4 Discussion

This study demonstrates that intraischemic hypothermia provides near complete protection of CA1 pyramidal cells from an episode of cerebral ischemia. Not only was there no significant CA1 cell loss 3 weeks post-occlusion if intraischemic hypothermia was administered, but also, typical behavioural impairments associated with ischemic injury were absent and CA1 synaptic transmission was preserved. While other experiments have utilized behavioural tests in conjunction with histological assessment to demonstrate both functional and morphological protection, this study is unique in that electrophysiological measures were combined with behavioural and histological evaluations of CA1 integrity. Functional measures of assessment can verify whether a putative protective treatment is efficacious. It is possible that some drug therapies, which appear to be protective histologically, may fail to yield functional protection because cells are in a transitional state from which they may or may not recover.

Early postischemic changes in behaviour are indicative of ischemic injury. Following a 5 minute occlusion, animals are typically inactive initially and exhibit a period of "postischemic hunch", a postural change characterized by kyphosis of the spine. This is reflected in Figure 3-2C, where the ischemic hunch posture lasted 1.5 - 2 hours in the NBT group. Following this period of inactivity, there is a second phase where animals become hyperactive. This behavioural activation is discussed in more detail in Chapter 5. In the intraischemic hypothermia group the postischemic behavioural activity pattern was altered. The peak behavioural

activation occurred sooner and within 3.5 - 4 hours post-occlusion the activity levels had returned to control levels.

Open field testing in this study was carried out from 3 to 10 days postischemia. If hypothermia had *not* preserved normal function, then testing during this time period would be more likely to detect an impairment since behavioural tests should be most sensitive before any substantial recovery of function has taken place. No significant differences in locomotor activity were found between the IBH and SHAM groups exposed to a novel open field, even during the first week postischemia. However, there was a non-significant tendency for the IBH group to display higher levels of locomotor activity than the SHAM group 3 and 7 days postischemia (Figure 3-3), whereas on day 10 full recovery had taken place. As shown in Figure 3-4B, the trend for slightly high activity scores in the open field was due to the behaviour of 3 animals in the IBH group, referred to as the IBH-high sub-group. The NBT group displayed very high activity levels and a much slower rate of habituation to the environment over all three test sessions. These results suggest that it is important to conduct behavioural tests (depending on task complexity) within the first weeks after occlusion in order to detect functional impairments. This would be essential in cases of partial protection produced by any type of therapeutic intervention.

It has previously been shown that the open field task is sensitive to hippocampal damage resulting from forebrain ischemia and impairments are unrelated to cell loss in other brain regions such as striatum (Mileson and

Schwartz, 1991). The present findings imply that hippocampal function is spared when hypothermia is administered during the five minute ischemic episode. Using the Morris water maze task, Green et al. (1992) have also suggested that intraischemic hypothermia preserves hippocampal function. However, their functional measure was not predictive of the histological outcome. In the present study both open field locomotor activity and the electrophysiological competence of CA1 pyramidal cells was predictive of dorsal CA1 cell ratings (Table 3-4). In Table 3-5 measurements from the functional and histological assessments of the two sub-divisions of the IBH group are presented. Animals in the IBH-high sub-group had *higher* activity scores in the open field on Day 3 than the IBH-low sub-group (see also Figure 3-4B), correspondingly fEPSP amplitudes and CA1 cell ratings were *lower* in the IBH-high sub-group. These findings reveal that the behavioural measure in the open field is able to predict small differences in the ultimate histological outcome (~ 84 % versus 97 % dorsal CA1).

Previous studies (Buzsáki et al., 1989; Urban et al., 1989, 1990; Jensen et al., 1991; Kirino et al., 1992) have utilized electrophysiological measures to study changes in neuronal transmission and to monitor the progression of cell death during the initial hours and days after an ischemic event. To the best of our knowledge this is the first report using extracellular recording techniques several weeks after occlusion to demonstrate that a putative therapy for ischemic injury has preserved normal synaptic function in the CA1 region. Measuring the

physiological properties of CA1 neurons has the advantage that recovery of function and compensatory mechanisms arising from extra-hippocampal brain structures which help reduce behavioural deficits several weeks after ischemia do not interfere with the *direct* functional assessment of CA1. Field responses recorded in stratum radiatum from the IBH group were similar to those from SHAM controls on measures of amplitude, area and slope. Again, as seen with early behavioural testing, there was a slight trend for the size of the CA1 field potentials to be reduced in the IBH group versus SHAM controls (see Fig. 4A). This slight reduction in field potential size most likely reflects a small loss of CA1 neurons in some animals which is consistent with the morphological outcome (i.e. the IBH-high group). Within the NBT group, there was a noticeable variation in response depending on where the slice had been taken along the septo-temporal axis of the hippocampus. Field responses in the majority of NBT slices were severely attenuated or completely absent. However, some temporal slices which correspond to the more ventral aspect of the hippocampus had much larger fEPSPs that in a few cases were near normal in size. These differences between dorsal and ventral CA1 were also observed in the histological assessment.

The single most important determinant of CA1 injury in this study was the brain temperature *during* occlusion. This was most obvious within the IBH group because of a greater range in intraischemic brain temperatures. When CA1 ratings for this group were compared to minute by minute brain temperatures

throughout the period of ischemia it was found that only one time point was predictive of histological outcome. The critical factor which determined whether an animal would have total or near total protection was the brain temperature 3 minutes into the occlusion ($r = 0.87$, $p < 0.01$, $n = 7$). Both *in vitro* and *in vivo* ischemia models have independently shown that 2.5 - 3 minutes appears to be a threshold point below which no permanent injury occurs, but beyond this time CA1 neurons are irreversibly damaged (Nowak et al., 1985; Kato et al., 1989; Raley and Lipton, 1990; Andou et al., 1992). Within the IBH group, the least protection (-1.7 mm: rating scores 8/12 and 9/12) was found in animals that had the highest brain temperatures following 3 minutes of ischemia (31.9 °C and 31.8 °C, respectively). Whereas the two animals with complete protection (-1.7 mm: rating scores 12/12) had the lowest brain temperatures at this time, 30.1 °C and 30.0 °C. Thus, complete protection against a 5 minute occlusion could be obtained if the brain temperature was cooled to a sufficient level before the three minute threshold point was crossed. Interestingly, Raley and Lipton (1990) have shown that hippocampal slices subjected to *in vitro* "ischemia" for 2 minutes will quickly recover protein synthesis to normal levels, whereas such treatment for a duration of 5 minutes will result in an extended period of protein synthesis inhibition. Intraischemic hypothermia, *in vivo*, may exert its protective action by preventing the pathogenesis which occurs after 2.5 - 3 minutes of ischemia thus allowing recovery of protein synthesis and normal neuronal function (see Widmann et al., 1993). In addition to the importance of intraischemic brain

temperature, postischemic temperature can also influence outcome. As seen in the NBT group, small variations in brain temperature during the first 90 minutes of reperfusion had significant effects on both functional and histological outcome.

A question raised by the present data is whether intraischemic hypothermia provides permanent neuroprotection. Some of the animals treated with intraischemic hypothermia may not have complete protection as evidenced by the observed trend for this group to display small, non-significant deficits in comparison to sham-operated controls on all three assessments. However, these slight differences were apparent within the first week after ischemia and were not exacerbated over time as would be expected if cell loss was continuing beyond 7 days post-occlusion. In addition to histological outcome, the electrophysiological recordings made 3 weeks postischemia indicated that synaptic transmission was normal which is suggestive of permanent protection of CA1 neurons. Consistent with our findings, Dietrich et al. (1993) have shown that when the brain is cooled to 30 °C during the period of ischemia histological protection is still observed 2 months later. Permanent functional as well as histological protection, resulting from hypothermic intervention during ischemia has also been demonstrated by Green and colleagues (1992) in a rat model of global ischemia.

In this study, open field behaviour and extracellular field recordings evoked in the CA1 dendritic field both predicted histological outcome. The

functional measures correlated significantly with each other and with dorsal CA1 ratings. It would seem that any one of these measures alone could adequately determine outcome following ischemia. However, this consistent relationship between all three outcome measures may only exist when a treatment has provided lasting neuroprotection. For example, other treatments such as drug therapy or postischemic hypothermia may not provide chronic protection, but only delay cell death (Dietrich et al., 1993; Colbourne and Corbett, 1994a). In view of these considerations it would seem advantageous to combine histopathological assessment with functional assessments using long survival times (e.g. at least several weeks). This approach could provide a more accurate indication of the viability of hippocampal neurons, particularly in pharmacological studies such as the next experiment.

4.1 Introduction

Delayed cell death resulting from cerebral ischemia is thought to be ultimately due to an excessive rise in free Ca^{2+} within vulnerable neurons, triggered by the excitatory neurotransmitter glutamate (Choi, 1988; Siesjö and Bengtsson, 1989). Initial optimism that NMDA receptor antagonists might be effective in treating stroke (Gill et al., 1987) has been tempered by the finding that the most potent of these compounds, MK-801, produced its neuroprotective action by inducing hypothermia (Buchan and Pulsinelli, 1990; Corbett et al., 1990; Nellgård et al., 1991). More recently, the AMPA antagonist, NBQX, was reported to provide robust protection against neuronal loss in several animal models, even with postischemic treatment (Sheardown et al., 1990; Buchan et al., 1991a; Diemer et al., 1992; Nellgård and Wieloch, 1992; Sheardown et al., 1993). Accordingly, it has been concluded that the AMPA receptor has a greater involvement in global ischemic injury than the NMDA receptor and stroke trials with AMPA receptor antagonists have been suggested (Buchan et al., 1993).

In the majority of studies in which NBQX has provided protection, however, temperature was not controlled during the postischemic period (Sheardown et al., 1990; Diemer et al., 1992; Sheardown et al., 1993). In view of the fact that many pharmacological studies of cerebral ischemia have been confounded by drug-induced hypothermia, it is essential that putative neuroprotective agents are examined closely to rule out similar interactions with brain temperature. In addition, recent data suggest that the preservation of CA1 neurons by several postischemic treatments may not be permanent (Morse and Davis, 1990; Dietrich et al., 1993; Colbourne and Corbett, 1994a). Since most pharmacological studies,

including those with NBQX, have utilized short survival times (1 week or less), the protection observed with this drug may also be transitory.

Although it has been reported that NBQX does not affect temperature (Sheardown et al., 1990), a significant decrease in temperature following NBQX administration has been noted when temperature has been monitored for prolonged periods (Suga and Nowak, 1992; Nurse and Corbett, 1993). Using the gerbil 2-vessel occlusion model, the postischemic temperature profiles of animals treated with NBQX were compared to those of vehicle controls. In order to determine if hypothermia contributed to the beneficial effect of NBQX a third group was given NBQX but their temperature was *regulated* from 1 to 24 hours postischemia, so that it mimicked the pattern in vehicle treated animals. In a second experiment, the NBQX temperature profile was simulated in the absence of drug treatment and the histological outcome was compared to that of NBQX treated gerbils at survival times of 4 and 10 days.

4.2 Methods

4.2.1 Brain Temperature Monitoring

Brain temperature was recorded on-line from AM-transmitter probes inserted through a guide-cannula into the dorsal striatum, as described in section 2.2. Two days prior to the administration of NBQX or induction of ischemia, baseline temperature was recorded for 3 hours in order to ensure readings were within the normal range. The sampling rate for all temperature recordings was 3/minute.

4.2.2 Temperature Effects of NBQX in Normal Animals

Two rodent species commonly used in models of ischemia, Sprague-Dawley rats (n = 4) and gerbils (n = 5), were examined for their temperature response to NBQX. Two days after initial temperature recording (see 4.2.1), temperature probes were reinserted into the guide-cannula. Following baseline recording for 1 hour, NBQX was administered as three separate intraperitoneal injections (3 x 30 mg/kg, i.p.). Injections were spaced 15 minutes apart. The drug was dissolved in NaOH (0.1 M) and diluted in 5.5% glucose, final pH was 8.5 ± 0.1. Brain temperature was monitored for 24 hours. One gram of NBQX (NNC 07-9202 acid) was donated by Novo-Nordisk, Copenhagen. All subsequent experiments used gerbils only.

4.2.3 Cerebral Ischemia

Gerbils were anaesthetized with 2% halothane in 30% O_2/70% N_2O and maintained at 1.5% throughout the operative procedure. The carotid arteries were isolated and occluded for 3 minutes at normothermia, as described in section 2.3. Sham-operated animals (n = 3) underwent carotid isolation without ischemia. Brain temperature continued to be monitored postischemically in unanaesthetized animals for up to 30 hours. During the initial 60 minutes of reperfusion, a 60 watt lamp was used to maintain normothermia (~ 37 °C), if necessary. Mild postischemic hyperthermia, which occurs spontaneously, was not prevented.

Experiment I.

Animals received either vehicle (5.5% glucose, pH 8.5, n = 7) or NBQX (30 mg/kg, i.p., n = 16) at 60, 75 and 90 minutes postischemia. NBQX treated animals were subdivided into two groups (n = 8 per group): NBQX and NBQX (regulated). Temperature regulation was discontinued in all groups 60 minutes after the occlusion, except the NBQX (regulated) group. In the NBQX (regulated) group the brain temperature was maintained at the same level observed in the vehicle treated group using an overhead lamp, with a variable height adjustment, until 24 hours post-occlusion. Following the period of temperature recording (24 - 30 hours), temperature probes were removed and the animals were returned to their home cages. Behavioural testing in the open field was conducted on days 3, 7 and 10. Following the last open field session the animals were given a lethal injection of barbiturate and perfused (see section 2.6).

Experiment II.

The above experiments were repeated to assess vehicle (n = 6) and NBQX (*no* temperature regulation, n = 9) treatments at a survival time of 4 days. A third treatment group was also added, simulating the NBQX-induced temperature profile in the absence of drug treatment (SimTP). The NBQX-induced temperature profile was simulated manually (SimTP) for 28 hours postischemia using (1) a fan placed above the cage and/or spraying room temperature water on the animal's back to lower temperature; (2) turning off the fan and/or using a 60 watt lamp to elevate temperature. Animals in the SimTP group had survival times of 4 days (n = 7) and 10 days (n = 10). Assistance with temperature regulation was provided by Suzanne Evans, Kathleen McKay and Dale Corbett.

Five additional animals were employed for electrophysiological recording 4 days postischemia. Two animals were occluded for 3 minutes and given NBQX (3 x 30 mg/kg) 1 hour postischemia. Two animals were implanted with a guide cannula for temperature recording, but did not have ischemia surgery. Brain probes were reinserted 2 days after baseline temperature recording and the animals placed in the recording boxes on AM receivers. After acquiring an hour of baseline data they were injected with the same dose of NBQX as the ischemic animals received. This group was used as a control for the long-lasting effects of NBQX and the possible toxicity of this compound with respect to hippocampal field potentials. A fifth animal was occluded for 3 minutes and then sacrificed for electrophysiology at the same time point as the other animals, 4 days postischemia/injection. This animal did not receive NBQX.

4.2.4 Histological Analysis

At either 4 or 10 days postischemia, animals received an overdose of sodium pentobarbital prior to perfusion fixation. Brain tissue was placed in 10% phosphate-buffered formalin (pH 7.4) overnight, *in situ*. The following day, the brain was extracted from the skull and stored in the same fixative. Blocks were dehydrated and embedded in paraffin. Sections were cut at 6 μm and subsequently stained with haematoxylin and eosin. Some of the histological processing was done by Suzanne Evans and Kathleen McKay. Three sectors in dorsal CA1 (lateral, middle and medial) were counted in each hemisphere at a level 1.7 mm posterior to bregma. Cell counts were performed by Dale Corbett who was blind to the treatment status of the animals. The six sectors were then

summed to yield a total CA1 score. CA1 cell counts from six control animals were pooled with the 3 sham-operated animals to provide an assessment of uninjured/control CA1.

4.2.5 Statistical Analysis

Total CA1 scores were analyzed with ANOVA, followed by Student-Newman-Keuls post hoc comparisons.

4.3 Results

When NBQX (3 x 30 mg/kg, i.p.) was given to non-ischemic animals (Figure 4-1), there was an initial drop in brain temperature of 2.0 °C in gerbils and 3.5 °C in rats. Three to 5 hours following drug administration, temperature partially recovered, but remained below the control level for longer than 24 hours. The changes recorded in brain temperature were also reflected in rectal temperature, which was sampled periodically in some animals (data not shown).

With postischemic NBQX administration, in the unregulated condition (Figure 4-2D), there was also an ~ 2 °C decrease in brain temperature below that observed in the vehicle treated group (Figure 4-2B). The NBQX temperature profile was altered by ischemia (compare Figure 4-1A and Figure 4- 2D). During ischemia and the initial 2 hours of reperfusion, the brain temperatures of NBQX (Figure 4-2D) and vehicle (Figure 4-2B) groups were not different. Thereafter, the temperature of NBQX treated animals was significantly reduced, 1.0 to 2.0 °C, with a maximal difference (~ 2.0 °C) evident 2.5 - 3 hours postischemia. Beginning 3 hours postischemia, the temperature gradually declined in *both* the NBQX and

vehicle treated groups, leveling off ~ 1.5 °C below the starting point at 15 hours postischemia. The slopes of the temperature profiles in the NBQX and vehicle treated groups were similar, with the NBQX group temperature profile being shifted 1.0 - 1.5 °C below the vehicle group. The brain temperature in the NBQX group was still 1.0 - 1.5 °C lower at 30 hours postischemia (Figure 4-2D). In an additional group treated with NBQX, brain temperature was manually regulated until 24 hours postischemia (Figure 4-2C) to prevent the drug-induced decrease in temperature. Interestingly, within 15 minutes following the termination of temperature regulation in this group (i.e. ~ 24 hours post-occlusion), brain temperature fell 1.0 °C and remained 1.0 - 1.5 °C below the vehicle group until the end of the recording session (Figure 4-2C). This new temperature was similar to the temperature recorded in the NBQX, non-regulated, group at this time (Figure 4-2D).

The mean CA1 cell counts and standard deviations for the three treatment groups: NBQX; NBQX regulated and untreated ischemic animals were: 218.5 ± 66.15; 62 ± 32.33 and 59.21 ± 57.18, respectively. Virtually all of the variance in histological outcome was attributable to the mild differences observed in postischemic brain temperature during the initial 24 hours of reperfusion. The number of viable CA1 neurons was inversely related to the mean brain temperature from 0 - 24 hours postischemia (Figure 4-3). ANOVA of the total CA1 cell counts for normal, vehicle, NBQX and NBQX (regulated) groups (Experiment I) yielded a significant treatment effect ($p < 0.0001$). Post hoc comparisons of total CA1 counts revealed that the NBQX group had a significantly improved histological outcome compared to both the NBQX (regulated) and vehicle groups ($p < 0.01$). However, the NBQX group did exhibit

a significant degree of cell loss in comparison to controls ($p < 0.01$). There was ***no*** statistically significant histological protection achieved with the same dose of NBQX when mild hypothermia was prevented by regulating brain temperature for 24 hours (see Figure 4-3).

When protection obtained with NBQX was compared to the level of protection obtained with a 28 hour temperature simulation (Figure 4-2E, Experiment II) it was found that both NBQX and the SimTP treatment produced substantial savings of CA1 neurons. These groups were not significantly different when histological assessment was made 4 days postischemically (Figure 4-4). Histological protection with both treatments, at 4 and 10 days postischemia, was significantly better than vehicle ($p < 0.01$ in all cases, except SimTP versus vehicle (10 day survival) $p < 0.05$).

Electrophysiological recordings from CA1 in hippocampal slices revealed a slight tendency for slices obtained from NBQX treated animals to have smaller field potentials 4 days after occlusion (Figure 4-5). The EPSP slope was significantly reduced, but the amplitude and area measurements did not reach significance. Field potentials recorded from the CA1 region in slices from an animal who had a 3 minute occlusion, but no treatment intervention, were severely attenuated at 4 days postischemia. The NBQX treatment in animals not receiving an ischemic insult did not appear to have any effect on the field potential.

Figure 4-1. Twenty-four hour temperature record of gerbils and rats given NBQX (3 x 30 mg/kg), but not receiving ischemia.

NBQX (3 x 30 mg/kg, i.p.) was administered to gerbils (A) and rats (B) starting 1 hour after the initiation of brain temperature recording. The arrow indicates the time of the first injection, the second and third injections were 15 minutes and 30 minutes later, respectively. The temperature profiles in each graph represent group means, with the shaded area depicting one standard deviation above and below the mean. Temperature data have been averaged over 10 minute intervals. The mean baseline brain temperature is indicated by the dashed line.

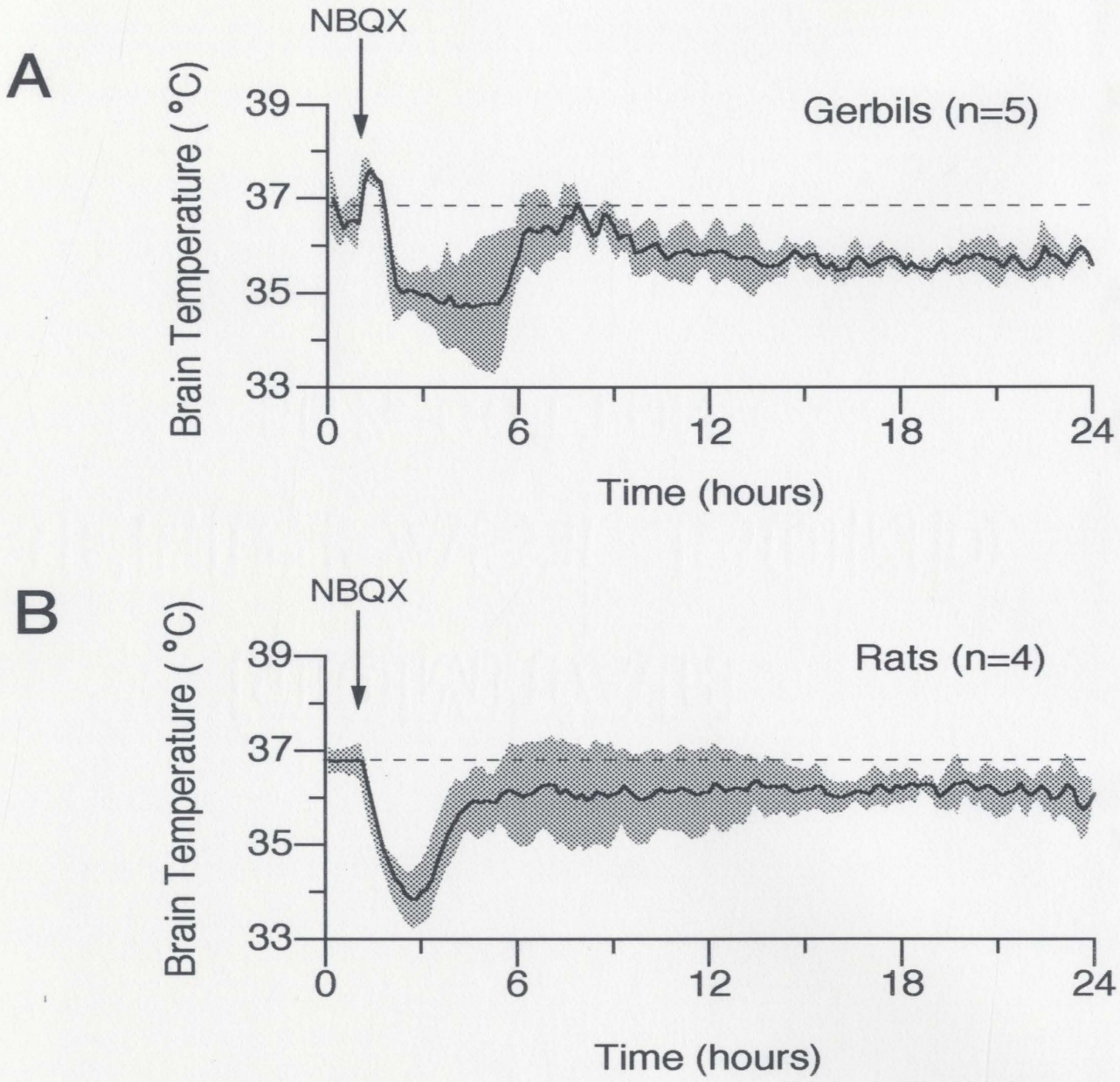

A
NBQX
Brain Temperature (°C)
39
37
35
33
Gerbils (n=5)
0
6
12
18
24
Time (hours)
B
NBQX
Brain Temperature (°C)
39
37
35
33
Rats (n=4)
0
6
12
18
24
Time (hours)

Figure 4-2. Brain temperature following ischemia/sham surgery.

Brain temperature (°C) is plotted with time 0 representing the beginning of reperfusion. The profiles are group means for each treatment, the standard deviation above and below the line is shown by the shaded area. The dashed line on each graph indicates mean control brain temperature (36.9 °C). Vehicle (3 x 5.5% glucose, i.p.) and NBQX injections (3 x 30 mg/kg, i.p.) were given starting 60 minutes after ischemia, each injection separated by 15 minutes. The heavy bar along the x-axis indicates the duration of postischemic temperature control, which was 60 minutes in all groups except C and E (see methods section 4.3.3: Experiment I and Experiment II). Note the drop in brain temperature in the NBQX (regulated) group at the end of temperature maintenance (C). All temperature data have been averaged over 10 minute intervals.

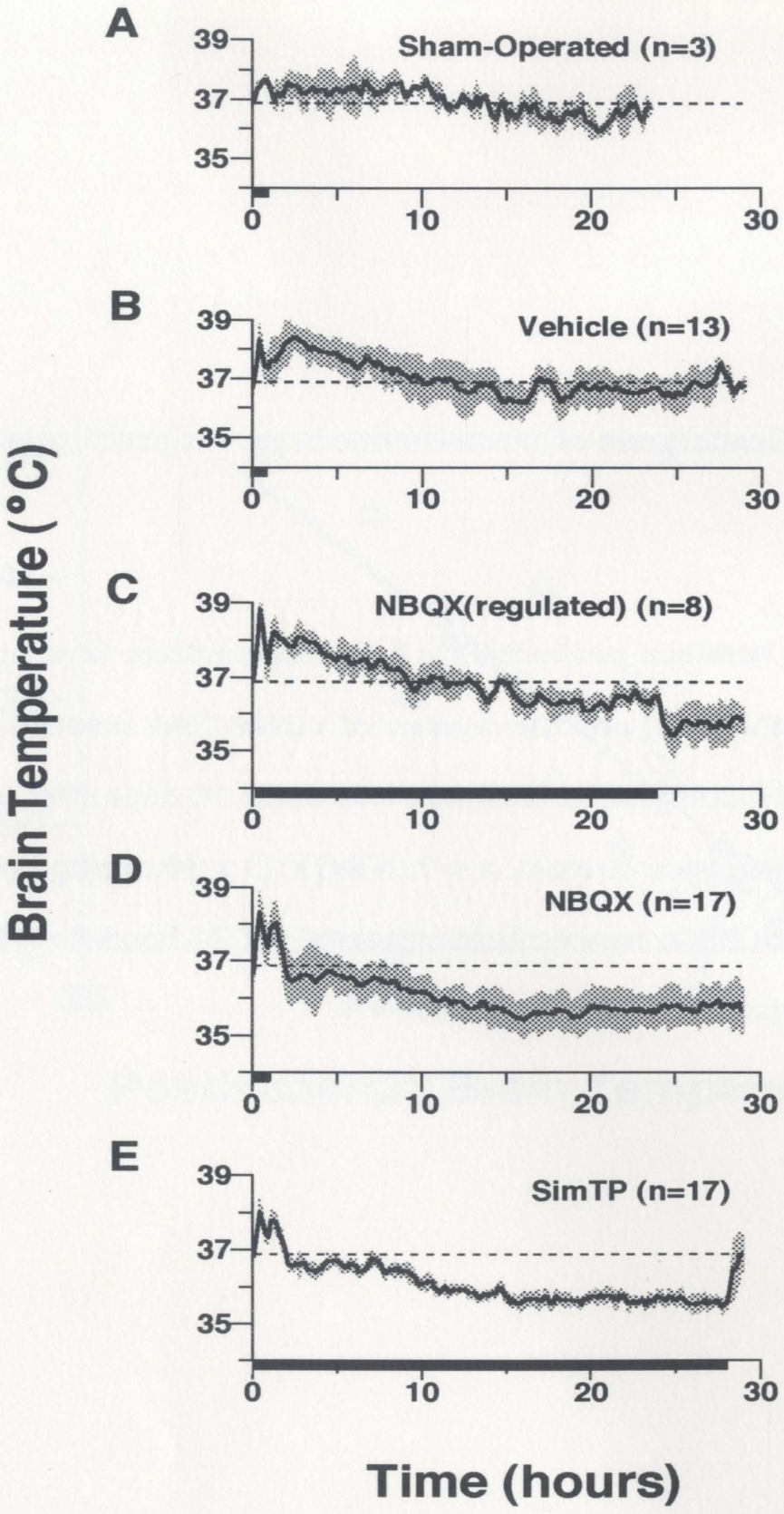

A
Sham-Operated (n=3)
B
Vehicle (n=13)
C
NBQX(regulated) (n=8)
D
NBQX (n=17)
E
SimTP (n=17)
39
37
35
0
10
20
30
Brain Temperature (°C)
Time (hours)

Figure 4-3. Scattergram of postischemic brain temperature and CA1 cell counts.

Relationship between postischemic brain temperature (averaged from 0 to 24 hours post-occlusion) and the number of viable CA1 neurons, $r = -0.901$, $df = 22$, $p < 0.0001$. Histological assessment was made 10 days after ischemia. Vehicle treated animals (open circles), $n = 7$; NBQX (3 x 30 mg/kg i.p., open triangles), $n = 8$; NBQX with brain temperature regulated for 24 hours to mimic the pattern in the vehicle group (solid triangles), $n = 8$.

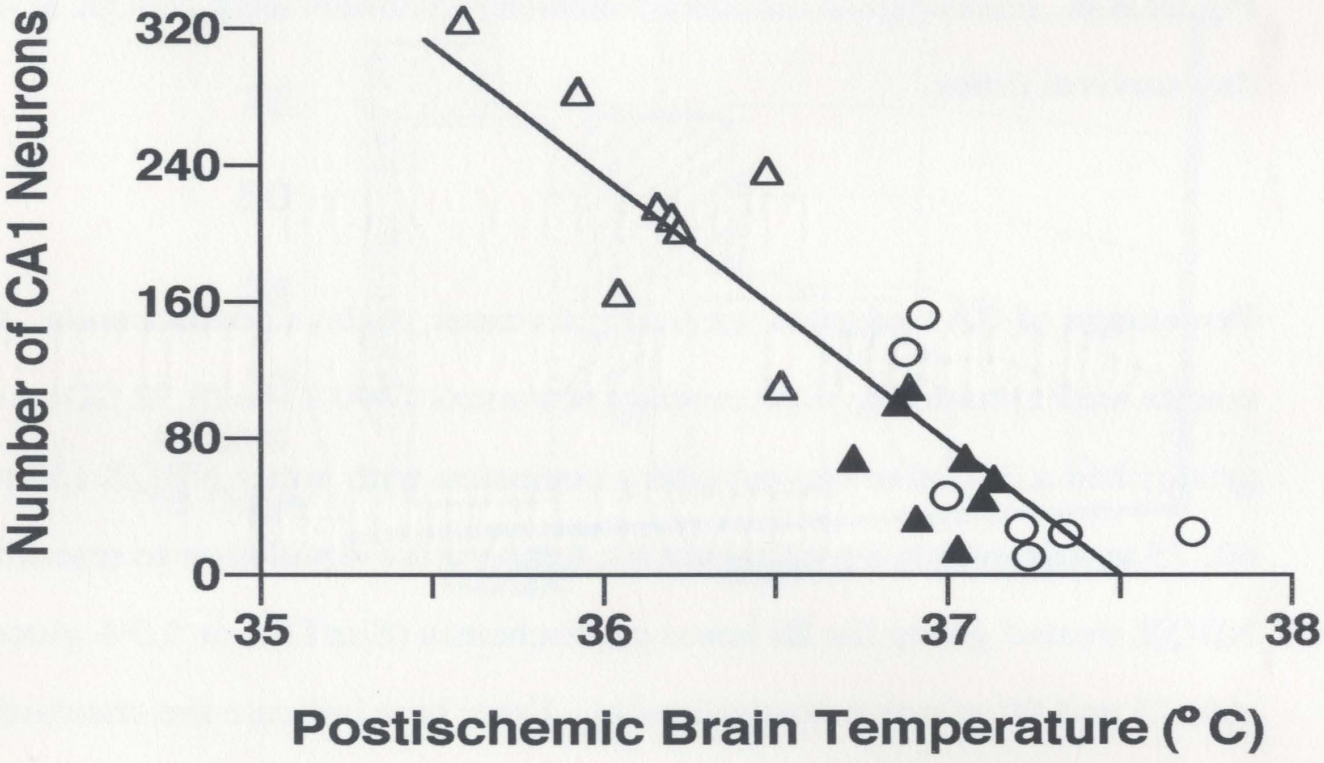

Number of CA1 Neurons
320
240
160
80
0
35
36
37
38
Postischemic Brain Temperature (°C)

Figure 4-4. Histological outcome following treatment with NBQX at 4 and 10 day survival times.

Percentage of CA1 neurons surviving 4 versus 10 days postischemia. CA1 cell counts are expressed as a percentage of control (290.11 ± 13.72 SD; n = 9). All groups had a 3 minute carotid artery occlusion with either NBQX (30 mg/kg i.p., 60, 75 and 90 minutes postischemia); temperature simulation to resemble the NBQX treated group for 28 hours postischemia (SimTP); or 5.5% glucose vehicle (60, 75 and 90 minutes postischemia). Error bars indicate the standard deviation (6 to 10 animals/group); * $p < 0.05$, ** $p < 0.01$ in comparison to vehicle treatment.

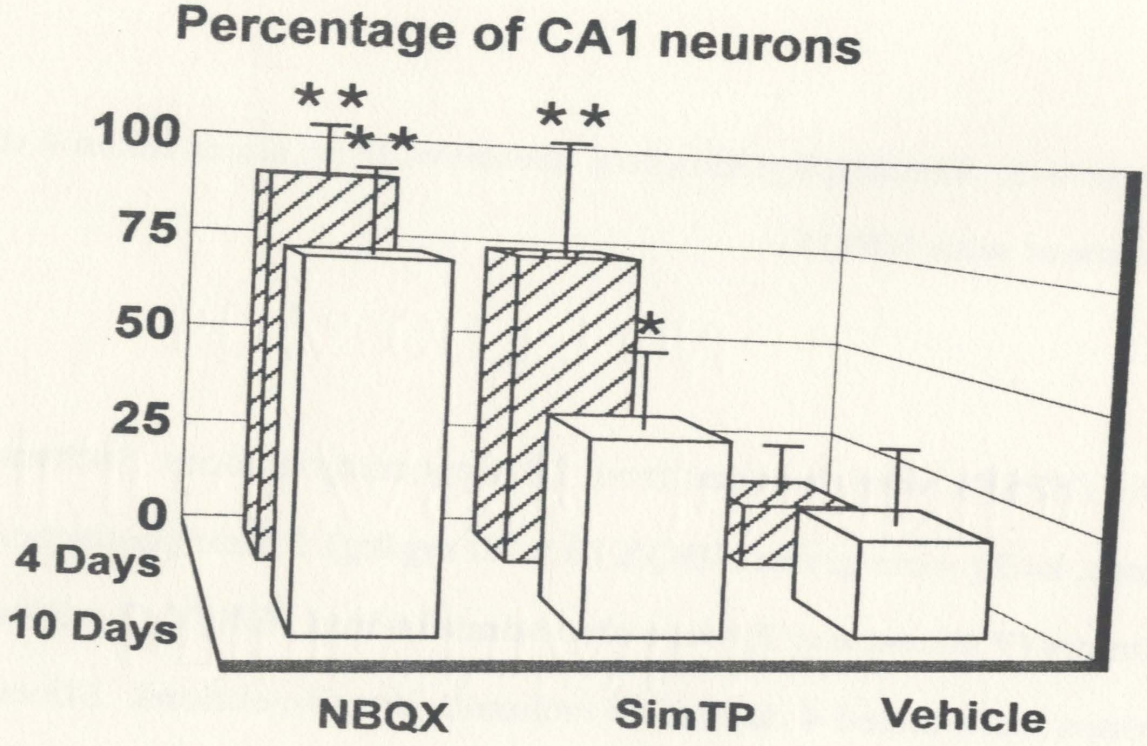

Percentage of CA1 neurons
100
75
50
25
0
4 Days
10 Days
NBQX
SimTP
Vehicle

Figure 4-5. Electrophysiological measures from slices taken 4 days after treatment with NBQX.

CA1 fEPSPs were recorded from 18 hippocampal slices. Ischemic animals (7 slices, n=2) were given NBQX (3 x 30 mg/kg) 1 hour postischemia. Control animals (7 slices; n = 2) were also administered NBQX (3 x 30 mg/kg). Both groups were tested 4 days after ischemia/drug-injections. Slices from an animal occluded for 3 minutes, but not treated postischemically (4 slices, n=1), were also examined to verify that the CA1 fEPSPs were virtually absent by 4 days after ischemia when no intervention was given. A, amplitude; B, slope; C, area. There was a significant treatment effect for all three measures $F_{2,15} = 13.528, 9.760, 11.601$, respectively; $p < 0.01$.

* $p < 0.05$, ** $p < 0.01$ Dunnett test.

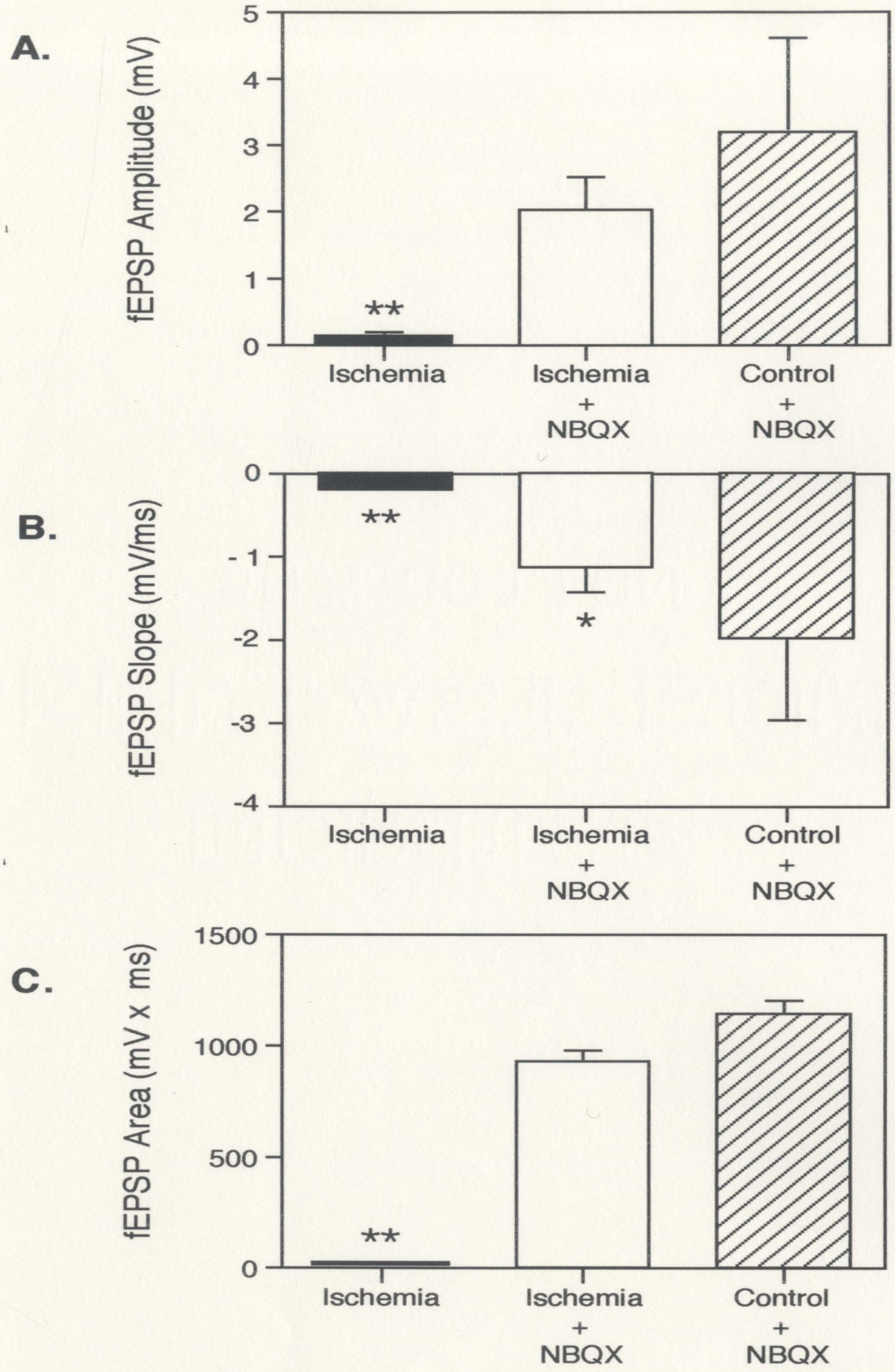
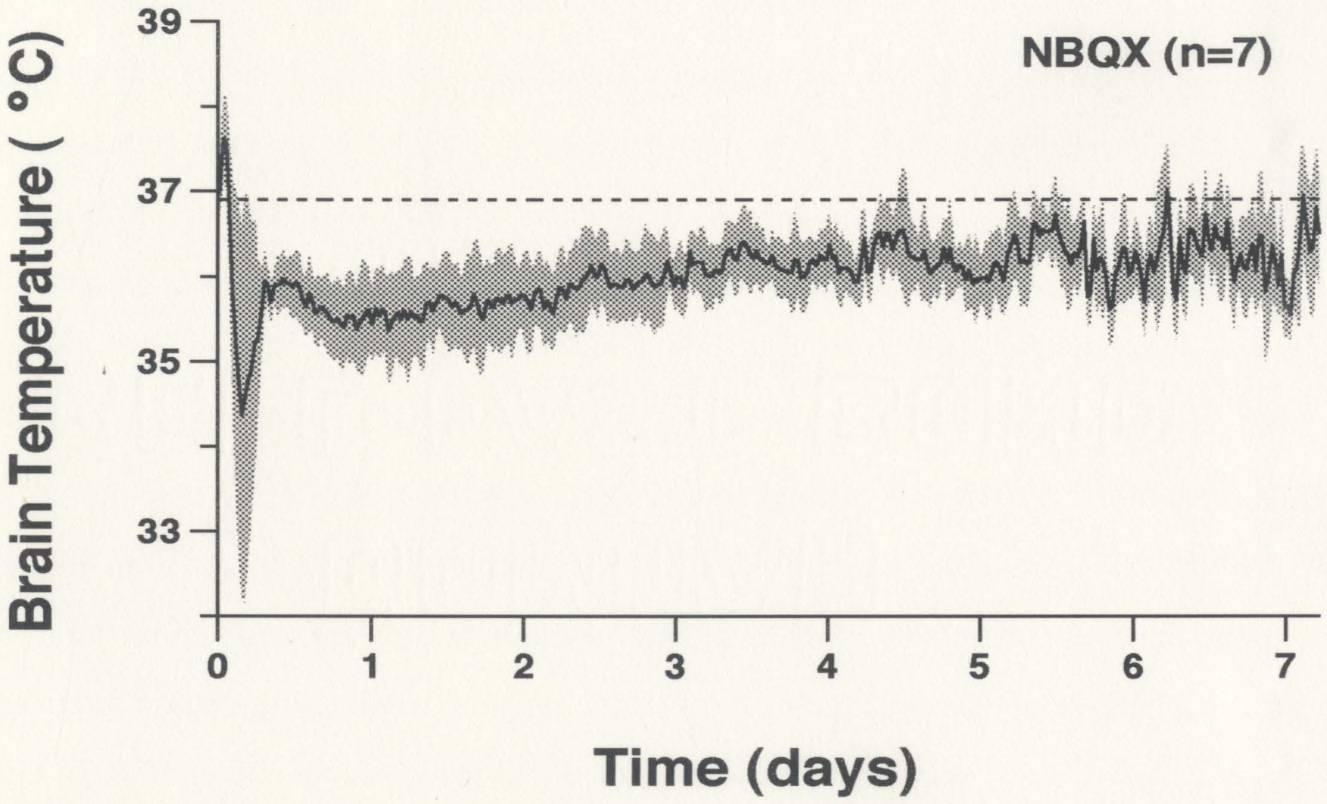

A.
fEPSP Amplitude (mV)
5
4
3
2
1
0
**
Ischemia
Ischemia + NBQX
Control + NBQX
B.
fEPSP Slope (mV/ms)
0
- 1
-2
-3
-4
**
*
Ischemia
Ischemia + NBQX
Control + NBQX
C.
fEPSP Area (mV x ms)
1500
1000
500
0
**
Ischemia
Ischemia + NBQX
Control + NBQX

Figure 4-6. Extended brain temperature recordings following postischemic treatment with NBQX.

Brain temperature recorded postischemically for 1 week following NBQX treatment (3 x 30 mg/kg, i.p.) administered at 60, 75 and 90 minutes postischemia (time 0 is the start of reperfusion). The shaded area represents the standard deviation above and below the mean. Temperature data have been averaged over 30 minute intervals. The dashed line indicates mean control brain temperature (36.9 °C).


NBQX (n=7)
Brain Temperature (°C)
39
37
35
33
0
1
2
3
4
5
6
7
Time (days)

4.4 Discussion

The results show that a dose of NBQX (3 x 30 mg/kg) which has previously been reported to be beneficial against ischemic injury (Sheardown et al., 1990; Buchan et al., 1991a; Diemer et al., 1992), has a mild hypothermic action that is linearly related to its neuroprotective efficacy. NBQX was not protective against injury following cerebral ischemia when the temperature was regulated to mimic that of the vehicle group. Furthermore, simulating the temperature profile induced by NBQX is sufficient to produce histological preservation of CA1 cells. However, there was a significant decline in the protection provided by the SimTP treatment between 4 and 10 days postischemia ($p < 0.01$). A similar trend of decreasing efficacy was also observed with NBQX. While not significant, this trend suggests that necrosis may progress if the survival time is lengthened to several weeks. While it remains possible that NBQX conveys a neuroprotective effect independent of temperature, it is likely that the protection provided by the SimTP treatment decayed at a faster rate than that of NBQX because the duration of mild hypothermia was shorter. In the present study, the brain temperature of the SimTP group was rewarmed at 28 hours after ischemia. However, it is evident that NBQX treated animals were still subnormal 30 hours postischemically (Figure 4-2E versus 4-2D). Subsequently, brain temperature was recorded for extended periods (up to 8 days) in animals treated with NBQX ($n = 7$). NBQX induced a state of mild hypothermia which lasted for ~ 4 - 5 days (Figure 4-5), thus increasing the period of mild hypothermia by at least 68 hours in comparison to the SimTP group. The long-lasting nature of this hypothermia may be due to a lingering precipitate of NBQX that is found in the intraperitoneal

cavity (Le Peillet et al., 1992; Buchan et al., 1993) and internal organs, such as the gall bladder, days to weeks after i.p. administration (unpublished observations). This precipitate might act as a depot, providing sustained release of NBQX in high enough concentrations to produce a hypothermic action for several days. Alternatively, the toxicity of the compound alone (Aoki et al., 1994; Xue et al., 1994) could explain its hypothermic action since non-specific drug toxicity can induce hypothermia (Gordon et al., 1988). In order to achieve identical histological protection by simulating NBQX-induced hypothermia, the simulation period may also have to be extended to several days. This was not possible in the present study because temperature was controlled manually and maintaining temperature at a slightly hypothermic level required constant attention (i.e. monitoring the temperature every 20 seconds and making adjustments every couple of minutes).

Recent findings from our laboratory (Colbourne and Corbett, 1994a) have shown that 12 hours of moderate hypothermia (32 °C) produced substantially less protection than 24 hours of hypothermia. The shorter duration of hypothermia resulted in a profound decline in protective efficacy between 10 and 30 day survival times. It seems that within limits, increasing either the degree or the duration of hypothermia should improve histological outcome. Nevertheless, the present results suggest that *mild* postischemic hypothermia, may not translate to permanent neuroprotection even if the duration of hypothermia is prolonged (i.e. several days).

In studies where survival times have been increased from 7 days to 1 month, neuroprotection with NBQX (3 x 30 mg/kg) is not maintained (Li and Buchan, 1995). The cascade of events resulting in neuronal necrosis may only be

temporarily interrupted during the postischemic hypothermic period. Once temperature is normalized these processes may resume, albeit at a slower rate. In the present study, and others (see Sheardown et al., 1990), where NBQX has been assessed following survival times of only 4 days, histological protection is often indistinguishable from cell counts/ratings from control animals. According to the findings presented here, the animals would have still been mildly hypothermic at this time, which could account for the near total histological preservation. However, in electrophysiological recordings from slices obtained from animals treated with NBQX 1 hour postischemia and surviving 4 days, the field responses tended to be smaller than control animals treated with NBQX. This direct measure of CA1 integrity suggests that while the histological appearance of the neurons is unchanged on day 4, synaptic responses are abnormal. However, this conclusion is tempered by the small group size in these experiments. The CA1 neurons might be in a transition state from which they will recover. However, the trend for cell loss to progress between the 4 and 10 day survival times and the results of Li and Buchan (1995) suggest that the possible impairment in electrophysiological functioning at day 4 is an indication that these cells are compromised and will eventually die. Treatments that provide apparently permanent protection, such as intraischemic hypothermia, do not result in reduced field potentials on day 4 (unpublished observations).

Data from the open field test were also collected from all animals in the NBQX study. Unfortunately, the behavioural responses cannot be compared to the electrophysiological data because of the confounding effects NBQX had on behaviour. Many animals lost a considerable amount of weight following treatment with NBQX and the locomotor activity in the open field was very

inconsistent in the groups treated with NBQX. This could be a result of toxic effects of the drug (Aoki et al., 1994; Xue et al., 1994) and/or the long-lasting pharmacological blockade of the AMPA receptor. In earlier studies using the non-competitive glutamate receptor antagonist MK-801 we found that the drug had a long-lasting pharmacological action which confounded the open field activity scores, and as with NBQX, the behavioural data could not be used. To circumvent this problem we changed the timing of our first open field test from 1 day after ischemia to 3 days postischemia. Unfortunately, with NBQX, 72 hours is obviously not long enough for the elimination of the drug and/or recovery from lingering toxicity. Due to a limited availability of the drug (1 gram), it was not possible to evaluate the level of behavioural protection provided by NBQX or long term (1month) electrophysiological and histological assessments as was originally intended. However, as stated above, the protection provided by NBQX declines with increased survival time (Li and Buchan, 1995).

Although the majority of the previous studies using NBQX have not regulated temperature during the postischemic period, a study by Buchan et al. (1991a) reported significant histological savings at 7 days when body temperature was regulated postischemically for 6 - 8 hours. However, as shown in Figure 2C, the temperature of NBQX treated animals drops almost immediately even when temperature regulation is terminated as late as 24 hours post-occlusion. It is also noteworthy that animals treated with NBQX demonstrate hypothermia even when they recover from the mild sedating effects of the drug and begin to show ambulation. Recovery of locomotion is often used as a marker of an animal's ability to thermoregulate after ischemia and a point at which temperature control is typically stopped. This is clearly an incorrect assumption.

Since NBQX administration can be delayed for 6, 12 or even 24 hours postischemia and still provide neuroprotection (Li and Buchan, 1993; Nurse and Corbett, 1993; Sheardown et al., 1993), then postischemic temperature regulation for less than 24 hours is likely insufficient if neuronal protection is due to hypothermia. The severity of the ischemia will probably determine the length of time that is necessary to regulate postischemic temperature and prevent hypothermia from confounding histological outcome. For example, following 5 minutes of ischemia, which produces > 95 % CA1 cell loss in our model, we have found NBQX treatment to be ineffective if administration is delayed until 6 hours after reperfusion (Nurse and Corbett, 1993). Therefore, with severe ischemia if NBQX is given within the first few hours of reperfusion then a 6 - 8 hour period of temperature regulation would probably suffice. However, after 3 minutes of ischemia which produces ~ 85 % CA1 neuronal loss in our model, treatment can be delayed for at least 6 hours and still provide histological protection (data not shown). However, the protection is correlated with the level of hypothermia induced and no histological protection is found following the 6 hour delayed administration if temperature is regulated from 6 to 24 hours postischemia (Nurse and Corbett, 1993). In models where CA1 cell loss is ~ 80 % or less (Sheardown et al., 1993), temperature regulation may need to be continued beyond 24 hours in order to prevent a confounding effect of hypothermia.

The present study also helps to reconcile some of the discrepancies between *in vivo* and cell culture models of ischemia and glutamate toxicity. Many *in vivo* studies comparing the efficacy of NMDA and AMPA receptor antagonists in global ischemia have found AMPA antagonists to offer considerably greater levels of neuroprotection (Nellgård and Wieloch, 1992;

Buchan et al., 1993; Sheardown et al., 1993; Lippert et al., 1994). Cell culture studies have either found no neuroprotective effects with AMPA antagonists alone (Kaku et al., 1991), or in cases where AMPA antagonists were effective the protection was not as great as that provided by NMDA antagonists (Lippert et al., 1994). However, both *in vitro* (Shuaib et al., 1993; Bruno et al., 1994) and *in vivo* (Busto et al., 1987; Dietrich et al., 1993; Coimbra and Wieloch, 1994; Colbourne and Corbett, 1994a,b; Nurse and Corbett, 1994; Colbourne and Corbett, 1995) studies have demonstrated dramatic protection with hypothermia. It is important to emphasize that the level of postischemic hypothermia induced by NBQX is milder than that in previous studies (Busto et al., 1989; Dietrich et al., 1993; Coimbra and Wieloch, 1994; Colbourne and Corbett, 1994a, 1995) using deliberate hypothermic manipulations (e.g. 32 - 34 °C). These results demonstrate the necessity of careful *postischemic* temperature assessment in pharmacological studies of cerebral ischemia since differences of only 1.0 - 2.0 °C, if prolonged, can significantly affect outcome. Further, the present study raises important questions about the wisdom of conducting costly clinical trials based upon current drug studies which are likely confounded by similar temperature effects and short survival times.

CHAPTER 5:

ACUTE AND CHRONIC LOCOMOTOR PATTERNS AS PREDICTORS OF ISCHEMIC OUTCOME

5.1 Introduction

The brain injury resulting from both global and focal ischemia produces changes in behaviour and/or impairments in normal function. Generally, the deficit observed corresponds to the brain region(s) affected by the ischemia. Damage to the hippocampus results in memory impairments and damage to the motor cortex leads to paralysis or weakness. In humans, ischemia often results in emotional lability and cognitive deficits (Walker et al., 1981; Zola-Morgan et al., 1986), as well as impairments in sensory and motor ability. In animal models of ischemia, changes such as hyperexcitability on handling (Smith et al., 1984a), increased startle (Truong et al., 1994), heightened locomotor activity (Chandler et al., 1985a; Poignet et al., 1989) and impaired performance on a number of tasks including the T-maze, radial arm maze, Morris water maze and passive avoidance have been reported (Volpe et al., 1984; Auer et al., 1989; Himori et al., 1990; Imamura et al., 1991; Karasawa et al., 1994).

One of the most frequently used behavioural signs of ischemic injury in the gerbil 2 vessel-occlusion model has been an increase in locomotor activity. However, there has been confusion in the literature as to how long this phenomenon lasts, with reports ranging from hours to months (Gerhardt and Boast, 1988; Kuroiwa et al., 1991a,b; Mileson and Schwartz, 1991; Babcock et al., 1993; Colbourne and Corbett, 1994a; Nurse and Corbett, 1994; Colbourne and Corbett, 1995). Our research group has recently reported that increased locomotion in an open field maze persists as long as 6 months after ischemia

(Colbourne and Corbett, 1994b). An explanation for these discrepancies can be found by analyzing the testing procedures used in the above experiments. In cases where the animals are tested in familiar environments (e.g. home cages), increases in locomotor activity are short-lived. However, if animals are tested in a novel environment, then locomotor changes can persist for months, depending on the frequency of exposure to the environment. The different testing procedures, which utilize novel versus familiar environments, may reveal two distinct functional abnormalities, an acute change that is apparent soon after ischemia and a chronic change that is triggered by novelty. Both acute and chronic changes in locomotor activity are thought to arise from hippocampal injury, specifically, damage to the CA1 subfield of Ammon's horn (Gerhardt and Boast, 1988; Wang and Corbett, 1990; Kuroiwa et al., 1991a,b; Mileson and Schwartz, 1991; Babcock et al., 1993).

In studies conducted over the last three years, there have been several interesting behavioural changes that have been noted following different ischemic conditions, such as occlusion duration and intraischemic brain temperature. The data reported in this chapter have been collected in different experiments and the results have been pooled in order to describe acute and chronic activity patterns induced by ischemia. These activity changes have not been well described in the literature and as a result there has been considerable confusion arising from conflicting results reported by different research groups (see Karasawa et al., 1994). Also, the value of the postischemic activity patterns

in predicting the degree of ischemic damage has not been appreciated by many researchers. In the following description of postischemic activity changes, measurements were made both in an environment the animals had been *habituated* to before the ischemic episode and an environment (i.e. open field) that was *novel*. The acute and chronic postischemic behavioural responses were compared following 1.5, 3.0 or 5.0 minute ischemic episodes, as well as under conditions of intraischemic hyperthermia and hypothermia.

5.2 Methods

All animals were prepared for brain temperature measurement as described in the general methods (section 2.2). Two days after cannula implantation, brain probes were inserted to monitor baseline temperature and activity for approximately 3 hours. During this three hour period, the animals were placed in plastic cages, measuring 21.0 x 15.2 x 18.5 cm, which they had not been exposed to before. These same cages were used to house the animals again in the early postischemic phase. All animals habituated to this environment very quickly and after the initial 30 - 45 minutes they usually spent the remainder of the 3 hour session sleeping/resting, with intermittent bouts of activity. At the end of the recording session, the brain probes were removed (under halothane anaesthesia) and the animals returned to the animal room.

Two days after the habituation session, cerebral ischemia or sham surgery was performed. In animals receiving an ischemic episode, the carotid arteries

were clamped for either 1.5, 3.0 or 5.0 minutes. Both rectal and brain temperature were monitored and maintained at normothermia throughout surgery (except in cases of hypo- and hyperthermic ischemia), as described in the general methods section. When intraischemic hyperthermia (38 °C) was induced, the brain temperature was raised to 38 °C before the initiation of ischemia and then kept at this level throughout the occlusion. When hypothermia was induced, the occlusion was started while brain and rectal temperatures were normothermic. Once the arterial clamps were applied, as described in the general methods (section 2.3), then the brain temperature was quickly cooled. Following surgery the anaesthestic nose cone and heating blankets were removed. The animals were placed on AM receivers in the plastic cages they had previously been habituated to, with the telemetry brain probe still in place. All postischemic brain temperature/activity measurements were made while the animal was in this environment. Following the acute postischemic monitoring period, animals were put back into their home cages and returned to the animal room.

Measurement of locomotor activity in a novel open field was initiated 3 days post-occlusion/sham surgery. The 10 minute test sessions were carried out in a sound attenuated room. After placing the animal on the floor of the maze and starting computerized data collection, the experimenter left the room. The animal's behaviour could be observed through a small window (31 x 31 cm). The computerized tracking system monitored the number of squares crossed during

each minute of the test session. Depending on the survival time, retesting in the same apparatus was performed 7 and 10 days post-occlusion.

5.3 Results

Locomotor hyperactivity was observed in all groups following ischemia/sham-surgery. In sham-operated animals the period of hyperactivity was very brief (Figure 5-1) and was observed immediately after the animals regained consciousness. A greater degree of acute behavioural activation was observed following ischemia (Figure 5-1). However, the pattern following 3 and 5 minutes of ischemia was biphasic, with an initial period of *decreased* locomotor activity before the period of hyperactivity began. Animals in the 3 and 5 minute ischemic groups usually righted themselves within 15 - 30 minutes post-occlusion, after which they assumed a hunched posture. The majority of animals remained inactive for approximately 2 - 3 hours. The duration of "ischemic hunch" was greater after 5 minutes than 3 minutes of ischemia and was not observed in animals who received a 1.5 minute occlusion (Figure 5-1). Once the "ischemic hunch" phase ended, all ischemic animals exhibited a period of increased locomotor activity that was significantly above the levels measured from sham-operated animals (Table 5-1). The duration of this heightened locomotor activity was related to the severity of ischemia, lasting 3 - 4 hours after a 1.5 minute occlusion and longer than 24 hours after a 5 minute occlusion. Brain temperature was also significantly elevated in the 3.0 and 5.0 minute occluded

groups (Table 5-2). However, brain temperature returned to control levels much more quickly than activity in these groups. Brain temperature is usually positively correlated with activity. However, as shown in the 5.0 minute group in Figure 5-1, this relationship does not always exist. Increased locomotor activity in animals subjected to 5 minutes of ischemia continued beyond 24 hours, in animals where postischemic recordings have been made for 3 days it has been observed that activity levels do not return to control values until ~ 42 - 48 hours post-occlusion.

The acute behavioural pattern consisted of repetitive circling of the cage with frequent rearings in the corners in a stereotyped fashion. While an animal was in this phase of behavioural activation many species typical activities, such as paper shredding, grooming, eating and drinking were either not performed, or were briefly attempted and then abandoned. This was especially true during the early stages of increased locomotor activity and gradually became less apparent. Ischemic durations of 1.5 minutes caused very brief alterations in typical gerbil behaviour. For example, an animal occluded for 1.5 minutes would normally resume paper shredding within 6 hours postischemia, whereas an animal occluded for 5 minutes would not resume paper shredding activity until ~ 30 - 48 hours postischemia. It was also observed that ischemic animals seemed to be hyper-reactive during the phase of behavioural activation. Certain acoustic stimuli (e.g. a high pitched tone) triggered a dramatic increase in locomotor

activity above that already being exhibited (Figure 5-2), whereas the response of sham animals to the same sound was almost undetectable.

Both intraischemic hypothermia and hyperthermia altered the acute postischemic behavioural profile (Figure 5-3). Lowering brain temperature during ischemia resulted in the peak behavioural activation occurring *earlier* in the postischemic period (see also Figure 3-2) and the behavioural activation attenuated more quickly. Intraischemic hyperthermia results in a greater duration of "ischemic hunch". However, temperature and activity were not monitored for long enough to precisely determine the length of acute postischemic hyperactivity in this group. It may have persisted for a longer interval than the 5.0 minute normothermic group, which display locomotor hyperactivity until ~ 48 hours post-occlusion. The heightened locomotor activity of the hyperthermic group during the first minute of the open field session (~ 72 hours postischemia) suggests that these animals may still be generally hyperactive at this time.

The first exposure to the open field apparatus was 3 days postischemia. All ischemic groups and shams started with similar levels of activity on test day 3, minute 1 (*except* the hyperthermic group, see results of intraischemic temperature manipulations). Sham-operated animals and the 1.5 minute occluded animal tended to habituate after the 2nd minute, showing decreased levels of activity with time. The groups occluded for 3 or 5 minutes exhibited what appeared to be a sensitization response, i.e., an increase in locomotor activity

which was most pronounced in the 5 minute occluded group (see total session scores in Figure 5-4 and activity scores per minute in Figure 5-5).

In addition to the activity measures, there were other subtle differences in behaviour. The motoric responses of animals subjected to ischemia for 3 or 5 minutes were less fluid than control animals, appearing slightly jerky. Normally, these differences in movement were subtle, however, in a few cases changes in movement following ischemia were more pronounced. In these extreme cases the animal's locomotor movements were obviously jerky, resembling the movements of a frog and always coincided with lower open field scores. Interestingly, movement disturbances have not been noted in ischemic animals when they are observed in their home cages immediately preceding exposure to the open field. Following the open field session, animals remained hyperexcitable for a short time (~ 5 minutes) before settling down. Post open field hyperexcitability appeared to be greater in 3 and 5 minute ischemic animals than in control animals, but this was not systematically studied.

The percentage of locomotion around the perimeter versus centre crossings was not systematically assessed. However, it was observed that 3 and 5 minute occluded animals exhibit a prominent pattern of continual forward locomotion around the perimeter, with very little or no interruption to engage in other behaviours, at least on their first exposure to the open field environment. In contrast, sham controls cross the centre of the maze as well as locomoting around the sides and frequently stop to rear or sniff.

As with the acute behavioural changes, temperature also influenced the chronic behavioural alterations following ischemia. Hyperthermia increased the degree of behavioural activation and hypothermia decreased the level of open field activity. In some cases, intraischemic hypothermia was so protective that the open field responses were identical to the sham-operated animals, in both the within session pattern and the absolute level of activity, which has already been discussed in Chapter 3 (see Figure 3-4B and 3-5B). Animals whose brain temperature was raised to 38 °C during ischemia were interesting in that they were the only group whose first minute open field scores on day 3 were not between 60 - 80 squares. On day 3, the hyperthermic group crossed an average of 130 squares during the first minute of the test session, minutes 2 and 3 were slightly lower, then a sensitization-like pattern was observed (Figure 5-6). Whereas the responses in the hypothermic group approached the sham-operated animals with time, the hyperthermic group remained higher than all other groups on all test days.

Figure 5-1. Post-surgery brain temperature and activity measurements

Mean brain temperature is plotted as a line with the reference on the left axis. Mean activity measurements are illustrated by the black bars with the standard deviation of the group indicated by the shaded area, the reference for activity levels is shown on the right axis. All data in the ischemic groups begin at the end of occlusion. Data for the sham-operated group is shown at a similar time with respect to length of surgery. The 1.5 minute occlusion data are based on n = 4 for the first 18 hours and n = 1 for 18 - 24 hours.

See Tables 5-1 and 5-2 for summary data and statistical comparisons.

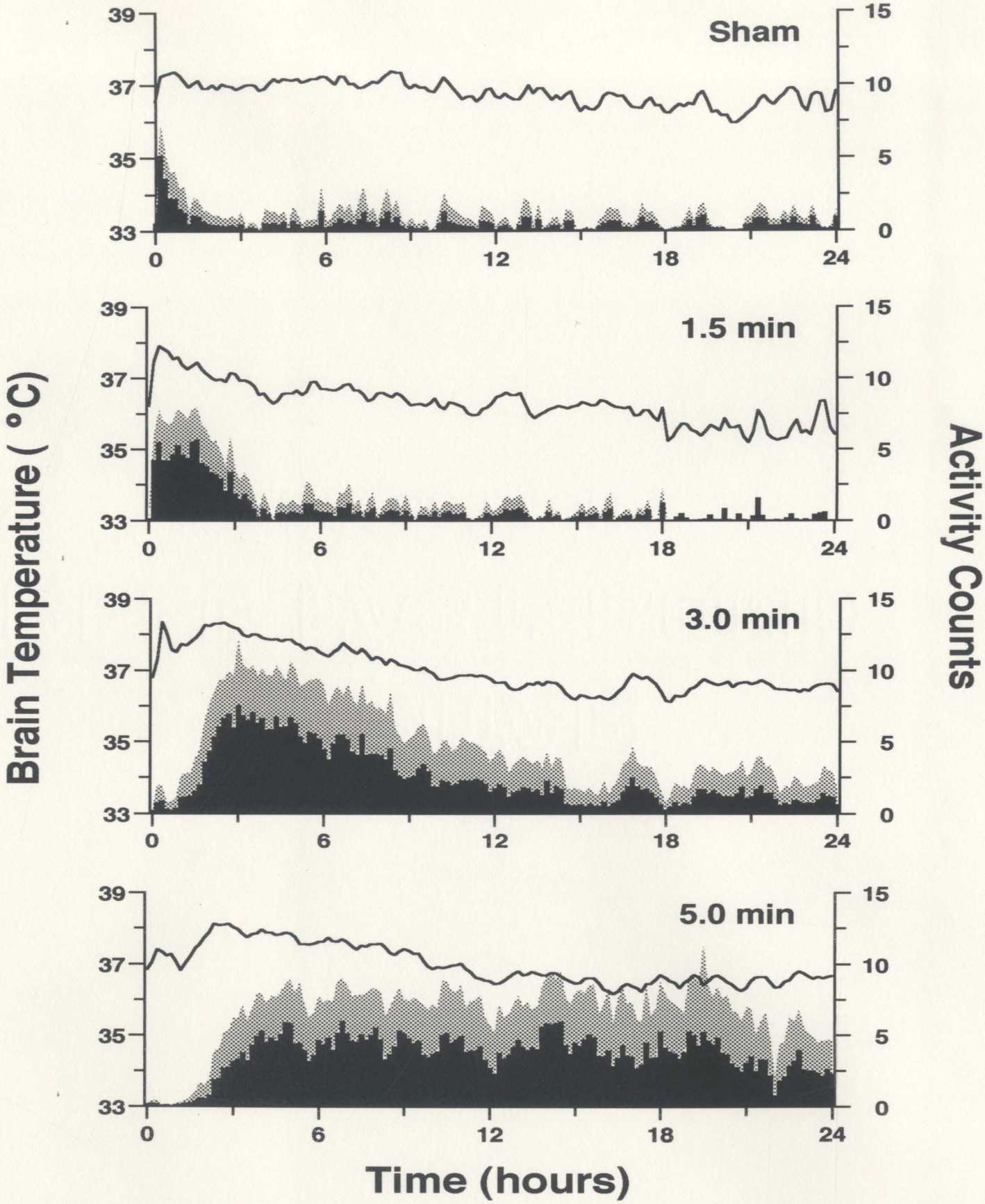

Sham
1.5 min
3.0 min
5.0 min
Brain Temperature (°C)
Activity Counts
Time (hours)
39
37
35
33
15
10
5
0
0
6
12
18
24

Table 5-1. Postischemic activity

Mean activity level averaged in 3 hour increments, from end of occlusion to 24 hours post-occlusion. Variance has been excluded from the table for clarity. The standard deviations for each group are plotted on the graphs in Figure 5-1. All post-hoc comparisons in the ischemic groups are to the sham-operated group (Dunnett *t*), $^{*}p < 0.05$, $^{**}p < 0.01$.

Time Post-Surgery	0 - 3 hours	3 - 6 hours	6 - 9 hours	9 - 12 hours	12 - 15 hours	15 - 18 hours	18 - 21 hours	21 - 24 hours
Sham-Operated	1.2	0.4	0.6	0.4	0.35	0.3	0.4	0.46
1.5 minute	3.9^{**}	0.8	0.6	0.3	0.35	0.3	1.3	0.24
3.0 minute	2.6^{*}	6.3^{**}	4.1^{*}	2.3^{*}	1.4	1.0	1.1	1.0
5.0 minute	0.4	4.2	4.9^{**}	4.3^{**}	4.3^{**}	3.8^{**}	4.1^{**}	2.7^{*}

Table 5-2. Postischemic temperature

Mean brain temperature for the sham-operated, 1.5, 3 and 5 minute occluded groups averaged in 3 hour increments, from end of occlusion to 24 hours post-occlusion. Group variance is the standard deviation. All post-hoc comparisons in the ischemic groups are to the sham-operated group (Dunnett *t*), $^{*}p < 0.05$, $^{**}p < 0.01$.

	0 - 3 hours	3 - 6 hours	6 - 9 hours	9 - 12 hours	12 - 15 hours	15 - 18 hours	18 - 21 hours	21 - 24 hours
Sham	37.1±.5	37.1±.6	37.1±.5	36.9±.5	36.7±.3	36.5±.3	36.4±.2	36.6±.2
1.5	37.3±.2	36.7±.4	36.6±.2	36.2±.3	36.3±.4	36.2±.4	36.3±1.1	35.6
3.0	37.9±.4**	37.8±.5*	37.4±.4	36.9±.4	36.5±.4	36.5±.4	36.5±.5	36.5±.4
5.0	37.2±.3	37.8±.4*	37.5±.2	36.9±.3	36.6±.4	36.4±.5	36.4±.6	36.6±.5

Figure 5-2. Increase in locomotor activity triggered by external noise

Brain temperature and activity in an ischemic animal during the early phase of behavioural activation. The occlusion duration was 3 minutes. Note the large increase in activity, lasting 5 minutes, which was triggered ~ 180 minutes after occlusion. The scale on the right y-axis is 5 times greater than in the activity plots in Figure 5-1. Other ischemic animals have shown occasional activity spikes outside the normal ischemic range, but not to the extent of the animal shown here. Usually unfamiliar sounds can trigger brief increases in activity in ischemic animals, ranging between 20 and 30 counts per minute.

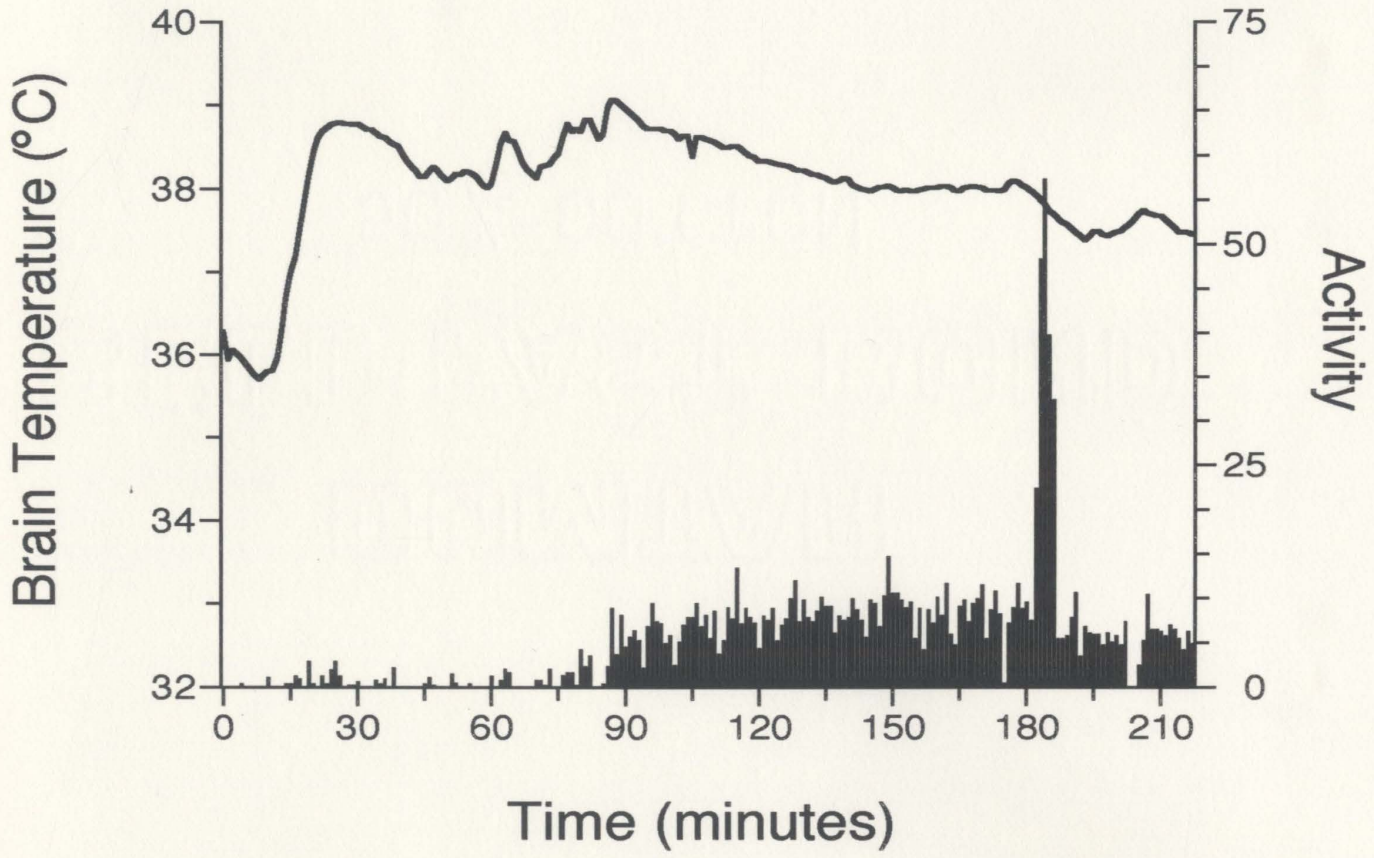
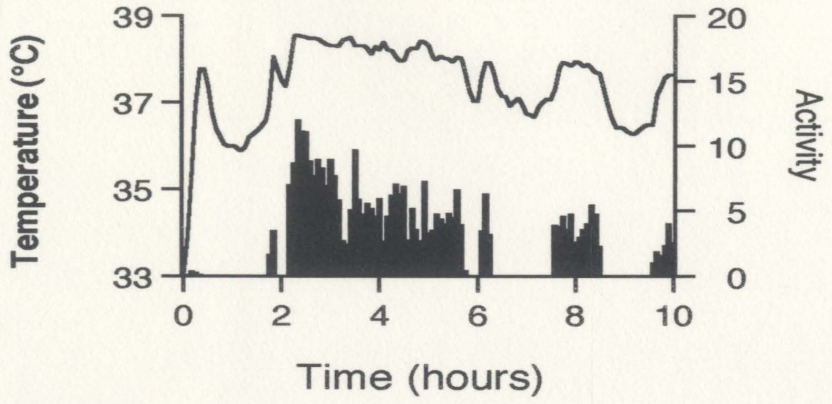

Brain Temperature (°C)
40
38
36
34
32
Activity
75
50
25
0
0
30
60
90
120
150
180
210
Time (minutes)

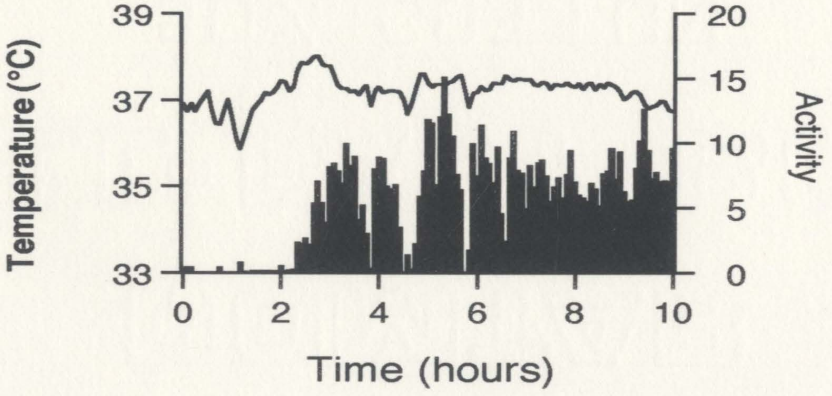
Figure 5-3. Intraischemic hypothermia, normothermia and hyperthermia- effects on postischemic temperature and activity

Post-surgery brain temperature and activity measurements in animals operated under brain temperature conditions of: A, intraischemic hypothermia (n = 1); B, intraischemic normothermia (n = 1); C, intraischemic hyperthermia (n = 3). Note that temperature and activity recording in the hyperthermic group (C) was discontinued at 9.5 hours postischemia (note the duration of the temperature plot on the graph in C).

A.


Temperature (°C)
39
37
35
33
20
15
10
5
0
Activity
0
2
4
6
8
10
Time (hours)

B.


Temperature (°C)
39
37
35
33
20
15
10
5
0
Activity
0
2
4
6
8
10
Time (hours)

C.

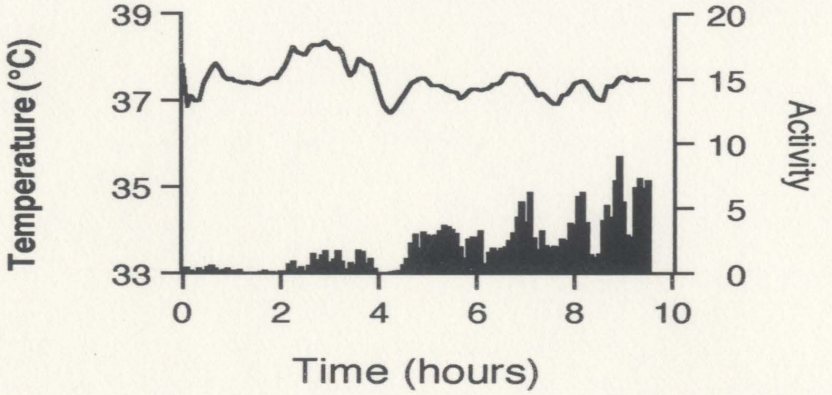

Temperature (°C)
39
37
35
33
20
15
10
5
0
Activity
0
2
4
6
8
10
Time (hours)

Figure 5-4. Total open field activity scores on days 3, 7 and 10. Sham-operated animals and animals occluded for 1.5, 3.0 and 5.0 minutes.

Sum of the number of squares crossed during each minute of the 10 minute sessions. Sham-operated animals (shaded bars), 1.5 minute occlusion (open bars), 3.0 minute occlusion (filled bars), 5.0 minute occlusion (hatched bars). Error bars indicate the standard deviation, post hoc comparisons of the ischemic groups to the sham-operated group were made using the Dunnett t, * $p < 0.05$ ** $p < 0.01$. In the following figure, Figure 5-5, the activity pattern during each session is plotted for each of the four treatment groups.

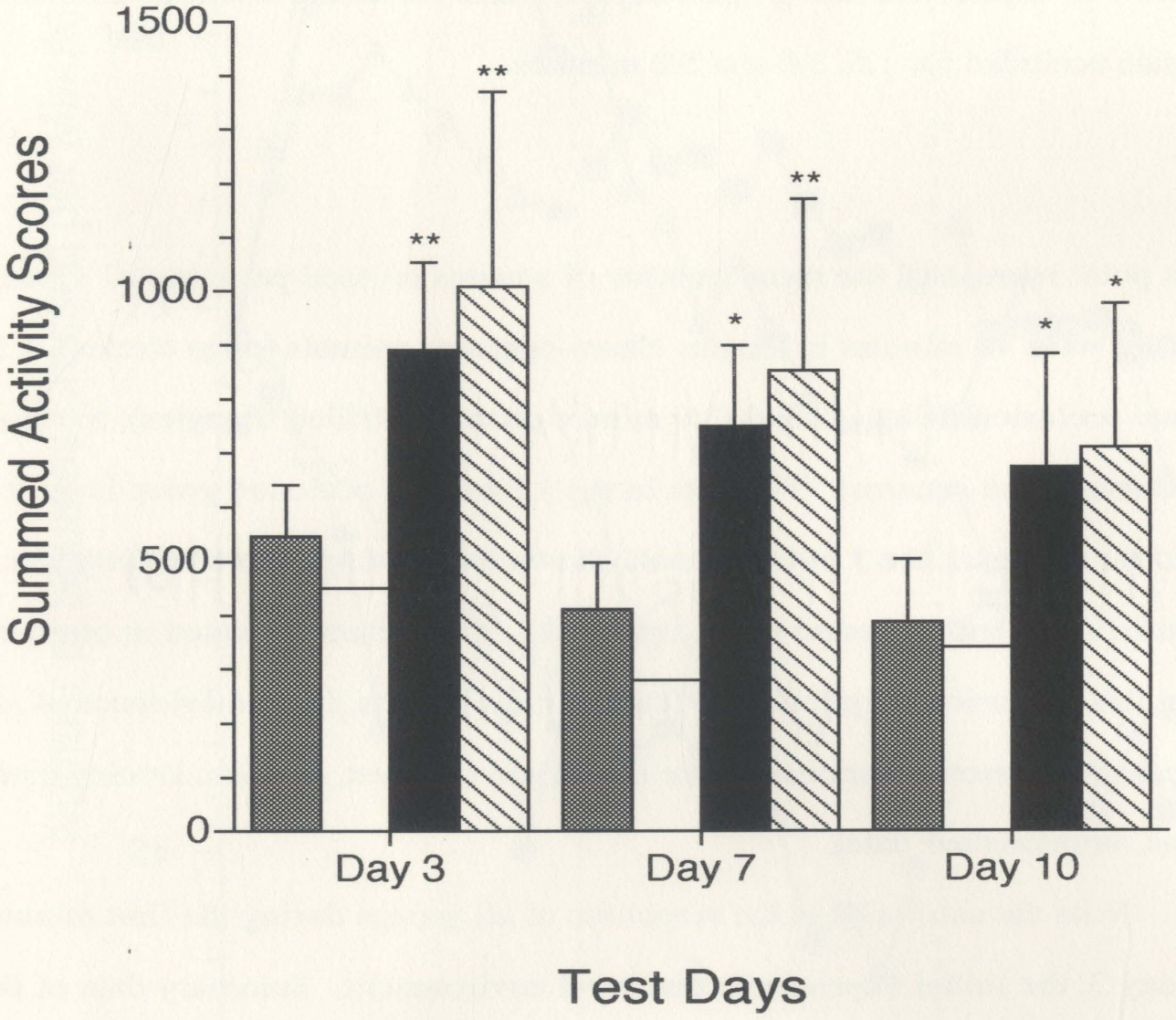

Summed Activity Scores
1500
1000
500
0
**
**
*
**
*
*
Day 3
Day 7
Day 10
Test Days

Figure 5-5. Open field line graphs, days 3, 7 and 10. Sham-operated animals and animals occluded for 1.5, 3.0 and 5.0 minutes

Each point represents the mean number of squares crossed per minute. Open field sessions were 10 minutes in length. Sham-operated animals (open circles), 1.5 minute occlusion (filled circles), 3.0 minute occlusion (filled triangles), 5.0 minute occlusion (filled squares). The data in the 1.5 minute occluded group is only based on 1 animal, the 3 other animals shown in the temperature/activity plot (Figure 5-1) were not tested in the open field. Other data collected in our lab using this occlusion duration have found similar results, i.e., no evidence of an increased locomotor response in the open field (Corbett, Crooks, Dooley and Evans, unpublished data).

Note the similarity in the responses of all groups during the first minute on test day 3, the initial exposure to the novel environment. Summary data of the total scores on each test day is presented in Figure 5-4, along with the standard deviation of each group. Error bars were not included in this graph in order to clearly show the group means and patterns of behaviour.

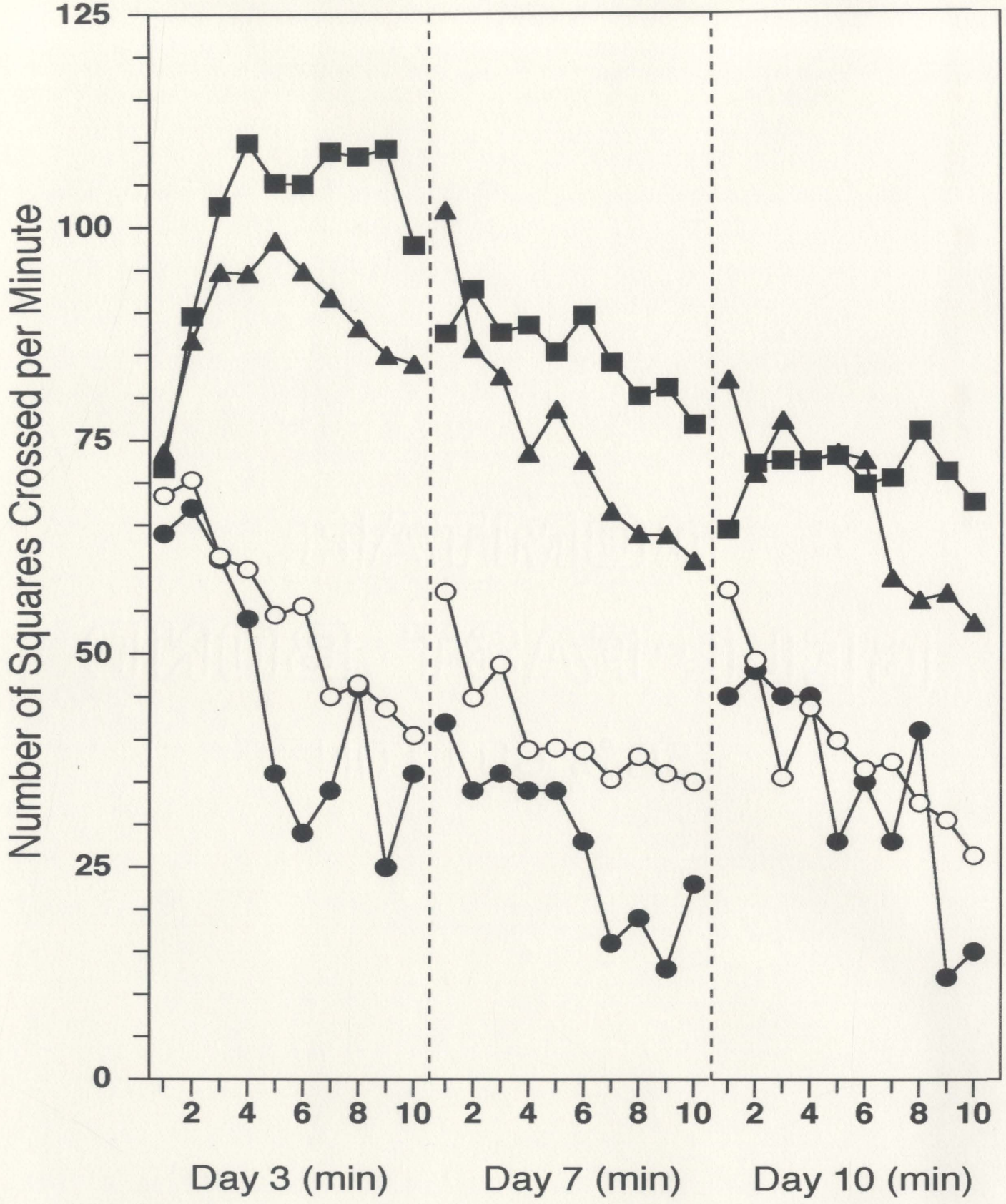

125
100
75
50
25
0
Number of Squares Crossed per Minute
2
4
6
8
10
Day 3 (min)
Day 7 (min)
Day 10 (min)

Figure 5-6. Open field line graphs, days 3, 7 and 10. Sham-operated animals and 5 minute occluded animals (hypothermic, normothermic and hyperthermic)

Open field activity on days 3, 7 and 10 post-surgery. Five minute occlusion, hyperthermia (squares- top left side filled), 5 minute occlusion, normothermia (filled squares), 5 minute occlusion, hypothermia (squares- bottom right side filled) and sham operated animals (open circles). Error bars were not included in this graph in order to clearly show the group means and patterns of behaviour. Note that the y-axis on this graph ranges from 0 to 150, whereas the y-axis in Figure 5-5 only ranged from 0 to 125.

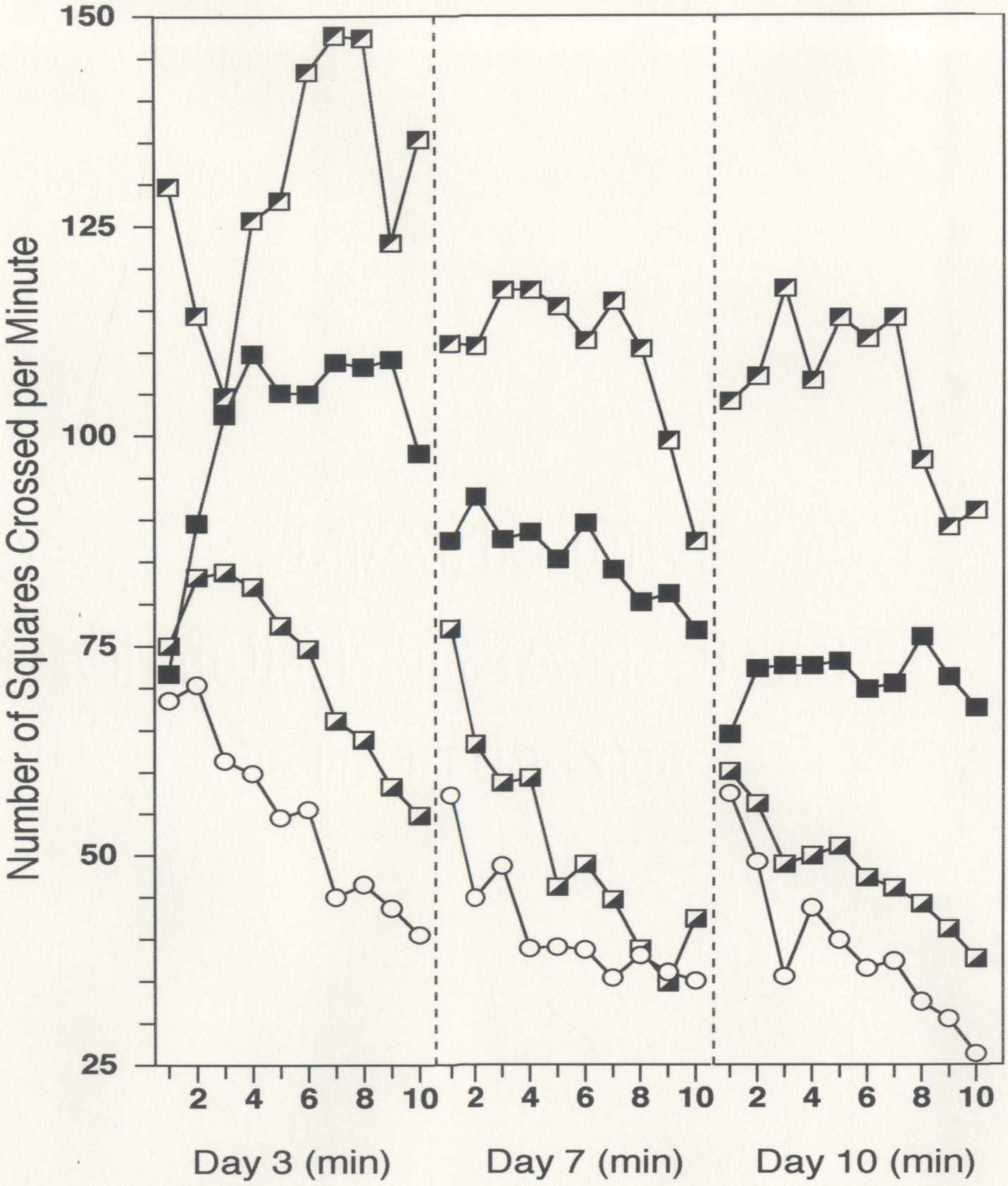
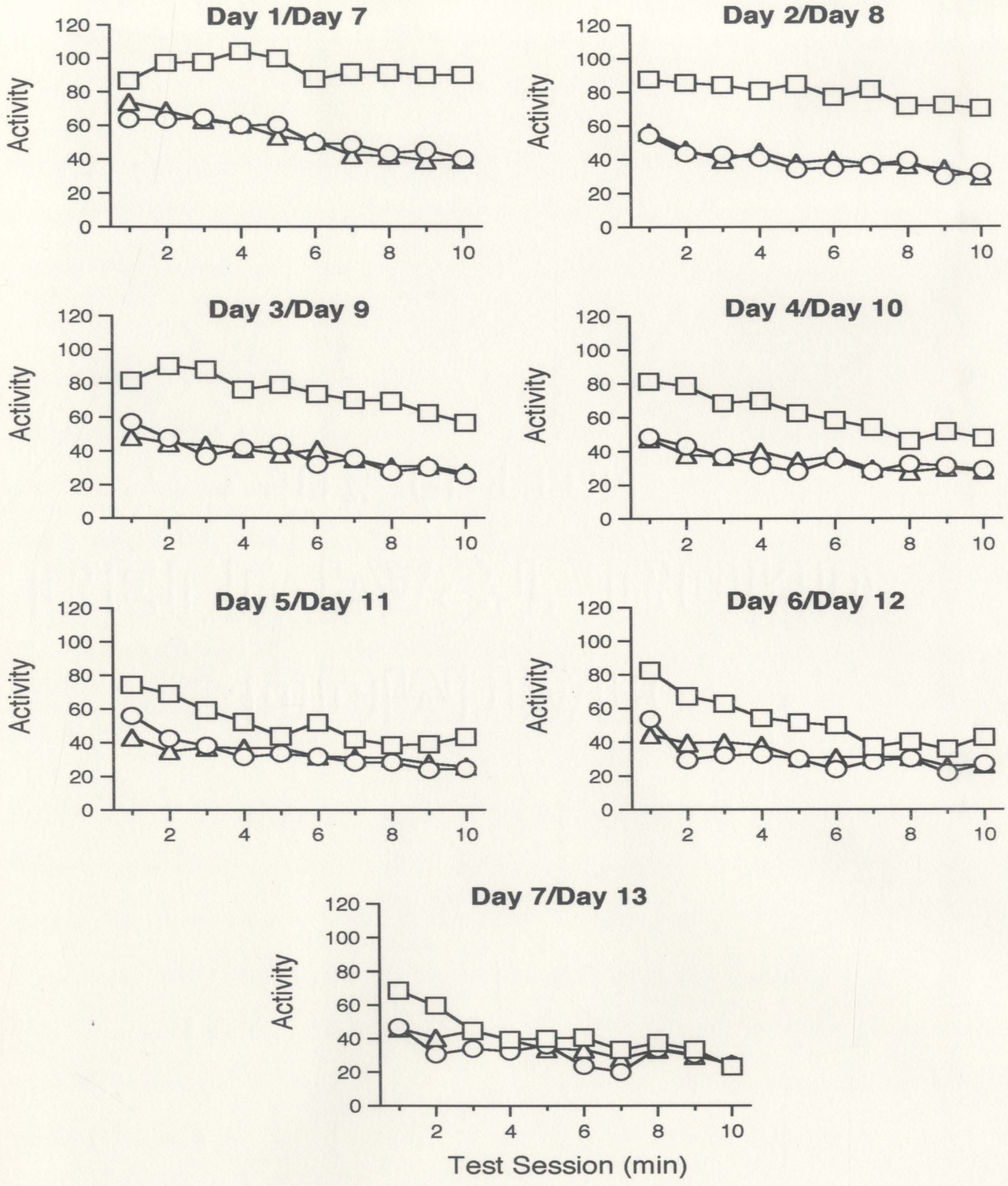

Number of Squares Crossed per Minute
150
125
100
75
50
25
2
4
6
8
10
Day 3 (min)
Day 7 (min)
Day 10 (min)

Figure 5-7. Unpublished data from Corbett, Hewitt, Wong and Nurse (1989), see discussion. Comparison of open field behaviour following a 5 minute ischemic episode when testing is started either 24 hours post-occlusion versus 7 days post-occlusion. Group A, 5 minute occlusion with daily testing beginning 24 hours post-surgery, open squares (n = 5). Group B, 5 minute occlusion with daily testing beginning at 7 days post-surgery, open circles (n = 5). Sham-operated animals, with testing beginning 24 hours post-surgery, open triangles (n = 8). Error bars have not been added in order to more clearly show the group means.


Day 1/Day 7
Day 2/Day 8
Day 3/Day 9
Day 4/Day 10
Day 5/Day 11
Day 6/Day 12
Day 7/Day 13
Activity
Test Session (min)
0
20
40
60
80
100
120
2
4
6
8
10

5.4 Discussion

Ischemia induces both an acute and a chronic increase in locomotor activity. The exact cause of both behavioural alterations is unknown, but the magnitude of the behavioural change is related to the severity of the ischemic insult. Due to the vulnerability of the CA1 area of the hippocampus to ischemia it has been proposed by many investigators that this region is responsible for the changes in behaviour. Abnormal activity in the CA1 cells may cause the acute behavioural effect (Kuroiwa et al., 1991a), loss of cellular function or CA1 cell death may be responsible for the deficits observed in the open field (Gerhardt and Boast, 1988; Wang and Corbett, 1990; Mileson and Schwartz, 1991; Babcock et al., 1993).

The pattern of *acute* postischemic behavioural activation is intriguing in that the animal's behaviour provided an index as to the severity of the ischemia. Ischemic durations of 1.5, 3 and 5 minutes produce clearly different behavioural profiles. The sham operated animals displayed a brief period of increased locomotor activity immediately following surgery, but this quickly recovered (i.e. average activity counts < 1). This post-anaesthesia activity elevation is commonly observed in gerbils. In the ischemic animals however, a different behavioural response was observed and both the *latency* to reach peak activity as well as the *duration* of locomotor activation varied depending on the length of the ischemic episode. Small differences in the ischemic duration (i.e. 1.5

versus 3 versus 5 minutes) had very marked differences on postischemic behaviour.

During the early postischemic period, the 3 and 5 minute ischemic groups initially displayed "ischemic hunch" before the phase of increased locomotor activity began. During this time the animals were conscious, but generally remained inactive. The period of ischemic hunch may reflect the length of time it takes for the brain to recover a relatively "normal" level of functioning. Ischemia causes a disturbance in ionic balance, a massive release of neurotransmitters, loss of energy reserves, hypoperfusion and depression of protein synthesis. The ischemic hunch phase may indicate the length of time it takes for the brain to recover from these perturbations. In magnetic resonance imaging studies, it has been found that recovery of high energy phosphates occurs within minutes following ischemia (Nowak et al., 1985; Haraldseth et al., 1992), so presumably ATP recovery would not explain the lengthy period of ischemic hunch. However, normalization of other functions, such as protein synthesis, can take several hours. In addition, the rate of recovery of protein synthesis varies with the length of ischemia and differences in intraischemic temperature. Following brief durations of ischemia (i.e. 2 minutes) the recovery of protein synthesis in the forebrain is much faster than following a 5 minute ischemic episode (Nakagomi et al. 1993). Interestingly, the prolonged suppression of protein synthesis during the postischemic period is reversed more quickly when intraischemic hypothermia is induced (Widmann et al., 1993).

Once behavioural activation began, the maximum level of activity was similar in all groups (a maximum of approximately 7 counts). This peak was observed in both the sham operated and the 1.5 minute occluded animals within the first hour following surgery, but the 1.5 minute group continued to display this high level of locomotor activity for a few hours. The 3 and 5 minute ischemic groups reached the peak at 3 and 5 hours, respectively. While the 3 minute group only spent approximately 2 hours at this high level of activity and then showed a gradual decline, the 5 minute group maintained this high activity level for almost 24 hours.

The cause of the acute increase in activity is unknown, but it has been speculated that abnormal activity in the CA1 area of the hippocampus is responsible (Kuroiwa et al., 1991a). Hossman's group measured locomotor changes after a 5 minute ischemic episode in gerbils, then 1 week later induced a second ischemic episode. The first 5 minute period of ischemia produced a dramatic increase in activity which lasted approximately 1 day (the greatest behavioural activation occurred during the first 12 hours) and ultimately 83 % of CA1 cells were lost. If a second 5 minute ischemic episode was induced 1 week later, there was only a brief increase in activity, which quickly subsided within 3 - 4 hours. Since the majority of CA1 cells would have died by 1 week postischemia the authors concluded that the presence of the CA1 neurons was necessary to cause postischemic hyperactivity. However, these data are open to other interpretations. First of all, many other brain regions, in addition to the CA1

region, are affected by an episode of forebrain ischemia, including the striatum, neocortex, septum, subiculum, CA2, CA3a and CA4 regions (Kirino, 1982; Crain et al., 1988; Akai and Yanagihara, 1993). Thus, a brain structure other than CA1 may be responsible for the behavioural changes. Alternatively, tolerance could develop to repeated ischemic episodes (Kitagawa et al., 1990) such that the magnitude of the behavioural response lessens after the second ischemic episode, irrespective of the loss of the CA1 neurons.

If CA1 neurons are responsible for the acute changes in activity following ischemia, this could result from stimulation of subicular neurons, which in turn have a prominent projection to the nucleus accumbens (Swanson and Cowan, 1977; Kelley and Domesick, 1982), a structure known to be involved in control of locomotor activity (Mogenson and Nielsen, 1984). Yang and Mogenson (1987) demonstrated that stimulation of subicular cells with NMDA results in motoric activation, which is blocked by concurrent application of a dopamine D2 antagonist into the nucleus accumbens. In the postischemic period, increased firing of CA1 neurons could activate the accumbens-mesencephalic locomotor system resulting in an increase in activity. A transient elevation in CA1 cell discharge could be responsible for the short period of behavioural activation seen after 1.5 minutes of ischemia (Nowak et al., 1985). The longer (3 and 5 minutes) durations of ischemia which induce delayed necrosis in CA1, could lead to an enhanced behavioural activation prior to the eventual death of the CA1 cells. There have been some reports of hyperexcitability in the CA1 region

postischemia (Suzuki et al., 1983). An earlier study (Brown et al., 1979) suggested that circling behaviour following carotid artery occlusion was due to epileptic activity. In a study by Mitani et al. (1989) using a gerbil model of global ischemia, carotid arteries were occluded for various durations (including 1.5, 3 and 5 minutes) and spontaneous extracellular unit activity was recorded postischemically in anaesthetized animals for 60 - 90 minutes. Interestingly, following a 1.5 minute occlusion there was a period of hyperexcitability in CA1 within the first hour. The spike discharges in this group were 4 times the frequency observed prior to ischemia. This period of high frequency discharges corresponds to the phase of increased behavioural activation observed after 1.5 minutes of ischemia (Figure 5-1). In this same study, Mitani and colleagues concluded that there was no hyperexcitability following 3 or 5 minutes of ischemia. However, as shown here, the phase of behavioural activation following occlusions of either 3 or 5 minute duration does not begin until a few hours after ischemia. Thus, it is possible that there were high frequency discharges in these groups but they might have been missed by terminating the recording session within 60 - 90 minutes of ischemia. There has been considerable debate over the issue of postischemic hyperexcitability. Many laboratories, even using periods of extended recording (Buzsáki et al., 1989), have been unable to replicate the original findings of Suzuki and colleagues (1983). These conflicting results could be due to species differences and/or differences in ischemic models. Gerbils were used in both the Suzuki and Mitani studies,

however, Buzsáki used the rat 4VO model. In our laboratory, it has been observed that rats (2VO model) do not exhibit the same level of postischemic behavioural activation as gerbils (Corbett and McKay, unpublished results). This might reflect a greater tendency for hyperexcitability in gerbils than in rats. However, rats do show an increased behavioural activation 24 - 72 hours postischemia. Correspondingly, Chang et al. (1989) have reported that there is increased firing of CA1 neurons on day 2 and day 3 after 2VO in rats, although not on day 1. Unfortunately, there have only been anecdotal reports linking increased behavioural activation to EEG changes in the CA1 region (Armstrong et al., 1989). Studies combining *in vivo* recording with detailed behavioural monitoring in freely moving animals, would help determine the extent to which neuronal activity in CA1 is involved in the behavioural activation observed after an ischemic episode.

There are many other explanations that could account for the increased activity in the early postischemic period. Cytokines, such as interleukin 1, are pyrogenic and cause behavioural arousal possibly mediated by the central actions of corticotrophin-releasing factor (CRF) (Berkenbosch et al., 1987, Sapolsky et al., 1987; Rothwell, 1989 and 1990). Concentrations of interleukins and CRF are known to increase in brain tissue after ischemia (Minami et al., 1992; Rothwell and Relton, 1993; Strijbos et al., 1994). Transmitters such as dopamine and glutamate have well-known effects on behavioural activation (Malec and Kleinrok, 1972; Pijnenburg et al., 1976; Donzanti and Uretsky, 1983). During

ischemia there is a massive release of glutamate (glutamate levels rise ~ 8 times above normal) and dopamine (300 - 500 % increase above normal, Globus et al., 1988a). While glutamate levels have been shown to return to control values soon after ischemia, a secondary rise in dopamine occurs during the early postischemic phase (Ahn et al., 1991). Unfortunately, long-term monitoring of transmitter levels, such as dopamine, during the postischemic period has not been carried out. This might be useful since the acute behavioural arousal induced by ischemia closely resembles the description of animals injected with psychomotor stimulants:

> " (Amphetamine) produces an increase in the incidence of forward locomotion, head movements, sniffing and rearing (i.e. the animal becomes generally hyperactive) and a concomitant decrease in the incidence of other behaviours, such as grooming...It should be noted that the pattern of locomotion produced by amphetamine is not normal in all respects, but is itself abnormally stereotyped", (p.161 and 162, Robinson and Becker, 1986).

Regardless of the mechanism for the acute behavioural change, it provides a useful measure of ischemic severity. Many models of cerebral ischemia, including the gerbil model used here, induce the ischemic episode under anaesthesia. This results in an inability to use behavioural signs (i.e. loss of

righting reflex) in order to determine the effectiveness of the occlusion. Other ischemia models, such as the rat 4VO model, are often carried out in the absence of anaesthetics. If certain critical behavioural symptoms are not observed (i.e. loss of the righting reflex) then animals are often discarded from the experiment. Since a small percentage of gerbils may have a functional circle of Willis, thus resulting in some blood flow via the collateral circulation, postischemic behavioural alterations could be used as a marker of effective or ineffective ischemia. In addition, many pharmacological studies now study the effects of delayed treatment (i.e. 12 - 24 hours postischemia). Therefore, the period of postischemic recovery prior to drug administration could be used to compare the behavioural responses among animals to ensure that different treatment groups had consistent ischemic injury before initiation of treatment. However, once drug treatments are administered, activity patterns can become confounded by side effects of the drugs, such as sedation. Indeed, many of the pharmacological agents that have been tested in animals models of ischemia (i.e. MK-801, NBQX, and the synthetic omega-conopeptide SNX-111) have sedating properties in the dose ranges that are protective.

Depending on the duration of the occlusion, within 1 - 2 days the behavioural activation declines and activity levels return to control values. In addition to the return to normal activity levels, species typical behaviours, such as grooming and paper shredding are also exhibited. A few days after the ischemic episode it would be difficult, if not impossible, to distinguish ischemic

from sham-operated animals by observing them in their home cages. However, if animals are placed in a novel environment, such as an open field apparatus, ischemic animals are readily distinguished from shams. The increased locomotor activity observed in an open field following occlusion durations of 3 and 5 minutes has been previously reported by many groups (Chandler et al, 1985a; Chandler et al., 1985b; Gerhardt and Boast, 1988; Babcock et al., 1993, , Mileson and Schwartz, 1991). A study by Wang and Corbett (1990), demonstrated that novelty is a requirement for this ischemia-induced change in behaviour. If animals were pre-exposed to the open field environment before the ischemic episode then the usual pattern of enhanced locomotor activity was not seen when the animals were re-exposed to the open field after ischemia. Thus, general locomotor hyperactivity is not the explanation for the enhanced behavioural activation in the open field since both ischemic groups, pre-exposed and naive, should have shown similar activity levels.

An accepted explanation that has been put forward for the behavioural response of ischemic animals to the open field test is that the loss of CA1 neurons alters processing of information through the tri-synaptic circuit of the hippocampus. This alteration in hippocampal processing may then lead to memory deficits, especially for spatial information, such that ischemic animals who have sustained damage to the CA1 sector do not habituate to novel environments as quickly as normal animals (Wang and Corbett, 1990; Babcock et al., 1993). If the animals are pre-exposed to the open field before ischemia, there

is no deficit because the animals have already habituated to the environment and stored this information elsewhere. The hippocampus is not believed to be a site for long-term memory storage (Scoville and Milner, 1957; Zola-Morgan et al., 1986). This interpretation of the open field deficit is consistent with findings from humans with hippocampal injury. For example, patient R.B. who had ischemic injury restricted to the CA1 field of the hippocampus as a result of cardiac arrest, suffered from anterograde amnesia deficits while "older" memories were left intact. The open field deficit has been repeatedly demonstrated in ischemic animals and has proved to be invaluable as an index of hippocampal injury since open field behaviour correlates highly with CA1 cell loss (Gerhardt and Boast, 1988; Mileson and Schwartz, 1991; Nurse and Corbett 1994) and not with damage to the caudate or cortex (Mileson and Schwartz, 1991). The open field test is also useful as a functional measure since deficits can be demonstrated for months after the ischemic event. In a long-term survival study, ischemic animals were tested in an open field 5, 10, 30 and 180 days postischemia (Colbourne and Corbett, 1994b; Colbourne and Corbett, 1995). Locomotor activity was significantly higher in the ischemic group than the sham-operated group on *all* test days. These data were interpreted as demonstrating a persistent memory deficit (lasting up to 6 months) in ischemic animals. However, given the simplicity of the open field test it is somewhat surprising that the animals have such difficulty habituating to this environment. Indeed, other studies have shown that ischemic animals have no detectable deficits in more

conventional tests of spatial memory, such as the Morris milk maze, if testing starts 21 days postischemia (Corbett et al., 1992).

Other results from an unpublished experiment by Corbett, Hewitt, Wong and Nurse (1989) raise questions about the exact nature of the open field deficit. In this unpublished study, two ischemic groups were compared to a group of sham-operated animals. Each ischemic group received 5 minute carotid artery occlusions. All groups were exposed to the open field daily, post-surgery, for 7 test days. The only difference between the two ischemic groups was the timing of the first exposure to the open field. In Group A open field testing was initiated 24 hours after ischemia, in Group B testing was delayed and the first exposure to the open field was 7 days postischemia. The sham-operated animals were tested starting 24 hours post-surgery. In Group A, the typical ischemic deficit was observed, which gradually recovered with daily testing. However, in Group B, both the absolute activity scores and the pattern of the response during each session were virtually identical to the sham-operated group. *There were no detectable differences between Group B and the sham operated animals* (see Figure 5-7), but the two 5 minute ischemic groups (Groups A and B) had significantly different behavioural profiles even though both groups had similar (moderate to severe) CA1 cell loss. What does this study suggest? Perhaps the open field deficit we routinely observe following ischemic durations of 3 minutes or longer is the result of *sensitization* triggered by novelty. If so, there may be a critical period following ischemia in which sensitization can take place. If the

animals are exposed to the open field after the critical period then they behave normally. In contrast, animals sensitized to the open field during the critical period may experience context-dependent conditioning so that subsequent intermittent exposures to the same environment (i.e. at 10 days or even 6 months post-occlusion) can trigger an increase in locomotor activity.

Context-dependent conditioning of locomotor activity can be induced pharmacologically, mainly by amphetamine-like stimulants and μ-opiates (Robinson and Becker, 1986) and also by stress (Antelman et al., 1980). Behavioural changes resulting from sensitization have been reported to last for months (Babbini et al., 1975; Kalivas and Stewart, 1991). If the postischemic deficit is a result of context-dependent conditioning it suggests that the problem is not an inability to learn or remember the environment, but instead a very strong conditioning response due to sensitization on the first exposure. Sensitization could be due to a generalization from the acute behavioural activation caused by ischemia to other stressors such as novelty, or it could be related specifically to CA1 neuronal loss.

Hippocampal lesions produced by methods other than ischemia have long been known to cause changes in locomotor activity (Kimble, 1963; Teitelbaum and Milner, 1963; Douglas and Isaacson, 1964; Jarrad, 1968). Increases in locomotor activation have been observed in both the animal's home cage and in novel environments, such as the open field test. As with ischemia, the exact cause of these behavioural changes is unknown. Glickman et al. (1970) have

shown that home cage locomotor activity is increased after hippocampectomy in gerbils, with assessments starting 17 days post-surgery. If these locomotor changes are due to the same mechanisms as the ischemia-induced behavioural alterations, then is the time course for acute home cage changes related to the magnitude of the hippocampal destruction? Following ischemia the hippocampal damage is much less extensive than in many studies of hippocampal lesions, with ischemia-induced necrosis confined primarily to the dorsal CA1 sector and the period of home cage activity changes is substantially shorter. As shown here, increasing durations of ischemia (i.e. 5 minutes versus 3 minutes) which increase hippocampal damage, lead to longer durations of elevated activity in familiar environments. Inducing ischemia (5 minutes duration) under slightly hyperthermic conditions, may have led to acute behavioural changes that persisted for at least 72 hours (rather than 48 hours) since the open field scores on test day 1, minute 1, were extremely high in this group. Hyperthermia also increased the extent of hippocampal damage, such that necrosis extended into the CA3 sector, which normally appears quite healthy after 5 minutes of ischemia at normothermia. If increasing degrees of hippocampal injury lead to longer periods of heightened locomotor activity in familiar environments, this may affect the time period in which deficits will be elicited in novel environments such as an open field. Babcock et al. (1993) induced 5 minute occlusions in gerbils, with temperature maintenance (38 °C), followed by open field testing at various times in the postischemic recovery period. In some groups testing was delayed until

13 days post-occlusion. In contrast to what we found in the unpublished 1989 study, Babcock et al. (1993) reported that the open field deficit was still apparent when initial testing began 13 days post-occlusion. There was no quantification of ischemic damage in this study, however, the representative photograph of the hippocampus from an ischemic animal appeared to have some injury in the CA3 sector as well as in the CA1 region. This degree of hippocampal injury is greater than the level of damage we obtained in our 1989 study and might have lead to a longer duration (i.e. 2 weeks versus less than 1 week) during which open field deficits would be elicited in novel environments.

Understanding the nature of a behavioural deficit following brain injury is a difficult undertaking. When the brain has been lesioned, researchers are not really studying the isolated function of the damaged region, but more specifically, how the remainder of the brain functions in the absence of this structure. In animal models, the task is further complicated by the inability to use many of the same tests that are used with humans since these often involve language. In addition, the testing protocol can have a large effect on the conclusions drawn from behavioural experiments. If locomotor activity had only been tested in familiar environments then it would have been concluded that the locomotor activity normalized within several hours to two days, depending on the duration of occlusion. This would have added to the conflicting reports in the literature, where groups comparing similar occlusion durations have reported vastly different time courses of behavioural activation (Gerhardt and Boast , 1988; Kuroiwa et al., 1991a,b; Colbourne and Corbett, 1995). Both acute and

chronic deficits may be related to CA1 cell loss, but each of these behavioural changes will have to be studied in more detail in order to determine the mechanism(s) underlying the locomotor activation. The changes in the early postischemic period may help to explain some of the neuronal events that underlie delayed neuronal death. Understanding the basis of these behavioural disturbances may lead to the development of new pharmacological therapies for ischemia. The open field test reveals that there are deficits which persist in ischemic animals *after* CA1 neurons have died, which is consistent with reports of long-lasting impairments in human patients (Zola-Morgan et al., 1986). It remains to be established whether the open field deficits that have persisted for months after the ischemic episode in animals are due to a persisting memory impairment, or whether this reflects a carry-over effect from earlier test sessions. Whatever the case, it has been shown in several experiments (see Chapter 3) that the magnitude of increased locomotor activity in the open field test (measured during the first week postischemia) corresponds to the size of the field responses recorded from the CA1 region and both functional measures relate to the magnitude of CA1 cell loss. Thus, the open field remains the best behavioural test to date for functionally assessing the magnitude of global ischemic injury.

CHAPTER 6: DISCUSSION

6.1 Summary of Major Findings

In Chapters 3 and 4, two different putative interventions to prevent ischemic injury, intraischemic hypothermia and NBQX, were found to provide protection against cell injury resulting from global ischemia. Although NBQX is a glutamate receptor antagonist, the protection it provided was through lowering brain temperature. Intraischemic hypothermia is a remarkably effective treatment. Even 3 weeks following the ischemic insult, both the functional measures and the CA1 cell ratings were indistinguishable from controls. This demonstrates that appropriate treatments can preserve function *and* prevent cell death. In the absence of temperature regulation, NBQX provided histological preservation 4 days postischemia. However, functional outcome based on electrophysiological recordings from the CA1 region, was not robust.

Intraischemic hypothermia preserved both behavioural and electrophysiological function. In previous studies of intraischemic hypothermia, behavioural tests done weeks after the ischemic insult have shown that *function* is preserved (Green et al., 1992). However, as discussed in Chapter 3, it may be more important to carry out behavioural tests soon after the ischemic episode, before any substantial recovery of function has taken place, since this may obscure an underlying deficit. For example, within the intraischemic hypothermia group CA1 cell ratings were lower in some animals than a sub-group of hypothermic animals with total or near total protection. It is unlikely that these

behavioural deficits in the IBH-high sub-group would have been detected with later testing. Even in untreated animals, the open field deficit following ischemia diminishes with increasing recovery time, *especially* if early testing has not been carried out (see Chapter 5).

Electrophysiological recording complements the behavioural testing in that it can be carried out at later survival times and the cell region of interest (i.e. CA1) can be recorded from directly. With behavioural tests one region cannot be tested in isolation and most often it is unclear which region(s) is contributing to a given behavioural deficit. In spite of these complications, behavioural testing permits the overall cognitive functioning to be evaluated, something not easily achieved with electrophysiology alone.

The NBQX data (Chapter 4) are quite important since they strongly suggest that direct blockade of AMPA receptors located on CA1 cell bodies is not responsible for the neuroprotection observed. Two lines of evidence help support this claim: (i) no protection was found when hypothermia was prevented after administration of NBQX and (ii) a precise simulation of the NBQX-induced temperature profile in the absence of the drug was neuroprotective. Of course, the fact that the degree of protection declined at a faster rate in the simulated temperature group than it did in the NBQX group raises the possibility that the drug offers an additional protective effect independent of a reduction in temperature. Thus, even if the simulated hypothermia had been maintained for the same duration as NBQX-induced hypothermia (i.e. ~ 100 hours) the protection may have still been inferior to that achieved with NBQX. However, the improved efficacy found with deliberate hypothermia (32 °C) when the

duration of treatment is lengthened from 12 - 24 hours (Colbourne and Corbett, 1994a) supports the hypothesis that the protection obtained with NBQX is due to a significantly greater duration of hypothermia. If so, keeping the temperature at a subnormal level for 4 - 5 days should achieve a comparable degree of protection to that observed with NBQX.

Regardless of the mechanism of action of NBQX, neuronal functioning (assessed electrophysiologically) is reduced at four days postischemia, a time when cell counts are indistinguishable from control animals. Therefore, NBQX does not provide the robust functional protection of intraischemic hypothermia, presumably because the degree of hypothermia achieved with NBQX is so mild, i.e., a decrease of only 1.0 - 1.5 °C. Indeed, increasing the level of hypothermia leads to increased neuronal and functional protection. For example 32 °C is far more effective than 34 °C (Colbourne and Corbett, 1995).

The changes in locomotor activity following ischemia were described in Chapter 5. Acute alterations in postischemic activity are a sensitive indicator of ischemia, even in cases of minimal injury. For example, animals exposed to intraischemic hypothermia displayed a brief burst in locomotor activity about 2 hours later, which quickly declined. Even in the IBH-low sub-group, which had virtually no injury in CA1 and normal open field behaviour, there was still an acute increase in activity which was not different from the IBH-high group (data not shown). Increased locomotor activity is also apparent in the 1.5 minute occluded group in which there is no necrosis in the CA1 region. Interestingly, the early activity patterns following 5 minutes of ischemia with intraischemic hypothermia did *differ* from the pattern following 1.5 minutes of ischemia at

normothermia, even though both groups had minimal CA1 injury. The 1.5 minute group did not exhibit ischemic hunch and the increase in activity peaked during the first hour. The intraischemic hypothermic group exhibited ischemic hunch and the peak behavioural activation occurred later. This suggests 5 minutes of ischemia with intraischemic hypothermia results in an insult that is greater in magnitude than an insult of 1.5 minutes duration at normothermia, but the reduced temperature enables recovery to take place. Moreover, the magnitude of the acute increase in locomotor activity is greatly enhanced with increasing occlusion durations. The difference between the 3 and 5 minute occlusion durations is quite striking, especially since the 3 minute duration is severe enough to kill > 80 % of dorsal CA1 neurons.

Changes in open field behaviour measured after ischemia are thought to result from impairments in spatial memory (Wang and Corbett, 1990; Babcock et al., 1993). However, this interpretation does not explain why animals with early exposure to the open field, i.e., 1 to 3 days postischemia, exhibit *higher* levels of activity on later test days than animals not previously exposed. It is possible that this effect is due to context-dependent sensitization and thus, dopamine antagonists, which block pharmacologically-induced sensitization (Stewart and Vezina, 1989), might block ischemia-induced sensitization. Such experiments are necessary if the ischemia-induced changes in the open field (and perhaps other behaviours) are to be understood.

6.2 Intraischemic versus Postischemic Treatments

As discussed in the previous section, 6.1, earlier reports suggesting that intraischemic hypothermia provides long-term benefit have been confirmed by the study presented in Chapter 3. The protection obtained with postischemic administration of NBQX, however, is questionable based on the results from the present studies, at best the protection provided is only partial. In view of these data it is unclear whether any purportedly protective pharmacological treatments administered during the postischemic period would achieve permanent neuroprotection.

The effectiveness of postischemic versus intraischemic treatments is dependent on the mechanisms involved in cell death and the mechanism of action of the protective treatment. In general, the events culminating in death of vulnerable neurons follow a three-step process:

Ischemia ——————→*Reperfusion* ——————→*Cell Death*

During the initial days following reperfusion, the repercussions of ischemia (and possibly postischemic events as well) become manifest and selective neuronal death results. Events occurring during the postischemic reperfusion period do have an impact on the *time course* of cell death as was demonstrated with both the postischemic administration of NBQX and the manual simulation of the NBQX temperature profile. It remains to be established that any postischemic treatment will result in lasting neuroprotection. This issue is dependent on

whether cell death results from the ischemia itself, or whether events in the postischemic period also injure neurons. Two theoretical models are outlined:

(i)	Irreversible injury induced by ischemia	⟶	Maturation period for the injury to result in cell death	⟶	Cell death
(ii)	Injury during the ischemic period	⟶	Exacerbation of injury during reperfusion	⟶	Cell death
		⟶	Postischemic intervention prevents the exacerbation of injury	⟶	Reduction in cell death

If scenario (i) is accurate then only intraischemic treatments will provide lasting protection. An intervention during the postischemic period may alter the time course of events culminating in necrosis, possibly by weeks or months, but will not irrevocably halt the processes culminating in cell death. Scenario (ii) however is more optimistic, it assumes that neuronal death results from the combined effects of ischemia and events during reperfusion. If this is the case, then a treatment which *effectively* reduces either the intraischemic or the postischemic process(es) will provide permanent neuroprotection. The strongest evidence in favour of the second scenario is the effectiveness of long duration

postischemic hypothermia (32 °C for 24 hours). This postischemic therapy provides an impressive degree of protection, but still the protection declines significantly between survival times of 1 month (~ 90 % protection of CA1 cells) and 6 months (~ 70 % protection of CA1 cells) (Colbourne and Corbett, 1995), and might continue to decline beyond this time. Long-term survival studies (i.e. 6 - 12 months) to assess the permanence of the neuronal protection obtained with intraischemic hypothermia will also need to be carried out in order to ensure that there is no significant decline. However, assessments made 2 months after intervention with intraischemic hypothermia (Green et al., 1992; Dietrich et al., 1993) suggest that the degree of CA1 cell preservation does *not* decrease with increasing survival times.

6.3 Hypothermia: Possible Mechanisms of Action

The mechanism(s) of action for the neuroprotective effects of hypothermia are unknown. It is possible that intraischemic and postischemic hypothermia do not necessarily work by the same mechanisms. Intraischemic hypothermia may lessen the degree of injury that a neuron sustains during the ischemic period. However, when postischemic treatments are employed, the neuron may have already sustained a level of injury that is sufficient to cause substantial cell loss in untreated animals. Therefore, postischemic hypothermia must either reduce the impact of the injury sustained and/or reduce the events associated with reperfusion that may also contribute to cell injury. There are many ways in which hypothermia could act to protect against ischemic damage, and the unrivaled

protection achieved with hypothermia (both intra- and post-ischemic hypothermia), in comparison to all currently used pharmacological treatments, may be due to *multiple* protective actions.

Hypothermia has been shown to stabilize neuronal membranes, thereby reducing leakiness and decreasing the magnitude of shifts in ionic balances, particularly Na^+ and K^+ (Astrup, 1982). Since maintenance of Na^+/K^+ homeostasis is the largest drain of metabolic energy in the brain (Astrup, 1982), stabilizing the tendency for membrane leakage of these ions will help to conserve ATP. Once synaptic transmission becomes depressed (~ 30 - 60 seconds after initiation of ischemia), 50 % of the remaining energy consumption is used to maintain Na^+/K^+ homeostasis (Whittam, 1962). Under hypothermic conditions, decreased utilization of ATP stores during the ischemic period should lead to an increase in the duration of ischemia that a cell can endure without injury. During anoxic/aglycemic conditions *in vitro*, hypothermia (30 - 31 °C) either prevents the occurrence of negative DC shifts (resembling spreading depression), or delays the time to the onset of these negative shifts (Hiramatsu et al., 1993; Taylor and Weber, 1993). This is believed to be due to decreased utilization of ATP during ischemia at hypothermic conditions. Under normothermic conditions, the appearance of negative DC shifts has been linked to irreversible neuronal injury (Somjen et al., 1990; Hossmann, 1994). In addition, if less ATP is used *during* ischemia then the accumulation of lactic acid will also be decreased, which could be protective by minimizing the ischemia-induced reduction in pH.

Another pathological change induced by ischemia, which is associated with irreversible injury, is prolonged inhibition of protein synthesis. Five minutes

of forebrain ischemia in the gerbil, at normothermia, will permanently suppress protein synthesis in vulnerable brain regions most notably the CA1 sector (Nowak et al., 1985; Nakagomi et al., 1993). In the rat 4VO model, 30 minutes of ischemia will also result in permanent suppression of protein synthesis if brain temperature is maintained at normothermic levels. However, decreasing the intraischemic brain temperature to 30 °C promotes recovery of protein synthesis in the CA1 region within 2 hours (Widmann et al., 1993). Thus, intraischemic hypothermia does not prevent the initial suppression of protein synthesis that is seen throughout the forebrain, but it results in an accelerated recovery.

Hypothermia has also been found to decrease transmitter release, with or without ischemia (Mitani and Kataoka, 1991; Hiramatsu et al., 1993). During ischemia, the extracellular accumulation of glutamate is thought to be primarily from non-vesicular release (Ikeda et al., 1989; Lobner and Lipton, 1990; Szatkowski et al., 1990; Zini et al., 1993). This Ca^{2+}-independent release of glutamate appears to be temperature sensitive, i.e., decreased temperature leads to decreased release of transmitter (Mitani and Kataoka, 1991). If glutamate does play a role in the subsequent cell death, then at least part of the protective action of hypothermia is attributable to a reduction in glutamate levels during ischemia. In addition, if there is less transmitter released during ischemia, then this will reduce the energy expenditure necessary during the postischemic period to *restore* homeostasis, thereby helping to better couple metabolic demand to blood flow.

Many of the same mechanisms outlined for intraischemic hypothermia could contribute to the protection offered by hypothermia postischemically. The

protective effect of even mild, postischemic hypothermia (a decrease of only 1 - 2 °C) is somewhat surprising. However, very small decreases in temperature, for example 1 °C, can decrease metabolic rate by 10 % (Gordon et al., 1984). Following reperfusion, blood flow is briefly increased above normal levels, followed by hypoperfusion about 10 minutes postischemia (Schmidt-Kastner and Freund, 1991). Since the level of blood flow during this period of hypoperfusion is normally inadequate to meet metabolic demand, postischemic hypothermia could help to couple the metabolic rate to the blood flow level by reducing metabolic demand.

A recent hypothesis to explain the death of CA1 neurons after ischemia has suggested that *synaptic activation* of CA1 cells during the *postischemic* period is the critical factor underlying cell death. For example, Crain et al. (1988) and Hsu and Buzsáki (1993) proposed that the early death of the hilar and CA3 interneurons (during the first 24 hours postischemia) increases excitatory transmission through the hippocampus and is responsible for the death of the CA1 neurons that becomes evident ~ 72 hours postischemia. Hypothermia following ischemia would reduce the excitatory drive on CA1 cells and could account for the observed protection. Although an appealing hypothesis, some pharmacological treatments that rescue hilar interneurons do not protect CA1 pyramidal cells (Honey et al., 1995).

6.4 Glutamate Excitotoxicity: Involvement of the AMPA Receptor?

The present experiments using the AMPA receptor antagonist, NBQX, have not yielded evidence favouring the glutamate excitotoxicity hypothesis. Previous pharmacological studies involving NBQX have been confounded by hypothermia, as were the original studies with the non-competitive NMDA receptor antagonist, MK-801. The subtlety of the hypothermia induced by NBQX may explain why it was missed with only one sampling of rectal temperature (Sheardown et al., 1990). The inability of NBQX to protect CA1 neurons when brain temperature is regulated suggests that activation of the AMPA receptor does *not* serve a critical role in ischemic cell death. However, the *duration* of any treatment is an important factor with respect to neuronal protection. Most pharmacological studies investigating ischemia employ a single dose of a drug. If neuroprotection is not obtained the drug is usually not pursued further. However, in order to protect neurons from ischemic damage, drug studies may need to be approached in the same way as recent experiments using postischemic hypothermia. Colbourne and Corbett (1994a) have shown a striking improvement in efficacy by increasing the duration of postischemic hypothermia from 12 hours to 24 hours. NBQX induces a prolonged period of hypothermia, but this temperature effect might be due to a non-specific effect of the drug, such as kidney toxicity (Xue et al., 1994). The half-life of NBQX (30 mg/kg, intravenous bolus) in the mouse is 4 hours (Dalgaard et al., 1994). In ischemia studies the higher dose and different route of administration of NBQX (90 mg/kg, i.p.) may result in a longer duration of action, but if the half-life is only a few hours then multiple injections of NBQX may be needed to maintain an

effective level of receptor blockade over ~ 24 - 48 hours. While theoretically possible, the toxicity of this compound would probably preclude repeated dosing.

In addition to the negative results reported in this thesis with NBQX, other problems with the original hypothesis of glutamate-AMPA toxicity have arisen. There has been difficulty in replicating the findings of Pellegrini-Giampietro et al. (1992, 1994) who found a decrease in the mRNA for the GluR2 subunit after ischemia. Thus, there may not be a reduction in the subunit which is responsible for the Ca^{2+}-impermeability property of the AMPA receptor (Diemer et al., 1994). Therefore, the hypothesis that postischemic activation of the AMPA receptor results in increased calcium entry through the associated ion channel may not be valid. This view is consistent with the lack of neuronal protection found with NBQX, when hypothermia is prevented.

6.5 Importance of Functional Assessments

Assessment of ischemic injury simply by counting neurons in the CA1 sector obviously does not provide the valuable information that can be obtained when functional tests are combined with a histological evaluation. In the absence of a reliable functional assessment, erroneous conclusions regarding the efficacy of a treatment can be easily made. For example, by assessing the protection afforded by NBQX at 4 days with cell counts as the outcome measure, it would have been concluded that NBQX provides virtually complete protection of CA1 neurons. However, the electrophysiological assessment at 4 days does

not support this conclusion and it appears that with increasing survival time histological protection is completely lost (Li and Buchan, 1995). In the mid-1980's cell death in the hippocampus was thought to be complete by ~ 72 hours and this led investigators to assess protective treatments histologically following survival times of 4 - 7 days. More recent data show that certain treatments can *postpone* the time course of cell death (i.e. Morse and Davis, 1990; Dietrich et al., 1993; Colbourne and Corbett, 1994a, 1995; Li and Buchan, 1995), however, from the data currently available, we do not know how long the cell death process can be extended. Colbourne and Corbett (1995) used a survival time of 6 months to assess the protective value of postischemic hypothermia. The protection of CA1 neurons declined from ~ 90 % at 30 days to ~ 70 % at 6 months, however, since only two time points were assessed it is not certain whether cell death was still continuing at 6 months, or whether the 20 % loss occurred soon after the 30 day survival time (perhaps between 1 and 2 months). Since it is unknown at what point it is reasonable to conclude *permanent neuroprotection* has been achieved, the addition of functional measures should help when assessing potentially protective therapies for ischemic injury. For example, if the animals in the above postischemic hypothermia study had been assessed electrophysiologically at 6 months and the resulting field potentials were ~ 70 % of normal, then this would represent stronger evidence that the level of protection was permanent. If field potentials were less than 70 %, this would indicate that a percentage of the CA1 cell population was non-functional and might be continuing to die. Interestingly, the behavioural measures recorded in the open field during the first week after ischemia in this study suggest that the

protection of postischemic hypothermia is incomplete, even though cell counts at this are 90% or greater. This indicates that early functional testing is a better predictor of eventual histological outcome than early histological assessment.

Other similar findings have recently been obtained in our laboratory in experiments with ischemic preconditioning (Corbett et al., 1995). Ischemic preconditioning is a phenomenon whereby brief durations of ischemia (e.g. 1.5-2 minutes) that are not lethal to CA1 cells "protect" against a subsequent ischemic episode (e.g. 5 minutes) that would ordinarily destroy 95 % of these neurons (Kitagawa et al., 1990). In our experiments animals were exposed to two 1.5 minute periods of ischemia, separated by an interval of 24 hours, followed 3 days later by a 5 minute occlusion. Field potentials recorded 4 days postischemia from animals who had received ischemic preconditioning were only ~ 50 % of normal, whereas the CA1 cell counts suggested that protection was ~ 90 % (Corbett et al., 1995). Open field activity levels in these preconditioned animals were as high as the levels in animals with near total CA1 destruction. Longer survival times assessed with the preconditioning treatment indicate that histological protection declines to 70 - 80 % of control at 10 days and ~ 50 % of control at 30 days (Crooks and Corbett, manuscript in preparation). As shown with NBQX, the ischemic preconditioning data also demonstrates a dissociation between indices of functional and histological protection.

The functional approach developed to evaluate potentially beneficial treatments in global ischemia could be improved by the addition of other behavioural tests and electrophysiological indices. However, if this approach is to be routinely used to assess a pharmacological treatment it is necessary to

keep the assessment measures simple, otherwise it becomes impractical for pharmacological studies where multiple groups of animals are required. While other types of behavioural tests (e.g. maze tests) have been used to evaluate animals following ischemia, none have been found to have the *sensitivity* of the open field test. In addition, many of these tests are either too labour intensive (e.g. T-maze and Morris milk maze) or do not distinguish between sham-operated and ischemic animals. However, additional electrophysiological tests, such as long-term potentiation (LTP), may prove useful. LTP is a long-lasting change in synaptic strength induced by a brief period of high frequency activation, that was originally described by Bliss and Lømo (1973). It is widely believed that LTP is a good neurobiological model for identifying molecular processes involved in long-term memory formation. LTP has been assessed in hippocampal slices taken from animals that were made ischemic *in vivo* (Hori and Carpenter, 1994), animals with chronic low blood flow (Sekhon et al., 1994), and animals subjected to traumatic brain injury (Miyazaki et al., 1992). In all cases, the histological appearance of CA1 and nearby neurons was unchanged from controls, whereas there was a significant reduction in the success of LTP induction. Thus, measuring the capacity of CA1 cells to express and maintain LTP could be used as a sensitive functional marker of CA1 neuronal integrity. In the early stages of cell death, synaptic currents may be normal but neuronal functions such as intracellular signaling may be abnormal.

The functional measures described in this thesis were designed to gauge hippocampal functioning after global ischemia. However, a similar approach could also be used in models of focal ischemia. Most focal models involve

occlusion of the middle cerebral artery which leads to cortical and varying degrees of striatal damage in the hemisphere ipsilateral to the occlusion. Typically, functional deficits, such as circling and contralateral paralysis or weakness are seen in these focal models.

A sensitive test for cortical damage is the measurement of forepaw dexterity, and the "staircase test" has been designed for this purpose in rodents (Montoya et al., 1991). This staircase test has recently been used in our laboratory to assess neuroprotection in a focal ischemia model (Peeling, Auer, Buchan, Saunders, Corbett and Palmer, manuscript in preparation) and appears to be more sensitive than routinely used histological endpoints, which measure infarct volumes. As in global ischemia, electrophysiological measures could be incorporated into focal studies by examining responses from cortical slices taken from the hemisphere affected by the occlusion. Regions that are infarcted would not be useful but cortical slices from regions bordering the infarct, the "ischemic penumbra", could be utilized to determine the size of the field potentials. In order to carry out a histological evaluation, in the same animals, slices could be immersion fixed after recording. Functional assessment is potentially more useful in focal ischemia models than it has been for global ischemia models. In global ischemia, the detection and quantification of CA1 neuronal loss is relatively simple due to the laminar organization of the hippocampus. However, the degree of injury in most focal studies is based solely upon calculating infarct volume with image analysis techniques. While the infarcted region (which appears unstained) is easily determined, quantification of partial neuronal loss in cell rich regions surrounding the ischemic core (i.e. the penumbra) is problematic. In

addition, surviving neurons, like those in global ischemia, may be viable, but functionally abnormal. Thus, functional outcome measures should be included in focal ischemia studies as well.

Any treatment which is being considered for clinical trials should not proceed until it has been demonstrated that it provides functional protection as well as histological protection comparable to what was demonstrated in the present experiments with intraischemic hypothermia. In addition, such treatments must also yield protection with survival times of at least several months, to reduce the likelihood that cell death is only being delayed. Without these minimal criteria being met, it is likely that laboratory derived therapies will fail when assessed clinically. This could lead to the conclusion that animal models are not relevant to human stroke. Instead, the discrepancies in neuroprotection may be due entirely to the use of inadequate assessment measures during preclinical (i.e. animal models) testing. Recognition of the importance of functional assessment and the demonstration of permanent versus transitory neuroprotection should allow basic science research to provide a truly effective therapy for acute stroke.

REFERENCES

Ahn SS, Blaha CD, Alkire MT, Wood E, Gray-Allan P, Marrocco RT, Moore WS (1991) Biphasic striatal dopamine release during transient ischemia and reperfusion in gerbils. *Stroke* **22**:674-679.

Akai F, Yanagihara T (1993) Identity of the dorsal hippocampal region most vulnerable to cerebral ischemia. *Brain Res* **603**:87-95.

Andou Y, Mitani A, Masuda S, Arai T, Kataoka K (1992) Re-evaluation of ischemia-induced neuronal damage in hippocampal regions in the normothermic gerbil. *Acta Neuropathol* **85**:10-14.

Antelman SM, Eichler AJ, Black CA, Kocan D (1980) Interchangeability of stress and amphetamine in sensitization. *Science* **207**:329-331.

Aoki M, Nomura F, Stromski ME, Tsuji MK, Fackler JC, Hickey PR, Holtzman D, Jonas RA (1994) Effects of MK-801 and NBQX on acute recovery of piglet cerebral metabolism after circulatory arrest. *J Cereb Blood Flow Metab* **14**:156-165.

Armstrong DR, Neill KH, Crain BJ, Nadler JV (1989) Absence of electrographic seizures after transient forebrain ischemia in the Mongolian gerbil. *Brain Res* **476**:174-178.

Astrup J, Siesjö BK, Symon L (1981) Thresholds in cerebral ischemia: The ischemic penumbra. *Stroke* **12**:723-725.

Astrup J (1982) Energy-requiring cell functions in the ischemic brain. Their critical supply and possible inhibition in protective therapy. *J Neurosurg* **56**:482-497.

Auer RN, Kalimo H, Olsson Y, Siesjö BK (1985) The temporal evolution of hypoglycemic brain damage. I. Light and electron-microscopic findings in the rat cerebral cortex. *Acta Neuropathol* **67**:13-24.

Auer RN, Jensen ML, Whishaw IQ (1989) Neurobehavioural deficit due to ischemic brain damage limited to half of the CA1 sector of the hippocampus. *J Neurosci* **9**:1641-1647.

Babbini M, Gaiardi M, Bartoletti M (1975) Persistence of chronic morphine effects upon activity in rats 8 months after ceasing the treatment. *Neuropharmacol* **14**:611-614.

Babcock AM, Baker DA, Lovec R (1993) Locomotor-activity in the ischemic gerbil. *Brain Res* **625**:351-354.

Benveniste H, Drejer J, Schousboe A, Diemer NH (1984) Elevation of the extracellular concentrations of glutamate and aspartate in the rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis. *J Neurochem* **43**:1369-1376.

Benveniste H, Jørgensen MB, Sandberg M, Christensen T, Hagberg H, Diemer NH (1989) Ischemic damage in hippocampal CA1 is dependent on glutamate release and intact innervation from CA3. *J Cereb Blood Flow Metab* **9**:629-639.

Benveniste H (1991) The excitotoxin hypothesis in relation to cerebral ischemia. *Cereb Brain Metab Rev* **3**:213-245.

Berkenbosch F, Van Oers J, Del Rey A, Tilders F, Beredovsky, H (1987) Corticotrophin releasing factor neurons in the rat are activated by interleukin-1. *Science* **238**: 524-526.

Bliss TVP, Lømo T (1973) Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. *J Physiol (Lond)* **232**:331-356.

Bothe H-W, Bosma H-J, Hofer H, Hossmann K-A, Angermeier WF (1986) Selective vulnerability of hippocampus and disturbances of memory storage after mild unilateral ischemia of gerbil brain. *Stroke* **17**:1160-1163.

Bratz E, Grossmann W (1923) Uber Ammonshornsklerose. *Z ges Neurol Psychiat* **81**:45-61. Referenced in: Schmidt-Kastner R, Freund TF (1991) Selective vulnerability of the hippocampus in brain ischemia. *Neurosci* **40**:599-636.

Brown AW, Levy DE, Kublik M, Harrow J, Plum F, Brierley JB (1979) Selective chromatolysis of neurons in the gerbil brain: A possible consequence of "epileptic" activity produced by common carotid artery occlusion. *Ann Neurol* **5**:127-138.

Bruno VMG, Goldberg MP, Dugan LL, Giffard RG, Choi DW (1994) Neuroprotective effect of hypothermia in cortical cultures exposed to oxygen-glucose deprivation or excitatory amino acids. *J Neurochem* **63**:1398-1406.

Buchan A, Pulsinelli WA (1990) Hypothermia but not the N-methyl-D-aspartate antagonist, MK-801, attenuates neuronal damage in gerbils subjected to transient global ischemia. *J Neurosci* **10**:311-316.

Buchan AM, Li H, Cho S, Pulsinelli WA (1991a) Blockade of the AMPA receptor prevents CA1 hippocampal injury following severe but transient forebrain ischemia in adult rats. *Neurosci Lett* **132**:255-258.

Buchan AM, Xue D, Huang Z-G, Smith K, Lesiuk H (1991b) Delayed AMPA receptor blockade reduces cerebral infarction induced by focal ischemia. *Neuroreport* **2**:473-476.

Buchan A (1992) Advances in cerebral ischemia: Experimental approaches. *Neurol Clin* **10**:49-61.

Buchan AM, Xue D, Slivka A (1992) A new model of temporary focal neocortical ischemia in the rat. *Stroke* **23**:273-279.

Buchan AM, Lesiuk H, Barnes KA, Li H, Huang Z-G, Smith KE, Xue D (1993) AMPA antagonists: Do they hold more promise for clinical stroke trials than NMDA antagonists? *Stroke* **24**(suppl 1):I148-I152.

Busto R, Dietrich WD, Globus MY-T, Valdés I, Scheinberg P, Ginsberg MD (1987) Small differences in intraischemic brain temperature critically determine the extent of ischemic neuronal injury. *J Cereb Blood Flow Metab* **7**:729-738.

Busto R, Dietrich WD, Globus MY-T, Ginsberg MD (1989) Postischemic moderate hypothermia inhibits CA1 hippocampal ischemic neuronal injury. *Neurosci Lett* **101**:299-304.

Buzsáki G, Freund TF, Bayardo F, Somogyi P (1989) Ischemia-induced changes in the electrical activity of the hippocampus. *Exp Brain Res* **78**:268-278.

Cammermeyer J (1961) The importance of avoiding 'dark' neurons in experimental neuropathology. *Acta Neuropathol* **1**:245-270.

Chen ST, Hsu CY, Hogan EL, Maricq H, Balentine JD (1986) A model of focal ischemia stroke in the rat: Reproducible extensive cortical infarction. *Stroke* **17**:738-743.

Chandler MJ, DeLeo J, Carney JM (1985a) An unanesthetized-gerbil model of cerebral ischemia-induced behavioral changes. *J Pharmacol Meth* **14**:137-146.

Chandler MJ, Hornbrook R, Carney JM (1985b) Adenylate cyclase activity and motor behavior following cerebral ischemia in the unanesthetized gerbil. *Life Sci* **37**:937-943.

Chang HS, Steward O, Kassell NF (1989) Decreases in excitatory synaptic transmission and increases in recurrent inhibition in the rat dentate gyrus after transient cerebral ischemia. *Brain Res* **505**:220-224.

Choi DW (1985) Glutamate neurotoxicity in cortical cell culture is calcium dependent. *Neurosci Lett* **58**:293-297.

Choi DW (1988) Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischemic damage. *Trends Neurosci* **11**:465-469.

Chopp M, Knight R, Tidwell CD, Helpern JA, Brown E, Welch KMA (1989) The metabolic effects of mild hypothermia on global cerebral ischemia and recirculation in the cat: Comparison to normothermia and hyperthermia. *J Cereb Blood Flow Metab* **9**:141-148.

Clifton GL, Taft WC, Blair RE, Choi SC, DeLorenzo RJ (1989) Conditions for pharmacological evaluation in the gerbil model of forebrain ischemia. *Stroke* **20**:1545-1552.

Coimbra C, Wieloch T (1994) Hypothermia ameliorates neuronal survival when induced 2 hours after ischaemia in the rat. *Acta Physiol Scand* **146**:543-544.

Colbourne F, Nurse SM, Corbett D (1993) Temperature changes associated with forebrain ischemia in the gerbil. *Brain Res* **602**:264-267.

Colbourne F, Corbett D (1994a) Delayed and prolonged post-ischemic hypothermia is neuroprotective in the gerbil. *Brain Res* **654**, 265-272

Colbourne F, Corbett D (1994b) Does prolonged hypothermia convey permanent neuroprotection? *Soc Neurosci Abst* **20**:83.10.

Colbourne F, Corbett D (1995) Delayed postischemic hypothermia: A six month survival study using behavioral and histological assessments of neuroprotection. *J Neurosci*, in press.

Corbett D, Evans S, Thomas C, Wang D, Jonas RA (1990) MK-801 reduces cerebral ischemic injury by inducing hypothermia. *Brain Res* **514**:300-304.

Corbett D, Evans SJ, Nurse SM (1992) Impaired acquisition of the Morris water maze following global ischemic damage in the gerbil. *Neuroreport* **3**:204-206.

Corbett D, Crooks P, Dooley P, Evans SJ, Nurse S (1995) Ischemic preconditioning conveys histological but not functional protection. *Soc Neurosci Abst* **21**:675.10.

Cotman CW, Monaghan DT, Ottersen OP, Storm-Mathisen J (1987) Anatomical organization of excitatory amino acid receptors and their pathways. *TINS* **10**:273-280.

Crain BJ, Westerkam WD, Harrison AH, Nadler JV (1988) Selective neuronal death after transient forebrain ischemia in the Mongolian gerbil: A silver impregnation study. *Neurosci* **27**:387-402.

Dalgaard L, Hjortkjaer RK, Regnier B, Nordholm L (1994) Pharmacokinetics of the neuroprotective glutamate antagonist NBQX (6-nitro-7-sulfamoyl-benzo(F)quinoxaline-2,3-dione) in mice, rats, and dogs. Interactions with probenecid. *Drug Metab Dispos* **22**:289-293.

Diemer NH, Jørgensen MB, Johansen FF, Sheardown M, Honoré T (1992) Protection against ischemic hippocampal CA1 damage in the rat with a new non-NMDA antagonist, NBQX. *Acta Neurol Scand* **86**:45-49.

Diemer NH, Rosdahl D, Seitzberg D, Nielsen M, Christensen T, Balchen T, Bruhn T, Jorgensen MB, Johansen FF (1994) Changes of AMPA and mGluR mRNA after cerebral ischemia in the rat. In: *Pharmacology of Cerebral*

Ischemia 1994 (Krieglstein J, Oberpichler-Schwenk H, eds), Wissenschaftliche Verlagsgesellschaft mbH Stuttgart, pp 119-129.

Dietrich WD, Busto R, Alonso O, Globus MY-T, Ginsberg MD (1993) Intraischemic but not postischemic brain hypothermia protects chronically following global forebrain ischemia in rats. *J Cereb Blood Flow Metab* **13**:541-549.

Donzanti BA, Uretsky NJ (1983) Effects of excitatory amino acids on locomotor activity after bilateral microinjection into the rat nucleus accumbens: Possible dependence on dopaminergic mechanisms. *Neuropharmacol* **22**:971-981.

Douglas RJ, Isaacson RL (1964) Hippocampal lesions and activity. *Psychonomic Science* **1**:187-188.

Gerhardt SC, Boast CA (1988) Motor activity changes following cerebral ischemia in gerbils are correlated with the degree of neuronal degeneration in hippocampus. *Behav Neurosci* **102**:301-303.

Gill R, Foster AC, Woodruff GN (1987) Systemic administration of MK-801 protects against ischemia-induced hippocampal neurodegeneration in the gerbil. *J Neurosci* **7**:3343-3349.

Ginsberg MD, Busto R (1989) Rodent models of cerebral ischemia. *Stroke* **20**:1627-1642.

Glickman SE, Higgins TJ, Isaacson RL (1970) Some effects of hippocampal lesions on the behavior of Mongolian gerbils. *Physiol Behav* **5**: 931-938.

Globus MY-T, Busto R, Dietrich WD, Martinez E, Valdes I, Ginsberg MD (1988a) Effect of ischemia on the *in vivo* release of striatal dopamine, glutamate, and γ-aminobutyric acid studied by intracerebral microdialysis. *J Neurochem* **51**:1455-1464.

Globus MY-T, Busto R, Dietrich WD, Martinez E, Valdes I, Ginsberg MD (1988b) Intra-ischemic extracellular release of dopamine and glutamate is associated with striatal vulnerability to ischemia. *Neurosci Lett* **91**:36-40.

Globus MY-T, Ginsberg MD, Busto R (1991) Excitotoxic index- a biochemical marker of selective vulnerability. *Neurosci Lett* **127**:39-42.

Gordon CJ, Long MD, Dyer RS (1984) Effect of ambient temperature on the hypometabolic and hypothermic effects of sulfolane in rats. *Arch Toxicol* **56**:123-127.

Gordon CJ, Mohler FS, Watkinson WP, Rezvani AH (1988) Temperature regulation in laboratory mammals following acute toxic insult. *Toxicol* **53**:161-178.

Green EJ, Dietrich WD, Dijk F van, Busto R, Markgraf CG, McCabe PM, Ginsberg MD, Schneiderman N (1992) Protective effects of brain hypothermia on behavior and histopathology following global cerebral ischemia in rats. *Brain Res* **580**:197-204.

Hansen AJ (1985) Effect of anoxia on ion distribution in the brain. *Physiol Rev* **65**:101-148.

Hara H, Onodera H, Kogure K (1992) Effects of hyperthermia on the effectiveness of MK-801 treatment in the gerbil hippocampus following transient forebrain ischemia. *Brain Res Bull* **29**:659-665.

Haraldseth O, Gronås T, Southon T, Thommessen L, Borchgrevink G, Jynge P, Gisvold SE Unsgård G (1992) The effects of brain temperature on temporary global ischemia in rat brain. A 31-phosphorous NMR spectroscopy study. *Acta Anaesthesiol Scand* **36**:393-399.

Himori N, Watanabe H, Akaike N, Kurasawa M, Itoh J, Tanaka Y (1990) Cerebral ischemia model with conscious mice involvement of NMDA receptor activation and derangement of learning and memory ability. *J Pharmacol Methods* **23**:311-327.

Hiramatsu K-I, Kassell NF, Lee KS (1993) Thermal sensitivity of hypoxic responses in neocortical brain slices. *J Cereb Blood Flow Metab* **13**:395-401.

Hollmann M, Hartley M, Heinemann S (1991) Ca^{2+} permeability of KA-AMPA-gated glutamate receptor channels depends on subunit composition. *Science* **252**:851-853.

Honey GD, Sowinski P, Meldrum BS (1995) Neuroprotection of hilar neurons following repetitive forebrain ischemia. *J Cereb Blood Flow Metab* **15**(Suppl 1):S198.

Hori N, Carpenter DO (1994) Functional and morphological changes induced by transient *in vivo* ischemia. *Exp Neurol* **129**:279-289.

Horn M, Schlote W (1992) Delayed neuronal death and delayed neuronal recovery in the human brain following global ischemia. *Acta Neuropathol* **85**:79-87.

Hossmann K-A (1994) Viability thresholds and the penumbra of focal ischemia. *Ann Neurol* **36**:557-565.

Hsu M, Buzsáki G (1993) Vulnerability of mossy fiber targets in the rat hippocampus to forebrain ischemia. *J Neurosci* **13**:3964-3979.

Ikeda M, Nakazawa T, Abe K, Kaneko T, Yamatsu K (1989) Extracellular accumulation of glutamate in the hippocampus induced by ischemia is not calcium dependent- *in vitro* and *in vivo* evidence. *Neurosci Lett* **96**:202-206.

Imamura L, Ohta H, Ni X-H, Matsumoto K, Watanabe H (1991) Effects of transient cerebral ischemia in gerbils on working memory performance in the delayed nonmatching to position task using a T-maze. *Japan J Pharmacol* **57**:601-608.

Iwai T, Niwa M, Yamada H, Nozaki M, Tsurumi K (1993) Hypothermic prevention of the hippocampal damage following ischemia in Mongolian gerbils: Comparison between intraischemic and brief postischemic hypothermia. *Life Sci* **52**:1031-1038.

Jarrard LE (1968) Behavior of hippocampal lesioned rats in home cage and novel situations. *Physiol Behav* **13**:65-70.

Jaspers RMA, Block F, Heim C, Sontag K-H (1990) Spatial learning is affected by transient occlusion of common carotid arteries (2VO): comparison of behavioural and histopathological changes after '2VO' and 'four-vessel-occlusion' in rats. *Neurosci Lett* **117**:149-153.

Jensen MS, Lambert JDC and Johansen FF (1991) Electrophysiological recordings from rat hippocampus slices following *in vivo* brain ischemia. *Brain Res* **554**:166-175.

Johansen FF, Jørgensen MB, Diemer NH (1985) Excitatory amino acids and interneuron loss. *Acta Neurol Scand* **72**(suppl):262.

Johansen FF (1993) Interneurons in rat hippocampus after cerebral ischemia. Morphometric, functional and therapeutic investigations. *Acta Neurol Scand* **88**(suppl 150):1-31.

Kaku DA, Goldberg MP, Choi DW (1991) Antagonism of non-NMDA receptors augments the neuroprotective effect of NMDA receptor blockade in cortical cultures subjected to prolonged deprivation of oxygen and glucose. *Brain Res* **554**:344-347.

Kaku DA, Giffard RG, Choi DW (1993) Neuroprotective effects of glutamate antagonists and extracellular acidity. *Science* **260**:1516-1518.

Kalivas PW, Stewart J (1991) Dopamine transmission in the initiation and expression of drug- and stress-induced sensitization of motor activity. *Brain Res Rev* **16**:223-244.

Kaplan TM, Lasner TM, Nadler JV, Crain BJ (1989) Lesions of excitatory pathways reduce hippocampal cell death after transient forebrain ischemia in the gerbil. *Acta Neuropathol* **78**:283-290.

Karasawa Y, Araki H, Otomo S (1994) Changes in locomotor activity and passive aviodance task performance induced by cerebral ischemia in Mongolian gerbils. *Stroke* **25**:645-650.

Kato H, Kogure K, Nakano S (1989) Neuronal damage following brief ischemia in the gerbil. *Brain Res* **479**:366-370.

Katoh A, Ishibashi C, Shiomi T, Takahara Y, Eigyo M (1992) Ischemia-induced irreversible deficit of memory function in gerbils. *Brain Res* **577**:57-63.

Kawamura S, Yashi N, Shirasawa M, Fukasawa H (1991) Rat middle cerebral artery occlusion using an intraluminal thread technique. *Acta Neurochir* **109**:126-132.

Kelley AE, Domesick VB (1982) The distribution of the projection from the hippocampal formation to the nucleus accumbens in the rat: an anterograde and retrograde horseradish peroxidase study. *Neurosci* **7**:2321-2335.

Kimble DP (1963) The effects of bilateral hippocampal lesions in rats. *J Comp Physiol Psych* **56**:273-283.

Kirino T (1982) Delayed neuronal death in the gerbil hippocampus following ischemia. *Brain Res* **237**:57-69.

Kirino T, Robinson HPC, Miwa A, Tamura A, Kawai N (1992) Disturbance of membrane function preceding ischemic delayed neuronal death in the gerbil hippocampus. *J Cereb Blood Flow Metab* **12**:408-417.

Kitagawa K, Matsumoto M, Tagaya M, Hata R, Ueda H, Niinobe M, Handa N, Fukunaga R, Kimura K, Mikoshiba K, Kamada T (1990) 'Ischemic tolerance' phenomenon found in the brain. *Brain Res* **528**:21-24.

Kuroiwa T, Bonnekoh P, Hossmann K-A (1991a) Locomotor hyperactivity and hippocampal CA1 injury after transient forebrain ischemia of gerbils. *Neurosci Lett* **122**:141-144.

Kuroiwa T, Bonnekoh P, Hossmann K-A (1991b) Therapeutic window of halothane anesthesia for reversal of delayed neuronal injury in gerbils: relationship to postischemic motor hyperactivity. *Brain Res* **563**:33-38.

Lekieffre D, Callebert J, Plotkine M, Boulu RG (1991) Enhancement of endogenous excitatory amino acids by theophylline does not modify the behavioral and histological consequences of forebrain ischemia. *Brain Res* **565**:353-357.

Lekieffre D, Ghribi O, Callebert J, Allix M, Plotkine M, Boulu RG (1992) Inhibition of glutamate release in rat hippocampus by kynurenic acid does not protect CA1 cells from forebrain ischemia. *Brain Res* **592**:333-337.

Le Peillet E, Arvin B, Moncada C, Meldrum BS (1992) The non-NMDA antagonists, NBQX and GYKI 52466, protect against cortical and striatal cell loss following transient global ischaemia in the rat. *Brain Res* **571**:115-120.

Levine S, Payan H (1966) Effects of ischemia and other procedures on the brain and retina of the gerbil (*Meriones unguiculatus*). *Exp Neurol* **16**: 255-262.

Li H, Buchan AM (1993) Treatment with an AMPA antagonist 12 hours following severe normothermic forebrain ischemia prevents CA1 neuronal injury. *J Cereb Blood Flow Metab* **13**:933-939.

Li H, Buchan AM (1995) Delayed treatment with NBQX or SNX-111 following transient forebrain ischemia in rats. Proceedings of the 30th Annual Canadian Congress of Neurological Sciences P-46, *Can J Neurol Sci* **22**(Suppl 1):S47.

Lippert K, Welsch M, Krieglstein J (1994) Over-additive protective effect of dizocilpine and NBQX against neuronal damage. *Eur J Pharmacol* **253**:207-213.

Ljunggren B, Norberg K, Siesjö BK (1974) Influence of tissue acidosis upon restitution of brain energy metabolism following total ischemia. *Brain Res* **77**:173-186.

Lobner D, Lipton P (1990) Sigma-ligands and non-competitive NMDA antagonists inhibit glutamate release during cerebral ischemia. *Neurosci Lett* **117**:169-174.

Longa EZ, Weinstein PR, Carlson S, Cummins R (1989) Reversible middle cerebral artery occlsuion without craniectomy in rats. *Stroke* **20**:84-91.

Ludvig N, Farias PA, Ribak CE (1991) An analysis of various environmental and specific sensory stimuli on the seizure activity of the Mongolian gerbil. *Epilepsy Res* **8**:30-35.

Malec D, Kleinrok Z (1972) The spontaneous motility of rats after intraventricular injection of dopamine. *Neuropharmacol* **11**:331-336.

Mayevsky A, Zipi B (1990) The Mongolian gerbil as a model for cerebral ischemia. In: *Cerebral Ischemia and Resuscitation* (Schurr A, Rigor BM, eds), CRC Press, Inc., Boca Raton, FL, pp 27-46.

Mileson BE, Schwartz RD (1991) The use of locomotor activity as a behavioral screen for neuronal damage following transient forebrain ischemia in gerbils. *Neurosci Lett* **128**:71-76.

Minami M, Kuraishi Y, Yabuuchi K, Yamazaki A, Sato M (1992) Induction of interleukin-1β mRNA in rat brain after transient forebrain ischemia. *J Neurochem* **58**:390-392.

Minamisawa H, Nordstrom CH, Smith M-L, Siesjö BK (1990a) The influence of mild body and brain hypothermia on ischemic brain damage. *J Cereb Blood Flow Metab* **10**:365-374.

Minamisawa H, Smith M-L, Siesjö BK (1990b) The effects of mild hyperthermia and hypothermia on brain damage following 5, 10 and 15 minutes of forebrain ischemia. *Ann Neurol* **28**:26-33.

Mitani A, Imon H, Katakoa (1989) High frequency discharges of gerbil hippocampal CA1 neurons shortly after ischemia. *Brain Res Bull* **23**:569-572.

Mitani A, Andou Y, Masuda S, Kataoka K (1991) Transient forebrain ischemia of three-minute duration consistently induces severe neuronal damage in field CA1 of the hippocampus in the normothermic gerbil. *Neurosci Lett* **131**:171-174.

Mitani A, Kataoka K (1991) Critical levels of extracellular glutamate mediating gerbil hippocampal delayed neuronal death during hypothermia: Brain microdialysis study. *Neurosci* **42**:661-670.

Mitani A, Andou Y, Kataoka K (1992) Selective vulnerability of hippocampal CA1 neurons cannot be explained in terms of an increase in glutamate concentration during ischemia in the gerbil: Brain microdialysis study. *Neurosci* **48**:307-313.

Miyazaki S, Katayama Y, Lyeth BG, Jenkins LW, DeWitt DS, Goldberg SJ, Newlon PG, Hayes RL (1992) Enduring suppression of hippocampal long-term potentiation following traumatic brain injury in rat. *Brain Res* **585**:335-339.

Mogenson GJ and Nielsen M (1984) Neuropharmacological evidence to suggest that the nucleus accumbens and subpallidal region contribute to exploratory locomotion. *Behav Neural Biol* **42**:52-60.

Montoya CP, Campbell-Hope LJ, Pemberton KD, Dunnett SB (1991) The staircase test: a measure of independent forelimb reaching and grasping abilities in rats. *J Neurosci Meth* **36**:219-228.

Morse JK, Davis JN (1990) Regulation of ischemic hippocampal damage in the gerbil: Adrenalectomy alters the rate of CA1 cell disappearance. *Exp Neurol* **110**:86-92.

Nakagomi T, Kirino T, Kanemitsu H, Tsujita Y, Tamura A (1993) Early recovery of protein synthesis following ischemia in hippocampal neurons with induced tolerance in the gerbil. *Acta Neuropathol* **86**:10-15.

Nellgård B, Gustafson I, Wieloch T (1991) Lack of protection by the N-methyl-D-aspartate receptor blocker dizocilpine (MK-801) following transient cerebral ischemia in the rat. *Anesthesiol* **75**:279-287.

Nellgård B, Wieloch T (1992) Postischemic blockade of AMPA but not NMDA receptors mitigates neuronal damage in the rat brain following transient severe cerebral ischemia. *J Cereb Blood Flow Metab* **12**:2-11.

Nowak TS Jr., Fried RL, Lust WD, Passonneau JV (1985) Changes in brain energy metabolism and protein synthesis following transient bilateral ischemia in the gerbil. *J Neurochem* **44**:487-494.

Nurse SM, Corbett D (1993) Does hypothermia contribute to the neuroprotective actions of NBQX? *Soc Neurosci Abst* **19**:683.3.

Nurse S, Corbett D (1994) Direct measurement of brain temperature during and after intraischemic hypothermia: Correlation with behavioral, physiological and histological endpoints. *J Neurosci* **14**:7726-7734.

Olney JW, Ikonomidou C, Mosinger JL, Frierdich G (1989) MK-801 prevents hypobaric-ischemic neuronal degeneration in infant rat brain. *J Neurosci* **9**:1701-1704.

Park CK, Nehls DG, Graham DI, Teasdale GM, McCulloch J (1988) The glutamate antagonist MK-801 reduces focal ischemic brain damage in the rat. *Ann Neurol* **24**:543-551.

Pellegrini-Giampietro DE, Zukin RS, Bennett MVL, Cho S, Pulsinelli WA (1992) Switch in glutamate receptor subunit gene expression in CA1 subfield of hippocampus following global ischemia in rats. *Prot Natl Acad Sci USA* **89**:10499-10503.

Pellegrini-Giampietro D, Pulsinelli WA, Zukin RS (1994) NMDA and non-NMDA receptor gene expression following global brain ischemia in rats: Effect of NMDA and non-NMDA receptor antagonists. *J Neurochem* **62**:1067-1073.

Pijnenburg AJJ, Honig WMM, Van der Heyden JAM, Van Rossum JM (1976) Effects of chemical stimulation of the mesolimbic dopamine system upon locomotor activity. *Eur J Pharmacol* **35**:45-58.

Poignet H, Beaughard M, Lecoin G, Massingham R (1989) Functional, behavioral, and histological changes induced by transient global cerebral ischemia in rats: Effects of cinnarizine and flunarizine. *J Cereb Blood Flow Metab* **9**:646-654.

Pulsinelli WA, Brierley JB (1979) A new model of bilateral hemispheric ischemia in the unanesthetized rat. *Stroke* **10**:267-272.

Pulsinelli WA, Brierley JB, Plum F (1982) Temporal profile of neuronal damage in a model of transient forebrain ischemia. *Ann Neurol* **11**:491-499.

Raley KM, Lipton P (1990) NMDA receptor activation accelerates ischemic energy depletion in the hippocampal slice and the demonstration of a threshold for ischemic damage to protein synthesis. *Neurosci Lett* **110**:118-123.

Robinson TE, Becker JB (1986) Enduring changes in brain and behavior produced by chronic amphetamine administration: A review and evaluation of animal models of amphetamine psychosis. *Brain Res Rev* **11**:157-198.

Rothman SM (1983) Synaptic activity mediates death of hypoxic neurons. *Science* **220**:536-537.

Rothman SM (1985) The neurotoxicity of excitatory amino acids is produced by passive chloride influx. *J Neurosci* **5**:1483-1489.

Rothman SM, Olney JW (1986) Glutamate and the pathophysiology of hypoxic-ischemic brain damage. *Ann Neurol* **19**:105-111.

Rothman SM, Olney JW (1987) Excitotoxicity and the NMDA receptor. *TINS* **10**:299-302.

Rothwell NJ (1989) CRF is involved in the pyrogenic and thermogenic effects of interleukin-1β in the rat. *Am J Physiol* **256**: E111-E115.

Rothwell NJ (1990) Central effects of CRF on metabolism and energy balance. *Neurosci Biobehav Rev* **14**: 263-271.

Rothwell NJ, Relton JK (1993) Involvement of interleukin-1 and lipocortin-1 in ischaemic brain damage. *Cerebrovasc Brain Metab Rev* **5**: 178-198.

Sapolsky R, Rivier C, Yamamoto G, Plotsky Y, Vale W (1987) Interleukin-1 stimulates the secretion of hypothalamic corticotrophin releasing factor. *Science* **238**: 522-524.

Schmidley JW (1990) Free radicals in central nervous system ischemia. *Stroke* **21**:1086-1090.

Schmidt-Kastner R, Freund TF (1991) Selective vulnerability of the hippocampus in brain ischemia. *Neurosci* **40**:599-636.

Scoville WB, Milner B (1957) Loss of recent memory after bilateral hippocampal lesions. *J Neurol Neurosurg Psychiat* **20**:11-21.

Sekhon LHS, Morgan MK, Spence I, Weber NC (1994) Chronic cerebral hypoperfusion and impaired neuronal function in rats. *Stroke* **25**:1022-1027.

Sheardown MJ, Nielsen EO, Hansen AJ, Jacobsen P, Honoré T (1990) 2,3-Dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline: A neuroprotectant for cerebral ischemia. *Science* **247**: 571-574.

Sheardown MJ, Suzdak PD, Nordholm L (1993) AMPA, but not NMDA, receptor antagonism is neuroprotective in gerbil global ischaemia, even when delayed 24 h. *Eur J Pharmacol* **236**:347-353.

Shuaib A, Sochocka E, Ishaqzay R, Hertz L, Code WE (1993) Protective effect of hypothermia during ischemia in neural cell cultures. *Neurochem Res* **18**:663-665.

Siesjö BK (1988) Historical overview. Calcium, ischemia, and death of brain cells. *Ann NY Acad Sci* **522**:638-661.

Siesjö BK, Bengtsson F (1989) Calcium fluxes, calcium antagonists, and calcium-related pathology in brain ischemia, hypoglycemia, and spreading depression: A unifying hypothesis. *J Cereb Blood Flow Metab* **9**:127-140.

Siesjö BK, Ekholm A, Katsura K, Theander S (1990) Acid-base changes during complete brain ischemia. *Stroke* **21**(Suppl III):194-198.

Siesjö BK (1992a) Pathophysiology and treatment of focal cerebral ischemia. Part I. Pathophysiology. *J Neurosurg* **77**:169-184.

Siesjö BK (1992b) Pathophysiology and treatment of focal cerebral ischemia. Part II: Mechanisms of damage and treatment. *J Neurosurg* **77**:337-354.

Silver IA, Erecinska M (1992) Ion homeostasis in rat brain in vivo: Intra- and extracellular [Ca2+] and [H+] in the hippocampus during recovery from short-term, transient ischemia. *J Cereb Blood Flow Metab* **12**:759-772.

Smith M-L, Auer RN, Siesjö BK (1984a) The density and distribution of ischemic brain injury in the rat following 2 - 10 min of forebrain ischemia. *Acta Neuropathol* **64**:319-332.

Smith M-L, Bendek G, Dahlgren N, Rosén I, Wieloch T, Siesjö BK (1984b) Models for studying long-term recovery following forebrain ischemia in the rat. 2. A 2-vessel occlusion model. *Acta Neurol Scand* **69**:385-401.

Somjen GG, Aitken PG, Balestrino M, Herreras O, Kawasaki K (1990) Spreading depression-like depolarization and selective vulnerability of neurons. A brief review. *Stroke* **21**:III179-III183.

Somjen GG, Aitken PG, Czeh G, Jing J, Young JN (1993) Cellular physiology of hypoxia of the mammalian central nervous system. In: *Molecular and Cellular Approaches to the Treatment of Neurological Disease* (Waxman, SG, ed), Raven Press Ltd, New York, pp 51-65.

Sommer W (1880) Erkrankung des Ammonshorns als aetiologisches Moment der Epilepsie. *Arch Psychiatry* **10**:631-675. Referenced in: Kirino T (1982) Delayed neuronal death in the gerbil hippocampus following ischemia. *Brain Res* **239**:57-69.

Stewart J, Vezina P (1989) Microinjections of SCH-23390 into the ventral tegmental area and substantia nigra pars reticulata attenuate the development of sensitization to the locomotor activating effects of systemic amphetamine. *Brain Res* **495**:401-406.

Strijbos PJLM, Relton JK, Rothwell NJ (1994) Corticotrophin-releasing factor antagonist inhibits neuronal damage induced by focal cerebral ischaemia or activation of NMDA receptors in the rat brain. *Brain Res* **656**:405-408.

Suga S, Nowak TS Jr. (1992) Pharmacology of the postischemic stress response: Effects of temperature on hsp70 expression after transient ischemia and hypothermic action of NBQX in the gerbil. In: *Pharmacology of Cerebral*

Ischemia 1992 (Krieglstein J, Oberpichler-Schwenk H, eds), Wissenschaftliche Verlagsgesellschaft mbH Stuttgart, pp 279-286.

Sutherland GR, Lesiuk H, Hazendonk P, Peeling J, Buist R, Kozlowski P, Jazinski A, Saunders JK (1992) Magnetic resonance imaging and ^{31}P magnetic resonance spectroscopy study of the effect of temperature on ischemic brain injury. *Can J Neurol Sci* **19**:317-325.

Sutherland GR, Peeling J (1994) *Progressing towards effective stroke treatment.* Upjohn Company of Canada, Don Mills, ON.

Suzuki R, Yamaguchi T, Choh-Luh L, Klatzo I (1983) The effects of 5-minute ischemia in Mongolian gerbils. II. Changes in spontaneous neuronal activity in cerebral cortex and CA1 sector of the hippocampus. *Acta Neuropathol* **60**:217-222.

Swanson LW, Cowan WM (1977) An autoradiographic study of the organization of the efferent connections of the hippocampal formation in the rat. *J Comp Neurol* **172**:49-84.

Szatkowski M, Barbour B, Attwell D (1990) Non-vesicular release of glutamate from glial cells by reversed electrogenic glutamate uptake. *Nature* **348**:443-446.

Taylor CP, Weber ML (1993) Effect of temperature on synaptic function after reduced oxygen and glucose in hippocampal slices. *Neurosci* **52**:555-562.

Teitelbaum H, Milner P (1963) Activity changes following partial hippocampal lesions in rats. *J Comp Physiol Psychol* **56**:284-289.

Truong DD, Matsumoto RR, Schwartz PH, Hussong MJ, Wasterlain CG (1994) Novel rat cardiac arrest model of posthypoxic myoclonus. *Mov Disord* **9**:201-206.

Tsubokawa H, Oguro K, Robinson HPC, Masuzawa T, Kirino T, Kawai N (1992) Abnormal Ca^{2+} homeostasis before cell death revealed by whole cell recording of ischemic CA1 hippocampal neurons. *Neurosci* **49**:807-817.

Urban L, Neill KH, Crain BJ, Nadler JV, Somjen GG (1989) Postischemic synaptic physiology in area CA1 of the gerbil hippocampus studied *in vitro*. *J Neurosci* **9**:3966-3975.

Urban L, Neill KH, Crain BJ, Nadler JV, Somjen GG (1990) Effects of transient forebrain ischemia in area CA1 of the gerbil hippocampus: An *in vitro* study. *Adv Exp Med Biol* **268**:491-500.

Valtysson J, Hillered L, Andine P, Hagberg H, Persson L (1994) Neuropathological endpoints in experimental stroke pharmacotherapy: The importance of both early and late evaluation. *Acta Neurochir* **129**:58-63.

Volpe BT, Pulsinelli WA, Tribuna J, Davis HP (1984) Behavioral performance of rats following transient forebrain ischemia. *Stroke* **15**:558-562.

Walker AE, Robins M, Weinfeld FD (1981) National Institute of Neurological and Communicative Disorders and Stroke: The national survey of stroke, clinical findings. *Stroke* **12**(suppl 1):I-13-I-31.

Wang D, Corbett D (1990) Cerebral ischemia, locomotor activity and spatial mapping. *Brain Res* **533**:78-82.

Warner MA, Nadler JV, Crain BJ (1990) Effects of NMDA receptor antagonists and body temperature in the gerbil carotid occlusion model of transient forebrain ischemia. In: *Current and future trends in anticonvulsant, anxiety, and stroke therapy*, Wiley-Liss, Inc., New York, pp 409-414.

Welsh FA, Sims RE, Harris VA (1990) Mild hypothermia prevents ischemic injury in gerbil hippocampus. *J Cereb Blood Flow Metab* **10**:557-563.

Whittam R (1962) The dependence of the respiration of brain cortex on active cation transport. *Biochem J* **82**:205-212.

Widmann R, Miyazawa T, Hossmann K-A (1993) Protective effect of hypothermia on hippocampal injury after 30 minutes of forebrain ischemia in rats is mediated by postischemic recovery of protein synthesis. *J Neurochem* **61**:200-209.

Xue D, Huang Z-G, Barnes K, Lesiuk HJ, Smith KE, Buchan AM (1994) Delayed treatment with AMPA, but not NMDA, antagonists reduces neocortical infarction. *J Cereb Blood Flow Metab* **14**:251-261.

Yang CR, Mogenson GJ (1987) Hippocampal signal transmission to the pedunculopontine nucleus and its regulation by D_2 receptors in the nucleus accumbens: an electrophysiological and behavioural study. *Neurosci* **23**:1041-1055.

Zini S, Roisin M-P, Armengaud C, Ben-Ari Y (1993) Effect of potassium channel modulators on the release of glutamate induced by ischaemic-like conditions in rat hippocampal slices. *Neurosci Lett* **153**:202-205.

Zola-Morgan S, Squire LR, Amaral DG (1986) Human amnesia and the medial temporal region: enduring memory impairment following a bilateral lesion limited to field CA1 of the hippocampus. *J Neurosci* **6**:2950-2967.

Zola-Morgan S, Squire LR, Rempel NL, Clower RP, Amaral DG (1992) Enduring memory impairment in monkeys after ischemic damage to the hippocampus. *J Neurosci* **12**:2582-2596.