PROTRACTED MILD HYPOTHERMIA PROVIDES LONG-TERM HISTOLOGICAL AND BEHAVIORAL PROTECTION FOLLOWING GLOBAL CEREBRAL ISCHEMIA

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By

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This thesis is dedicated to Refger IV.

ABSTRACT

Global cerebral ischemia (cardiac arrest) often results in profound, but selective, hippocampal CA1 injury. This cell death, which normally occurs hours to days later, induces profound anterograde amnesia in humans with habituation and working memory impairments in rodents.

In this thesis, the gerbil model of global ischemia was used. Under Halothane anesthesia, both carotid arteries were isolated and briefly occluded. Without intervention, near-total CA1 loss ensues with resultant memory impairments.

In the first experiment a novel brain temperature system was compared to rectal and skull measurements. Brain temperature dropped during ischemia even though rectal and skull temperatures were maintained. Subsequently, prolonged Halothane anesthesia was found to enhance this dissociation. Thus, rectal and skull readings are not reliable indices of brain temperature during ischemia and anesthesia. Brain temperature must be controlled to avoid the confounding protective effects of intra-ischemic hypothermia.

While the protective effects of mild intra-ischemic hypothermia are well documented, the value of postischemic cooling is less clear. Therefore, rigorous brain temperature measurement, lengthy survival times and behavioral tests were used to clarify the effects of postischemic hypothermia. Prolonged cooling (32°C for 12 hr) initiated 1 hour after normothermic ischemia was highly neuroprotective at 10 and 30 day survival against 3 minutes of ischemia, but provided only a mild, transient savings

against a 5 minute episode. Habituation impairments, in open field tests, were also reduced in cooled gerbils. Notably, 24 hours of hypothermia initiated 1 hour after a 5 minute occlusion rescued almost all CA1 neurons (90%) with 30 day survival.

More recently, significant CA1 savings were found with clear reductions in habituation (open field) and working memory (T-maze) impairments for up to 6 months postischemia (5 min) with 1 hour delayed hypothermia (32°C for 24 hr). While CA1 protection at 6 months (≈70%) was less than with 1 month survival it nonetheless showed effective and very persistent benefit. Hypothermia (32°C for 24 hr), when started 4 hours postischemia, saved ≈12% of CA1 cells at 6 months with mild behavioral benefit. Hypothermia (34°C from 1-25 hr postischemia) also reduced habituation impairments and rescued ≈60% of CA1 neurons with 1 month survival.

In summary, these data indicate that delayed mild postischemic hypothermia is an efficacious and persistent neuroprotectant deserving of clinical investigation.

[Key words: cerebral ischemia, postischemic hypothermia, open field, T-maze, CA1, delayed neuronal death]

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LIST OF ABBREVIATIONS AND SYMBOLS USED

2-VO	Two vessel occlusion in the rat
4-VO	Four vessel occlusion in the rat
AMPA	D,L-α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid
ATP	Adenosine triphosphate
CONT	Untreated ischemic group
CT	Choice trial
DND	Delayed neuronal death
EAA	Excitatory amino acid
FT	Forced trial
HYPO	Ischemia treated with hypothermia
ITI	Intertrial interval
MK-801	(+)-5-methyl-10,11-dihydro-5H-di [a,d] cycloheptene-5, 10-imine (dizocilpine maleate)
NBQX	2,3,-Dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline
NMDA	N-methyl-D-aspartate
SHAM	Surgery, but no ischemia

Chapter 1 (General Introduction)

1.1 Introduction

Stroke is a devastating neurological condition which results from an impaired blood flow (ischemia) to the brain and/or cerebrovascular hemorrhage. Cerebral ischemia may be global such as that occurring during cardiac arrest, or focal which often occurs because of a middle cerebral artery blockage. Similar neuronal injury also results from periods of hypoxia or severe hypoglycemia. While the apoplexy of distant times has remained relatively unchanged, it is becoming increasingly more prevalent as the population ages. Stroke is the leading cause of chronic disability and the third most frequent cause of death in Canada.

Currently, there are four distinct approaches to fighting stroke, of which prevention has the greatest potential. Accordingly, the control of risk factors, such as quitting smoking, has been clearly beneficial. The use of Aspirin (Antiplatelet Trialists' Collaboration, 1988) and Ticlopidine (Harbison, 1992) has also reduced stroke incidence by inhibiting platelet aggregation (i.e., clot formation). A second strategy is to limit the duration and severity of ischemia through prompt cardiopulmonary resuscitation. In addition, there are clot dissolving drugs (e.g., tissue plasminogen activator (TPA), Haley, 1993) that limit the period of occlusion. Third, one may reduce ischemic damage by counteracting the intra- and postischemic metabolic disturbances. Unfortunately, the latter research area has so far failed to yield an effective post-stroke clinical therapy.

Finally, one may improve recovery of function (e.g., physical therapy).

The objective of this thesis was to limit the cellular damage and behavioral impairments that occur after global ischemia (third approach) in the gerbil. I hypothesized that delayed, but prolonged, postischemic hypothermia would persistently attenuate ischemic injury and associated behavioral impairments. Furthermore, the efficacy of hypothermia was thought to depend upon: severity of ischemia, duration and degree of cooling, initiation delay and survival time assessment.

1.2 Global Cerebral Ischemia

The central nervous system is exceptionally vulnerable to global ischemia. The people resuscitated from lengthy global ischemia usually have very severe and widespread cerebral injury. Briefer episodes (e.g., 5 - 10 min) produce severe, but more restricted neuronal injury (e.g., hippocampal CA1 region) which results in severe memory impairments. It is these patients that will likely benefit most from postischemic intervention. Prior to clinical study, however, any potential therapy necessarily requires extensive animal testing.

1.2.1 *Models*

There are many animal models of global ischemia (see Ginsberg and Busto, 1989). Three common *in vivo* models are: the rat two (2-VO, Eklöf and Siesjö, 1972) and four vessel occlusion models (4-VO, Pulsinelli and Brierley, 1979) and the gerbil model (Levine and Payan, 1966; Kirino, 1982). In the rat one must occlude the four major arteries (4-VO; vertebral and carotid) or combine systemic hypotension with bilateral carotid artery occlusion (2-VO) to consistently produce severe global ischemia. Occlusion of the carotids alone will not sufficiently reduce blood flow because of collateral flow from the vertebral arteries through the posterior communicating arteries. Thus, it is necessary to block this compensatory flow by either cauterizing the vertebral arteries or producing systemic hypotension. Most gerbils lack communicating arteries in the circle of Willis (Levine and Sohn, 1969; Levy and Brierley, 1974; Ito et al., 1975)

and, therefore, occlusion of both carotid arteries will severely restrict blood flow to the forebrain. As such, this model has the advantage of simplicity and brief anesthesia. Ischemic durations of 3 to 5 minutes in gerbils (Mitani et al., 1991; Andou et al., 1992; see Chapters 2 to 5) and about 10 minutes in 2-VO (Smith et al., 1984) and 15 - 20 minutes in 4-VO models (Pulsinelli and Brierley, 1979) will consistently produce severe, but selective, neuronal loss similar to human cardiac arrest survivors (Zola-Morgan et al., 1986; Petito et al., 1987).

In addition to these commonly used *in vivo* models there are several common *in vitro* models. These involve glucose and oxygen deprivation of hippocampal slices (e.g., Whittingham et al., 1984) or cortical neuronal/glial cultures (e.g., Goldberg and Choi, 1993). While these systems do not truly mimic ischemia they have the advantage of greater simplicity and control of experimental variables.

1.2.2 *Histological Injury*

In addition to hippocampal CA1 injury, brief *in vivo* ischemia also kills CA2, hilar and subicular neurons (Kirino, 1982; Pulsinelli et al., 1982; Smith et al., 1984; Akai and Yanagihara, 1993; Matsuyama et al., 1993). Other brain regions are also susceptible, especially with more severe ischemia (i.e., neocortical, striatal, thalamic and cerebellar Purkinje neurons). Fortunately, most vulnerable neurons do not die during ischemia, but instead recover only to die several hours to days later, thereby providing an opportunity for therapeutic intervention.

The most thoroughly investigated of these vulnerable groups are the CA1 neurons. Following brief ischemia in rats (Pulsinelli et al., 1982), gerbils (Kirino, 1982) and humans (Petito et al., 1987) CA1 cells transiently recover, but subsequently die 24 to 72 hours later. Until a few hours before their death these neurons appear normal with Nissl staining. Neurons then undergo typical ischemic changes (Brown and Brierley, 1972) characterized by swollen cell bodies, eccentric nuclei and clumped chromatin followed by a loss of structural integrity and eventual phagocytosis (i.e., necrosis). A careful analysis can reveal more subtle structural damage at earlier survival times (e.g., dendritic beading; Hori and Carpenter, 1994). In addition, there are several other early markers of impending ischemic injury such as disrupted microtubules (Matesic and Lin, 1994) and behavioral abnormalities (discussed later).

The mechanisms underlying selective vulnerability and delayed neuronal death (DND) are not clear. However, some of the ischemic perturbations that may culminate in death are known (section 1.3) and these are potential targets for limiting injury.

1.2.3 *Behavioral Impairments*

Global ischemia is a devastating condition, not simply because of cell loss, but because of the functional impairments that ensue. Often, brief ischemia results in a profound and typically permanent anterograde amnesia; an inability to form declarative memories. For example, an ischemic episode rendered patient R.B. severely amnesic until his death 5 years later when, upon autopsy, a circumscribed CA1 lesion was noted

(Zola-Morgan et al., 1986). Prior to R.B.'s death, careful neuropsychological evaluation revealed extensive impairments on tasks such as paired associate learning, story and diagram recall, but not general intelligence or motivation.

Similar to human declarative memory impairments, rats and gerbils also show deficits on learning and memory tasks following global ischemia (e.g., habituation and working memory failure). In rodents these are typically assessed with tests such as the Morris water maze, T-maze, radial arm maze and open field.

Gerbils show large increases in spontaneous locomotor activity within a few hours of ischemia (Chandler et al., 1985; Gerhardt and Boast, 1988; Kuroiwa et al., 1991; unpublished data). While early motor hyperactivity (< 24 hr) probably reflects an acutely injured brain, later activity increases have been ascribed to a failure to habituate to novel environments (Wang and Corbett, 1990; Babcock et al., 1993). This was shown by exposing gerbils to an open field for 5 days (10 min sessions) prior to ischemia and then testing them for 5 days postischemia. Pre-ischemic habituation blocked the hyperactivity normally observed following ischemia since the open field was then familiar (Wang and Corbett, 1990). Babcock and colleagues (1993) also linked the postischemic hyperactivity with memory failure. They observed increased exploratory activity following ischemia that eventually returned to the level of normal gerbils with repeated open field exposure. Following this apparent recovery they introduced normal and ischemic animals to a semi-novel environment and found greater exploration in the ischemic group. Thus, ischemic gerbils still showed an impaired habituation to novelty.

Similarly, late ischemic impairments were apparent when testing was delayed until day 13 and 14 (learning a new environment), but not when testing was repeated from the first postischemic day (eventual habituation). If open field activity was simple motor hyperactivity, then these groups should have been the same on days 13 and 14. Finally, Mileson and Schwartz (1991) showed that the amount of exploration was related to the extent of hippocampal CA1 damage and not to other areas such as the striatum and thus, a potential index of ischemic CA1 injury.

Working memory deficits following ischemia have been shown in the T-maze (Ordy et al., 1988; Volpe et al., 1988, 1992; Hagan and Beaughard, 1990; Imamura et al., 1991). For example, impaired choice accuracy in a forced alternation T-maze was noted in rats up to 10 weeks postischemia (Hagan and Beaughard, 1990). In this test animals completed pairs of forced (FT) and choice trials (CT). On the FT they were randomly allowed into an arm by a door blocking the opposite arm. Following this (minimal delay) they had a choice of arms to enter, but are only rewarded if they entered the previously blocked arm. Working memory is assessed since animals must remember the direction of the previous FT on the CT, but do not use this information for subsequent trials. When delays (30 and 60 sec) were introduced between the FT and CT (intertrial interval, ITI) ischemic gerbils were impaired. However, the effects of greater ITIs on performance was confounded by a residual impairment from the no-delay training. Imamura and colleagues (1991) trained gerbils prior to ischemia and then tested them from 1 - 3 days and at 1 month following ischemia. Gerbils showed a significant early impairment which

recovered by 1 month. Volpe and colleagues (1988, 1992; Ordy et al., 1988) have also shown working memory (10 - 180 sec ITIs) impairments with pre-ischemic trained rats tested in a T-maze from 1 to 4 months following ischemia.

Other impairments have been demonstrated with the Morris water maze (Auer et al., 1989; Hagan and Beaughard, 1990; Jaspers et al., 1990; Corbett et al., 1992; Green et al., 1992) and the radial arm maze (Volpe et al., 1984; Davis et al., 1987; Gionet et al., 1991; Kiyota et al., 1991). However, these impairments (on all memory tests) often show substantial recovery, and the most persistent deficits have only been noted for up to about 4 months postischemia. Thus, it is unknown whether any functional deficits in rodents are permanent, as they are in humans and primates (Zola-Morgan et al., 1986, 1992).

While the aim of clinical intervention is to lessen these functional impairments, most researchers only quantify CA1 injury (usually by cell counts), assuming that this relates linearly to learning and memory ability. This view is perhaps overly simplistic since there are several factors which potentially obfuscate this relationship. For example, other vulnerable neurons (e.g., hilar, CA2 and subiculum) are components of the circuitry subserving learning and memory and yet these regions are rarely assessed. Furthermore, we cannot assume that the degree of CA1 injury accurately predicts that of other hippocampal regions. In addition, neurons may look normal with Nissl stains but not function properly (Bothe et al., 1986; Jaspers et al., 1990; Lyeth et al., 1990, Corbett et al., unpublished data). For example, Hori and Carpenter (1994) found dendritic beading in CA1 neurons which had normal looking cell bodies. This injury was reflected by

impaired long term potentiation 1 day following ischemia while gross histological injury was noted about 5 days later. Furthermore, a residual mild injury, such as following a partially effective postischemic neuroprotectant, may result in a very delayed CA1 injury which can take months to mature, but is reflected in early functional testing (sections 4.3 and 5.3). If this is the case, then early behavioral impairments may signal eventual neuronal death. Conversely, undetectable neuronal savings may improve function. For example, Grotta and colleagues (1988) found behavioral protection with pre-ischemic nicardipine (Ca^{++} channel blocker), but did not observe histological benefit. Similarly, compensatory factors (e.g., receptor supersensitivity, sprouting, circuitry redundancy) may facilitate behavioral recovery despite neuronal loss. Therefore, one must rigorously determine performance, in addition to histological assessment, to be certain of a therapy's benefit. Quantitative histology is important since cell loss, even when without obvious functional effect, may have detrimental consequences later in life or on other more demanding tasks. Ideally, studies should combine rigorous histological measurement in several vulnerable areas with extensive behavioral testing. Such a combination should predict efficacy in humans better than either histology or animal behavior alone.

1.3 Metabolic Perturbations

The brain, because of an inability to store glycogen, its high metabolic demand and high O_2 requirement, can only tolerate very brief ischemia before there is a rapid exhaustion of oxygen and glucose substrates with an accumulation of waste (e.g., lactate). This results in a marked decrease in ATP synthesis which impairs the Na^+ - K^+ pump responsible for maintenance of ionic gradients. For a brief period (≈ 1 - 2 min depending on ischemic severity) acidity increases and there is a gradual accumulation of $[K^+]_e$. This produces a slow depolarization followed by a series of action potentials. Suddenly, there is a quick cessation of neural activity (anoxic depolarization) as Na^+ and Ca^{++} rapidly enter while K^+ exits the cell along their concentration gradients (positive feedback) (e.g., see Siesjö et al., 1989). These ions pass through voltage gated and leak channels, in addition to receptor operated channels (glutamatergic). Ionic flux may also occur via the Na^+/H^+ and $3Na^+/Ca^{++}$ electrogenic exchangers which reverse when the membrane is depolarized (e.g., see Karmazyn and Moffat, 1993).

Glutamate (an EAA) is released in excessive quantities during ischemia (Benveniste et al., 1984; Hagberg et al., 1985; Globus et al., 1988; Mitani et al., 1990) probably by Ca^{++} dependent vesicular release (Drejer et al., 1985) and/or reverse operation of the glutamate uptake carrier (Ikeda et al., 1989; Szatkowski et al., 1990; Attwell et al., 1993). It is thought that the rise in extracellular glutamate levels triggers neuronal depolarization via non-NMDA receptor (e.g., AMPA) activation which permits

an influx of Na^+ and an efflux of K^+ (Mayer and Westbrook, 1987). This depolarization causes Mg^{++} to vacate the ion channel of the NMDA receptor complex and allows entry of Na^+ and Ca^{++}, while K^+ exits. While anoxic depolarization will occur without glutamate receptor activation, it is thought that prolonged activation of NMDA receptors allows a greater, more lethal Ca^{++} influx.

Entry of Cl^- along with Na^+ causes water entry by osmosis. In some neurons this is sufficient to produce lysing. In murine cortical culture, brief exposure to glutamate or oxygen/glucose deprivation induces an osmotic death in some neurons while most undergo a delayed degeneration (Choi, 1985; Choi, 1987; Goldberg and Choi, 1993). The osmotic lysing, but not the DND, can be prevented by replacing Na^+ or Cl^- with impermeant ions, while the late component can be arrested by removing Ca^{++} from the extracellular space.

Like glutamate or "ischemia" exposure *in vitro*, most CA1 neurons recover quickly upon blood flow restoration and only a few cells die by osmotic lysing. Energy production (phosphocreatine, ATP and glucose levels) returns to near-normal quickly upon reperfusion, well before the DND in the vulnerable structures such as the striatum and CA1 (Pulsinelli and Duffy, 1983). The subsequent gradual progression to necrosis is believed to critically depend on the massive intracellular Ca^{++} accumulation that occurred during ischemia. Unfortunately, early restoration of calcium homeostasis is followed by many hours of altered Ca^{++} regulation such that postischemic CA1 activation (depolarization) causes an increased Ca^{++} entry compared to non-ischemic CA1 neurons

(Andiné et al., 1988, 1992; Silver and Erecinska, 1992). It is not fully understood how Ca^{++} triggers eventual neuronal disintegration, but it is thought to be due to over-stimulation of lipases, proteases (e.g., calpain activation can damage cytoskeletal elements; Matesic and Lin, 1994) and endonucleases. The summation of these elevated Ca^{++} transients may also contribute to the massive accumulation of Ca^{++} noted around the time of cellular disintegration (Deshpande et al., 1987; Dux et al., 1987).

The NMDA channel is an important route of Ca^{++} entry. However, activation requires concurrent depolarization (via AMPA conductance) to relieve the Mg^{++} voltage-dependent block. Originally, it was thought that glutamate over-excited the neuron (CA1) and this lead to prolonged NMDA activity and, therefore, lethal Ca^{++} influx (glutamate excitotoxicity theory; Rothman and Olney, 1986). However, there are several major inconsistencies with this theory. First, initial reports of CA1 hyperactivity (Suzuki et al., 1983) have not always been confirmed (Buzsàki et al., 1989; Mitani et al., 1990; Imon et al., 1991). Second, glutamate levels rise to the same levels and the same time course in non-vulnerable (e.g., CA3, dentate) and vulnerable neurons (CA1) during ischemia which then normalizes soon after reflow and prior to the putative hyperactivity (Mitani et al., 1992). Third, the distribution of NMDA receptors does not explain neuronal susceptibility (e.g., hilar neurons have fewer NMDA receptors than CA1 while the dentate has more than CA1; Bekenstein et al., 1990). Before dismissing the theory, however, some evidence suggests that ischemia alters the AMPA receptor subunit composition in a way that permits Ca^{++} flux (Hollmann et al., 1991; Pellegrini-

Giampietro et al., 1992, but see Diemer et al., 1994 for contrasting data). Perhaps, this explains the increased Ca^{++} transients that occur following ischemia. Normal, and especially increased activity could then trigger enough Ca^{++} influx to kill the neuron. The cause of an altered AMPA subunit composition is unknown, but it may be triggered by the Ca^{++} injury received during ischemia.

One further manifestation of ischemic injury is a persistent protein synthesis inhibition. While this occurs in all neuronal populations following ischemia only the non-vulnerable cells (e.g., CA3 and dentate) recover (within a few hr) while a persistent impairment remains in susceptible neurons (i.e., CA1) (Bodsch et al., 1985; Thilmann et al., 1986; Araki et al., 1990; Widmann et al., 1991). Interestingly, ischemia decreases eukaryotic initiation factor 2 activity (Hu and Wieloch, 1993) which is known to be important in protein synthesis initiation (Ray et al., 1992). Perhaps, a Ca^{++} dependent kinase phosphorylates this initiation factor (Schatzman et al., 1983) thereby providing a mechanism of interaction between altered Ca^{++} homeostasis and impaired protein synthesis.

The detrimental effects of elevated Ca^{++} activity coupled with impaired protein synthesis may culminate in eventual cellular disintegration as damage exceeds the critical limit in vital structures. Damage to intracellular structures such as the mitochondria would further impair an already compromised system (positive feedback). The elucidation of such thresholds (therapeutic windows) is an exceedingly important goal of stroke research. Unfortunately, the point of no return may be well passed prior to actual

cell death since irreversibility is defined by the most vulnerable, essential structure which is not necessarily the membrane integrity. Thus, there will be a marked loss of neuroprotective ability with reperfusion time as this point of irreversible injury is surpassed. Since many systems are "attacked" by over-activation of Ca^{++} regulated enzymes it is likely that the most efficacious therapy will have to be applied early and counteract the entire gamut of abnormalities which injure the cell (e.g., Ca^{++} overload and protein synthesis inhibition).

1.4 Therapeutic Interventions

1.4.1 *Pharmacology*

From the previous discussion it would appear that pharmacological interventions directed at blocking both NMDA and AMPA receptors would be neuroprotective. However, positive reports with NMDA antagonists (e.g., MK-801; Gill et al., 1987, 1988) have been confounded by the findings of drug induced hypothermia (Buchan and Pulsinelli, 1990; Corbett et al., 1990). More recent enthusiasm (Sheardown et al., 1990) with the AMPA receptor antagonist, NBQX, has been similarly confounded since Nurse and Corbett (submitted) have conclusively shown that NBQX induces a protracted mild cooling which appears to explain all of NBQX's reported efficacy. Thus, it seems that NMDA and AMPA antagonists are ineffective against global ischemia when brain temperature is adequately regulated. Other strategies to limit Ca^{++} accumulation have also been assessed. L- (e.g., nimodipine, Auer, 1993 for a review) and P-type (e.g., SNX-111, Valentino et al., 1993) calcium channel antagonists have been reported to be beneficial. However, the most effective compound, SNX-111, appears to cool animals for a protracted time. Furthermore, protection with SNX-111, and NBQX, is transient (< 28 days, A. Buchan, personal communication).

To summarize, this diverse pharmacology has yet to produce a compound that clearly provides lasting protection which is devoid of a drug-induced side effect (e.g., hypothermia or improved cerebral blood flow). The failure of such compounds to provide

clear cut neuroprotection represents a serious challenge to current beliefs about how ischemic injury occurs (glutamate excitotoxicity theory).

1.4.2 *Hypothermia*

1.4.2.1 Intra-Ischemic Hypothermia

It has been known for many years that hypothermia during ischemia is dramatically protective (Bigelow et al., 1950; Pontius et al., 1954). For example, cooling to 27°C was used to extend the safe period of blood flow occlusion with cardiac repair (Lewis et al., 1954).

More recently, the beneficial effects of milder temperature reductions were demonstrated in models of global ischemia (Busto et al., 1987; Green et al., 1992; Nurse and Corbett, 1994). For example, 31°C hypothermia completely and persistently mitigated the behavioral (open field tests), electrophysiological and histological consequences of a 5 minute occlusion in gerbils (Nurse and Corbett, 1994).

The protective mechanism of intra-ischemic hypothermia is not known (for review see Ginsberg et al., 1992). It does not significantly affect the reduction in cerebral blood flow during ischemia nor does it affect the end depletion of energy (i.e., phosphocreatine, ATP and high energy adenylates) (Busto et al., 1987, 1989). However, it does slightly retard the initial decline in these molecules (Chopp et al., 1989; Welsh et al., 1990). This is probably of minor importance as Kristián and colleagues (1992)

showed that mild cooling had only a minimal effect (≈ 30 sec) on the time to anoxic depolarization. Alone, this cannot account for the remarkable potency of intra-ischemic hypothermia, as it would only extend the safe period of ischemia by a short time. Furthermore, cooling did not affect the amount of Ca^{++} accumulation during ischemia (Kristián et al., 1992), which is believed to be the key contributor to DND.

Hypothermia does significantly blunt the intra-ischemic rise in EAAs (glutamate and aspartate) and promotes the normalization in the first few hours postischemia (Busto et al., 1989; Mitani and Kataoka, 1991). However, since these EAAs are thought to promote injury by increasing Ca^{++} entry, which hypothermia does not markedly alter (Kristián et al., 1992), it is possible that reduced EAA release is an epiphenomenon and not the true mechanism of neuroprotection. Perhaps, cooling works at steps beyond the initial Ca^{++} entry, such as inhibiting the enzymes responsible for protein synthesis inhibition and later Ca^{++} homeostasis deregulation. If so, intra-ischemic cooling has the advantage of attenuating injurious events downstream of Ca^{++} influx, while blockade of a few routes of Ca^{++} entry (e.g., EAA antagonists) are ineffective. For example, intra-ischemic hypothermia was found to promote the recovery of ubiquitin synthesis (Yamashita et al., 1991) which is important in degradation of abnormal proteins. Cooling was also found to attenuate the inhibition of protein kinase C (PKC) following ischemia (Cardell et al., 1991). It is believed that PKC affects gene expression, neuronal regulation and release of neurotransmitters (Nishizuka, 1986) and thus, its inhibition could markedly impair neuronal viability. Finally, intra-ischemic cooling was shown to

promote the recovery of protein synthesis in CA1 (Widmann et al., 1993).

1.4.2.2 Postischemic Hypothermia

Unfortunately, since most strokes occur without warning, the value of intra-ischemic hypothermia is limited to elective surgery. While postischemic hypothermia could be of greater clinical significance, its efficacy is somewhat controversial. Early reports with post-stroke cooling were encouraging. In 1958 Williams and Spencer reported good recovery in four individuals with immediate post-cardiac arrest cooling (24 - 72 hr at 30 - 34°C). They noted that similar untreated patients rarely lived. Benson and colleagues (1959) also improved survival of cardiac arrest patients with delayed hypothermia (3 hr - 8 days at 30 - 32°C). Likewise, immediate postischemic hypothermia (18 - 36 hr at 31 - 33°C) reduced the mortality of dogs subjected to a 10 minute circulation occlusion (Zimmerman and Spencer, 1959). Wolfe (1960) also reduced mortality in dogs subjected to a 5 minute ventricular fibrillation by lowering temperature to 31°C (for ≈ 24 hr) soon after resuscitation. However, these results were tempered by poor controls and a lack of clear benefit. More recently, Michenfelder and colleagues (1977, 1979) found deleterious effects (e.g., edema and death) of postischemic hypothermia (29°C for 48 hr) in cats and monkeys with a middle cerebral artery occlusion. Michenfelder et al. (1980) also reported adverse effects (e.g., cardiovascular collapse) in the dog (24 hr at 29°C). The lack of convincing experimental data, management problems and the occurrence of side effects led to the abandonment of

postischemic hypothermia. However, the degree of cooling is an important consideration. Serious complications, such as cardiac arrhythmias occur more often at temperatures below 30°C (Bailey et al., 1954; Lewis et al., 1954). This, along with severe ischemia, species differences and sometimes lengthy delays before intervention, may have overwhelmed the beneficial effects of hypothermia in some of these early studies.

Accordingly, more recent work investigated mild postischemic hypothermia and found it to be beneficial when initiated within 30 minutes of brief ischemia (Busto et al., 1989; Chopp et al., 1991). Subsequently, several groups have even shown CA1 neuroprotection with delayed (beyond 30 min) postischemic hypothermia both in gerbil (Carroll and Beek, 1992; Hoffman and Boast, 1992) and in rat (Coimbra and Wieloch, 1992, 1994). Carroll and Beek (1992) subjected gerbils to 5 minutes of ischemia and found that 6 hours of hypothermia (rectal temperature = 28 - 32°C) initiated 1 hour after occlusion resulted in approximately 50% CA1 preservation with 4 day survival. Similarly, Hoffman and Boast (1992) reduced CA1 loss in gerbils (sacrificed at 4 days) with 5 hours of hypothermia (rectal = 32°C) which started 1 hour after a 5 minute ischemic episode. Coimbra and Wieloch (1992) found CA1 protection with 5 hours of hypothermia (rectal and skull ≈ 33°C) initiated 2 hours after 10 minutes of 2-VO ischemia in rats that survived for 1 week. Subsequently, these authors (1994) even documented CA1 savings when initiation was delayed as late as 12 hours postischemia (7 day survival). Furthermore, like Carroll and Beek (1992), Coimbra and Wieloch (1994) also showed greater CA1 savings with quicker intervention and longer hypothermic

periods (≤ 5 hr). Unfortunately, all of this work has been cast in doubt by the findings of Dietrich and colleagues (1993) who reported that postischemic hypothermia only delays cell death. In their study, postischemic hypothermia (3 hr) resulted in CA1 preservation (10 min of 2-VO) when animals were sacrificed at short (3 and 7 days) but not after a longer survival time (2 months). Thus, the current view is that postischemic hypothermia conveys little in the way of permanent neuroprotection.

However, many of the processes believed to culminate in CA1 death, such as impaired protein synthesis and altered Ca^{++} homeostasis, continue for many hours (or days) after ischemia. It is reasonable, therefore, that a neuroprotectant will have to be administered over a protracted period to be truly beneficial. In addition, early positive reports with postischemic hypothermia (e.g., Williams and Spencer, 1958; Benson et al., 1959) utilized protracted hypothermia (24 hr or longer). Similarly, drugs that provide protection (i.e., NBQX and SNX-111) do so by prolonged cooling. Since humans can tolerate days (and even weeks, see Hendrick, 1959) of mild hypothermia (> 32°C) it makes sense to continue cooling not for a few hours, but for many hours, perhaps days, to thoroughly investigate the effectiveness of mild postischemic hypothermia. To do so, however, a well characterized and consistent model of global ischemia is needed.

Chapter 2 (Temperature Correlations)

2.1 Introduction

Brief global ischemia produces a profound loss of hippocampal CA1 neurons which is mitigated by mild hypothermia (e.g., 29 - 33°C) and exacerbated by hyperthermia either during ischemia or in the early recirculation period. In order to avoid the confounding effects of temperature when quantifying ischemic severity or testing potential anti-ischemic therapies, it is necessary to monitor brain temperature during surgery as well as in the postischemic period. For example, it was shown that the reported protective action of MK-801 (Gill et al., 1987, 1988) was largely due to hypothermia (Buchan and Pulsinelli, 1990; Corbett et al., 1990). Similarly, the beneficial effects of NBQX (Sheardown et al., 1990) appear to be due to hypothermia (Nurse and Corbett, submitted).

Most investigators now rely on skull temperature (i.e., probe inserted under skin or into the temporalis muscle) to approximate brain temperature since rectal temperature does not always faithfully reflect brain temperature (Busto et al., 1987). In the rat, rectal, skull and brain temperatures were compared during ischemia (Busto et al., 1987; Minamisawa et al., 1990). Brain temperature was also described in the gerbil (Neill et al., 1990), but no systematic comparisons of brain, rectal and skull temperatures were provided. In addition, little information concerning postischemic temperature is available.

In this study (Colbourne et al., 1993) we used a wireless, AM transmitter-based brain temperature monitoring system (Mini-Mitter, Sunriver, OR, USA) that allowed

continuous brain temperature measurement during surgery and in conscious, freely moving animals. We compared rectal and skull readings with brain temperature during and after ischemia in the gerbil.

2.2 Methods

2.2.1 *Subjects*

Ten female, Mongolian gerbils (High Oak Ranch Ltd., Baden, ONT, Canada), weighing between 75 and 115 g were used. Animals were individually housed under diurnal light conditions with food and water freely available. All procedures were approved by the Memorial University of Newfoundland animal care committee in accordance with the guidelines of the Canadian Council on Animal Care.

2.2.2 *Baseline Brain Temperature Monitoring*

Five days prior to ischemia the gerbils were anesthetized with Somnotol (65 mg/kg, i.p.) and a 20 gauge stainless steel guide cannula (6.0 mm) was lowered to the dural surface overlying the left frontal cortex. This was approximately 1 mm anterior and 1 mm lateral to bregma. Three inverted nylon screws (4 mm) were then glued to the skull. The cannula was attached with dental cement and a stylet was inserted to prevent infection and to block the cannula.

Two days later gerbils were briefly anesthetized with 1.5% Halothane in 70% N_20 and 30% O_2. Eight mm brain temperature probes (Mini-Mitter, model XM-FH, Mini-Mitter Co., Inc., Sunriver, OR, USA) were inserted to sample cortical temperature. Probes were taped to the dental cap and anesthesia was discontinued. The gerbils were then placed into individual plexiglass cages (20 cm long X 14 cm wide X 17 cm high)

resting on AM receivers (Mini-Mitter, model RA-1010) interfaced to a computer. Brain temperature was recorded every 20 seconds for 3 hours to provide baseline normal temperature. Temperature data were analyzed and exported with DataQuest III software (Data Sciences, Inc., St. Paul, MN, USA).

2.2.3 *Cerebral Ischemia*

Two days after baseline temperature was recorded, the gerbils were anesthetized with 1.5% Halothane in 30% O_2 and 70% N_2O. The Halothane was delivered through a mask equipped Fluovac Scavenger System (Stoelting Co., Chicago, IL, USA). Rectal temperature was monitored and maintained at approximately 37.5°C with a homeothermic blanket control unit (Harvard Apparatus, South Natick, MA, USA). Skull temperature was measured by a 30 gauge thermocouple probe (Omega Engineering, Stamford, CT, USA) inserted subcutaneously and contralateral to the guide cannula. Skull temperature was maintained at 37.5°C by wrapping a heated water flow-through blanket (Mul-T-Pads, model TP-3E, Gaymar Industries Inc., Orchard Park, NY, USA) snugly around the dorsal and lateral aspects of the head. A small opening in the water blanket allowed passage of the brain probe. This permitted brain temperature measurement without interfering with the water blanket heating system. Brain temperature was measured in 5 gerbils during surgery and in all animals after surgery.

Once the temperature probes were in place, a ventral midline neck incision was made. The common carotid arteries were then isolated and occluded for 5 minutes using

micro-arterial clamps (Fine Science Tools, Vancouver, BC, Canada). At the end of occlusion the clamps were removed and the arteries checked for reflow. The incision was sutured and anesthesia discontinued. The entire procedure took approximately 20 minutes. Gerbils were then placed in their individual cages.

Following surgery rectal temperatures (Digi-Sense, Cole-Parmer Instrument Company, Chicago, IL, USA) were recorded at 0.5, 1, 2 and 3 hours after ischemia. Skull temperature measurement continued until the animal displayed signs of discomfort (typically 15 - 30 min after occlusion). Finally, brain temperature was monitored continuously for 3 hours following ischemia.

2.2.4 *Histology*

Gerbils were sacrificed 10 days after ischemia with an overdose of sodium pentobarbital. They were then transcardially perfused with 30 ml of 0.9% heparinized saline followed by 30 ml of 10% phosphate-buffered formalin. Brains were then stored in the same fixative. One day prior to sectioning brains were immersed in a solution of 20% sucrose in 10% formalin prior to being frozen and sectioned at 10 µm. Cells were stained with cresyl violet.

CA1 pyramidal neurons were examined in three equal sectors (each 0.4 mm long) corresponding to medial, middle and lateral portions of the CA1 cell layer at approximately 1.7 mm posterior to Bregma (Loskoto et al., 1975). These three sectors were adjacent to subiculum (medial), at the apex of CA1 (middle) and next to CA2/CA3

(lateral). Each sector was rated, by two experimentally naive observers, on a five-point scale as follows: 90-100% normal cells = 4; 60-89% = 3; 30-59% = 2; 6-29% = 1; and 0-5% = 0. The rating scores from all six (3 per hemisphere) sectors were added to yield a cumulative CA1 cell rating with a maximum score of 24 (i.e., a normal CA1) (Colbourne and Corbett, 1992). Unpublished data (N = 56, from Colbourne and Corbett, 1992) has shown that this rating score correlates highly with CA1 cell counts, $r = 0.973$, $p = 0.0001$. In addition, both our rating and counting methods include only viable-looking neurons (i.e., well defined nucleus, distinct cellular membrane and not shrunken).

2.3 Results

The mean CA1 cell rating (out of 24) was 1.2 ± 1.14 SD, indicating severe loss of CA1 neurons.

The mean brain temperature recorded 2 days prior to occlusion varied between 36.5 - 37.2°C. During surgery, skull and rectal temperatures (Fig. 2.1 and Table 2.1) were maintained near 37.5°C. Nevertheless, brain temperature fell by 1.5°C during occlusion (Fig. 2.1) before quickly returning to normal values during reperfusion.

Rectal temperatures recorded 30 and 60 minutes after occlusion were approximately 38°C. Thereafter (2 and 3 hr postischemia), rectal temperature increased to nearly 39°C. After an initial decline at the end of occlusion, skull temperature quickly rose to approach 38°C. Brain temperature also increased above baseline values and was virtually identical to skull temperature at 30 minutes (Fig. 2.1). Brain temperatures recorded 30, 60, 120 and 180 minutes into the postischemic period were significantly higher than the median (36.7°C) pre-ischemic brain temperature (t_8 = 2.33 to 3.20, $p <$ 0.05).

Table 2.1: Rectal temperatures (mean ± SEM) during surgery (SS = start of surgery, SO = start of occlusion, EO = end of occlusion) and at the specified postischemic intervals (30, 60, 120 and 180 min).

SS	SO	EO	30 min	60 min	120 min	180 min
37.51 ± 0.47	37.78 ± 0.26	37.44 ± 0.32	38.10 ± 0.44	37.91 ± 0.40	38.37 ± 0.34	38.90 ± 0.37

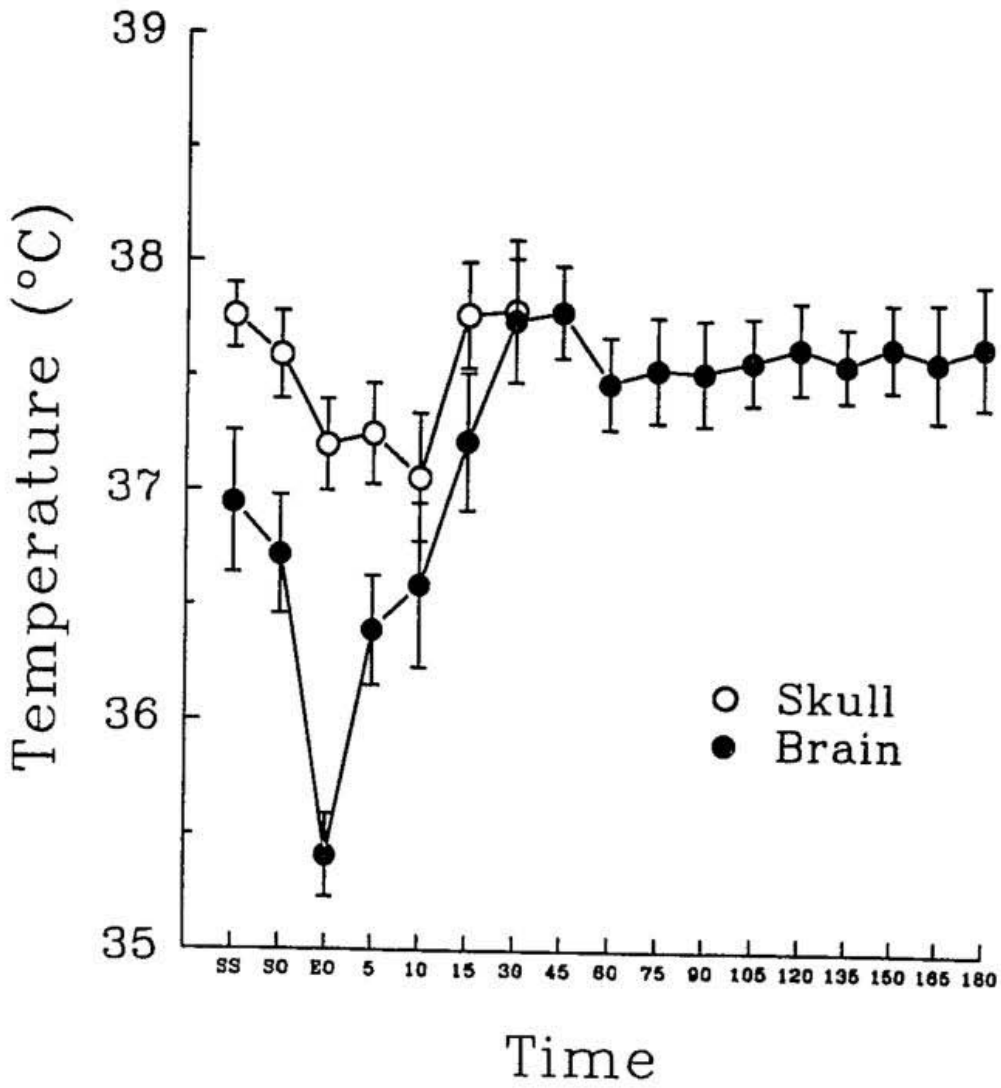


Figure 2.1: Brain and skull temperatures (mean ± SEM) during surgery (SS = start of surgery, SO = start of occlusion, EO = end of occlusion) and after recirculation. Brain temperature is averaged over either 5 or 15 minute intervals.

2.4 Conclusions

Maintaining skull and rectal temperatures at normal values during occlusion did not prevent a 1.5°C fall in brain temperature. Therefore, skull and rectal temperatures were not accurate indices of brain temperature during ischemia. Similar decreases in ischemic brain temperature were noted in studies using rat (Busto et al., 1987; Minamisawa et al., 1990) and gerbil (Mitani and Kataoka, 1991). While it is desirable to maintain constant brain temperature during occlusion this is difficult to achieve unless surgery is performed in a temperature controlled, humidified chamber to prevent evaporative heat loss (Minamisawa et al., 1990). It is likely that evaporative heat loss through the nasal and oral cavities coupled with cessation of cerebral blood flow accounted for the observed intra-ischemic decline in brain temperature. However, this small decline in brain temperature did not prevent a severe and consistent loss of CA1 neurons.

Skull temperature was approximately 0.5 - 1.0°C above brain temperature during the first 15 minutes of reperfusion and at 30 minutes the two temperatures were nearly identical. Rectal temperature was also within 1.0°C of brain temperature at the times sampled in the postischemic period. Thus, in the awake, untreated gerbil both rectal and skull temperatures may provide an approximation of brain temperature, especially 15 minutes or more into reperfusion. However, certain drugs such as MK-801 (Buchan and Pulsinelli, 1990; Warner et al., 1991) and NBQX (Nurse and Corbett, submitted) affect

temperature for many hours after ischemia. These temperature changes may easily be missed by sampling rectal or skull temperature at a few arbitrary time points.

Several authors have noted a mild postischemic hyperthermia in the gerbil as assessed with rectal, skull or brain probes (Kuroiwa et al., 1990; Neill et al., 1990; Warner et al., 1990). The average increase in brain temperature reported is about 0.7°C (Neill et al., 1990) which corresponds well with the degree of brain hyperthermia (0.9°C) observed in the present study (Fig. 2.1).

In summary, this study has shown that maintaining normal skull and/or rectal temperatures during ischemia does not result in maintenance of normal brain temperature. If the head is not warmed during surgery, the drop in brain temperature may be greater than the 1.5°C decline noted in our experiment. Larger reductions in brain temperature during or after ischemia can markedly attenuate the ischemic insult. This hypothermic blunting may in turn act synergistically or additively with neuroprotective drugs to yield a substantial, though somewhat artifactual, degree of protection. Our findings suggest that investigators should not rely exclusively on intermittent rectal or skull temperature measurements, but instead determine brain temperature directly. Furthermore, this system is ideal for continuous, non-stressful, brain temperature measurement during and for many hours after ischemia.

Chapter 3 (Postischemic Hyperthermia)

3.1 Introduction

Five minutes of global ischemia in the gerbil usually produces severe and consistent hippocampal CA1 loss. Hyperthermia either during or after ischemia is believed to aggravate this injury. Several authors have noted that gerbils experience transient hyperthermia of approximately 0.5 - 1.5°C within the first few hours after ischemia (section 2.3; Kuroiwa et al., 1990; Neill et al., 1990; Warner et al., 1991). Remarkably, Kuroiwa and colleagues (1990) reported near complete CA1 preservation by preventing this postischemic hyperthermia. Postischemic temperature was assessed by skull and rectal probes and maintained by a heating pad under extended Halothane anesthesia. However, rectal and skull temperatures do not necessarily reflect brain temperature (Chapter 2). Thus, it is possible that the extended anesthesia, as used by Kuroiwa and colleagues (1990), may foster dissociations between brain and skull/rectal temperatures.

In this study (Colbourne et al., 1993) we measured brain, skull and rectal temperatures before, during and for several hours after 5 minutes of ischemia in gerbils. Some animals were kept under Halothane anesthesia for 85 minutes after ischemia so that rectal and skull temperatures could be maintained at normal values. In this way, we assessed the effects of preventing the immediate postischemic hyperthermia, as measured with rectal and skull probes, on brain temperature and subsequent CA1 integrity.

3.2 Methods

3.2.1 *Subjects*

Twenty female gerbils, weighing between 64 and 110 g were assigned to one of two treatment groups: control (CONT; N = 7) and extended anesthesia (EXT; N = 13).

3.2.2 *Temperature Monitoring*

Procedures for rectal, skull and brain temperature monitoring/control were as described previously (section 2.2.2).

3.2.3 *Cerebral Ischemia*

Many surgical procedures were similar to previous work (section 2.2.3). Rectal and skull temperatures were maintained near 37.5°C and 37.0°C, respectively. Both were continuously monitored to ensure steady temperature control. Once the rectal, skull and brain probes were in place the common carotid arteries were isolated. After 30 - 35 minutes of anesthesia the arteries were occluded for 5 minutes. Both the occlusion and subsequent anesthesia were performed under 1.0% Halothane. In CONT animals anesthesia was discontinued and gerbils, with brain probes in place, were placed in their cages. Brain temperature was then monitored for 4 hours and 25 minutes. In the EXT group, gerbils were maintained with 1.0% Halothane for 85 minutes after occlusion while their skull and rectal temperatures were kept at normal and their brain temperature was

monitored. After 85 min, anesthesia was discontinued and gerbils were placed in cages for 3 more hours of brain temperature recording.

Rectal temperature (Digi-Sense) was recorded 1, 2 and 3 hours after ischemia in CONT animals. In EXT gerbils, rectal temperature was simultaneously recorded (blanket unit) with skull values during the 85 minutes of postischemic anesthesia. Rectal temperature was then sampled with the Digi-Sense probe 2 and 3 hours after ischemia.

3.2.4 *Histology*

Histological assessment after 10 day survival was identical to previous work (section 2.2.4). Briefly, the CA1 layer was rated on a scale from 0 (complete CA1 loss) to 24 (a normal CA1).

3.3 Results

Normal brain temperature, recorded 2 days prior to occlusion, was 37.08 ± 0.50 SD, similar to our previous findings (section 2.3).

The degree of spontaneous postischemic hyperthermia in CONT animals (peak brain temperature = 37.88°C at 35 min postischemia and mean brain temperature of the first 85 min after occlusion = 37.31°C) was also similar to previous work (section 2.3). Maintaining postischemic skull and rectal normothermia, under Halothane anesthesia, prevented this temperature rise (Table 3.1). Regardless, the average CA1 ratings (out of 24) were almost identical at 7.08 ± 7.60 SD and 6.00 ± 5.51 SD in the EXT and CONT groups, respectively. Cell damage ranged from 0 (completely damaged) to 23 (no damage).

In spite of rectal and skull normothermia (Table 3.1) several animals, in both groups, displayed significant brain hypothermia. In animals that experienced normothermic (brain) ischemia there was severe CA1 cell death, whereas animals that experienced prolonged cerebral hypothermia during and/or after ischemia had reduced CA1 damage (Fig. 3.1 and 3.2).

Mean brain temperature (N = 20), during occlusion, was significantly correlated with histological damage, $r = -0.487$, $p = 0.029$. Mean brain temperature (N = 20) for the initial 85 minutes of reperfusion was also statistically related to histological outcome, $r = -0.558$, $p = 0.011$. Thus, it appears that the mild brain hypothermia either during ischemia

or in the first 85 minutes of reperfusion afforded histological protection. However, mean brain temperature (N = 20) averaged over a 3 hour period starting 86 minutes after ischemia was not related to histological outcome, $r = -0.120$, $p = 0.613$. Since rectal and skull temperatures were normothermic during anesthesia there was no meaningful relationship between these and histological outcome. There was also no significant relationship between postanesthetic rectal readings and CA1 rating (statistics not shown).

Table 3.1: Brain, skull, and rectal temperatures (mean ± SD), respectively, before (first 30-35 min of anesthesia), during and after occlusion (first 85 min of recirculation and the subsequent 3 hr) in EXT and CONT animals. Some values are post-anesthesia rectal recordings at 1 (a), 2 (b) and 3 hours (c), respectively.

		PRE-OCCLUSION	OCCLUSION	0 - 85 MIN	86 - 265 MIN
EXT	Brain	36.37 ± 0.11	35.18 ± 1.11	36.23 ± 0.77	37.58 ± 0.44
	Skull	36.99 ± 0.12	36.76 ± 0.33	37.14 ± 0.04	
	Rectal	37.78 ± 0.40	37.63 ± 0.25	37.41 ± 0.03	37.95 ± 1.18[b]
					37.69 ± 0.73[c]
CONT	Brain	36.74 ± 0.09	35.00 ± 1.06	37.31 ± 0.89	37.94 ± 0.73
	Skull	37.18 ± 0.17	36.87 ± 0.35		
	Rectal	37.87 ± 0.24	37.59 ± 0.24	37.06 ± 0.65[a]	37.63 ± 0.78[b]
					38.43 ± 0.93[c]

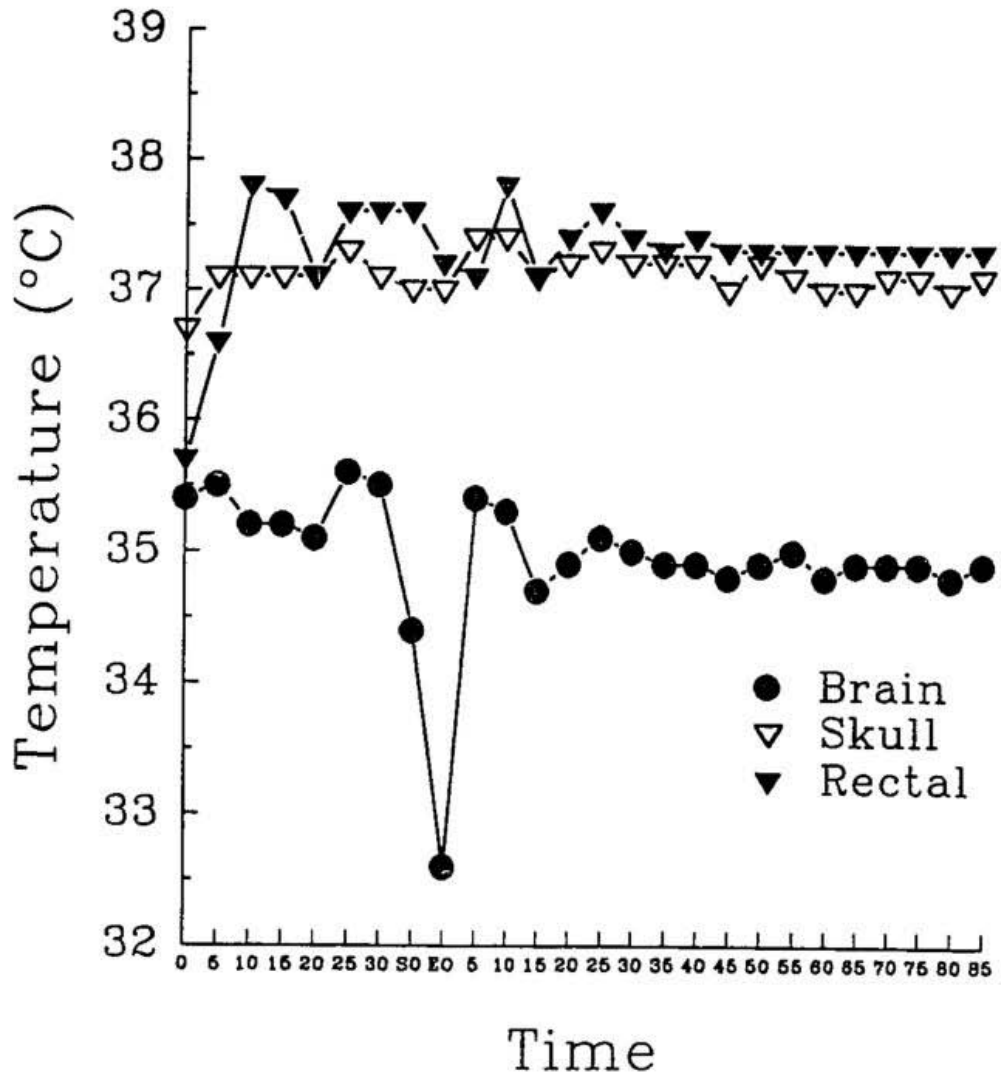


Figure 3.1: Rectal, skull and brain temperatures of an EXT gerbil which had near complete CA1 preservation. Note the prolonged mild brain hypothermia with skull and rectal normothermia (SO = start of occlusion, EO = end of occlusion).

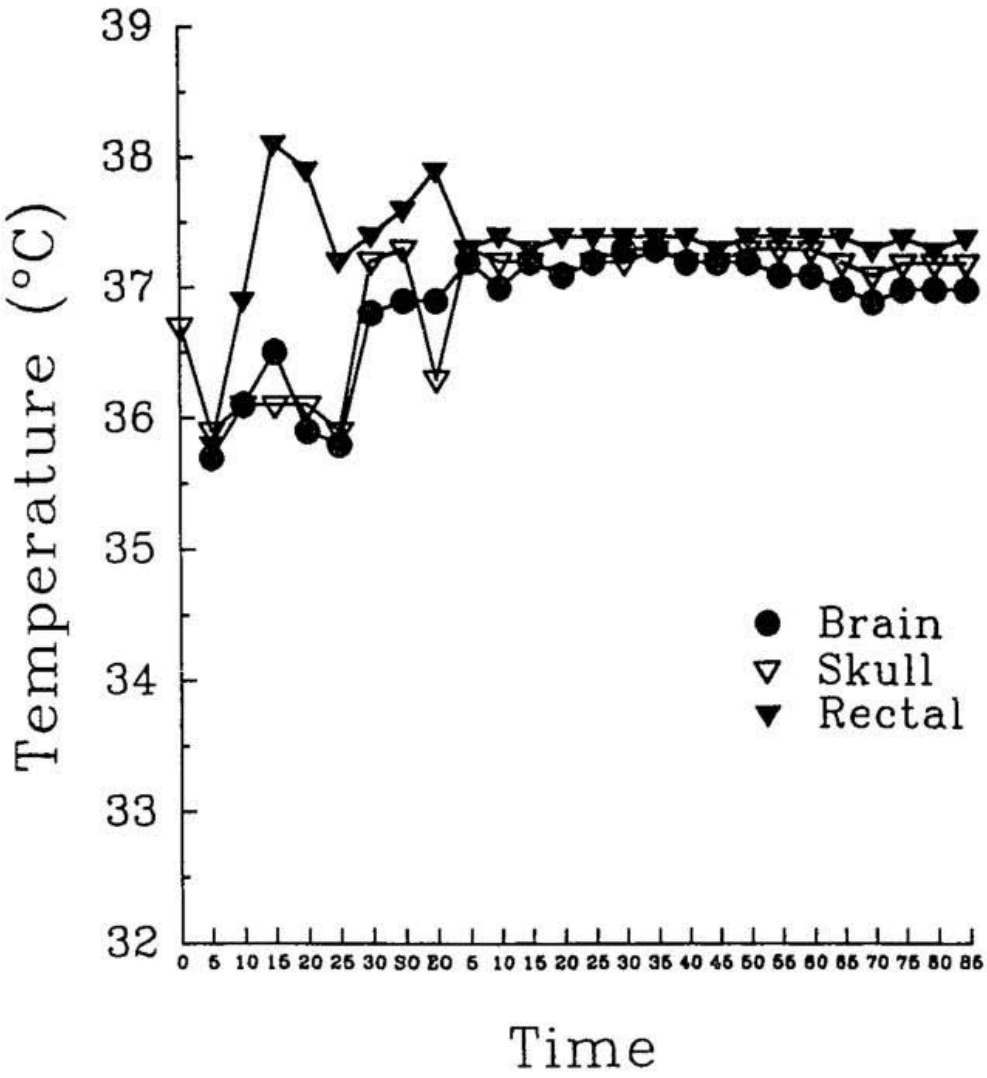


Figure 3.2: Rectal, skull and brain temperatures of a normothermic EXT gerbil with severe CA1 damage (SO = start of occlusion, EO = end of occlusion).

3.4 Conclusions

There was marked CA1 cell loss in both the EXT and CONT groups, however, the variability was somewhat high. More recent data (sections 4.3 and 5.3) indicate that more severe and consistent CA1 necrosis is produced with regulation of brain rather than skull temperature. However, in this study we attempted to approximate the procedures used by Kuroiwa et al. (1990) who did not measure brain temperature.

This experiment shows that brain temperature can markedly dissociate from both skull and rectal readings during ischemia surgery with lengthened anesthesia in the gerbil. We also show that 5 minutes of ischemia in those EXT gerbils with an intra-ischemic brain temperature range of 35.62 - 36.78°C and a postischemic range of 36.48 - 37.25°C results in severe CA1 necrosis (2.83 ± 2.48 SD, N = 6). This result contrasts with that of Kuroiwa, Bonnekoh and Hossmann (1990), where they found robust CA1 preservation by preventing the spontaneous postischemic rise in temperature normally associated with 5 minute occlusions in gerbils. They used various durations of extended Halothane anesthesia (up to 85 min) and a rectal heating pad to maintain *rectal* and *skull* normothermia during and after ischemia. However, *brain* temperature was not assessed. Importantly, we observed brain hypothermia, with skull and rectal normothermia, in several animals, which was quite dramatic in 2 of the 13 EXT gerbils (Fig. 3.1). Perhaps Kuroiwa et al. (1990) also induced protracted mild brain hypothermia in many of their extended anesthesia animals. This brain hypothermia, rather than prevention of

postischemic hyperthermia, may have afforded CA1 protection in their study. Without brain temperature data, they cannot conclude that immediate postischemic hyperthermia, as compared to normothermia, is required for severe CA1 damage after a 5 minute occlusion. We may not have encountered as many gerbils with such dissociations because our heating system utilizes rectal and skull *blankets* rather than a single heating *pad*. We used the cranial blanket since the protruding brain probe elevates the head above the body blanket, thereby reducing its effectiveness. Our dual heating system would likely reduce the incidence of brain temperature dissociations.

The degree of postischemic brain hyperthermia in CONT animals was less than the approximately 1.5°C rise in rectal and skull temperatures noted by Kuroiwa et al. (1990). However, the degree of postischemic hyperthermia observed in the present study is comparable to other findings in the gerbil (Neill et al., 1990). Perhaps the greater postischemic rise in their study was partially due to the stress of taking multiple rectal and skull readings. Our brain temperature system does not cause any noticeable stress and, therefore, would not likely alter temperature.

In conclusion, 5 minutes of normothermic ischemia followed by 85 minutes of normothermic recirculation resulted in severe CA1 necrosis. Thus, immediate spontaneous postischemic hyperthermia was not necessary for severe CA1 damage. This does not indicate that postischemic hyperthermia is not important. It is likely that greater or more protracted temperature rises after ischemia would aggravate damage, perhaps through recruiting additional brain regions to necrosis. For example, Mitani and Kataoka

(1991) showed that an elevation of brain temperature to 39°C increased CA1 cell loss. Postischemic hyperthermia may also have greater influence on more moderate insults (e.g., 3 min occlusion in gerbils). Finally, this study shows that prolonged mild brain hypothermia may occur with extended anesthesia, resulting in histological protection. These results and our previous study (Chapter 2) suggest that brain temperature should be determined in any ischemia study, especially those utilizing extended periods of anesthesia or other procedures that might promote skull/rectal and brain temperature dissociations.

Chapter 4 (Prolonged Postischemic Hypothermia)

4.1 Introduction

Global cerebral ischemia produces severe CA1 loss with concomitant impairments in new learning and habituation. Several groups have suggested that mild postischemic hypothermia attenuates this neuronal loss even, in some cases, when delayed for several hours after ischemia (section 1.4.2.2). Unfortunately, these conclusions have been cast in doubt by the findings of Dietrich and colleagues (1993) who reported that postischemic hypothermia only delayed cell death. In their study, immediate postischemic hypothermia (3 hr duration) resulted in CA1 preservation when animals were sacrificed at short (3 and 7 days) but not after a long survival time (2 months). Thus, the current view is that postischemic hypothermia conveys little in the way of permanent neuroprotection.

One question raised by the above studies concerns the duration of postischemic hypothermia which has often been 6 hours or less. It may be that a more prolonged duration of hypothermia is required to offset the myriad of disturbances resulting from ischemia. Indeed, certain neuroprotective drug treatments that appear to work via hypothermia (e.g., MK-801) have a long half-life (Buchan and Pulsinelli, 1990). The lack of functional assessment is another limitation of the above studies since one may achieve some behavioral protection irrespective of gross histological outcome (section 1.2.3).

In the first experiment (Colbourne and Corbett, 1994) we assessed whether 12

hours of hypothermia (brain = 32°C) initiated 1 hour after either 3 or 5 minutes of normothermic ischemia (brain) would reduce damage in several areas of the vulnerable CA1 region in the gerbil. Open field tests, which assessed exploration in a novel environment, determined hippocampal function since functional, and not histological outcome, is the more important clinical endpoint. Finally, histological outcome was evaluated at 10 and 30 days after occlusion because hypothermia may delay and not simply reduce neuronal necrosis. In the second experiment (Colbourne and Corbett, 1994) we used 24 hours of hypothermia beginning 1 hour after 5 minutes of ischemia in animals that survived for 30 days. This was to determine if 24 hours of hypothermia was more effective than a 12 hour duration.

4.2 Methods

4.2.1 *Subjects*

Eighty-two female gerbils were included in this study. Gerbils were approximately 12 to 13 weeks old and weighed about 55 g at the time of ischemia.

4.2.2 *Temperature Monitoring*

Procedures for rectal and brain temperature measurement were similar to previous work (section 2.2). However, a 5.0 mm guide cannula was used. This placed the probe tip in the anterior dorsal striatum at approximately the same depth as the hippocampus.

During these and subsequent experiments, the brain probes were more thoroughly cleaned (e.g., 30 min in 95% ethanol) prior to insertion. Without this precaution infection may occur. In earlier studies infection was occasionally found in the immediate area surrounding the probe tract (i.e., cortex and dorsal striatum). Regardless, no discernable effect upon CA1 outcome was found. Nonetheless, it makes sense to sterilize the probes as well as place them in a structure distant from the area of interest (i.e., CA1).

4.2.3 *Cerebral Ischemia*

Two days after measuring normal brain temperature, gerbils were subjected to 3 or 5 minutes of bilateral carotid artery occlusion under Halothane anesthesia. Both rectal and brain temperatures were regulated during ischemia surgery by a homeothermic rectal

heating blanket (rectal temperature during occlusion was approximately 38.6°C) and a heated water blanket wrapped around the gerbil's head. Rectal temperature was kept at this level to help maintain normal brain temperature when arterial clips were applied. A 60 watt lamp (positioned over the ventral surface of the head) was used to warm the incision area.

Both carotid arteries were checked for immediate reflow following clip removal. Several gerbils (7/89) were excluded because this did not occur (all were exposed to 5 min of ischemia). Following verification of reflow, animals were sutured, anesthesia was discontinued, and gerbils were placed in their individual boxes for brain temperature measurement. If necessary, a 60 watt heating lamp was used within the first hour of recirculation to ensure a brain temperature of at least 37.0°C.

One hour after ischemia all gerbils were anesthetized with Halothane for about 5 minutes while their backs and abdomens were shaved. Upon return to their boxes, control (ischemia but no hypothermia) and sham operated gerbils were quickly rewarmed to 37.0°C (brain) using a 60 watt lamp, which was then discontinued. In contrast, hypothermic gerbils were manually subjected to prolonged hypothermia upon return to their boxes. Hypothermia was induced by an overhead fan and intermittent cold (≈ 4.0°C) water spray. Gerbils were cooled by approximately 1.0°C/10 minutes to 32.0°C and then maintained around this value (within 0.5°C) until they were slowly rewarmed (use of fan and spray were reduced and if necessary an overhead heating lamp was turned on) by 1.0°C/10 minutes to 37.0°C at the end of the hypothermic period. With this

procedure the gerbils experienced whole body hypothermia and thus rectal and brain temperatures are similar (unpublished observations).

4.2.4 *Open Field Testing*

Exploration was evaluated by exposing gerbils (experiment 1) to an open field test 3, 7 and 10 days after ischemia for 10 minutes per trial (section 1.2.3; Colbourne and Corbett, 1992). The open field chamber measured 72 x 76 x 57 cm and was divided into 25 squares by an image tracking system that recorded the total number of squares crossed per session (HVS Systems, Kingston, UK). The open field was situated under two 60 watt lamps in a sound attenuated room. Distinctive features of the room (e.g., computer equipment, video camera, shelves) remained constant throughout testing and there were no special markings within the box. Gerbils with an intact hippocampus show less exploration over test days while ischemic gerbils, with CA1 damage, exhibit heightened activity.

4.2.5 *Histology*

Gerbils were sacrificed either 10 or 30 days after ischemia in accordance with previous work (section 2.2.4). Adjacent frozen coronal sections (10μm) were collected and stained with cresyl violet or cresyl violet/phloxine. Bilateral cell counts of viable-looking CA1 neurons in cresyl violet sections were performed blind to treatment conditions.

In the cresyl violet sections CA1 pyramidal cells were counted in the medial, middle and lateral sectors of CA1 (summed for analysis) at -1.7 mm and in a sector at the apex of CA1 at -2.2 mm to bregma (Loskoto et al., 1975). Total cell counts across all sectors in both hemispheres were used for data analysis.

It should be noted that these cell counts correlate highly with the previous rating scale. The main reason for this change was because counting is more widely accepted than rating systems.

Adjacent phloxine stained sections, which demarcated acidophillic neurons (with cytorrhexis and karyorrhexis) as red, were assessed by categorizing the sections as displaying none, some or many red cells. Tissue staining quality (indistinct cellular boundaries) did not allow for exact acidophillic cell counts. In addition, most red "cells" would not have been counted in cresyl violet sections as they would have appeared abnormal or too indistinct. While phloxine staining may largely demarcate neurons already dead ("cell corpses") prior to perfusion, it may also mark cells that would progress to death if the animals survived longer (Auer et al., 1985).

4.2.6 *Experiment 1*

There were 8 groups including: 4 hypothermic groups (12 hr duration) with either 3 or 5 minutes of ischemia and either 10 or 30 day survival (HYPO-3-10, N = 8; HYPO-3-30, N = 9; HYPO-5-10, N = 7; and HYPO-5-30, N = 7) and similarly 4 control groups (CONT-3-10, N = 8; CONT-3-30, N = 8; CONT-5-10, N = 7; and CONT-5-30, N

= 8). CA1 neurons were counted in sectors that were each 0.4 mm long at both -1.7 and -2.2 mm to bregma. Total cell counts in these groups were compared to sham-operated animals reported previously (Colbourne and Corbett, 1992).

4.2.7 *Experiment 2*

Two groups were exposed to 5 minutes of ischemia with either 24 hours of hypothermia which started 1 hour after ischemia (HYPO(2), N = 8), or not (CONT(2), N = 8). A third group (N = 4) of sham operated gerbils (SHAM(2)) was also added. Gerbils survived for 30 days. All other procedures were similar to experiment 1 except CA1 neurons were only assessed at -1.7 mm to bregma at a higher magnification (0.2 mm long sectors) which permitted easier and faster neuronal counts. This experiment was to quickly determine if longer duration hypothermia provided greater CA1 neuroprotection.

4.2.8 *Statistics*

Total CA1 cell counts (cresyl violet) were analyzed with a three factor ANOVA and specific contrasts. Since the significance trend in posterior CA1 was similar to anterior CA1 only the latter results are presented. Phloxine data were not statistically analyzed, but are presented as frequency data (i.e., number of gerbils per group with numerous acidophillic cells) to complement cresyl violet data where appropriate.

Open field data were analyzed with a three factor ANOVA. Groups were collapsed over survival times since this factor could not influence prior behavioral

testing. In addition, Greenhouse-Geisser adjusted degrees of freedom were used in comparisons involving the day factor because of a significant sphericity test ($p < 0.0001$).

Where appropriate, simple effects and planned comparisons were calculated without α adjustment for multiple comparisons, which was initially set at 0.05. For each ANOVA the homogeneity of variance assumption was evaluated by calculating F_{max} which is defined as the largest cell variance divided by the smallest cell variance in the analysis. Where F_{max} was greater than 9 in the overall ANOVA, indicating serious heterogeneity of variance, we halved the α level for main effects and interactions. In addition, we used the pooled variances from only those cells compared in subsequent contrasts, instead of the usual pooled error variance determined from the overall ANOVA. If F_{max} was greater than 3 for the simple effect or comparison we used an α level of 0.025, otherwise it was 0.05 (Keppel, 1991). All data are expressed as the mean ± SD.

4.3 Results

4.3.1 *Experiment 1*

Mean brain temperature, collected 2 days before ischemia, was 36.47 ± 0.36°C SD, with a group range of 36.25 to 36.77°C.

Intra-ischemic brain temperature was maintained close to this normal range. Hypothermic animals and their respective CONT groups (i.e., HYPO-5-10 vs CONT-5-10, HYPO-5-30 vs CONT-5-30, HYPO-3-10 vs CONT-3-10 and HYPO-3-30 vs CONT-3-30) were similarly maintained during ischemia ($p \geq 0.1991$). Likewise, postischemic (first hr of recirculation) temperature contrasts were not significantly different between HYPO and CONT groups ($p \geq 0.3324$), except for HYPO-3-10 gerbils who displayed significantly more hyperthermia (≈ 0.5°C) than CONT-3-10 gerbils ($p = 0.0080$) (Fig. 4.1, Table 4.1, 4.2 and 4.3).

Both occlusion and postischemic (first hr) brain temperatures (Fig. 4.1, Table 4.1, 4.2 and 4.3) were consistently higher in groups exposed to 3 minutes of ischemia ($p < 0.0001$ for main effects). Temperatures of day 10 versus 30 animals during ischemia ($p \geq 0.3406$) and in the first hour of recirculation ($p \geq 0.1141$) were not significantly different, except for CONT-3-10 gerbils who were maintained slightly (0.23°C), but significantly ($p = 0.0083$) higher during occlusion than the CONT-3-30 group.

Postischemic (1 - 13 hr) brain temperature was easily and precisely regulated (section 4.2.3) in all HYPO groups (Fig. 4.1). During this period all CONT groups

exhibited hyperthermia (peak = 38.2°C) that was maximal around 2 hours after occlusion. This hyperthermia dissipated over many hours. Finally, all groups (CONT and HYPO) displayed fairly similar temperature profiles beyond 13 hours after ischemia.

Delayed postischemic hypothermia (12 hr) reduced the increased exploration associated with both 3 ($p = 0.0105$) and 5 ($p = 0.0174$) minute ischemic episodes over all test days, but was not statistically significant on each day (Fig. 4.2, Table 4.4). Habituation over days occurred ($p < 0.0001$) and this was approximately equal among groups ($p = 0.8916$). Groups subjected to 3 minutes of ischemia showed less activity, indicating less impairment, than did those occluded for 5 minutes ($p = 0.0119$). This trend was consistent over days, but was significant only on days 3 ($p = 0.0244$) and 10 ($p = 0.0097$) and not significant on day 7 ($p = 0.1226$).

Five minutes of normothermic ischemia (CONT-5) resulted in near-complete anterior and posterior CA1 loss (Fig. 4.3 and Table 4.5). Damage in CONT-5 animals was similar with 10 and 30 day survival ($p = 0.4651$). Hypothermia significantly reduced CA1 loss with 10 ($p = 0.0066$) and 30 ($p = 0.0016$) day survival. However, neuronal protection observed with 30 day survival was less than that at 10 days, suggesting some progression of cell loss after day 10. This difference only approached statistical significance in anterior CA1 ($p = 0.0530$) and was not significant in posterior CA1 ($p = 0.1688$). However, phloxine stained tissue revealed mild to intense (50 or more cells) acidophillic staining (anterior and posterior CA1) in several HYPO-5-30 gerbils.

Three minutes of ischemia (CONT-3) also resulted in severe anterior and

posterior CA1 loss with no statistically significant difference ($p = 0.1531$) between CONT-3-10 and CONT-3-30 groups (Fig. 4.3 and Table 4.5). Hypothermia, unlike the modest protection in the 5 minute occluded groups, greatly attenuated CA1 loss against 3 minutes of ischemia at 10 ($p < 0.0001$) and 30 days ($p = 0.0004$). There was no decrement in effectiveness from 10 to 30 day survival ($p = 0.5219$). In agreement with this, phloxine staining revealed only a few acidophillic CA1 neurons in 2 of 9 HYPO-3-30 gerbils.

Day 3 open field scores (N = 62 per day) predicted days 7 ($r = 0.6101$, $p < 0.0001$) and 10 scores ($r = 0.4685$, $p = 0.0001$). Day 7 results were also significantly related to day 10 scores ($r = 0.7795$, $p < 0.0001$). Open field performance (days 3, 7 and 10; multiple regression) also predicted histological outcome in: anterior CA1 ($r = -0.5302$, $p = 0.0002$) and posterior CA1 ($r = -0.5406$, $p = 0.0002$). Finally, anterior CA1 counts were highly correlated with posterior CA1 ($r = 0.9668$, $p < 0.0001$).

4.3.2 *Experiment 2*

Brain temperature measured 2 days before ischemia was 36.34 ± 0.42°C, similar to the first experiment. Brain temperature during ($F[1,14] = 0.38$) and for the first hour after ischemia ($F[1,14] = 1.19$, $p = 0.2928$) was not significantly different between ischemic groups, and also similar to the previous experiment (Table 4.1). Postischemic temperature was regulated as desired in HYPO(2) gerbils (Fig. 4.4). Postischemic hyperthermia occurred in CONT(2) animals similar to experiment 1. This gradually

declined but remained above the temperature of SHAM(2) gerbils for the entire 26 hour monitoring period.

Ischemia (CONT(2)) caused profound CA1 loss (vs SHAM(2), $F[1,10] = 1303.96$, $p < 0.0001$, $\alpha = 0.025$) (Fig. 4.5). Postischemic hypothermia (HYPO(2)) provided consistent and robust CA1 preservation (vs CONT(2), $F[1,14] = 1114.64$, $p < 0.0001$, $\alpha = 0.025$) to a degree that HYPO(2) and SHAM(2) groups were not significantly different ($F[1,10] = 3.8$, $p = 0.0798$). Phloxine staining did not reveal any acidophillic CA1 neurons in HYPO(2) gerbils, suggesting persistent neuroprotection.

Table 4.1: Mean (± SD) brain temperature during "occlusion" and in the first hour of recirculation. Groups are classified according to treatment (HYPO, CONT or SHAM), duration of ischemia (**D**, 0, 3 or 5 min) and survival time (**S**, 10 or 30 days). See Tables 4.2 and 4.3 for statistical comparisons.

Treatment	D	S	"Occlusion"	First Hour
Experiment 1				
HYPO	5	10	36.13 ± 0.54	37.11 ± 0.31
		30	36.44 ± 0.63	37.03 ± 0.15
	3	10	36.83 ± 0.19	37.96 ± 0.68
		30	36.76 ± 0.21	37.68 ± 0.53
CONT	5	10	36.49 ± 0.45	37.16 ± 0.25
		30	36.21 ± 0.64	37.03 ± 0.25
	3	10	36.89 ± 0.12	37.49 ± 0.56
		30	36.66 ± 0.18	37.49 ± 0.57
Experiment 2				
HYPO(2)	5	30	36.33 ± 0.20	37.09 ± 0.19
CONT(2)	5	30	36.39 ± 0.15	37.25 ± 0.37
SHAM(2)	0	30	36.84 ± 0.18	37.14 ± 0.20

Table 4.2: Statistical comparisons for the mean occlusion temperature ($\alpha = 0.025$ for all main effects and interaction, and $\alpha = 0.05$ for all specific comparisons) (T = treatment, D = duration of ischemia, S = survival time, H = HYPO, C = CONT and, for example H510 refers to HYPO-5-10).

Comparison	df	*F*	*p*
T	1,54	0.04	0.8388
D	1,54	20.0	0.0000
S	1,54	0.42	0.5176
T x D	1,54	0.16	0.6863
T x S	1,54	3.33	0.0735
D x S	1,54	0.59	0.4442
T x D x S	1,54	1.01	0.3205
H510 vs H530	1,12	0.98	0.3406
H510 vs C510	1,12	1.85	0.1991
H530 vs C530	1,13	0.50	0.4915
H310 vs H330	1,15	0.41	0.5337
H310 vs C310	1,14	0.68	0.4246
H330 vs C330	1,15	1.23	0.2853
C510 vs C530	1,13	0.95	0.3473
C310 vs C330	1,14	9.43	0.0083

Table 4.3: Temperature comparisons for the mean first postischemic hour ($\alpha = 0.025$ for main effects and interactions and $\alpha = 0.05$ for all specific comparisons, except H510 vs H530 which is 0.025).

Comparison	df	*F*	*p*
T	1,54	3.90	0.0535
D	1,54	55.69	0.0000
S	1,54	2.60	0.1125
T x D	1,54	5.30	0.0253
T x S	1,54	0.52	0.4727
D x S	1,54	0.06	0.8079
T x D x S	1,54	1.18	0.2825
H510 vs H530	1,12	0.44	0.5206
H510 vs C510	1,12	0.13	0.7216
H530 vs C530	1,13	0.00	0.9785
H310 vs H330	1,15	2.82	0.1141
H310 vs C310	1,14	9.55	0.0080
H330 vs C330	1,15	1.00	0.3324
C510 vs C530	1,13	1.11	0.3123
C310 vs C330	1,14	0.00	0.9834

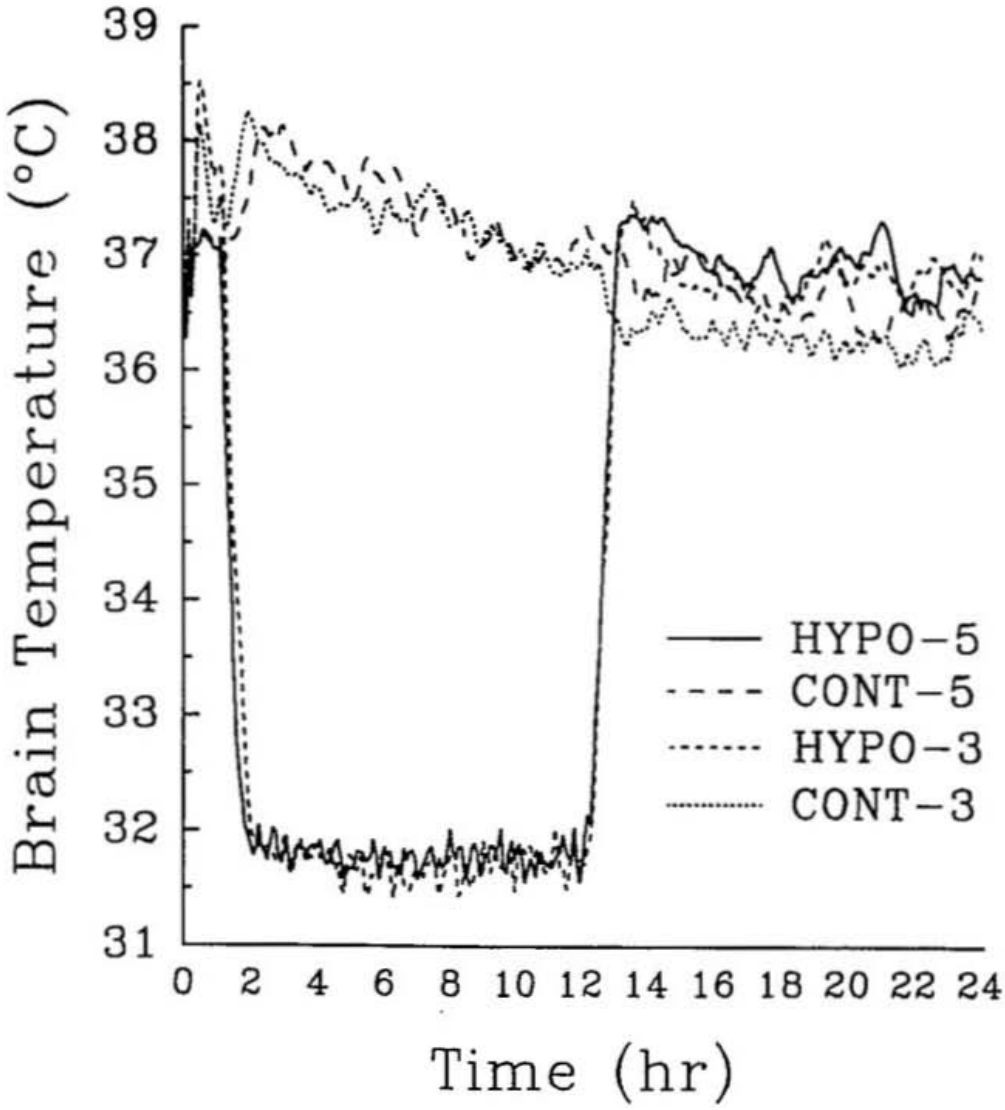
Table 4.4: Statistical outcome for the open field data (Fig. 4.2). Effects or specific comparisons involving treatment (T) or duration (D) factors have 1 and 58 df, except those involving the test day factor (O) where the df are 1.53 and 88.81 (Geisser-Greenhouse). These adjusted df were used because of a significant sphericity test ($p < 0.0001$). All comparisons have $\alpha = 0.05$ except for T(5) at days 7 and 10 where $\alpha = 0.025$. Note that numbers in parentheses refer to occlusion duration (i.e., 3 or 5 min).

Effect	*F*	*p*
T	12.93	0.0007
D	6.75	0.0119
T x D	0.00	0.9816
O	46.51	0.0000
O x T	3.02	0.0674
O x D	0.52	0.5474
O x T x D	0.07	0.8916
T(5) at day 3	7.02	0.0103
T(5) at day 7	2.48	0.1207
T(5) at day 10	3.08	0.0845
T(3) at day 3	423.16	0.0000
T(3) at day 7	2.15	0.1484
T(3) at day 10	5.00	0.0291
D at day 3	5.34	0.0244
D at day 7	2.46	0.1226
D at day 10	7.15	0.0097

Table 4.5: Statistical outcome for anterior CA1 cell counts (Fig. 4.3). All comparisons, except H310 vs H330 and C510 vs C530, have an $\alpha = 0.025$.

Effect	df	*F*	*p*
T	1,54	85.64	0.0000
D	1,54	53.66	0.0000
S	1,54	0.03	0.8571
T x D	1,54	22.49	0.0000
T x S	1,54	3.66	0.0612
D x S	1,54	6.19	0.0159
T x D x S	1,54	0.19	0.6609
H510 vs H530	1,12	4.61	0.0530
H510 vs C510	1,12	10.76	0.0066
H530 vs C530	1,13	15.87	0.0016
H310 vs H330	1,15	0.43	0.5219
H310 vs C310	1,14	99.25	0.0000
H330 vs C330	1,15	20.02	0.0004
C510 vs C530	1,13	0.57	0.4651
C310 vs C330	1,14	2.28	0.1531
C510 vs C310	1,13	5.79	0.0318
C530 vs C330	1,14	2.87	0.1124

Figure 4.1: Brain temperature during (0 hr) and after ischemia (24 hr) in HYPO-5, CONT-5, HYPO-3 and CONT-3 groups in experiment 1. Groups are collapsed over survival times for graphical simplicity and since these differences were negligible. Data are averaged every 5 minutes from the start of ischemia to 24 hours after occlusion. The occlusion time (at 0 hr) is an average of only 3 minutes in HYPO-3 and CONT-3 groups. Also see Table 4.1 for occlusion and first hour mean temperatures.


Brain Temperature (°C)
39
38
37
36
35
34
33
32
31
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (hr)
HYPO-5
CONT-5
HYPO-3
CONT-3

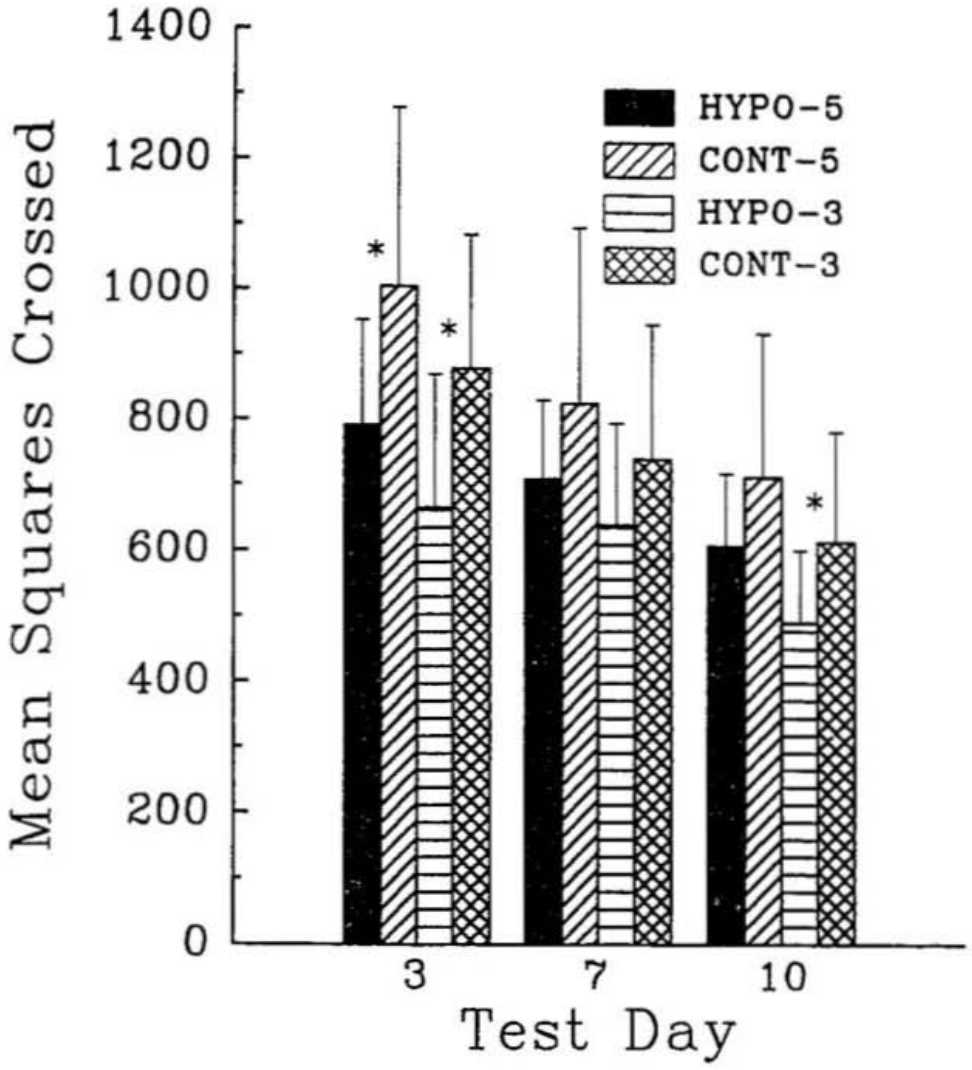


Figure 4.2: Open field activity scores (mean ± SD) for HYPO-5, CONT-5, HYPO-3 and CONT-3 groups on days 3, 7 and 10 after ischemia (experiment 1). Note that HYPO gerbils exhibited less exploration than their respective CONT animals (* denotes significant difference). In addition, animals occluded for 5 minutes show greater behavioral impairment than those occluded for 3 minutes.

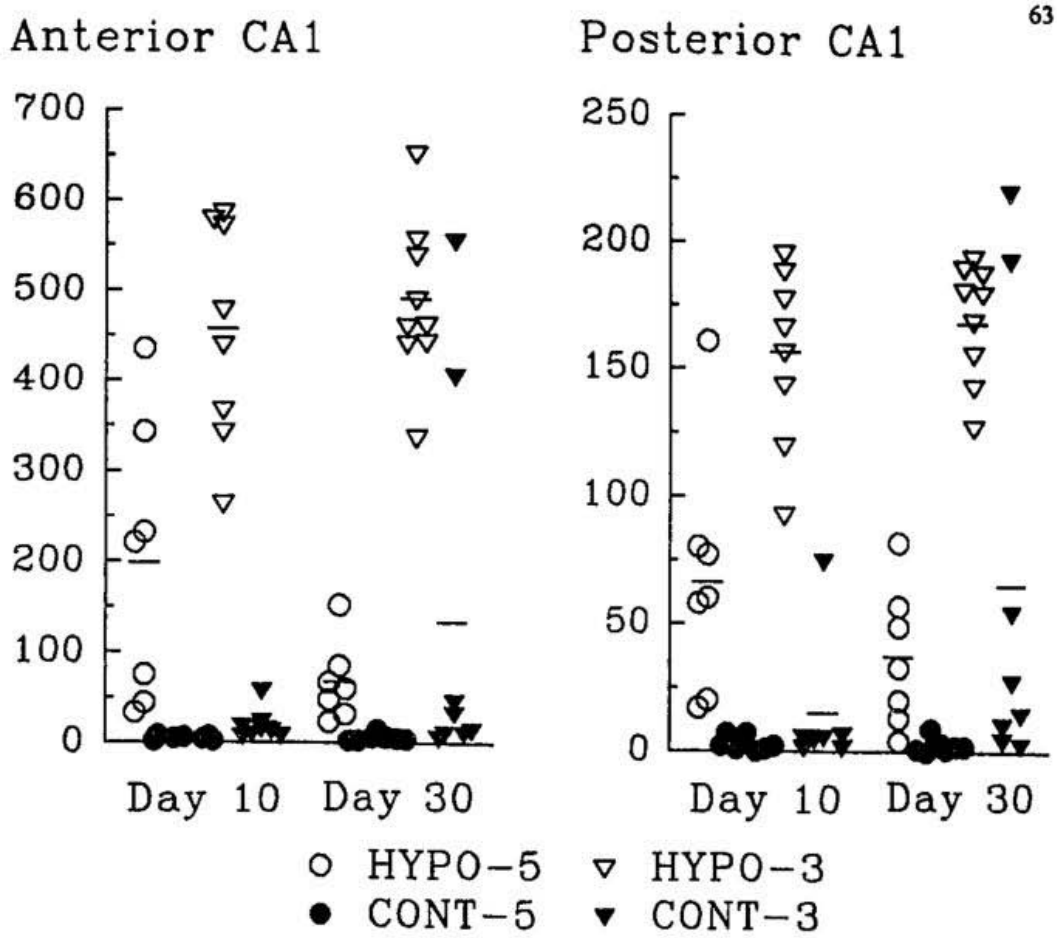


Figure 4.3: Hippocampal CA1 cell counts at -1.7 mm (anterior) and -2.2 mm (posterior) to bregma (experiment 1). Hypothermia significantly reduced CA1 loss against 5 minutes of ischemia with 10 and 30 day survival. Protection was better with 10 day survival, indicating progression of cell loss after 10 days. Hypothermia substantially reduced CA1 loss against 3 minutes of ischemia with 10 and 30 day survival. Protection against 3 minutes of ischemia did not decline from 10 to 30 days. Horizontal bars are group means. Sham operated gerbils, obtained from Colbourne and Corbett (1992) (N = 5), had 570.8 ± 39.75 SD and 184.4 ± 21.54 SD cells at the anterior and posterior levels, respectively.

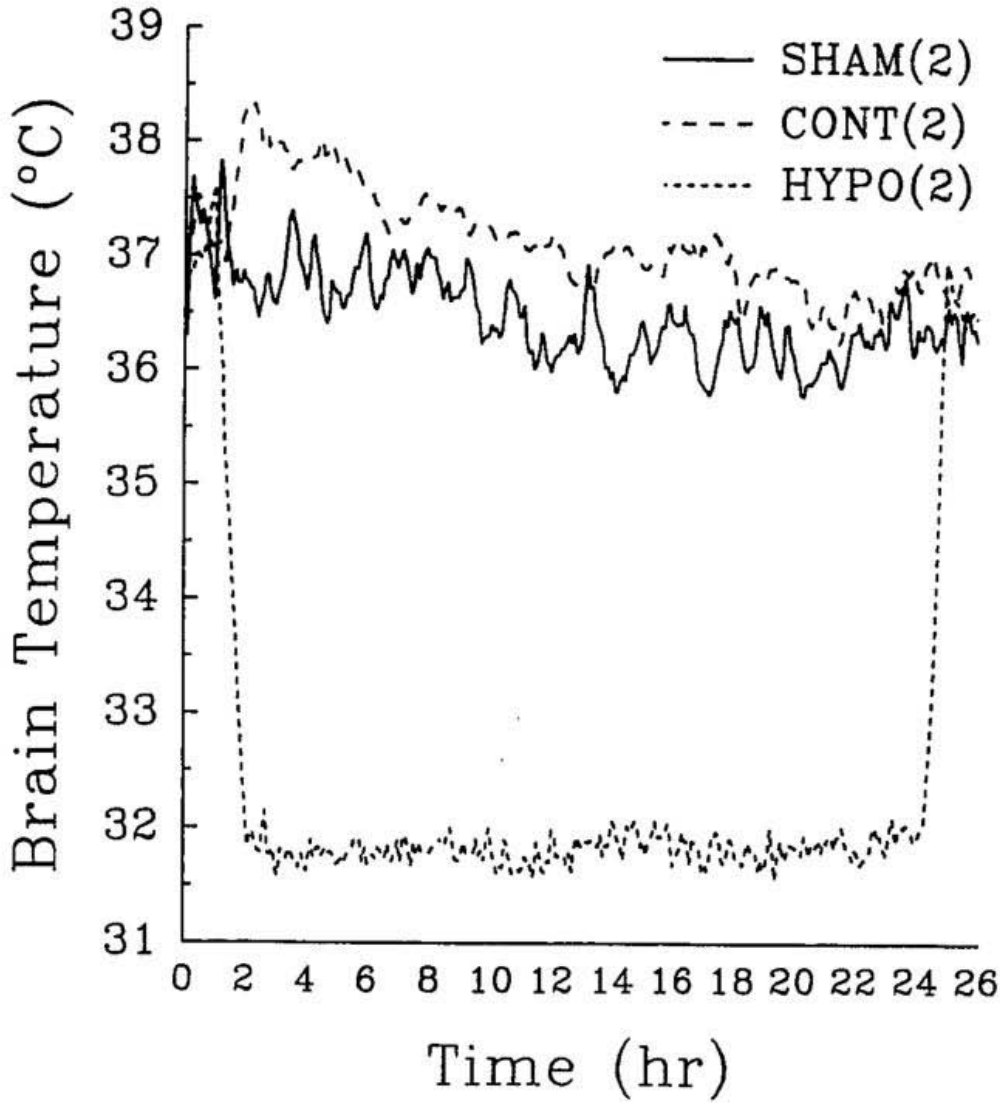


Figure 4.4: Brain temperature during (0 hr) and after ischemia (26 hr) in HYPO(2), CONT(2) and SHAM(2) groups in experiment 2. Data are averaged every 5 minutes from ischemia (at 0 hr) to 26 hours after occlusion. Also see Table 4.1 for occlusion and first hour mean temperatures.

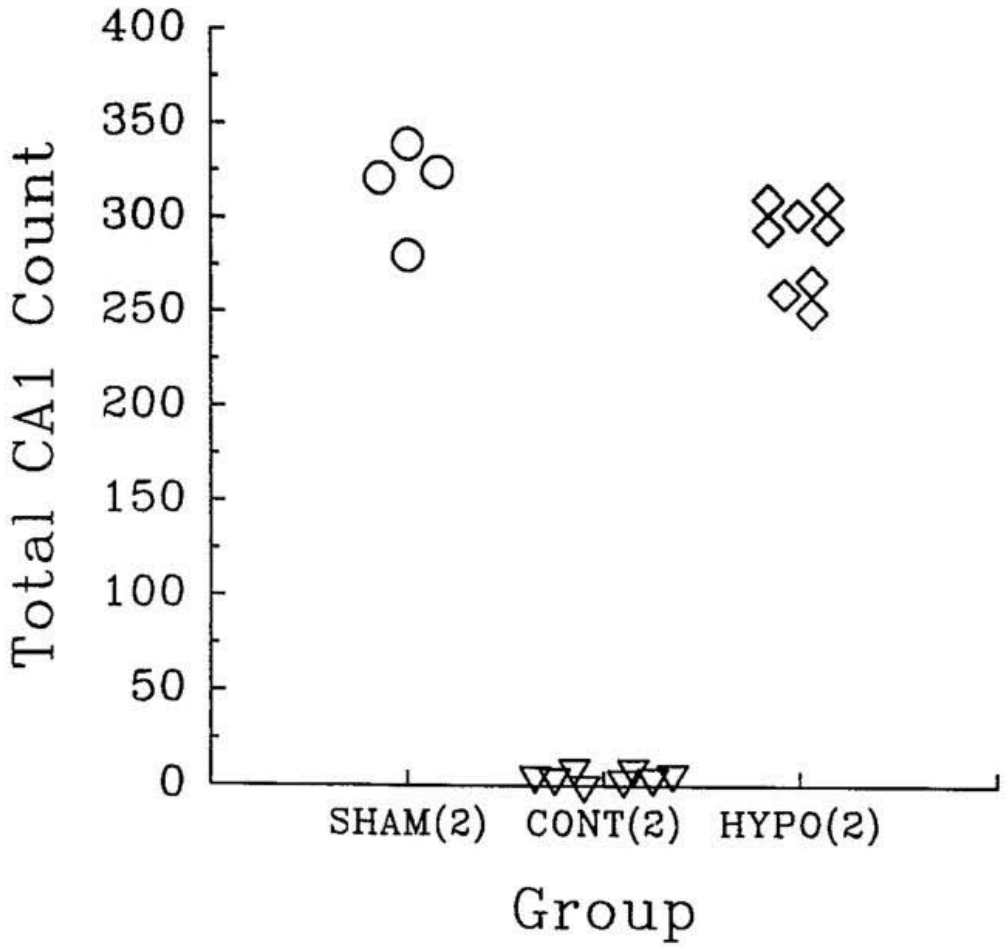


Figure 4.5: Hippocampal CA1 cell counts (-1.7 mm) in experiment 2. Twenty-four hours of postischemic hypothermia prevented CA1 necrosis when assessed 30 days after ischemia. Neuronal protection, at 30 days, was much greater with 24 hours of hypothermia (≈ 90%) than with the 12 hour duration (≈ 15%) used in experiment 1 (see Fig. 4.3).

4.4 Conclusions

Twelve hours of delayed postischemic hypothermia significantly reduced anterior and posterior CA1 loss against 5 minutes of ischemia following both 10 and 30 day survival. However, hypothermia may have delayed some CA1 loss in HYPO-5 gerbils since cell counts at 30 days were somewhat less than those observed with 10 day survival. In addition, phloxine staining suggested that either there was recent cell loss or that necrosis would progress if the HYPO-5-30 gerbils survived longer. This same hypothermic treatment was much more effective against 3 minutes of ischemia where CA1 loss was substantially reduced with no decrement in effectiveness over survival days. Absence of significant phloxine staining in HYPO-3-30 gerbils further suggests that this protection was permanent or very long lasting.

In experiment 2, 24 hours of postischemic hypothermia provided much greater neuroprotection (≈ 90%) against a 5 minute ischemic episode than the 12 hour duration (≈ 15%) with 30 day survival. Since brain temperature during and for the first hour after ischemia was similar in both experiments we can conclude that 24 hours of hypothermia is much more effective than 12 hours.

In conjunction with reduced hippocampal damage (experiment 1), HYPO groups also displayed less exploration on all open field test days. Nonetheless, both HYPO and CONT groups showed habituation over test days. All animals may eventually habituate to this simple test because the entire hippocampus is not damaged (i.e., ventral CA1) and

other systems undoubtedly facilitate recovery of habituation. Additional behavioral testing at later postischemic times is required in order to demonstrate permanent functional protection since postischemic hypothermia may delay cell loss. This could result in short term behavioral protection that dissipates as necrosis progresses. However, as expected of a hippocampal dependent task, open field test scores (days 3, 7 and 10) predicted histological outcome in CA1. In addition, open field scores about equally predicted CA1 histological outcome in both 10 and 30 day survival groups ($r \approx -0.53$). This suggests that either there was minimal progression of cell loss from 10 to 30 days and/or more likely, behavioral impairments occurred early and preceded gross histological injury. For example, we (Colbourne and Corbett, 1992) have observed open field impairments as early as 1 day following brief ischemia (3 min) which is at a time when only minimal CA1 loss has occurred.

One important issue raised by the present findings and by Dietrich and collaborators (1993) is the importance of prolonged survival time to test for permanent protection. While we noted a suggestion of prolonged DND from 10 to 30 days after 5 minutes of ischemia in experiment 1 it may not have been significant because most cell loss may have occurred prior to day 10 (i.e., 3 to 10 days after ischemia) and was no longer apparent by 10 to 30 days. Additional cell loss could also have occurred after 30 days if the animals survived longer. Pilot data (unpublished observations) with less than 6 hours of hypothermia showed no benefit (at 10 days), while 12 hours (Experiment 1) provided mild and 24 hours (Experiment 2) provided almost complete CA1 protection.

Therefore, if the duration of hypothermia is too brief (e.g., 1 - 3 hr) one might simply delay cell loss, or see no benefit at all as in the studies by Busto and colleagues (Busto et al., 1989; Dietrich et al., 1994) and Welsh and Harris (1991). More severe ischemia (5 min in gerbils) requires a longer hypothermic period (24 hr) to prolong cell survival. Similarly, 2 hours of immediate postischemic hypothermia reduced CA1 loss (7 day survival) against 8 but not 12 minutes of ischemia in the rat 2-VO model (Chopp et al., 1991).

Another important question is whether hypothermia is neuroprotective when delayed for several hours after an ischemic episode. This protection could depend critically on ischemic severity and survival time assessment. For example, hypothermia, with greater delays, may only "protect" against mild ischemia. However, longer duration hypothermia, as used in the present study, may expand this time window. Thus, careful evaluation with longer hypothermic periods and prolonged survival times is essential.

This study also shows a prolonged ischemia-induced hyperthermic period (Fig. 4.4). Kuroiwa et al. (1990) showed that prevention of postischemic rectal and skull hyperthermia (with Halothane) within the first 85 minutes after ischemia greatly attenuated CA1 loss. Their data must be qualified, however, since we (Chapter 3) found that the use of extended Halothane anesthesia to maintain rectal and skull normothermia may, in fact, cause mild brain hypothermia which reduces CA1 loss. Five minutes of ischemia with postischemic brain normothermia (85 min) did not reduce CA1 loss. While it is likely that protracted hyperthermia is detrimental, especially against mild ischemia, it

is not the primary cause of CA1 loss following severe (5 min) ischemia. For example, 9 ischemic control gerbils (5 min) selected from Experiments 1 and 2 with a similar postischemic temperature profile (36.87°C; average of complete postischemic monitoring period) to SHAMs (36.48°C) had complete CA1 loss. Thus, it is highly unlikely that the hypothermic protection observed in Experiments 1 and 2 was simply due to prevention of postischemic hyperthermia. After all, a temperature of 36.87°C is within the normal range. This does, however, raise the issue of degree of hypothermia. For example, would 34°C hypothermia be as neuroprotective as 32°C?

Even though brain temperature was carefully regulated there were some notable group differences in experiment 1. First, animals exposed to 3 minutes of ischemia had a mean occlusion temperature that was slightly higher (≈ 0.5°C) than those occluded for 5 minutes. Brain temperature was more difficult to maintain during 5 minutes of ischemia since it tended to decline as a function of ischemia duration even though both the body and head were actively heated. Second, CONT-3-10 gerbils were maintained slightly warmer (0.23°C) during ischemia than the CONT-3-30 group. However, these groups were not significantly different in histological outcome. Third, all HYPO-3 and CONT-3 groups were consistently warmer than HYPO-5 and CONT-5 groups in the first hour of recirculation. Three minute occluded gerbils did recover (i.e., righted, but sat hunched and inactive) faster than those occluded for 5 minutes, but there were no obvious activity differences, within the first hour, that accounted for the different temperature profiles (unpublished observations). One possibility is that the decoupling of oxidative

metabolism from protein synthesis could thermodynamically lead to heat production. Protein synthesis is depressed for many hours after ischemia while oxidative metabolism recovers much sooner (within minutes) (section 1.3). Ischemia of 3 and 5 minute durations may differentially affect the recovery of these processes (faster recovery of oxidative metabolism with briefer ischemia) and lead to greater immediate hyperthermia in the 3 minute groups. After about 1 hour all gerbils show prolonged increases in activity and these seem highly correlated (positive) with brain temperature (unpublished data). Thus, the postischemic hyperthermia that occurred after 1 hour in all CONT groups may be partially due to this hyperactivity. Fourth, HYPO-3-10 gerbils displayed more postischemic hyperthermia (first hour) than the CONT-3-10 group. Regardless, the greater postischemic rise in the HYPO group would only, at worse, reduce the beneficial effect observed in the present study. Finally, normal temperature was lower in these 2 experiments than in previous work (sections 2.3 and 3.3). This apparent drop in normal temperature (0.5°C) is probably because this study measured striatal temperature while the former assessed cortical temperature. The slight difference could also be due to improved calibration.

In summary, prolonged hypothermia (12 hr) initiated 1 hour after either 3 or 5 minutes of normothermic ischemia reduced CA1 loss and the associated habituation impairment in the gerbil. Protection against 3 minutes of ischemia was robust and persistent while neuroprotection against a 5 minute insult appeared to result from a combination of true neuronal preservation with perhaps some delay of eventual cell

necrosis. Importantly, experiment 2 showed that 24 hours of hypothermia provided much more robust and persistent CA1 savings against 5 minutes of ischemia, indicating the usefulness of protracted hypothermic durations.

Chapter 5 (Hypothermia and Functional Outcome)

5.1 Introduction

While the previous data are encouraging, further work is needed to clarify the efficacy of postischemic hypothermia. Perhaps the most controversial issue is whether CA1 neurons are permanently saved. Dietrich and colleagues (1993) only briefly rescued CA1 neurons with 3 hours of immediate postischemic hypothermia. We too (section 4.3) found somewhat transient protection when 12 hours of cooling was initiated 1 hour following severe ischemia (5 min occlusion in gerbil). However, hypothermia was more effective against milder ischemia (3 min occlusion) and a longer hypothermic period (24 hr) provided greater and more persistent (30 day) CA1 benefit against a severe insult (5 min). It appears that the degree and permanence of CA1 protection critically depends on the ischemic severity and the duration of hypothermia. Nonetheless, it is not yet known if hypothermia, even when protracted, is indeed able to permanently salvage CA1 neurons since cell loss may continue beyond 30 days postischemia.

Functional outcome is another unresolved endpoint of paramount importance since one should not rely solely on histology to predict behavioral performance (section 1.2.3). Delayed hypothermia (section 4.3.1) did reduce early habituation impairments (days 3, 7 and 10 postischemia) in the gerbil. However, it is unknown whether the functional deficits in untreated animals would show complete recovery with extended survival, and if not, would postischemic hypothermia attenuate such impairments. Thus,

long-term functional assessment is necessary.

Finally, two important parametric questions, indubitably entwined with the issue of permanence, are the degree and therapeutic onset of hypothermia that are most effective. Since life-threatening complications (e.g., cardiac arrhythmias) occur more often at temperatures below 30°C it is reasonable to limit study to the 32 - 35°C hypothermic range. With respect to therapeutic window, it is likely that greater benefit will arise with quicker intervention. This was evident in early studies (Benson et al., 1959) and in recent work (Carroll and Beek, 1992; Coimbra and Wieloch, 1994). While protracted hypothermia (24 hr) may extend this window, its limits are unknown.

In this study (Colbourne and Corbett, 1994, submitted), we assessed whether 24 hours of hypothermia (32°C) initiated 1 hour after a severe 5 minute stroke in gerbil would reduce CA1 loss with 6 month survival. Exploration in an open field was determined on days 5, 10, 30 and 180 postischemia. Acquisition of a win-shift strategy in a T-maze was measured at 1, 3 and 5 months, while working memory delays were assessed subsequent to each acquisition. Finally, the number of gerbils to learn a win-win rule was determined. Several other groups were included to better characterize hypothermic protection. These were: a 4 hour delayed hypothermic treatment (24 hr at 32°C), a 34°C hypothermic group (from 1 - 25 hr) and a postischemic stress group.

5.2 Methods

5.2.1 *Subjects*

Fifty-one female, gerbils were included in this study. Three died during ischemia and one CONT gerbil was excluded because of spontaneous postischemic hypothermia (due to illness). Groups were: SHAM (N = 10; 6 month survival), CONT (N = 14; 6 month survival), HYPO(1-25) (N = 9; 6 month survival), HYPO(4-28) (N = 8; 6 month survival), HYPO-34 (N = 5; 30 day survival) and STRESS (N = 5; 10 day survival). Animals were approximately 15 weeks old and weighed about 54 g at the time of ischemia.

5.2.2 *Temperature Monitoring*

Rectal and brain temperature measurement/control was similar to previous experiments (section 4.2).

5.2.3 *Cerebral Ischemia*

Ischemia surgical procedures were identical to those in section 4.2. Briefly, gerbils were subjected to 5 minutes of ischemia or sham operation under Halothane anesthesia 2 days following normal temperature measurement. Following anesthesia, gerbils recovered in individual boxes resting on telemetry receivers. Gerbils were warmed with an overhead lamp if their brain temperature fell below 37.0°C during the

first postischemic hour.

Most gerbils were shaved at 1 hour after ischemia and then returned to their boxes. Brain temperature was monitored until 26 hours postischemia in sham operated (SHAM) and ischemic gerbils (no treatment; CONT). Hypothermic groups (HYPO(1-25) and HYPO-34) were cooled beginning 1 hour after ischemia to 32 and 34°C, respectively. These groups were later warmed to 37°C at 25 hours post occlusion and then monitored for 1 more hour. Another group (HYPO(4-28)) was shaved 4 hours after ischemia and then subjected to 24 hours of hypothermia (32°C). These animals were monitored until 29 hours postischemia. The final group (STRESS) was subjected to a similar procedure as HYPO(1-25) gerbils (intermittent water spray and fan), but was not cooled (temperature was maintained similar to CONT gerbils by a periodic warm air supply and a lamp).

5.2.4 *Open Field Testing*

Gerbils were placed in a novel open field (section 4.2.4) for ten minutes on the fifth postischemic day. Thereafter, depending on survival time, retesting occurred on days 10, 30 and 180. An image tracking system recorded the number of squares crossed per minute.

Unpublished open field data (Experiment 2 from Chapter 4; SHAM (N = 4), CONT (N = 8) and HYPO(1-25) (N = 8)) were included in the present analysis to increase the statistical power. These groups were very similar (temperature profiles, age,

weight and open field scores) to the present groups, except they were only tested on days 5, 10 and 30. Inclusion of this older data was deemed acceptable based upon these similarities and the fact that both experiments were within a 6 month period.

5.2.5 *T-maze Testing*

Only SHAM, CONT and HYPO(1-25) gerbils were tested in the T-maze. The other groups were not tested either because of their survival time or to limit the already extensive amount of testing. The maze measured 47 cm (stem) by 30 cm (each arm) by 10 cm wide. Extra-maze cues (window, lighting, experimenter) remained constant throughout testing. Gerbils were not food deprived, but were instead given a preferred treat (1/2 sunflower seed) for correct responses. To facilitate training, sunflower seeds were removed from the regular diet 27 days after surgery. Gerbils were habituated to the T-maze on days 31, 32 and 33 (2 x 5 min per day). During habituation seeds were initially spread over the entire maze floor, but were then progressively localized to the reward cups.

Training began on day 34 and consisted of 10 pairs of forced (FT) and choice (CT) trials. Gerbils were sequentially run in groups of 4 or 5. On the FT, gerbils were randomly forced into either the right or left arm by a door blocking the opposite arm. There were 5 right and 5 left-turn FTs per day. Once the gerbil entered the forced arm and received a reward, it was captured by a sliding door. Gerbils were allowed 15 seconds (5 sec minimum) to eat this reward before being returned to the start area.

Fifteen seconds following the FT response the start box door was opened and gerbils were allowed to enter either of the choice arms. Gerbils received another reward only if they entered the opposite arm (WIN-SHIFT strategy). Gerbils were trained to criterion, which was ≥ 80% correct over 3 consecutive days (5 day minimum). One SHAM gerbil was excluded because it completed < 25 of the first 50 trials. Once animals reached criterion, delays were imposed between the FT and CT. Delay testing consisted of consecutive 1, 2, 3, 5 and 0 minute delays over the following 5 days (only one time per day). During these delays animals remained in the start area. A 0 minute day (same as training) was given after the 5 minute delay day to test whether delay testing would cause animals to forget the win-shift rule. Thus, good performance on this 0 min day would ensure that any prior delay impairment was due to an impaired working memory and not because of unlearning the rule.

Training was repeated at 3 and 5 months postischemia (minimum of 3 days). Delay testing was 5 and 0 minutes at 3 months and 1, 2, 3, 5 and 0 minutes at 5 months. One CONT gerbil was excluded from further T-maze training after the first phase because of the tendency to bite the experimenter.

At the end of the 5 month testing (0 min delay day) another 2 training days were given. This ensured both high accuracy and similar performance among the 3 groups. Training was then changed to a WIN-WIN strategy. Gerbils had to re-enter the FT arm to get rewarded. Gerbils were trained up to a maximum of 250 trials (10 per day).

The T-maze was chosen because it allowed for repeated memory assessment over

the course of several months. Importantly, this maze also readily permitted manipulations (i.e., delays, rule change) aimed at detecting more subtle differences in hippocampal function (e.g., due to mild protection and/or recovery processes).

5.2.6 *Histology*

Gerbils were sacrificed with an overdose of Somnotol and then perfused with 15 ml of heparinized saline followed by 50 ml of 10% phosphate-buffered formalin. Brains were later embedded in paraffin, sectioned at 6 microns and stained with haematoxylin and eosin. This method produced higher quality sections than the previous frozen technique. Viable looking neurons (distinct cell membrane and nucleus; not eosinophilic) were counted in medial, middle and lateral sectors (each 0.2 mm long) of CA1 at -1.7 mm to bregma (Loskoto et al., 1975). Counts were summed over left and right hemispheres and expressed as a percent of normal (i.e., SHAM).

Similarly, viable neurons were counted in the medial, middle and lateral sectors of dorsal CA1 at 2.2 mm posterior to bregma. A middle CA1 sector (at apex of pyramidal cell layer) was also assessed at 2.8 mm posterior to bregma. Bilateral ventral CA1 sectors were counted at these levels.

5.2.7 *Statistics*

Statistical procedures were similar to previous work (section 4.2.8).

Open field data and T-maze delay data (1 and 5 months) were analyzed with

mixed factorial ANOVAs for each test period. The number of trials to reach criterion and the mean temperature data were analyzed with between group ANOVAs. The number of gerbils that acquired the WIN-WIN strategy was examined by Fisher exact tests.

The CA1 data (% normal) were analyzed with 1 factor ANOVAs for each of the medial, middle and lateral sectors (6 month survival groups). The percent protection in HYPO(1-25) and HYPO-34 gerbils (medial, middle and lateral sectors) were compared (t-test) to the 30 day survival hypothermic (32°C) group (Experiment 2 in Chapter 4). Finally, CA1 sector comparisons were performed via paired t-tests with the above α criterion. Only the most important statistical data for the -2.2 and -2.8 mm histology data are given since it was similar to that of the -1.7 mm level.

Data are given as the mean ± SD.

5.3 Results

Normal brain temperature was 36.22°C ± 0.30 SD with no significant differences among groups ($F[5,45] = 0.14$).

Brain temperature during ischemia/sham occlusion was maintained close to normal (Table 5.1 and Fig. 5.1). There were no significant differences between ischemic groups during occlusion ($F[1,45] < 1$) or in the first postischemic hour (t-tests, $p \geq 0.1631$). Ischemic CONT gerbils showed a mild, but prolonged spontaneous hyperthermia for the entire monitoring period as previously noted (Chapter 4). This was mimicked in the STRESS group. The three hypothermic groups were regulated to the desired temperatures (i.e., 32 or 34°C).

Both ischemia and hypothermia affected body weight. Ischemia (CONT and STRESS) induced an approximate 4 g weight loss by the first postischemic day, while hypothermia (32°C) produced about an 8 g loss (HYPO(1-25) and HYPO(4-28)). The HYPO-34 group lost approximately 5 g. By the fifth postischemic day, when open field testing started, gerbils had regained most or all of their pre-ischemic weight and the groups were similar from then on. Hypothermia did not produce any other noticeable side effects.

Ischemic CONT gerbils explored more than SHAM gerbils on all test days ($p \leq 0.0001$) (Fig. 5.2 and Tables 5.2 to 5.5). In addition, the pattern of habituation (Group X Min interaction) was often different, especially on the first test day ($p < 0.0001$). Here,

the CONT group showed an elevated and persistent activity level, while SHAM animals habituated quickly. The STRESS procedure did not alter behavioral outcome as these gerbils were significantly impaired (vs SHAM; $p \leq 0.0007$), and not statistically different from CONT gerbils ($p > 0.7$). Thus, untreated ischemia produced very persistent habituation impairments as revealed by simple open field testing.

Hypothermia (HYPO(1-25)) significantly reduced ischemic impairments on all test days (vs CONT; $p \leq 0.0171$). The HYPO(1-25) gerbils did, however, explore significantly more than SHAM gerbils on days 5 ($p = 0.0363$) and 10 ($p = 0.0057$), but not quite at 30 ($p = 0.0516$) and 180 days ($p = 0.0836$). Hypothermia (34°C) also reduced ischemic impairments (vs CONT), but this was only significant on days 5 ($p = 0.02$) and 10 ($p = 0.01$) and not day 30 ($p = 0.1337$). In general, the HYPO(1-25) and HYPO-34 groups performed similarly. Thus, hypothermia introduced 1 hour after ischemia provided persistent functional benefit. When treatment was delayed until 4 hours after occlusion (HYPO(4-28)) protection was only observed on day 180 ($p = 0.008$) and not on days 5, 10 and 30 ($p \geq 0.4266$).

The number of trials to criterion (Fig. 5.3 and Table 5.6) in the T-maze (win-shift) revealed that CONT gerbils were significantly slower on initial learning than both SHAM ($p = 0.0088$) and HYPO(1-25) groups ($p = 0.0062$). Retraining at 3 ($p = 0.03$) and 5 months postischemia ($p = 0.0775$) also revealed somewhat slower learning in the CONT group (vs SHAM), but these were not significant. There were no significant differences between HYPO(1-25) and SHAM gerbils at either 1, 3 or 5 months ($p \geq 0.077$). Finally,

HYPO(1-25) and CONT groups were similar at 3 and 5 months ($p \geq 0.3633$). Thus, ischemia caused an initial acquisition impairment that recovered with repeated testing. Postischemic hypothermia decreased this impairment.

Performance on the delay tests (1, 2, 3 and 5 min) was variable at 1 month (Fig. 5.4). Overall, CONT gerbils showed greater impairments over delays than the SHAM group ($F[1,26] = 8.54, p = 0.0071$). The HYPO(1-25) group was not significantly different than either the CONT ($F[1,26] = 3, p = 0.0954$) or SHAM group ($F[1,26] = 1.29, p = 0.2665$). However, there was a trend for a progressively greater impairment in CONT animals (vs SHAM), which HYPO(1-25) reduced. Delay testing at 3 and 5 months was quite variable and did not show significant group differences (statistics not shown). In addition, the three groups performed equally well (≈ 95% correct) on the final three training days (no delay) which were given just after the 5 minute delay at 5+ months ($F[2,27] = 0.17, p = 0.8483$). Thus, there was a substantial functional recovery in the T-maze (win-shift).

Switching the rule to win-win did reveal group differences (Fig. 5.5). Eight of 9 SHAM gerbils learned this strategy by 250 trials, while only 4 of 9 HYPO(1-25) gerbils and no CONT gerbil reached criterion. Thus, untreated ischemia resulted in a long-lasting learning impairment ($p < 0.0001$) in the T-maze that was previously not evident with repeated win-shift training. The HYPO(1-25) group had significantly reduced impairments (vs CONT, $p = 0.0211$). Importantly, the HYPO(1-25) group performance was not quite as good as normal animals (vs SHAM, $p = 0.0656$). During the first 2

weeks of win-win training the three groups performed similarly. At that time all groups performed at chance levels. SHAM, and to a lesser extent HYPO(1-25) gerbils, then progressively learned while CONT animals tended to persevere at 50 to 60% correct. Finally, the latencies to complete the FT and CT in the T-maze were similar among the 3 groups over all testing times (data not shown), thus indicating that the observed differences were not due to motor impairment or differences in motivation.

Five minutes of normothermic ischemia (CONT) induced severe cell loss (≈ 97% loss) in the medial, middle and lateral CA1 sectors (-1.7 mm, Fig. 5.6 and Table 5.7). This was not altered by the STRESS procedure. Previously (section 4.3.2), postischemic hypothermia (from 1 - 25 hr at 32°C) was found to significantly attenuate this loss with 30 day survival in the medial, middle and lateral sectors (-1.7 mm, 87.21 ± 14.29, 91.27 ± 3.65 and 93.57 ± 7.33 SD % of normal, respectively) with no significant sector differences. We now find robust protection (vs CONT) in all sectors with 6 month survival ($p < 0.0001$). However, unlike at 30 days, protection was regional, with greater savings in the middle (vs medial, $t_8 = 3.75$, $p = 0.0056$) and lateral sectors (vs medial, $t_8 = 5.82$, $p = 0.0004$; vs middle $t_8 = 2.31$, $p = 0.0494$). There was also a significant decline in CA1 savings from 30 to 180 day survival in the medial ($t_{15} = 3.35$, $p = 0.0044$, $\alpha = 0.025$), but not the middle ($t_{15} = 1.31$, $p = 0.2074$, $\alpha = 0.025$) or lateral sectors ($t_{15} = 0.63$). A mild, but significant CA1 savings ($p \leq 0.0015$) even occurred when hypothermia was started 4 hours postischemia (HYPO(4-28)) with no region effect. However, this was significantly less than the HYPO(1-25) group ($p \leq 0.0018$). Milder hypothermia (HYPO-

34) initiated at 1 hour postischemia was also significantly protective at 30 day survival ($t_{17} \geq 6.11, p < 0.0001, \alpha = 0.025$). However, 34°C cooling was not quite as effective as 32°C (30 days). This was significant in medial CA1 ($t_{11} = 4.34, p = 0.0012$), but not in middle ($t_{11} = 2.14, p = 0.0557, \alpha = 0.025$) or lateral CA1 ($t_{11} = 2.09, p = 0.0601, \alpha = 0.025$). Furthermore, medial CA1 was significantly less protected than the middle ($t_4 = 5.26, p = 0.0062$) and lateral CA1 ($t_4 = 8.8, p = 0.0009$) in the HYPO-34 group. Protection in the middle and lateral sectors were not significantly different.

A similar trend occurred in dorsal CA1 (medial, middle and lateral sectors) at -2.2 mm to bregma (Fig. 5.7), except hypothermic protection was even more pronounced. In medial CA1 both the HYPO(1-25) and HYPO-34 treatments were more effective than at the anterior CA1 level. In addition, CA1 protection did not significantly decline in any sector at this level from 30 (Experiment 2, Chapter 4) to 180 day survival with the HYPO(1-25) treatment ($t_{15} \leq 1.96, p \geq 0.0685, \alpha = 0.025$). Protection was also better in the HYPO(4-28) group than at -1.7 mm. Even though protection with 34°C cooling was better than at -1.7 mm, this group was still significantly less protected (medial and middle CA1) than with 32°C hypothermia at 1 month ($t_{11} \geq 5.87, p \leq 0.0001$).

Damage at -2.8 mm to bregma (Fig. 5.8) in the CONT and STRESS groups was somewhat less than at the more anterior levels. All three hypothermic groups were significantly ($p < 0.0001$) protected (vs CONT). The percentage of remaining CA1 neurons was approximately that of the preceding level for the HYPO(1-25) group. While there was about 20% more protection in the HYPO-34 group, protection was still

significantly less than that of the 30 day survival, 32°C group ($t_{11} = 2.28$, $p = 0.0436$). Notably, the HYPO(4-28) treatment rescued a similar percentage of neurons (40%) at the -2.2 and -2.8 mm to bregma levels.

There was only minimal (≈ 10%) to no damage in the ventral CA1 regions at -2.2 and -2.8 mm and therefore, the effects of hypothermia was negligible (data not shown).

Day 5 ($r = -0.614$, $p < 0.0001$), 10 ($r = -0.688$, $p < 0.0001$), 30 ($r = -0.516$, $p = 0.0006$) and 180 ($r = -0.432$, $p = 0.0053$) open field scores (sum of 10 min sessions) significantly correlated with histological outcome at 6 months (total % normal at -1.7 mm). The number of trials to reach criterion (1+ month) also significantly predicted cell counts (-1.7 mm, $r = -0.527$, $p = 0.0019$). Finally, behavioral scores were inter-correlated, especially the day 5, 10, 30 and 180 open field scores and the 1 month T-maze acquisition data ($r = 0.424$ to 0.8333, $p \leq 0.0157$).

Table 5.1: Mean (± SD) brain temperature during ischemia/sham occlusion and in the first postischemic hour. Ischemic groups were not statistically different.

Treatment	"Occlusion"	First Hour
SHAM	36.63 ± 0.18	37.30 ± 0.25
CONT	36.43 ± 0.21	37.10 ± 0.35
STRESS	36.40 ± 0.14	37.03 ± 0.16
HYPO(1-25)	36.38 ± 0.17	36.98 ± 0.07
HYPO-34	36.34 ± 0.15	36.99 ± 0.26
HYPO(4-28)	36.43 ± 0.20	36.92 ± 0.11

Table 5.2: Statistics for the open field test on day 5 (Fig. 5.2). The group main effect has 5,64 df, while the minute comparisons have 4.73,302.48 df and the interactions have 23.63,302.48 df. Specific contrasts have 1,64 df. All comparisons have an $\alpha = 0.05$, except HYPO-34 vs CONT which is 0.025.

Comparison	*F*	*p*
Group	7.15	0.0000
Minute	12.28	0.0000
Group x Minute	5.58	0.0000
SHAM vs CONT	28.36	0.0000
SHAM vs CONT (Min)	15.00	0.0000
STRESS vs SHAM	12.74	0.0007
STRESS vs CONT	0.01	0.9374
HYPO(1-25) vs SHAM	4.58	0.0363
HYPO(1-25) vs CONT	10.54	0.0019
HYPO-34 vs SHAM	0.85	0.3595
HYPO-34 vs CONT	5.7	0.0200
HYPO(4-28) vs SHAM	11.61	0.0011
HYPO(4-28) vs CONT	0.56	0.4554

Table 5.3: Statistics for the open field test on day 10 (Fig. 5.2). The group main effect has 5,65 df, while the minute comparisons have 4.95,321.73 df and the interaction have 24.75,321.73 df. Specific contrasts have 1,65 df. All comparisons have an $\alpha = 0.05$.

Comparison	*F*	*p*
Group	10.29	0.0000
Minute	64.16	0.0000
Group x Minute	1.27	0.1778
SHAM vs CONT	40.71	0.0000
SHAM vs CONT (Min)	0.67	0.6439
STRESS vs SHAM	14.84	0.0003
STRESS vs CONT	0.12	0.7262
HYPO(1-25) vs SHAM	8.18	0.0057
HYPO(1-25) vs CONT	12.66	0.0007
HYPO-34 vs SHAM	2.76	0.1014
HYPO-34 vs CONT	7.05	0.0100
HYPO(4-28) vs SHAM	24.53	0.0000
HYPO(4-28) vs CONT	0.00	0.9733

Table 5.4: Statistics for the open field test on day 30 (Fig. 5.2). The group main effect has 4,61 df, while the minute comparisons have 6.03,367.73 df and the interactions have 24.11,367.73 df. Specific contrasts have 1,61 df. All comparisons have an $\alpha = 0.05$.

Comparison	*F*	*p*
Group	5.47	0.0008
Minute	82.77	0.0000
Group x Minute	1.74	0.0178
SHAM vs CONT	20.40	0.0000
SHAM vs CONT (Min)	2.07	0.0557
HYPO(1-25) vs SHAM	3.94	0.0516
HYPO(1-25) vs CONT	6.57	0.0129
HYPO-34 vs SHAM	2.31	0.1340
HYPO-34 vs CONT	2.31	0.1337
HYPO(4-28) vs SHAM	7.50	0.0081
HYPO(4-28) vs CONT	0.64	0.4266

Table 5.5: Statistics for the open field test on day 180 (Fig. 5.2). The group main effect has 3,36 df, while the minute comparisons have 4.33,155.78 df and the interactions have 12.98,155.78 df. Specific contrasts have 1,36 df. All comparisons have an $\alpha = 0.05$.

Comparison	*F*	*p*
Group	7.23	0.0006
Minute	70.36	0.0000
Group x Minute	1.87	0.0370
SHAM vs CONT	20.43	0.0001
SHAM vs CONT (Min)	3.16	0.0134
HYPO(1-25) vs SHAM	3.17	0.0836
HYPO(1-25) vs CONT	6.25	0.0171
HYPO(4-28) vs SHAM	1.82	0.1853
HYPO(4-28) vs CONT	7.87	0.0080

Table 5.6: Statistical outcome for the number of trials to criterion in the T-maze at 1, 3 and 5 months (win-shift) (Fig. 5.3).

Comparison	df	*t*	*p*	α
Month 1				
SHAM vs CONT	21	2.89	0.0088	0.025
SHAM vs HYPO(1-25)	16	0.03	0.9775	0.05
CONT vs HYPO(1-25)	21	3.04	0.0062	0.025
Month 3				
SHAM vs CONT	20	2.34	0.0300	0.025
SHAM vs HYPO(1-25)	16	1.89	0.0770	0.025
CONT vs HYPO(1-25)	20	0.57	0.5724	0.025
Month 5				
SHAM vs CONT	20	1.86	0.0775	0.025
SHAM vs HYPO(1-25)	16	1.10	0.2875	0.025
CONT vs HYPO(1-25)	20	0.93	0.3633	0.05

Table 5.7: Statistical outcome in CA1 (-1.7 mm) for SHAM, CONT, HYPO(1-25) and HYPO(4-28) groups at 6 months (Fig. 5.6).

Comparison	df	t	p	α
Medial CA1				
SHAM vs CONT	22	31.96	0.0000	0.025
SHAM vs HYPO(1-25)	17	5.33	0.0001	0.025
SHAM vs HYPO(4-28)	16	19.65	0.0000	0.05
CONT vs HYPO(1-25)	21	6.54	0.0000	0.025
CONT vs HYPO(4-28)	20	3.91	0.0009	0.025
HYPO(1-25) vs HYPO(4-28)	15	3.79	0.0018	0.025
Middle CA1				
SHAM vs CONT	22	34.52	0.0000	0.025
SHAM vs HYPO(1-25)	17	2.15	0.0466	0.025
SHAM vs HYPO(4-28)	16	15.83	0.0000	0.05
CONT vs HYPO(1-25)	21	7.31	0.0000	0.025
CONT vs HYPO(4-28)	20	3.69	0.0015	0.025
HYPO(1-25) vs HYPO(4-28)	15	4.28	0.0007	0.025
Lateral CA1				
SHAM vs CONT	22	19.56	0.0000	0.025
SHAM vs HYPO(1-25)	17	1.12	0.2790	0.025
SHAM vs HYPO(4-28)	16	10.23	0.0000	0.05
CONT vs HYPO(1-25)	21	8.29	0.0000	0.025
CONT vs HYPO(4-28)	20	4.2	0.0004	0.025
HYPO(1-25) vs HYPO(4-28)	15	4.7	0.0003	0.025

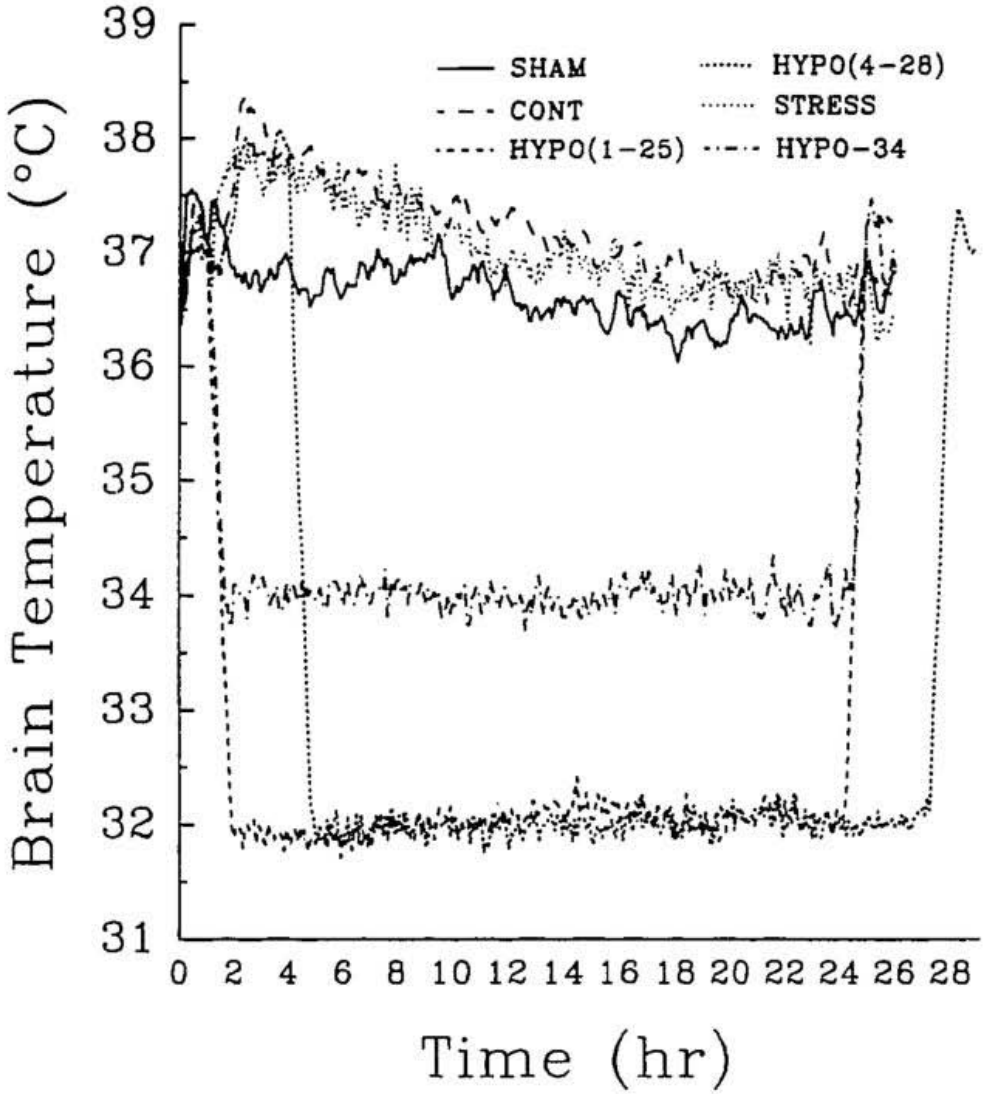


Figure 5.1: Brain temperature during (0 hr) and for up to 29 hours postischemia. Data was collected every 20 seconds and averaged over every 5 minutes. See Table 5.1 for mean (± SD) occlusion and first hour temperatures.

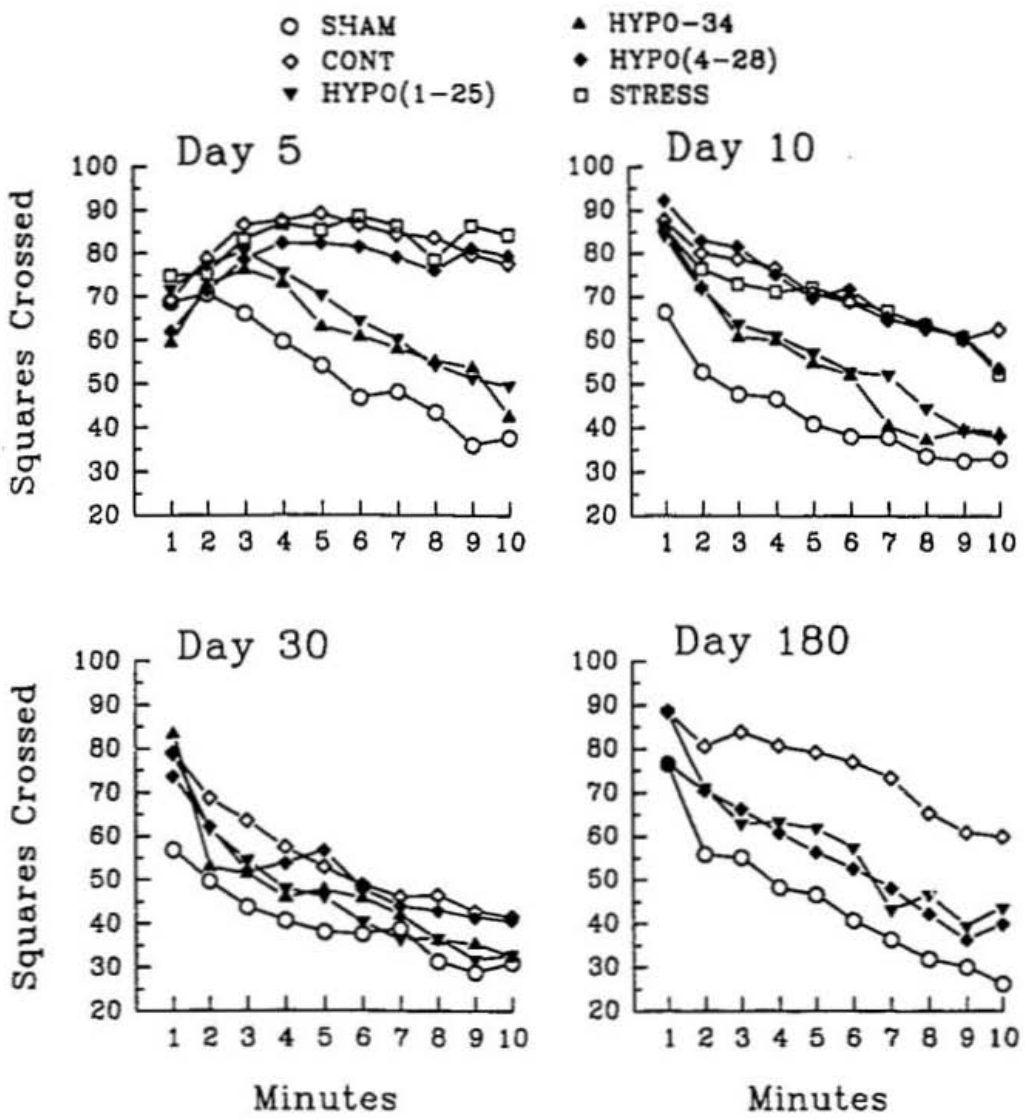


Figure 5.2: Mean open field activity scores (squares crossed per minute) on day 5, 10, 30 and 180 postischemia. See Tables 5.2 to 5.5 for statistics.

Figure 5.3: Trials to reach criterion scores for SHAM, CONT, and HYPO(1-25) groups in the T-maze at 1, 3, and 5 months postischemia. Ischemia (CONT) produced an initial impairment that recovered with repeated testing. Hypothermia (HYPO(1-25)) blunted this impairment. Note that all groups performed similarly at 5 months postischemia.

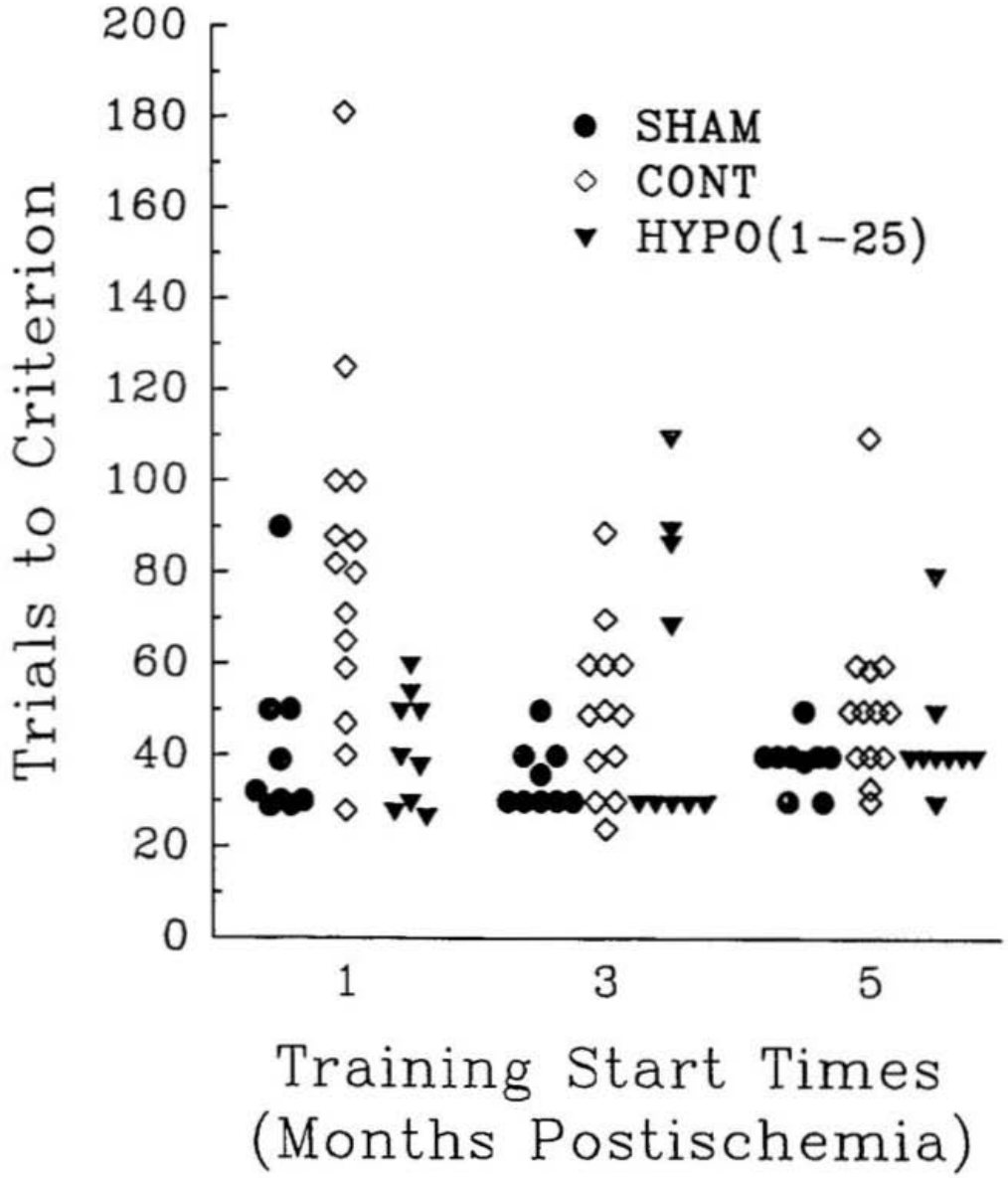
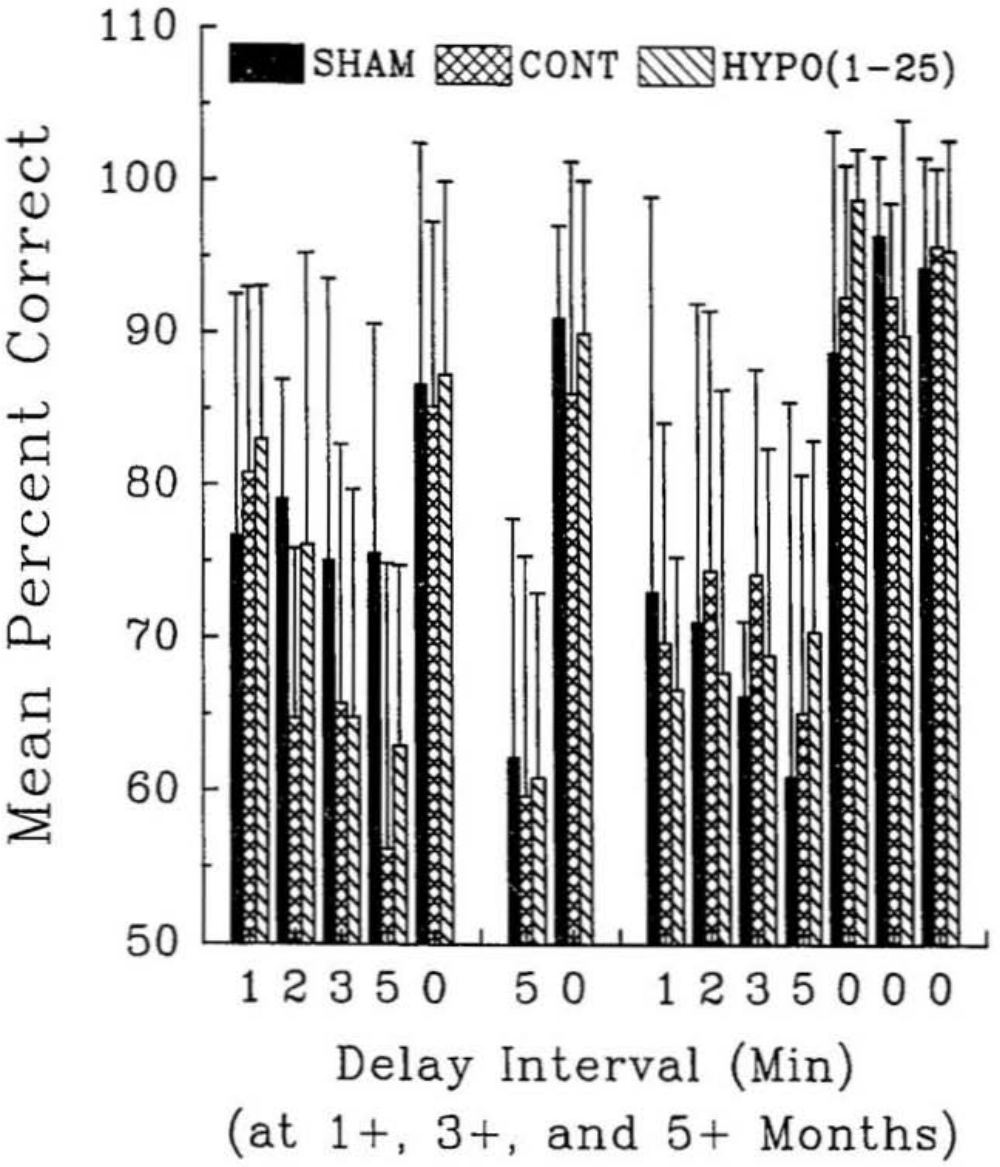

200
180
160
140
120
100
80
60
40
20
0
Trials to Criterion
SHAM
CONT
HYPO(1-25)
1
3
5
Training Start Times
(Months Postischemia)

Figure 5.4: Delay performance (% correct on CT) at 1+, 3+, and 5+ months postischemia. Note that CONT gerbils show greater impairments with longer delays than SHAM and HYPO(1-25) groups at 1+ months postischemia. Delay performance at 3+ and 5+ months after ischemia did not reveal any group differences. In addition, the last three 0 minute delay days at 5+ months postischemia showed very similar performances (≈ 95% correct) among the 3 groups.


110
100
90
80
70
60
50
SHAM
CONT
HYPO(1-25)
Mean Percent Correct
1 2 3 5 0
5 0
1 2 3 5 0 0 0
Delay Interval (Min)
(at 1+, 3+, and 5+ Months)

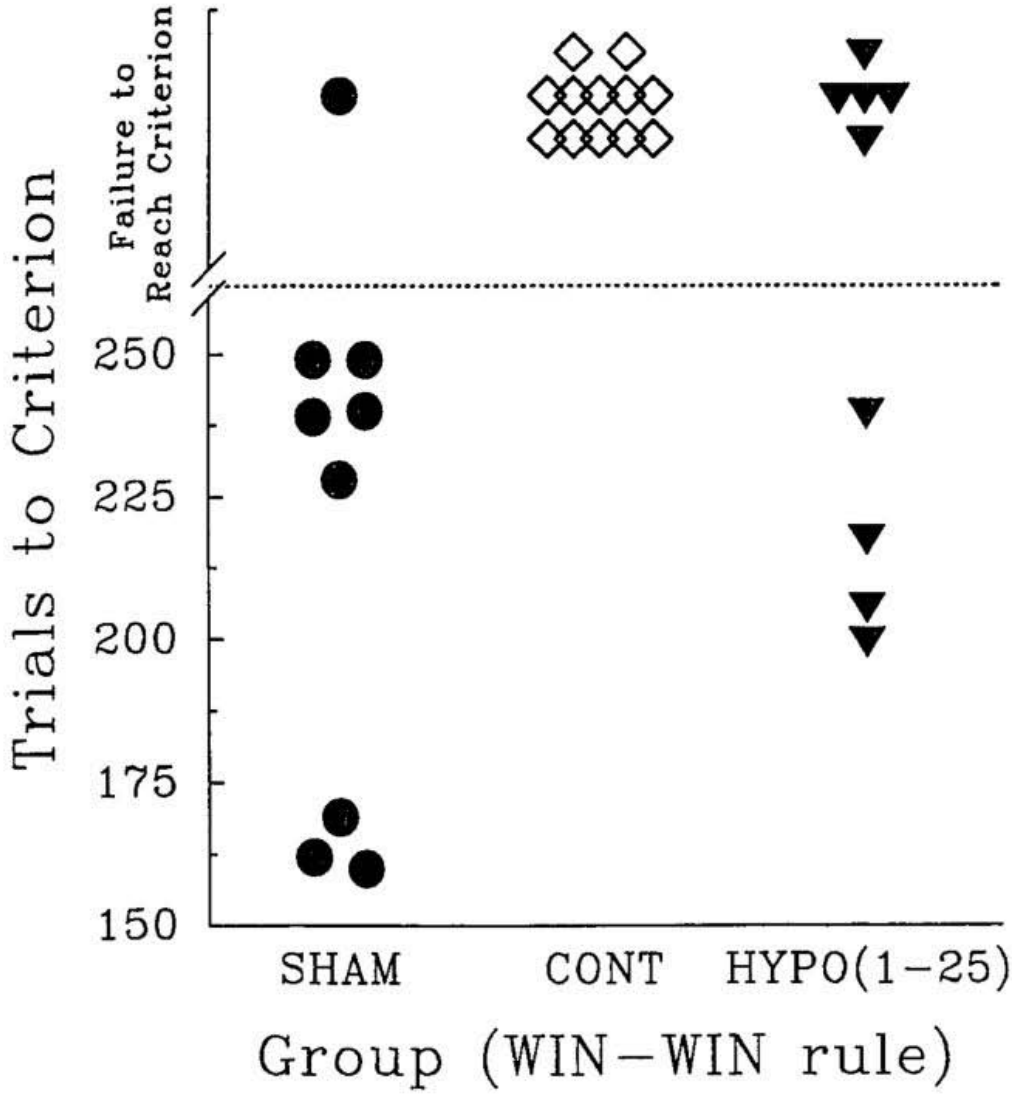


Figure 5.5: Trials to reach criterion scores in the win-win strategy at 5+ months postischemia. Note that all CONT and some HYPO gerbils failed to reach criterion.

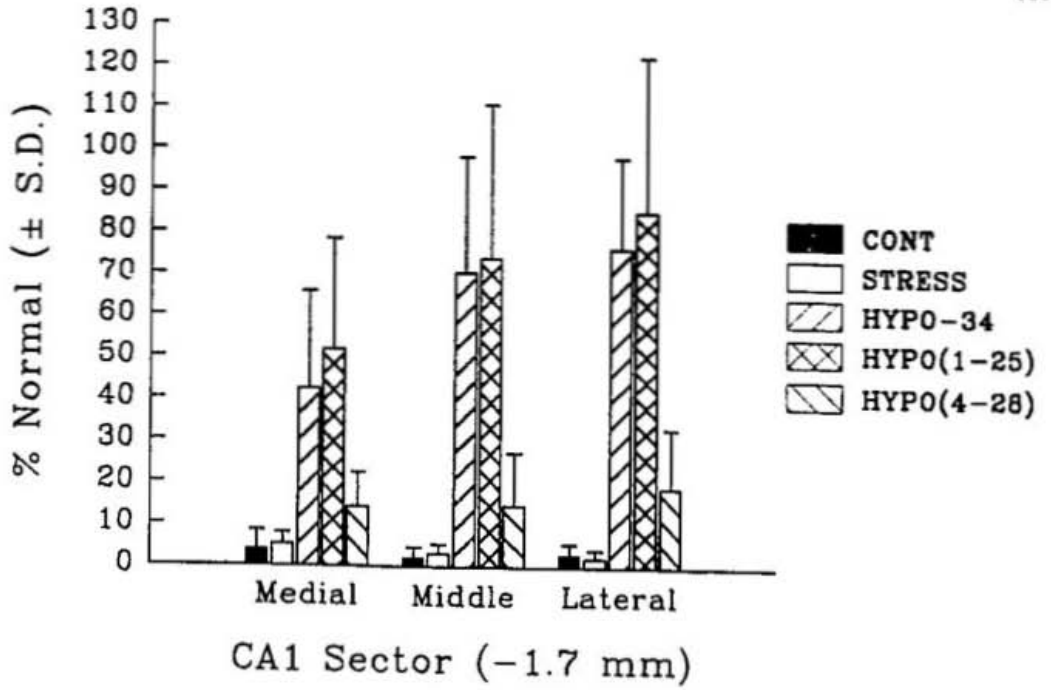


Figure 5.6: Percent (mean ± SD) of normal CA1 counts (medial, middle and lateral at -1.7 mm to bregma) in CONT (6 month survival), HYPO(1-25) (6 months), HYPO(4-28) (6 months), STRESS (10 days), and HYPO-34 groups (30 days). Note that cooling, when started at 1 and even 4 hours postischemia significantly reduced CA1 loss. This was greater in the more lateral sectors for the HYPO(1-25) and HYPO-34 groups. Hypothermia (1-25 hr at 32°C) provided 87%, 91%, and 94% CA1 savings at 1 month (sections 4.3.2 and 5.3). Allowing gerbils to survive for 6 months resulted in a further progression of necrosis (significant in the medial sector only). The 34°C group was also significantly protected, but significantly less so than the 1 month survival 32°C group in medial CA1.

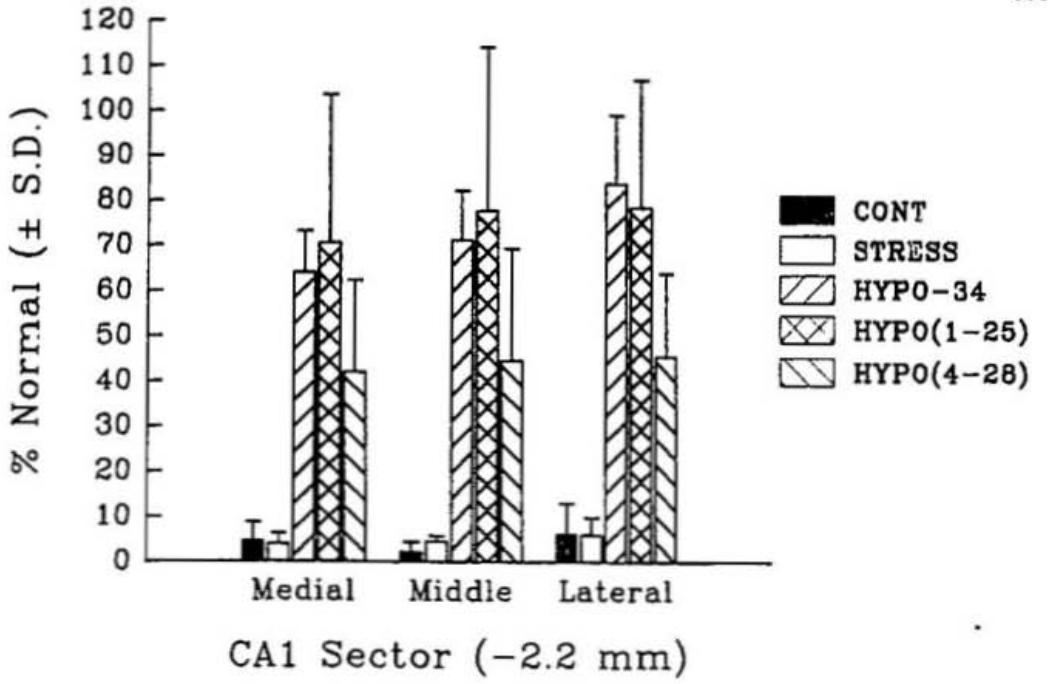


Figure 5.7: Percent (mean ± SD) of normal CA1 counts (medial, middle and lateral) at the -2.2 mm level. Ischemia (CONT and STRESS) produced severe CA1 loss in all three sectors. Hypothermia (1-25 hr at 32°C, 30 day survival, section 4.3.2) resulted in cell counts that were 87.56 ± 5.67, 103.08 ± 6.56 and 95.24 ± 7.79 % of normal in the medial, middle and lateral sectors, respectively.

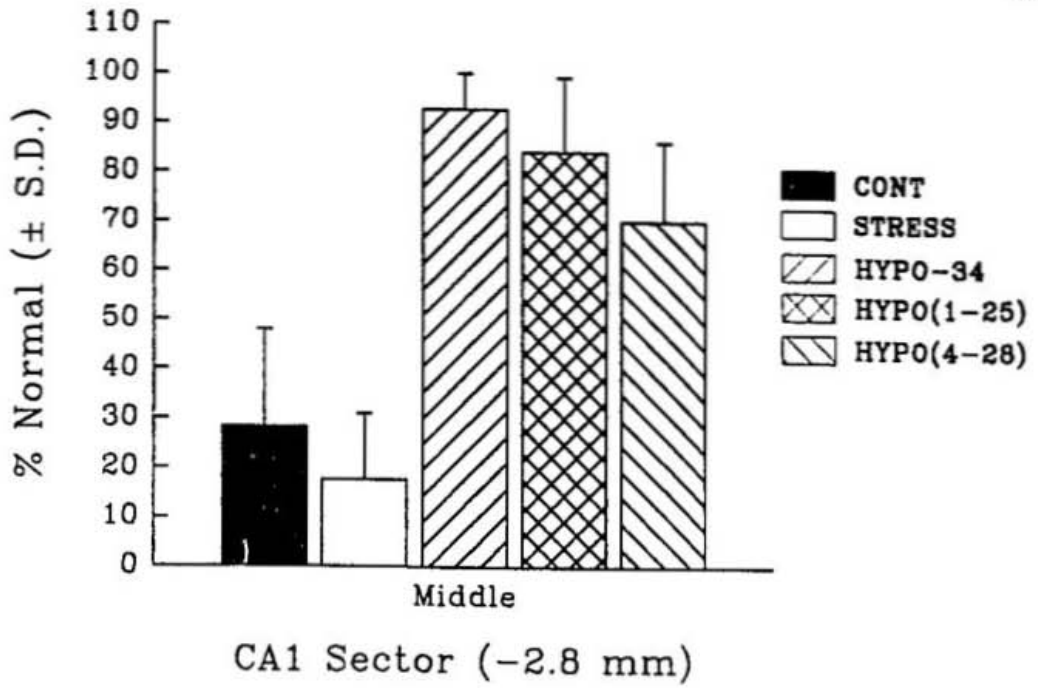


Figure 5.8: Percent (mean ± SD) of normal counts in the middle CA1 sector at -2.8 mm to bregma. Ischemic damage (CONT and STRESS) was somewhat less at this level compared to the previous two anterior levels. Protection by postischemic hypothermia was robust and significant. Hypothermia (1-25 hr at 32°C, section 4.3.2) resulted in complete CA1 protection (101.56 ± 6.43 % of normal) with 30 day survival. The 6 month survival group was slightly, but significantly less protected than the 30 day survival animals ($t_{15} = 2.99$, p - 0.0092, $\alpha = 0.025$).

5.4 Conclusions

A 24 hour hypothermic period (32°C) initiated 1 hour after severe ischemia (5 min) was markedly and persistently (6 month) beneficial. However, CA1 protection (-1.7 mm) was regional, with greater savings in the more lateral sectors. Furthermore, medial CA1 was significantly less protected at 6 months than with 30 day survival (section 4.3.2) which indicates that CA1 neurons may continue to die beyond a 1 month survival. However, unless animals were sacrificed with a very long survival time (perhaps years) it is unknown if cell loss would progress beyond 6 months. Thus, the robust and very long-lasting protection observed, especially in middle and lateral CA1, suggests that it may indeed be permanent. This is contrary to the findings of Dietrich and colleagues (1993) who concluded that immediate postischemic hypothermia (3 hr) was only transiently effective (< 2 months) in the rat 2-VO. This discrepancy is most likely attributable to the fact that we used a much longer hypothermic period, and not simply because of differences in ischemic severity due to model and species differences. First, we had previously shown that a 24 hour cooling period was approximately 6 times more effective than a 12 hour interval (section 4.3). Thus, it makes sense that a very brief temperature reduction would be minimally effective. Second, the degree of CA1 damage (≈ 90 %) produced by Dietrich et al. (1993) was less than that produced by our 5 minute occlusion in the gerbil (≈ 97%). Thus, the better protection observed in this study is not because of a milder insult.

Posterior CA1 counts (-2.2 and -2.8 mm) displayed a similar trend to the anterior level. However, posterior CA1 was more amenable to treatment, especially in the HYPO(4-28) group. This is probably due to less severe ischemia at more posterior (and ventral) levels. This concurs with our previous findings (section 4.3) of greater savings against 3 (vs 5) minutes of ischemia. Overall, these results underscore the importance of evaluating several CA1 regions and levels.

Untreated ischemia (CONT) caused severe and persistent habituation impairments in the open field. This was not just motor hyperactivity since normal and ischemic gerbils were similarly active for the first few minutes of the first test session (Fig. 5.2; section 1.2.3). Normal animals then quickly habituated while CONT gerbils did not, instead they showed elevated and persistent activity levels. On days 10 and 30 the CONT group was still impaired relative to SHAMs, but did show clear habituation trends. By 180 days it seemed that CONT gerbils forgot most of this learning since they then displayed an elevated exploration level and a different rate of habituation than the SHAM group. Thus, with repeated early testing open field deficits appear to partially recover (Wang and Corbett, 1990; Colbourne and Corbett, 1992; Babcock et al., 1993; section 4.3.2). Similarly, initial working memory impairments in the T-maze (win-shift) recovered with extensive testing (see Imamura et al., 1991). Recovery is made possible because the entire hippocampus is not damaged (e.g., more posterior and ventral CA1, CA3 and dentate) and other brain structures (e.g., cingulate cortex; Sutherland et al., 1988) can compensate for learning deficits especially when testing is repeated often and in close

succession. By using longer inter-test intervals, which made it more difficult to remember previous test sessions, we observed persistent memory impairments in the open field (absolute amount and pattern of exploration). Likewise, an enduring learning impairment was observed on the T-maze win-win strategy even though there was prior recovery (i.e., CONT gerbils eventually learned) with the win-shift rule. The win-win strategy was more difficult to acquire because all gerbils had to first unlearn the win-shift rule. Since all CONT gerbils failed to learn the win-win strategy, including those with win-shift scores similar to SHAM and HYPO(1-25) gerbils, we conclude that the ischemic win-win impairment is not due to a selective overtraining in the CONT group as such, but to a residual learning deficit. Thus, while substantial recovery of function is sometimes noted (e.g., Corbett et al., 1992), more difficult and extensive testing can reveal enduring memory impairments in the ischemic rodent. This is similar to findings in humans and monkeys who show permanent declarative learning impairments following ischemia (section 1.2.3).

Ischemic CONT gerbils were also impaired (vs SHAM) when longer ITIs were imposed after the first win-shift acquisition. Thus, even though all groups had reached criterion, the CONT group still showed a working memory impairment. However, delay testing at 3 and 5 months did not reveal any significant differences. While recovery was expected on the no delay win-shift training, it was thought that long ITIs (especially 5 min) would sufficiently tax memory to reveal persistent ischemic impairments as the win-win training did. The most likely explanation for this failure is the apparent lack of

motivation in all groups during the delays. Notably, gerbils would often sleep during the delay, but not during the no-delay condition. This was not as common at 1+ month postischemia. This motivation problem may be circumvented by using food deprivation instead of sunflower seed treats.

Postischemic hypothermia (HYPO(1-25)) was an unquestionably effective and persistent behavioral neuroprotectant since it significantly and chronically reduced the open field habituation impairments and reduced the win-shift and win-win T-maze acquisition deficits. However, since this treatment did not completely attenuate hippocampal damage these gerbils were not always as good as SHAMs. The HYPO(1-25) gerbils explored significantly more than SHAMs on days 5 and 10 in the open field even though CA1 counts should have been near normal at those times (≥ 90%). Similarly, untreated gerbils are impaired in the open field when tested as early as 1 day following ischemia (Wang and Corbett, 1990; Colbourne and Corbett, 1992) even though CA1 necrosis would be minimal at that time. Therefore, early functional testing seems to be a better indicator of eventual histological outcome than early histology itself! Perhaps this is because the structural/metabolic derangements that culminate in eventual necrosis also impair neuronal function. Since these perturbations can take months to mature it is not sufficient to rely solely on cell counts as an estimate of neuroprotection.

Milder hypothermia (34°C) also significantly attenuated CA1 loss and associated habituation impairments. However, CA1 savings were significantly less than with 32°C cooling (1 month survival) in the medial (-1.7 and -2.2 mm) and middle (-2.2 and -2.8

mm) sectors. One might expect this necrosis to progress such that at a 6 month survival CA1 counts could be substantially less. However, CA1 loss in the HYPO-34 group may have matured faster than the HYPO(1-25) group and both could eventually have similar savings. Since the HYPO-34 (1 month survival) and HYPO(1-25) (6 month survival) groups had similar CA1 protection, it cannot be concluded that 32°C cooling is better. Furthermore, if early open field performance is a good indication of final histological outcome then it follows that the HYPO(1-25) and HYPO-34 groups were similarly protected (final outcome). If small differences exist, more extensive testing (in the open field or T-maze) or larger group sizes may be able to distinguish between such gradations in injury.

Hypothermia (32°C) also reduced CA1 loss when started at 4 hours postischemia, but was clearly less effective than intervening at 1 hour. The HYPO(4-28) group also showed mild behavioral protection, but only at 6 months postischemia and not earlier. It is conceivable that the few remaining CA1 neurons were not initially functioning properly, but later contributed to the observed recovery. However, since this group was not tested in the T-maze the CONT and SHAM groups are not perfect controls and it cannot be absolutely concluded that 4 hour delayed hypothermia is of functional benefit. Thus, there is a critical period in the first few hours following ischemia when hypothermia is most effective. Notably, this time frame is different from that shown by Coimbra and Wieloch (1994) who found protection with a 5 hour hypothermic duration when started as late as 12 hours after ischemia. However, since this study utilized a short

survival time (7 days) and a brief hypothermic period the protection may have dissipated with longer survival times. Thus, the real therapeutic window probably ends within a few hours of ischemia, except perhaps against a very mild insult or in the more posterior levels of CA1.

The stress of the cooling procedure per se did not improve outcome since the STRESS and CONT groups had similar CA1 damage and open field performance. However, postischemic stress may be detrimental. In humans hypothermia would be induced with anesthetics and muscle relaxants (e.g., see Clifton et al., 1992) and hence, it would be important to compare hypothermia with and without such drugs. It is possible that reduced stress would augment the present degree of protection, while increased stress would not increase an already maximal CA1 injury.

In summary, ischemia produced profound hippocampal CA1 loss which resulted in chronic habituation and working memory impairments in the gerbil. Delayed (1 and 4 hr) but protracted (24 hr) postischemic hypothermia (32 or 34°C) significantly reduced ischemic injury (behavioral and histological) for up to 6 months postischemia. Greater protection was achieved with quicker intervention and it appears that 32°C hypothermia was better than 34°C. While, it is not certain that this protection is permanent, the long-lasting savings presently found makes this an ideal candidate for clinical investigation.

Chapter 6 (General Discussion)

6.1 Introduction

There are two primary conclusions from this thesis. First, the gerbil can be used to provide a valuable, highly reproducible model of global ischemia with histological and behavioral endpoints similar to those observed in human cardiac arrest victims. Second, protracted postischemic cooling significantly and persistently attenuates the behavioral and histological consequences of global ischemia. Protection depends on the severity of ischemia, duration and degree of cooling, initiation delay and survival time. Thus, hypothermia (32°C) is most effective when induced quickly following global ischemia and maintained for at least 24 hours.

6.2 Gerbil Model of Global Ischemia

With careful brain temperature maintenance severe CA1 injury results in gerbils exposed to normothermic ischemic periods as brief as 3 - 5 minutes (Chapters 2 to 5). This is similar to the pathology found after brief global ischemia in humans (section 1.2). Brain temperature must be regulated in rodents because, unlike humans, they easily become hypothermic (small head) during ischemia and this markedly attenuates injury (Chapters 2 and 3). Since brain temperature clearly dissociates from rectal and skull readings one cannot rely solely on these indirect measures to gauge ischemic severity. Thus, strict brain temperature control is essential when comparing ischemic durations (e.g., 3 vs 5 min) and across studies. Extensive brain temperature measurement is also of utmost importance when assessing drugs (e.g., Corbett et al., 1990; Nurse and Corbett, submitted) since they may promote temperature dissociations and/or prolonged temperature effects. In this regard, the Mini-Mitter / Data Sciences system is ideal since it allows for extended and continuous, stress-free brain temperature measurement in the awake, freely moving animal. Without anesthesia, it would be exceedingly difficult to conduct temperature-controlled experiments with intermittent rectal or skull measurements.

Even with strict normothermia and severe CA1 loss, mortality rates during 3 or 5 minutes of ischemia were low (< 5%). Since almost all gerbils survived brief ischemia and did not show overt motor impairments or evidence of seizures they were ideal for

repetitive behavioral testing over long survival times. This is in contrast to the more invasive rat 2-VO and 4-VO models where there is often a tendency for postischemic seizure activity and poor survival (up to 50% mortality).

Early open field testing revealed a striking habituation impairment in the ischemic gerbil (sections 1.2.3, 4.3.1 and 5.3; Colbourne and Corbett, 1992). However, when testing is repeated within a short period (e.g., 1 week) ischemic gerbils do show recovery, eventually to the level of SHAMs (Wang and Corbett, 1990; Babcock et al., 1993). From this alone, it might be concluded that exploration impairments are fleeting. However, this is not the case since more enduring deficits were apparent with greater inter-test intervals (section 5.3). A similar situation occurred with win-shift training in the T-maze where initial ischemic acquisition impairments recovered with extensive testing while subsequent win-win training revealed a residual learning impairment (section 5.3). Thus, like humans and primates (section 1.2.3), the gerbil showed persistent behavioral abnormalities (habituation and working memory impairments) which stemmed from hippocampal injury. From this (Chapters 4 and 5) and previous studies (section 1.2.3) it is clear that memory impairments are not static, but somewhat variable depending on the test conditions and the postischemic interval. As such, the full breadth of ischemic functional impairments are only revealed by repeated and long-term assessment. Thus, one-time "snapshot" memory testing, while informative, is undoubtedly of limited value.

6.3 Postischemic Hypothermia

Delayed postischemic cooling significantly attenuated ischemic CA1 loss and associated functional impairments (open field and T-maze). However, this critically depended on several parameters. First, better protection occurred against milder ischemia. This was shown in Chapter 4 where 12 hours of postischemic cooling almost completely attenuated cell loss against 3 minutes of ischemia but was only transiently effective against a 5 minute insult. This illustrates the importance of the use of several ischemic durations since the benefit of a partial neuroprotectant may be missed against a severe insult, while studies with only mild ischemia may lead to overly optimistic conclusions. Since stroke severity is quite variable in humans, it is important to test putative neuroprotectants against similar gradations of ischemia.

Second, extending the period of cooling from 12 to 24 hours increased protection by approximately 6 fold against a 5 minute occlusion with 1 month survival (section 4.3). Since CA1 death normally matures over several days it is possible that an even longer hypothermic period (e.g., 3 days) would be more effective. Evidence supporting this comes from Nurse and Corbett (personal communication) who found a transient (< 10 days) CA1 savings (3 min occlusion in gerbils) with a 24 hour period of mild cooling (≈ 1.5°C drop). However, when they gave NBQX it induced the same temperature reduction, but for about 4 days. This period of subnormal temperature resulted in a longer lasting benefit (30 day survival). These data are also interesting in that they show that

even a very mild cooling can have significant benefit against a moderate insult. This suggests that just reducing hyperthermia may also be beneficial. While such a therapy would certainly not be harmful, its usefulness is probably restricted to mild insults or borderline ischemic areas since prevention of immediate postischemic hyperthermia was ineffective against a severe 5 minute insult (Chapter 2). In addition, more protracted variations (≈ 1°C) in postischemic hyperthermia also did not affect CA1 injury after a 5 minute occlusion (section 4.4). Thus, more severe ischemia requires a deeper (section 5.3) and a longer cooling period (section 4.3).

As expected, greater protection occurred with quicker intervention. In fact, 24 hours of hypothermia was up to 5 times better when initiated at 1 hour than at 4 hours postischemia. Thus, cooling must be initiated within a few hours of ischemia to achieve substantial benefit. Finally, histological protection was both regional and dynamic. For instance, hypothermic (32°C from 1 - 25 hr) protection at 30 days was found to progress such that at 6 months postischemia there was a significant decline in the medial, but not the middle or lateral CA1 (-1.7 mm). An explanation for this regional vulnerability within CA1 is not forthcoming from these experiments. Perhaps this sector effect is due to some intrinsic property (e.g., different connections from medial to lateral) of CA1 neurons. It is also possible that regional differences in blood flow or intracerebral temperature gradients could contribute to the observed differences. Finally, a maturation effect must be considered. For example, it is possible that medial CA1 simply progresses to necrosis faster than lateral CA1.

Overall, these data underscore the necessity of assessing several CA1 regions with a long recovery period since a short survival time may lead to completely erroneous conclusions. Like memory impairments, gross histological injury may evolve over many months, and not just a few days (Murdrick and Baimbridge, 1989; Dietrich et al., 1993; Fukuda et al., 1993; Chapters 4 and 5).

6.4 Possible Mechanisms of Neuroprotection

The mechanisms underlying the protective effect of postischemic hypothermia are not clear. However, CA1 neurons do show an enhanced postischemic sensitivity to normal synaptic input such that depolarization causes a much increased Ca^{++} entry than when applied before ischemia. Thus, even though glutamate levels return to normal after ischemia CA1 cells appear supersensitive to any input. Ischemia triggers some factor(s) that leads to this altered Ca^{++} homeostasis (perhaps AMPA subunit changes), which in turn may initiate a cascade of events (over-activation of lipases, endonucleases and proteases) that culminate in eventual death (section 1.3). Therefore, it is possible that one of the protective mechanisms of hypothermia was a reduction of the "normal" synaptic drive on CA1 from the perforant path and CA3 (from less glutamate release) at a time when these neurons are in a highly vulnerable state. Perhaps, the prolonged duration of hypothermia also helped the cells recover normal Ca^{++} homeostasis. Hypothermia may also inhibit the destructive enzymes (e.g., calpain) responsible for cellular damage (section 1.3).

Another possibility is that hypothermia improves the recovery of postischemic protein synthesis as intra-ischemic hypothermia does (Widmann et al., 1993). This could be due to improved Ca^{++} regulation which would decrease the inhibition of protein synthesis initiation.

It is also possible that cooling reduces CA1 loss by a host of mechanisms such as

reduced intracranial pressure (less edema) which would increase cerebral blood flow (Dempsey et al., 1987; Marion et al., 1993; Shiozaki et al., 1993) and help prevent secondary ischemia due to poor reflow (Imdahl and Hossmann, 1986). Cooling would also reduce the metabolic needs of CA1 neurons and thus, promote coupling of blood flow to demand. Like intra-ischemic cooling (Dietrich et al., 1990; Karibe et al., 1994), prolonged postischemic hypothermia may also reduce blood-brain barrier damage which can further aggravate ischemic injury.

Regardless of the exact factors contributing to cell death it is likely that they start early and continue over many hours especially when ischemia is prolonged (5 min in gerbils). Our data clearly show much greater benefit with 24 hours (vs 12 hr) of hypothermia which indicates that some key event contributing to cell loss continues beyond the first 13 hours after ischemia. Furthermore, much greater benefit occurs with quicker intervention. Thus, it is likely that the most important components of hypothermic neuroprotection are events that start during or soon after ischemia and continue for many hours, such as altered Ca^{++} homeostasis and impaired protein synthesis.

6.5 Limitations and Future Directions

There are several straightforward parametric questions that deserve further investigation. First, are longer hypothermic periods (e.g., 48 hr) more beneficial? This is extremely important since stroke therapy should be maximized for efficacy, but minimized for side-effects. Second, a better understanding of the therapeutic window is essential. For instance, it may be of no use cooling a stroke victim with intervention delays greater than 12 hours. Third, is hypothermia indicated for other types of strokes (e.g., haemorrhagic or focal) (e.g., Baker et al., 1992; Karibe et al., 1994)? Fourth, are other vulnerable structures (e.g., hilus, CA2, striatum) protected and what are their therapeutic windows? In this regard, it appears that postischemic hypothermia does rescue CA2 neurons (10 and 30 day survival; unpublished data with a 12 hr duration starting 1 hr after ischemia). Furthermore, Coimbra and Wieloch (1994) reduced striatal injury with postischemic cooling. However, this must be assessed with a longer survival time than 7 days. Fifth, how does a slower rate of cooling and rewarming affect outcome? This is clinically important since humans cannot be cooled as quickly as small rodents. It is likely that very slow cooling (1°C/hr) will be less effective, while slow rewarming may be more beneficial as it prolongs the hypothermic period and reduces rewarming shock. Since humans will be cooled with anesthetics and muscle relaxants, the effects of these agents should also be determined. Finally, more behavioral testing and longer survival assessments (e.g., 1 year) would also be useful in determining the true neuronal savings.

The greatest potential limitation of this thesis is whether results in the gerbil will apply to the human stroke victim (global ischemia). Thus, prior to a clinical investigation any potential therapy should pass several criteria. First, it should be clearly shown to be efficacious in animal studies. In the gerbil, postischemic hypothermia does significantly reduce both the functional and histological sequelae of global ischemia similar to the so-called gold standard of neuroprotection, intra-ischemic hypothermia (Buchan, 1992). Second, the therapy must be safe. Humans have been repeatedly shown to readily tolerate protracted **mild** hypothermia without serious side effects (section 1.4.2.2; see below). Finally, the putative neuroprotectant must be applicable to the human stroke condition (i.e., postischemic administration). Again hypothermia is suitable since many victims can be cooled within 4 hours of the stroke.

Interestingly, several groups have investigated prolonged cooling in humans for the treatment of elevated intracranial pressure and traumatic brain injury with some success (Sedzimir, 1959; Clifton et al., 1992; Marion et al., 1993; Shiozaki et al., 1993). It is noteworthy that Guy Clifton (personal communication) is now cooling head-injured patients for 48 hours at 32°C or 72 hours at < 35°C! Now is the time for similar trials in stroke patients.

6.6 Summary

These data conclusively show that delayed postischemic hypothermia can substantially and persistently improve functional and histological outcome. Such findings dictate the use of prolonged temperature control (≥ 24 hr) in all pharmacological experiments where a mechanism of action is being sought. Furthermore, such studies must employ longer survival times (at least 30 days) and behavioral tests to truly assess neuroprotection. For example, finding robust histological protection with a 4 day survival time may be a poor indication of actual benefit whereas the use of quantitative behavioral testing appears to more accurately reflect the final outcome.

The magnitude and persistence of hypothermic neuroprotection is sufficient to justify clinical investigation following global ischemia. However, when initiation of hypothermia is delayed even longer (e.g., 12 hr) after ischemia or when ischemia is too severe (e.g., 10 min in gerbils) hypothermia may not, by itself, reduce damage. In such cases, combination therapy of postischemic hypothermia and a neuroprotective drug may prove useful. It is hoped that a better mechanistic understanding of postischemic cooling will lead to selective pharmacology that may augment or even replace cooling. Until then, however, postischemic hypothermia is the clinical candidate of choice.

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