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Brain Research 654 (1994) 265–272

BRAIN RESEARCH

Research report

Delayed and prolonged post-ischemic hypothermia is neuroprotective in the gerbil

Frederick Colbourne *, Dale Corbett

Division of Basic Medical Sciences, Faculty of Medicine, Memorial University of Newfoundland, St. John's, NF A1B 3V6, Canada

Accepted 10 May 1994

Abstract

Global ischemia, in the gerbil, produces profound hippocampal CA1 loss which leads to functional abnormalities (e.g. habituation impairment). In experiment 1, gerbils were subjected to 3 or 5 min of normothermic (brain) ischemia. Hypothermic groups were cooled to 32°C for 12 h beginning 1 h after ischemia, while control groups (no hypothermia) regulated their own temperature. Exploration in a novel open field was assessed on days 3, 7 and 10 following ischemia and CA1 neurons were counted after 10- or 30-day survival. Both ischemia durations produced severe CA1 necrosis which resulted in increased open field activity. Hypothermia attenuated this behavioral pattern and substantially reduced CA1 necrosis against 3 min of ischemia when assessed at 10 and 30 days, but was only partially effective against a 5 min occlusion where, in addition, some cell death appeared to be delayed rather than prevented. In experiment 2, gerbils were occluded for 5 min and survived for 30 days. Twenty-four hours of hypothermia initiated 1 h after ischemia resulted in near total preservation of CA1 neurons. Thus, increasing the duration of post-ischemic hypothermia from 12 to 24 h produced much greater neuroprotection against severe ischemia. Prolonged post-ischemic hypothermia may be a valuable intervention in stroke patients.

Key words: Cerebral ischemia; Gerbil; Post-ischemic hypothermia; Delayed neuronal death; Open field

1. Introduction

In humans and rodents a brief episode of global forebrain ischemia produces selective and often extensive neuronal loss in several vulnerable brain structures (e.g. hippocampal CA1) [17,19,25,30]. Fortunately, cell death does not occur immediately, but is delayed for hours to days. This process, termed delayed neuronal death (DND) [19,25], provides an opportunistic time window when some intervention may reduce cell loss and associated learning and memory deficits.

The most effective treatment at present is intra-ischemic hypothermia, which has been repeatedly shown to confer remarkable histological and behavioral protection [6,15,24]. Post-ischemic hypothermia has also been found to be beneficial when initiated within 30 min of reperfusion [5,8]. More recently, however, several groups have shown CA1 neuroprotection with delayed (beyond 30 min) post-ischemic hypothermia both in gerbil [7,16] and in rat [9]. Carroll and Beek [7] subjected gerbils to 5 min of ischemia and found that 6 h of hypothermia (rectal temperature = 28–32°C) initiated 1 h after occlusion resulted in approximately 50% CA1 preservation with 4 day survival. Similarly, Hoffman and Boast [16] reduced CA1 loss in gerbils (sacrificed at 4 days) with 5 h of hypothermia (rectal = 32°C) which started 1 h after a 5 min ischemic episode. Coimbra and Wieloch [9] found CA1 protection with 5 h of hypothermia (rectal and skull ≈ 33°C) initiated 2 h after 10 min of 2-vessel occlusion (2-VO) ischemia in rats that survived for 1 week. Unfortunately, these findings have been cast in doubt by the findings of Dietrich and colleagues [14] who reported that post-ischemic hypothermia only delays cell death. In their study, post-ischemic hypothermia (3 h) resulted in CA1 preservation when animals were sacrificed at short (3 and 7 days) but not a long survival time (2 months). Thus, the current view is that post-ischemic hypothermia conveys little in the way of permanent neuroprotection.

* Corresponding author. Fax: (1) (709) 737-7010; E-mail: fcolbourne@kean.ucs.mun.ca

SSDI 0006-8993(94)00613-H

One question raised by the above studies concerns the duration of post-ischemic hypothermia which has often been 3 h or less. It may be that a more prolonged duration of hypothermia is required to offset the myriad of intracellular disturbances resulting from ischemia. Indeed, certain neuroprotective drug treatments that appear to work via hypothermia (e.g. MK-801) have a long half-life [4].

Brain temperature must be monitored to accurately gauge ischemic severity. Importantly, several studies have shown that rectal and skull temperatures are *not* always adequate indices of brain temperature during ischemia [6,12]. Therefore, it is necessary to maintain intra-ischemic brain normothermia in order to properly assess the efficacy of post-ischemic treatments.

In the first experiment we assessed whether 12 h of hypothermia (brain = 32°C) initiated 1 h after either 3 or 5 min 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 [27], 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. Results of preliminary data have appeared in abstract form [11]. In the second experiment we used 24 h of hypothermia beginning 1 h after 5 min of ischemia in animals that survived for 30 days. This was to determine if 24 h of hypothermia was more effective than a 12 h duration.

2. Materials and methods

2.1. Subjects

Eighty-two female, Mongolian gerbils (High Oak Ranch Ltd., Goodwood, Ont.) were included in this study. Animals were housed individually, with food and water ad lib. Gerbils were approximately 12–13 weeks old, and weighed about 55 g at the time of ischemia. All procedures were approved by the Memorial University of Newfoundland animal care committee, and in addition, were in accordance with the guidelines of the Canadian Council on Animal Care.

2.2. Brain temperature

Procedures for brain temperature measurement were similar to previous work [12,13]. Briefly, gerbils were implanted with a 5.0 mm guide cannula above the dural surface overlying the left frontal cortex. Two days later 8.0 mm brain temperature probes (Mini-Mitter, model XM-FH, Mini-Mitter Co. Inc., Sunriver, OR) were inserted and 3.0 h of continuous normal brain (anterior dorsal striatum) temperature was collected in awake, freely-moving animals. Since Minamisawa and colleagues [22] have shown temperature gradients at different depths in the brain, care was taken to place the probes at approximately the same depth as the hippocampus.

2.3. Ischemia

Two days after measuring normal brain temperature, gerbils were subjected to 3 or 5 min of bilateral carotid artery occlusion, using micro-arterial clips (Fine Science Tools, Vancouver, BC), under 1.5% halothane in 70% N_2O and 30% O_2. 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) (Harvard Apparatus, South Natick, MA), and a heated water flow-though blanket (Mul-T-Pads, model TP-3E, Gaymar Industries Inc., Orchard Park, NY) 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 W 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 individual plexiglass boxes resting on telemetry receivers (Mini-Mitter, Model RA-1010) for brain temperature measurement. If necessary, a 60 W 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 min 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 W lamp, which was then discontinued. In contrast, hypothermic gerbils were subjected to prolonged hypothermia upon return to their boxes. Hypothermia was induced by an overhead fan and intermittent cold ($\approx$ 4.0°C) water spray. Gerbils were cooled by approximately 1.0°C/10 min to 32.0°C and then maintained around this value 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 min 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).

2.4. Behavioral testing

Exploration was evaluated by exposing gerbils (experiment 1) to an open field test 3, 7 and 10 days after ischemia for 10 min per trial [10,27]. The open field chamber measured 72×76×57 cm and was divided into 25 equal 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 W lamps in a sound attenuated room. Distinctive features of the room (e.g. computer equipment) remained constant throughout testing and there were no special markings within the box. Gerbils with an intact hippocampus show less exploration (number of squares crossed) over test days while ischemic gerbils, with CA1 damage, exhibit heightened activity [27].

2.5. Histology

Gerbils were sacrificed either 10 or 30 days after ischemia with an overdose of sodium pentobarbital in accordance with previous work [12,13]. Adjacent frozen coronal sections (10 μm) were collected and stained with Cresyl violet or Cresyl violet/phloxine. Bilateral counts (added for analysis) of viable-looking neurons (well-defined nucleus, distinct cellular membrane and not shrunken) in Cresyl violet sections were performed by an observer (F.C.) blind to treatment conditions.

In the Cresyl violet sections CA1 pyramidal cells were counted in

the medial, middle and lateral sectors of CA1 (added for analysis) at −1.7 mm, and in a sector at the apex of CA1 at −2.2 mm to bregma [21].

Adjacent phloxine stained sections, which demarcated acidophillic neurons (with cytorrhexis and karyorrhexis) as red, were assessed by categorizing the sections as displaying no, 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 [2].

2.6. Experiment 1

There were 8 groups including: 4 hypothermic groups (12 h duration) with either 3 or 5 min 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 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 [10].

2.7. Experiment 2

Two groups were exposed to 5 min of ischemia with either 24 h of hypothermia which started 1 h 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). This experiment was to determine if longer duration hypothermia provided greater CA1 neuroprotection.

2.8. Statistics

Total CA1 cell counts (Cresyl violet) were analyzed with 3-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 3-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 [18]. All data are expressed as the mean ± S.D. Some statistics are omitted to reduce unnecessary content. Interested readers are encouraged to write for a copy.

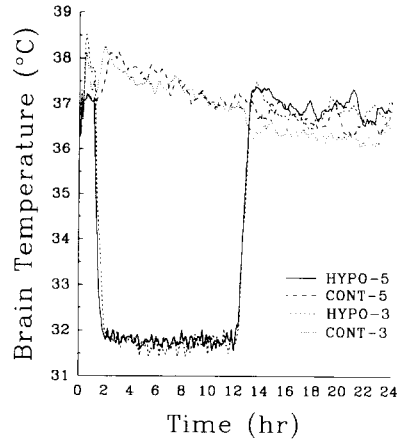

Fig. 1. Brain temperature during (0 h) and after ischemia (24 h) in HYPO-5, CONT-5, HYPO-3 and CONT-3 groups in experiment 1. Groups are collapsed over survival times for graphical simplicity and since these differences were negligible. Data are averaged every 5 min from the start of ischemia to 24 h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

3. Results

3.1. Experiment 1

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

Intra-ischemic brain temperature was maintained close to this normal range (Fig. 1 and Table 1). Hy-

Table 1
Mean (±S.D.) brain temperature (°C) during occlusion and in the first hour of recirculation

T	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 ± 0.31
CONT(2)	5	30	36.39 ± 0.15	37.25 ± 0.43
SHAM(2)	0	30	36.84 ± 0.18	37.14 ± 0.30

Groups are classified according to treatment (T, HYPO or CONT), duration of ischemia (D, 0, 3 or 5 min) and survival time (S, 10 or 30 days).

pothermic 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, post-ischemic (first hour 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 ($\approx$ 0.5°C) than CONT-3–10 gerbils ($P = 0.0080$) (Fig. 1 and Table 1).

Both occlusion and post-ischemic (first hour) brain temperatures (Fig. 1 and Table 1) were consistently higher in groups exposed to 3 min 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.

Post-ischemic (1–13 h) brain temperature was easily and precisely regulated (see section 2.3) in all HYPO groups (Fig. 1). During this period all CONT groups exhibited hyperthermia (peak $\approx$ 38.2°C) that was maximal around 2 h after occlusion. This hyperthermia dissipated over many hours. Finally, all groups (CONT and HYPO) displayed fairly similar temperature profiles beyond 13 h after ischemia.

Delayed post-ischemic hypothermia (12 h) reduced the increased exploration associated with both 3 ($P = 0.0105$) and 5 ($P = 0.0174$) min ischemic episodes over all test days, but was not statistically significant on each day (Fig. 2). Habituation over days occurred (day main effect, $P < 0.0001$) and this was approximately equal among groups (3-way interaction, $P = 0.8916$). Groups subjected to 3 min of ischemia showed less activity, indicating less impairment, than did those occluded for 5 min (duration main effect, $P = 0.0119$). 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 ($P = 0.1226$).

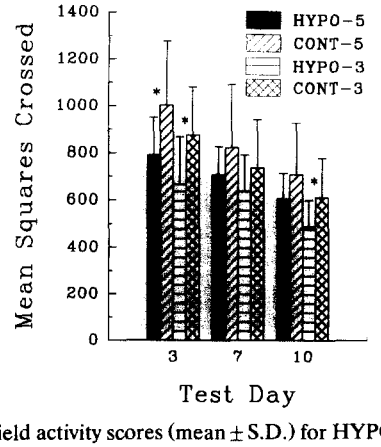

Fig. 2. Open field activity scores (mean ± S.D.) for HYPO-5, CONT-5, HYPO-3 and CONT-3 groups on days 3, 7 and 10 after ischemia (experiment 1). Note that HYPO gerbils exhibited less exploration than their respective CONT animals (* denotes significant difference). In addition, animals occluded for 5 min show greater behavioral impairment than those occluded for 3 min.

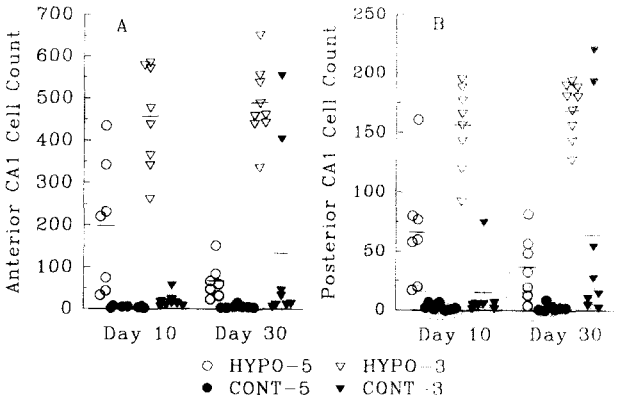

Fig. 3. Hippocampal CA1 cell counts at −1.7 mm (A) and −2.2 mm (B) to bregma (experiment 1). Hypothermia significantly reduced CA1 loss against 5 min of ischemia with 10- and 30-day survival. Protection was better with 10-day survival, indicating progression of cell loss after 10 days. Hypothermia substantially reduced CA1 loss against 3 min of ischemia with day 10 and 30 survival. Protection against 3 min of ischemia did not decline from 10 to 30 days. Horizontal bars are group means. Sham-operated gerbils, obtained from Colbourne and Corbett [10] ($n = 5$), had 570.8 ± 39.75 S.D. and 184.4 ± 21.54 S.D. cells in A and B, respectively.

Five minutes of normothermic ischemia (CONT-5) resulted in near-complete anterior and posterior CA1 loss (Fig. 3). Damage in CONT-5 animals was maximal with 10 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 ($P = 0.053$; $\alpha = 0.025$), 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. 3). Hypothermia, unlike the moderate protection in the 5 min occluded groups, greatly attenuated CA1 loss against 3 min 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

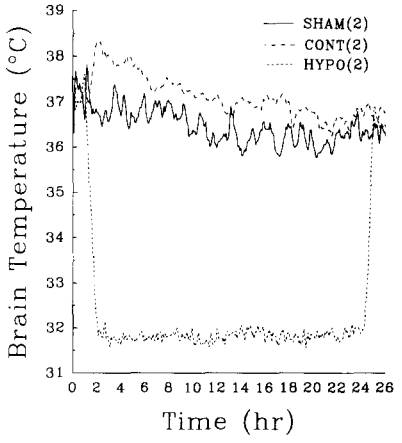

Fig. 4. Brain temperature during (0 h) and after ischemia (26 h) in HYPO(2), CONT(2) and SHAM(2) groups in experiment 2. Data are averaged every 5 min from ischemia (at 0 h) to 26 h after occlusion. Also see Table 1 for occlusion and first hour mean temperatures.

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 ($r = 0.9668$, $P < 0.0001$).

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 and for the first hour after ischemia was also similar to the previous experiment, with no significant difference between ischemic groups ($P > 0.29$) (Table 1). Post-ischemic temperature was regulated as desired in HYPO(2) gerbils (Fig. 4). Post-ischemic 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 h monitoring period.

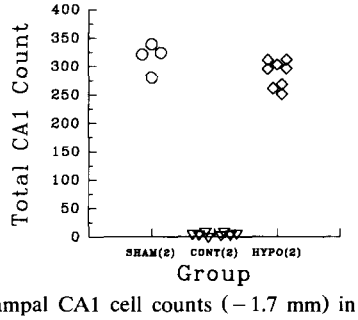

Fig. 5. Hippocampal CA1 cell counts (−1.7 mm) in experiment 2. Twenty-four hours of post-ischemic hypothermia prevented CA1 necrosis when assessed 30 days after ischemia. Neuronal protection, at 30 days, was much greater with 24 h of hypothermia (≈ 90%) than with the 12 h duration (≈ 15%) used in experiment 1 (see Fig. 3).

Ischemia caused profound CA1 loss (SHAM(2) vs. CONT(2); $P < 0.0001$) (Fig. 5). Post-ischemic hypothermia provided consistent and robust CA1 preservation (CONT(2) vs. HYPO(2); $P < 0.0001$) to a degree that HYPO(2) and SHAM(2) groups were not significantly different ($P = 0.0798$). Phloxine staining did not reveal any acidophillic CA1 neurons in HYPO(2) gerbils, suggesting persistent neuroprotection.

4. Conclusions

Twelve hours of post-ischemic hypothermia significantly reduced anterior and posterior CA1 loss against 5 min 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 min 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 is permanent or very long lasting.

In experiment 2, 24 h of post-ischemic hypothermia provided much greater neuroprotection (≈ 90%) against a 5 min ischemic episode than the 12 h 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 h of hypothermia is more effective than 12 h.

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 these habituation processes. Additional behavioral testing at later post-ischemic times is required in order to demonstrate permanent functional protection since post-ischemic 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 gerbils ($r \approx -0.53$). This suggests that either there was mini-

mal progression of cell loss from 10 to 30 days and/or more likely, behavioral impairments occurred early and were detected by the open field tests.

One important issue raised by the present findings and by Dietrich and collaborators [14] is the importance of prolonged survival time to ensure permanent protection. Dietrich and colleagues [14] reported a protracted CA1 DND with 3 h of immediate post-ischemic hypothermia in rats exposed to 10 min of 2-VO ischemia. Hypothermia reduced CA1 cell loss when assessed at 3 and 7 days, but not at 2 months following occlusion. Thus, cell loss could have been extended from a few days to several weeks. While we noted a suggestion of prolonged DND from 10 to 30 days after 5 min of ischemia in experiment 1 it may not have been significant because most cell loss may have occurred prior to day 10 (i.e. 3–10 days after ischemia), and was no longer apparent by 10–30 days. Additional cell loss could also have occurred after 30 days if the animals survived longer. Pilot data (unpublished observations) with less than 6 h of hypothermia showed no benefit (at 10 days), while 12 h (experiment 1) provided mild and 24 h (experiment 2) provided almost complete CA1 protection. Therefore, if the duration of hypothermia is too brief (e.g. 1–3 h) one might simply delay cell loss, or see no benefit at all as in the studies by Busto and colleagues [5,14] and Welsh and Harris [28]. More severe ischemia (5 min in gerbils) requires a longer hypothermic period (24 h) to reduce and not just delay cell loss. Similarly, 2 h of immediate post-ischemic hypothermia reduced CA1 loss against 8 but not 12 min of ischemia in the rat 2-VO model [8].

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). Kuroiwa et al. [20] showed that prevention of post-ischemic rectal and skull hyperthermia (with halothane) within the first 85 min after ischemia greatly attenuated CA1 loss. Their data must be qualified, however, since we [13] 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 post-ischemic 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 similar post-ischemic temperature profiles (36.87°C; average of complete post-ischemic 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 post-ischemic 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.

The mechanisms underlying the protective effect of post-ischemic hypothermia are not clear. However, CA1 neurons do show an enhanced post-ischemic sensitivity to normal synaptic input [1,26] such that depolarization causes a much increased Ca^{2+} entry than when applied before ischemia. Thus, even though glutamate levels return to normal after ischemia [3,23] CA1 cells appear supersensitive to any input. This enhancement begins approximately 1 h after ischemia and persists for many hours. Andiné and colleagues [1] showed that the degree of extracellular Ca^{2+} shift was positively correlated with eventual cell death. Similarly, Silver and Erecinska [26] argued that the change in Ca^{2+} homeostasis, and the increased intracellular Ca^{2+} content may be a cause of cell necrosis. Ischemia sets in motion some factor(s) that leads to this altered Ca^{2+} homeostasis, which in turn may initiate a cascade of events that culminate in eventual death. 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 (resulting in less glutamate release) at a time when these neurons are in a highly vulnerable state. Perhaps, the prolonged duration of hypothermia (i.e. 12 and especially 24 h) also helped the cells recover normal Ca^{2+} homeostasis. Another possible mechanism may be that hypothermia improves the recovery of post-ischemic protein synthesis even in vulnerable areas such as CA1 [29]. It is possible that hypothermia reduces CA1 loss by a host of unknown mechanisms. Regardless of the exact factors contributing to cell death it is likely that they continue over many hours especially when ischemia is prolonged (5 min in gerbils). Our data clearly show much greater benefit with 24 h of hypothermia which indicates that some key event contributing to cell loss continues beyond the first 13 h after ischemia.

Even though brain temperature was carefully regulated there were some notable group differences in experiment 1. First, animals exposed to 3 min of ischemia had a mean occlusion temperature that was slightly higher (≈ 0.5°C) than those occluded for 5 min. Brain temperature was more difficult to maintain during 5 min 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 min occluded gerbils did recover (i.e. righted, but hunched and inactive) faster than those occluded for 5 min, but there were no obvious activity differences, within the first hour, that accounted for the different temperature profiles (unpublished observations). We have no explanation for this difference. After about 1 h all gerbils show prolonged increases in activity and these seem highly correlated (positive) with brain temperature (unpublished data). Thus, the post-ischemic hyperthermia that occurred after 1 h in all CONT groups may be partially due to this hyperactivity. Fourth, HYPO-3–10 gerbils displayed more post-ischemic hyperthermia (first hour) than the CONT-3–10 group. Regardless, the greater post-ischemic rise in the HYPO group would only, at worse, reduce the beneficial effect observed in the present study.

In summary, prolonged hypothermia (12 h) initiated 1 h after either 3 or 5 min of normothermic ischemia reduced CA1 loss and the associated habituation impairment in the gerbil. Protection against 3 min of ischemia was robust and persistent while neuroprotection against a 5 min 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 h of hypothermia provided much more robust and persistent CA1 savings against 5 min of ischemia, indicating the usefulness of protracted hypothermic durations.

This study shows that post-ischemic hypothermic intervention is remarkably beneficial, and suggests that it may be a useful clinical intervention following ischemic injury. However, when initiation of hypothermia is delayed even longer (e.g. 6 h) after ischemia or when ischemia is too severe (e.g. > 5 min in gerbils) hypothermia may not, by itself, reduce damage. In such cases, combination therapy of post-ischemic hypothermia and a neuroprotective drug may prove useful.

Acknowledgements

Research support was provided by a grant from the Medical Research Council of Canada awarded to D.C. Studentship support was provided to F.C. by fellowships from Memorial University and the Medical Research Council of Canada. The authors thank B. Howell and S. Evans for excellent technical assistance. We also thank Dr. J. Evans and Dr. M. Howe for useful statistical discussion and S. Nurse for helpful comments on the manuscript.

References

[1] Andiné, P., Jacobson, I. and Hagberg, H., Enhanced calcium uptake by CA1 pyramidal cell dendrites in the post-ischemic phase despite subnormal evoked field potentials: excitatory amino acid receptor dependency and relationship to neuronal damage, *J. Cereb. Blood Flow Metab.*, 12 (1992) 773–783.

[2] Auer, R.N., Kalimo, H., Olsson, Y. and Siesjö, B.K., The temporal evolution of hypoglycemic brain damage. I. Light- and electron-microscopic findings in the rat cerebral cortex, *Acta Neuropathol. (Berl.)*, 67 (1985) 13–24.

[3] Benveniste, H., Drejer, J., Schousboe, A. and Diemer, N.H., Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis, *J. Neurochem.*, 43 (1984) 1369–74.

[4] Buchan, A. and Pulsinelli, W.A., Hypothermia but not the *N*-methyl-D-aspartate antagonist, MK-801, attenuates neuronal damage in gerbils subjected to transient global ischemia. *J. Neurosci.*, 10 (1990) 311–6.

[5] Busto, R., Dietrich, W.D., Globus M.Y.-T. and Ginsberg, M.D., Post-ischemic moderate hypothermia inhibits CA1 hippocampal ischemic neuronal injury, *Neurosci. Lett.*, 101 (1989) 299–304.

[6] Busto, R., Dietrich, W.D., Globus M.Y.-T., Valdés, I., Scheinberg, P. and Ginsberg, M.D., Small differences in intra-ischemic brain temperature critically determine the extent of ischemic neuronal injury, *J. Cereb. Blood Flow Metab.*, 7 (1987) 729–38.

[7] Carroll, M. and Beek, O., Protection against hippocampal CA1 cell loss by post-ischemic hypothermia is dependent on delay of initiation and duration, *Metabolic Brain Disease*, 7(1) (1992) 45–50.

[8] Chopp, M., Chen, H., Dereski, M.O. and Garcia, J.H., Mild hypothermic intervention after graded ischemic stress in rats, *Stroke*, 22 (1991) 37–43.

[9] Coimbra, C. and Wieloch, T., Hypothermia ameliorates neuronal survival when induced 2 hours after ischaemia in the rat, *Acta Physiol. Scand.*, 146 (1992) 543–544.

[10] Colbourne, F. and Corbett, D., Effects of D-amphetamine on the recovery of function following cerebral ischemic injury, *Pharmacol. Biochem. Behav.*, 42 (1992) 705–710.

[11] Colbourne, F. and Corbett, D., Delayed and prolonged post-ischemic brain hypothermia is neuroprotective in gerbils, *Soc. Neurosci. Abstr.*, 19 (1993) 1670.

[12] Colbourne, F., Nurse, S.M. and Corbett, D., Temperature changes associated with forebrain ischemia in the gerbil, *Brain Res.*, 602 (1993) 264–267.

[13] Colbourne, F., Nurse, S.M. and Corbett, D., Spontaneous post-ischemic hyperthermia is not required for severe CA1 ischemic damage in gerbils, *Brain Res.*, 623 (1993) 1–5.

[14] Dietrich, W.D., Busto, R., Alonso, O., Globus, M.Y.-T. and Ginsberg, M.D., Intra-ischemic but not post-ischemic brain hypothermia protects chronically following global forebrain ischemia in rats, *J. Cereb. Blood Flow. Metab.*, 13 (1993) 541–549.

[15] Green, E.J., Dietrich, W.D., van Dijk, F., Busto, R., Markgraf, C.G., McCabe, P.M., Ginsberg, M.D. and Schneiderman, N., Protective effects of brain hypothermia on behavior and histopathology following global cerebral ischemia in rats, *Brain Res.*, 580 (1992) 197–204.

[16] Hoffman, C.A. and Boast, C.A., Delayed hypothermia does not contribute to neuroprotection in gerbils, *Soc. Neurosci. Abstr.*, 18 (1992) 1587.

[17] Ito, U., Spatz, M., Walker, J.T. and Klatzo, I., Experimental cerebral ischemia in Mongolian gerbils, *Acta Neuropathol. (Berl.)*, 32 (1975) 209–223.

[18] Keppel, G., *Design and Analysis: a Researcher's Handbook*, Prentice-Hall, Englewood Cliffs, NJ, 1991.

[19] Kirino, T., Delayed neuronal death in the gerbil hippocampus following ischemia, *Brain Res.*, 239 (1982) 57–69.

[20] Kuroiwa, T., Bonnekoh, P. and Hossmann, K.-A., Prevention of post-ischemic hyperthermia prevents ischemic injury of CA1 neurons in gerbils, *J. Cereb. Blood Flow Metab.*, 10 (1990) 550–556.

[21] Loskoto, W.J., Lomax, P. and Verity, M.A., *A Stereotaxic Atlas of the Mongolian Gerbil Brain (Meriones unguiculatus)*, Ann Arbor Science Publishers, Ann Arbor, MI, 1975.

[22] Minamisawa, H., Mellergård, P., Smith, M.-L., Bengtsson, F., Theander, S., Boris-Möller, F. and Siesjö, B.K., Preservation of brain temperature during ischemia in rats, *Stroke*, 21 (1990) 758–64.

[23] Mitani, A. and Kataoka, K., Critical levels of extracellular glutamate mediating gerbil hippocampal delayed neuronal death during hypothermia: Brain microdialysis study, *Neuroscience*, 42 (1991) 661–70.

[24] Nurse, S. and Corbett, D., Direct measurement of brain temperature during and after intra-ischemic hypothermia: correlation with behavioural, physiological and histological endpoints, *J. Neurosci.*, in press.

[25] Pulsinelli, W.A., Brierley, J.B. and Plum, F., Temporal profile of neuronal damage in a model of transient forebrain ischemia, *Ann. Neurol.*, 11 (1982) 491–498.

[26] Silver, I.A. and Erecinska, M., Ion homeostasis in rat brain in vivo: intra- and extracellular $[Ca^{2+}]$ and $[H^+]$ in the hippocampus during recovery from short-term, transient ischemia, *J. Cereb. Blood Flow Metab.*, 12 (1992) 759–772.

[27] Wang, D. and Corbett, D., Cerebral ischemia, locomotor activity and spatial mapping, *Brain Res.*, 533 (1990) 78–82.

[28] Welsh, F.A. and Harris, V.A., Post-ischemic hypothermia fails to reduce ischemic injury in gerbil hippocampus, *J. Cereb. Blood Flow Metab.*, 11 (1991) 617–620.

[29] Widmann, R., Miyazawa, T. and Hossmann, K.-A., Protective effect of hypothermia on hippocampal injury after 30 minutes of forebrain ischemia in rats is mediated by post-ischemic recovery of protein synthesis, *J. Neurochem.*, 61 (1993) 200–9.

[30] Zola-Morgan, S., Squire, L.R. and Amaral, D.G., Human amnesia and the medial temporal region: enduring memory impairment following a bilateral lesion limited to field CA1 of the hippocampus, *J. Neurosci.*, 6(10) (1986) 2950–2967.