The Journal of Neuroscience, November 1995, 15(11): 7250–7260

Delayed Postischemic Hypothermia: A Six Month Survival Study Using Behavioral and Histological Assessments of Neuroprotection

Frederick Colbourne and Dale Corbett

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

In the gerbil, brief global forebrain ischemia induces profound habituation and working memory impairments that stem from delayed hippocampal CA1 death. Short duration postischemic hypothermia has been shown to reduce CA1 loss, but such reports are controversial, as it is thought that protection may be transient. The purpose of this study was to investigate whether prolonged postischemic hypothermia provided long-term CA1 and functional neuroprotection.

Previously, 90% of anterior CA1 neurons were rescued (30 d survival) when 24 hr of hypothermia (32°C) was induced 1 hr following a 5 min occlusion that otherwise produced more than 95% loss (Colbourne and Corbett, 1994). We now find about 70% CA1 savings with this same hypothermic treatment in gerbils that survived for 6 months postischemia. While this is a significant reduction from 30 day survival (medial CA1 only), it nonetheless shows, for the first time, persistent, if not permanent neuroprotection, especially in middle and lateral CA1. In addition, in nontreated animals, ischemia impaired learning in an open field and T-maze for up to 6 months. Postischemic hypothermia significantly reduced these deficits. Hypothermia (32°), when initiated 4 hr after ischemia, rescued ≈ 12% of CA1 neurons at 6 months with a slight behavioral benefit. Milder hypothermia (34°C, 1–25 hr postischemia, 30 d survival) also reduced habituation impairments and saved ≈ 60% of CA1 neurons. Similar trends were found at more caudal CA1 levels.

These results clearly show that postischemic hypothermia provides effective and long-lasting neuroprotection, which depends upon the delay to initiation, duration, and degree of cooling and survival time. The protracted functional and histological benefit observed justifies further basic and clinical investigation.

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

The CNS is exceptionally vulnerable to global ischemia. Even a brief episode produces severe hippocampal CA1 injury, which results in a devastating anterograde amnesia (Zola-Morgan et al., 1986). Like humans, ischemic rats and gerbils also show profound learning deficits (habituation and working memory impairments) that have been demonstrated with tests such as the T-maze (Volpe et al., 1988, 1992) and open field (Wang and Corbett, 1990; Babcock et al., 1993).

While the aim of clinical intervention is to lessen functional impairment, most researchers only quantify CA1 injury (cell counts), assuming that this relates linearly to learning and memory. This view is perhaps overly simplistic, since there are several factors that potentially obfuscate this relationship. For example, other vulnerable regions (e.g., hilus) are components of the circuitry subserving learning and memory, and yet these are rarely assessed. In addition, neurons may exhibit a normal histological appearance but not function properly (Bothe et al., 1986; Hori and Carpenter, 1994). Therefore, one must rigorously determine performance, in addition to histological assessment, to be certain of a therapy's benefit.

Importantly, most CA1 neurons survive ischemia only to succumb within a few days (Kirino, 1982; Pulsinelli et al., 1982). This fortuitous delay allows for opportunistic interventions directed at preventing ongoing necrosis and associated functional impairments. Perhaps the best therapy is intraischemic hypothermia, which has been shown to be remarkably protective (Bigelow et al., 1950; Pontius et al., 1954; Busto et al., 1987; Green et al., 1992; Nurse and Corbett, 1994).

Several groups have also examined (≤ 7 d survival) brief periods (≤ 6 hr) of mild postischemic hypothermia (≈ 32°C). Immediate, and even delayed (a few hours) cooling significantly reduced CA1 loss (Busto et al., 1989; Carroll and Beek, 1992; Coimbra and Wieloch, 1994). However, such results are questionable, since Dietrich and colleagues (1993) found that immediate postischemic hypothermia (3 hr at 30°C) delayed but did not prevent (2 month survival) CA1 loss in rats. We (Colbourne and Corbett, 1994) also found a transient protection (< 30 d) with a 12 hr hypothermic period (32°C) initiated 1 hr following a severe (97% CA1 loss) 5 min occlusion in the gerbil. Extending hypothermia from 12 to 24 hr resulted in dramatic (90%) and long-lasting (30 d) CA1 savings. However, it is possible that this protection would have dissipated with longer survival times.

Delayed postischemic hypothermia also reduced habituation impairments in the gerbil (Colbourne and Corbett, 1994). This was determined by open field tests given within 10 d postischemia. However, it is unknown whether functional deficits in untreated animals would show complete recovery with extended survival (e.g., Corbett et al., 1992), and if not (e.g., Ordy et al., 1988), would postischemic hypothermia attenuate such impairments?

Received Mar. 10, 1995; revised June 5, 1995; accepted July 5, 1995.

Research support was provided by grants awarded to D.C. from the Medical Research Council of Canada and the Heart and Stroke Foundation of Newfoundland. Studentship support was provided to F.C. from the MRC. We thank Bonnie Colbourne, Sue Evans, and Kathy McKay for temperature control assistance, and Suzanne Nurse for comments on the manuscript.

Correspondence should be addressed to Frederick Colbourne, University of Calgary, 3330 Hospital Drive N.W., Calgary, Alberta, Canada T2N 4N1.

Since the value of postischemic hypothermia remains controversial, we assessed whether 24 hr of hypothermia (32°C) initiated 1 hr after a 5 min ischemic episode in gerbil would reduce CA1 loss with 6 month survival. Learning ability (open field and T-maze) was also repeatedly assessed up to 6 months postischemia. Several other groups were included to better characterize neuroprotection. These were: a 4 hr delayed hypothermic treatment (32°C), a 34°C group (from 1–25 hr), and a postischemic stress group.

Preliminary results were presented in abstract form (Colbourne and Corbett, 1994).

Materials and Methods

Subjects. Experimental work was done according to the guidelines of the Canadian Council on Animal Care and, in addition, was approved by the Memorial University Animal Care Committee. Fifty-one female, Mongolian gerbils were used (High Oak Ranch, Baden, ONT). Three died during ischemia. Animals were approximately 15 weeks old and weighed about 54 gm at the time of ischemia. Groups were: SHAM (N = 10; 6 month survival), ISC (N = 14; 6 month survival), HYPO(1–32) (N = 9; 6 month survival), HYPO(4–32) (N = 8; 6 month survival), HYPO(1–34) (N = 5; 30 d survival) and STRESS (N = 5; 10 d survival). Note that for the hypothermic (HYPO) groups the numbers in parentheses refer to the postischemic start time and the degree of cooling, respectively.

Brain temperature. Brain temperature measurement was similar to previous experiments (Colbourne et al., 1993a,b, Colbourne and Corbett, 1994). Briefly, gerbils had a 5.0 mm guide cannulae implanted to the dural surface 4 d prior to ischemia. Two days later, baseline striatal (approximate depth of CA1) temperature was collected for 3 hr in awake, freely moving animals using a miniature AM brain probe (Mini-Mitter, brain probe model XM-FH, Mini-Mitter Co., Inc., Sunriver, OR).

Ischemia. Surgical procedures to induce ischemia were identical to Colbourne and Corbett (1994). Gerbils were subjected to 5 min of ischemia (ISC) or sham operation (SHAM) 2 d following normal temperature measurement. Ischemia was produced, under 1.5% Halothane in 30% O_2 and 70% N_2O, by temporary occlusion of both carotid arteries. Rectal and brain temperatures were maintained during surgery with an overhead lamp, a homeothermic body blanket (Harvard Apparatus, South Natick, MA), and a heated water blanket (Mul-T-Pads, model TP-3E, Gaymar Industries Inc., Orchard Park, NY). The latter was wrapped around the dorsal and lateral aspects of the head. Anesthetic time was approximately 20 min.

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 (abdomen and back) at 1 hr after ischemia under brief Halothane anesthesia and then returned to their boxes. Brain temperature was monitored until 26 hr postischemia in SHAM and ISC gerbils. Hypothermic groups [HYPO(1–32) and HYPO(1–34)] were cooled beginning 1 hr after ischemia to 32 and 34°C (± 0.2°C), respectively. These groups were later warmed to 37°C at 25 hr postocclusion and then temperature was monitored for 1 more hour. Another group [HYPO(4–32)] was shaved at 4 hr postischemia and then subjected to 24 hr of hypothermia (32°C). These gerbils were monitored until 29 hr postischemia. In all hypothermic groups temperature was lowered and later rewarmed by 1°C/10 min. Hypothermia was manually produced by intermittent water spray (≈ 4°C) and the frequent use of an overhead fan. If necessary, a lamp was used to warm the gerbils. Great care (continuous observation) was taken to ensure precise temperature control during the entire regulation period (see Colbourne and Corbett, 1994). The last group (STRESS) was subjected to a similar procedure as HYPO(1–32) gerbils (intermittent water spray and fan), but was not cooled. Their temperatures were maintained similar to ISC gerbils by a periodic warm air supply (hair dryer) and a lamp.

Open field testing. Gerbils were exposed to a novel open field (72 × 76 × 57 cm) for 10 min on the fifth postischemic day. Thereafter, depending on survival time, retesting occurred on days 10, 30, and 180. An image-tracking system (HVS Systems, Kingston, UK) divided the open field into 25 squares and recorded the number crossed per minute. The open field was in a sound-attenuated room with lighting conditions and environmental cues held constant throughout testing.

Unpublished open field data [Experiment 2; SHAM (N = 4), ISC (N = 8) and HYPO(1–32) (N = 8)] from Colbourne and Corbett (1994) were included in the present analysis. These groups were very similar (temperature profiles, age, weight) to the present groups, except they were only tested on days 5, 10, and 30.

Experiments by Wang and Corbett (1990) and Babcock et al. (1993) have shown that the open field is a sensitive indicator of a gerbil's ability to habituate to novelty, and thus, this test gauges hippocampal function (also see Chandler et al., 1985; Gerhardt and Boast, 1988; Mileson and Schwartz, 1991). We find that gerbils display bursts of hyperactivity (depending on ischemic severity) starting about 1 hr following ischemia, and this persists for approximately 24 hr (unpublished continuous observations). However, this subsides by the time the first open field test is given. At that time (i.e., > 24 hr), increased activity in a novel open field is largely due to an impaired habituation process and not motor hyperactivity (Wang and Corbett, 1990; Babcock et al., 1993).

T-maze testing. Only SHAM, ISC, and HYPO(1–32) gerbils were tested in the T-maze. The maze measured 47 cm (stem) by 30 cm (each arm) by 10 cm wide. Extramaze cues (window, lighting, experimenter) remained constant throughout testing. Gerbils were not food deprived, but were instead given a preferred treat (½ sunflower seed) for correct responses. To facilitate training, sunflower seeds were removed from the regular diet 27 d after surgery. Gerbils were habituated to the T-maze on days 31, 32, and 33 (2 × 5 min per day). During habituation, seeds were initially distributed throughout the maze, 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 four to five. On the FT, gerbils were randomly allowed into either the right or left arm by a door blocking the opposite arm. There were five right and five 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 sec (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 free to enter either arm. 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. However, regardless of performance, gerbils were trained for a minimum of 5 days to reduce the likelihood of reaching criterion by chance. 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 min delays over the following 5 d (only one time per day). During these delays, animals remained in the start area. A 0 min day (same as training) was given after the 5 min 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 min at 3 months and 1, 2, 3, 5, and 0 min at 5 months. One ISC gerbil was excluded from further T-maze training after the first phase because of a tendency to bite.

At the end of the 5 month testing phase (0 min delay day) another 2 training days were given. This ensured both high accuracy and similar performance among the three groups. Training was then changed to a WIN-WIN strategy. In other words, gerbils had to reenter the FT arm to get rewarded. Gerbils were trained up to a maximum of 250 trials (10 per day) on this task.

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 stored in formalin until subsequently embedded in paraffin, sectioned at 6 μm, and stained with haematoxylin and eosin. The number of remaining 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 posterior to bregma (Loskoto et al., 1975). These sectors were determined by placing a microscope grid a few cells medial from CA2 neurons (lateral sector), which are distinguished by their larger size, at the apex of CA1 (middle sector) and on the upswing of CA1 in an area clearly distinct from subiculum (medial sector). Counts were summated 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

Table 1. Mean (± SD) brain temperature (°C) during ischemia/sham occlusion and in the first postischemic hr

Group	"Occlusion"	First hour
Sham	36.63 ± 0.18	37.30 ± 0.25
ISC	36.43 ± 0.21	37.10 ± 0.35
Stress	36.40 ± 0.14	37.03 ± 0.16
HYPO(1–32)	36.38 ± 0.17	36.98 ± 0.07
HYPO(1–34)	36.34 ± 0.15	36.99 ± 0.26
HYPO(4–32)	36.43 ± 0.20	36.92 ± 0.11

Ischemic groups were not statistically different.

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 2.2 and 2.8 mm (see Fig. 6).

Statistics. Statistical procedures were as described previously (Colbourne and Corbett, 1994). Briefly, for each factorial ANOVA the homogeneity assumption was assessed by calculating F_{max} (largest s^2/smallest s^2). If this was larger than 9, the α level was lowered to 0.025 for main effects and interactions. In addition, t tests were used instead of the usual specific contrasts to reduce the influence of heterogeneity. For all specific comparisons we used an α of 0.025 if F_{max} was larger than 3 (see Keppel, 1991). Greenhouse-Geisser adjusted degrees of freedom were used in comparisons involving the time factor (minutes within a test session) because of potential violations of the homogeneity assumption.

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 one-factor ANOVAs for each of the medial, middle, and lateral sectors (6 month survival groups). The percent protection in HYPO(1–32) and HYPO(1–34) gerbils (medial, middle, and lateral sectors) were compared (t test) to the 30 d survival hypothermic (32°C) group (Experiment 2 in Colbourne and Corbett, 1994). Finally, CA1 sector comparisons were performed via paired t tests with the above α criterion. Data are presented as the mean ± SD.

Results

Brain temperature/body weight

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

Brain temperature during ischemia/sham occlusion was maintained close to normal (Table 1 and Fig. 1). There were no significant differences between ischemic groups during occlusion

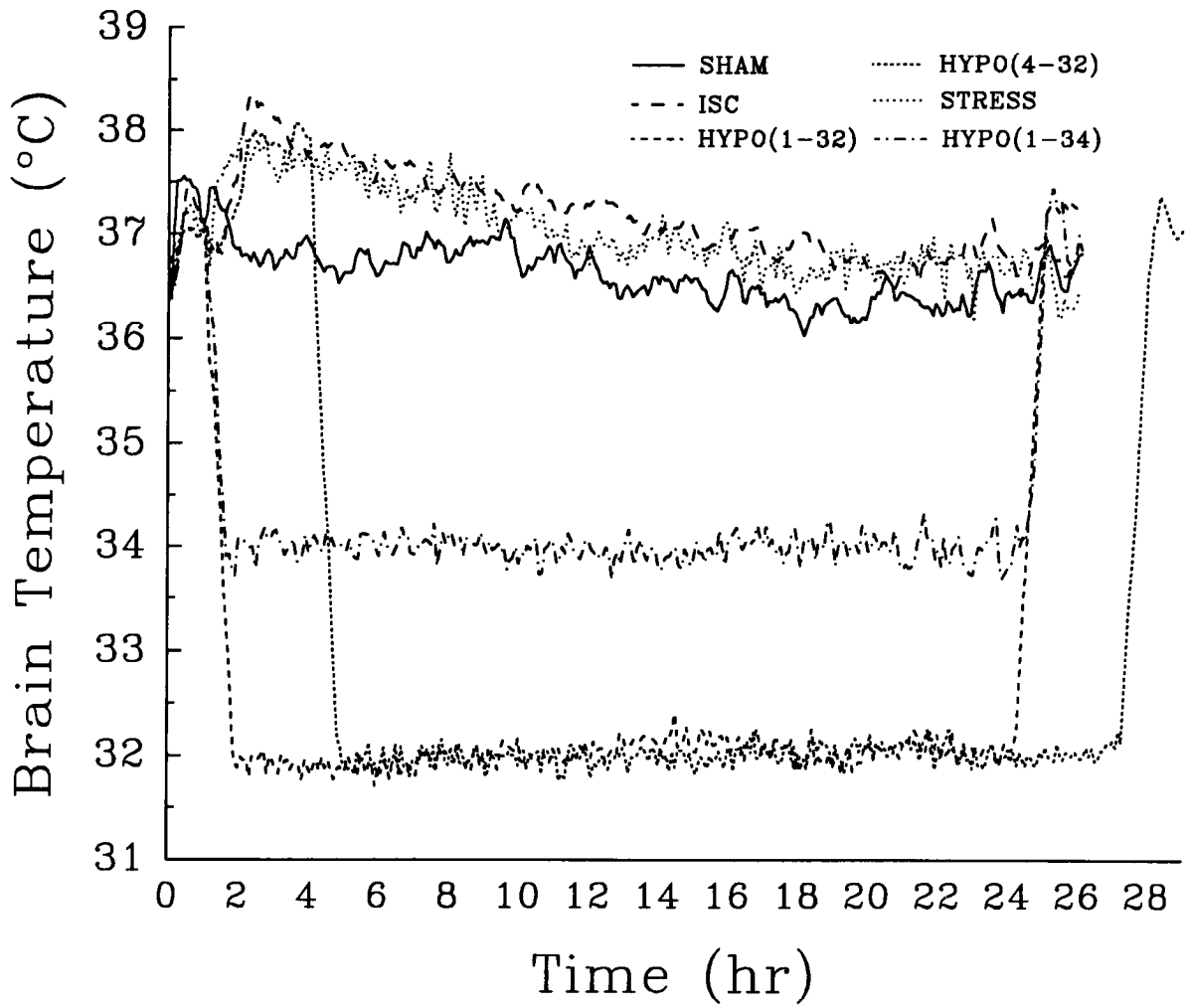

Figure 1. Brain temperature during (0 hr) and for up to 29 hr postischemia. Data were collected every 20 sec and averaged over every 5 min. See Table 1 for mean (± SD) occlusion and first hour temperatures. When appropriate temperature was manipulated by manual control of a fan, lamp, and a cold-water spray. Note that the hyperthermia observed in the ISC group beginning at about 2 hr is at least partially due to bursts of hyperactivity which diminish over the first day (unpublished data).

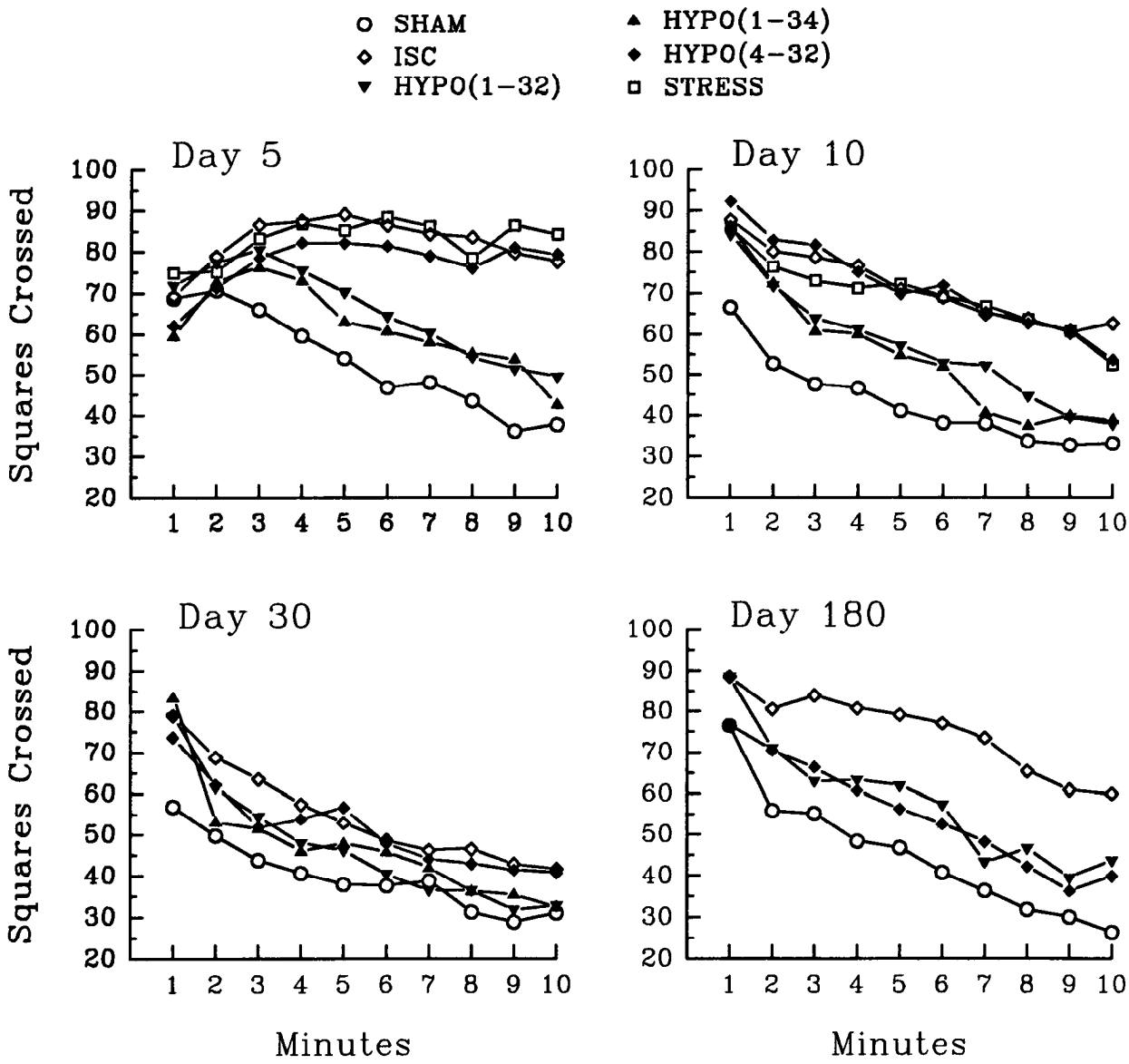

Figure 2. Mean open field activity scores (squares crossed per minute) on days 5, 10, 30, and 180 postischemia. Note that not all groups were tested on each day (see Materials and Methods). As further evidence supporting the use of the open field as a habituation test note that SHAM and ISC gerbils were similarly active during the first minute on day 5. Activity in SHAMs then steadily declined (habituation), while the ISC group had an elevated and persistent activity level (learning impairment). Therefore, these data illustrate the need to use test sessions greater than a few minutes since performance among groups was similar on the first few minutes of the first test session.

[$F(1,45) < 1$] or in the first postischemic hour (t tests, $p \geq 0.1631$). Ischemic ISC gerbils showed a mild, but prolonged spontaneous hyperthermia for the entire monitoring period as previously noted (Colbourne and Corbett, 1994). 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 (ISC and STRESS) induced an approximate 4 gm weight loss by the first postischemic day, while hypothermia (32°C) produced about an 8 gm loss [HYPO(1–32) and HYPO(4–32)]. The HYPO(1–34) group lost approximately 5 gm. By the fifth postischemic day, when open-field testing started, gerbils had regained most or all of their preischemic weight and the groups were similar from then on. Hypothermia did not produce any other noticeable side effects.

Open field testing

Ischemic gerbils (ISC) explored the open field more than SHAM animals on all test days ($p \leq 0.0001$) (Fig. 2). In addition, the pattern of habituation (group × min interaction) was different, especially on the first test day. Here, the ISC and SHAM groups initially (first min) explored to a similar extent. Then the ISC group showed an elevated and persistent activity level, while SHAM animals habituated quickly [$F(4.73,302.48) = 15$, $p < 0.0001$]. 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 ISC gerbils. Thus, untreated ischemia produced a persistent habituation impairment as revealed by simple open-field testing.

Hypothermia [HYPO(1–32)] significantly reduced ischemic impairments on all test days (vs ISC; $p \leq 0.0171$). The

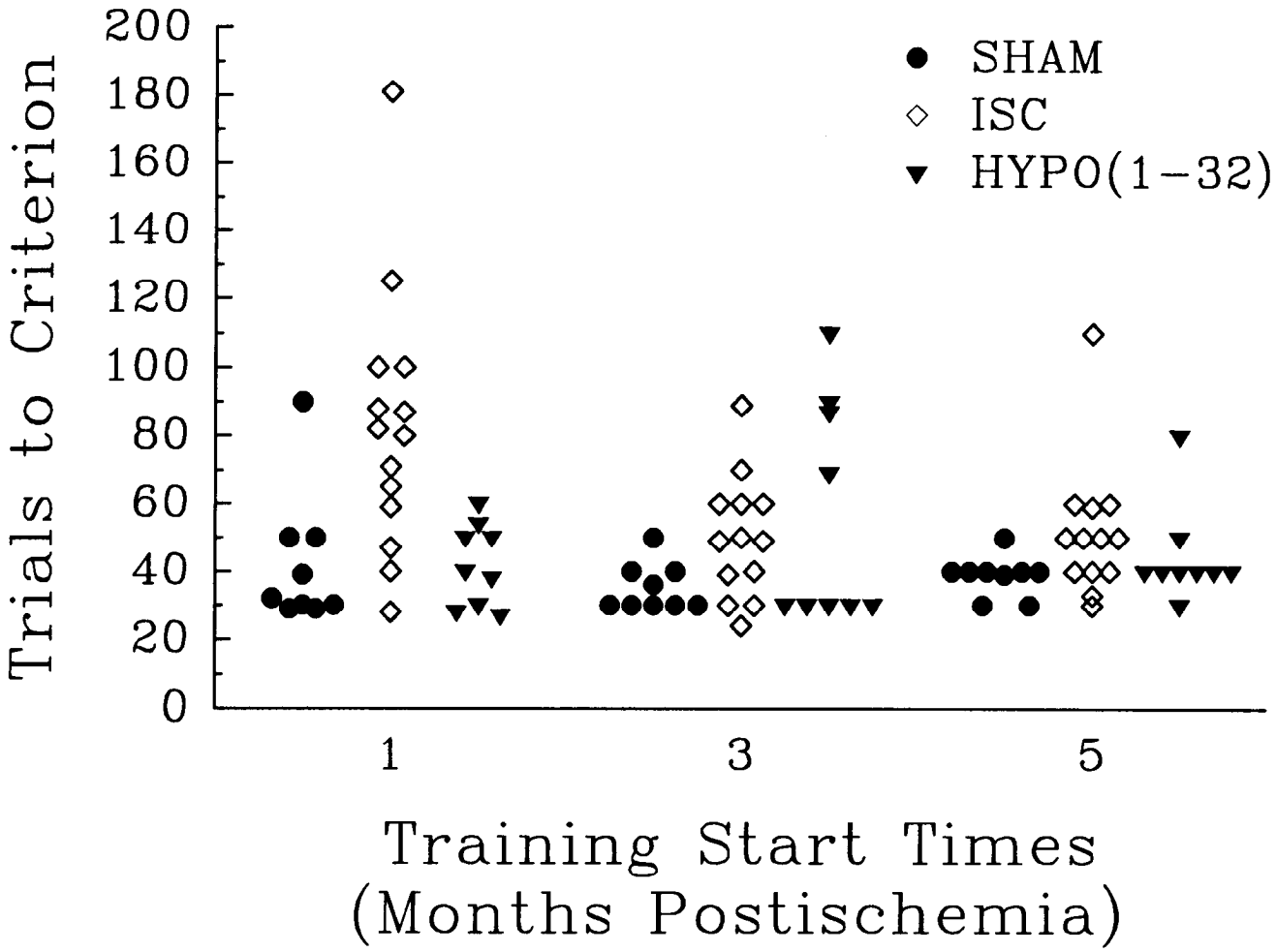

Figure 3. Trials to reach criterion (win-shift) scores for SHAM, ISC, and HYPO(1–32) groups in the T-maze at 1, 3, and 5 months postischemia. Ischemia (*ISC*) produced an initial acquisition impairment that hypothermia [HYPO(1–32)] blunted. This ischemic deficit (*ISC*) recovered with repeated testing since all groups performed similarly at 3 and 5 months. Importantly, there was a subset of ISC gerbils who were not impaired even at 1 month. This occasional lack of an apparent behavioral correlate of obvious hippocampal damage was not surprising since it also occurs with other tests (e.g., water maze, open field) and species (e.g., rats, humans).

HYPO(1–32) gerbils did, however, explore significantly more than SHAM gerbils on days 5 [$F(1,64) = 4.58$, $p = 0.0363$] and 10 [$F(1,65) = 8.18$, $p = 0.0057$], but not quite at 30 [$F(1,61) = 3.94$, $p = 0.0516$] and 180 d [$F(1,36) = 3.17$, $p = 0.0836$]. Hypothermia (34°C) also reduced ischemic impairments (vs ISC), but this was only significant on days 5 [$F(1,64) = 5.7$, $p = 0.02$, $\alpha = 0.025$] and 10 [$F(1,65) = 7.05$, $p = 0.01$] and not day 30 [$F(1,61) = 2.31$, $p = 0.1337$]. In general, the HYPO(1–32) and HYPO(1–34) groups performed similarly. Thus, hypothermia introduced 1 hr after ischemia provided persistent functional benefit. When treatment was delayed until 4 hr after occlusion [HYPO(4–32)] behavioral protection was only observed on day 180 [$F(1,35) = 7.87$, $p = 0.008$] and not on days 5, 10, and 30.

T-Maze testing

The number of trials to criterion (Fig. 3) in the T-maze (win-shift) revealed that ISC gerbils were significantly slower on initial learning than both SHAM [$t(21) = 2.89$, $p = 0.0088$, $\alpha = 0.025$] and HYPO(1–32) groups [$t(21) = 3.04$, $p = 0.0062$, $\alpha = 0.025$]. Retraining at 3 [$t(20) = 2.34$, $p = 0.03$, $\alpha = 0.025$] and 5 months postischemia [$t(20) = 1.86$, $p = 0.0775$, $\alpha = 0.025$] also revealed somewhat slower learning in the ISC group (vs SHAM), but this was not significant. There were no significant differences between HYPO(1–32) and SHAM gerbils at either 1, 3, or 5 months ($p \geq 0.077$). Finally, HYPO(1–32) and ISC 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. 4). Overall, ISC gerbils showed greater impairments over delays than the SHAM group [$F(1,26) = 8.54$, $p = 0.0071$]. The HYPO(1–32) group was not significantly different than either the ISC [$F(1,26) = 3$, $p = 0.0954$] or SHAM group [$F(1,26) = 1.29$, $p = 0.2665$]. However, there was a trend for a progressively greater impairment in ISC animals (vs SHAM), which was reduced by hypothermia [HYPO(1–32)]. Delay testing at 3 and 5 months was quite variable and did not show significant group differences (data not shown). In addition, the three groups performed equally well ($\approx$ 95% correct) on the final 3 training days (no delay), which were given upon completion of the 5 min delay test at 5+ months [$F(2,27) = 0.17$,

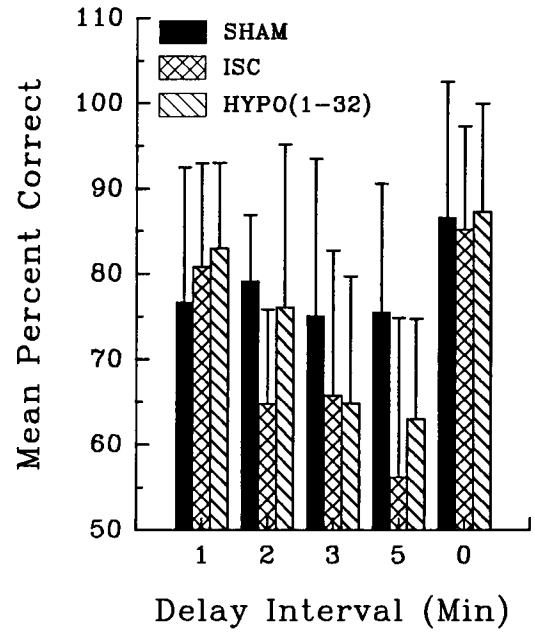

Figure 4. Delay performance (mean ± SD % correct on CT) on 1, 2, 3, 5, and 0 min delays days at 1+ months postischemia (win-shift). Note that ISC gerbils show greater impairments with longer delays than SHAM and HYPO(1–32) groups. Delay performance at 3+ and 5+ months after ischemia did not reveal any group differences (data not shown).

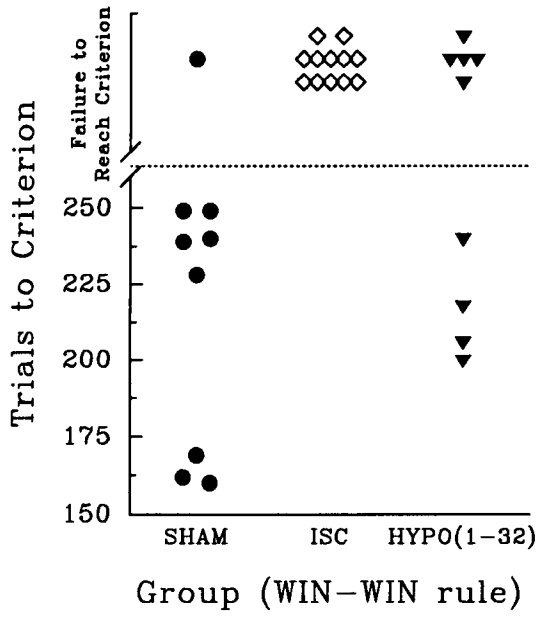

Figure 5. Trials to reach criterion scores for the win-win strategy at 5+ months postischemia. Untreated ischemia (ISC) resulted in a substantial impairment that was attenuated by hypothermia [HYPO(1–32)]. While this task did distinguish groups, it did not clearly predict the degree of neuronal savings within the HYPO(1–32) group. Perhaps this incongruity underscores the necessity of both histological and behavioral assessments (see introductory paragraphs). Finally, note that this more difficult test did not have a similar subset of nonimpaired ISC animals as did the win-shift task (Fig. 3).

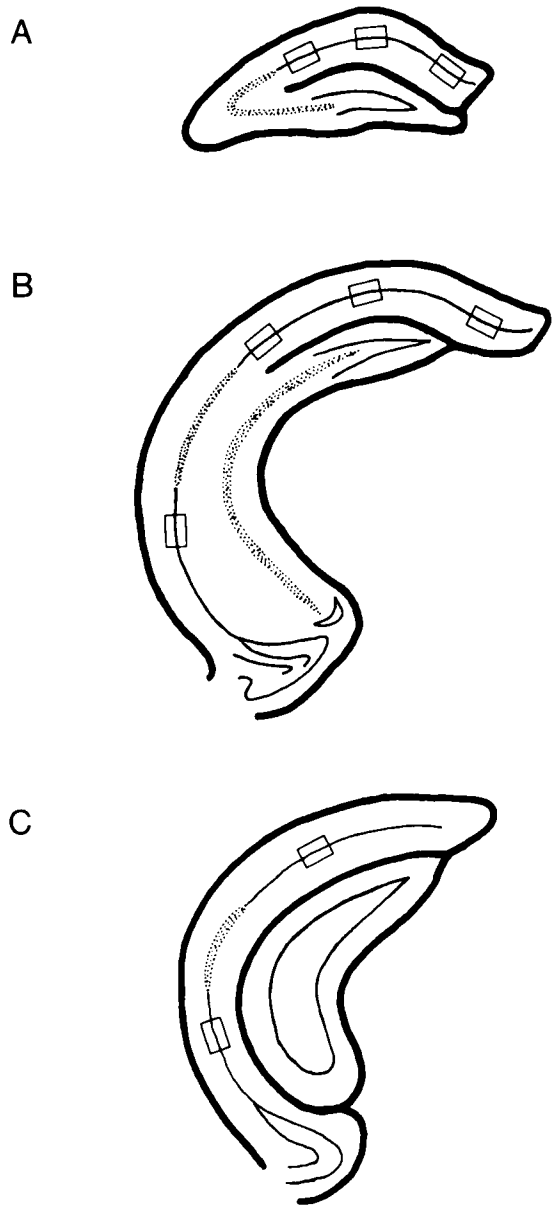

Figure 6. Necrosis was assessed by counting the number of healthy neurons (see text for further details) across the rostral (*A*), middle (*B*), and posterior (*C*) levels of the hippocampus in the CA1 sectors delineated by the *rectangles*. The *stippled pattern* indicates the CA2/CA3 regions.

$p = 0.8483$]. Thus, there was substantial functional recovery in the T-maze (win-shift strategy).

Reversing the rule to win-win did reveal group differences (Fig. 5). Eight of nine SHAM gerbils learned this strategy within 250 trials, while only four of nine HYPO(1–32) gerbils and no ISC gerbil reached criterion. Thus, ISC gerbils, who previously showed recovery with repeated win-shift training, were now impaired ($p < 0.0001$). The HYPO(1–32) group was significantly better (vs ISC, $p = 0.0211$), but was not quite as good as SHAM animals ($p = 0.0656$). During the first 2 weeks of win-win training the three groups performed similarly (% correct). At that time all groups performed at chance levels. SHAM, and to a lesser extent HYPO(1–32) gerbils, then progressively attained criterion, while ISC animals tended to perseverate at 50 to 60% correct. Finally, the latencies to complete the FT and CT in the

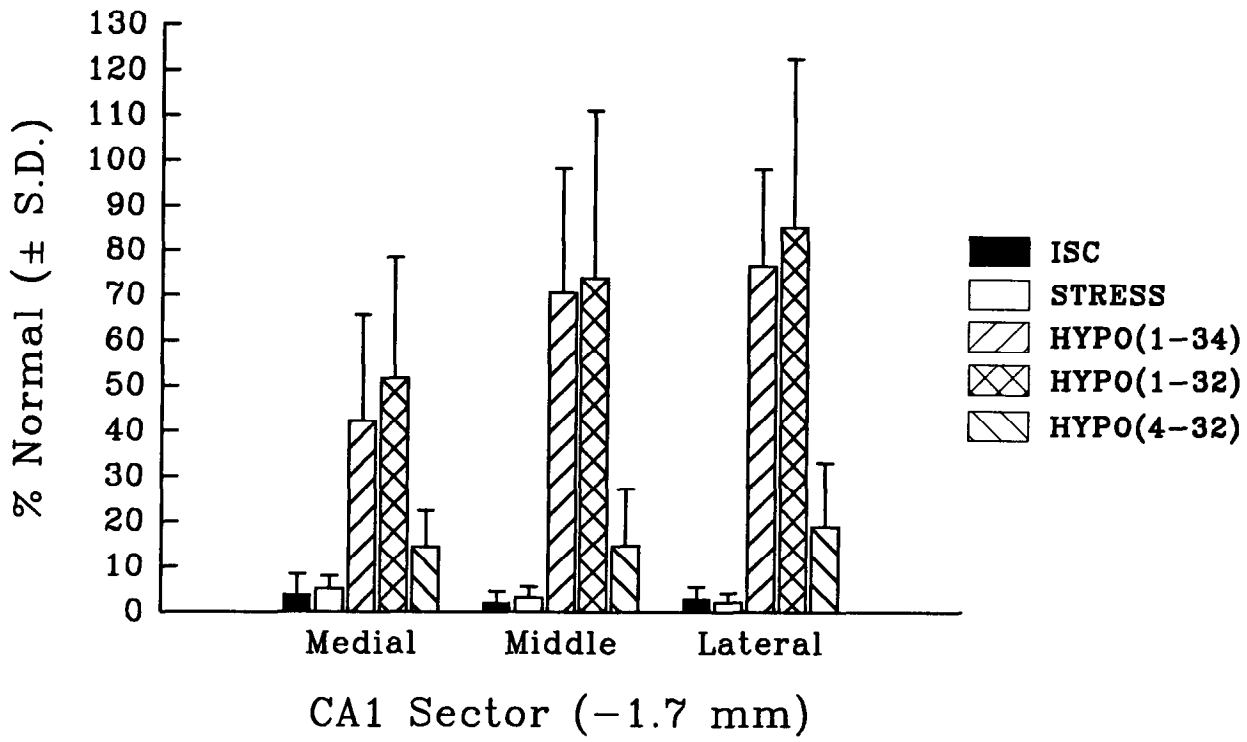

Figure 7. Percent (mean ± SD) of normal (SHAM) CA1 counts (medial, middle, and lateral at −1.7 mm to bregma) in ISC (6 month survival), HYPO(1–32) (6 month survival), HYPO(4–32) (6 month survival), STRESS (10 day survival), and HYPO(1–34) (30 d survival) groups. Untreated ischemia (ISC, STRESS) induced near-total CA1 cell loss. Postischemic hypothermia (32 or 34°C), when started at 1 and even 4 hr following occlusion, significantly reduced CA1 loss in all sectors. Previously, hypothermia (1–25 hr at 32°C) provided 87%, 91%, and 94% CA1 savings with 1 month survival (from Colbourne and Corbett, 1994). Allowing gerbils to survive for 6 months resulted in a further progression of necrosis in medial CA1. The 34°C group was also significantly less protected in medial CA1.

T-maze were similar among the three groups over all testing times (data not shown), thus indicating that the observed differences were not due to motor impairment or differences in motivation.

Histology

Five minutes of normothermic ischemia (ISC) induced severe cell loss (≈ 97% loss) in the medial, middle, and lateral CA1 sectors (1.7 mm posterior to bregma). This was not altered by the STRESS procedure. Previously (Colbourne and Corbett, 1994), postischemic hypothermia (from 1–25 hr at 32°C) was found to significantly attenuate this loss with 30 d survival in the medial, middle, and lateral sectors (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 ISC) in all sectors with 6 month survival [$t(21) \geq 6.54$, $p < 0.0001$, $\alpha = 0.025$] (Fig. 7). However, unlike at 30 d, 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 d 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 [$t(20) \geq 3.91$, $p \leq 0.0015$, $\alpha = 0.025$] even occurred when hypothermia was started 4 hr postischemia [HYPO(4–32)] with no significant sector differences. However, this was significantly less than the HYPO(1–32) group [$t(15) > 3.79$, $p \leq 0.0018$, $\alpha = 0.025$]. Milder hypothermia [HYPO(1–34)] initiated at 1 hr postischemia was also significantly protective at 30 d 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 d). 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(1–34) 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. 8), except hypothermic protection was even more pronounced. In medial CA1 both the HYPO(1–32) and HYPO(1–34) 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 (Colbourne and Corbett, 1994) to 180 day survival with the HYPO(1–32) treatment [$t(15) \leq 1.96$, $p \geq 0.0685$, $\alpha = 0.025$]. Protection was also better in the HYPO(4–32) 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. 9) in the ISC and STRESS groups was somewhat less than at the more anterior levels. All three hypothermic groups were significantly ($p < 0.0001$) protected (vs ISC). The percentage of remaining CA1 neurons was approximately that of the preceding level for the HYPO(1–32) group. While there was about 20% more protection in the HYPO(1–34) group, protection was still significantly less than that of the 30 day survival, 32°C group [$t(11) = 2.28$,

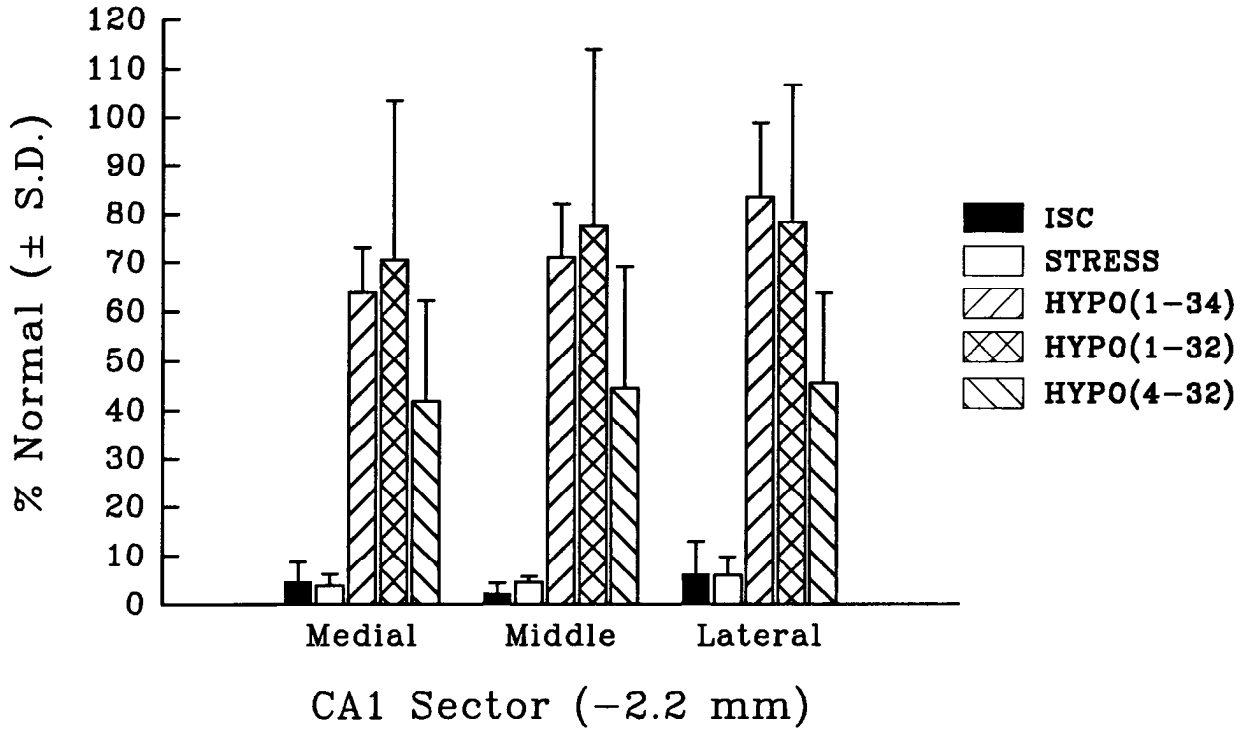

Figure 8. Percent (mean ± SD) of normal CA1 counts (medial, middle, and lateral) at the −2.2 mm level. Ischemia (ISC and STRESS) produced severe CA1 loss in all three sectors. Unpublished data (from Colbourne and Corbett, 1994) showed that hypothermia [HYPO(1–32), 30 day survival] resulted in cell counts 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.

$p = 0.0436$]. Notably, the HYPO(4–32) 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 were negligible (data not shown).

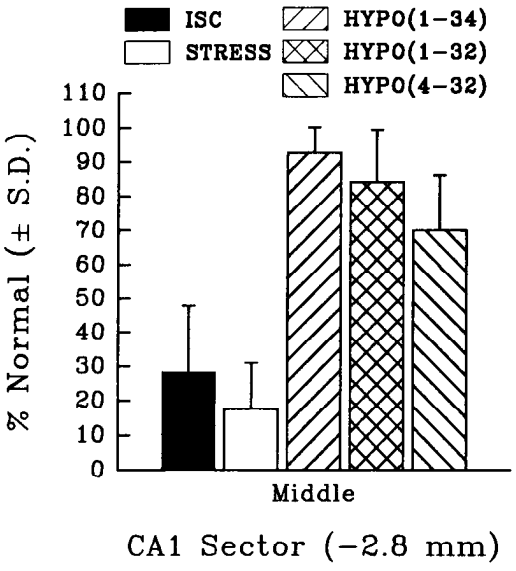

Figure 9. Percent (mean ± SD) of normal counts in the middle CA1 sector at −2.8 mm to bregma. Ischemic damage (ISC and STRESS) was somewhat less at this level compared to the previous two anterior levels. Protection by postischemic hypothermia was robust and significant. Unpublished data (from Colbourne and Corbett, 1994) showed that hypothermia [HYPO(1–32)] resulted in complete CA1 protection (101.56 ± 6.43% of normal) with 30 d survival. The 6 month survival group was slightly, but significantly less protected than the 30 d survival animals [$t(15) = 2.99$, $p = 0.0092$, $\alpha = 0.025$].

Correlations

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 in all gerbils) significantly correlated with histological outcome at 6 months (total % normal at the −1.7 mm level). The number of trials to reach criterion in the T-maze (win-shift) at 1 month also significantly predicted these cell counts ($r = -0.527$, $p = 0.0019$). Finally, behavioral scores were intercorrelated, 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, $p \leq 0.0157$).

Discussion

A 24 hr hypothermic period (32°C) initiated 1 hr 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 d survival, which indicates that CA1 neurons may continue to die beyond 1 month postischemia. 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 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. First, we had previously shown that a 24 hr cooling period was approximately 6 times more effective than a 12 hr interval (Colbourne and Corbett, 1994). Thus, it makes sense that a very brief temperature reduction (e.g., 3 hr) like that used by Dietrich and colleagues (1993) 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 min 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. However, posterior CA1 was more amenable to treatment, especially in the HYPO(4–32) group. This is probably due to less severe ischemia at more posterior (and ventral) levels. This concurs with our previous findings (Colbourne and Corbett, 1994) of greater savings against 3 (vs 5) min of ischemia. Overall, these results underscore the importance of evaluating several CA1 regions and levels.

Ischemia (ISC) caused severe and persistent habituation impairments in the open field. However, with repeated early testing, these deficits appear to partially recover (Wang and Corbett, 1990; Colbourne and Corbett, 1992, 1994; Babcock et al., 1993). 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 (CA3, dentate, and more posterior and ventral CA1) 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 intertest 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., ISC 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 ISC gerbils failed to learn the win-win strategy, including those with win-shift scores similar to SHAM and HYPO(1–32) gerbils, we conclude that the ischemic win-win impairment is not due to a selective overtraining in the ISC group, but to a residual learning deficit. Thus, while substantial recovery of function is often 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 (Zola-Morgan et al., 1986, 1992).

Postischemic hypothermia [HYPO(1–32)] was an unquestionably effective and persistent behavioral neuroprotectant since it significantly and chronically reduced the open field habituation impairments and 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(1–32) 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 d 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 function. Since these perturbations can take months to mature, it is not sufficient to rely solely on cell counts to estimate 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, since the HYPO(1–34) (1 month survival) and HYPO(1–32) (6 months) 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(1–32) and HYPO(1–34) groups were similarly protected (final outcome). If small differences exist, more extensive behavioral testing, larger group sizes or a 6 month HYPO(1–34) group may distinguish such gradations in injury.

Hypothermia (32°C) also significantly reduced CA1 loss when started at 4 hr postischemia, but was clearly less effective than intervening at 1 hr. The HYPO(4–32) group also showed mild behavioral protection, but only at 6 months 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 ISC and SHAM groups are not perfect controls and it cannot be absolutely concluded that 4 hr delayed hypothermia is of functional benefit. Thus, there is was critical period in the first few hr following ischemia when intervention was most effective.

The stress of the cooling procedure per se did not improve outcome since the STRESS and ISC 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 (see Clifton et al., 1992) and, hence, it is 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.

From these experiments it is clear that further investigation is warranted. 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 since this may depend on many factors (e.g., ischemic severity). We have shown that a 24 hr hypothermic period must be initiated within 4 hr after severe ischemia to have significant benefit, which may explain some of the early unconvincing clinical reports with postischemic cooling where therapy was often delayed for many hours (Williams and Spencer, 1958; Benson et al., 1959).

An understanding of how hypothermia reduces CA1 loss could be of tremendous value as it may lead to a more selective and effective therapy. Presumably, postischemic hypothermia protects by several mechanisms. Perhaps the two most important factors contributing to ischemic injury in vulnerable brain regions (e.g., CA1) are an altered Ca^{2+} homeostasis (Andiné et al., 1988, 1992; Silver and Erecinska, 1992), and a persistent protein synthesis inhibition (Bodsch et al., 1985; Thilmann et al., 1986; Araki et al., 1990). Both of these derangements continue for many days (perhaps months) after ischemia and it is

possible that the cumulative deleterious effects of increased Ca^{2+} entry coupled with an impaired protein synthesis results in excessive damage (via lipases, proteases, endonucleases), which the cell is unable to repair. Prolonged hypothermia may reduce the excitatory drive on CA1 neurons, which would attenuate the toxic Ca^{2+} buildup at a time when cooling is promoting recovery of protein synthesis (Widmann et al., 1993). This would explain why hypothermia must begin within a few hours of ischemia to be persistently neuroprotective since the events that culminate in neuronal injury begin during ischemia, and the cell may be irreversibly injured within a few hours.

In summary, these data conclusively show that delayed postischemic hypothermia can substantially improve functional and histological outcome with long survival times. These findings dictate the use of prolonged temperature control ($\geq$ 24 hr) in all pharmacological experiments where a mechanism of action is sought. Furthermore, such studies must employ longer survival times ($\geq$ 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 and electrophysiology (Nurse and Corbett, 1994) appears to more accurately reflect the final outcome.

Delayed (1 and 4 hr) and protracted (24 hr) postischemic hypothermia (32 or 34°C) significantly reduced ischemic impairments (behavioral and histological) for up to 6 months postischemia. While, it is not certain that this protection is permanent, the long-lasting savings presently found makes this an ideal candidate for clinical investigation.

References

Andiné P, Jacobson I, Hagberg H (1988) Calcium uptake evoked by electrical stimulation is enhanced postischemically and precedes delayed neuronal death in CA1 of rat hippocampus: involvement of *N*-methyl-D-aspartate receptors. J Cereb Blood Flow Metab 8:799–807.

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

Araki T, Kato H, Inoue T, Kogure K (1990) Regional impairment of protein synthesis following brief cerebral ischemia in the gerbil. Acta Neuropathol 79:510–505.

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

Benson DW, Williams GR, Spencer FC, Yates AJ (1959) The use of hypothermia after cardiac arrest. Anesth Analg 38:423–428.

Bigelow WG, Callaghan JC, Hopps JA (1950) General hypothermia for experimental intracardiac surgery. Ann Surg 132:531–537.

Bodsch W, Takahashi K, Barbier A, Grosse Ophoff B, Hossmann K-A (1985) Cerebral protein synthesis and ischemia. Prog Brain Res 63: 197–210.

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

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

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

Carroll M, Beek O (1992) Protection against hippocampal CA1 cell loss by post-ischemic hypothermia is dependent on delay of initiation and duration. Metabol Brain Dis 7:45–50.

Chandler MJ, DeLeo J, Carney JM (1985) An unanesthetized-gerbil model of cerebral ischemia-induced behavioral changes. J Pharm Methods 14:137–146.

Clifton GL, Allen S, Berry J, Koch SM (1992) Systemic hypothermia in treatment of brain injury. J Neurotrauma 9:487–495.

Coimbra C, Wieloch T (1994) Moderate hypothermia mitigates neuronal damage in the rat brain when initiated several hours following transient cerebral ischemia. Acta Neuropathol 87:325–331.

Colbourne F, Corbett D (1992) Effects of *d*-Amphetamine on the recovery of function following cerebral ischemic injury. Pharmacol Biochem Behav 42:705–710.

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

Colbourne F, Corbett D (1994) Does prolonged postischemic hypothermia convey permanent neuroprotection. Soc Neurosci Abstr 20: 189.

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

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

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

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

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

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

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

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

Keppel G (1991) Design and analysis: a researcher's handbook. Englewood Cliffs, NJ: Prentice-Hall.

Kirino T (1982) Delayed neuronal death in the gerbil hippocampus following ischemia. Brain Res 239:57–69.

Loskoto WJ, Lomax P, Verity MA (1975) A stereotaxic atlas of the Mongolian gerbil brain. Ann Arbor, MI: Ann Arbor Science Publishers.

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

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

Ordy JM, Thomas GJ, Volpe BT, Dunlap WP, Colombo PM (1988) An animal model of human-type memory loss based on aging, lesion, forebrain ischemia, and drug studies with the rat. Neurobiol Aging 9:667–683.

Pontius RG, Bloodwell RD, Cooley DA, De Bakey ME (1954) The use of hypothermia in the prevention of brain damage following temporary arrest of cerebral circulation: experimental observations. Surg For 5:224–228.

Pulsinelli WA, Brierley JB, Plum F (1982) Temporal profile of neuronal damage in a model of transient forebrain ischemia. Ann Neurol 11:491–498.

Silver IA, Erecinska M (1992) 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 12:759–772.

Sutherland RJ, Whishaw IQ, Kolb B (1988) Contributions of cingulate cortex to two forms of spatial learning and memory. J Neurosci 8(6): 1863–1872.

Thilmann R, Xie Y, Kleihues P, Kiessling M (1986) Persistent inhibi-

tion of protein synthesis precedes delayed neuronal death in postischemic gerbil hippocampus. Acta Neuropathol 71:88–93.

Volpe BT, Waczek B, Davis HP (1988) Modified T-maze training demonstrated dissociated memory loss in rats with ischemic hippocampal injury. Behav Brain Res 27:259–268.

Volpe BT, Davis HP, Towle A, Dunlap WP (1992) Loss of hippocampal CA1 neurons correlates with memory impairment in rats with ischemic or neurotoxin lesions. Behav Neurosci 106:457–464.

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

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

Williams GR, Spencer FC (1958) The clinical use of hypothermia following cardiac arrest. Ann Surg 148:462–466.

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

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