Behavioral Testing Does Not Exacerbate Ischemic CA1 Damage in Gerbils

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Background and Purpose—Previous research studying ablative lesions has suggested that functional use may exacerbate brain injury. If true, this would have considerable ramifications not only for the mechanistic understanding of neuronal injury but also for the clinical use of physiotherapy. In this report the hypothesis that behavioral use of brain tissue exacerbates ischemic hippocampal injury was tested.

Methods—Gerbils were subjected to sham operation or 5 minutes of normothermic ischemia. To produce borderline hippocampal CA1 injury and enhance susceptibility to exacerbation, 2 of 3 ischemic groups were cooled (>48 hours) beginning at 6 hours after ischemia. Increased use of the hippocampus was produced by a battery of tests involving 3 novel small mazes, a T maze, and an open field. One hypothermic group was not tested and served as a control.

Results—Behavioral testing failed to worsen ischemic damage since neuronal loss in the behaviorally tested and untested hypothermic groups was 12% and 8%, respectively, while that in the untreated ischemic group was 81% at a 1-month survival. Accordingly, protected CA1 cells tolerated the neuronal activity associated with behavioral testing. Concomitant with marked CA1 neuroprotection, a significant reduction in behavioral deficits with the hypothermic treatment was observed. Importantly, behavioral testing was found to transiently elevate brain temperature.

Conclusions—CA1 neuronal survival was unaffected by behavioral testing or the associated mild fever. Hypothermia delayed for 6 hours provided sustainable CA1 neuroprotection. (Stroke. 1998;29:1967-1971.)

Key Words: cerebral ischemia • fever, stress-induced • hippocampus • hypothermia • gerbils
after an acclimation period of 4 to 6 weeks (weight, ~54 g). There were 4 groups: SHAM (n = 14) (sham operation), ISCH (n = 12) (normothermic ischemia), ISCH+HYPO (n = 20) (ischemia plus hypothermia), and ISCH+HYPO-NT (n = 20) (ischemia plus hyperthermia, not tested) (see below for complete description of groups). All groups except ISCH+HYPO-NT were given behavioral tests of learning and memory. During the experiment, animals had free access to food and water and were housed individually under diurnal light conditions (lights on at 8 AM and off at 8 PM). This experiment conformed to the guidelines of the Canadian Council on Animal Care as well as the standards of the Animal Care Committee of the University of Calgary.

Ischemia and Temperature Control

Gerbils were implanted (65 mg/kg IP sodium pentobarbitol [Somnotol]) with a guide cannula as described previously.\(^1\) After 2 days, telemetry brain temperature probes (model XM-FH, Mini-Mitter Co) were inserted under brief halothane anesthesia. Animals recovered in individual cages (23 cm wide×14 cm high×31 cm long) that rested on RLA-1020 receivers interfaced to a computer running DataQuest IV (DataSciences, Int). Baseline temperature (2-second average from anterior dorsal striatum) and general motor activity (30-second sum) were recorded every 30 seconds and averaged every 5 minutes.

Ischemia or sham occlusion surgery was done between 8 AM and 1 PM 4 days after cannula implantation. Briefly, gerbils were anesthetized with halothane (2.0% induction, 1.5% maintenance, 0.5% during ischemia) followed by a midline neck incision, isolation of both carotid arteries, and subsequent occlusion (except for SHAM) with microaneurysm clips for 5 minutes followed by verification of reflow. Core temperature was kept near 37°C by a heating blanket, and brain temperature was regulated to 36.4°C by an overhead infrared lamp.\(^1\)

After sham operation, the gerbils were returned to their cages for monitoring. After ischemia, animals were shaved to facilitate subsequent temperature regulation for 2 to 3 days in their cages. One group of ischemic animals (ISCH) had their temperature regulated at a mild hyperthermic pattern\(^14,15\) for 24 hours and were then kept from falling below 35.5°C for an additional 24 hours. This was to minimize variability. The ISCH+HYPO and ISCH+HYPO-NT groups were subjected to a similar pattern for the first 6 hours. At that time they were slowly cooled (1.0°C/30 min) to 32°C and kept at that temperature for 24 hours. Gerbils were then rewarmed (1.0°C/30 min) to 34°C and kept at that temperature for 24 hours. After this, animals were rewarmed to 35°C and kept at 35°C and 36°C for 12 hours. All groups were monitored for ~6.5 days, at which time they were briefly anesthetized and their brain probes were removed.

Temperature regulation after surgery was achieved in the awake, freely-moving animal by a precise (±0.2°C) servo-controlled system that used infrared lamps (175 W), fans, and fine water misters.\(^1\) Gerbils had free access to food (rat chow) and water throughout this experiment. After surgery, for the first 4 days only, gerbils were given ~7 g of mixed hamster food (Staple VME Diet, Hagen) daily. This seed mixture is preferred over their regular diet and has the advantage of not getting soaked by the water misters.

Behavioral Testing

Gerbils in the SHAM, ISCH, and ISCH+HYPO groups were repeatedly tested, while gerbils in the ISCH+HYPO-NT group were not. Since tests of spatial learning (small mazes, open field) and working memory (T maze) require the use of the hippocampus, the ISCH+HYPO and ISCH+HYPO-NT groups were familiarized to a T maze (60 cm stem length, 30 cm arm lengths, 10 cm wide, 12 cm high) for three 5-minute sessions per day. During the next 6 days, gerbils were given 10 pairs of trials per day in which they received sunflower seeds (not food deprived) as reward. This T-maze procedure, which measures spatial working memory, was previously used in the gerbil and found to reflect hippocampal integrity.\(^13,12\)

Histology

Gerbils were killed at 30 to 31 days after ischemia by an overdose of Somnotol and transcardiac perfusion with 30 mL of heparinized saline and 120 mL of 4.0% buffered formaldehyde. Brains were left in situ overnight in fixative before removal. They were then processed, embedded in paraffin, and coronally sectioned at 6 μm. Sections were stained with hematoxylin and eosin. Viable (not eosinophilic) CA1 neurons were counted in medial (next to subiculum), middle, and lateral (adjacent to CA2) grids (each 0.2 mm long) at ~1.7 and ~2.2 mm to bregma,\(^13\) as described previously.\(^13\) The number of viable neurons was compared between groups by a 1-factor ANOVA (for each CA1 region) with specific group contrasts.

Sections of the heart, liver, lung, kidney, and adrenal gland were also collected, grossly examined, processed, and stained with hematoxylin and eosin.

Results

Basal brain temperature collected the day before ischemia/sham occlusion surgery was similar between groups, with a range of 36.4°C to 36.7°C, which is similar to previous work.\(^14,16,19\) Brain temperature during and after ischemia (Figure 1) was regulated as desired (see Materials and Methods). There were no significant differences between the ISCH (35.9±0.6°C) (~SD), ISCH+HYPO (35.9±0.4°C), and ISCH+HYPO-NT (36.0±0.3°C) groups during occlusion. The temperature of these groups was also similar (~<0.25°C difference) for the first 6 hours after ischemia, at which time cooling was induced in the hypothermia groups, as intended.
See Materials and Methods for description of groups.

Attenuated fever that recovered to the level of the other groups the small mazes, while the ISCH SHAM and ISCH groups displayed a similar febrile response to

Data are averaged over the 3 sessions on each test day. The start of each maze session, which is also denoted by a bar.

Notable, however, was the finding that exposure to the small mazes caused a rise in brain temperature that began with or immediately after placement in the small mazes and continued for a brief time after maze exposure. These temperature elevations, seen at hyperthermia were similar in SHAM and ISCH groups, and focal ischemia and traumatic brain injury. In this study, the brief elevations in temperature associated with behavioral testing were not usually >38°C, and they occurred ≥4 days after ischemia and therefore were unlikely to influence outcome.

Exposure to the small mazes on days 4, 5, and 6 elevated brain temperature. Others have also noted that stressful situations, such as a novel open field, can increase temperature. Briefly, untreated ischemia resulted in impairments on all tests, and these deficits were attenuated by hypothermic treatment. Notable, however, was the finding that exposure to the small mazes caused a rise in brain temperature that began with or immediately after placement in the small mazes and continued for a brief time after maze exposure. These temperature elevations, seen at approx. 102, 126, and 150 hours after ischemia (Figure 1), were due to placing the gerbils in the novel mazes. While hyperthermia was similar in SHAM and ISCH groups, it was initially (day 4 after ischemia) blunted in the ISCH+HYPO group (Figure 2). Temperature during these maze sessions did not correlate with CA1 damage (r=0.03).

Extensive (overall counts averaged ~19% of normal) CA1 damage (Figure 3) occurred in the ISCH group (P<0.0001). Hypothermia significantly (P<0.0001) and almost completely attenuated CA1 injury in all sectors and levels at a 30-day survival. There were no significant (F1,56 <1) CA1 differences between ISCH+HYPO and ISCH+HYPO-NT groups, and therefore behavioral testing did not lessen CA1 neuroprotection.

**Discussion**

The hypothesis that hippocampal-dependent tasks might promote CA1 neurodegeneration after ischemia and hypothermic intervention was not supported because behavioral testing did not affect neuroprotection. Earlier or more prolonged testing than that presently used might reveal a detrimental effect. Clearly, any such effect would be difficult to discern from behaviorally induced increases in brain temperature. Delayed hyperthermia is known to increase brain injury after global and focal ischemia and traumatic brain injury. In this study, the brief elevations in temperature associated with behavioral testing were not usually >38°C, and they occurred ≥4 days after ischemia and therefore were unlikely to influence outcome.

One SHAM gerbil was excluded because its dental cap and probe became loose and fell off by day 4 after ischemia. In total, 4 hypothermic gerbils died. One ISCH+HYPO gerbil died unexpectedly at 19 days of unknown cause. This gerbil had completed behavioral testing and had an intact CA1 zone (only signs of autolysis and not necrosis) that was similar to others in its group. The histology data were not included because of the shorter survival time. Three ISCH+HYPO-NT gerbils died during or soon after hypothermia of unknown cause. Histopathological examination of sections from the heart, lung, liver, kidney, and adrenal gland of the first 26 gerbils entered into the study did not reveal any group differences or deleterious effects of cerebral ischemia or cooling. The only deleterious effect of hypothermia was a temporary loss of weight (~4 g by day 7), which has been repeatedly found. It is possible that the animals that died prematurely (ISCH+HYPO-NT) did not have sufficient energy reserves to tolerate this lengthy hypothermic treatment.

The behavioral data are presented elsewhere. Briefly, untreated ischemia resulted in impairments on all tests, and these deficits were attenuated by hypothermic treatment. Notable, however, was the finding that exposure to the small mazes caused a rise in brain temperature that began with or immediately after placement in the small mazes and continued for a brief time after maze exposure. These temperature elevations, seen at ~102, 126, and 150 hours after ischemia (Figure 1), were due to placing the gerbils in the novel mazes. While hyperthermia was similar in SHAM and ISCH groups, it was initially (day 4 after ischemia) blunted in the ISCH+HYPO group (Figure 2). Temperature during these maze sessions did not correlate with CA1 damage (r=0.03).

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**Figure 2.** Brain temperature (°C) averaged every 5 minutes from 15 minutes before the small-maze sessions to 30 minutes after the end of each session. The time on the x axis is relative to start of each maze session, which is also denoted by a bar. Data are averaged over the 3 sessions on each test day. The SHAM and ISCH groups displayed a similar febrile response to the small mazes, while the ISCH+HYPO group initially had an attenuated fever that recovered to the level of the other groups on day 6 after ischemia. The greatest temperature rise occurred near the end of each maze session and dissipated thereafter. See Materials and Methods for description of groups.

**Figure 3.** CA1 cell counts (percentage of SHAM ±SD) at 1.7 and 2.2 mm posterior to bregma. Normal-looking neurons were counted in medial, middle, and lateral sectors, each being 0.2-mm lengths of the CA1 pyramidal layer. Six-hour delayed hypothermia significantly blunted injury (vs ISCH, P<0.0001) in all sectors at both levels, with no significant differences between the ISCH+HYPO and ISCH+HYPO-NT groups. Only the medial −1.7 mm sector of the ISCH+HYPO group had counts significantly lower than SHAM. See Materials and Methods for description of groups.
erved not only with early rehabilitation efforts but also in mechanistic studies of neuronal injury. Notably, several other factors may explain the different conclusions of this study and that of Kozlowski et al. First, these studies used different models of brain injury. Second, behavioral testing was initiated immediately after injury in the cortical lesion study, whereas it was begun 4 days after ischemia in the present study and behavioral manipulations/testing were much briefer in the present study. Thus, quicker and longer behavioral testing paradigms may have a greater detrimental effect. Further experiments are needed to determine this.

This study also shows that 6-hour delayed hypothermia can significantly and persistently (30 days) reduce CA1 neuronal injury after forebrain ischemia in the gerbil. This is a substantial improvement over previous work in which we found very limited CA1 protection with a 4-hour delayed hypothermic intervention (24 hours’ duration) after ischemia. The superior efficacy in the present study was likely due to the use of a longer duration of hypothermia (>48 hours).

In summary, the data show that behavioral testing and the associated transient fever did not lessen the neuroprotective effects of prolonged postischemic hypothermia. It remains possible that behavioral pressure may have greater detrimental effects following the use of less efficacious neuroprotectors or after other types of stroke (eg, focal ischemia). Such studies must necessarily control brain temperature if mechanistic conclusions are to be made regarding the effect of neuronal use on brain injury.

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References


This study tested the hypothesis that behavioral testing after transient global ischemia in gerbils exacerbates CA1 hippocampal damage. This interesting question is the result of previous data from other laboratories showing that “excessive rehabilitation” procedures initiated immediately after focal brain injury aggravate outcome.1,2 In the present study, behavioral tests requiring the use of the hippocampus failed to worsen CA1 hippocampal damage, while postischemic hypothermia improved histopathological and behavioral outcome.

The question of whether repeated episodes of behavioral testing adversely influence ischemic outcome is of obvious clinical interest in terms of rehabilitation strategies to promote functional recovery after central nervous system injury. Using the present study protocol, the authors could not demonstrate that cognitive testing worsened outcome. Significant differences between the study design and previous investigations,1,2 in which the extensive use of a forelimb led to more cortical damage, most likely explain the negative findings. First, unlike previous studies, behavioral testing in this study was initiated days after the ischemic insult. Second, the overuse of 1 limb in previous studies was continuous for an extended period, while the present behavioral testing procedures were not considered extensive. Thus, the degree and temporal profile of the induced behavioral stress as well as the type of brain injury (ie, global versus focal) may be important variables in determining whether use-dependent exaggeration of neuronal injury can be demonstrated.

It is known that elevations in core and brain temperature occur in rodents during periods of increased motor activity. Thus, the present observation that behavioral testing transiently increased brain temperature in sham and ischemic gerbils is not surprising. Whether activity-induced mild hyperthermia is involved in the detrimental consequences of previous casting studies is, however, extremely interesting. We know from previous work that the postinjured brain is extremely sensitive to delayed temperature elevations.3 Also, recent clinical data indicate that elevations in brain temperature occur after traumatic or ischemic injury.4,5 Thus, it is possible that activity-induced increases in local brain temperature could conceivably lead to aggravation of tissue injury in brain regions surrounding a focal lesion.

A variety of abnormal conditions, including the uncoupling of cerebral blood flow and metabolism, the generation of ischemic depolarizations as well as elevations in extracellular levels of neurotransmitters, and abnormal gene expression, have been proposed to contribute to the vulnerability of the ischemic penumbra.6 On the basis of the present discussion, future investigations are required to determine whether elevations in local brain temperature contribute to penumbral vulnerability after ischemic stroke and/or whether temperature elevations participate in the use-dependent exaggeration of neuronal injury. If, for example, regional brain hyperthermia is shown to occur as a consequence of flow-metabolism uncoupling, selective brain hypothermia could be used to inhibit this hyperthermic response to injury.

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References
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