Behavioral Testing Does Not Exacerbate Ischemic CA1 Damage in Gerbils

Frederick Colbourne, PhD; Roland N. Auer, MD, PhD; Garnette R. Sutherland, MD

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.

Key Words: cerebral ischemia • fever, stress-induced • hippocampus • hypothermia • gerbils

A recent study by Kozlowski et al1 suggested that the behavioral use of neurons aggravated cortical injury. These investigators used an electrolytic sensorimotor cortex lesion in rat immediately followed by prolonged casting of the normal ipsilateral limb and subsequently increased use of the affected limb contralateral to the lesion. They found that the increased use of the affected limb resulted in a significantly greater degree of cortical damage. The authors concluded that use of compromised neurons worsened injury.

These provocative results, if true, have profound implications for functional activity (eg, physiotherapy, speech therapy) after stroke. Global cerebral ischemia in rodents, which models human cardiac arrest encephalopathy, is a clinically relevant model ideally suited to study the effects of delayed behavioral testing. Notably, brief global ischemia produces a 2- to 3-day delayed loss of hippocampal CA1 neurons.2–4 Indeed, there is some evidence that the typical loss of CA1 cells is, in part, mediated by functional activity since CA1 neurons can be irreversibly depolarized and killed by low-frequency afferent stimulation during the initial few days after ischemia, and this is related to abnormal [Ca2+] regulation.5–8 Accordingly, the activation of “place cells”9,10 in spatial environments (ie, exploration tests) and the induction of long-term potentiation or similar mechanisms during learning and memory11 may intolerably strain damaged CA1 neurons, since memory formation depends on glutamate release and calcium entry in these cells.12

Untreated global ischemia in rodent models, such as the gerbil, produces near-total CA1 loss before the fourth day,2,3,13 which has a typical all-or-none pattern of injury.14 Thus, it is difficult to assess the effects of delayed behavioral testing on untreated global ischemia. Since prolonged postischemic hypothermia partially protects CA1 neurons,14–17 these incompletely protected CA1 neurons can be used to assess the effects of delayed functional testing. Moreover, some hypothermia-treated CA1 neurons persistently show signs of nonlethal injury (ie, dilated endoplasmic reticulum and mitochondria, intranuclear vacuoles, mitochondrial autolysosomes),13 and this vulnerability may result in enhanced susceptibility to the normal physiological stimulation that occurs during hippocampal-dependent learning and memory tasks. Accordingly, we hypothesized that testing of spatial learning and working memory may have a detrimental effect on hypothermic neuroprotection.

Materials and Methods

Subjects
Female Mongolian gerbils were obtained from High Oak Ranch (Baden, Ontario, Canada) at ~11 to 12 weeks of age and were used

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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 pentobarbital [Somnotol]) with a guide cannula as described previously. 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.

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 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 warmed (1.0°C/30 min) to 34°C and kept at that temperature for 24 hours. After this, animals were warmed to 35°C and kept between 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. 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 group comparison examined the hypothesis that behavioral testing may diminish hypothermic CA1 neuroprotection. Behavioral data are presented elsewhere. The SHAM, ISCH, and ISCH+HYPO groups were exposed to 3 (A, B, and C) initially novel and distinct mazes (22 cm wide×32 cm long×15 cm high) on days 4 to 6 after ischemia/sham occlusion surgery. Gerbils were placed in each maze for 15 minutes once per day (ie, 3 mazes per day) over 3 consecutive days. The 3 maze sessions per day, which took place between 1 and 4 PST, were separated by ~1 hour (start to start). Each maze had a unique internal design thought to be distinguishable by the gerbils. Accordingly, 3 (versus 1 or 2) mazes were used to stimulate greater use of the hippocampus.

Figure 1. Brain temperature during (0 hours) and after ischemia/sham occlusions presented as 5-minute averages. Postischemic temperature was regulated to the desired levels in the ISCH, ISCH+HYPO, and ISCH+HYPO-NT groups (see Materials and Methods) by the combined use of an overhead lamp, fan, and water misting system. The increases in temperature in the SHAM, ISCH, and ISCH+HYPO groups at 102, 126, and 150 hours were due to placing the gerbils in the small mazes (see Figure 2).

On the morning of day 8 after ischemia/sham operation, gerbils (SHAM, ISCH, and ISCH+HYPO) were placed in an open field (1×1-m box; 60 cm high) for three 10-minute sessions (1-hour intertest interval). Ischemic hippocampal damage causes increased and persistent locomotion in this test, which is dependent on novelty. The morning of days 9 and 10, SHAM, ISCH, and ISCH+HYPO gerbils 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.

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 from bregma, as described previously. 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

Baseline 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. 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.
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 transient loss of weight (=4 g by day 7), which has been repeatedly found.14-16,19 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.79 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).

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 (F,1,56<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 global24-26 and focal ischemia27,28 and traumatic brain injury.29 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.30-32 and this is thought to be a fever.30,33,34 Our data suggest that the CA1 zone is unimportant in this type of febrile response since SHAM and ISCH groups had very similar fevers during and after the maze sessions over the 3 test days. Notably, Morrow and colleagues35 have argued that the hippocampus is the site of glucocorticoid negative feedback that acts to dampen stress-induced fever. Our data appear to contradict this since extensive CA1 damage, which eliminates most of the hippocampal output, should eliminate glucocorticoid feedback. If so, fevers would have been more pronounced or prolonged over the course of the 3 sessions on each day (3-hour span), and this was not the case. Finally, the initial blunting of stress-induced fever in the ISCH+HYPO groups is a novel and surprising finding. Further experiments will be needed to determine the mechanism.

Hyperthermia may have confounded the results of Kozlowski et al.,1 since immediate postinjury casting of the normal limb, which increased use of the affected limb on a continuous basis, may have persistently elevated brain temperature (by stress and/or increased movement activity), and this confounding parameter, unmeasured in their experiment, may be responsible for the greater lesion size. Clearly, the detrimental effects of hyperthermia must be carefully consid-
The superior efficacy in the present study was likely due to models of brain injury. Second, behavioral testing was and Drs Fangwei Yang and Dubravka Rakic. The authors acknowledge the technical support of Bonnie Colbourne fellowship support from the Heart and Stroke Foundation of Canada. Dr Colbourne gratefully acknowledges postdoctoral injury after forebrain ischemia in the gerbil. This is a significantly and persistently (30 days) reduce CA1 neuronal and that of Kozlowski et al. First, these studies used different 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 posts ischemic hypothermia. It remains possible that behavioral pressure may have greater detrimental effects following the use of less efficacious neuroprotectants 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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Editorial Comment

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. 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, 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. Also, recent clinical data indicate that elevations in brain temperature occur after traumatic or ischemic injury. 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. 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.

W. Dalton Dietrich, PhD, Guest Editor
Departments of Neurological Surgery and Neurology
University of Miami School of Medicine
Miami, Florida

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