Research report

Delayed transient ischemic attacks kill some CA1 neurons previously salvaged with postischemic hypothermia: neuroprotection undone

Suzanne B. De Bow, Frederick Colbourne*

Center for Neuroscience and Department of Psychology, University of Alberta, P217 Biological Sciences Building, Edmonton, Alta, Canada T6G 2E9

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Abstract

Delayed hypothermia reduces ischemic hippocampal CA1 injury. However, there are residual structural and functional abnormalities. Therefore, we studied whether these apparently vulnerable rescued neurons are susceptible to secondary insults. All gerbils were subjected to normothermic forebrain ischemia (ISC, 5 min) or SHAM operation. Gerbils were treated with mild hypothermia (HYPO; 33.8°C for 24 h) beginning 12 h after surgery, or they remained normothermic (NORMO). Then 5 and 6 days following ISC/SHAM operation gerbils received sublethal transient ischemic attacks (TIA, 1.5 min) or sham (SH) surgeries. Behavioral testing was done and animals survived for 30 days for quantification of medial, middle and lateral CA1 sector cell death. The SHAM groups were not significantly different. The ISC–NORMO group lost 87.3% (of SHAM) of medial CA1 neurons, which was not significantly exacerbated in the ISC–NORMO–TIA group (91.1%, $P = 0.633$). However, the ISC+HYPO+TIA group (58.8% loss) had significantly more cell death than the ISC+HYPO+SH group (42.8%; $P = 0.035$), although CA1 protection was still better than in ISC+NORMO groups ($P < 0.001$). Trends were similar in middle and lateral CA1, but the deleterious effects of TIAs were not statistically significant. Behavioral testing did not distinguish groups with or without TIA, but did reveal deficits in ISC+NORMO groups and protection in ISC+HYPO groups. These data, like previous ultrastructural findings, show that while most hypothermia-rescued CA1 neurons are healthy, some are susceptible. Perhaps other neuroprotectants, especially weaker ones, might be undone by delayed insults (e.g. TIA, fever).

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1. Introduction

Forebrain ischemia results in delayed degeneration of hippocampal CA1 sector neurons in both humans [31] and rodents [26,32]. This delayed neuronal death is believed to be due, in part, to an increase in glutamate release during ischemia [34] activating both N-methyl-D-aspartate and α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptors. Notably, cell death is preceded by downregulation of the AMPA receptor subunit GluR2, making the channel permissive to Ca$^{2+}$ and Zn$^{2+}$ entry, and therefore continuing toxicity [35]. Further support for this comes from studies that show that depolarization of postischemic CA1 neurons leads to cell death, which is related to abnormal regulation of Ca$^{2+}$ [1,2,34,37].

Intra-ischemic hypothermia permanently reduces CA1 neuronal death and is unparalleled in efficacy [20]. Brief postischemic hypothermia only transiently reduces cell death [19]. Prolonged postischemic hypothermia (e.g. 24–48 h) can substantially attenuate CA1 neuronal death, even when delayed 12 h after ischemia [7,8,13,24]. However, some initially salvaged neurons eventually die [8,13]. Furthermore, some ‘rescued’ CA1 cells are morphologically abnormal (e.g. organelle dilations, mitochondrial injury [13]). In addition, while prolonged hypothermia induced after global ischemia (1-h delay) attenuates GluR2 down-regulation in gerbils and promotes an eventual full recovery, there was still a significant GluR2 down-regulation...
that lasted for several days [23]. These and likely other abnormalities in rescued CA1 neurons may account for the very slow cell death in some neurons, possibly by repeated neuronal activation (e.g. spatial learning tasks). Furthermore, one would expect that such abnormal neurons might be especially sensitive to subsequent ischemic insults, including insults that would not normally kill CA1 neurons. This is supported by the fact that a single TIA (2 min) acts synergistically with GluR2 downregulation (via antisense oligodeoxynucleotide knockdown) to kill CA1 neurons [30]. Thus, hypothermia-rescued CA1 neurons, which have a transient GluR2 downregulation among other abnormalities, should be especially vulnerable to delayed transient ischemic attacks (TIAs) that would not normally kill CA1 cells. A loss of previously protected CA1 neurons has been demonstrated recently [22]. In that study, environmental enrichment delayed for 3 days following ischemia partially diminished neuroprotection achieved earlier with ischemic preconditioning.

In order to test the resiliency of hypothermia-treated CA1 neurons after ischemia, gerbils were subjected to delayed TIAs (1.5 min) on days 5 and 6 after they were subjected to a severe global ischemic insult (5 min) that was treated with prolonged postischemic hypothermia. A 12-h intervention delay was used in order to produce a partial sparing of CA1 neurons [13] and thus optimize the conditions for assessing delayed TIAs. Prolonged hypothermia induced soon after ischemia would be more resilient to exacerbation as most cells are rescued permanently [13]. We chose two 1.5-min TIAs since they alone do not kill CA1 neurons in normal gerbils, but do sufficiently prime the cells to produce ischemic preconditioning [15]. The TIAs were administered on days 5 and 6 because of several cellular abnormalities, discussed above, known to occur at this time and since the hypothermia treatment extended over 3 days. The 24-h interval between TIAs was chosen to avoid the obviously deleterious effects of repeated, brief and untreated ischemic insults when they occur closely together [29]. Thus, we sought to determine whether there is an enhanced and persistent vulnerability of salvaged CA1 neurons to otherwise non-lethal TIAs.

2. Materials and methods

2.1. Subjects

A total of 76 (see Fig. 1 for groups and numbers) female Mongolian gerbils (64.8±6.0 g (mean±S.D.); Charles River, Quebec, Canada) were used in accordance with the Canadian Council for Animal Care guidelines and the Biosciences Animal Policy and Welfare Committee at the University of Alberta. Three gerbils were excluded due to surgical complications. All gerbils were on a 12-h dark–light cycle with food and water ad libitum.

The gerbil was chosen because of our extensive ex-perience with this model of global ischemia and delayed hypothermic neuroprotection [13].

2.2. Ischemia and temperature control

Gerbils were first implanted with sterilized telemetry probes (model TA10TA-F20, Data Sciences, Int., St. Paul, MN, USA), which were placed into the peritoneal cavity under sodium pentobarbital anesthesia (65 mg/kg i.p., and 0.05 mg/kg s.c. atropine sulphate). Additionally, a 20-gauge stainless steel guide cannula was implanted on the dural surface overlying the frontal cortex of the left hemisphere (~1 mm anterior and lateral to bregma). The cannula was placed such that an inserted 30-gauge thermocouple probe (Model HYP1-30-1/2-T-G-60-SMP-M, Omega Engineering, Stamford, CT, USA) sampled dorsal striatal temperature during ischemia and sham surgery. Upon removal of the thermocouple probe following surgery, a dummy cannula was inserted to prevent infection. Following this surgery all gerbils were individually housed in cages and placed upon receivers (RPC-1, Data Sciences) which sampled core temperature every 30 s. The data collected the day immediately before ischemia and sham surgery served as baseline.

Brain temperature telemetry probes are susceptible to damage following prolonged use in a gerbil, and thus core telemetry probes were used in this study. During ischemia in gerbils core temperature does not necessarily predict brain temperature [9], but the relationship is good in conscious postischemic rodents [17]. Brain temperature control during injury reduces variability in this model, and thus both brain and core temperature measurements were taken during the time of ischemia.

Four days following core probe and brain cannula implantation, gerbils were anesthetized with 4% (1.5–2% maintenance) isoflurane anesthetic in 70% N₂O and 30% O₂. Brain and core temperature were monitored and maintained with an infrared heat lamp near 36.5 and 37.5 °C, respectively. Striatal temperature was measured.

![Fig. 1. Diagram illustrating treatment groups and numbers. Animals were randomly assigned to receive SHAM surgery or ISC (5-min occlusion). Then 12 h after surgery, hypothermia treatment began in half of the animals, and the other half was regulated as described in Section 2.2. At 5 and 6 days following ISC and SHAM surgery, animals were again randomly assigned to receive a 1.5-min TIA or SH insult on each day.](image-url)
via the thermocouple probe, and core via the telemetry abdominal probe. Gerbils were randomly assigned to receive ischemia (5-min bilateral common carotid artery occlusion, ISC) or sham surgery (arteries were isolated but not occluded, SHAM). Both common carotid arteries were occluded in ISC gerbils using micro-arterial clamps. At the end of the occlusion, the clamps were removed, the arteries visually inspected for reflow, the midline incision sutured, the thermocouple probe removed and anesthesia discontinued.

All gerbils received post-ischemic temperature control (Fig. 2), excluding untreated SHAM animals as they were allowed to self regulate (SHAM+NORMO). In an effort to minimize variability, untreated ischemia groups (ISC+NORMO) were regulated for 12 h to mimic a mild hyperthermia pattern that spontaneously occurs [7,8] and this was also done in the ISC groups that were cooled. Cooling in the hypothermia groups (ISC+HYPO, SHAM+HYPO) was delayed until 12 h after reperfusion or sham occlusion [13]. Hypothermia-treated gerbils were cooled slowly at a rate of 1 °C per 30 min to 33 °C (for 24 h) then slowly warmed at the same rate to 35 °C (for 24 h). At this time, gerbils were warmed to 36 °C and maintained between 36 and 37 °C for 12 h. Core telemetry probes were removed 9 days following ischemia under brief isoflurane anesthesia. Hypothermia was produced with an automated system that uses infrared lamps to heat and fine water mist and fans to cool [10].

2.3. Delayed transient ischemic attacks

At 5 and 6 days following ischemia or sham surgery, all groups were further divided to receive a TIA (SHAM+NORMO+TIA, SHAM+HYPO+TIA, ISC+NORMO+TIA, ISC+HYPO+TIA) or sham surgery (SHAM+NORMO+SH, SHAM+HYPO+SH, ISC+NORMO+SH, ISC+HYPO+SH). All procedures were done as described above for ischemia; however, occlusion of the carotid arteries in the TIA groups lasted 1.5 min on both days. The SH group animals did not have their arteries occluded, but they were isolated. Brain temperature was measured and maintained at ~36.5 °C throughout the TIA/SH surgeries. These insults have been repeatedly used by others [15] to induce tolerance to subsequent lethal insults, but they do not kill CA1 neurons in naïve animals.

2.4. Behavioral testing

At 4 days following ischemia or sham occlusion, gerbils were placed in a novel maze (a black Plexiglas box with a
Looking (Cresyl violet stain) pyramidal neurons in the injury was quantified by counting the number of viable NORMO groups; however protection was not perfect.

Brain temperature is normally lower than core temperature when measured with telemetry probes. Accordingly, brain temperature was maintained very near normothermia of 36.4°C [8].

3. Results

Baseline core temperature did not differ among groups (Table 1). Brain temperature during the 5-min ischemic insult or sham occlusion and the 1.5-min TIAs or sham occlusions were also very similar among groups (Table 1). Core temperature following ischemia (Fig. 2) was regulated as described in Section 2.2. There was no mortality associated with the treatments.

There were no significant differences among SHAM groups for CA1 cell counts in the medial (F4, 21 = 1.583, P = 0.228), middle (F4, 21 = 1.489, P = 0.251) and lateral CA1 sectors (F4, 21 < 1), and thus these groups were combined for subsequent statistical analyses (likewise for behavioral tests; statistics not shown). In addition, an unblinded examination of SHAM+TIA groups revealed no obvious signs of injury in CA1, nor in other hippocampal areas. In the ANOVAs that included the combined SHAM and all ISC groups there were significant group main effects for medial (F4, 72 = 5.595, P < 0.001), middle (F4, 72 = 50.667, P < 0.001) and lateral CA1 sector cell counts (F4, 72 = 62.581, P < 0.001). Extensive damage occurred in all sectors of CA1 in ISC+NORMO+SH and ISC+NORMO+TIA groups (P < 0.001 vs. SHAM; Figs. 3 and 4). However, these groups were not significantly different (P ≥ 0.586) showing that delayed TIAs did not aggravate untreated ischemic injury, which was near maximal anyway. Hypothermia treatment markedly reduced CA1 injury in both ISC+HYPO+SH and ISC+HYPO+TIA groups (P < 0.001 for all sectors vs. ISC+NORMO groups); however protection was not perfect (P < 0.001 vs. SHAM). Moreover, these two groups dif-

2.5. Histology

Gerbils were sacrificed 30 days after ISC/SHAM operation with an overdose of sodium pentobarbital (0.1 ml) and perfused with saline followed by 10% neutral-buffered formalin. Brains were then processed, frozen, and 10-μm coronal sections were taken with a cryostat. Ischemic injury was quantified by counting the number of viable-looking (Cresyl violet stain) pyramidal neurons in the lateral (next to CA2), middle (apex of CA1), and medial (adjacent to subiculum) subsections of CA1 (each 0.2-mm long; 400× light microscopy) at −1.7 mm to bregma as previously done [8]. The experimenter conducting cell counts was blinded to the subjects' treatment. CA1 cell counts were analyzed with ANOVA followed by planned comparisons (Fisher L.S.D. test).

Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline core temp. (°C)</th>
<th>ISC/SHAM brain temp. (°C)</th>
<th>TIA/SH brain temp. (°C), day 5</th>
<th>TIA/SH brain temp. (°C), day 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM+NORMO+SH</td>
<td>37.6 ± 0.2</td>
<td>36.2 ± 0.7</td>
<td>36.3 ± 0.7</td>
<td>36.2 ± 0.3</td>
</tr>
<tr>
<td>SHAM+NORMO+TIA</td>
<td>37.5 ± 0.3</td>
<td>36.2 ± 0.5</td>
<td>35.8 ± 0.5</td>
<td>36.0 ± 0.2</td>
</tr>
<tr>
<td>SHAM+HYPO+SH</td>
<td>37.5 ± 0.3</td>
<td>36.2 ± 0.5</td>
<td>36.2 ± 0.4</td>
<td>36.3 ± 0.5</td>
</tr>
<tr>
<td>SHAM+HYPO+TIA</td>
<td>37.5 ± 0.3</td>
<td>36.2 ± 0.4</td>
<td>35.5 ± 1.0</td>
<td>36.0 ± 0.6</td>
</tr>
<tr>
<td>ISC+NORMO+SH</td>
<td>37.4 ± 0.3</td>
<td>36.1 ± 0.6</td>
<td>36.3 ± 0.5</td>
<td>36.3 ± 0.4</td>
</tr>
<tr>
<td>ISC+NORMO+TIA</td>
<td>37.5 ± 0.0</td>
<td>35.9 ± 0.4</td>
<td>35.9 ± 0.5</td>
<td>35.8 ± 0.4</td>
</tr>
<tr>
<td>ISC+HYPO+SH</td>
<td>37.5 ± 0.3</td>
<td>35.9 ± 0.6</td>
<td>35.9 ± 0.6</td>
<td>36.2 ± 0.4</td>
</tr>
<tr>
<td>ISC+HYPO+TIA</td>
<td>37.4 ± 0.3</td>
<td>35.9 ± 0.5</td>
<td>36.3 ± 0.5</td>
<td>36.4 ± 0.5</td>
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Brain temperature is normally −1°C lower than core temperature when measured with telemetry probes. Accordingly, brain temperature was maintained very near normothermia of 36.4°C [8].
Fig. 3. CA1 cell counts expressed as a mean percentage of SHAM (% ± S.D.). SHAM groups were not significantly different from each other. Cell counts in the untreated ischemia groups (ISC+NORMO+SH/TIA) were not significantly different. However, the ISC+HYPO+TIA had significantly fewer remaining neurons in medial CA1 with similar trends in middle and lateral CA1. See Fig. 4 for representative photomicrographs.

Fig. 5. Mean activity counts (sampled every 30 s and averaged) measured with the core telemetry probe in the small mazes on days 4 and 9 following ISC/SHAM surgery. On day 4 both ISC+NORMO+TIA/SH groups were significantly hyperactive compared with SHAMs, and both ISC+HYPO groups. The TIA or SH interventions occurred on days 5 and 6. On day 9 ISC+NORMO+TIA/SH groups were significantly hyperactive compared to SHAMs and ISC+HYPO+TIA/SH groups. The TIA treatments did not affect activity levels.

The primary findings of this study are that: (i) some hypothermia-salvaged CA1 neurons are susceptible to spatial habituation impairment. Both ISC+HYPO+SH and ISC+HYPO+TIA groups did not differ significantly from each other (P=0.410). These hypothermia treated groups were significantly less active than ISC+NORMO groups (P=0.008) and not significantly different from SHAM (P=0.427), which suggests a normal habituation pattern. Thus, testing in the small maze on day 4 suggests untreated ischemic animals did not differ from each other prior to secondary TIA. Statistical results of activity levels during the novel maze session on day 9 were very similar (statistics not shown). Accordingly, ISC+NORMO groups were still impaired whereas ISC+HYPO groups were normal. The TIA insults did not worsen habituation ability.

An ANOVA on the average performance (percent correct) in the T-maze did not yield an overall significant effect (F_{4, 64}=2.106, P=0.090). Nonetheless, the ISC+NORMO groups (~76% success) were less successful (i.e. impaired working memory) than SHAMs (~82%) and the ISC+HYPO groups (~82%) were like SHAMs although they were not statistically better than ISC+NORMO groups. The TIAs did not affect either NORMO or HYPO groups. Removal of a few histological outliers did result in significant deficits of ISC+NORMO groups, but this did not affect the pattern of results; notably the TIAs did not worsen working memory performance in the T-maze.

4. Discussion

The primary findings of this study are that: (i) some hypothermia-salvaged CA1 neurons are susceptible to
delayed, normally sublethal, TIAs following hypothermic neuroprotection, and (ii) many hypothermia-salvaged neurons are resilient to TIAs. Since secondary TIAs did not worsen CA1 cell death after untreated ischemia it is likely that the secondary insults killed hypothermia-rescued cells in the ISC+HYPO+TIA group. In this study, hypothermia treatment was delayed for 12 h as this procedure provides an intermediate level of neuroprotection with a continuing, but partial, loss of CA1 neurons with time [13]. In addition, some of the salvaged CA1 cells have residual morphological abnormalities (e.g. mitochondrial autolysosomes), which would be expected to compromise neuronal survival (e.g. impaired metabolism). Furthermore, since 1-h delayed cooling does not fully prevent ischemia-induced downregulation of the AMPA channel GluR2 subunit [23], one would expect a greater residual downregulation with the 12-h delayed cooling. While these abnormalities likely contribute to sustained vulnerability in rescued CA1 cells, further studies are needed to prove the mechanism(s), which may also include, but are not limited to: hemodynamic alterations [16], enhanced excitotoxicity (e.g. greater or more sustained elevations in glutamate) [29], and alterations in gene expression. Additionally, future studies must examine the resiliency of cytoprotection in different brain regions (e.g. striatum) to various cellular stressors (e.g. brief focal ischemia).

The reduction in CA1 survival because of delayed TIAs was significant in the medial CA1 zone. This is in agreement with data showing that postischemic hypothermia results in more persistent neuroprotection in middle and lateral CA1 sectors than in medial CA1 [8]. Less efficacious treatments, more severe or earlier secondary insults would likely affect all CA1 zones. The loss of neuroprotection in ischemic preconditioned animals given postischemic environmental enrichment [22] was greater than that presently found with delayed TIAs after hypothermic neuroprotection even though both studies utilized a similar ischemia model. This could be due to the fact that ischemic preconditioning is less neuroprotective or that environmental enrichment was a more severe stress than TIAs, which also might be due to enrichment therapy being introduced 3 days after ischemia in that study [22]. Conversely, more effective therapies (e.g. intra-ischemic hypothermia, early and prolonged postischemic hypothermia) are less likely to be affected. Indeed, Colbourne et al. [12] failed to lessen hypothermic neuroprotection with a battery of behavioral tests starting on day 4 after ischemia. In that study, cooling was begun 6 h after ischemia and resulted in ~90% protection of CA1.

In spite of the TIAs reducing cell survival there were no obvious behavioral ramifications. Perhaps those additional CA1 neurons that died did not initially contribute to functional performance. For instance, they may have suffered from significant electrophysiological abnormalities. While an earlier study [21] found no such abnormalities in CA1 neurons rescued with postischemic hypothermia, it is important to note that protection was better in that study (early treatment intervention) and only a few neurons were examined making it impossible to generalize to all rescued neurons from various treatments. It is also possible that the exaggerated loss of CA1 neurons was not sufficient to allow easy detection, at least not with the present tests. More demanding tests, such as the win-stay variant of the T-maze [3,8] used by Farrell et al. [22], might reveal behavioral effects. Additionally, such tests would be expected to show greater (and statistically significant) working memory deficits in untreated ischemic gerbils than that presently observed. In a previous study [8] untreated ischemic gerbils were significantly impaired in the T-maze win-shift task. Perhaps the difference is due to total amount of hippocampal injury, which was somewhat less in this study, and/or the use of a different supplier of gerbils.

Interestingly, Farrell and colleagues [22] observed improved functional outcome in an ischemic preconditioned group that was also given environmental enrichment, a group that had fewer surviving CA1 neurons than the preconditioned alone treatment. The effects of enrichment are thought to be due to a variety of cellular processes (e.g. sprouting) throughout the brain [27]. Thus, various beneficial effects of enrichment outside of the CA1 zone and within surviving CA1 neurons could compensate for the increased loss of hippocampal CA1 neurons and it cannot be concluded that those lost neurons either had disturbed hippocampal function or would not have eventually contributed to proper hippocampal function.

Fever aggravates global ischemic injury when it occurs within the first day [4]. We did not observe fever after the brief TIAs. However, it is possible that fever repeatedly accompanied the environmental enrichment paradigm used by Farrell et al. [22]. Animals commonly experience stress-induced fever with behavioral manipulations (e.g. small mazes [12], present study—data not shown). Repeated changes in the enrichment cage (e.g. novel objects) might repeatedly cause stress-induced fever along with other physiological changes that could account for the exacerbated injury in that study. This would only occur in the ischemic preconditioned group and not in the untreated ischemic gerbils since it is the preconditioned group that has salvaged and vulnerable CA1 neurons.

Aggressive motor rehabilitation (constraint-induced movement therapy) has also been shown to worsen cortical brain injury when it is administered soon after the lesion [6,18,25,28]. This effect seems to be dependent on glutamate [33] and thus may share common mechanisms (usedependency) as the lessening of CA1 neuroprotection in the present study. It is also possible that constraint-induced movement therapy aggravates brain injury by causing a localized rise in brain temperature in the vulnerable tissue (~0.7 °C elevation; De Bow et al., unpublished data). This possibility is being explored.

In summary, not all CA1 neurons salvaged by neuro-
protective interventions are tolerant of delayed insults. Presently, some CA1 neurons were killed off by quite delayed TIAs. Accordingly, the clinical efficacy of treatments administered after global and focal ischemia might be undone by insults that would otherwise remain untreated. Such ‘mild’ insults may necessitate further therapy. Similarly, aggressive rehabilitation therapies may need to be delayed to avoid losing some previously saved tissue. Finally, the present paradigm may prove useful in determining whether cytoprotective strategies are truly global ischemia, J. Neurosci. 19 (1999) 4200–4210.

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