Post-ischemic diazepam does not reduce hippocampal CA1 injury and does not improve hypothermic neuroprotection after forebrain ischemia in gerbils

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Abstract

The hippocampal CA1 sector is especially vulnerable to brief forebrain ischemia. Excitotoxicity is widely thought to contribute to this cell death. Accordingly, drugs that presumably counteract excitotoxicity, such as GABAergic agonists, have been repeatedly tested and found to reduce CA1 cell loss. Post-ischemic diazepam reduces CA1 injury. However, diazepam also causes hypothermia, which by itself is neuroprotective. Most studies fail to adequately control for this confound. In this study, we tested whether diazepam reduces injury in temperature controlled gerbils subjected to brief forebrain ischemia. Furthermore, we tested whether diazepam augments hypothermic neuroprotection. All gerbils were implanted with a core temperature telemetry probe and a cannula for the subsequent insertion of a thermocouple probe to measure ischemic brain temperature. Subsequently, they were given a 5-min normothermic ischemic insult. In Experiment 1, two groups of gerbils were given 10 mg/kg doses of diazepam (i.p.) at both 30 and 90 min post-ischemia. Temperature was maintained in one group by heating lamps. Another group was administered saline. Diazepam reduced cell death at 7 days post-ischemia when the drug-induced hypothermia was permitted, but not when it was prevented. In Experiment 2, four groups of ischemic gerbils were treated starting at 12 h post-ischemia with prolonged hypothermia, diazepam and the combination or saline treatment. Hypothermia, but not diazepam, provided partial neuroprotection and diazepam did not augment hypothermic neuroprotection. Thus, neuroprotection with diazepam is solely due to hypothermia. These data do not support the clinical use of diazepam as a neuroprotectant after global ischemia.

Keywords: Ischemia; Neuroprotection; GABA; Valium; Hypothermia

1. Introduction

Cerebral ischemia is a leading cause of mortality and morbidity. Global ischemia, such as occurs with cardiac arrest, causes the excessive release of excitatory amino acids (glutamate) within the brain and selective death of hippocampal CA1 neurons if the insult is brief [2]. Glutamate is strongly implicated in ischemic cell death including the delayed neuronal death (DND) of hippocampal CA1 sector neurons that follows transient forebrain ischemia in rodents. CA1 sector cells typically succumb 2–4 days post-ischemia [16,19]. The time until onset of CA1 sector DND allows an opportunity for interventions to counter the mechanisms of impending cell death. Presently, post-ischemic hypothermia provides the best long-term functional and histological protection after global ischemia [5,7,15]. For instance, prolonged mild hypothermia persistently reduces CA1 zone DND even when cooling begins up to 12 h post-ischemia [8]. Furthermore, recent clinical trials report a favorable neurological outcome and reduced mortality with delayed mild hypothermia after cardiac arrest [1,24].

The use of benzodiazepines, particularly the full agonist diazepam [21–23] is another promising neuroprotective strategy after global ischemia. Benzodiazepines act on the GABA\textsubscript{A} receptor, enhancing GABA neurotransmission and thereby increasing inhibition. This inhibition could potentially attenuate excitotoxicity following ischemia. However, diazepam-induced hypothermia [13,22] must also be considered. Several studies have attempted to dissociate the
direct neuroprotective effects of diazepam from hypothermia. For instance, diazepam reduced CA1 sector DND after direct microinjection of diazepam into the hippocampus where it would not cause systemic hypothermia [21]. Another study found that diazepam was more effective than a similar externally applied cooling paradigm [22]. Finally, one group regulated the temperature of the diazepam-treated animals at normothermic levels and found some, albeit a reduced amount of CA1 sector protection [13]. Notably, that study regulated the diazepam-treated gerbils at normothermia and not at the mild hyperthermic level that characterizes the early reperfusion period in the gerbil model.

In this study, we assessed the neuroprotective efficacy of diazepam given following transient forebrain ischemia in the gerbil. The first study determined the magnitude of diazepam-induced hypothermia and tested whether diazepam, given at 30 and 90 min post-ischemia, reduces cell death with and without the concomitant drug-induced hypothermia. Thus, one diazepam-treated group was kept at an identical temperature as the saline-treated ischemic gerbils. The second study assessed the efficacy of a 12-h delayed diazepam treatment given alone and in combination with prolonged mild hypothermia. Thus, these studies tested whether diazepam reduces CA1 sector cell death when given alone and in combination with induced hypothermia where diazepam was theorized to provide additive or synergistic neuroprotection. Diazepam was chosen for three reasons. First, it has been repeatedly shown to reduce CA1 DND and appears to be a true neuroprotectant. Second, diazepam has a favorable safety profile allowing it to be given to many patients with different types of ischemic insults. Finally, diazepam or other benzodiazepines could be administered with hypothermia as a secondary neuroprotective agent and as a way to facilitate cooling (e.g., diminishing shivering, reducing stress).

2. Methods

2.1. Subjects

Seventy adult (4–6 months) female Mongolian gerbils, obtained locally, weighing 55–80 g at the time of the first surgery were used. Females were chosen because most of our previous work on hypothermic neuroprotection has been done in females [4–6]. All gerbils were housed individually and given food and water ad libitum. All experimental procedures were in compliance with the Canadian Council for Animal Care Guidelines, and were approved by the Biosciences Animal Policy and Welfare Committee at the University of Alberta.

2.2. Temperature and activity measurement

Each gerbil was implanted with a core temperature telemetry probe (model TA10TA-F20; Transoma Medical, St. Paul, MN, USA) and a 5.0-mm guide cannula overlying the dura (≈ 1 mm anterior and 1 mm to the left of bregma) as previously described [10]. The latter was used for subsequent measurement of striatal brain temperature with a thermocouple probe (model HYP 1-30-1/2-T-G-60-SMP-M, Omega Engineering, CT, USA). The surgery was performed under either sodium pentobarbital (65 mg/kg i.p.) or 2% isoflurane anesthetic (4% induction, 70% N2O, 30% O2). After surgery, gerbils were placed on receivers interfaced to a computer running A.R.T. software (Transoma Medical) that recorded temperature and activity every 30 seconds [9]. Activity counts, which were determined from movement of the probe over the receiver, give a relative measure of whole body movement [6]. Core temperature was monitored for 24 h prior to ischemia and this period served as a baseline. Finally, in the second experiment, our temperature regulation software [9] recorded the amount of time the fan, lamp and spray was used out every 300 s (5 min). This data was then used to reflect the difficulty in regulating temperature.

2.3. Ischemia

Gerbils were anesthetized with isoflurane (2% maintenance) 4 days following the telemetry probe surgery. After insertion of the thermocouple probe into the dorsal striatum, a 2-cm incision was made along the ventral midline of the neck. The common carotid arteries were isolated and occluded for 5 min using micro aneurysm clips under reduced isoflurane levels (usually 0.5–1%). Brain temperature was regulated at normothermic levels (≈ 36°C) using an overhead infrared lamp (175 W). Brain temperature was regulated after removal of the clips. Reperfusion was visually confirmed after removal of the clips. Marcaine (Sanofi, Markham, ON, Canada) was applied and the wound was sutured closed, after which antibiotic ointment was applied topically.

2.4. Diazepam treatment and temperature regulation

In Experiment 1, diazepam (10 mg/kg i.p.) or saline was given at 30 and 90 min post-ischemia. The saline (SAL-NORMO, N=8) group was regulated at a temperature level that spontaneously occurs in untreated ischemic gerbils [5]. Half of the diazepam-treated gerbils were allowed to spontaneously cool (DZP-HYPO, N=10) while the others (DZP-NORMO, N=9) were maintained at the same temperature as SAL-NORMO gerbils. One additional gerbil (DZP-HYPO, N=9) was excluded due to a technical error.

In Experiment 2, four groups of ischemic gerbils received either saline or diazepam (10 mg/kg i.p.) at 12 and 13 h post-ischemia and were kept warm or subjected to prolonged mild hypothermia. Thus, the four groups were: SAL-NORMO (N=10), SAL-HYPO (N=10), DZP-NORMO (N=10) and DZP-HYPO (N=9). Hypothermia was induced slowly at a rate of 1 °C per 30 min to a core temperature of 33°C as previously done [8]. They
were maintained at this temperature for 24 h at which time they were warmed (1 °C/30 min) to 35 °C and held at this level for 24 h before warming slowly to normothermia. The SAL-NORMO and DZP-NORMO groups were temporarily maintained at a level of mild hyperthermia as in Experiment 1. All gerbils were shaved to facilitate cooling and prevent knowledge of group identity. Three additional gerbils were excluded due to technical problems (e.g., probe battery failure).

2.5. Histology

Gerbils were euthanized 7 days post-ischemia with an overdose of Somnotol (~ 80 mg/kg i.p.) and transcardially perfused using saline followed by 10% phosphate-buffered formalin. Ten-micrometer sections of the hippocampus were cut using a cryostat and stained with cresyl violet. Viable cells were counted bilaterally in the medial, middle and lateral sections of the CA1 area of the hippocampus at 1.7 and 2.2 mm posterior to bregma as previously described [5]. We used a brief (7 days) survival time in order to maximize the chance of finding a beneficial effect of diazepam because use of only long survival times might overlook a transient protective effect.

2.6. Statistics

The data in Experiment 1 were analyzed with a one-way ANOVA (SPSS v. 12) followed by contrast tests (planned comparisons). Note that this maximized the likelihood of detecting a beneficial effect of diazepam (i.e., lessening the chance of a Type II error that more likely occurs with stringent post-hoc tests). In Experiment 2, we used a two-way ANOVA except for the device usage (e.g., fan) that was analyzed as a specific contrast between SAL-HYPO and DZP-HYPO groups in a one-way ANOVA.

3. Results

3.1. Experiment 1

Baseline data for temperature and activity were similar among groups (data not shown). Brain temperature was maintained at normothermia during ischemia (group means ranged from 35.90 to 36.10 °C). Diazepam caused moderate core hypothermia that persisted for approximately 12 h (Fig. 1). This was mostly prevented in the DZP-NORMO group.

Fig. 1. Core temperature (°C, left) and movement-related activity data (right) in Experiments 1 (top) and 2 (bottom). Core temperature was measured with an implanted telemetry probe. Changes in the signal strength of this probe (i.e., movement over the receiver) were used to determine activity levels by the telemetry software. Forebrain ischemia in gerbils causes hyperactivity [6]. Hypothermia and diazepam treatments attenuate this hyperactivity. Limited data are shown in order to highlight early group differences.
Relative activity levels were measured with the telemetry system via detecting signal strength changes of the probe over the receiver. The SAL-NORMO group was hyperactive especially at 2–4 h post-ischemia. Diazepam caused marked hypoactivity that returned to ischemic hyperactive levels by about 8 h post-ischemia in the DZP-NORMO group. The DZP-HYPO group was hypoactive until about 10 h post-ischemia.

In Experiment 1 (Fig. 2), diazepam reduced anterior \((t_{23} = -4.073, p < 0.001)\) and posterior hippocampal CA1 cell death \((t_{23} = -4.514, p < 0.001)\) but only if hypothermia was permitted to occur (DZP-HYPO). Anterior \((t_{23} = -0.590, p = 0.561)\) and posterior \((t_{23} = -1.247, p = 0.225)\) CA1 sector injury was not significantly reduced in DZP-NORMO gerbils. These animals were treated with diazepam and kept at a hyperthermic level that spontaneously occurs in the untreated ischemic gerbils. The DZP-HYPO gerbils, which were allowed to spontaneously cool, had significantly more anterior \((t_{23} = 3.590, p = 0.002)\) and posterior CA1 \((t_{23} = 3.368, p = 0.003)\) neurons than DZP-NORMO gerbils, which were temperature controlled. One DZP-HYPO gerbil died within 24 h of ischemia of unknown cause.

3.2. Experiment 2

Brain temperature was maintained at normothermia during ischemia (group means ranged from 35.96 to 36.32 °C). Core temperature was regulated very near desired values (Fig. 1). However, the DZP-NORMO group was slightly cooler than desired as it was very difficult to maintain the temperature of diazepam-treated animals.

The amount of time each device was active (i.e., lamp or fan on, spray valve open) over every 5 min period was recorded and the average usage per 5 min from 12 to 16 h post-ischemia was analyzed. The data for the SAL-HYPO and DZP-HYPO groups are presented in Fig. 3 for 12–16 h post-ischemia (i.e., during and for a brief period following the induction of hypothermia). The induction of hypothermia was facilitated by use of diazepam as the DZP-HYPO...
group required significantly less fan ($t_{35} = 9.673$, $p < 0.001$) and spray usage ($t_{35} = 6.339$, $p < 0.001$) and more lamp usage ($t_{35} = -4.793$, $p < 0.001$) than the SAL-HYPO group.

As in Experiment 1, SAL-NORMO gerbils were hyperactive after ischemia. At 12 h post-ischemia, the DZP-NORMO, SAL-HYPO and DZP-HYPO groups all became hypoactive relative to SAL-NORMO gerbils. The DZP-NORMO gerbils returned to hyperactive levels by approximately 24 h post-ischemia (12 h post diazepam injection). The hypothermia groups remained hypoactive until after re-warming.

Diazepam did not significantly affect anterior (saline vs. diazepam main effect: $F_{1,34} = 1.05$, $p = 0.313$) or posterior CA1 sector cell death ($F_{1,34} = 1.12$, $p = 0.297$; Fig. 2). Conversely, hypothermia significantly reduced cell death in anterior ($F_{1,34} = 18.85$, $p < 0.001$) and posterior CA1 ($F_{1,34} = 40.46$, $p < 0.001$). The drug–temperature interaction was not significant for either anterior ($F_{1,34} < 1$) or posterior CA1 ($F_{1,34} < 1$). Thus, whereas hypothermia reduces cell death, diazepam does not when given to temperature maintained gerbils. Diazepam also fails to provide additional benefit to an induced hypothermia treatment.

4. Discussion

Post-ischemic diazepam significantly reduced CA1 sector cell death only when hypothermia was permitted. Expressed as a percentage of normal CA1 sector cell counts (taken from Ref. [10]) the DZP-HYPO group in Experiment 1 had approximately 73% and 68% of CA1 sector neurons remaining at the anterior and posterior CA1 levels, respectively. Indeed, we chose a 12-h delay in order to provide an intermediate level of protection [8,10] and as it is more clinically relevant than early interventions. The addition of diazepam did not statistically increase the protection observed with externally induced hypothermia treatment (12 h delay).

Our results from Experiment 1 are similar to those of Dowden et al. [13] who found that forced normothermia in diazepam-treated gerbils diminished neuroprotection. However, in that study, diazepam given while normothermia was maintained still provided significant benefit. Perhaps, the residual protection in that study resulted from the slightly lower temperature of diazepam-regulated gerbils compared to saline-treated ischemic animals that were hyperthermic. Hyperthermia spontaneously occurs following forebrain ischemia in gerbils [5] and likely contributes to cell death [14]. Presently, DZP-NORMO and SAL-NORMO gerbils were maintained at a mild hyperthermic level in Experiment 1 and there was no significant protective effect. It is important to note that the present insult did not produce quite as much CA1 injury as that produced by Dowden et al. [13], and thus differences in insult severity cannot explain the lack of protection in our study.

Diazepam-induced hypothermia significantly reduced CA1 sector neurons remaining at the anterior and posterior CA1 levels, respectively. Indeed, we chose a 12-h delay in order to provide an intermediate level of protection [8,10] and as it is more clinically relevant than early interventions. The addition of diazepam did not statistically increase the protection observed with externally induced hypothermia treatment (12 h delay).
brief cooling does not protect permanently even when cooling begins immediately after global ischemia.

In Experiment 2, we compared four groups to test for additive or synergistic effects of diazepam and hypothermia treatments. Diazepam failed to protect when given alone and failed to provide additional benefit to that provided by hypothermia alone. It was clear, however, that the DZP-HYPO group was easier to cool than SAL-HYPO gerbils. This suggests that the stress of induced hypothermia with fans and water spray, which presumably diazepam counters, does not negatively affect hypothermic neuroprotection. These data also suggest that the countering effects of diazepam on early or late excitotoxicity are not sufficient to salvage CA1 neurons.

There were small, non-significant trends towards greater CA1 sector cell survival with diazepam treatment (e.g., SAL-NORMO vs. DZP-NORMO). We expect that substantially greater group sizes would be required to statistically prove such small effects. Nonetheless, had these been significant, further study would have to determine whether a small amount of protection translates into improved functional recovery (e.g., spatial memory) and whether the neuroprotection would sustain at longer survival times. Finally, despite using a very high dose of diazepam, it is possible that longer-acting benzodiazepines or additional treatments with diazepam might provide greater, statistically significant benefit. Such studies will have to carefully prevent hypothermia.

Our results do not easily explain why diazepam was neuroprotective when directly microinjected into the hippocampus [21], but this may relate to dosing differences or insult severity. Furthermore, it has been argued that externally induced hypothermia is not as protective as diazepam treatment with concomitant hypothermia [22]. There are several problems with this comparison [13]. First, the hypothermic profile of diazepam-treated gerbils was not identical to the control group, which were forcibly cooled [22]. Second, rectal temperature measurements are stressful. For example, rectal temperature measurements can cause a stress-induced fever in normal and ischemic gerbils [3]. This stress would presumably be blocked by diazepam and left unabated in controls. Thus, the different stress response in diazepam and vehicle-treated gerbils makes a direct comparison tenuous. Mildly hypothermic gerbils (e.g., 32 °C) are generally inactive, but not sedated like diazepam-treated gerbils (e.g., 2 × 10 mg/kg). For instance, hypothermic gerbils typically stay in a hunched posture with occasional eating and drinking, whereas diazepam-treated gerbils lie without limb support for hours. Third, the repeated rectal temperature measurements used by Schwartz-Bloom et al. [22] would not, in our extensive experience with induced hypothermia in gerbils, be sufficient to allow for precise temperature control as a gerbil’s temperature can rapidly fluctuate at hypothermic levels (e.g., by several °C in a few min). Finally, early diazepam and hypothermia treatments may act additively or synergistically, although they do not when given at 12 h post-ischemia. Hence, the data suggest that diazepam simply protects against CA1 sector DND via hypothermia.

It has been suggested that maintenance of normothermia reduces diazepam’s sedative action [20]. However, neither this study nor a previous report [13], both of which directly measured activity continuously via telemetry, support this contention. Fig. 1 illustrates that DZP-NORMO gerbils were hypoactive for nearly as long as DZP-HYPO gerbils. The slightly longer period of hypoactivity in the DZP-HYPO group is undoubtedly due to continuing hypothermia at that time as diazepam is wearing off. Hypothermia alone reduces activity as seen previously [6] and as shown in Experiment 2.

In summary, post-ischemic diazepam does not reduce CA1 sector DND when temperature is adequately controlled after forebrain ischemia. Recently, similar results were found with post-ischemic diazepam administration after the onset of focal cerebral ischemia in rat [17]. Furthermore, diazepam does not augment hypothermic neuroprotection suggesting that it does not provide even a weak protective effect. Our results do not exclude the use of diazepam or other benzodiazepines as an adjunct therapy with hypothermia allowing easier induction of hypothermia with less stress. However, further studies should investigate combination therapies of prolonged hypothermia and a truly neuroprotective agent. Unfortunately, it is difficult to know which drugs truly salvage cells after ischemia because most are not thoroughly assessed (e.g., temperature confounds are not ruled out) [11]. Indeed, the present findings emphasize the need for precise temperature measurement and control in all cytoprotection efficacy studies. This incomplete assessment of experimental cytoprotective drugs in animal models has likely led to the premature advancement of many drugs to clinical trials where they have failed as diazepam has for out-of-hospital cardiac arrest in humans [18].

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