Introduction

Several large epidemiological studies have found that stroke outcome is worse in heavy drinkers and that this effect persists even when adjusted for the high proportion of hypertensive subjects among this group. Previous high levels of ethanol consumption is a significant risk factor for stroke recurrence, and autopsy studies have found that cerebral infarction is more common at an earlier age in alcoholics than in non-alcoholics. Chronic alcoholics also typically exhibit generalized cortical atrophy and pronounced hippocampal degeneration, both of which are neuroanatomical areas with high densities of NMDA receptors. Recently, ethanol administration has been linked to increased sensitivity of the hippocampus to NMDA-related excitotoxicity. Using the technique of in vivo microdialysis, this study attempted to determine the extent to which NMDA excitotoxic processes might be altered and whether histological and neurobehavioral measures might map onto putative exacerbated stroke outcomes in ethanol-treated subjects.

Materials and Methods

Injection schedule: Institutional ethics approval was given for this study. Twenty-seven male Mongolian gerbils (Tumblebrook Farms, USA) weighing 60-80 g were housed individually with free access to food and water, and maintained on a 12:12 h light:dark cycle. Three groups (n = 9 each) received 4 g/kg, 1 g/kg and 0 g/kg ethanol in 1 ml of saline for 21 days. Injections (s.c.) were made with a 30 gauge needle and the sites of injection were rotated in order to minimize discomfort.

Blood alcohol determinations: A second group of gerbils (n = 30), litter-mates of those used injected as above, were sacrificed for blood ethanol determinations. Blood alcohol determinations were made by sacrificing probe subjects (n = 5 per group) 1, 6, 12 and 18 h post-injection for the high dose group. For the low dose group, blood alcohol was determined only at 1 and 6 h post-injections. At criterion times, animals were anesthetized with an overdose of pentobarbitol and the chest cavity was exposed. The left ventricle was pierced with a heparinized phlebotomy needle with attached vial and 1 ml venous blood was collected. The whole blood was centrifuged at 2500 \times g for 15 min and plasma aliquots were exposed to commercially available alcohol dehydrogenase Ektachem slide strips (Kodak Co., Rochester, N.Y.). The enzyme strip reaction was allowed to proceed to its endpoint and was measured colorimetrically with an Ektachem colorimeter at 340 nm giving a reading in mM ethanol/1 plasma.
Ischemia surgery: Nine days after the termination of alcohol treatment, each gerbil was subjected to bilateral carotid artery occlusion. A 9 day rest period was required to allow for weight recovery in all animals since one of the effects of chronic alcohol consumption is weight loss. A single ischemic insult of 5 min was utilized; this results in small but detectable deficits both structurally and functionally. Transient ischemia was produced by methods described in detail elsewhere. In brief, a warming blanket and a heating lamp were used in combination to maintain the cerebral temperature between 36.5 and 37.5°C at all times since hypothermia can have neuroprotective effects. Before transfer of the animal to a stereotaxic frame a midline neck incision was used to expose the carotid arteries. The two arteries were isolated with threads so that they could be easily manipulated once the animal was in the stereotaxic frame. Care was taken to ensure that the threads did not hamper blood flow to the brain in any way before the actual occlusion. At the appropriate time the carotid arteries were carefully occluded, with aneurysm clips, for 5 min.

In vivo microdialysis procedure: Following the isolation of the carotid arteries the animals were placed in a stereotaxic frame (David Kopf, USA) for positioning of the microdialysis probe. The probe was exposed and a burr hole was drilled over the right hemisphere. An in vivo microdialysis probe mounted on guide clips was then slowly lowered into the CA1 subfield of the hippocampus with coordinates 1.7 mm lateral and 1.5 mm posterior to bregma at a depth of 2 mm according to the gerbil atlas.

Once the polycarbonate-polyether copolymeric probe (membrane length 2 mm, CMA 12, Stockholm, Sweden) was at its target, the gerbils remained anesthetized for 2 h prior to the collection of baseline samples. Modified Ringer’s lactate solution (NaCl 145 mM, KCl 2.7 mM, CaCl2 1.2 mM, MgCl2 1.2 mM, pH 7.4) was infused at a rate of 2 μl/min using an infusion pump (CMA 100 microinjection pump), and the fluid was collected in 10 min samples (20 μl per sample). A heat lamp was used to warm the microdialysis fluid to approximately the same temperature as the brain. After the collection of three baseline samples, the aneurysm clips were applied. A 2 min delay between onset of ischemia and collection of sample number 4 was introduced to allow time for the ischemic perfusate to reach the sample vial.

At the end of the ischemic period the clips were removed and an additional six samples were collected. The scalp and neck were then carefully sutured and the animal was allowed to recover for one month before histology: the effects of ischemia may be underestimated in animals sacrificed <10 days after ischemic procedures. During this recovery period gerbils were closely monitored for evidence of brain infection, which none developed.

HPLC procedure: The microdialysis fluid was immediately transferred on ice for HPLC measurements (Waters, Toronto, Canada) by a rapid HPLC-EC method. Briefly, pre-column derivatization of amino acids with o-phthalaldehyde (OPA)/β-mercaptoethanol (BME) was performed prior to electrochemical detection with a 715 Ultra Wisp Sample Processor (Waters, Toronto, Canada). The Ultra Wisp dispenses the reagent into the microdialysis perfusate and mixes it thoroughly in a 200 μl loop. The injection was made precisely 2 min following the reaction time. The mobile phase consisted of 0.10 M disodium hydrogen orthophosphate, 0.13 mM ethylenediamine tetraacetic acid—sodium salt and 20% methanol. External standards of 0.625, 1.25, 2.5, 25, 100 and 1200 pM/10 μl glutamate were used for all the samples.

Histopathology: One month post-ischemia each surviving animal was anesthetized and perfused, and the brain was extracted and cryoprotected according to methods described previously. The brains were then cut into 40 μm coronal slices, and stained with a modification of the silver impregnation method. Representative sections were cut through the cortex, striatum, hippocampus and thalamus, and distally through the substantia nigra and the medial geniculate nucleus according to the gerbil atlas. Neuronal damage was assessed by light microscopy in a blind manner using the following scoring system: 0, no damage; 1, <25% damage; 2, 25–75% damage; 3, 75% damage; 4, infarction. The correct positioning of the microdialysis probe was also verified and all probes reached their targets.

Results

Blood alcohol concentrations reached equilibrium levels ~1 h after the s.c. injections. The high dose resulted in immediate loss of the righting reflex in all subjects, while low doses made subjects ataxic (Table 1). Intergroup comparisons of glutamate release were made by first converting post-ischemic sample

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time post-injection</th>
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<tbody>
<tr>
<td>4 g/kg</td>
<td>1 h  6 h  12 h  18 h</td>
</tr>
<tr>
<td>1 g/kg</td>
<td>76.8 ± 8.1 83.0 ± 2.5 23.6 ± 2.4 0.0 ± 0.0</td>
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Values are the mean ± s.e.m. expressed in mM/l plasma (n = 5 per group).
concentrations for each gerbil (n = 6) into a percentage of the average concentration of combined samples 1-3 (pre-ischemia), so that each animal functioned as its own baseline. Three gerbils in the high dose group and one in the low dose group died during the surgical procedure due to accidental carotid rupture. An ANOVA with repeated measures on sampling time (with statistical correction for unequal sample sizes) revealed an overall significant group × time interaction for glutamate release (F(10,100) = 3.18, p < 0.001). Neuman–Keuls comparisons revealed decreased glutamate release in both the high dose (p = 0.03) and low dose (p = 0.04) groups at 40 min compared with saline-injected controls (Fig. 1).

The average damage score for each of the three groups was ~1 in the CA1 and CA4 subfields of the hippocampus and 0.5 in the striatum indicating approximately 25% cell loss in all three groups. There were no detectable intergroup differences in brain damage in any of the six regional brain areas examined. Intergroup comparisons of reference and working memory performance on a radial arm maze10 one month post-ischemia to test for neurobehavioral deficits revealed no differences.

Discussion

The significantly decreased glutamate release immediately post-ischemia which was seen in both groups treated with ethanol could be interpreted as an ethanol-induced decrease in the number of post-synaptic receptors in the hippocampus.9 In this respect the low dose was above a critical threshold since alcohol exerts dose-dependent responses.7 Since the hippocampus is rich in NMDA receptors,8 a reduction in the number of post-synaptic NMDA receptors in the hippocampi of intermittently ethanol-injected animals could result in compensatory decreased extracellular glutamate release into the synaptic cleft during ischemia. Since glutamate released during ischemia may exacerbate neuropathological outcomes,12 attenuated glutamate release in ethanol-treated animals would have been expected to result in improved cytological outcome.

Conclusion

The lack of significant intergroup differences on either histological or neurobehavioral measures may suggest a functional dissociation of glutaminergic mechanisms involvement in the pathogenesis of aggravated stroke outcomes with alcoholism. By default, the findings could lend support for the alternative calcium hypothesis of neuronal cell death,18 given that increased voltage-gated calcium channels have also been found to follow repeated ethanol exposure.19

References


ACKNOWLEDGEMENTS: Supported by a grant from the Heart and Stroke Foundation (A.S.). Parts of this article were presented in November 1996 at the XXVI Annual Meeting of the Society for Neuroscience in Washington, DC.

Received 15 July 1997; accepted 5 August 1997