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Research report

Neuroprotective effects of estrogen treatment on ischemia-induced behavioural deficits in ovariectomized gerbils at different ages

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ABSTRACT

Although much is known about the protective effect of acute estrogen therapy in cerebral ischemia, relatively little is known about its effect on functional outcome at different ages. The impact of age is, however, important on the efficacy of steroids in the central nervous system. We investigated whether a single dose of estradiol pre-treatment would be neuroprotective in young (4 months), middle-aged (9 months) and old (18 months) female gerbils following 10 min global brain ischemia. Apoptotic and necrotic cells were labelled and quantified in the affected hippocampus; exploratory activity, attention and memory functions were tested using open field, spontaneous alternation, novel object recognition and hole-board test. Age effect and treatment effect were analysed. High single dose (4 mg/kg b.w.) of estradiol pre-treatment exposed a marked neuroprotective effect against hippocampal cell loss in all age groups. In behavioural tests, however, age-related differences could be observed. In middle-aged and old animals the worsening in memory function following ischemia was more prominent compared to that in the young ones. In the Y-maze and the novel object recognition tests the middle-aged, in the hole-board test (investigating working memory and total time) the old gerbils had the worst functional outcome. Only reference memory in hole-board test did not change by age. Estrogen improved memory performances in all the tests at every age. We can conclude that age of experimental animals is a factor worsening the outcome following brain ischemia. A single-dose estrogen therapy prevents the lesion-induced behavioural dysfunctions and the hippocampal cell loss.

1. Introduction

In the clinical practice usually the elderly subjects are affected by ischemic stroke, while preclinical animal data are collected mostly from different stroke models of young animals. Very few systemic studies do exist on ageing animals and their behavioural consequences in response to brain ischemia.

The ageing brain reacts in a different manner to ischemic brain injury compared to young's, as there are age-related structural and functional changes in the brain [3,6,18,24]. Increased neuronal loss [2,10,32,33,35], increased glial scar formation [33], early macrophage activation [33], decreased plasticity of the cerebrovascular wall [34] together with worst functional recovery [2,33,56] are associated with ageing.

Local neurosteroid production in the hippocampus together with the circulating gonadal hormones plays an essential role in brain functions, such as in synaptic plasticity and memory formation [14,36]. Changes in synaptic density during estrous cycle and following OVX are attributed to estradiol [36]. Exogenous estradiol also increases the synaptic density even following one single dose of estradiol [38], and enhances hippocampus dependent learning by modifying GABA-ergic and cholinergic release and receptors [11,17].

In cerebral ischemia estrogen moderates blood–brain barrier dysfunction [26], reduces excitotoxicity [7,22,52], and inflammation [22,44,46], functioning as antioxidant [7], increases cerebral blood flow [22,23,31], and increases the expression of cell-survival mediators (such as bcl-2) together with inhibiting death-promoting cascades (p75, caspase-3, caspase-12, TNF-α, IL-1 and IL-6, etc.) [28]. Estrogen therapy can lead to enhanced recovery following ischemic events of brain tissue in young animals [22,28,41,46] based on mainly chronic assessment that is a model of

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perimenopausal estrogen supplementation. The efficacy of a single-dose estrogen administration after experimental brain ischemia is not well documented. Further unsolved question is the estrogen effect on cerebral ischemia at different ages and its impact on functional outcome, as the ageing brain can react differently to any drug that had been protective in the young [12,16,47,49]. In case of estradiol therapy, as estrogen receptor expression is altered with reproductive senescence, its efficacy is also doubtful [22,27,43].

The aim of our study was to compare therapeutic effect of a single-dose estradiol (E2) pre-treatment on different behavioural performances after brain ischemia in young, middle-aged and elderly female gerbils. In our study we used an accepted method by Chen et al. [4]. We chose to examine a single-dose therapy as it is clinically more relevant than chronic treatment – in preventing or threatening global cerebral hypoperfusion and ischemia, such as related to cardiac and vascular surgery, etc.

2. Materials and methods

2.1. Animals

Female Mongolian gerbils of 4 (4 mo, young), 9 (9 mo, middle-aged) and 18 (18 mo, aged) months of age were used for these experiments. Animals were housed in an air-conditioned room at 22 ± 1 °C with a 12 h light/dark cycle (light on at 07.00 a.m.). Food and tap water were available ad libitum. In all animal groups bilateral ovariectomy was performed to eliminate endogenous estradiol production. For the experiment ovariectomized (OVX) female gerbils were randomly assigned into three experimental groups, 15 animals per group: 1. control animals (sham operation (carotid exposure without clipping the arteries) + vehicle treatment; abbreviated as sham-veh or sham), 2. ischemia affected animals (ischemia + vehicle; abbreviated as isch-veh or ischemic), 3. estrogen-treated gerbils (ischemia + estradiol treatment, abbreviated as isch-E2 or estrogen treated). We used five animals from each group for histological examinations, their brain samples were collected on post-operative day 4. The rest of gerbils from each group were designed for behavioural tests starting on post-operative day 7. General locomotor activity and hyperactivity were evaluated in novelty-induced exploration and in spontaneous alternation tests, effects of ageing and hippocampal damage were estimated by spontaneous alternation and novel object recognition tests. Both memory functions (working and reference memories) as well as spatial learning capacity of hippocampal and cortical structures were observed in hole-board spatial learning task. On post-operative day 12 these gerbils were sacrificed. All the experimental procedures carried out on animals had been approved by the Animal Examination Ethical Council of the Animal Protection Advisory Board at the Semmelweis University, Budapest.

2.2. Ovariectomy (OVX) and transient bilateral carotid clipping

Animals were anaesthetised initially with 4% halothane in a 30% O_2/70% N_2O mixture and the anaesthesia was maintained during the course of experiment with 1.5–2.5% halothane breathing spontaneously via facemask. Bilateral OVX surgery was carried out through two small lateral abdominal incisions and both right and left horns of the uterus were exposed. A ligature of non-absorbable silk filament was placed around the two horns of the uterus and both ovaries to avoid bleeding, then ovaries were carefully removed, the uterus remained intact. Two weeks later using the same anaesthesia, brain ischemia was induced as follows. The common carotid arteries were exposed through a cervical incision at the ventral midline and were carefully separated from the surrounding tissue and vagal nerves. Both arteries were clipped with atraumatic aneurysm clips (Codman, Johnson and Johnson, Le Locle, NE, Switzerland) for 10 min. Following the occlusion clips were removed to restore blood flow. The same surgical procedure was performed on the sham-operated group, but without the actual ligation. Thirty minutes before the surgery sham-operated and untreated ischemic animals were injected intraperitoneally with vehicle solution (50% alcohol and 50% normal saline) in a dose of 0.4 ml/100 g body weight, while estradiol treated ischemic animals subjected to ischemia were injected by 0.4 mg/100 g body weight 17β-estradiol (4 mg/kg body weight, Sigma Chemical Co. St Louis, MO, USA) dissolved in the vehicle solution (0.1% estradiol solution).

2.3. Brain histology, TUNEL and caspase-3 double labelling

On the 4th post-operative day animals (five from each group) were sacrificed (decapitation was performed under deep Halothane/O_2/N_2O anaesthesia) and brains were removed and immersion fixed in buffered paraformaldehyde (in 10% buffered paraformaldehyde for 2 days and in 4% buffered paraformaldehyde for another 5 days) then embedded into paraffin. From the dorsal hippocampus region (starting at the level −2.2 mm to bregma) three paralleled coronal 10-μm-thick sections were collected 0.1 mm from each other. The apoptotic and necrotic cells were labelled by TUNEL (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labelling) and caspase-3 immunostaining [42,48,51].

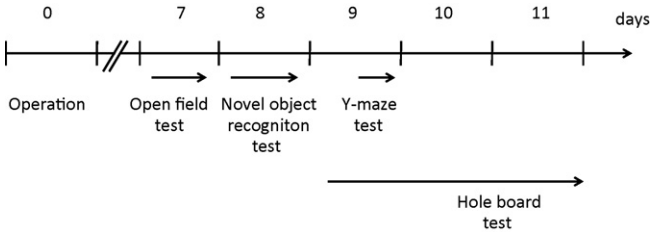

Fig. 1. Time line of behavioural tests.

Briefly, TUNEL reaction-mixture (In Situ Cell Death Detection Kit, Roche, Germany; 60 min) was used for immunostaining according to the manufacturer's protocol. After rinsing with phosphate buffered saline (PBS), sections were incubated in caspase-3 primary antibody solution (diluted 1:100, RnD Systems, Germany; 60 min) followed by rinsing with phosphate buffered saline and incubation with fluorescence conjugated goat anti-rabbit secondary antibody (diluted 1:100, Alexa 568, Molecular Probes; 40 min).

For quantitative analysis of apoptotic and necrotic cells, randomly selected non-overlapping areas (0.15 mm^2) of the hippocampal CA1 and CA2 zones were selected, TUNEL and TUNEL–caspase-3 double-labelled cells were counted using 60× objective, and an average number was calculated based on all sections in each individual. We took five hippocampal images within each brain hemispheres for a total of 10 images per coronal slice using three parallel sections from each animal. The TUNEL and caspase-3 positive cells were counted automatically with Image J 1.37 software (NIH, USA). Data were analysed blind to exclude the operator bias. All images were taken with the same settings of confocal microscopy (Bio-Rad MRC 1024 confocal system, Bio-Rad Corp., Hertfordshire, England on a Nikon Optiphot inverted microscope, Donsanto Corp., Nattick, Massachusetts, USA).

2.4. Behavioural tests

All animals were previously habituated to experimental manipulations by 1 week long daily handling before behavioural tests. For age-related behavioural differences gerbils were tested for general and exploratory activity, attention, short- and long-term memory functions. The adaptation of these behavioural tests to gerbils was described previously [51]. Briefly, novelty-induced exploration was examined on post-operative day 7, followed by novel object recognition on day 8, and by spontaneous alternation on day 9. Accommodation to the hole-board spatial learning task was started on day 8, while the spatial learning test itself was carried out on days 9–11. Animals were food deprived from post-operative day 8 (after the novel object recognition test) to the 11th post-operative day, receiving restricted food supply, while their body weights were maintained on the 95–98% of their original weights (Y-maze test was made after the hole-board test, when animals were fed already). In this way the animals were motivated enough to collect the reward, i.e. sunflower seed in the hole-board arena. On post-operative day 12, animals were sacrificed the same way as for brain histology. For the time line of the behavioural tests see Fig. 1.

Novelty-induced exploratory activity was carried out in a cylindrical open field (diameter 80 cm) surrounded by a wall of 35 cm high. Animals were placed in the centre of arena and during a 3 min period the following behaviours were recorded: latency time to start exploration (s), horizontal activity (crossing) – number (scores) of lines crossed between sectors in the outer and inner circles were recorded separately, vertical activity (number of rearing) – number and duration of standing up into an upright position, frequency and duration of face washing and body grooming. In addition activity was calculated by the following equation:

$$\text{activity} = \text{crossing inner} + \text{crossing outer} + (\text{number of rearing} \times 2).$$

Spontaneous alternation [51] is mainly a hippocampus-dependent behaviour serving for assessing attention toward novelty and working memory. This test was estimated in a black plastic Y-maze with sawdust on the floor. The arms were 50 cm long, 30 cm high and 10 cm wide and converged at 120° angle. Each animal was placed at the centre of the maze and allowed to move freely (3 min). Alternation was defined if the animal entered the arm different from the two previously entered arms; an error was recorded if the animal went back to either of the two arms just previously visited. The percentage of relative alternation was calculated from the ratio of the number of alternations divided by the number of total arm enters – 2. The value was multiplied by 100.

Novel object recognition [51] was tested in a habituated open field arena. During the first trial (5 min) two identical objects were placed into the arena and gerbils were allowed to freely explore them. After a 90 min delay the animals were tested for another 5 min. During this second trial one object from the first trial (familiar object) was replaced by a novel object. Frequency (score) and duration (second) of visiting the objects were registered. The total number and duration of visits towards both objects served for general exploratory activity. The percentage of *recognition*