Effect of acute estrogen pre-treatment on the expression of apoptotic and cerebral plasticity genes and adaptive behavior following global cerebral ischemia in gerbils at different ages.

Adaptation of behavioral tests to gerbils.

PhD thesis

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1. INTRODUCTION

Ischemic stroke is one of the main causes of death and disability in recent clinical practice, and the old are primarily affected. In basic research, however, mainly young animals are used. During aging the brain undergoes several structural and functional changes, and reacts differently to ischemic brain injury compared to the young brain. For this reason, a drug should be evaluated in older animals to ensure efficacy, particularly if the mechanism of action is suspect to be affected by age. In case of estrogen therapy, where receptor expression decreases by age, it is important to test its effect at different ages following brain injury. In addition, mechanisms of action are commonly studied but it is very important to understand how a treatment changes the functional outcome in the animals after stroke.

Although much is known about the protective effect of estrogen therapy in cerebral ischemia, relatively little is known about its effect on functional outcome in the elderly and the old due to acute treatment. There are only a few experimental data on the effect of acute estradiol treatment in ischemic animals, and no age effect has been described before. Following brain ischemia estrogen decreases infarct volume, moderates blood-brain-barrier dysfunction, reduces excitotoxicity, increases cerebral blood flow, and increases the expression of cell survivor mediators, such as bcl-2 in young animals. Although this data is mainly related to chronic estrogen therapy, there are also some publications on acute estrogen treatment, which models stroke therapy, rather than menopause with chronic estrogen substitution.
Determining the effect of acute estrogen treatment on apoptotic genes’ expression is essential to understand the protective effect of the therapy. The role of anti-apoptotic bcl-XL, or pro-apoptotic bax expression has not been determined in a model of acute estrogen therapy after brain ischemia.

Estrogens are also known to increase synaptic density in the intact brain, but its effect on cerebral plasticity following ischemic insult had not been described.

2. AIMS

The aims of my work were the followings:

- To standardize some behavioral tests on Mongolian gerbils that are used previously in other species (open field, Y-maze, novel object recognition, hole-board spatial learning).
- To determine if these tests are reliable at different ages.
- To standardize these tests to 10 min global cerebral ischemia in gerbil.
- To determine the effect of a single-dose estradiol pre-treatment on functional outcome in gerbils.
- To compare therapeutic effect of a single-dose estradiol pre-treatment on different behavioral tasks, and hippocampal damage after brain ischemia in young, middle-aged and elderly gerbils.
To determine the effect of acute estrogen treatment in ischemic animals on the expression of apoptotic (bcl-XL, bax) and cerebral plasticity (synapsin-I, GAP-43 /growth-associated protein 43/, nestin) genes.

3. MATERIALS AND METHODS

In our work I used Mongolian gerbils (Meriones unguiculatus) for all studies. To investigate intact animals’ behavior 4-, 9- and 18 months old (n=10 in every group), to investigate the effect of brain ischemia, 9 months old male gerbils were used (n=10 in every group). Sham operated animals were used as controls in this test. Hippocampal damage was verified by TUNEL-caspase-3 staining on the post operative day 4\textsuperscript{th} (n=5 in every group).

To assess the neuroprotective potential of a high, single-dose of estradiol we used 4-, 9- and 18 month old ovariectomized female gerbils. We studied behavioral differences (n=10 in every group) and histological changes (n=5 in every group) in sham operated, ischemic and ischemic+estrogen treated animals at each ages.

To evaluate the changes in apoptotic and cerebral plasticity genes’ expression following cerebral ischemia and estrogen pre-treatment we used 4 month old ovariectomized gerbils (n=5 in every group), and hippocampal samples were analyzed using real-time PCR.

Behavioral tests were started on post operative day 7\textsuperscript{th}, histology and real-time PCR was done on post operative day 4\textsuperscript{th}.
3.1. Behavioral tests

To investigate animals’ exploratory activity and habituation we used the open field test, where horizontal and vertical activity and grooming were recorded over 3 min. In the Y maze test we studied short term memory function using alternation between three possible arm choices over 3 mins. An arm alternation was defined as the animal entering a different arm from the two previous arms entered. In the novel object recognition test we analyzed long term memory function. During the first trial (5min) two identical objects were placed in the open field arena, which became familiar to the animal after 5 min. After a delay of one and a half hours, in the second trial, one of the familiar objects was replaced by a novel object and long-term memory was determined from the relative time spent exploring the novel object. Spatial learning ability was studied in a hole-board apparatus with 16 holes (4x4 arrays) in the floor and with 4 rewards (food) to find. In this task we registered the total time to collect rewards. We calculated the short-term and long-term memory parameters of the gerbils during three days three times a day.

3.2. Surgery

Animals were anesthetized with Halothane and a mixture of O$_2$/N$_2$O. Global cerebral ischemia was established by clipping both common carotid arteries for 10 minutes with atraumatic microaneurysm clips (Codman, Johnson and Johnson, Le Loche, Switzerland). During sham operation the preparation of the carotid artery was done without the actual clipping. In case of female gerbils ovariectomy was
performed 3 weeks prior to brain ischemia via a lateral abdominal incision. Acute estrogen therapy, 4 mg/kg body weight 17β-estradiol (Sigma Chemical Co., St. Louis, MO, USA) was given intraperitoneally 30 min prior to ischemia.

3.3. Histology

Four days after ischemia or sham operation animals were euthanized. Hippocampal damage was studied on paraffin embedded sections to determine the effectiveness of the brain injury and/or estrogen therapy. To visualize cell loss, TUNEL-caspese-3 double labeling (TUNEL reaction mixture, In Situ Cell Death Detection Kit, Roche, Germany and anti-caspase-3 antibody, RnD systems, Germany with Alexa 568, Molecular Probes, USA) was used on 3 serial 10 µm sections. Cells were count in the CA1 and the CA2 regions. If cells were TUNEL positive, they were counted as necrotic, if cells were TUNEL positive and also caspase-3 positive, they were counted as apoptotic. For this analysis a laser confocal microscope (Bio-Rad MRC 1024 confocal system, Bio-Rad Corp., Hertfordshire, England on a Nikon Optiphot inverted microscope, Donsanto Corp., Nattick, Massachusetts) and image analyzer software (Image J 1.37 software, NIH, USA) were used.

3.4. RNA isolation and real-time PCR

Total cellular mRNA was isolated from the hippocampus 4 days after ischemia or sham operation. The expression of bcl-XL, bax, GAP-43, synapsin-I and nestin genes was analyzed using quantitative
real-time RT-PCR and TaqMan® Gene Expression Assays (Applied Biosystems). The quantification of gene expression levels was determinate by ddCT method.

3.5. Statistics

To analyze behavioral data we used one/two way ANOVA or two-factorial ANOVA analysis followed by Tukey’s post hoc test depending on what kind of statistics the data needed. In case of 2 groups (ischemia and sham operation in male gerbils) student’s t test, Kruskal-Wallis rank sum test (with Dunn test) were used.

For histological analysis: in case of two groups student’s t test was used; but in case of more groups one way ANOVA was chosen with Tukey’s post hoc analysis.

To analyse differences in gene expression, one way ANOVA was used.

For statistical significance p<0.05 was the criterion.

4. RESULTS

- The following behavioral tests are successfully adopted to gerbil: open field exploration, alternation in Y-maze, novel object recognition and hole-board spatial learning task. Animals were active and inquisitive enough in every test. Their memory function could be easily determined in these tests and they could understand their `mission` in the spatial learning task.
• Animals had stable behavior between 4 and 18 month of age. This indicated that the reliability of these tests is good during the examined period.

• Ten min of global cerebral ischemia resulted in worse functional outcome in every test. In the open field arena animals were more active (mainly in the outer circle), but they did not explore their surrounding as much as intact gerbils did (activity was mainly running around and around). Short term and long term memory functions together with spatial learning were significantly impaired after brain injury. In the Y-maze test ischemic animals could not remember which arm they had just visited. In the novel object recognition test, 1.5 hours following the first trial, injured gerbils could not discriminate the `familiar` object from the `novel` object. In the hole-board task every parameter deteriorated, so total time to collect rewards was longer, short and long-term memory worsened in stroke animals compared to control sham operated animals.

• A single, high-dose of estradiol-pretreatment improved behavioral deficits in each test. Estrogen treated animals had similar behavior than sham operated control gerbils. This change in function was accompanied by significantly less injury in the hippocampus (both TUNEL-positive necrotic and TUNEL-caspase double labeled apoptotic cells were significantly less in the treated group).

• Age-related differences were not observed in hippocampal cell loss. In behavioral tests, however, age did impact outcome. 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 animals were worse. 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 with age. Estrogen ameliorated functional deficits in all tests at every age; no age effect was observed together with treatment.

- Expression of the anti-apoptotic bcl-Xl and the cerebral plasticity marker synapsin-I and nestin increased with acute estrogen pre-treatment in ischemic animals. No change, however, in bax or GAP-43 expression was detected in estrogen treated animals compared to ischemic gerbils.

5. CONCLUSIONS

On the basis of these results of our experiments I can conclude, that there are many reliable hippocampus-dependent memory tests (Y maze, novel object recognition and hole-board task) for Mongolian gerbils, even at the age of 18 months. We also can say that a high, single dose of estradiol is effective at different ages (4-, 9- and 18 months of age) following 10 min global cerebral ischemia in gerbils as functional outcome was significantly better and cell loss was also significantly reduced than in the ischemic group. This therapy has anti-apoptotic effect and increases cerebral plasticity, which play an important role in cytoprotection or cerebroprotection.
6. PUBLICATIONS

6.1. Publications directly related to my thesis


6.2. Publications not related to my thesis

