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DIFFERENT *c- fos* ACTIVATION IN RAT HIPPOCAMPAL CA1 NEURONS AND DENTATE GRANULE CELLS AFTER GLOBAL AND PRECONDITIONED CEREBRAL ISCHEMIA

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We investigated the activation of c-fos early gene in the hippocampal region CA1 and gyrus dentatus in two groups of rats. The first group, exposed to global ischemia (during 10 minutes of occlusion of four vessels) was sacrificed 60 minutes after reperfusion, and the second group was first exposed to transient (3-4 minutes) ischemia (preconditioned or tolerant rats) and after 72 hours was again exposed to global ischemia in the same way as the first group. Immunohistochemistry for c-fos protein was performed using the avidin-biotin peroxidase method. Analysis of results included morphological, semiquantitative analysis and t-test of differences between GD and CA1 region of the hippocampus. Results showed a significantly more intense c-fos activation in GD than in the CA1 region in both groups, with global ischemia and preconditioned rats. This indicates different pattern of c-fos activation in investigated brain regions in relation to time factors, and also indicates a strong impact of ischemic preconditioning on c-fos activity in both investigated regions. Our results clearly show that in the future studies of c-fos activation in the brain a very careful experimental design related to the control of distinct regional and time effects needs to be performed.

Key words: CA1 hippocampal region, cerebral ischemia, c-fos activation, dentate gyrus, preconditioned ischemia, rat

INTRODUCTION

After the discovery of a class of early genes, which are not induced as a consequence of cell growth, but are directly regulated by growth factors (Cochran *et al*., 1983), numerous studies showed rapid activation of the proto-oncogene *c-fos* in brain (Herrera and Robertson, 1996). The activation of *c-fos* immediate early gene in the brain was reported after various stimuli such as: noxious or mechanical brain injury, brain ischemia, stress, pharmacological activation, brain

development (related to synaptogenesis and differentiation rather than to mitosis) (Herrera and Robertson, 1996; Schonthal, 1990; Weinberg *et al*., 2007; Girotti and Spencer, 2007; Leifer and Kowall, 1993). Many studies have shown that prolonged *c-fos* activation lead to neuronal death (Gubits *et al*., 1993; Kasof *et al.*, 1995; Morgan and Curran, 1991). The selective neuronal death can be prevented by a short period of ischemia (preconditioning) and subsequent longer ischemia few days later (Somer *et al.*, 1995; Kirino, 2002).

In rats occlusion of all four major arteries supplying the brain produces global ischemia. Global ischemia activates immediate early genes (*c-fos*) in the rat brain which encode transcription factors that play an important part in the signal cascade between extracellular messengers and long term changes in the cell phenotype (Sanders *et al*., 2008). There are many models of global ischemia (Pulsinelli, 1985) and it is very difficult to determine clearly the border between injury (lethal or recovering changes) caused by these different models. Transient cerebral ischemia produces induction of *c-fos* proto-oncogene and this expression could play an important role in the cell recovery from ischemia by up-regulating late gene expression for survival (Cho *et al*., 2001).

In transient global ischemia some parts of the hippocampus are spared (Kirino and Sano, 1984) and different parts of the brain are *c-fos* activated (Aden *et al*., 1994). *In situ* hybridization study shows that 24 h recirculation after 20 min four vessel occlusion ischemia is followed by an increase of *c-fos* positive neurons in the hippocampus, with a peak between 24 and 48 hours (Jorgensen *et al*., 1989). Induction of *c-fos* immediate early gene is restricted to the less vulnerable regions (dentate gyrus) (Kiessling *et al*., 1993).

Ischemic preconditioning produces bilateral ischemic tolerance which shows significant protection against neuronal damage especially in the hippocampus (Belayev *et al*., 1996; Heurteaux *et al*., 1995). The increase of *c-fos* expression in global ischemia could be prevented by a mild preconditioning insult. This induction of tolerance lead to the protection of vulnerable neurons (Truettner *et al*., 2002). In different hippocampal neurons *c-fos* expression could be seen in all sectors peaking in vulnerable CA1 pyramidal neurons and in the dentate gyrus (Bottiger *et al*., 1999). Investigations of immediate early gene expression after cerebral ischemia in gerbils show that *c-fos* is present in reversibly damaged CA1 neurons (Tomimoto *et al.*, 1999). The increase in expression of stress-related, neurotrophic and immediate early genes in response to a mild preconditioning insult may help to mediate the protection of vulnerable neurons to subsequent lethal ischemic insult. This may be important for tissue recovery, as well as for neuropsychiatric symptoms after stroke (Johansson *et al*., 2000).

In this study we compared 60 minutes after ischemic attack the effects of transient global ischemia and of preconditioned transient global ischemia on *c-fos* expression in neurons of the hippocampus (CA-1 region and dentate gyrus) of rats.

MATERIALS AND METHODS

We used 15 Wistar rats (9 females, 6 males; 200-635 gr) which were kept in cages at room temperature, food and water *ad libitum*. Experiments were performed with approval of the Animal Use Committee.

Ischemic attack according to the Pulsinelli method (1982):

– Global (total) ischemia (6 rats) caused by ligature of all four major blood vessels (vertebral coagulation with bilateral reversible ligatures of the carotid artery by paraffin threads for 10 minutes) and perfusion (sacrifiction) 60 minutes after ischemic attacks (Rats: R6, R10, R32, R33, R34, R35).

– Ischemic tolerant group (6 preconditioned rats) had the first ischemia caused by transient obliteration of all four vessels for a period of 3-4 minutes (ishemic tolerant attack) and after 72 hours global ischemia, produced as described above, for a period of 10 minutes and perfusion (sacrification) 60 minutes after the second attack (Rats: T2, T3, T4, T5, T6, T7).

– Control-1 (2 rats): control group with no intervention (R7, R8).

– Control-2 (1 rat): vertebral coagulation with 3-4 minutes ligature of both carotid arteries without 10 minutes re-ligature on both of them. This rat was a control for the tolerant group of rats (T9).

One hour after ischemic attacks the animals were perfused 60 minutes with 4% paraformaldehide with 1% glutaraldehide. Brains were removed, postfixed in the same fixative overnight and cryoprotected by immersion in 20% sucrose. Thereafter, 50 μm thick cryostat sections were prepared for the free floating sections immunohistochemistry using avidin-biotin peroxidase (ABC, Vectastain) method. Briefly, sections were washed in 0.1 M phosphate-buffered saline (PBS), incubated in Triton-X-100 and 10% normal goat serum, and subsequently incubated 48 h with specific *c-fos* antibody (Santa Cruz, 1:10 000). The next step was incubation in secondary anti-rabbit antibody for 1 hour and in avidin-biotin peroxidase for 1 hour. Phosphate-buffered saline (0.1 M, pH 7.26) was used to wash sections between all steps. The antigen-antibody complexes were visualized by 3,3'-diaminobenzidine and 0.03% H_2O_2. The sections were mounted and covered by DePeX.

Digital photos were used for morphological studies of *c-fos* immunoreactivity by the Analysis program (magnification 20x). Semiquantitative analysis, i.e. measuring of *c-fos* protein density in the gyrus dentatus and CA1 was done by "Scion Image 2000" program (based on NIH program). Analyzed data are: mean density and integrated density of *c-fos*-positive neurons in CA1 region and gyrus dentatus of rat hippocampus. Mean density presents average immunoreactivity within specific regions, and *integrated density* is immunoreactivity in the specific areas with background subtracted.

The statistical method used was standard t-test.

RESULTS

Morphological analysis

In the first group of 6 rats (R6, R10, R32, R33, R34, R35) with global 10 minute ischemia and perfused after 60 minutes, there was a very positive bilateral *c-fos* reaction in the dentate gyrus and a mild reaction in the CA1 hippocampal region.

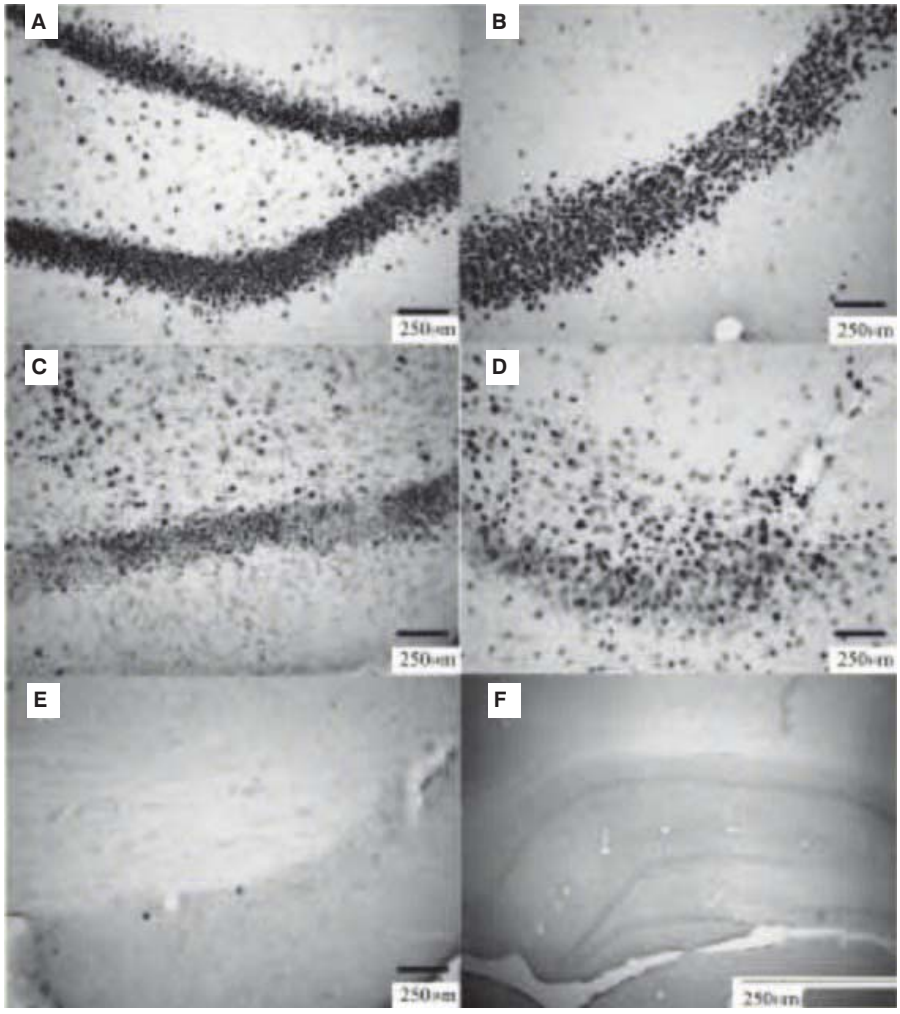

Figure 1. Reaction of *c-fos* in the hippocampal CA1 region and dentate gyrus. Observe a more intensive reaction in the dentate gyrus than in CA region in both experimental groups. Also, preconditioned rats show lower intensitiy of *c-fos* reaction than global ischemia group. A. Global ischemia-dentate gyrus; B. Global ischemia – CA1 region; C. Tolerant (preconditioned) group-dentate gyrus; D. Tolerant group CA1 region; Hippocampal regions of E. semicontrol, and of F. Total control

The second or ischemic tolerance group of 6 rats (T2, T3, T4, T5, T6, T7) had transient ischemia (3-4 minutes) and again (after 72 hours) global ischemia for 10 minutes, with perfusion after 60 minutes. The positive bilateral strong reaction reaction was in the hippocampus, both in the dentate gyrus and CA1 region.

The third or total-control group of 2 rats (R7, R8), and the fourth or semi-control group (one rat - T9), i.e. control for tolerant group showed the low *c-fos* reaction in all observed neural structures.

Semiquantitative analysis

Table 1. Mean density of *c-fos* neurons in global ischemia group

	Mean	SD	SE
CA1	73.922	6.5069	1.6801
Gyrus dentatus	99.0366	15.123	2.1387

The t-test (–6.234) showed statistically highly significant ($p<0.01$) greater mean density of *c-fos* neurons in dentate gyrus than in CA1 region.

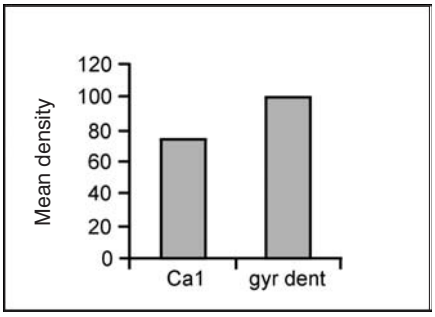

Figure 2. Mean density of *c-fos* neurons in the global ischemia group

Table 2. Integrated density of *c-fos* neurons in global ischemia group

	Mean	SD	SE
CA1	22.2293	22.0374	5.69
Gyrus dentatus	105.5254	43.4887	6.1502

The experimental value of Mann-Whitney U Test ($Z=-4.78$) showed greater, statistically highly significant ($p<0.01$) integrated density of c-fos neurons in dentate gyrus than in CA1 region.

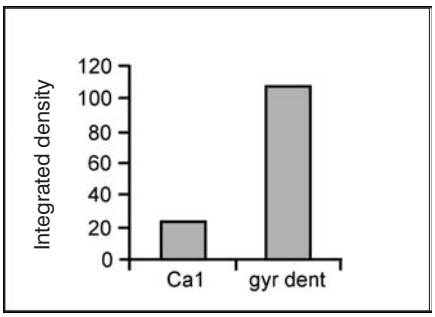

Figure 3. Integrated density of *c-fos* neurons in the global ischemia group

Table 3. Mean density of *c-fos* neurons in the preconditioned (tolerant) group

	Mean	SD	SE
CA1	79.6329	5.7155	2.1603
Gyrus dentatus	89.0841	10.6959	1.4555

Using the t-test (t=-2.284) in the tolerant group we found a statistically significant difference ($p<0.05$), with greater mean density of *c-fos* neurons in the gyrus dentatus than in CA1 region.

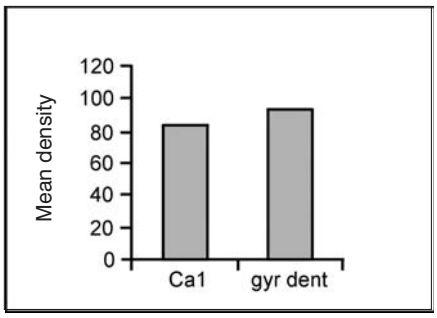

Figure 4. Mean density of *c-fos* neurons in the preconditioned (tolerant) group

Table 4. Integrated density of *c-fos* neurons in the tolerant group

	Mean	SD	SE
CA1	40.4871	27.8684	10.5333
Gyrus dentatus	82.368	62.1965	8.4639

Comparing the integrated density in the gyrus dentatus and in CA1 of the tollerant group rats, Mann Whitney U Test did not show a statistically significant difference ($Z=-1.22$; $p>0.05$) between these two regions.

DISCUSSION

The present study demonstrates the influence of global ischemia and of ischemic preconditioning on the immediate early gene expression *c-fos* in the rat hippocampus. The reaction was evaluated after a single (10 minutes) ischemic attack and after repeated (3 minutes ischemia and after 72 hours again for 10 minutes) ischemic attacks (tolerant group). Morphological analysis after a single attack shows more positive *c-fos* reaction in the hippocampus, as compared to the preconditioned rats. Our finding of a stronger reaction in dentate gyrus in both experimental groups is well related to the finding that *c-fos* expression is focused in the resistant area of dentate gyrus (Dragunow *et al*., 1993).

In the investigated animal model of transient global ischemia, neurons show a very positive *c-fos* reaction in dentate gyrus while in the CA1 region the reaction was low. This seems different from the results of other authors (Kirino and Sano, 1984; Pulsinelli, 1985). They found that neurons in the hippocampus selectively die, faster in CA1 than in the dentate gyrus. Late – onset *c-fos* induction was observed in the CA1 region after 30 minutes of global ischemia, strong labelling was in dentate gyrus 1 hour after ischemia, and CA1 neurons were labelled intensely after 3 days (Neumann-Haefelin *et al.,* 1994). Induction of *c-fos* occurred in the areas surviving transient cerebral ischemia promptly after 1 to 6 hours in pyramidal cells in hippocampus, but it is weak or absent in the part of the hippocampus known to develop delayed neuronal death (Takemoto *et al*., 1995). In the context of reaction to stress is interesting that in adrenalectomizied rats corticosterone injection caused early decrease of *c-fos* activity restricted only to dentate gyrus after 0.8 hours (Hansson and Fuxe, 2008). Our semiquantitative data show significant differences of both, mean and integrated density of *c-fos* positive neurons between CA1 and gyrus dentatus in the group of global ischemia (Figures 2 and 3). Both densities are much higher in gyrus dentatus than in CA1region. In the tolerant group of rats, the mean density is higher in the gyrus dentatus than in CA1 region, but integrated densities are similar. As the integrated density is immunoreactivity which is background subtracted, it means that *c-fos* immunoreactivity is not significantly different between CA1 region and the gyrus dentatus in the tolerant group of rats. Ischemic tolerance was also found in the cerebral cortex, basal ganglia and thalamus (Kitagawa *et al*., 1991). Recent experimental data have demonstrated that the selective neural death can be prevented by short period of ischemia and this tolerance shows significant protection especially in the hippocampus (Belayev *et al.,* 1996).

Our finding of decreased *c-fos* immunoreactivity related to time passed after ischemic attack (72 hours) was by different ways confirmed by other authors. Fos-like immunoreactivity after transient global ischemia was seen in several CA1 pyramidal cells, while neurons in dentate gyrus show higher density of immunoreactivity (Tseng *et al.,* 1997). After 20 min of transient ischemia, activation

was most intense in dentate gyrus after 30 min and delayed in CA3, and a moderate increase was after 2 hours after ischemia with the second peak in CA1 at 24-48 h postischemia (Wessel *et al,* 1991). Few neurons in the pyramidal layer of CA1 expresed *c-fos* as early as 24 hours, but most intense labelling in this region was at 72 h of recirculation (Jorgensen *et al.,* 1989). After 30 min four vessels ischemia, delayed cell death appeared in CA1 region of rat hippocampus with increases up to 72 hours (Grimaldi *et al*., 1990) which corresponds to our findings. Six hours after reperfusion *c-fos* immunoreactivity was greatly diminished in all areas of the hippocampus (Cho *et al.,* 2001). All these data generally suggest the importance of timing in studies of early genes activation in different brain regions. Early impairement of spiny CA3 cells and hilar neurons after ischemia may be causal to delayed neuronal death in CA1 principal cells (Hsu and Buzsaki, 1993). Delayed CA1 pyramidal cell death is caused by postischemic CA1 hyperactivity, primary generated by dentate gyrus granule cells activity. This explains why granule cells destruction prior to ischemic insult fully protects CA1 cells from ischemic insult (Johansen *et al.,* 1987).

CONCLUSION

The morphological and semiquantitative study of *c-fos* induction after cerebral ischemia shows significant differences between gyrus dentatus and CA1 region of rat hippocampus. Earlier and more intense activity was found in dentate gyrus than in CA1 neurons. Short transient ischemia with subsequent (after 72 hours) global ischemia probably induced a mechanism of tolerance which can have a protective role. This protective role can be seen in our experiment as decreased *c-fos* immunoreactivity in preconditioned (tolerant) group. Our study clearly showed significant regional and timing impact on *c-fos* activation in different brain regions, as a very significant but complex factor and its details remain to be elucidated.

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RAZLIKA U AKTIVACIJI *c-fosa* U HIPOKAMPALNIM CA1 NEURONIMA I GRANULARNIM NEURONIMA *GYRUS DENTATUS*-a KOD JEDNOKRATNE I PONOVLJENE ISHEMIJE MOZGA PACOVA

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SADRŽAJ

Istraživana je aktivacija ranog gena *c-fos* u CA1 regionu hipokampusa i u *gyrus dentatus*-u u dve grupe odraslih pacova. Jedna grupa je bila podvrgnuta globalnoj cerebralnoj ishemiji (tokom 10 minuta okluzije sva četiri krvna suda) i 60 minuta posle reperfuzije pacovi su bili žrtvovani. Druga grupa je bila prvo 3-4 minuta podvrgnuta tranzitornoj ishemiji (rezistentna grupa) i opet je posle 72 sata bila izložena globalnoj ishemiji na isti način kao i prva grupa. Imunohistohemijska analiza reakcije na *c-fos* protein je izvršena avidin – biotin metodom. Analiza rezul-

tata obuhvatila je morfološku i semikvantitativnu analizu i t-test u izračunavanju značajnosti razlika u reaktivnosti između *gyrus dentatus*-a i CA1 regiona hipokampusa. Rezultati su u obe grupe pacova, kako posle globalne ishemije, tako i posle tranzitorne i potom globalne ishemije (rezistentna grupa), ukazali na signifikantno veću *c-fos* aktivaciju u *gyrus dentatus*-u nego u CA1 regionu. Takođe je u rezistentnoj grupi *c-fos* aktivacija bila mnogo slabija nego u grupi podvrgnutoj samo globalnoj ishemiji. Sve ovo ukazuje na različit tok procesa *c-fos* aktivacije u istraživanim regionima mozga i u različitim vremenskim fazama eksperimenta. Takođe je dokazan i jak uticaj ishemičkog prekondicioniranja na ovu aktivaciju u oba istraživana regiona. Iz naših rezultata proizlazi da buduće studije moraju biti pažljivo osmišljene, tako da budu kontrolisani regionalni i vremenski efekti. Tek takva istraživanja bi nas približila mogućem korišćenju uticaja aktivnosti *c-fos* gena u zaštiti moždanih struktura od efekata ishemije.