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The Role of C-Fos Protein, Somatostatin and Neuropeptide Y in the Pathogenesis of Ischemic Brain Injuries Based on Animal Model of Cerebral Ischemia

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A B S T R A C T

The aim of this study was to define all the areas of changes in expression of nuclear c-Fos protein (c-Fos), cytoplasmic somatostatin (SS) and neuropeptide Y (NPY) in rat brain during experimental ischemia. Using the immunohistochemical method, brain mapping (based on the atlas by Paxinos & Watson) of immunoreactivity for c-Fos, SS and NPY in 39 rats, was studied in telencephalon, diencephalon and midbrain after resistant and transitory ischemia. The first experimental group (R group) was exposed to resistant ischemia by occlusion (10 minutes) of four vessels according to the Pulsinelli method. The second group was first exposed to transitory (4 minutes) ischemia (preconditioning) and, after 72 hours, to total ischemia as in the R group. There was a statistical difference between the R and T group in the c-Fos reaction, especially in the parietofrontal cortex, anterior amygdaloid area, claustrum, reuniens nucleus and suprachiasmatic nucleus. The dominant immunohistochemical reactivity was found for c-Fos protein, and the most reactive in terms of co-localization of c-Fos with SS and NPY was periventricular area of hypothalamus. The mapping showed that both, phylogenetically new as well as phylogenetically older brain structures reacted immunohistochemically. The results of our study, regarding the impact of preconditioning with a short period of ischemia on c-Fos activity and co-localization of c-Fos with SS and NPY immunoreactivity, showed the need for future studies of brain neuropeptides related to regional and time effects, and indicated brain structures which may require pharmacological targeting to achieve neuroprotective level of proto-oncogene activity in populations at risk.

***Key words**: brain, transitory ischemia, proto-oncogenes, c-Fos, somatostatin, neuropeptide Y*

Introduction

In spite of serious methodological problems and extrapolations in drawing analogies between animal and human models, experimental brain lesions have significantly improved our knowledge of molecular biology, diagnostic techniques and both prevention and medication management of numerous medical disorders[1,2]. In that sense, the focus of our study was on the regional expression of nuclear c-Fos protein (c-Fos), and cytoplasmic somatostatin (SS) and neuropeptide Y (NPY) during resistant and transitory experimental ischemia in rat brains.

Within the central nervous system (CNS), numerous stimuli, such as ischemia we used in our experiment, may induce immediate early genes (IEGs) expression. IEGs represent an immediate response mechanism that is activated at the transcription level in the first round of

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response to stimuli, before any new proteins are synthesized. The members of »fos« and »jun« family are the prototypes of IEGs who serve as transcription factors and code for proteins that help to regulate cell growth and differentiation. They are also considered as proto-oncogenes given that upon activation, a proto-oncogene (or its product) may become a tumor-inducing agent[3]. Though the role of activation of c-Fos gene and c-Fos protein in central nervous system (CNS) is not completely clear so far, the expression of c-Fos gene has been frequently used as a marker for research on neuronal activity in different brain areas[4,5].

NPY is a 36-amino acid peptide found in the hypothalamus, brainstem, spinal cord, and several limbic structures and is involved in the regulation of stress, eating, reward and sleep as well as anxiety and depression[6–8]. NPY neurons innervate CRF (corticotropin-releasing factor) containing neurons in the paraventricular nucleus of the hypothalamus, and NPY administration increases hypothalamic CRF levels and promotes CRF release[9]. A neuroprotective role of NPY under physiological has been suggested given that changes in NPY levels have been observed in different pathological conditions such as brain ischemia and neurodegenerative diseases implying that that NPY and NPY receptors may represent pharmacological targets in different pathophysiological conditions in the CNS[10]. Patients diagnosed with major depression who commit suicide were reported to have a reduction in NPY levels in the cortex, cerebellum and caudate nucleus[11]. Chronic administration of antidepressant drugs increased NPY concentrations in the neocortex and hippocampus in rats, and lower CSF NPY was found in first episode depressed patients compared with recurrent depressed patients[12,13]. A functional polymorphism in the NPY gene was found to be associated with alcohol dependence and recent evidence implicates NPY in both neurogenesis and involvement in schizophrenia and Alzheimer's-type dementia[14,15].

SS is a hypothalamic tetradecapeptide that is located principally in the nerve endings of the median eminence and in neurosecretory neurons located in the paraventricular nucleus. SS inhibits anterior pituitary secretion of adrenocorticotropic hormone, thyrotropin, growth hormone (GH), and prolactin and alters release of catecholamine neurotransmitters[9]. Five receptor subtypes have been cloned, and receptor-specific ligands have been developed. SS was so named because of its action in inhibiting the release of immunoreactive GH, a function that is sub-served by SS-2 receptors. In rats, SS delays the extinction of active avoidance behavior and antagonizes amnesia induced by electric shock[16]. Alterations in the concentration of SS have been associated with a number of conditions in which cognitive dysfunction is present, including Huntington's disease, Parkinson's disease, multiple sclerosis, Alzheimer's disease, and schizophrenia[17–22]. Decreases in SS are highly correlated with decreases in acetylcholinestrase, suggesting a close relationship between the cholinergic and somatostatinergic systems in long-term effects of brain lesions[23]. Decreased concentrations of SS are inconsistently found in patients with depression and central injection of SS in rats causes decreased slow wave and REM sleep, altered appetite and locomotor activity, impaired cognition and sensitivity to pain[24–28].

It is evident that our understanding of the neurobiology of neuropeptides has increased remarkably during the past decade, and there is already considerable evidence that these neuroregulators are involved in both the pathophysiology of certain major neuropsychiatric disorders and the mechanism of action of some of the drugs used to treat these disabling illnesses[29]. In the scope of our study, of special interest are findings concerning SS and NPY immunoreactivity (IR) following ischemia where increased synthesis of the neuropeptides is considered as a neuronal preservation mechanism[30–35].

Given the possible protective role of c-Fos, SS and NPY, the aim of this study was to define all the areas of their expression and co-localization in rat brains during ischemia.

Materials and Methods

This research involved 39 brains of adult Wistar rats studied in resistant and transitory ischemia and basically divided into the group with resistant ischemia (R) and the group with transitory ischemia (T) – preconditioned group. Animal experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

The material for c-Fos analysis was divided into 2 subgroups: a) the subgroup of 6 brains from R-group and 6 brains from T group. In these subgroups semi-quantitative measurements of c-Fos protein in cells was performed by the analysis of photographs with the Scion J program and transforming them into binary image (example in Figure 1); b) the subgroup of 5 brains from R group, and 5 brains from T group, both used for mapping.

The material where SS was analyzed, involved 8 brains – 3 from R group and 5 from T-group. The material for the analysis of NPY included 9 brains – 5 in R group and 4 in T group. Both materials (for SS and NPY analysis) were photographed and mapped to establish areas of CC and NPY expression and co-localization with C-FOS protein.

Rats of both sexes weighting 200–260 g were anesthetized by ketamine (100 mg/kg), placed in a stereotaxic frame, and both vertebral arteries were electro-cauterized. On the next day, rats were anesthetized with halothane (1.5–2.0%) in air for 4–5 min and surgically prepared for four-vessel occlusion ischemia by Pulsinelli[36]. R group was exposed to total ischemic attack caused by ligature of all four major blood vessels according to the Pulsinelli method (vertebral coagulation with bilateral reversible ligatures of the carotid artery by paraffin threads for 10 minutes) and perfusion (sacrification) 60 minutes after ischemic attacks. T group had the first

ischemia caused by transitory obliteration of all four vessels for a period of 4 minutes. After 72 hours, T group was exposed to the total ischemia, as described above, for a period of 10 minutes followed by perfusion (sacrification) 60 minutes after the second attack During surgical procedures, the body temperature of all the experimental animals was kept on 37 °C by heating lamps. All experimental animals were sacrificed by intracardiac perfusion with 4% paraformaldehyde after being anesthetized by ketamine (100 mg/kg). Brains were removed, post-fixed in the same fixative solution overnight and cryo-protected by immersing the tissues in 20% sucrose. Finally, 60ìm free-floating sections were cut. Immunohistochemistry was performed using the avidin-biotin peroxidase method (Vectastain ABC, Vector Labs. Inc., Burlingame, CA, USA) with some modifications. Sections were washed in 0.1M phosphate-buffered saline (PBS), pH 7.26 incubated in PBS containing 0.5% Triton-X-100 and 10% normal goat serum for 60 min, and subsequently with c-Fos antibodies sc-52 (lot#D1503 rabbit policlonal IgG Sant Biotechnology, Inc., Santa Cruz, USA) in 1:10000 solution for 48h. The SS and NPY antibodies of the same producer were used in 1:4000 solution[37].

Permanent histological preparation was obtained by the Permount medium (Fisher) and analyzed on the »Leica« microscope. Photographs were made by the CCD digital camera, and analyzed by the »Scion Image 2000« program (based on NIH program) program for transforming pictures into binary image (example in Figure 1) to obtain numerical values of coloration for consequent statistical analysis.

Fig. 1. An example of the binary image of nucleus paraventricularis hypothalami (transitory ischemia, magnification 20x).

For brain mapping we used the maps, (example in Figure 2), from the atlas by Paxinos & Watson[38]. Given the differences in intensity of immunoreactive coloration

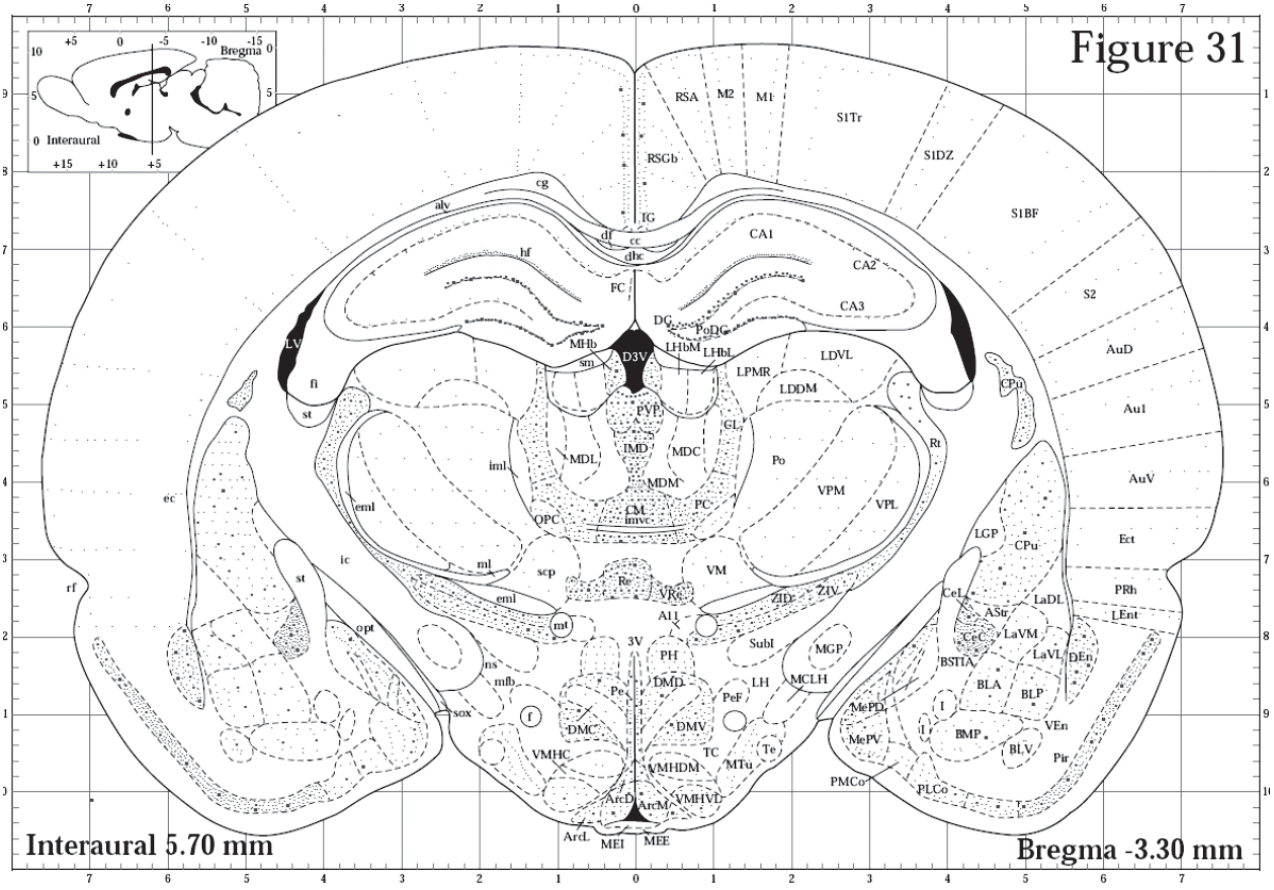

Fig. 2. An example of the map where dots denote C-Fos immunoreactive cells

TABLE 1
THE INTENSITY OF C-FOS IMMUNOREACTIVITY OF NEURONS IN RESISTANT (R) AND TRANSITORY ISCHEMIA (T)

Locations	Resistant ischemia (R)			Transitory ischemia (T)			T	Sig
	Min	Med	Max	Min	Med	Max		
Medial prefrontal cortex	4.00	44.00	84.00	17.00	130.50	244.00	–1.103	>0.05
Parietofrontal cortex	2.00	32.00	62.00	23.00	190.00	357.00	–2.363	<0.05*
Pyriform cortex	31.00	91.50	152.00	6.00	158.50	311.00	–0.893	>0.05
Anterior amygdaloid area	20.00	33.00	46.00	23.00	75.00	127.00	–2.678	<0.05*
Claustrum	25.00	54.50	84.00	18.00	205.50	393.00	–1.785	<0.05*
Accumbens nucleus	10.00	206.50	413.00	21.00	47.00	73.00	0.210	>0.05
Reuniens nucleus	20.00	46.00	72.00	29.00	118.50	208.00	–2.363	<0.05*
Anterior septal nucleus	9.00	61.00	113.00	14.00	59.00	104.00	0.420	>0.05
Supraoptic nucleus	15.00	107.50	200.00	7.00	186.00	365.00	–1.575	>0.05
Suprachiasmatic nucleus	50.00	143.50	237.00	51.00	162.00	273.00	–1.575	>0.05
Paraventricular nucleus of hypothalamus	28.00	193.00	358.00	97.00	206.50	316.00	–0.945	>0.05
Periventricular nucleus of hypothalaums	21.00	146.00	271.00	28.00	68.50	109.00	–0.368	>0.05
Anterior hypothalamic area	23.00	49.50	76.00	23.00	60.00	97.00	–1.628	>0.05

Min – minimum, Med – median, Max – maximum, T – Wilcoxon T, Sig – significance of T

in T and R group both between slices, and between the structures within one slice, we used two point values (Adobe Photoshop 1 px and 3px) in order to localize a positive cell reaction. Immunopositive c-Fos reaction in the cortex clearly represented or corresponded to the laminar organization of cortex or to histological structure of investigated regions and served as the basis for the map description and analysis.

Results

The dominant immunohistochemical reactivity was found for c-Fos protein and the most reactive in terms of co-localization of c-Fos with SS and NPY was periventricular area of hypothalamus.

Positive immunoreactivity (IR) for c-Fos protein was obtained on following locations in cortical, hypothalamic and septal areas: medial prefrontal cortex, parietofrontal cortex, pyriform cortex, anterior amygdaloidea area, claustrum, accumbens nucleus, reuniens nucleus, anterior septal nucleus, supraoptic nucleus, suprachiasmatic nucleus, paraventricular nucleus of hypothalamus, periventricular nucleus of hypothalamus, anterior hypothalamic area,.

Fig. 3. C-Fos protein immunoreactivity in nucleus supraopticus (transitory ischemia, magnification 20 x).

C-Fos IR was found in motor cortex, mainly in layers II and III, and in sensory cortex in layers V and VI. In hypothalamus, most intense IR was found in paraventricular, periventricular, supraoptic and suprachiasmatic nuclei, and less in preoptic area and lateral hypothalamus. In thalamus, most intensive reaction was found in centromedial, lateroposterior, paracentral, and intermediodorsal groups of nuclei and in reuniens nucleus. Significant c-Fos IR was also found in pyriform cortex, caudoputamen, claustrum, amygdaloid body (mostly central amygdaloid nucleus), and endopyriform nuclei.

The comparison of intensity for c-Fos IR in neurons of R and T groups, based on the Wilcoxon *T* test (Table 1), showed significant differences in parietofrontal cortex, anterior amygdaloidea area, claustrum, reuniens nucleus and suprachiasmatic nucleus. The comparison was based on the Wilcoxon *T* test where the test statistic was reported as a value of T with critical value of 1.645 at the 0.05 alpha level of significance.

SS-IR neurons were found in layers II and III of granular, motor and visual cortex, in layers V and VI of sensory and secondary auditory cortex, in dentate gyrus, in CA1, CA2, CA3 hippocampal cell groups, endopiriform nuclei, periventricular and paraventricular area of hypothalamus, amygdaloid body (mainly in central nucleus), and finally, in claustrum and caudoputamen, with prominent differences between R and T groups.

For NPY-IR neurons, there were no significant differences between R and T groups of rats. Strong reactivity

for NPY was found in caudoputamen, dentate gyrus, amygdaloid body (mainly in central nucleus), periventricular and paraventricular area of hypothalamus, layers V and VI of sensory and secondary auditory cortex, and layers II and III of motor and visual cortex. Less reactive were CA1, CA2, and CA3 hippocampal regions.

Discussion

Though experimental ischemia in rats is not an ideal model for vascular brain lesions, it has proved to be a valid animal model for further research on the evolution of ischemic injuries and possible strategies in the treatment of cerebrovascular diseases[39].

A more prominent c-Fos IR during transitory ischemia in comparison with SS IR and NPY IR observed in our study is probably the result of late gene activation typical of SS and NPY, which is of significance for further advances in our knowledge of neuroprotection. From anatomical and embryonic point of view, a positive reaction in our study was found in both evolutionary older and evolutionary younger brain structures. However, in a functional sense, vital centers enabling functioning in hard conditions, such as stress caused by ischemia in our experiment, remained well preserved contrary to less important structures such as nuclei related to hunger or sexual behavior. In general, C-Fos IR is associated with suffering in a certain brain structure. However, considering SS and NPY IR, which are associated with late response genes, it is still difficult to speak of causality considering our rudimentary understanding of the association between pathological processes and the changes in concentrations of these proteins. Given the anatomical areas which showed immunoreactivity to proto-oncogenes during transitory ischemia, the results of our study showed that it is necessary to keep on further research on all the aspects of the expression of proto-oncogenes and neuropeptides, primarily in physiological, biochemical and pharmacological domains since this research provides an accurate mapping of all the brain areas to be protected in various populations at risk cerebral ischemia.

Proto-oncogenes act as an allergic reaction, an exaggerated defensive response leading to the destruction of affected tissue. Therefore, it is of substantial interest to find out at what level the proto-oncogene activity is neuroprotective, and at what level this activity causes neuronal death[40]. Other interesting experiments on c-Fos expression in neurons, showed that glial cell reaction found in ischemia and other lesions may be considered as protective biological response[41,42]. Research evidence has indicated the possibility of prevention of selective neuronal death by preconditioning by a short period of ischemia and its relationship with proto-oncogene activity[43–45]. In line with previously mentioned are the results of our study regarding the impact of preconditioning on c-Fos activity and co-localization of c-Fos with SS and NPY immunoreactivity in experimental ischemia in rat brains showing the need for future studies of brain neuropeptides activation related to ischemic and other injuries with a very careful experimental design related to the control of distinct regional and time effects. In general, all the medical conditions leading to blood flow reduction may have the benefit of such a research given that atherosclerosis and thrombosis are frequent epidemiological problem causing ischemia not only in brain, but also in heart, kidneys and other organs affected by metabolic disorders, bad dietary habits, stress, inadequate blood pressure, endocrine diseases or other conditions.

In spite of extensive research, a complex interaction of c-Fos gene, SS and NPY is so far not completely clarified[46,47]. New advances in this domain require further research not only on proto-oncogenes related to tumor proliferation or ischemia but also on cell reaction to stress, that would extend our knowledge of cell defensive mechanisms and how to improve them. We must also take into consideration the role of widespread neuropeptide immunoreactivity not only in neurological diseases but in diseases of other organ systems as well[48–51].

Conclusion

Our brain mapping indicated areas of regional expression and co-localization of nuclear c-Fos protein (c-Fos), somatostatin (SS) and neuropeptide Y (NPY) during resistant and transitory experimental ischemia in rat brains. The results of our study, regarding the impact of preconditioning with a short period of ischemia on c-fos activity and co-localization of c-Fos with SS and NPY immunoreactivity in experimental ischemia in rat brains, showed the need for future studies of brain neuropeptides activation with a careful experimental design related to the control of distinct regional and time effects. Finally, our findings indicated brain structures which may require pharmacological targeting to achieve neuroprotective level of proto-oncogene activity in order to prevent ischemic injuries in populations at risk.

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ULOGA C-FOS PROTEINA, SOMATOSTATINA I NEUROPEPTIDA Y U PATOGENEZI ISHEMIJSKOG OŠTEĆENJA MOZGA NA ŽIVOTINJSKOM MODELU CEREBRALNE ISHEMIJE

S A Ž E T A K

Cilj je ovog istraživanja bio utvrditi sva područja promjene u ekspresiji c-Fos nuklearnog proteina (c-Fos), citoplazmičkom somatostatina (SS) i neuropeptida Y (NPY) u mozgu štakora tijekom eksperimentalne ishemije. Uz korištenje imunohistokemijskij metoda, mapiranje mozga na imunoreaktivnost za c-Fos, SS i NPY u 39 štakora, obavljeno je za telencefalon, diencefalon te srednji mozak tijekom trajne i prolazne ishemije. Prva eksperimentalna skupina (R grupa) izložena je trajnoj ishemiji okluzijom (10 minuta) četiri žile prema Pulsinelli metodi. Druga skupina je prvi put izložena prolaznoj (4 minute) ishemiji te nakon 72 sata, potpunoj ishemiji kao u R grupi. Ustanovljena je statistička razlika između R i T grupe u c-Fos reakciji, pogotovo u parietofrontalnom korteksu, prednjem amigdaloidnom području, klaustrumu, reuniens nukleusu i suprahijazmatičnom nukleusu. Dominantna imunohistokemijska aktivnost ustanovljena je za c-Fos protein, a najreaktivnija u smislu kolokalizacije svih triju proteina bila je u periventrikularnom području hipotalamusa. Mapiranje je pokazalo na su imunohistokemijski podjednako reagirala filogenetski mlađa kao i starija područja. Rezultat našeg istraživanja u vezi sa utjecajem predtretiranja kratkim periodom ishemije na aktivnost c-Fos proteina i kolokalizacije c-Fos, SS i NPY imunoreaktivnosti pokazuju potrebu za daljnjim studijama moždanih neuropeptida povezanih sa regionalnim i vremenskim učincima te ukazuju na moždane strukture na koje bi se moglo ciljano farmakološki djelovati kako bi se postigla neuroprotektivna razina proto-onkogene aktivnosti u rizičnih populacija.