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Changes in NGF/c-Fos colocalization in specific limbic structures of juvenile and aged rats after open field stimulation

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Changes in NGF release during stressful events have been associated with the activation of neurons expressing NGF receptors. This study examined the influence of acute stress-induced stimulation on NGF/c-Fos colocalization in the following limbic regions: the paraventricular (PV) nucleus of the hypothalamus, medial (MeA) nucleus of the amygdala, and CA3 hippocampus. Juvenile (P21) and aged rats (P360) were exposed to a 15-minute acute open field (OF) test. Double immunofluorescence staining, used to detect NGF-ir and c-Fos-ir cells, revealed a higher percentage of NGF/c-Fos-ir neurons in the P21 control group than in the P360 control group. Under OF acute stimulation, a statistically significant ($p < 0.05$) increase of NGF/c-Fos level in CA3 of juvenile animals and in PV and CA3 of the aged rats was observed. These observations indicate that the investigated structures in both age groups show a different response to acute OF stimulation. Acute OF affects the levels of NGF/c-Fos more significantly in aged rats. (Folia Morphol 2009; 68, 3: 129–134)

Key words: paraventricular nucleus, medial amygdaloid nucleus, CA3, limbic system, open field, stress

INTRODUCTION

The structures belonging to the hypothalamic-pituitary-adrenal (HPA) axis are subject to diverse changes during stress stimulation, which might be caused by the involvement of endogenous factors like nerve growth factor (NGF) [8, 12, 14, 27, 28]. It is commonly believed that NGF mediates growth and survival of neurons and promotes their repair and remodelling. NGF is required to control synaptic function and plasticity, and to sustain neuronal cell survival, its morphology, and differentiation [1, 3, 8, 29]. Recently a wider involvement of NGF with physiological and pathophysiological processes has been observed [11, 22].

It is thought that NGF plays a key role in the degeneration process, and this link can be ascribed to reduced activation of NGF signalling pathways [16]. Several studies have also found that deregulation of NGF expression is strongly implicated in the etiology of such mental illnesses as schizophrenia and depression [2, 8, 39, 44, 49].

Stressors can affect the amount of NGF in the brain [1, 27, 44, 50]. It is a proven fact that exposure to stressors, regardless of age, can lead to significant changes of NGF levels in the brain [3, 8, 12, 20]. A well-researched property of NGF is its participation in the development of the central nervous system (CNS); however, other important functions of this protein in the mature organism have also

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been extensively studied [13, 19]. The role of NGF in the ageing of the CNS is still unclear. Nevertheless, it is postulated that NGF is implicated in the decreased physiological regulation in response to various stressors [41, 44, 50]. Taking into account psychophysiological changes in different periods of life, we studied the activity of NGF-ir cells (measured by c-Fos protein activation) in the rat limbic system during the open field (OF) test. Since OF represents a psychological stressor, it is possible to observe the animal engaging itself in active exploration of the novelty [31, 40].

The research, carried out in two critical ontogenetic periods (on juvenile and aged rats), was designed to investigate the influence of acute OF stimulation on NGF/c-Fos-ir colocalized cells in stress-related limbic structures, namely the paraventricular (PV) nucleus of the hypothalamus, medial (MeA) nucleus of the amygdala, and the CA3 region of the hippocampus.

MATERIAL AND METHODS

Animals

Twenty male Wistar rats were divided into two groups: juvenile (P21; P — postnatal day) and aged (P360) rats. Each group consisted of non-stressed (remained in their home cage until perfusion) control rats (n = 5) and experimental rats (n = 5) exposed (once for 15 min) to an acute OF stressor. The rats were kept singly in plastic cages, and were given free access to water and food pellets. The animal treatment and care complied with the requirements of guidelines for laboratory animals established by the National Institute of Health as well as by the Local Ethical Committee of the Medical University of Gdańsk.

Open field test

The OF test procedure applied to this experiment was described in our previous study [5, 26]. An open field area constituted a wooden white box (100 × × 100 × 40 cm) illuminated by a 500 W halogen light. Each animal was gently placed in the centre of the box to provoke stress reaction.

Experimental procedure

The rats underwent the open field test between 9:00 a.m. and 2:00 p.m. Every individual was exposed to a 15-minute acute stressor, after which it was placed back in its home cage. The control animals remained in their home cages until perfusion. After 90 minutes of the final exposure all rats were deeply anaesthetized with a lethal dose of Nem-butal (80 mg/kg/body weight), and then perfused transcardially with 0.9% saline solution with heparin followed by 4% paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.4). The brains were postfixed in 4% paraformaldehyde for 3––4 hours. Afterwards they were stored overnight at 4°C in 0.1 M phosphate buffer containing 10% sucrose and next 30% sucrose until they sunk. Coronal 40-μm-thick serial sections of brain were cut with a JUNG 1800 cryostat (Leica, Germany).

Immunohistochemistry

Adjacent sections were processed for NGF and c-Fos using double immunohistochemical methods. After unmasking the antigen in 0.01 sodium citrate buffer (pH 6.0, 74°C for 40 min), the sections were blocked in 2% normal donkey serum for 2 hours and then incubated with a cocktail of primary antibodies containing goat anti-cFos IgG (Santa Cruz Biotechnology; dilution 1:250) and rabbit anti-NGF (Chemicon; dilution 1:500) for 3 days at +4°C. Following multiple rinses in phosphate buffered saline ph 7.4 (PBS), the samples were incubated for 2–3 hours at room temperature with appropriate secondary antibodies, such as Cy3-conjugated donkey anti-rabbit (Jackson ImmunoResearch; dilution 1:600) and Alexa Fluor 488-conjugated donkey anti-goat (Molecular Probes; dilution 1:150). Immunohistochemically stained slides were examined with a Bio-Rad Radiance 2100 confocal system mounted on a Nikon Eclipse 600 fluorescent microscope equipped with a Krypton/Argon laser. Confocal microscopy images, studied with the use of 40× and 60× objective lenses, were obtained due to optimal iris adjustment for each magnification.

Quantitative analysis

All studied structures were sampled randomly for the purposes of quantifying their immunoreactivity. Their representative sections constituted the test areas (0.01 mm^2), where the total number of NGF-ir cells amounted to at least 100. The percentage of NGF/c-Fos-ir cells in relation to all NGF-ir cells was estimated individually for every animal. Statistical analysis of the raw data, performed separately for the two age groups (P21 and P360), was carried out by Statistica v. 7.1 software. The mean value of each parameter, along with standard deviation, was calculated for these groups and the findings were presented on

a graph. Depending on the results of ANOVA analysis (done by means of Bartlet and Leven tests), further statistical evaluation made to assess differences between the experimental groups was carried out by either ANOVA with post hoc least significant difference test or by nonparametric Kruskall-Wallis test, followed by a multiple comparison test. The level of significance was set at 0.05. The percentage of NGF/c-Fos-ir cells for each individual examined structure (PV, MeA, CA3) was collated with respect to the age and response to OF stress stimulation.

RESULTS

Double-immunofluorescence staining for NGF and c-Fos revealed that in P21 non-stressed control rats the percentage of NGF/c-Fos-ir colocalization amounted to 11% in the PV nucleus of the hypothalamus, and about 4% in the other studied areas (MeA and CA3). In the P360 control group, the level of NGF/c-Fos-ir colocalized neurons achieved about 2.5% in all investigated structures (Figs. 1A–C). Comparison between the P21 and P360 control groups indicated a statistically significant ($p < 0.05$) higher percentage of NGF/c-Fos-ir colocalization in P21 in PV, MeA, and CA3 (Figs. 1A–C).

In contrast to the control, in the group of juvenile (P21) rats acute OF stress stimulation caused an increase in the percentage of NGF/c-Fos-ir colocalization in all examined structures, but the change was statistically significant ($p < 0.05$) only in the CA3 region of the hippocampus, where it reached 7.5% (Figs. 1A–C).

Single exposition to OF stressor in P360 led to a statistically significant ($p < 0.05$) rise in the percentage of NGF/c-Fos-ir colocalization in PV and CA3, in which it came to 10% and 7%, respectively. The level of NGF/c-Fos-ir in MeA, however, did not significantly alter (Figs. 1A–C). Another observation made on the groups of juvenile (P21) and aged (P360) animals under OF stress revealed no statistically significant differences in the percentage of NGF/c-Fos-ir in all the investigated structures (Figs. 1A–C).

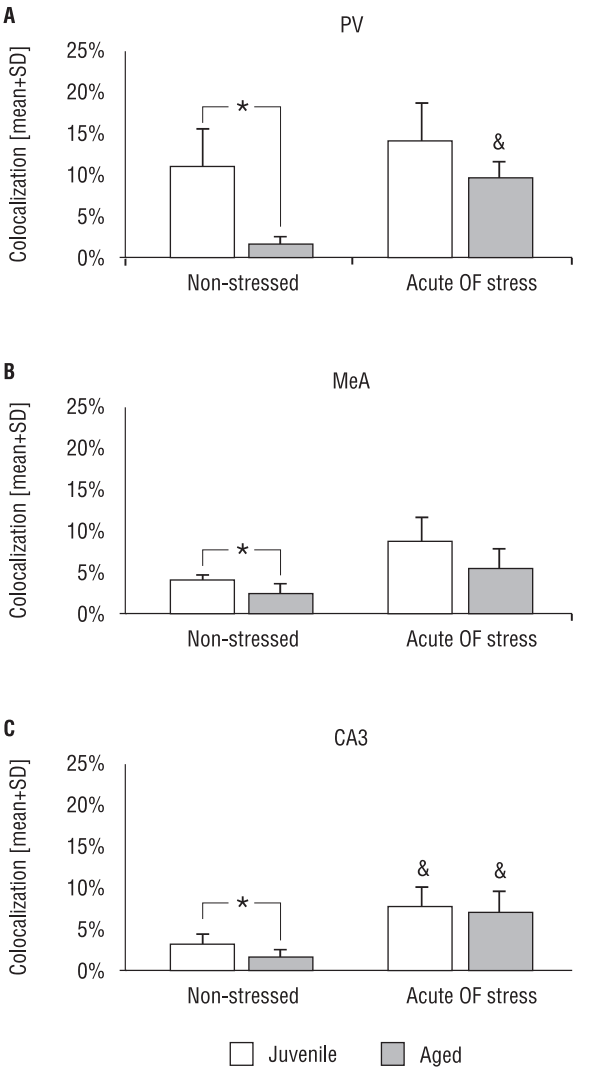

Figure 1. Percentage of NGF/c-Fos-ir colocalized cells in juvenile (P21) and aged (P360) rats, examined in two groups: non-stressed and subjected to acute open field test; **A.** PV — paraventricular nucleus; **B.** MeA — medial amygdaloid nucleus; **C.** CA3 — hippocampal region; *juvenile *vs*. aged rats ($p < 0.05$); &non-stressed *vs*. acute open field (OF) test ($p < 0.05$).

DISCUSSION

Forced confrontation with novelty is stressful for rodents [40]. A wide range of psychological stimuli, including the open field test, induce neuronal activation, so c-fos mRNA or c-Fos protein can be used as its marker [4, 5, 15, 17, 25, 46, 48]. The level of c-Fos may be parallel to the level of HPA axis activity in the structures of the limbic system [18, 32, 37].

It has been reported that stress-stimulation, involving complex neuronal networks, may affect the synthesis of NGF [1, 3, 50]. Moreover, the activation of the NGF gene is suggested to be mediated by Fos proteins, which interact with the AP-1 (cellular transcriptional factor) site of NGF promoter. Since NGF induces protooncogenes Fos, this interaction could illustrate an important pathway in NGF expression [33, 36, 45, 47].

On the basis of double-immunofluorescence staining analysis we found NGF/c-Fos-positive neurons in all investigated areas in the P21 and P360

control rats. Comparison between juvenile and aged animals revealed a statistically significant higher percentage of NGF/c-Fos colocalized cells in the P21 control group in all studied structures, especially in the PV nucleus of the hypothalamus. Due to the fact that the PV nucleus is the integration centre, responding to stimulatory and inhibitory inputs, and playing a key role in the regulation of the HPA axis [23, 35, 38, 43, 52], we assume that the higher percentage of NGF/c-Fos cells observed in non tested juvenile rats can be a consequence of intense cognitive activity, characteristic of a young animal's behaviour. Thus, our findings may reflect the impact of enhanced environmental exploration on activity NGF-ir cells in the control juvenile rats.

The open field test represented such a situation in which the rat could engage in active exploration of the novelty [40]. In the group of juvenile (P21) rats exposed to acute OF stimulation, we recorded an increased percentage of NGF/c-Fos colocalized cells in all structures, but the increase was statistically significant only in the CA3 region of the hippocampus. In the group of aged rats, however, the percentage of NGF/c-Fos cells visibly increased in PV and CA3 ($p < 0.05$) after acute OF stress. Activity-dependent NGF release in the hippocampus (being a site of anxiety and fear circuits), particularly in CA3 (a region additionally susceptible to damage at the beginning of life and while aging) as well as in PV (a nucleus providing the neuroendocrine response to stimuli of psychological nature) was reported [2, 7–10, 51].

The above-mentioned observations together with our findings prove that a single OF stimulation activates NGF-ir neurons in the limbic system [14, 21, 35]. The increase in the percentage of NGF/c-Fos neurons we found in our research after acute psychological stress might be the upshot of greater demand for NGF protein in the activated structures, which in turn may modulate the response of HPA axis to stress [50]. Furthermore, the increase of NGF/c-Fos we noted could be attributed to NGF brain neuroprotective effects induced by stress [8, 27].

In the groups of animals exposed to acute OF stressor, as opposed to the non-stressed groups, the percentage of NGF/c-Fos increased more significantly (mainly in PV and CA3) in aged rats than in juvenile rats. These results confirm that neuronal response to acute OF stress is crucially determined by the age of the animals [12, 30]. There is also evidence for a dysfunctional activation of the HPA axis during stress in aged animals [42], in addition to some documentation advocating that the aging process is associated with enhanced responsiveness of the HPA axis to acute stress [6, 24]. Taking all data into account, it can be deduced that juvenile and aged animals differ in their pattern of response to exposition to novelty and bright light in the open field arena. It is worth mentioning here that NGF release in acute stress events participates in neuroregulation during the aging process [3, 34].

While collating the results of our study on the two age groups of rats, we made yet another observation: In contrast to the controls, in the groups after acute OF stimulation we did not find any significant age-dependent changes in the percentage of NGF/c-Fos-ir neurons in the structures involved in the processing of emotional cues. These findings imply that, despite different initial pools of NGF/c-Fos colocalized cells, both juvenile and aged rats achieved similar levels of NGF/c-Fos-ir neuronal expression because of acute open field stimulation. All this indicates dissimilar dynamics of NGF/c-Fos changes in P21 and P360.

CONCLUSIONS

This study demonstrates that acute OF stressor influences the NGF/c-Fos-ir double staining cells in stress-related limbic structures differently in juvenile and aged groups of rats. Acute OF stimulation affects the levels of NGF/c-Fos more significantly in aged rats, which is probably connected with neuroprotective effects of NGF during the aging process.

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