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Changes in NGF/c-Fos double staining in the structures of the limbic system in juvenile and aged rats exposed to forced swim test

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This study aimed to investigate the influence of acute (a single 15 min) and chronic (15 min daily for 21 days) exposure to forced swim (FS) test on nerve growth factor (NGF)/c-Fos cells in hypothalamic paraventricular (PV) and supraoptic (SO) nuclei, the central (CeA) and medial (MeA) amygdaloid nuclei and CA3-hippocampus in juvenile (P28) and aged (P360) rats. The double-immunofluorescence (-ir) method was used to detect NGF-ir and c-Fos-ir cells. The amount of NGF/c-Fos-ir cells in relation to all NGF-ir cells is shown as a percentage. In the acute FS test an increase in NGF/c-Fos-ir cells ($P<0.05$) was observed in all studied structures of juvenile rats and in the PV and SO of the aged individuals. After chronic FS stress, the NGF/c-Fos-ir ratio remained unaltered (except in the SO) in P28, but it increased ($P<0.05$) in all investigated regions in P360 compared with the controls. The findings may reflect the state of molecular plasticity within the limbic hypothalamic-pituitary-adrenocortical (HPA) axis in both age groups, yet the phenomenon of habituation in NGF/c-Fos-ir after chronic FS exposure was observed only in juvenile animals.

Key words: NGF, c-Fos, stress, forced swim test, limbic system

INTRODUCTION

Stress stimulation *via* a complex network of signals involving neurons can influence the synthesis of nerve growth factor (NGF) (Alleva and Santucci 2001, Aloe et al. 2002, Von Richthofen et al. 2003). The transcriptional mechanisms involved in the regulation of the NGF gene are still largely unknown (only one regulatory element, AP-1, has been fully characterized) (Onteniente et al. 1994, Semkova and Krieglstein 1999). Cellular AP-1 transcription factor was made up of different Fos family members (Whitmarsh and Davis 1996, Tong et al. 2002). AP-1 activity was induced by a wide array of stimuli including growth factors, cytokines, neurotransmitetters and cellular stress (Angel and Karin 1991, Karin 1995). NGF gene expression is correlated with several proto-oncogenes encoding proteins of the Fos family (Sofroniew et al. 2001). The c-fos early gene participates in the transduction of extracellular stimuli by modulating the transcription of other genes, including the NGF gene (Hengerer et al. 1990). On the other hand it has been demonstrated that NGF in turn can induce the c-fos gene both *in vitro* and *in vivo* (Ginty et al. 1994, Giovannelli et al. 2000).

Traditionally, NGF is believed to foster the growth and survival of neurons, as well as to promote their repair and remodeling. It is also required to control synaptic function and plasticity, and has been proved to induce morphological differentiation (Alleva and Santucci 2001, Aloe et al. 2002, Lessmann et al. 2003, Branchi et al. 2004). Recently, it has been demonstrated that NGF is involved in physiological and pathophysiological processes in cells connected with apoptosis and neurodegeneration. Hennigan and coauthors (2007) and Chang and others (2008) report that its action depends highly on the neurochemical context, the physiological state of neurons, and/or connections with other brain structures. Several other studies also found that NGF may be strongly linked with men-

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tal illnesses such as schizophrenia and depression (Branchi et al. 2004, Schulte-Herbrüggen et al. 2006, Pohl et al. 2007, Valvassori et al. 2008).

Exposure to acute and chronic stressors, regardless of age, can lead to significant changes of NGF levels in the brain (Hadjiconstantinou et al. 2001, Chen et al. 2006). As documented by Chen and colleagues (2006) and Grace and others (2008), NGF is essential for the development of the central nervous system (CNS); however, its role in the ageing of the CNS is still elusive. It is postulated that impaired efficiency in the physiological regulation of the response to various stressful stimuli is related to a decreased level of NGF (Schulte-Herbrüggen et al. 2006, Ríos et al. 2007). Changes in NGF release during stressful events can influence the basal activity of neurons expressing NGF-receptors (Spillantini et al. 1989, Hellweg et al. 1997, Ríos et al. 2007), which seems to be more relevant in the early period of life (Aloe et al. 2002, Pizarro et al. 2004). The fluctuation in NGF concentrations in the brain of developing organisms can lead to depressive reactions in adult life (Spear 2000, Aloe et al. 2002, Pohl et al. 2007).

Taking into account psychophysiological changes in two critical periods of life, we studied the activity of NGF-ir cells (measured by c-Fos protein activation) in the limbic system of juvenile and adult rats during the forced swim test – an animal model employed to study anxiety and depression-like behaviour (Kalueff and Tuohimaa 2004). The research was designed to investigate the influence of stress duration (acute and repeated forced swim stimulation), in two critical ontogenetic periods (in juvenile and aged rats), on the NGF/c-Fos-ir double staining cells in stress-related limbic structures: the paraventricular (PV) and supraoptic (SO) nuclei of the hypothalamus, central (CeA) and medial (MeA) nuclei of the amygdala and the CA3 region of the hippocampus.

METHODS

Animals

Thirty male Wistar rats were divided into two groups: juvenile (P28; P – postnatal day) and aged (P360) rats. The care and treatment of the rats were in accordance with the 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. Each group consisted of non-stressed (remained in their home cage until perfusion) control rats (n=5) and two groups of experimental rats (n=5 each), which were exposed to either acute forced swim (FS) stress (once for 15 min) or to chronic FS stress (15 min daily for 21 days).

Forced Swim Test (FS)

The forced swim test was carried out in a glass cylinder (45 cm high, 20 cm in diameter) filled with water (22°C) up to a height of 30 cm. After the testing procedure, the rats were returned to their respective home cages. If some individuals at the earliest age were not able to complete the 15 min FS test, that is when the tip of their nose did not remain above the water level, the length of the test was reduced. The experiments were conducted between 09:00 AM and 02:00 PM.

Experimental procedure

The animals were sacrificed on postnatal day 28 (P28) and 360 (P360). Ninety minutes after the final exposure, all the rats were deeply anesthetized with a lethal dose of Nembutal (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 until they sank, and then were kept overnight at 4°C in 0.1 M phosphate buffer containing 10% sucrose and 30% sucrose. Coronal 40-μm-thick serial sections of brain were cut with a JUNG 1800 cryostat (Leica, Germany).

Immunohistochemistry (IHC)

Adjacent sections were processed using double immunohistochemical methods for NGF and c-Fos. 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 (NDS) for 2 h, and then incubated at 4°C for 3 days in a cocktail of primary antibodies containing goat anti-cFos IgG (Santa Cruz Biotechnology, sc-52-G; dilution 1:250) and rabbit anti-NGF (Chemicon, AB927; dilution 1:500). Following multiple rinses in PBS, the sections were incubated again, but this time for 2–3 hours at room temperature in appropriate secondary antibodies, namely Cy3-conjugated donkey anti-rabbit (Jackson

ImmunoResearch, 711-165-152; dilution 1:600) and Alexa Fluor 488-conjugated donkey anti-goat (Molecular Probes, A11055; dilution 1:150).

Immunohistochemically-stained slides were examined under a Nikon Eclipse-600 fluorescence microscope with Radiance 2100 confocal system (Bio-Rad, UK), equipped with a Krypton/Argon laser. Confocal microscopy images were obtained through 40× and 60× objective lenses, and additionally, optimal iris was used for magnification purposes.

Quantitative analysis

In order to quantify immunoreactivity, test areas (0.01 mm²) from representative sections of the studied structures were chosen randomly. In every test area total number of NGF-ir, c-Fos-ir and NGF/c-Fos-ir colocalized neurons were counted. The following parameters were estimated for each animal: percentage of NGF-ir cells in relation to all immunoreactive cells (NGF+c-Fos), percentage of c-Fos-ir cells in relation to all immunoreactive cells, and percentage of NGF/c-Fos-ir cells in relation to all NGF-ir cells. The raw data were analyzed using Statistica v. 7.1 software. Statistical analysis was performed separately for the two age groups (P-28 and P-360). The mean value of each parameter along with standard deviation were calculated for these groups and the findings were presented on a graph.

Statistical evaluation was based on the results of the ANOVA analysis (done by means of Bartlett and Leven tests). To assess differences between the studied groups (control vs. FS-acute vs. FS-chronic in every studied structure), either ANOVA with *post hoc* least significant difference test, or nonparametric Kruskal-Wallis test followed by multiple comparison test were applied. The significance level was set at $P<0.05$.

RESULTS

Changes in c-Fos-ir and NGF-ir total cell percentage

In the non-stress groups, a relatively low percentage of c-Fos-immunoreactive cells was observed in all studied structures in juvenile (P28) and aged (P360) animals; however, this amount was higher in the first group. Compared to the controls of juvenile and aged rats, under acute and chronic FS stimulation the percentage of c-Fos-ir cells increased, which was most discernible in the PV nucleus. Chronic FS, by comparison with acute stress, led to a decrease in the percentage of c-Fos-ir cells in juveniles, but in the group of aged animals it remained unaltered in PV and SO, or it increased (CeA, MeA, CA3) (Table I).

All the investigated structures of non-stress P28 and P360 control groups revealed a very high percentage of NGF-immunoreactive cells, oscillating between 95 and 100. Under acute and chronic FS stimulation, the percentage of NGF-ir cells did not change significantly compared to the controls of juvenile and aged rats. In addition, no visible differences between acute and chronic FS stress conditions were noted (Table I).

The influence of the stress duration and animal age on the percentage of NGF/c-Fos-ir double staining cells

The analysis of double immunofluorescence staining for NGF and c-Fos in the control groups revealed a low percentage of NGF/c-Fos-ir in relation to all NGF-ir cells in all the investigated structures, both in juvenile (P28) as well as in aged (P360) rats. A higher percentage of double staining NGF/c-Fos-ir cells was, however, observed in juvenile animals, especially in the PV (11.1%) and SO (7.6%) nuclei of the hypothalamus, while in the other studied areas (MeA, CeA, and CA3) the figure was approximately 3.7%. The P360 control group revealed about 2.1% NGF/c-Fos-ir double staining neurons in all investigated structures (Fig. 1).

In juvenile (P28) rats, acute FS stress stimulation induced a statistically significant ($P<0.05$) increase in the percentage of NGF/c-Fos-ir in all the investigated structures, among which the PV (27.5%) and SO (19.4%) nuclei of the hypothalamus displayed the strongest double staining. The calculations concerning the remaining areas under study were as follows: MeA – 15.6%, CeA – 12.1%, and CA3 – 9% of NGF/c-Fos-ir. After repeated FS stress stimulation, there were no statistically significant changes in the percentage of NGF/c-Fos, except for SO (19.4%). When comparing the influence of acute stress with chronic stress, it must be noted that the latter led to a statistically significant ($P<0.05$) decrease in NGF/c-Fos-ir cells in the whole investigated limbic region. This observation, however, did not relate to SO and CeA (Fig. 1).

A single exposure to FS stress resulted in a statistically significant ($P<0.05$) increase in the percentage of

NGF/c-Fos-ir double staining in PV (22.4%) and in SO (10.7%) in aged (P360) rats.

A similar tendency was observed in other investigated regions of the limbic system (MeA, CeA and CA3), but it was not statistically significant. As a result of chronic FS exposure, the percentage of NGF/c-Fos-ir cells rose markedly ($P<0.05$) in all the studied regions. To be precise, the rise in PV equalled 20.4%, in SO – 8.3%, CeA – 10.2%, MeA – 19.5%, and in CA3 – 11%. The comparison of the findings that emerged from FS tests on the impact of acute and chronic stress revealed, with the exception of SO, no statistically significant differences in NGF/c-Fos-ir (Fig. 1).

Figure 2 shows exemplary NGF-ir, c-Fos-ir, and double immunostaining with NGF/c-Fos neurons in various investigated structures (PV, SO, MeA and CA3) of juvenile (P28) and aged (P360) rats.

DISCUSSION

Similar to Dayas and coworkers (2001), in our study c-Fos-ir was used to determine neuronal activity in the

Table I

Percentage of NGF-ir and c-Fos-ir cells in relation to all immunoreactive cells in the control and after acute and chronic forced swim test (FS) in juvenile (P28) and aged (P360) rats

structure	group	% NGF-ir cells	% c-Fos-ir cells
	P28		
PV	control	98.5	12.5
	FS acute	99.5	27.9
	FS chronic	95.9	16.2
SO	control	96.9	10.4
	FS acute	100.0	19.4
	FS chronic	99.6	16.0
CeA	control	100.0	3.6
	FS acute	100.0	12.1
	FS chronic	98.1	6.3
MeA	control	98.5	5.5
	FS acute	100.0	15.6
	FS chronic	99.1	8.9
CA3	control	99.7	3.7
	FS acute	100.0	9.0
	FS chronic	99.6	5.1

structure	group	% NGF-ir cells	% c-Fos-ir cells
		P360	
PV	control	99.0	3.1
	FS acute	99.3	22.9
	FS chronic	98.0	22.1
SO	control	100.0	1.8
	FS acute	99.3	11.3
	FS chronic	96.9	11.1
CeA	control	99.4	3.2
	FS acute	99.6	7.1
	FS chronic	96.7	13.1
MeA	control	98.9	3.6
	FS acute	99.8	10.8
	FS chronic	96.9	22.0
CA3	control	99.5	2.0
	FS acute	99.6	5.9
	FS chronic	97.0	13.6

forced swim test and showed that the percentage of c-Fos-ir cells increased after both acute and chronic FS stimulation in juvenile and in aged rats. Whereas all the investigated structures of the brain exhibited this phenomenon, the PV displayed the most considerable growth percentage of c-Fos-ir cells. The rise in c-Fos in various limbic areas of rats followed by a number of stressors (acute and/or chronic) had been observed by different authors quite frequently before (e.g. Campbell and Merchant 2003, Westenbroek et al. 2003, Badowska-Szalewska et al. 2005, 2006, Boguszewski and Zagrodzka 2005, Romeo et al. 2008). Since it had been proved that the PV nucleus plays a key role in the hypothalamic-pituitary-adrenocortical (HPA) axis regulation as well as in the hypothalamic response to stimulatory and inhibitory inputs (Herman and Cullinan 1997, Sawchenko et al. 1996, Ons et al. 2004, Yanagita et al. 2007), our findings also confirm the involvement of the investigated areas of the limbic system in the response to FS stress.

Furthermore, we noted that after chronic FS stress, juvenile and aged rats showed dissimilar neuronal activation. The percentage of c-Fos-ir cells decreased in juvenile animals, while in the group of aged individuals it remained at the same level or increased. As it is thought that a decrease in c-Fos is a result of adaptive responses to chronic stress (Girotti et al. 2006, McQuade et al. 2006), the present study indicates that this kind of stressor modulates HPA axis activity differently in prepubertal and aging rats. The persistent

high level of c-Fos-ir after chronic FS simulation is probably connected with prolonged hormonal stress response, since the stressor in question seems to present a heavy load, at least for the reason of physical effort, for aged animals.

Many other researchers have observed fluctuations in the brain concentration of NGF during exposure to different stressors (Scaccianoce et al. 2000, Alleva and Santucci 2001, Hadjiconstantinou et al. 2001, von Richthofen et al. 2003, Marais et al. 2008). Our previous study on adult rats exposed to the open field (OF) test demonstrated that the number of NGF-ir cells in the hypothalamus, amygdala and hippocampus increased (from a moderate to a high level) after acute and chronic OF stimulation (Badowska-Szalewska et al. 2005, 2006). In this experiment, however, detailed calculations showed that the percentage of NGF-ir cells was high in the juveniles as well as in the aged control rats, and it remained at a high level under acute and chronic FS stimulation. This corroborates a strong involvement of NGF in the normal functioning of neurons (Chen et al. 1995, Levi-Montalcini et al. 1996, Lee et al. 1998, Zhu et al. 2006). What is more, our experimental data confirm the findings reported earlier by Scaccianoce and colleagues (2000), who demonstrated that NGF concentrations in the hippocampus and basal forebrain in unstressed aged rats do not differ from those in the corresponding structures of young animals.

Changes in the levels of NGF expression are regulated in an activity-dependent manner by various neurochemical mediators involved in a particular stress (Lindholm et al. 1993 Scaccianoce et al. 2000). It is supposed that NGF, as a factor stimulating transcription of certain proteins, plays a role in the plasticity of the limbic system in different periods of life, thereby influencing the development and functioning of neurons in juveniles and preventing their degeneration in the aged brain (Thoenen 1995, McAllister et al. 1999, Aloe et al. 2002, Lee et al. 2005). The invariable high concentration of NGF-ir cells in this study indicates that acute and chronic FS stimulation did not result in changes in the percentage of NGF-ir neurons.

Our investigation results, obtained through double-immunostaining analysis, revealed a low percentage of NGF/c-Fos-ir cells in all tested structures in juveniles and aged control rats, although the number was higher in the former group, especially in PV and SO hypothalamic nuclei. We assume that the greater percentage of

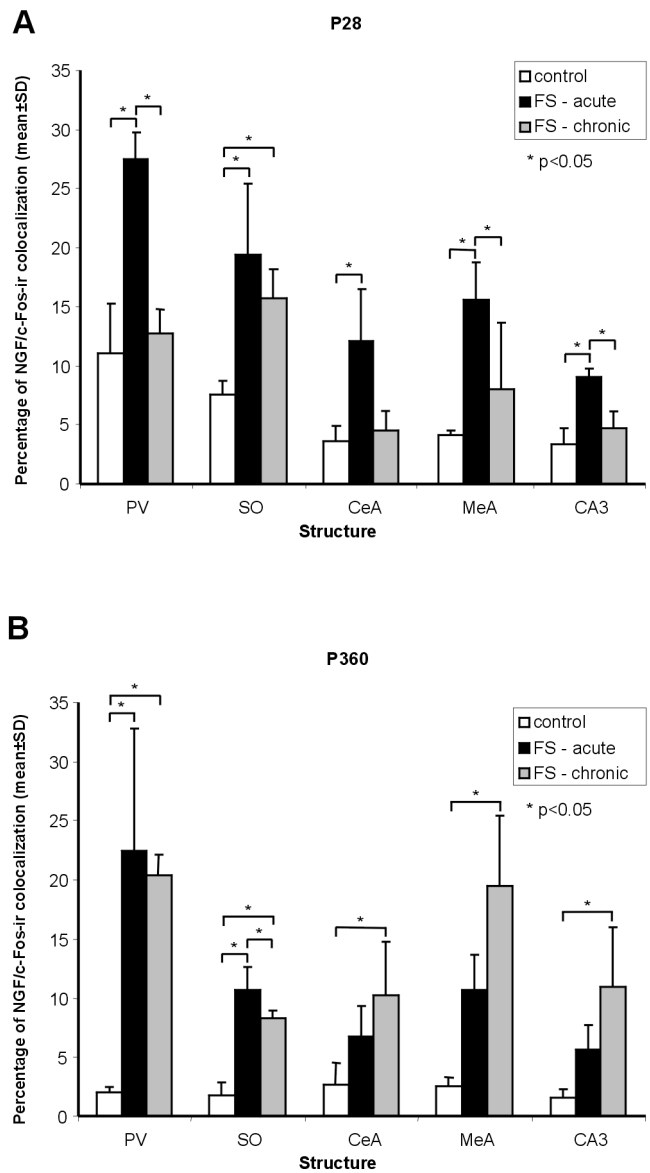

Fig. 1. Percentage of NGF/c-Fos-ir cells colocalization in relation to all NGF-ir cells after acute and chronic forced swim test (FS). (A) 28-day-old (juvenile) animals; (B) 360-day-old (aged) rats. (PV) paraventricular nucleus; (SO) supraoptic nucleus; (CeA) central amygdaloid nucleus; (MeA) medial amygdaloid nucleus; (CA3) hippocampal region.

NGF/c-Fos cells observed in non-tested juvenile rats is probably the result of high environmental exploration, which is a characteristic feature of young animals. Our findings may therefore reflect the impact of the enhanced activity of control juvenile rats on the number of NGF-ir cells.

Under FS acute stimulation, a statistically significant (P<0.05) increase of NGF/c-Fos-ir was observed in all

studied structures in juvenile animals, and in the PV and SO nuclei of the hypothalamus in aged rats. These data may suggest that a single FS stimulation, which activated NGF-ir neurons, predominantly affects the activity of such limbic areas as PV and SO (Cirulli 2001, Yanagita et al. 2007). Strong activity in the nuclei of the periventricular zone of the hypothalamus after FS stimulation was observed both in young and aged rats (Alleva and Santucci 2001, Habib et al. 2001, Branchi et al. 2004, Engelmann et al. 2004), which explains why the animals develop an active strategy of swimming in the FS test. The increase in NGF/c-Fos double staining

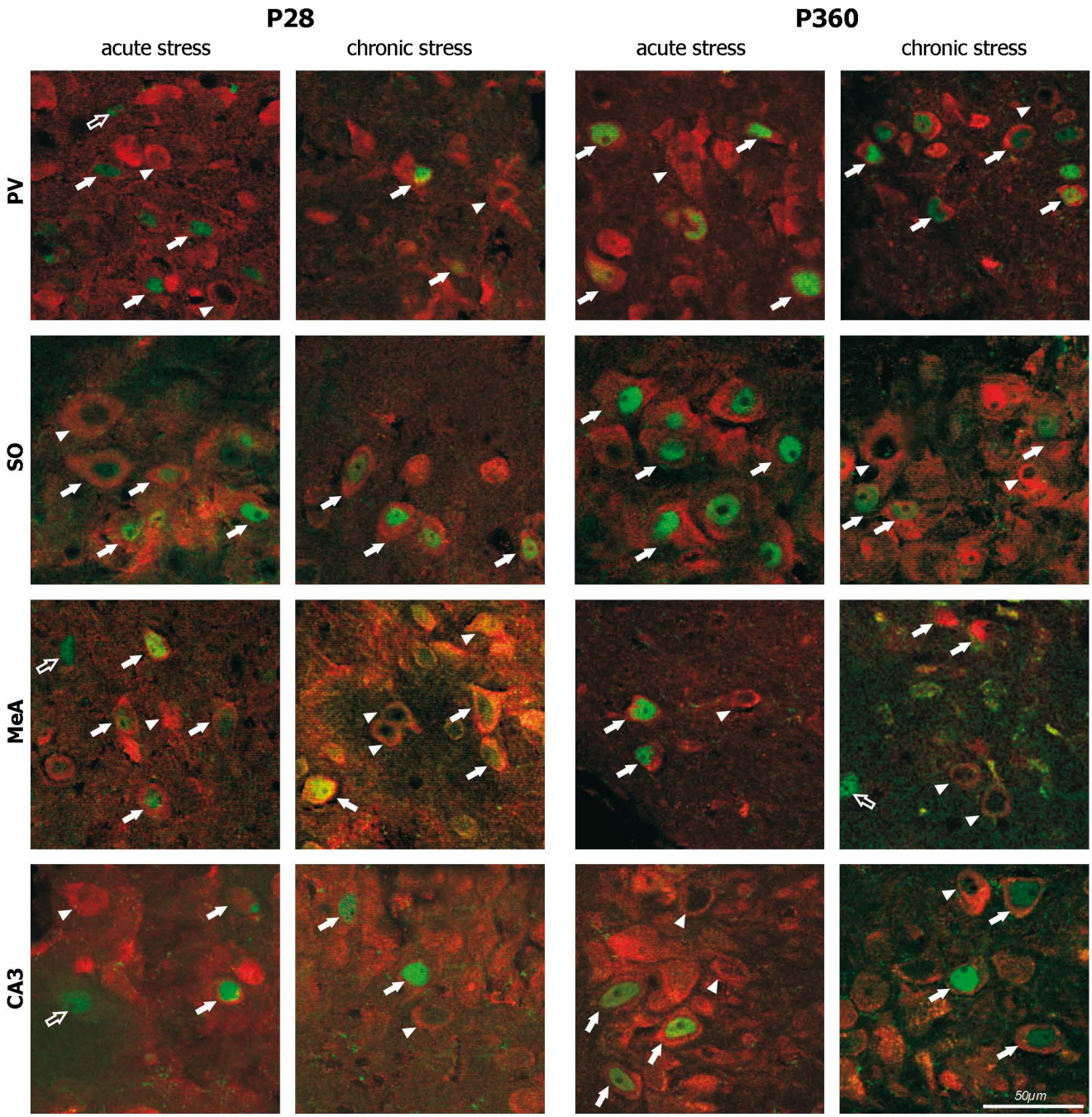

Fig. 2. NGF-immunoreactive (red) (arrow heads), c-Fos-immunoreactive (green) (transparent arrow) and double immunostaining with NGF/c-Fos-immunoreactive (arrow) neurons after forced swim test (FS) in juvenile (P28) and aged (P360) rats. (PV) paraventricular nucleus; (SO) supraoptic nucleus; (MeA) medial amygdaloid nucleus; (CA3) hippocampal region; Scale bar is 50 µm.

neurons we found in our research after acute psychophysiological stressor might be caused by greater demand for NGF protein, which in turn can modulate the response of HPA axis to stress (von Richthofen et al. 2003). With relation to these findings, it is supposed that NGF/c-Fos expression triggered by acute FS stress stimulation has a positive influence on neuronal circuits and microplasticity (Aloe et al. 2002). Moreover, it has been scientifically noted that response to neurogenic stressors is more rapid and conspicuous in the case of juvenile rats than in fully matured adults (Chen et al. 1995, Marais et al. 2008). In our experiment, the neuronal response of juvenile rats to acute FS stressors was also stronger than that of aged rats. It could therefore be concluded that the effects of acute FS stress on NGF/c-Fos-ir are crucially governed by age.

The CA3 hippocampus is a region susceptible to the loss of the dendritic tree (Brunson et al. 2001, 2005, Vyas et al. 2002). Since the slightest change in NGF/c-Fos-ir under acute FS stress was recorded in the CA3 region of the hippocampus in both juvenile and aged rats, we may assume that the applied FS stimulation is not a burden factor for CA3 neurons in either age group. Taking into consideration the fact that c-fos genes activate NGF receptors though several transitional biochemical reactions that occur in the cell, we can only speculate that the observed increase of NGF/c-Fos-ir due to acute FS stimulation indicates an increase in the amount of active NGF-ir neurons in juvenile and aged animals (Semkova and Krieglstein 1999).

Compared with controls, in the group of juvenile rats exposed to chronic FS stress, the percentage of NGF/c-Fos-ir cells remained statistically unaltered (except in the SO). As opposed to acute FS stimulation, chronic FS stress caused a decrease in the percentage of NGF/c-Fos-ir cells in all investigated structures in juvenile rats. This may denote the habituation phenomenon of juvenile animals to FS exposure (Stone et al. 2007). Neuroendocrine changes in the limbic HPA-axis activity, being in all probability linked with the process of adaptation to repeated FS stress in young rats (Dunn and Swiergiel 2008), could be deemed relevant to the explanation of the diminution in the percentage of NGF/c-Fos-ir cells. Strictly speaking, glucocorticoids, which are elevated during stress, may have reduced NGF synthesis in the group of juvenile animals in our research (Barbany and Persson 1993, Ueyama et al. 1997). Another explanation for the reduced NGF/c-Fos concentration after chronic stress can be that NGF content was influenced by the changes in neuronal activity which occurred during the stress test (Knipper et al. 1994, Hellweg et al. 1997, von Richthofen et al. 2003).

In contrast with the juvenile individuals, the percentage of NGF/c-Fos-ir clearly increased after chronic FS in aged rats in all investigated regions, especially in PV and MeA nuclei. Assuming that NGF is expressed by excited cells (which express Fos protein), we conclude that a higher NGF/c-Fos-ir may indicate a lack of inhibition of FS stress response, and may also suggest that the activated cells of synthetic NGF induce neuroprotective effects during chronic stress in the aged brain (Branchi et al. 2004, Lang et al. 2004, McEwen 2008). Another observation we made in our studies was an unchanged percentage of NGF/c-Fos-ir in aged rats exposed to chronic FS stress, as opposed to those under acute stress. Ageing is associated with decreased efficiency in the physiological regulation of the response to stress stimuli. There is evidence that HPA axis dysfunction in aged animals is caused by stress (Schulte-Herbrüggen et al. 2006, McEwen 2008). Scaccianoce and coauthors (2000) demonstrated that HPA axis activation induced by NGF is significantly reduced in aged rats, thus we suppose that regulation of the HPA axis during ageing is inadequate. In addition, ageing impairs activation of transcription factors in the stress response-triggered recovery processes (Gu et al. 1998, Wurtwein et al. 1998). Despite the significant findings that emerged from our experiment, we have not been able to answer the question of whether this age-dependent process is protective or harmful to the aged brain.

In summary, the percentage of NGF-ir neurons in the analyzed structures in the control groups was high and, generally, did not change under FS stimulation (acute and chronic) in both age groups examined. However, the number of c-Fos-ir neurons increased following stressful stimulation. It was also observed that the majority of c-Fos-ir cells were also NGF-ir. In the light of these findings, we can postulate that changes in the percentage of NGF/c-Fos colocalization are probably caused by the changes of the number of c-Fos-ir cells, but not by that of NGF ones. Assuming that stress stimulation induces NGF to initiate trascriptional mechanisms leading to c-Fos production (through AP-1 factor) we suspect that NGF/c-Fos colocalization is, for the neuron, functionally important. The question, which yet requires further research, is what effect do the changes in NGF/c-Fos double staining cells under FS stimulation have on neuronal functioning?

CONCLUSION

This study demonstrates that stress-related limbic structures respond to FS stimuli differently, showing disparate levels of NGF/c-Fos-ir double staining cells in juvenile and aged rats. It is probably connected with different functions of NGF in juvenile (development, trophic effects) and aged (protection against neurodegeneration) animals. These findings may reflect the state of molecular plasticity within the limbic HPA-axis in both age groups; however, the phenomenon of habituation in NGF/c-Fos-ir after chronic FS exposure was observed only in juvenile animals.

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