Original Article

Age-Related (Aged vs. Adult) Comparison of the Effect of Two Mild Stressors on the Nerve Growth Factor (NGF) in the Rat Hypothalamic Supraoptic Nucleus (SON) – Immunohistochemical Study

(nerve growth factor / supraoptic nucleus / mild stress / adulthood / ageing / immunohistochemistry)

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Abstract. The ontogenetic period of life and stress can have different effects on the nerve growth factor (NGF) in the hypothalamus. The aim of our study was to investigate the influence of two mild stressors, acute and chronic exposure to forced swim (FS) or high-light open field (HL-OF), on neurons containing NGF. Immunofluorescence staining was used to reveal the density of NGF-immunoreactive (ir) cells in the hypothalamic supraoptic nucleus (SON) in adult (postnatal day 90; P90) and aged (P720) rats. The P90 and P720 rats that were subjected to acute and chronic FS showed no differences in the density of NGF-ir neurons in the SON compared with non-stressed rats. However, a significant increase in NGF-ir cells was noted after acute but not after chronic HL-OF only in P90 rats. What is more, there were no age-related (P90 vs. P720) changes in the density of NGF-ir neurons in non-stressed and FS- or HL-OF-stressed rats. Our results indicate that acute HL-OF was the only factor inducing changes in the density of NGF-ir neurons in the SON of adult rats. This could be related to the neuroprotective role of NGF-ir cells in response to acute HL-OF. The absence of age-dependent changes in the density of NGF-ir neurons may indicate that the ageing processes in SON do not generate changes in the NGF immunoreactivity of its neurons.

Introduction

The hypothalamic supraoptic nucleus (SON) consists of magnocellular neurons with synthesized neurohormones arginine-vasopressin (AVP) and oxytocin (OX), and transports them to the neural lobe of the pituitary form of the neurosecretory hypothalamo-neurohypophysial system (Swanson and Sawchenko, 1983; Armstrong, 1995). Within the posterior pituitary, these hormones are stored in axon terminals, from which they are secreted into the circulation system after various stimuli (Cunningham and Sawchenko, 1991). Because of this, SON plays an important role in neuroendocrine stress regulation (Engelmann and Ludwig, 2004; Landgraf and Neumann, 2004), and therefore possesses a high degree of structural plasticity in response to stimulation (Miyata et al., 1994a; Hatton, 1997). It has been claimed that the neuroendocrine secretion functions of AVP hypothalamic neurons are closely connected with the nerve growth factor (NGF) (Scaccianoce et al., 1993).

NGF is produced in the structures of the central nervous system not only in the development period, but also throughout adult life and during ageing. Multiple functions are regulated by endogenous NGF signalling, such as induction of enhanced neuronal growth and differentiation, facilitating neuronal survival, its remodelling and repair (Sofroniew, 2001; Lessmann et al., 2003). Moreover, by being involved in neuroendocrine secretion (Aloe et al., 2002; Webster Marketon and Glaser, 2008), NGF can participate in the neuronal response to stress stimulation (Scaccianoce et al., 2000; Von Richthofen et al., 2003) by taking part in the process of regulating the hypothalamic-pituitary-adrenal (HPA) axis activity (Cirulli and Alleva, 2009). In the hypothalamus, NGF might re-establish hormonal and neuropeptide pools (e.g. AVP, corticotrophin-releasing hormone – CRH) against homeostasis disturbance (Scaccianoce et al., 1993; Cirulli and Alleva, 2009).

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Abbreviations: ANOVA – analysis of variance, AVP – arginine-vasopressin, CRH – corticotrophin-releasing hormone, FS – forced swim test, HPA – hypothalamic-pituitary-adrenal axis, HL-OF – high-light open-field test, ir – immunoreactive, NGF – nerve growth factor, OX – oxytocin, PVN – paraventricular nucleus of the hypothalamus, SON – supraoptic nucleus.

Stress affects the limbic structures, including the hypothalamus, throughout an individual's life differently (Pedersen et al., 2001; Lupien, 2009). Reports referring to the changes (decrease or lack) in NGF expression in the process of ageing are not consistent either in non-stressed rats (Lärkfors et al., 1987; see: Kelly et al., 2000; see: Bimonte-Nelson et al., 2008) or in rats submitted to stress stimulation (Scaccianoce et al., 2000; Sofroniew et al., 2001). In addition, there is a lack of available data about age-related changes in the NGF immunoreactivity (-ir) in the hypothalamic SON under forced swim (FS) or high-light open-field (HL-OF) stress models, which may provoke emotional responses such as fear and anxiety in rats (Dal-Zotto et al., 2000; Bouwknecht et al., 2007).

Considering the fact that SON is closely related to the stress response (Miyata et al., 1994a; Engelmann et al., 1999; Engelmann and Ludwig, 2004) and assuming that NGF plays a significant role in its secretion function, the aim of our study was to investigate whether the SON differs in terms of the density of NGF-ir labelled neurons following exposure of adult, postnatal day 90 (P90) versus aged (P720) rats to the acute and chronic FS or HL-OF stressor.

Material and Methods

Animals

All experimental procedures and both 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 (opinion date: 090106; opinion number: 3/6). Adult (P90; P – postnatal day) and aged (P720) male Wistar Han rats were used for the experiments. All rats were bred until they reached the appropriate age under standard conditions in the Tri-City Academic Laboratory Animal Centre – Research and Services Centre. Two weeks before the experiments started, the rats were taken to the Department Animal Centre. All rats were housed socially in polysulphone cages (T. IV, 56 cm × 36 cm × 20 cm + 7 cm cage lid) containing dust-free sawdust on the floor (3–4 animals per cage). The rats were kept in air-conditioned rooms under a constant temperature (22 ± 2 °C), a humidity of 55 ± 10 % and a lighting regimen (light on from 7:00 a.m. to 7:00 p.m.) with free access to water and food pellets. Both food and water given to the animals were previously autoclaved and water was available *ad libitum*.

Model of the test

The P90 and P720 rats were divided into control groups and experimental groups. The control groups consisted of non-stressed animals that were handled daily for a few minutes by the same operator and remained in their home cage (in the same conditions as the experimental ones) until being anaesthetised. The experimental groups were exposed to acute or chronic stress in the forced swim (FS) test or to acute or chronic stress in the high-light open-field (HL-OF) test. Each of the P90 groups contained 6–7 rats, whereas the P720 groups consisted of 7–8 animals. Acute stress stimulation was conducted once in a 15–20 min session, whereas the chronic test was conducted once a day in 15–20 min sessions for 21 consecutive days at the same time – between 9:00 am and 2:00 pm. After the end of the tests, the rats were returned to their respective home cages for 90 minutes before the start of the sacrifice.

FS was chosen as a combination of a psychological (novelty, water) and physical (exercise – swimming, temperature) naturalistic, mild stressor, because it was proved to be an effective tool for identifying different pathways of coping in an unavoidable situation (Dal-Zotto et al., 2000; Stone et al., 2007). The HL-OF psychological test was used to examine the reaction to high-intensity light in an open-field area (unfriendly, potentially threatening environment), which is an aversive stimulus that provokes emotional stress responses (Hall et al., 2000; Bouwknecht et al., 2007). Procedures and conditions for the test models applied were described earlier (Badowska-Szalewska et al., 2015).

Tissue preparation and immunohistocheminal methods

The detailed procedure of tissue collection and preparation as well as the procedure of immunohistochemical labelling for NGF were described previously (Badowska-Szalewska et al., 2015). A primary polyclonal rabbit anti-NGF antibody (Chemicon International Inc., Billerica, MA, AB927; dilution 1 : 500) was used and then secondary antibodies: Cy3-conjugated goat anti-rabbit (Jackson ImmunoResearch Laboratories Inc., West Baltimore Pike, PA, 711-165-152; dilution 1 : 600) were applied. Controls for the immunohistochemistry, negative for any reactivity, were obtained by repeating the same procedure with omission of the primary or secondary antibodies and did not show any signals. The specificity of the NGF antibody applied here was tested by Western blotting by Conti et al. (2009).

Quantitative analysis

The newCAST ver.4.4.4.0 (Visiopharm, Hørsholm, Denmark) image analysis system based on an Olympus BX51 microscope equipped with DP72 camera (Olympus, Tokyo, Japan) was used to assess the density of NGF-immunostaining cells in the SON nucleus examined. The SON area was selected on the basis of the rat brain atlas by Paxinos and Watson (2006): bregma points from –0.60 to –1.56.

The regions of interest were outlined at 4× magnification. Computer-aided estimation was used to calculate the number of NGF-ir profiles of cells in the SON. Cell profiles were counted in a single layer of cells under a 40× objective lens separately for each structure. The program we used automatically selects at random 60 %

of the total area of the section of the structure on a given slide. The results were then recalculated proportionally into 100 % of the field structure on the slide. The total number of cells obtained was converted into a surface area of 1 mm². Four to six sections of the same structure from each rat were evaluated and the data were averaged. The results were grouped and analysed statistically for each structure separately.

Statistical analysis

All statistical analyses were carried out using InStat (GraphPad Software Inc, La Jolla, CA, 1998), version 3.0. After passing a normality test (Kolmogorov-Smirnoff), the differences between the groups studied (intact-control, acutely stressed and chronically stressed) in the age groups (adult – P90 rats vs. aged – P720 rats) were assessed by using a parametric analysis of variance (ANOVA). For the individual differences, the post-hoc Tukey multiple comparison test was applied. The precise F value of ANOVA and q value of the Tukey test as well as the corresponding approximate P levels were provided. The entire process of statistical inference was performed at a significant level of $P < 0.05$. The results of the immunohistochemical study were expressed as a mean density (number of labelled NGF-ir cells/mm^2) ± standard deviation (SD).

Results

The density of NGF-ir cells in the SON of P90 rats in the control group and after FS or HL-OF stimulation

Analysis of variance (ANOVA) revealed statistically significant changes in the density of NGF-ir cells between the groups (control, FS acute, FS chronic, HL-OF acute, HL-OF chronic) of adult (P90) rats studied ($F = 4.365$, $P = 0.0078$, $P < 0.01$). Analysis of immunostained sections, derived from adult (P90) non-stressed rats, demonstrated that NGF-ir cells are arranged relatively homogenously in the SON. The NGF immunostaining was distributed in the cytoplasm of cells (data not shown).

The post-hoc Tukey test analyses showed that after applying the acute and chronic FS stress stimulus there were no statistically significant changes in the density of NGF-ir cells in the SON (acute: $q = 2.813$, $P > 0.05$; chronic: $q = 0.878$, $P > 0.05$) in adult rats in relation to non-stressed P90 rats (Fig. 1). However, acute but not chronic HL-OF led to a marked increase in the density of NGF-ir cells in the SON (acute: $q = 4.811$, $P < 0.05$; chronic: $q = 0.105$, $P > 0.05$) when compared with the control rats (Figs 1, 2 A,A'–C). In addition, we detected a statistically significant decrease in the density of NGF-ir neurons in the SON ($q = 4.888$, $P < 0.05$) after chronic HL-OF in relation to acute HL-OF stimulation (Figs. 1, 2B–C).

The density of NGF-ir cells in the SON of P720 rats in the control group and after FS or HL-OF stimulation

ANOVA demonstrated no statistically significant differences between the groups studied (control, FS acute, FS chronic, HL-OF acute, HL-OF chronic) in the density of NGF-ir cells in aged (P720) rats for the SON ($F = 2.377$, $P = 0.071$, $P > 0.05$). In comparison with non-stressed rats, acute and chronic FS or HL-OF stimulation did not cause any significant changes in the density of NGF-ir cells despite the tendency towards the increase in the density of NGF-ir neurons in response to acute HL-OF (Figs. 1, 2D,D'–F).

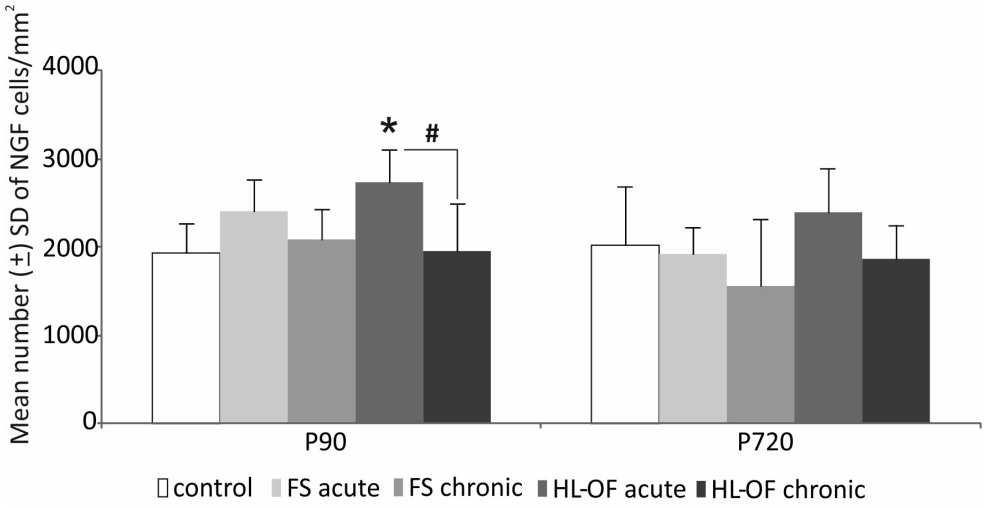

Fig. 1. The effects of FS or HL-OF on the density of NGF-ir neurons in the hypothalamic SON in adult (P90) and aged (P720) rats. The figure shows the comparison of experimental groups vs. control groups, chronic stress groups vs. acute stress groups and P90 vs. P720 rats. Statistical analyses were performed by ANOVA with Tukey's post hoc test. A significant increase in the density of NGF-ir cells after acute HL-OF compared with the control group in P90 rats is indicated by * ($P < 0.05$). A significant decrease in the density of NGF-ir cells after chronic HL-OF compared with acute HL-OF in P90 rats is indicated by # ($P < 0.05$). N = 6–7 (P90 groups) and N = 7–8 (P720 groups).

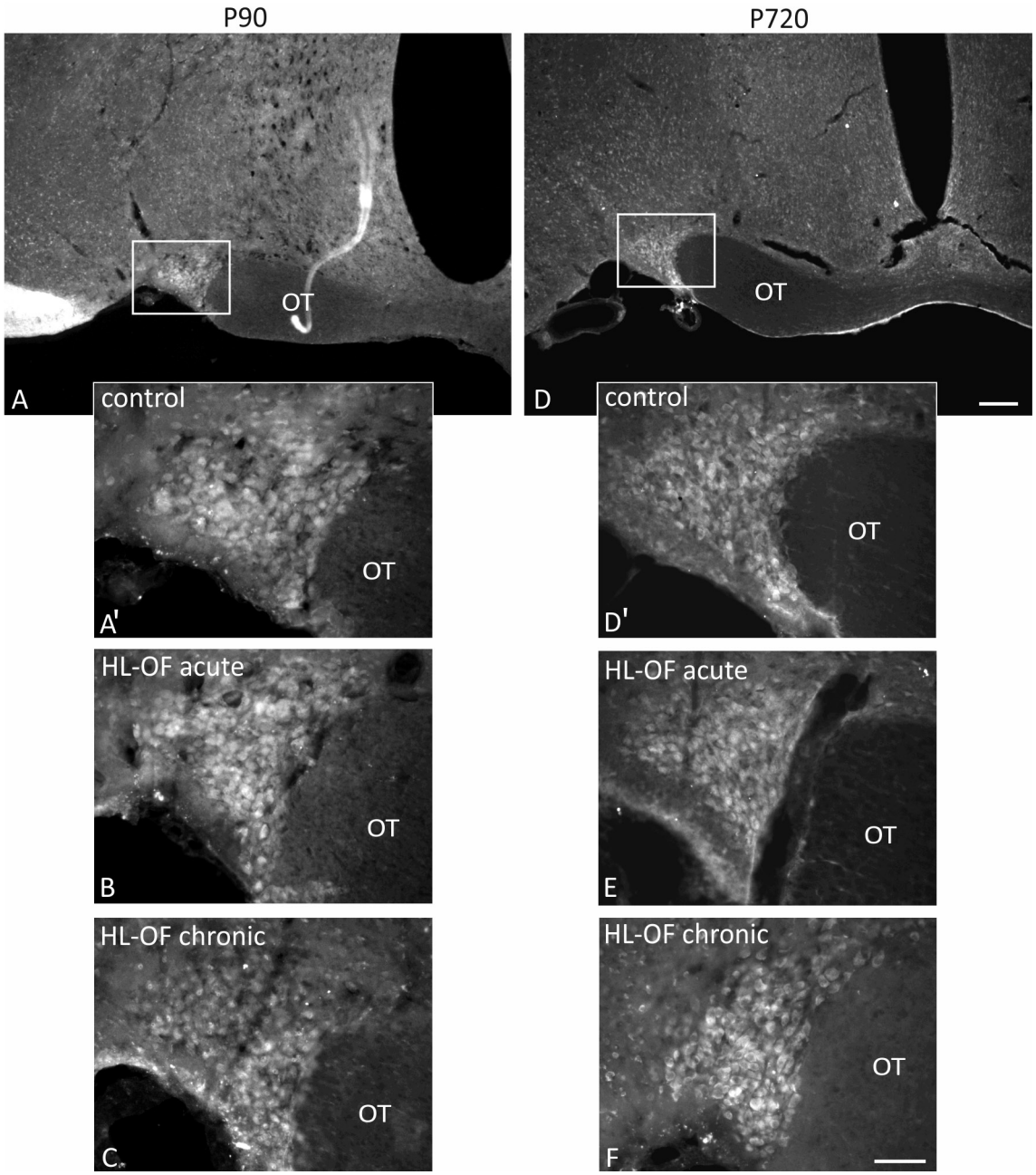

Fig. 2. Distribution of NGF-ir neurons in the hypothalamic SON. NGF-ir neurons in the SON in adult (P90) (A,A'–C) and aged (P720) (D,D'–F) rats from the control groups (A,A', D,D') and from the groups exposed to acute (B, E) or chronic (C, F) HL-OF stress. The figure shows a significant increase in the density of NGF-ir neurons after acute HL-OF vs. control and decrease after chronic HL-OF vs. acute HL-OF in P90 rats. OT – optic tract. Scale bar = 200 μm (A, D) and 100 μm (A',D', B–C, E–F).

The density of NGF-ir cells in the SON in relation to age (P90 vs. P720)

Post-hoc Tukey test analyses revealed no statistically significant differences in the density of NGF-ir cells between the non-stressed P90 control group in comparison with the P720 control group of rats ($q = 0.412$, $P > 0.05$) (Fig. 1). Similarly, statistically non-significant changes in the density of NGF-ir neurons in relation to the age (P90 vs. P720) in both FS- (acute: $q = 2.587$, $P > 0.05$; chronic $q = 2.77$, $P > 0.05$) and HL-OF- (acute: $q = 1.820$, $P > 0.05$; chronic: $q = 0.466$, $P > 0.05$) stressed rats were detected (Fig. 1).

Discussion

The lack of age-dependent change in the basal density of NGF-ir in the SON

Immunohistochemical studies indicated the presence of NGF-ir neurons in the SON of non-stressed rats; their density was quite high and comparable in P720 vs. P90

rats. NGF-ir in the hypothalamus had been previously detected in non-stressed adult and aged rodents (Aloe et al., 1990; Badowska-Szalewska et al., 2015). Moreover, in our earlier semi-quantitative data referring to 180-day-old rats, numerous NGF-ir neurons in the SON were also observed (Badowska-Szalewska et al., 2006).

There are contradictory reports concerning the changes in basal NGF expression in brain structures related to the ageing process. Most researchers claim that ageing selectively influences the changes in basal NGF expression depending on the analysed structure (Katoh-Semba et al., 1998; Niewiadomska et al., 2006; Perovic et al., 2013). Like other researchers (Lee et al., 1998; Pereira et al., 2005), we can assume that NGF observed in neurons of the non-stressed rats indicates engagement of the factor for normal functioning of cells; making the endocrine function of SON neurons effective and acting in neuronal protection (e.g. prevention of glutamate and nitric oxide cytotoxicity, glucose deprivation, or Ca^{2+} influx) (see: Sofroniew et al., 2001), at least in adult rats. The lack of changes in the density of NGF-ir cells in P720 rats versus P90 rats may suggest that the density of NGF-ir cells is sufficient for the structure to function effectively. On the other hand, it is known that in senescence, the neurodegeneration processes and deficiencies connected with them are significantly amplified (see: Sofroniew et al., 2001); therefore, the lack of evidence for a decrease in the basic NGF-ir cells in aged rats in our study does not exclude possible failures of NGF signalling.

Acute and chronic FS do not influence the change in the density of NGF-ir neurons in the SON of P90 and P720 rats

It has been observed that exposure to some stressors, e.g. chronic osmotic (Luo et al., 1994; Miyata et al., 1994b), acute (2 h) restraint (Miyata et al., 1994a; Krause et al., 2011), acute social defeat (Engelmann et al., 1999), acute high-light open-field (Badowska-Szalewska et al., 2006), or 10-min forced swim (Wotjak et al., 1998) can activate the SON. Different kinds of stress stimuli, mainly of an emotional nature, may affect the NGF expression in the hypothalamus (Aloe et al., 1990, 2002; Alleva and Santucci, 2001). It is accepted that there exists a mutual influence of HPA axis activity and NGF on the hypothalamus (Watkins, 1995).

We have noted that adult (P90) and aged (P720) rats exposed to acute and chronic FS showed no significant disparities in the density of NGF-ir cells in the SON compared to the non-stressed groups of rats. This confirms our previous studies in which neither acute nor chronic FS caused marked changes in NGF-ir cells in another hypothalamic nucleus – paraventricular (PVN) – in adult and aged rats (Badowska-Szalewska et al., 2015). In view of our current results, we believe that acute and chronic FS are not able to induce changes in NGF-ir neurons in the SON of adult and aged rats. What is more, FS stress does not represent an additional risk factor in the ageing process in respect of its impact on the density of NGF-ir neurons in the SON.

Swimming is considered a psychophysical stressor that rats can meet in their habitat (Dal-Zotto et al., 2000). The natural stressors may provoke evolutionally developed compensatory homeostatic mechanisms (e.g. antioxidant processes) (Plata-Salamán et al., 2000; Pedersen et al., 2001; Pajović et al., 2006), which might counteract changes in the density of NGF-ir cells in the SON. What is more, our observations might suggest that the degree of HPA axis activation caused by FS was insufficiently strong to induce significant differences in the density of NGF-ir cells. Thus we believe that the FS used in our study (due to its naturalistic character, relatively weak adversity and possibly short period of action – 15–20 min exposure) was not an aggravating factor for either adult or aged rats with regard to its influence on the NGF-ir neurons in the SON, because the rats were able to control stressful situations.

Acute HL-OF has significant impact on the density of NGF-ir cells in the SON in adult (P90) but not in aged (P720) rats

Our study showed a significant increase in the density of NGF-ir cells in the SON after exposure to acute HL-OF of the P90 group tested but no substantial change in P720 in relation to the non-stressed rats. Furthermore, we found a marked decrease in NGF-ir cells after chronic HL-OF compared to acute stress in P90 rats. However, it was previously observed that acute and chronic HL-OF induced no significant changes in the number of NGF-ir-supraoptic neurons in 6-month-old rats (Badowska-Szalewska et al., 2006). Two components determine the adversity of HL-OF: "open field" – new, unknown, potentially dangerous surroundings without the possibility to escape, and "bright light" – because rats are nocturnal animals and tend to avoid brightly lit places (Hall et al., 2000; see: Bouwknecht et al., 2007).

We can assume that the probable reason for the significant increase in the density of NGF-ir cells in the SON of adult rats after acute HL-OF, as well as in the earlier study, could be due to the higher (compared to FS) activation of the HPA axis (Badowska-Szalewska et al., 2015). Indirectly, it is supported by the fact that the density of the c-Fos-ir cells (c-Fos expression can be taken as an index of neuronal/trans-synaptic activations in the stress-related circuitry – Kovács, 1998; Thrivikraman et al., 2000) in PVN, which is the site where HPA axis activation is initiated, was higher in the rats that were under the effect of acute HL-OF in the P90 age groups, whereas in P720 rats this difference was statistically non-significant in comparison with acute FS (see Badowska-Szalewska et al., 2016, supplementary data). The activation of the stress axis is able to affect the functioning of the neuroendocrine response system as a reaction to acute HL-OF. Hypersecretion of neuropeptides or hormones, e.g. glutamate (a major excitatory transmitter in the SON – Stern et al., 1999), might have

affected the SON and cause an increase in NGF immunoreactivity in their neurons. The synthesis of supraoptic NGF may have in turn modulated the response of HPA axis to stress (Taglialatela et al., 1991; Von Richthofen et al., 2003). Because NGF may play a functional role in stress-coping responses (Aloe et al., 2002), an increase in NGF-ir cells after acute HL-OF seems to be neuroprotective against the destructive influence of stress hormones for adult rats (Alleva et al., 1996; Cirulli and Alleva, 2009).

The lack of changes in the density of NGF-ir neurons observed after chronic HL-OF in adult rats could indicate the fact that the process of HPA axis activity was not continued for a longer time probably due to glucocorticoid action. Like others (e.g. Dubovicky and Jezova, 2004; Cirulli and Alleva, 2009), we can assume that the result of chronic exposure to HL-OF was an adaptive response of NGF-ir neurons (habituation) in the SON, and therefore, this process demonstrates the NGF-dependent plasticity of the SON.

The lack of significant differences in the density of NGF-ir cells of aged rats treated with acute or chronic HL-OF may suggest that as far as the influence on the density of NGF-ir neurons in the SON is concerned, HL-OF was not an aggravating factor for aged rats. It is also possible that a large number of NGF-ir neurons could ensure functioning of the ageing structure not only in the physiological conditions, but also during stress. Taking the data into account, it can also be deduced that adult and aged rats differed in their patterns of response to exposure to HL-OF.

The lack of age-dependent (P90 vs. P720) changes in the density of cells containing NGF-ir in FS- and HL-OF-stressed rats in the SON

It is widely believed that brain ageing is connected with morphological and biochemical changes in neurons (Kelly, 2000). In addition, the ageing process is usually associated with a lower capability of coping with stress (Pedersen et al., 2001; Pardon, 2007), which is due to disruption of all the mechanisms that participate in the HPA axis stress response (Pedersen et al., 2001; Meyza et al., 2007).

Despite the increase in the density of NGF-ir neurons after acute HL-OF in P90 rats, when comparing P90 with P720 acute and chronic FS- or HL-OF-stressed rats, it was observed that the density of NGF-ir cells in the SON was not altered. However, an age-related (P90 vs. P720) decrease in the density of NGF-ir neurons was previously observed in other limbic structures: PVN and some of the hippocampal regions in rats stressed in the same way (Badowska-Szalewska et al., 2015). This may suggest that age-related changes in NGF immunoreactivity appearing in stressed rats depend on the structure being analysed. However, it cannot be excluded that the lack of age-dependent changes in the density of NGF-ir neurons in FS- and HL-OF-stressed rats does not necessarily mean a lack of disturbances in supraoptic NGF signalling.

Conclusions

Forced swim test (acute and chronic) was not an aggravating factor with regard to its influence on the density of NGF-ir neurons in the SON for the adult and aged rats. It is likely that natural stimulation can provoke homeostatic mechanisms preventing these changes. However, acute HL-OF was the only factor inducing changes (increase) in the density of NGF-ir cells in the SON of adult rats. This could be related to the neuroprotective role of NGF-ir neurons in response to acute HL-OF. The differences observed in the influence of acutely acting FS and HL-OF on the density of NGF-ir SON neurons in adult rats might have been caused by the different degree of adversity of these stressors.

The absence of age-dependent changes in the density of supraoptic NGF-ir neurons is presumably connected with the involvement of the neurons in important but age-different functions and it may point to the fact that ageing processes in the SON do not generate changes in the NGF immunoreactivity of its neurons.

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