The influence of mild stressors on neurons containing interleukin-1β in the central (CeA) and medial (MeA) amygdala in the ageing process of rats

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Proinflammatory cytokine – interleukin 1β (IL-1β) plays an important role in stress reactions in the structures of limbic system. The impact of stress on IL-1β may depend on the ontogenetic age. The study examined the influence of acute and chronic exposure to forced swim (FS) or high-light open-field (HL-OF) stressors on neurons containing IL-1β. Double immunofluorescence staining was used to reveal the density of IL-1β/NeuN (NeuN – a neuronal nuclear marker) – immunoreactive (ir) cells in the amygdaloid central (CeA) and medial (MeA) nuclei, which are closely involved in the regulation of emotional stressors and hypothalamic-pituitary-adrenal axis (HPA) activation. Adult (P90; P – postnatal day), middle-aged (P360), and aged (P720) male Wistar Han rats were used in these experiments. We observed an age-dependent increase in the basal density of IL-1β/NeuN-ir cells in CeA and MeA in P90 vs. P360 and P360 vs. P720 rats. Neither acute nor chronic FS caused significant changes in the density of IL-1β-ir neurons in any of the investigated nuclei in P90, P360, and P720 rats as compared with the non-stressed groups. However, chronic but not acute HL-OF caused a marked increase in the density of IL-1β/NeuN-ir cells in the CeA and MeA of P360 rats and in MeA of the P720 animals. Moreover, chronic HL-OF led to an increase in the density of IL-1β-ir neurons in relation to acute HL-OF in the CeA and MeA of both P360 and P720 rats. Our results may indicate the involvement of IL-1β neurons in the development of ageing processes in CeA and MeA. Furthermore, our results point out that chronic HL-OF is an aggravating factor that induces an increase in the density of IL-1β/NeuN-ir cells in the MeA and/or CeA of middle-aged and aged rats. The increase is possibly due to insufficient control of the HPA axis associated with involutional ageing processes and seems to be a common denominator of the ageing process and stress.

Key words: interleukin-1β, neuron, amygdala, mild stress, ageing

INTRODUCTION

The amygdala is one of the limbic structures integrating emotional and sensory information for the expression of fear and anxiety; it is widely regarded as being involved in the emotion of fear and with unconditioned and conditioned responses to fearful stimuli (Carter et al. 2004, Phelps and LeDoux 2005). The amygdala also plays an important role in the central integration of stress reactions (Dayas et al. 1999, Herman et al. 2003); it regulates the hypothalamic-pituitary-adrenocortical (HPA) axis which has an excit-

atory effect on it (Vyas et al. 2002, Myers et al. 2014). The influence of the amygdala on the HPA system is largely mediated by the central (CeA) and medial (MeA) amygdaloid nuclei, representing the principal amygdalar projection neurons to basal forebrain, hypothalamus and brainstem structures (Dayas et al. 1999, Herman et al. 2003). These nuclei are also engaged in the mechanisms of amygdala neuroplasticity during stress (see Dayas et al. 1999, Nikolaev et al. 2002, Matys et al. 2004, Shoji and Mizoguchi 2010).

Proinflammatory cytokine – interleukin 1β (IL-1β) plays a significant role in the biology of limbic system structures (Kronfol and Remick 2000, Vitkovic et al. 2000). It is produced and released not only by non-neuronal cells but also from neurons (Müller 1997, Pearson et al. 1999, see Szelényi 2001). Apart from its

contribution to immunoregulation, IL-1β, can affect neuroendocrine mechanisms and participates in the physiological regulation of diverse cellular processes (Boutin et al. 2003, Srinivasan et al. 2004, Yirmiya and Goshen 2011) in healthy structures (Vitkovic et al. 2000, Tonelli and Postolache 2005, Khairova et al. 2009) and also under conditions of damage (inflammatory), disease (depression, anxiety) and stress (Friedman 2001, Srinivasan et al. 2004, Hayley 2011). By taking part in HPA axis regulation (Turnbull and Rivier 1999, Grinevich et al. 2001, Elenkov and Chrousos 2002), IL-1β is involved in physiological and behavioral responses to physical or psychological stressors (Kronfol and Remick 2000, Herman et al. 2003, Goshen and Yirmiya 2009). Moreover, the IL-1β signalling pathway seems to play a prominent role in the development of neurodegeneration processes (Allan et al. 2005).

It is commonly believed that the influence of stress stimulation on the changes in IL-1β expression depends on the nature and duration of stressors (Deak et al. 2005), the region in which they are measured (O'Connor et al. 2003), ontogenetic period of life (Muray and Lynch 1998) and control over the stressor (Kronfol and Remick 2000). Stress affects the structures of the limbic system in different ways throughout an individual's life (Moynihan 2003, Lupien 2009). This is caused by changes in the functioning of the HPA axis, which in turn induce changes in the release of stress hormones (Fuchs and Flügge 2003, McEwen 2010). These changes are particularly important in the process of ageing (Pedersen et al. 2001, Pardon 2007).

Ageing is one of the ontogenic periods of importance with respect to the morphological, biochemical and functional changes which occur within the limbic system (Pardon 2007, Lupien 2009) including amygdala (Garrido 2011). An ageing-induced increase in oxidative stress and an important decline in neurotransmission induce a progressive loss of neuronal function (Finkel and Holbrook 2000, Segovia et al. 2001). In addition, ageing causes organisms to become vulnerable to stress, which might be mediated by dysfunction of the brain system regulating emotional and stress responses (Shoji and Mizoguchi 2010). Studies indicated that ageing increased anxiety-like behavior in rats (Boguszewski and Zagrodzka 2002, Meyza et al. 2011).

There is evidence that aged animals would be more sensitive to the effects of mild stress than adult ones and suggests a possible role for IL-1β (Buchanan et al. 2008). What is more, it has been determined that mild chronic stress may have a powerful effect on the amygdala by impairing memory through non-hippocampal mechanisms, such as enhanced emotionality, especially in aged rats (Conrad et al. 1999, Shoji and Mizoguchi 2010). However, the precise role of stress-induced IL-1β changes in the ageing limbic system structures is not completely defined. In particular, the changes IL-1β in neurons (in which IL-1β plays an important role), caused by the ageing process and by simultaneous load in the form of emotional stimulation, such as forced swim (FS) or high light open-field (HL-OF) in the amygdaloid CeA and MeA – structures involved in responses to emotional stressors, fear and anxiety, have never been tested.

An analysis of all the above has led us to the aim of our study, that is, to answer the question of whether the ageing process in rats has an influence on the density of neurons containing IL-1β-ir in CeA and MeA. A further question is whether exposure to mild acute and chronic stress stimulation can cause changes in the density of IL-1β-ir neurons in CeA and MeA in adult (P90), middle-aged (P360) and aged (P720) rats. FS and HL-OF have been chosen, because they are commonly known as stress stimuli which can induce depression symptoms in rats (Dal-Zotto et al. 2000, Hale et al. 2006).

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), middle-aged (P360) 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 Center. All rats were housed socially in polysulfone 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 AM to 7:00 PM) with free access to water and food pellets. Both the food and water given to the animals were previously autoclaved and water was available *ad libitum*.

Model of the test

The P90, P360 and P720 rats were divided into control groups and experimental groups. The control groups comprised 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 anaesthetized. 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 5–6 rats, whereas the P360 and P720 groups consisted of 7–9 animals.

Acute stress stimulation was conducted once in a 15-minute session, whereas the chronic test was conducted once a day in 15-minute sessions for 21 consecutive days at the same time – between 9:00 AM and 2:00 PM. After the tests, the rats were returned to their respective home cages.

Forced swim (FS) test

FS was chosen as the mix of a psychological (novelty, water) and physical (exercise – swimming, temperature) naturalistic, mild stressor, because it was proven 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 rats were placed individually in a glass cylinder (45 cm high, 20 cm in diameter) filled with clear, fresh water (at 22°C) up to the height of 30 cm.

High-light open-field (HL-OF) test

The HL-OF psychological test was used to examine the reaction to high-intensity light in open field area (unfriendly, potentially threatening environment), which is an aversive stimulus that provokes emotional stress responses (Hale et al. 2006, Bouwknecht et al. 2007). The apparatus for the test consisted of a 100×100×40 cm wooden box illuminated with a 500--watt halogen lamp. In order to provoke a stress reaction, each animal was gently placed in the centre of the open field arena which, after each test, was cleaned with water and 70% ethanol.

Tissue collection and preparation

After stressor termination, the rats were returned to their home cages for ninety minutes before the start of the killing (based on Sugama et al. 2011). Afterwards, the rats were deeply anaesthetized with a lethal dose of Nembutal (80 mg/kg body weight), and then perfused transcardially with a 0.9% saline solution with heparin, followed by a 4% paraformaldehyde solution in a 0.1M phosphate buffer (pH 7.4). The brains were post-fixed in 4% paraformaldehyde for 3–4 hours, and were kept overnight at 4ºC in a 0.1 M phosphate buffer containing 15% sucrose and, subsequently 30% sucrose, until they sank. Coronal 30-µm thick serial sections of the brain were cut with a JUNG 1800 cryostat (Leica, Germany).

Immunohistochemistry

Sampled sections throughout the length of the structures being analyzed were stained for IL-1β and NeuN (neuronal marker) with the use of double immunohistochemical methods. Free-floating sections were blocked in 10% normal goat serum (NGS) for 2 hours and then incubated for 3 days at 4°C in a mixture of primary polyclonal rabbit anti-IL-1β antibody (Endogen, USA: PR 427B; dilution 1:100), monoclonal mouse anti-NeuN antibody (Chemicon-Milipore: #MAB 377; dilution 1:500) and 0.3% Triton X-100. After multiple rinses in phosphate buffered saline (PBS), the sections were incubated (for 2–3 hours at room temperature) with a mixture of appropriate secondary antibodies: Cy3-conjugated goat anti-rabbit (Jackson ImmunoResearch Laboratories, Inc: #111-165-144; 1:600 dilution) and Alexa Fluor 488-conjugated goat-anti-mouse (Molecular Probes, Eugene, OR, USA: A-11001; 1:150 dilution). Finally, the sections were washed, mounted on slides and coverslipped with Kaiser's Glyceringelatine (#A12083, Merck, Germany). Controls for the immunohistochemistry, which were negative for any reactivity, were obtained by repeating the same procedure with the omission of the primary or secondary antibodies. The specificity of the IL-1β

antibody had been tested previously by Western blotting (Badowska-Szalewska et al. 2014).

Quantitative analysis

The newCAST ver.4.4.4.0 (Visiopharm, Denmark) image analysis system, based on a Olympus BX51 microscope equipped with a DP72 (Olympus, Japan) camera, was used to assess the density of IL-1β/NeuN-ir double-staining cells in the CeA and MeA nuclei of amygdala examined. These CeA and MeA areas were selected on the basis of the rat brain atlas (Paxinos and Watson 2007): Bregma points from −1.72 to −2.76 (for CeA) and Bregma points from −1.92 to −3.12 (for MeA).

The regions of interest were outlined at 4-time magnification. Computer-aided estimation was used to calculate the number of IL-1β/NeuN-ir profiles of cells in the CeA and MeA. Cell profiles were counted separately in a single layer of cells under a 40x objective lens in each structure. Only clearly labeled IL-1β/NeuN-ir cells were counted. The program used for this purpose automatically chose a random 60% area of the section of the structure on a given slide. The results were then calculated to account for 100% of the field structure on the slide. The total number of cells obtained was converted into a surface area of 1mm² (optical density). Four to six sections of the same structure from each rat were evaluated and the data were averaged. The results were grouped and analyzed statistically for each structure in turn.

Statistical analysis

After passing a normality test (of Kolmogorov-Smirnoff), the differences between the groups studied (intact-control, acutely stressed and chronically stressed) in the age groups (adult – P90 rats *vs.* middle-aged – P360 rats and middle-aged – P360 *vs.* aged – P720 rats) were assessed using an F-test parametric analysis of variance (ANOVA) and *post-hoc* Tukey multiple comparison test. All statistical analyses were carried out by using InStat (GraphPad Software Inc, 1998), version 3.0. For the individual differences, the precise q-value of the Tukey test, as well as the corresponding approximate P-level was provided. The whole 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 double-labelled IL-1β/NeuN-immunoreactive cells/mm²) ±standard deviation (SD).

RESULTS

The density of IL-1β/NeuN-ir cells in the CeA and MeA of P90 rats in the control group and after FS or HL-OF stimulation

One-way Analysis of Variance (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 IL-1β/NeuN-ir cells in adult (P90) rats both for the CeA ($F=1.158$, $P=0.3538$) and for the MeA ($F=0.036$, $P=0.9973$). The analysis of double-stained sections (IL-1β/NeuN) derived from the P90 non-stress rats revealed that IL-1β-ir neurons appeared in the CeA and MeA in a very low density (Figs 1A–B, 2A, 3A).

The adult (P90) rats that underwent acute or chronic FS stimulation showed no significant changes in the density of IL-1β-ir neurons in the CeA (acute: $q=0.00$, $P>0.05$; chronic: $q=0.905$, $P>0.05$) and MeA (acute: $q=0.437$, $P>0.05$; chronic: $q=0.437$, $P>0.05$) compared to the control group (Figs 1A–1B, 2A–2B, 3A–3B). Furthermore, acute and chronic HL-OF did not cause any significant changes in the density of IL-1β/NeuN-ir cells in CeA (acute: $q=1.460$, $P>0.05$; chronic: $q=1.449$, $P>0.05$) and in MeA (acute: $q=0.397$, $P>0.05$; chronic: $q=0.228$, $P>0.05$) of P90 rats (Figs 1A–B, 2A, 2C, 3A, 3C).

The density of IL-1β/NeuN-ir cells in the CeA and MeA of P360 rats in the control group and after FS or HL-OF stimulation

The one-way ANOVA test revealed statistically significant changes between the groups studied (control, FS acute, FS chronic, HL-OF acute, HL-OF chronic) in the density of IL-1β/NeuN-ir cells in middle-aged (P360) rats for the CeA ($F=13.721$, $P<0.0001$) as well as for the MeA ($F=29.735$, $P<0.0001$). In P360 non-stress control rats, IL-1β/NeuN-ir cells that often appeared in the CeA and MeA were observed (Figs 1A–1B, 2D, 3D).

Post-hoc Tukey multiple comparison test showed that in response to both acute and chronic FS stimulation, there were no significant changes in the density of IL-1β-ir neurons in any of the amygdaloid nuclei investigated: CeA (acute: $q=1.301$, $P>0.05$; chronic: $q=2.826$,

P>0.05) and MeA (acute: *q*=1.977, *P*>0.05; chronic: *q*=0.265, *P*>0.05) as compared with the control group in P360 rats (Figs 1A–1B, 2D–2E, 3D–3E). P360 rats that underwent acute HL-OF stimulation demonstrated no statistically significant differences in the density of IL-1β/NeuN-ir cells in the CeA (*q*=2.733, *P*>0.05) and MeA (*q*=2.752, *P*>0.05) in relation to the control rats (Fig. 1A–1B). However, chronic HL-OF led to a marked increase in the density of IL-1β-ir neurons in the CeA (*q*=9.647, *P*<0.001) and MeA (*q*=11.982, *P*<0.001) in

relation to non-stressed rats and in comparison with acute HL-OF stimulation (CeA: *q*=6.586, *P*<0.001; MeA: *q*=8.373, *P*<0.001) (Figs 1A–B, 2D, 2F, 3D, 3F).

The density of IL-1β/NeuN-ir cells in the CeA and MeA of P720 rats in the control group and after FS or HL-OF

The one-way ANOVA test revealed statistically significant changes between the groups studied (control,
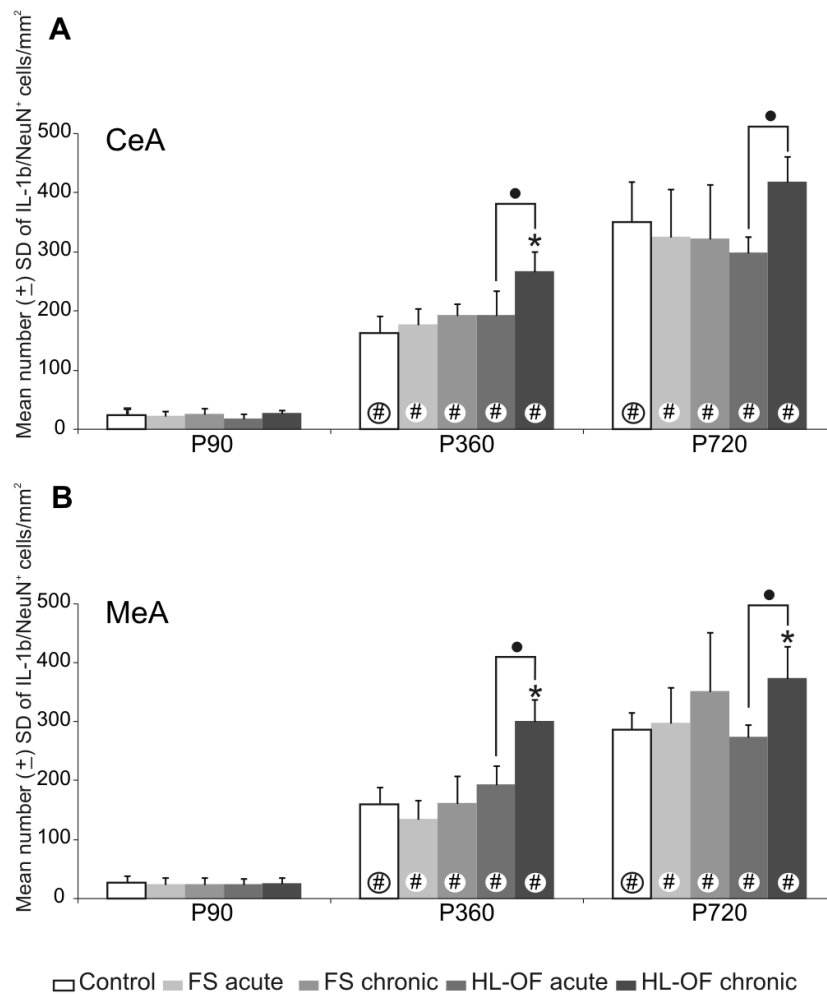

Fig. 1. The effects of FS or HL-OF on the density of IL-1β/NeuN-ir cells in the CeA and MeA nuclei of amygdala in relation to age. The effects of acute and chronic exposure to forced swim (FS) or high-light open-field (HL-OF) stimulation on the density of IL-1β/NeuN-immunoreactive (ir) cells in the central (CeA) (A) and medial (MeA) (B) nuclei of the amygdala in adult (P90), middle-aged (P360) and aged (P720) rats. The figure shows the comparison of experimental groups *vs.* control groups, chronic stress groups *vs.* acute stress groups, P90 *vs.* P360 and P360 *vs.* P720 rats. Statistical analyses were performed by analysis of variance (ANOVA) with Tukey's *post hoc* test. A significant increase in the density of IL-1β/NeuN-ir cells after chronic HL-OF compared with the control groups is indicated by *(*P*<0.05). A significant increase in the density of IL-1β/NeuN-ir cells under chronic stress (HL-OF) compared with acute HL-OF stress is indicated by • (*P*<0.05). A significant increase in the density of IL-1β/NeuN-ir cells in P360 *vs.* P90 and P720 *vs.* P360 rats is indicated by # (*P*<0.05).

CeA
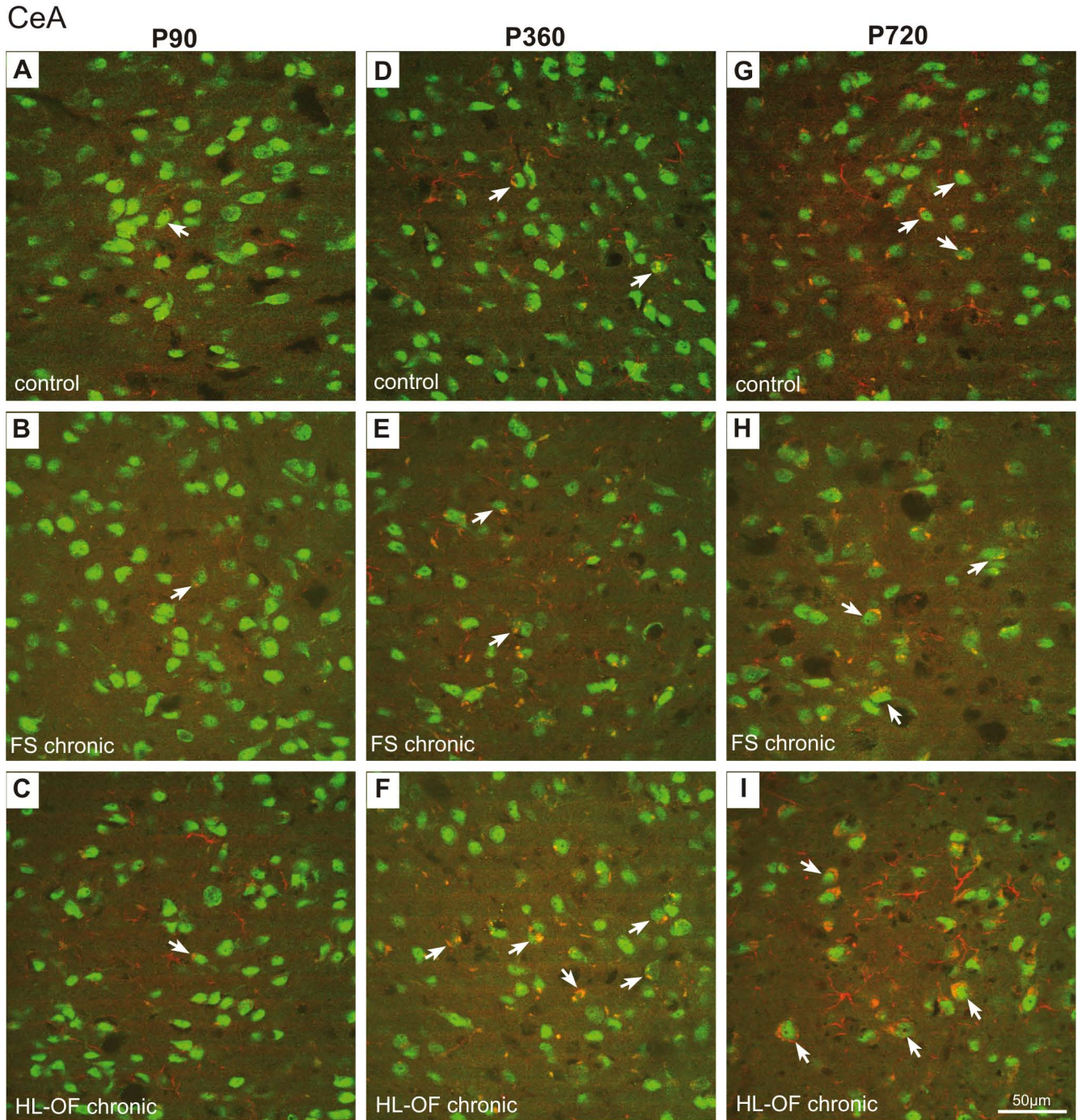

Fig. 2. Distribution of IL-1β/NeuN-ir cells in the CeA nucleus of amygdala. IL-1β-ir cells (red), NeuN-ir cells (green), and double IL-1β/NeuN-immunostaining cells (yellow-orange; arrow) in the central nucleus of amygdala (CeA) in adult (P90) (A–C), middle-aged (P360) (D–F) and aged (P720) (G–I) rats from the control groups (A, D, G) and from the groups exposed to chronic forced swim (FS) (B, E, H) or chronic high-light open-field (HL-OF) (C, F, I) stress. The figure shows an age-dependent (P90 *vs.* P360 *vs.* P720) increase in the density of IL-1β/NeuN-ir cells in the controls and chronic FS or HL-OF stressed rats, and increase in the density of IL-1β-ir neurons in P360 rats after chronic HL-OF *vs.* control. Scale bar is 50 μm.

FS acute, FS chronic, HL-OF acute, HL-OF chronic) in the density of IL-1β-ir neurons in aged (P720) rats for the CeA ($F=13.721$, $P<0.0001$) and MeA ($F=29.735$, $P<0.0001$). In the analysis of immunostained sections derived from aged (P720) non-stressed rats, it was observed that IL-1β-ir neurons very often appeared in the CeA and MeA (Figs 1A–1B, 2G, 3G).

Post-hoc Tukey test analyses showed that after applying the acute and chronic FS stress stimulus, there were no significant changes in the density of IL-1β/NeuN-ir cells in the CeA (acute: $q=1.041$, $P>0.05$; chronic: $q=1.187$, $P>0.05$) and MeA (acute: $q=0.660$, $P>0.05$; chronic: $q=3.244$, $P>0.05$) in aged rats in relation to non-stressed aged rats (Figs 1A–1B, 2G–2H, 3G–3H). Similarly, acute HL-OF did not cause any changes in the density of IL-1β-ir neurons in either the CeA ($q=2.148$, $P>0.05$) or MeA ($q=0.575$, $P>0.05$) (Fig. 1A–1B). However, chronic HL-OF led to a marked increase in the density of IL-1β/NeuN-ir cells in the MeA ($q=4.634$, $P<0.05$), but not in the CeA ($q=2.997$, $P>0.05$) when compared with the control rats (Figs 1A–1B, 2G, 2I, 3G, 3I). In addition, we detected a statistically significant increase in the density of IL-1β-ir neurons both in the CeA ($q=5.001$, $P<0.01$) and MeA ($q=5.071$, $P<0.01$) after chronic HL-OF in relation to acute HL-OF stimulation (Fig. 1A–1B).

The density of IL-1β/NeuN-ir cells in the CeA and MeA in relation to age (P90 *vs.* P360) and (P360 *vs.* P720)

In the non-stress groups, post-hoc Tukey test analyses showed a significant increase in the density of IL-1β/NeuN-ir cells in the CeA ($q=7.745$, $P<0.001$) and MeA ($q=7.872$, $P<0.001$) of middle-aged (P360) rats as compared with adult (P90) animals (Figs 1A–1B, 2A, 2D, 3A, 3D). Similarly, a significant age-related (P360 *vs.* P720) increase in the density of IL-1β-ir neurons in the CeA ($q=11.547$, $P<0.001$) and MeA ($q=9.027$, $P<0.001$) in the non-stress groups was detected (Figs 1A–1B, 2D, 2G, 3D, 3G).

FS and HL-OF stressed rats were found to produce statistically significant age-dependent changes in the density of IL-1β/NeuN-ir cells in the CeA and MeA. In the CeA, both FS (acute and chronic) and HL-OF (acute and chronic) stressed rats showed an increase in the density of IL-1β-ir neurons, which was higher (acute FS: $q=8.304$, $P<0.001$; chronic FS: $q=9.259$,

$P<0.001$; acute HL-OF: $q=9.003$, $P<0.001$; chronic HL-OF: $q=13.252$, $P<0.001$) in middle-aged (P360) stressed rats than in adult (P90) tested animals (Figs 1A, 2B, 2E, 2C, 2F). In the MeA, a higher value in the density of IL-1β-ir neurons was noted in acute ($q=6.588$, $P<0.01$) and chronic FS ($q=8.023$, $P<0.001$), as well as in acute ($q=9.618$, $P<0.001$) and chronic HL-OF ($q=17.410$, $P<0.001$) P360 stressed rats than in the P90 stressed animals (Figs 1B, 3B, 3E, 3C, 3F).

The density of IL-1β/NeuN-ir cells showed statistically significant changes in relation to age when comparing P360 with P720 FS or HL-OF stressed rats. The value was significantly higher in the CeA of both FS (acute: $q=8.852$, $P<0.001$; chronic: $q=7.985$, $P<0.001$) and HL-OF (acute: $q=6.135$, $P<0.01$; chronic: $q=9.183$, $P<0.001$) aged (P720) stressed rats than in the middle-aged (P360) stressed animals (Figs 1A, 2E, 2H, 2F, 2I). The density was also higher in the MeA of FS (acute: $q=11.590$, $P<0.001$; chronic: $q=12.261$, $P<0.001$) and HL-OF (acute: $q=5.226$, $P<0.05$; chronic: $q=5.189$, $P<0.05$) P720 stressed rats in comparison with P360 stressed animals (Figs 1B, 3E, 3H, 3F, 3I).

DISCUSSION

Age-dependent increase in basal density of IL-1β/NeuN-ir cells in the CeA and MeA

In the control groups, quantification by immunohistochemistry analysis showed that IL-1β within amygdaloid CeA and MeA neurons occurred in adult, middle-aged and aged rats, but the density of IL-1β/NeuN-ir was significantly different. A striking, age-dependent increase in the basal density of IL-1β-ir neurons in the adult *vs.* the middle-aged and middle-aged *vs.* aged rats was observed. Furthermore, in contrast to P360 and P720 rats, there were only a few IL-1β/NeuN-ir cells in P90 animals. However, contrary to our findings, Sugama and others (2011) determined that in the absence of stress, IL-1β-ir cells did not colocalize with NeuN-positive cells in the amygdala of adult rats.

The neuronal localization of IL-1β in the structures of limbic system (hypothalamic paraventricular nucleus – PVN, hippocampal subfields, somatosensory cortex) had been detected under physiological conditions in adult non-stressed animals before (Watt and Hobs 2000, Kwon et al. 2008, Hallett et al. 2010, Badowska-Szalewska et al. 2014). Moreover, our pre-
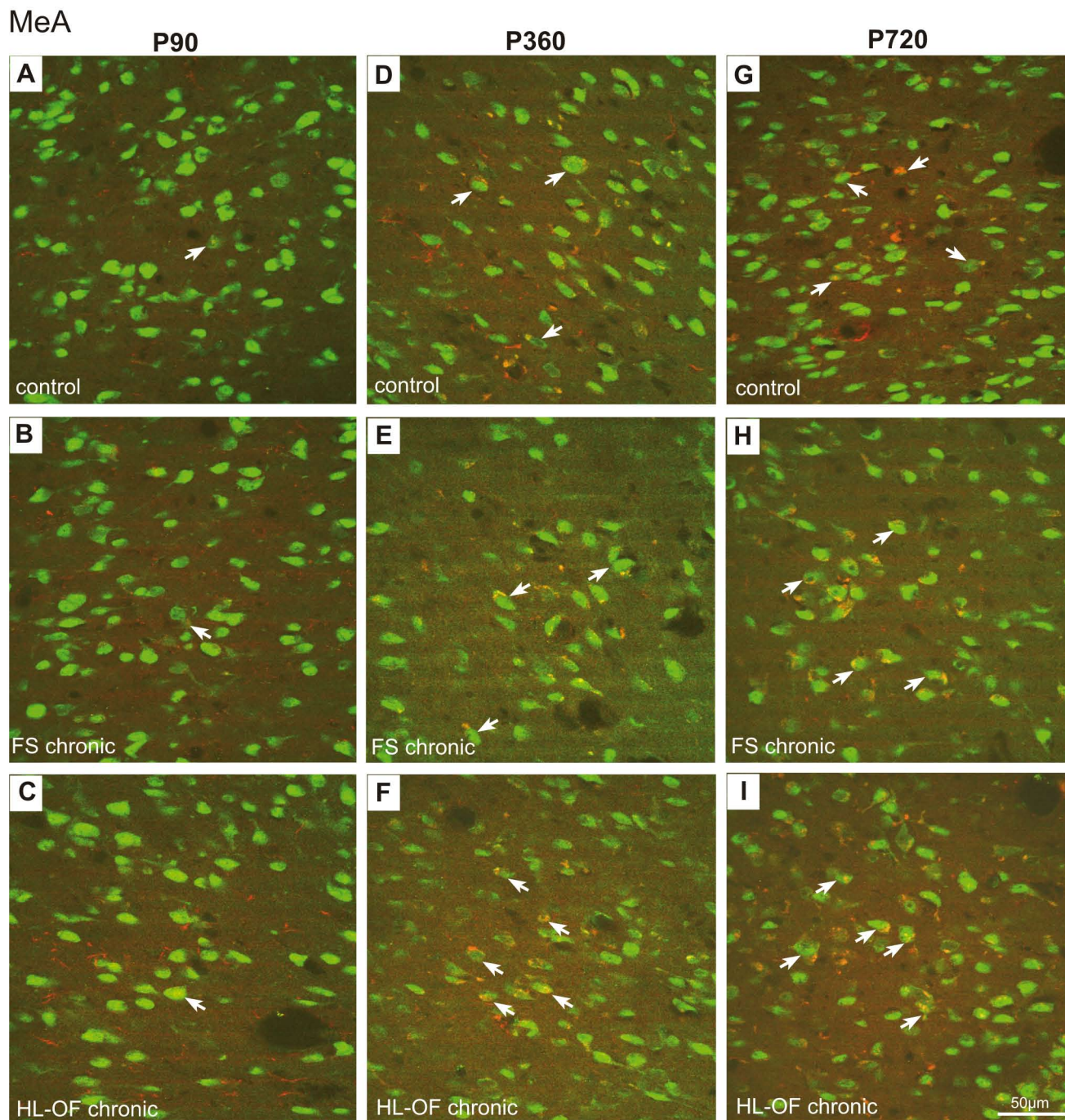
Fig. 3. Distribution of IL-1β/NeuN-ir cells in the MeA nucleus of amygdala. IL-1β-ir cells (red), NeuN-ir cells (green), and double IL-1β/NeuN-immunostaining cells (yellow-orange; arrow) in the medial nucleus of amygdala (MeA) in adult (P90) (A–C), middle-aged (P360) (D–F), and aged (P720) (G–I) rats from the control groups (A, D, G) and from the groups exposed to chronic forced swim (FS) (B, E, H) or chronic high-light open-field (HL-OF) (C, F, I) stress. The figure shows an age-dependent (P90 *vs.* P360 *vs.* P720) increase in the density of IL-1β/NeuN-ir cells in the controls and FS or HL-OF stressed rats, and increase in the density of IL-1β-ir neurons in P360 and P720 rats after chronic HL-OF *vs.* control. Scale bar is 50 μm.

vious preliminary semiquantitative data demonstrated a basal concentration of IL-1β/NeuN-ir neurons in the amygdaloid CeA and MeA in middle-aged rats (Badowska-Szalewska et al. 2009). This may correspond to the role of IL-1β in neurons. It is believed that IL-1β in neurons can directly influence neuronal activity through the ability to modulate neuronal ion channels and thereby, for example, to regulate neuronal survival or to provide metabolic support for neurons (stimulate glucose uptake, the release of certain neurotransmitters and modulators) (see Vitkovic et al. 2000, Srinivasan et al. 2004, Viviani and Boraso 2011).

Ageing is associated with the elevated production and secretion of IL-1β in the structures of the limbic system (Krabbe et al. 2004, see Viviani and Boraso 2011) and this increase influences the neuronal function impairments (Yirmiya and Goshen 2011). High levels of IL-1β concentration in ageing has deleterious effects on neurons, causing neuronal injury by increasing calcium influx and reactive oxygen production (Murray and Lynch 1998, Kelly et al. 2001, Viviani and Boraso 2011). Age-related higher basal IL-1β-ir neurons in rats have been reported before by us and others, in the hippocampus, hypothalamus (PVN), and striatum (Campuzano et al. 2009, Badowska-Szalewska et al. 2014). However, no studies have previously investigated IL-1β-ir in amygdaloid neurons in the senescence.

A significant increase in the density of neurons containing IL-1β-ir with ageing in non-stressed animals in CeA and MeA was probably correlated with biochemical and morphological alterations in their neurons (in the structure and the permeability of plasma membranes) (Scahill et al. 2003, see Viviani and Boraso 2011). This may reflect neuronal dysfunction. Based on these findings, we can assume that a dynamic age-related increase in IL-1β-ir neurons in the CeA and MeA points rather to the advancing development of the degeneration processes of these structures than to neuroprotection (Lucas et al. 2006, Campuzano et al. 2009).

Acute and chronic FS does not influence the change in the density of IL-1β-ir neurons in CeA and MeA in P90, P360 and P720 rats

Among the negative environmental factors enhancing the vulnerability of the brain to ageing is stress

(Garrido 2011). Both psychophysical and psychological stimuli can induce IL-1β production and secretion in various brain structures, which in turn activates the HPA axis (Turnbull and Rivier 1999, see Goshen and Yirmiya 2009, Gądek-Michalska and Bugajski 2010) through stimulation of the hypothalamic corticotrophin-releasing factor (CRF) and pituitary adrenocorticotropic hormone (ACTH) secretion (Sapolsky et al. 1987). It has been observed that exposure to some types of psychophysical acute or repeated stressors (e.g. immobilisation, tail and foot shock) can affect IL-1β expression in brain structures (within the hippocampus or hypothalamus) (Nguyen et al. 2000, Kwon et al. 2008, O'Connor et al. 2003), while others cannot (e.g. restraint, forced swim) (Deak et al. 2003, 2005). However, these experiments were conducted only on adult animals.

In our research the adult (P90) as well as middle-aged (P360) and aged (P720) rats that underwent acute or chronic exposure to FS showed no significant disparities in the density of IL-1β/NeuN-ir cells in CeA and MeA in comparison to the non-stressed groups of rats. This is consistent with our previous study, in which neither acute nor chronic FS caused any changes in IL-1β/NeuN-ir cells of other limbic structures: the PVN of the hypothalamus in adult and aged rats, and in hippocampal subfield of adult, middle-aged and aged rats (apart from the increase in IL-1β-ir neurons in aged CA3 and dentate gyrus) (Badowska-Szalewska et al. 2013, 2014). In view of our current results, we believe that neither acute nor chronic FS are able to induce changes in IL-1β-ir neurons in the CeA and MeA of adult rats, nor in elderly 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 IL-1β-ir neurons in the CeA and MeA.

Swimming might be a frequent cause of stress for rats in their natural environment (Dal-Zotto et al. 2000), so swimming in the FS test might be a typical situation for them. It is possible that a natural stressor may provoke compensatory (evolutionarily developed) homeostatic mechanisms (involving glucocorticoids, CRF, antioxidant activity) (Plata-Salamán et al. 2000, Pedersen et al. 2001, Pajović et al. 2006), which consequently might prevent changes (increase) in the density of IL-1β-ir neurons in the CeA and MeA. Pajović and others (2006) noted that 3 weeks of repeated forced swimming enhanced antioxidant activity in the hippocampus. Furthermore, Dal-Zotto and others (2000)

demonstrated great differences in the sensitivity of various behavioral and physiological responses to FS in adult rats (for example, despite the effect of FS on behavioral changes, the authors noted no significant effect of single or repeated exposure to FS on the secretion of adrenocorticotropic hormone – ACTH). Secondly, it is likely that the period of single application (15 minutes) of the stressor action might have been too short to cause changes in the density of IL-1β-ir neurons. For example, at least 2 hours of exposure to acute cold or immobilization led to an increase in oxidative processes (Pajović et al. 2006). Thirdly, it is likely that changes in the IL-1β-ir neurons after acute FS may occur at a later time than assayed in our study. Because, after both acute and chronic FS any changes have been noticed in the density of IL-1β/NeuN-ir cells, we can presume that the stressor used (due to its naturalistic character) was not an aggravating factor for adult and ageing rats with regard to its influence on the density of IL-1β-ir neurons in the CeA and MeA.

Chronic but not acute HL-OF influences the density of IL-1β-ir neurons in the MeA and/or CeA during senescence

The open field is a factor in which the rat can engage itself in active exploration of an unfriendly novelty (Dubovicky and Jezova 2004). Additionally, high-intensity light, which is used in the test as an aversive stimulus, provokes emotional responses and anxiety-like behavior (Prut and Belzung 2003, Hale et al. 2006).

In our study, we noted the lack of changes in the density of IL-1β/NeuN-ir cells after exposure to acute HL-OF in both the CeA and MeA in all age groups tested. In contrast, chronic HL-OF caused a statistically significant increase in the density of IL-1β-ir neurons in the CeA and MeA of P360 rats and in the MeA of P720 animals in relation to the controls. However, an increase in IL-1β/NeuN-ir cells in comparison to acute HL-OF was observed in both nuclei in the senescence.

According to Plata-Salamán and others (2000), IL-1β levels do not change in adult rats after acute and chronic naturalistic stressor exposure (predator) in the amygdala and another brain regions. In addition, no changes in IL-1β levels were observed in rats that were exposed to acute visual, olfactory, and auditory stressors in combination with transport/novelty situation

(see Deak et al. 2003). On the other hand, Goshen and Yirmiya (2009) quote examples in which milder stressors (cold stress, social isolation) in other limbic structures (hippocampus or hypothalamus) can activate the IL-1β, but only when they act chronically. Previously, it was also shown that chronic (but not acute) HL-OF induced a density increase of IL-1β-ir neurons in the PVN of P90 rats and in the hippocampus of P360 and P720 rats (Badowska-Szalewska et al. 2013, 2014). We therefore believe that with respect to the density of IL-1β-ir neurons in the CeA and MeA, the acute HL-OF did not affect the changes in the number of neurons containing IL-1β-ir.

The reason why acute HL-OF did not affect IL-1β-ir neurons in the CeA and MeA is that (as with FS) the period of single application of the stressor or time interval between the end of single application of HL-OF and killing, might have been too short to cause stress-induced immunohistochemical alterations in the density of IL-1β-ir neurons in the CeA and MeA. Nguyen and others (1998) reported that acute inescapable tail shock had no effect on IL-1β protein levels in some brain regions when measured immediately, or 2 hours or later after exposure to the stressor. But the authors observed an increase in IL-1β protein 2 hours after administering the stressor in the hypothalamus and hippocampus of adrenalectomized rats. Consequently, we can also take into account the possibility that glucocorticoids secreted during acute HL-OF may have a tonic inhibitory effect on a potential increase in the density of IL-1β-ir neurons.

The increase in the density of IL-1β-ir neurons after chronic HL-OF in CeA and MeA indicates that in senile rats, long-term aversive stimulus, such as a high intensity of light in the open field, is a factor causing an upregulation of IL-1β-ir neurons in the amygdaloid CeA and MeA. It is believed that secretion of glucocorticoids and neurotransmitters, released during long-term unpredictable stress, can increase IL-1β production (Elenkov and Chrousos 2002, Sorrells and Sapolski 2007, see García-Bueno et al. 2008). CeA and MeA are able to process signals related to the stress levels of glucocorticoids because they express glucocorticoid receptors (Herman et al. 2005). Prolonged secretion of glucocorticoids as a result of chronic HL-OF stress might therefore have caused the increase in the density of neurons containing IL-1β in MeA and CeA in the senescence rats. Because a similar scheme of changes in IL-1β-ir neurons in response to chronic HL-OF was

detected in the hippocampus of 1-year-old (Badowska-Szalewska et al. 2013) and 2-year-old rats (Badowska-Szalewska et al. 2014), it indicates that during the ageing process chronic HL-OF is a factor causing an upregulation of IL-1β/NeuN-ir cells in the structures of the limbic system involved in the mechanisms of emotional response to stress.

The ageing process is associated with a lower capability of coping with stress (Pedersen et al. 2001, Pardon 2007). Ageing causes disruption to all the mechanisms that participate in the HPA axis stress response (Pedersen et al. 2001, Meyza et al. 2007). This is probably related to insufficient control of HPA axis activation by the central stress system, including the amygdala, whose reactivity in senility changes fundamentally (Pedersen et al. 2001). Taking the above into consideration, we presume that an increase in the density of IL-1β-ir neurons in the CeA and MeA of P360 and P720 groups of rats exposed to chronic HL-OF might have been caused by a disruption of HPA axis regulation due to the ageing process. Moreover, our results may suggest that chronic HL-OF could lead to an amplification of this increase in the density of IL-1β-ir CeA and MeA neurons caused by ageing, which in turn may influence the change in the HPA axis functioning, probably through dysregulation excitatory control exerted by the amygdala (Vyas et al. 2002).

Ageing is characterized by a decreased ability to maintain homeostasis and, consequently, less efficient adaptation to change (de Kloet et al. 2005). Therefore, we can assume that from the point of view of the influence on IL-1β-ir CeA and MeA neurons, both P360 and P720 rats seem to be unable to adapt to the continued presence of a HL-OF stressor. The increase in the density of neurons containing IL-1β, resulting from chronic HL-OF, may contribute to amplification of the neurodegenerative changes in the CeA and MeA caused by ageing.

CONCLUSIONS

An age-dependent (P90 *vs.* P360 and P360 *vs.* P720) increase in the basal density of IL-1β/NeuN-ir cells detected in the CeA and MeA may indicate the involvement of IL-1β neurons in the development of ageing processes in the structures.

Our results have shown that neither acute nor chronic FS are an aggravating factor in the impact on the density of IL-1β/NeuN-ir cells in the CeA and MeA in adult rats, as well as during the ageing process in P360 and P720 rats. Presumably, this occurs because this natural stimulation can provoke homeostatic mechanisms counteracting the changes in the density of IL-1β-ir CeA and MeA neurons.

We have demonstrated that chronic, but not acute HL-OF, was the decisive factor inducing changes in the density of MeA and/or CeA IL-1β/NeuN-ir cells of ageing rats. The increase in the density of IL-1β-ir neurons in the MeA and/or CeA after chronic HL-OF in P360 and P720 rats is presumably caused by the insufficient control of HPA axis associated with involutional ageing processes and seems to be the common denominator of the ageing process and chronic stress.

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