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Research report

The effect of age on the dynamics and the level of c-Fos activation in response to acute restraint in Lewis rats

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Abstract

Recent studies have reported an age-related increase of anxiety in rodents with a concomitant decrease in neuronal activity in some of the key structures of the fear/anxiety circuit. In the present study we present evidence that distinct parts of this circuit are differentially affected by age in Lewis rats. The effect of ageing is observed both at the actual level of neuronal activation and its time-course. While the structures belonging to the HPA axis react with a bigger neuronal activation and almost no change in the shape of dynamics curve in response to restraint, the structures involved in higher processing of emotional cues (amygdala and hippocampus) become deficiently activated with age despite their generally higher basal level of activation.

Keywords: Stress; Ageing; HPA axis; Amygdala; Hippocampus; c-Fos protein; Lewis rats

1. Introduction

Since the discovery of c-Fos as a functional marker of neuronal activation, expression of this protein has been used in many studies to identify cells and specific brain circuits that are responsive to various stimuli [23,31,35]. With this method it has been demonstrated that an increase in anxiety is associated with increased c-Fos expression in specific brain areas implicated in the regulation of emotional behavior including the amygdala, hypothalamus and hippocampus [8,9,22,29,31,35].

The anxiety level is known to be affected by age in humans as well as in animals. In recent years several studies have reported increased anxiety with age in rodents using a variety of well-validated tests of anxiety [4,6,17,24–28]. It could therefore be hypothesized that the increased anxiety in older rats should be associated with elevated neuronal activation in key parts of the fear/anxiety circuit. To the best of our knowledge, only a very few papers have directly addressed this question [6,34]. Salchner et al. [34] found that in spite of an age-related reduction of social interaction, indicative of increased anxiety [16], old rats exhibited a lower test-induced c-Fos response than young rats in the amygdala, periaqueductal grey and paraventricular hypothalamic nucleus. Based on this finding, the authors suggested that reduced social interaction does not reflect enhanced anxiety in old rats. Similar results were described by Boguszewski and Zagrodzka [5]. In this preliminary study c-Fos expression in the amygdala was significantly lower in old rats compared with young rats after the open field test and immobilisation, despite demonstrating an increased level of anxiety as measured in a set of standard laboratory tests and evaluated by principal component analysis [6]. One of the possible explanations of these somewhat unexpected results points to age-related differences in dynamics of c-Fos production. Most comparative studies of gene expression during aging have been conducted at a single time point usually matched to the maximum mRNA or protein concentration in young animals. Studies of the dynamic curve of *c-fos* mRNA content as a function of time after pentylenetetrazole (PTZ)-induced seizures have shown, that maximum *c-fos* concentration in the hippocampus and cortex of 3-month-old rats occurred 1 h post injection and 3 h later returned to basal levels. In contrast, in old rats (20 and 30 months old) there was a gradual increase in the levels of *c-fos* mRNA content, which reached a maximum 3 h after PTZ administration and returned to basal levels by approximately 15 h [33,36,42].

The aim of the present experiment is to compare the time-course of c-Fos protein expression in response to acute restraint in young and old rats. Acute restraint is an extremely stressful

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stimulus known to evoke robust and widespread *c-fos* expression in numerous stress-specific and anxiety related structures [9].

2. Material and methods

Twelve young (YA, 3 months old) and 12 old (OA, 21–23 months old) male Lewis rats (from a breeding colony established at the Medical Academy, Warsaw, Poland) were housed in groups of two to three rats per cage (control animals housed separately from the experimental ones), with unlimited access to water and standard laboratory rat chow, in L:D 12:12 conditions, with lights on at 7:00 a.m. At the age of 3 and 21–23 months, respectively, the rats were subjected to acute restraint, for a duration of 15 min, in a clear Plexiglass ventilated tube, 20 cm long, 6.5 cm inner diameter, with adjustable length according to the size of the animal and tails protruding. The size of the tube restricted movements in all directions but did not interfere with respiration. After the acute restraint procedure, rats were returned to their home cages. The rats (three animals per time point) were sacrificed with an overdose of chloride hydrate anaesthesia (>360 mg/kg) at 90 min, 150 min or 240 min after the beginning of the test and perfused transcardially with ice-cold phosphate buffered saline (PBS, pH 7.4 Sigma) followed by 4% paraformaldehyde (POCh) solution. Control animals (three animals per age group) were sacrificed directly from their home cages. Their brains were dissected and postfixed in 4% paraformaldehyde solution overnight and thereafter in 20% and 30% sucrose (Sigma) solutions. The brains were deep frozen and stored at −72 °C until the day of sectioning in the cryostat (−21 °C). Forty-micrometer thick coronal sections were taken and subjected to standard c-Fos immunocytochemistry according to the procedure described in detail before [35].

c-Fos stained brain slices were microphotographed and bilaterally assessed for c-Fos activation using ImageJ software (WCIF, Toronto, Canada) in the amygdaloid complex, including basolateral (BLA), central (CeA), medial (MeA) and cortical (CoA) nuclei, the hippocampus (CA1, CA2 and CA3 fields) and in the hypothalamus including the paraventricular (PVN), dorsomedial (DMH) and arcuate (Arc) nuclei. Each structure was assesed on the basis of measures from three neighboring brain slices. For each brain structure, the number of c-Fos immunopositive nuclei was counted and divided by the area occupied by this structure (in arbitrary units). The subnuclear division was obtained by comparison with the adjacent, Nissl-stained sections. Slices from the brains of three OA animals had to be excluded from the study post mortem due to the occurrence of tumors in the hypothalamus (one animal from the control group and one from 150 min) and in the olfactory bulbs (one animal from 240 min group).

The study was conducted in accordance with the Polish Law on Animal Protection and the guidelines established by the Declaration of Helsinki concerning the care and use of animal in research.

Statistical analysis was performed using MANOVA and subsequent *post-hoc* Fischer/NIR tests. Values were considered significant if $p < 0.05$.

3. Results

In this study, a structure specific difference in the dynamics of neuronal activation induced by acute restraint stress in young adult (YA) as compared with old (OA) rats was observed. The differences in relative activation (based on comparison of exact counts of c-Fos positive nuclei at a given time point with baseline activation) were observed between the two age groups at all experimental time points (90, 150 and 240 min). The effect of age was significant ($F(1, 32) = 382.35$, $p < 0.001$). So was the effect of time points ($F(3, 32) = 14.21$, $p < 0.001$). The interaction of age and time points occurred to be structure specific ($F(27, 288) = 5.57$, $p < 0.001$).

The *post-hoc* analysis revealed that the time-course of neuronal activation in response to acute restraint in structures of the hypothalamic-pituitary-adrenocortical (HPA) axis (the paraventricular, arcuate and dorsomedial nuclei of the hypothalamus)

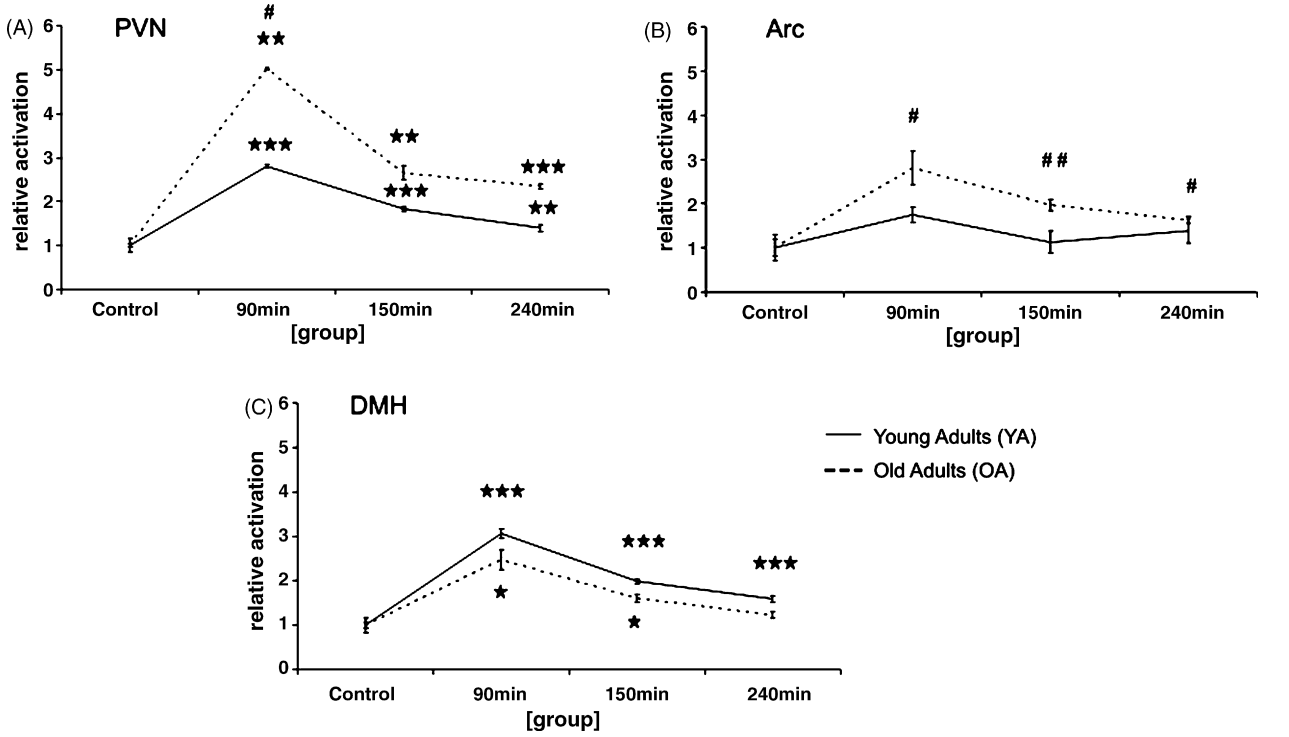

Fig. 1. The dynamics of neuronal activation assessed by c-Fos expression in (A) paraventricular (PVN), (B) arcuate (Arc), and (C) dorsomedial nuclei of the hypothalamus (DMH) at 90 min, 150 min and 240 min after the onset of acute restraint. Continuous line represents YA. Broken line represents OA. Values are presented as mean ± S.E.M. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ for comparison between YA and OA; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ for comparison with baseline within one age group.