



Research report

Age increases anxiety and reactivity of the fear/anxiety circuit in Lewis rats

Ksenia Z. Meyza, Pawel M. Boguszewski, Evgeni Nikolaev, Jolanta Zagrodzka*

Nencki Institute of Experimental Biology, Polish Academy of Sciences, 3 Pasteur St. 02-093 Warsaw, Poland

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ABSTRACT

A growing body of data indicates that changes in emotional behavior occur with age. Young Lewis rats are known to display hypofunction of the HPA axis. With age the reactivity of this axis is thought to increase with a concomitant rise in anxiety. In the current study, we investigate how and if the pattern of neuronal activation (measured as c-Fos protein expression) in Lewis rat brains changes with age and in response to novel environments differing in aversiveness. We found that distinct parts of the fear/anxiety circuit (i.e., the amygdalar complex, hippocampus and hypothalamus) undergo diverse age-related changes in response to behavioral challenges. While in the hypothalamus an increase in responsivity to mild stressors was observed with age, no such effect was present in the hippocampus. The amygdalar complex (especially the medial and cortical nuclei) on the other hand exhibited an age-dependent decrease in neuronal activation to mild stressors. This was accompanied by a marked increase in anxiety not correlated with a decline in locomotor activity.

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1. Introduction

It is well documented that ageing is associated not only with a decline in cognitive functions but also with emotional changes. The character of these changes is however ambiguous. Many gerontological surveys indicate an increase in anxiety and depression in the elderly [1,2], yet the impact of risk factors like loneliness or disability as compared to ageing itself remain unknown. On the other hand, psychological research [3–5] suggests that emotion regulation and mood management improve with age and older adults experience less negative affect. Moreover, several recent fMRI studies have shown an ageing-dependent loss of amygdala reactivity and amplified activity in the prefrontal cortex, in response to negative stimuli [6–10]. It should be pointed out however, that the studies mentioned above were all performed with the use of rather low-stressful stimuli (emotion-laden pictures from International Affective Pictures System (IAPS) [11] and words from Affective Norms for English Words (ANEW) database [12] as well as questionnaires with emotionally charged hypothetical problems). The above results are not in line with commonly recognized increases in anxiety among the elderly and with data obtained in animal studies of ageing. Most authors that have addressed this question in rodents have reported an age-related increase in anxiety/hyperemotionality upon exposure to mild stress (e.g., a novel situation) [13–20].

Little is known about the neurobiological mechanisms that underlie the different emotional responses of young and old animals subjected to stressogenic stimuli. Yet there is extensive evidence indicating that various kinds of stressors induce neuronal activation (with an immediate early gene *c-fos* mRNA and protein expression employed as a functional marker) in the brain structures involved in the regulation of emotions [21–23]. The most important regions among these structures include the amygdalar complex [24] and the hippocampus [25]. In Lewis rats, used in the current study, behavioral arousal may also be dependent on the activity of their hypothalamo–pituitary–adrenal (HPA) axis [26]. Thus the activity of the hypothalamic regions may also be crucial for the control of the emotional behavior of these rats.

It might be supposed that the increased emotional reactivity of old rats is linked to the higher activation of these key structures in the fear/anxiety circuit. Those few studies that have dealt with the issue report a far more complicated pattern of age-related neuronal changes. While some researchers have observed a decline in the neuronal reactivity of aged rats [27,28], others have shown that distinct parts of the fear/anxiety circuit may be affected diversely by age [29].

The aim of the present experiment is to investigate the effect of age on the behavioral and neuronal response to a wide spectrum of stressful conditions. These range from low aversive spontaneous exploration of the novel environment of the Hole Board arena, through mildly stressful Open Field with Illuminated Center and Elevated Plus Maze tests, to highly stressful Acute Restraint procedure. In a previous study on psychogenetically selected Roman High Avoidance (RHA/Verh) and Roman Low Avoidance (RLA/Verh) rats, we found this set of tests to be a useful and effective measure

* Corresponding author. Tel.: +48 225892246; fax: +48 228225342.

E-mail address: j.zagrodzka@nencki.gov.pl (J. Zagrodzka).

of emotional reactivity/anxiety level differences in behavioral and molecular responses [30]. The main advantage of this methodology is that it permits the investigation of (possible) functional heterogeneity within the structures belonging to the fear/anxiety circuit, as a function of a full range of aversiveness of novel behavioral challenge. It also allows for a complex profiling of the emotional reactivity of the animals used in the study. We applied Principal Component Analysis (PCA) on the set of behavioral measures from novel environment tests (HB, OF and EPM) to trace the possible differences in motivation factors driving the behavior of young and old Lewis rats. PCA is considered a particularly beneficial statistical tool for the interpretation of behavioral data since it allows for extraction of presumably independent factors reflecting different drives constituting behavior [31–34]. In our experiment, factor analysis was applied for three main reasons: to identify the relationship between specific test indices and factors such as motor activity, anxiety and exploratory drive, to take account of the individual differences between subjects – it is well known that marked variation in behavioral impairments is seen between individuals of the same aged rat population [35,36], and finally, to assess the applicability of behavioral tests and settings used to the investigation of both young and aged animals behavior.

2. Materials and methods

2.1. Animals

A total of 50 males, inbred Lewis rats from the breeding colony at the Medical University of Warsaw were randomly assigned into two, equally numbered groups. The first group was behaviorally tested at the age of approx. 3 months (Young Adults, YA, $n=25$, 312 ± 7 g). The second group was kept in the animal house of the Nencki Institute of Experimental Biology, PAS until the age of 20–22 months and then behaviorally tested (Old Adults, OA, $n=23$, as two animals had to be excluded from the study due to movement impairment, 523 ± 13 g). Both groups were handled and habituated (daily, Monday through Friday) to the experimental room 3 weeks prior to testing. Additionally, the OA group was handled regularly throughout their lives (twice a week). The animals were housed in groups of 2–3 littermates per cage, with unlimited access to water and standard laboratory rat chow (Labofeed B Standard, Morawski, Kcynia, Poland), in light:dark 12:12 conditions, with lights on at 8:00AM.

2.2. Behavioral testing

At the age of either 3 or 20–22 months the rats were randomly assigned to 5 groups. The control group (YA, $n=5$ and OA, $n=4$) was killed directly from their home cages on day 1, before the onset of any other behavioral testing. Rats from the first three experimental groups (each consisting of $n=5$, named after the last test in the series of three: OF, EPM and HB) were tested in three consecutive tests: Open Field with Illuminated Center (OF), Elevated Plus Maze (EPM) and Hole Board test (HB). The tests had a different order in each group (for experimental design see Fig. 1). To minimize the effect of previous experiences on the behavior in consecutive tests, seven day intervals were applied between them (between the tests 4 habituation sessions took place).

At the end of the behavioral testing animals from the fifth group (YA, $n=5$ and OA, $n=4$), handled in the same way as the other rats, were subjected to the Acute Restraint procedure. The behavioral data from the first 3 experimental groups was

collected (in MPEG-2 format) and analyzed using a video-based, automated Ethovision System (Noldus, Wageningen, NL) and in case of nose-poke activity in the HB test (behavior difficult to assess by means of an automated system) with an observer-based program (BehaView, <http://www.pmbogusz.net/>).

2.2.1. Open Field with Illuminated Center (OF)

The test arena was a black painted square (90 cm × 90 cm), enclosed by walls (30 cm height) with a 50 W halogen bulb suspended 30 cm above the center (900 lx at the bottom of the cage, directly under the bulb) as the only source of light. The animal was placed in the border zone facing one of the corners and its movements were recorded for 10 min. Two zones, the illuminated center zone (a circle directly corresponding to the brightly lit part of the arena) and the border zone, were drawn according to previously selected criteria [14]. The following parameters were taken into analysis – in the illuminated center: number of entries to zone, time spent, distance moved and movement duration; in the border zone: distance moved and movement duration; in the whole arena: total distance moved, total movement duration and mean velocity.

2.2.2. Elevated Plus Maze (EPM)

A black wooden apparatus [37] consisting of two enclosed arms (10 cm × 50 cm × 30 cm) and two open arms (10 cm × 50 cm) connected with a central platform (10 cm × 10 cm) located 70 cm above the floor was used. The rats were introduced to the closed arm of the maze via a lifted door and their exploration was recorded for 5 min. The testing was done in a dimly lit (30 lx at the maze level) area surrounded with non-transparent, grey curtains in order to limit any additional spatial stimuli. The following parameters were calculated – in closed arms: number of entries, total time spent, distance moved and movement duration; in open arms: number of entries, total time spent, distance moved and movement duration; in the central platform: time spent; in the whole arena: total distance moved, total movement duration, total number of entries to open and closed arm and ratio of entrances to open/closed arms.

2.2.3. Hole Board (HB)

The HB test was performed in a 60 cm × 60 cm × 30 cm box with grey walls and four equidistant, 1 cm deep holes (3 cm in diameter) in the central part of a black-painted floor as previously described [30]. The testing took place in a room lit by two 80 W light bulbs (70 lx). At the beginning of the test the rat was placed in one of the corners of the arena and allowed to explore freely for 10 min. The following parameters were analyzed – in central part of the apparatus: number of entrances, time spent, distance moved and movement duration; in the border zone: distance moved and movement duration; in the whole arena: total distance moved, movement duration, mean velocity and number of nose pokes into holes in the arena floor.

2.2.4. Acute Restraint (immobilization, IM)

The Acute Restraint test was performed using a clear Plexiglas ventilated tube, 20 cm long, 6.5 cm inner diameter, with adjustable length according to the size of the animal and tail protruding. The size of the tube restricted movement in all directions but did not interfere with respiration [29,30]. The animals were kept in the apparatus for 15 min. No behavioral parameters were recorded.

2.3. Immunocytochemistry

Rats from all experimental groups were killed 90 min after the beginning of the final test (OF, EPM, HB or IM) with an overdose of chloride hydrate anesthesia (>360 mg/kg) and perfused transcardially with ice-cold phosphate buffered saline (PBS, pH=7.4 Sigma) followed by 4% paraformaldehyde (Sigma). The control group was killed directly from their home cages. The brain of each animal was removed from the skull and postfixed as previously described [29,30]. The brains were deep

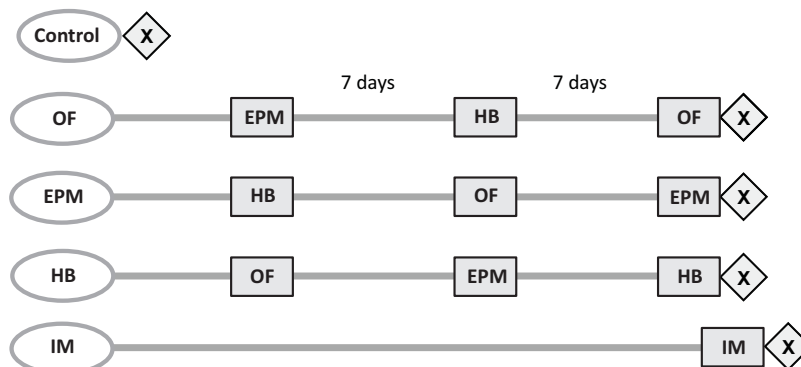


Fig. 1. Experimental design: white ellipses indicate group labeling, grey boxes show behavioral tests. X the moment of lethal anesthesia and perfusion.

Table 1
Behavioral parameters and their orthogonal loadings for the Open Field with Illuminated Center test.

Behavioral parameters	Young adults		Old adults	
	Factor 1 (68%) Locomotor activity	Factor 2 (21%) Anxiety	Factor 1 (82%) Locomotor activity	Factor 2 (15%) Anxiety
Number of entries to illuminated center		0.83		0.92
Time spent in the illuminated center [s]		0.96		0.93
Distance moved in the illuminated center [cm]		0.92		0.93
Movement duration in the illuminated center [s]		0.98		0.93
Distance moved in the border zone [cm]	0.97		0.94	
Movement duration in the border zone [s]	0.80		0.96	
Total distance moved [cm]	0.91		0.83	0.54
Total movement duration [s]	0.69		0.90	
Mean velocity [cm/s]	0.91		0.83	0.55

frozen and stored at -72°C until the day of sectioning into $40\ \mu\text{m}$ thick coronal sections. Slices were subjected to free-floating c-Fos immunocytochemistry according to Savonenko [38].

c-Fos stained brain slices were microphotographed and assessed for c-Fos protein expression using ImageJ software (WCIF, Toronto, Canada) in the amygdalar complex, including the basolateral complex (BLA), central (CeA), medial (MeA) and cortical (CoA) nuclei, the dorsal hippocampus (CA 1, 2 and 3 fields) and in hypothalamic paraventricular nucleus (PVN), dorsomedial nucleus (DMH) and arcuate nucleus (Arc). Each structure was assessed on the basis of bilateral measures from 3 brain slices (6 measurements per rat per structure). For each, the number of c-Fos immunopositive nuclei was counted and divided by the area occupied by this structure on the particular slice (data shown in arbitrary units). Then, the 6 measurements were averaged and a single value per rat was used for statistical comparisons. The threshold for immunostained nuclei recognition was set for each structure (the amygdalar complex, hippocampus and hypothalamus) separately and was verified on a randomly picked square for the number of recognized nuclei (compared with manual counting). The area and exact shape of investigated structures were evaluated using the adjacent, Nissl-stained sections.

Three rats from the YA (two in control and one in the IM group) and two rats from the OA group (one from control and HB groups) had to be excluded from the study due to a post mortem finding of tumors.

2.4. Statistical analysis

2.4.1. Behavioral data

The behavioral parameters from the OF, EPM and HB tests, after testing the hypothesis of normal distribution and homogeneity of variance (homoscedasticity) by means of a Shapiro–Wilk W test and Lilliefors test, were analyzed separately for YA and OA rats by means of Principal Component Analysis (PCA, STATISTICA, 5th edition) with a normalized varimax orthogonal rotation of the factor matrix. This rotation was chosen because it emphasizes the variance on the new axes, which was necessary to extract the uncorrelated factors reflecting different drives constituting behavior of a given group of rats. The number of variables/parameters from a given test never exceeded the number of cases ($n=9$ for OF, $n=13$ for EPM and $n=10$ for HB, as compared with $n=15$ animals in each age group). This should produce a stable factor structure [14,32]. The labeling of the extracted factors for HB test was confirmed by performing correlation analysis with factorial values for factors extracted for OF test.

The number of extracted factors was assessed using the Kaiser criterion (eigen value >1). The loadings exceeding the value of 0.5 (and -0.5 respectively) were

Table 2
Behavioral parameters and their orthogonal loadings for the Elevated Plus Maze test.

Behavioral parameter	Young adults			Old adults		
	Factor 1 (51%) Anxiety	Factor 2 (30%) Locomotor activity	Factor 3 (9%) Risk assessment	Factor 1 (74%) Anxiety	Factor 2 (13%) Locomotor activity	Factor 3 (9%) Risk assessment
Number of entries to closed arms		0.77			0.61	0.62
Total time spent in closed arms [s]	-0.74		-0.64	-0.58		-0.78
Distance moved in closed arms [cm]		0.86			0.91	
Movement duration in closed arms [s]	-0.59	0.62			0.98	
Number of entries to open arms	0.94			0.83		
Total time spent in open arms [s]	0.96			0.92		
Distance moved in open arms [cm]	0.95			0.94		
Movement duration in open arms [s]	0.94			0.93		
Total distance moved [cm]		0.92		0.69	0.58	
Total movement duration [s]		0.91		0.56	0.67	
Total number of entries to zones	0.63	0.64		0.63	0.50	0.57
Ratio open/total entries	0.92			0.76		
Time spent in central platform [s]			0.93			0.97

shown in Tables 1–3. The parameters, which were included in the PCA and had high loadings to extracted factors, were subjected to statistical analysis of between-age group differences by means of a Student's *t*-test. For confirmation of the lack of the effect of order of testing on these parameters, two-way (age \times group) ANOVA with post-hoc Bonferroni test was used. Differences were considered significant if $p < 0.05$.

2.4.2. c-Fos protein expression

The c-Fos protein expression data was tested for normal distribution and the homogeneity of variance by means of Shapiro–Wilk W and Lilliefors tests. Statistical analysis was performed for each of the investigated brain structures separately using UNIANOVA with Bonferroni post hoc test for assessment of the effect of age and different behavioral tests. Differences were considered significant if $p < 0.05$.

The study was conducted in accordance with the Polish Law on Animal Experimentation and the EU Directive 86/609/EEC and was approved by the Local Ethical Committee.

3. Results

3.1. Behavior

3.1.1. Open Field with Illuminated Center (OF)

Young Lewis rats, as compared to old individuals, explored the testing arena more extensively in all its zones, including the brightly illuminated center. The duration of illuminated center exploration was significantly higher for YA rats than for OA rats ($F(1,28)=4.34$, $p < 0.05$, Fig. 2A). The distance moved and movement duration in the border zone, as well as in the total arena, were also higher for YA individuals ($F(1,28)=52.56$, $F(1,28)=27.01$, $F(1,28)=34.92$, $F(1,28)=22.76$, respectively, all $p < 0.001$). The mean velocity of movement in the arena also declined significantly with age ($F(1,28)=34.42$, $p < 0.001$).

Principal Component Analysis yielded two distinct factors driving the behavior of YA and OA (Table 1). The parameters describing the activity of the animal in the border zone and the whole arena (distance moved, movement duration and velocity) formed the first

Table 3
Behavioral parameters and their orthogonal loadings for the Hole Board test.

Behavioral parameters	Young adults		Old adults	
	Factor 1 (44%) Exploration	Factor 2 (40%) Locomotor activity	Factor 1 (50%) Exploration	Factor 2 (39%) Locomotor activity
Number of entries to the central part	0.62		0.93	
Time spent in central part [s]	0.95		0.96	
Distance moved in the central part [cm]	0.93		0.96	
Movement duration in the central part [s]	0.95		0.99	
Distance moved in the border zone [cm]	−0.74	0.64		0.86
Movement duration in the border zone [s]	−0.81	0.54	−0.52	0.78
Total distance moved [cm]		0.96		0.96
Total movement duration [s]		0.93		0.90
Mean velocity [cm/s]		0.96		0.96
Number of nose pokes		0.60	0.78	

factor, labeled the “Locomotor activity” in both YA and OA (explaining respectively 68% and 82% of variance in behavioral parameters). The second factor received the highest loadings from those parameters that described activity in the illuminated center (number of entries, time spent, distance moved and movement duration) and was therefore labeled “Anxiety”. The percent of variance explained by this factor was higher for YA than OA (21% vs. 15%) yet the

assignment of parameters to the factor suggests a different factorial structure in OA, given that (unlike in YA) both total distance moved and velocity parameters load to it.

3.1.2. Elevated Plus Maze (EPM)

Given the choice of either exploring the open arms or remaining in the enclosed arm where they were initially put, the YA explored the open arms of the maze more frequently and for longer periods of time than OA ($F(1,28)=10.70$, $p<0.01$, Fig. 2B and $F(1,28)=5.78$, $p<0.05$ respectively). The ratio of entrances to open/closed and open arms and number of entrances to all arms was also higher in YA ($F(1,28)=10.09$, $p<0.01$), as was the distance moved within open arms and in the whole maze ($F(1,28)=4.74$, $p<0.01$) – despite no differences in movement duration in the closed arms section ($F(1,28)=2.09$, $p>0.05$).

The factor analysis reveals that the behavior in the EPM is differentially driven by three distinct factors and dependent on age (Table 2). The first factor, interpreted as showing anxiety level (with positive loadings from parameters describing the behavior of animals in the open arms of the maze and negative loadings from time spent and, in case of YA, duration of movement in the closed arms), in YA explained 51% and in OA as much as 74% of total variability in the test. The second factor, related to locomotor activity, in YA explained as much as 30% of variability, while in OA only 13%. Moreover in OA, some characteristic parameters (total distance moved and total movement duration) for Locomotor activity in YA also had positive loadings on the Anxiety factor. The third extracted factor, mainly correlated with time spent in the central platform (thus reflecting risk assessment behavior), explained a similar degree of variance in YA and OA rats (9% in both age groups). In OA rats it received positive loadings from typically locomotor parameters such as the number of entries to closed arms and total number of entries to all zones. In both cases the third factor showed a similar loading as those for the Locomotor activity factor. No such effect was observed for YA.

3.1.3. Hole Board (HB)

In the hole board arena YA showed more intense exploratory behavior, as measured by the number of nose pokes into the holes ($F(1,28)=49.12$, $p<0.001$, Fig. 2C), the number of entrances to the central part of the arena ($F(1,28)=22.98$, $p<0.001$), as well as in the distance moved within its borders ($F(1,28)=29.94$, $p<0.001$). Throughout this time YAs also remained more active in the border zone ($F(1,28)=27.39$, $p<0.001$).

The factor analysis revealed two uncorrelated factors driving the behavior of YA and OA in the HB test. The proportion of variation explained by the two factors, as well as assignment of particular parameters, was nevertheless different for YA and OA (Table 3). The first factor, receiving loadings from parameters describing the activity of the animals in the central part of the arena (interpreted

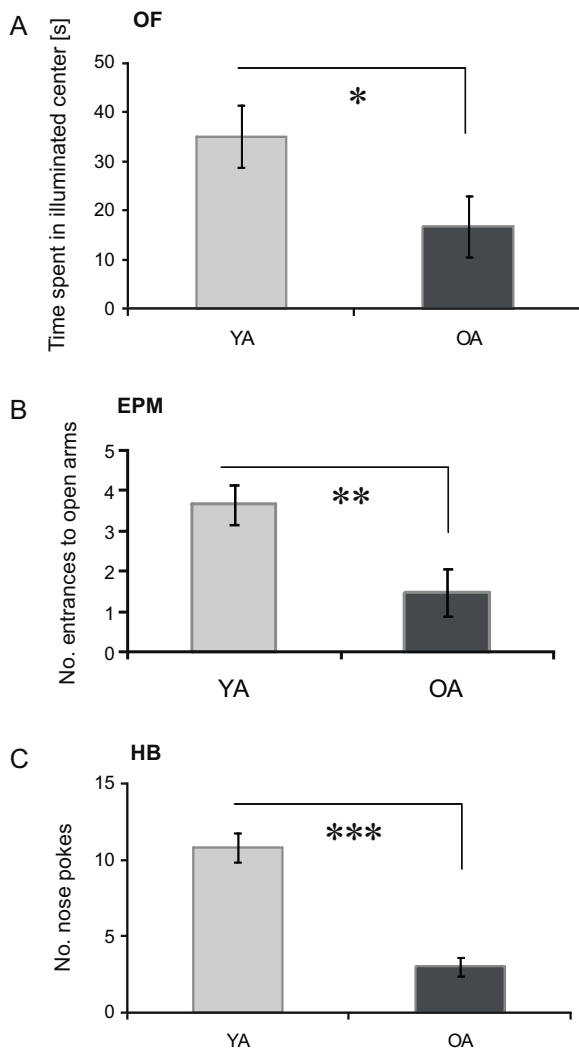


Fig. 2. Differences in the behavior of YA (light grey) and OA (dark grey) in the A. Open Field: time spent in the illuminated center, B. Elevated Plus Maze: number of entrances to open arms, C. Hole Board: number of nose pokes. Values are presented as mean ± SEM, *, ** and *** represent respectively $p<0.05$, $p<0.01$ and $p<0.001$.

as “Exploration”), explained 50% and 44% of variance respectively. There was a striking difference in assignment of the nose poke parameter. In OA it loaded to Exploration factor, while in YA this parameter was assigned to the second factor, labeled “Locomotor activity”. Locomotor activity (with loadings from parameters describing activity in the border zone and the whole arena) displayed an equivalent degree of variance in YA and OA rats (40% and 39%).

For confirmation of factor labeling we have performed a cross-correlation of HB Locomotor activity with OF Anxiety and HB with OF Locomotor activity. In OA this analysis yielded much weaker Pearson coefficients ($r = -0.13$ and 0.09 , $p > 0.05$) than a cross-correlation between HB Exploration with OF Locomotor activity and OF Anxiety ($r = 0.63$ and 0.55 , $p < 0.05$). In YA neither of the correlations were significant.

3.1.4. Order of testing

The design of the study, where each of the animals from OF, EPM and HB groups was tested in each of the three tests measuring the behavior in novel environment, required testing of the hypothesis of the possible effect of the order of tests as well as past experience on behavioral parameters describing the activity in a given test. Two-way ANOVA (age \times group) showed a significant effect of age but not group for parameters describing the activity in the OF and EPM tests, e.g. YA spent more time in the illuminated center of the OF than OA ($F(1,24) = 5.36$, $p < 0.05$) regardless of which group they belonged to ($F(2,24) = 1.37$, $p > 0.05$). Similar, they entered open arms more often than OA ($F(1,24) = 9.23$, $p < 0.01$), regardless of the group assignment ($F(2,24) = 0.62$, $p > 0.05$). The number of nose pokes though seemed to be affected both by age and the past experience ($F(1,23) = 72.08$, $p < 0.001$ and $F(2,23) = 3.51$, $p < 0.05$, with a significant interaction of the two variables, $F(2,23) = 4.31$, $p < 0.05$). Bonferroni test nevertheless did not show any significant differences between the groups within each of the age groups. Only differences between age groups (YA performing more nose pokes than OA) were statistically significant.

3.2. c-Fos protein expression

3.2.1. Control group

A significant, age-related difference in the basal c-Fos expression was observed in the amygdalar complex of YA and OA rats ($p < 0.05$, Fig. 3). ANOVA with Bonferroni post hoc test revealed that in the basolateral complex, medial and cortical nuclei of the amygdala of aged rats we found more c-Fos immunopositive cells under the control conditions than in young rats (Fig. 3A, C and D). This effect was not observed in the basal expression of c-Fos protein in central nuclei of the amygdala as well as the hypothalamus and hippocampus ($p > 0.05$, Figs. 4 and 5, Control bars).

3.2.2. Experimental conditions

In general, a distinct pattern of neuronal activation was observed for YA and OA rats in response to novel behavioral stimuli. ANOVA yielded significant effects of age ($F(1,33) = 17.44$, $p < 0.001$), test ($F(4,33) = 9.794$, $p < 0.001$) and structure ($F(9,297) = 144.21$, $p < 0.001$), as well as the interaction of structure and test ($F(36,297) = 3.48$, $p < 0.001$), and an interaction of age \times test \times structure ($F(36,297) = 1.64$, $p < 0.05$). Since the effect of structure was profound we decided to show the results of ANOVA with Bonferroni post hoc test performed for each of the brain structures separately.

3.2.2.1. The amygdalar complex. In the basolateral complex of the amygdala ANOVA yielded significant effects of age ($F(1,33) = 14.69$, $p < 0.001$) and test ($F(4,33) = 3.88$, $p < 0.05$), as well as age \times test interaction ($F(4,33) = 4.42$, $p < 0.01$). Apart from the aforementioned difference in basal c-Fos expression ($p < 0.05$, Fig. 3A), an age-specific difference in neuronal activation was noted upon highly stressful stimulation, the Acute Restraint test (IM, $p < 0.05$). The main age-related change in neuronal activation was such that while in YA all behavioral challenges evoked equally high activation of BLA (although not reaching significance when compared to baseline), in the OA animals the OF, EPM and HB exposure resulted in

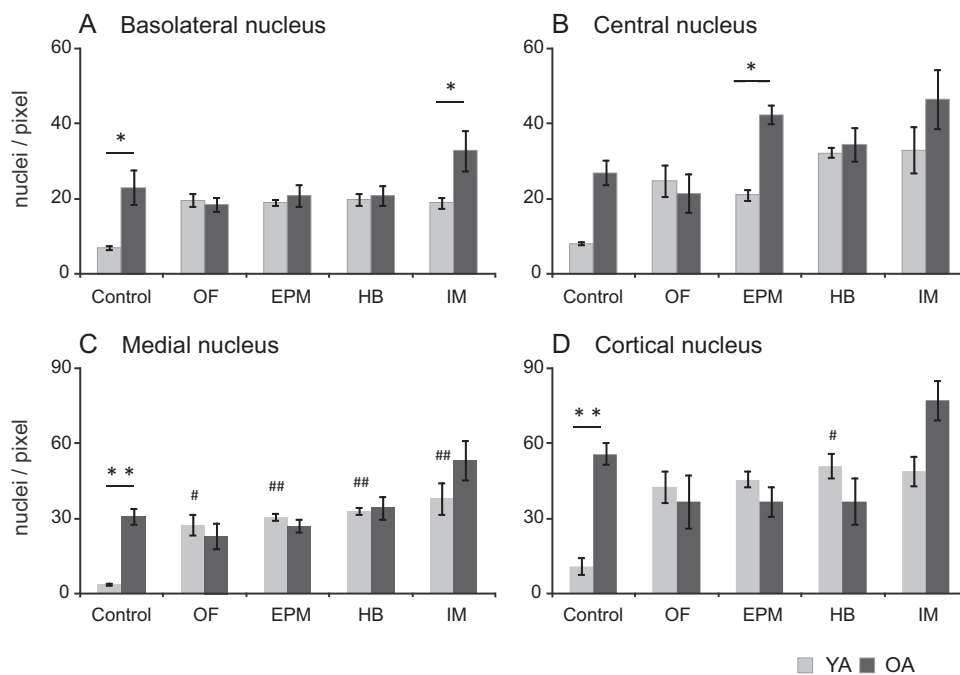


Fig. 3. c-Fos protein expression measured after home cage, Open Field with illuminated center, Elevated Plus Maze and Hole Board exposure as well as after acute restraint (IM) in A. basolateral complex, B. central nucleus, C. medial nucleus and D. cortical nucleus of the amygdalar complex in YA (light grey bars) and OA (dark grey bars) rats. Values are presented as mean \pm SEM. # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ for comparison of Control and Experimental treatment in YA. * indicates $p < 0.05$ for between age-group comparison.

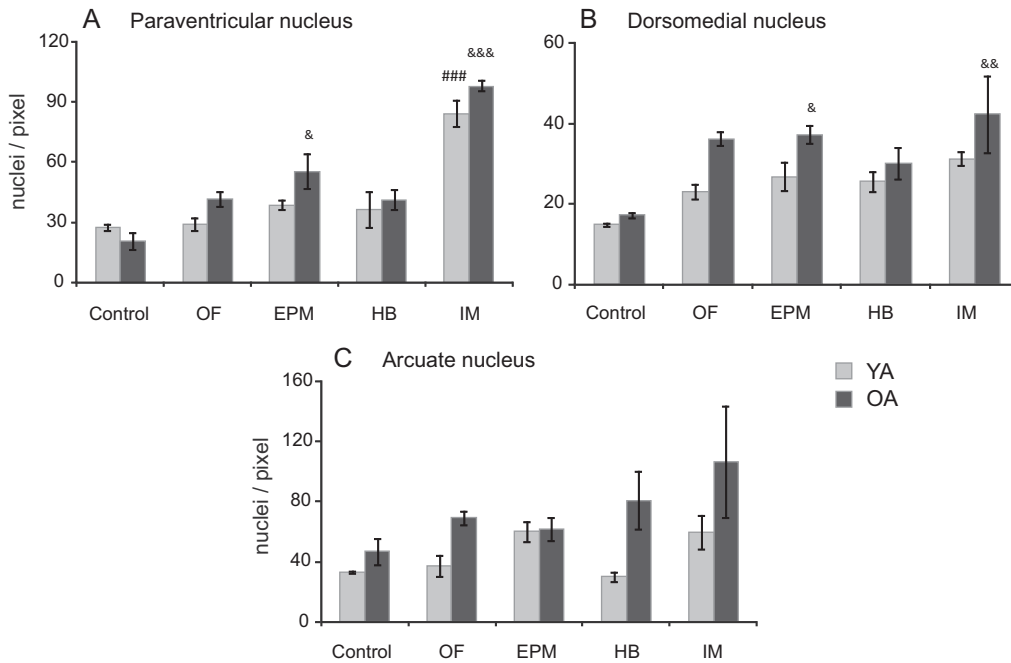


Fig. 4. c-Fos protein expression measured after home cage, Open Field with illuminated center, Elevated Plus Maze and Hole Board exposure as well as after acute restraint (IM) in A. paraventricular, B. dorsomedial and C. arcuate nucleus the hypothalamus in YA (light grey bars) and OA (dark grey bars) rats. Values are presented as mean ± SEM. ### $p < 0.001$ for comparison of Control and Experimental treatment in YA. * $p < 0.05$ and *** $p < 0.001$ for comparison of Control and Experimental treatment in OA.

significantly smaller activation rate than the Acute Restraint (IM, $p < 0.01$, not indicated).

For the central nucleus of the amygdala ANOVA yielded similar significant effects of age ($F(1,33) = 13.11$, $p < 0.001$) and test ($F(4,33) = 6.59$, $p < 0.001$), as well as age × test interaction ($F(4,33) = 2.98$, $p < 0.05$) as in BLA. Bonferroni post hoc test revealed that the neuronal activation was higher in OA, as compared with YA, only after exposure to the EPM test ($p < 0.001$).

The activation of the medial nucleus of the amygdala after the behavioral challenge was strong in YA and much weaker in OA. ANOVA yielded significant effects of age ($F(1,33) = 6.71$, $p < 0.05$) and test ($F(4,33) = 10.44$, $p < 0.001$), as well as age × test interaction ($F(4,33) = 4.43$, $p < 0.01$). The higher c-Fos protein expression was observed in OA only for the control conditions ($p < 0.05$, Fig. 3C). In YA all behavioral challenges evoked similar, high activation, as compared with the control group ($p < 0.001$). In OA rats, on the other

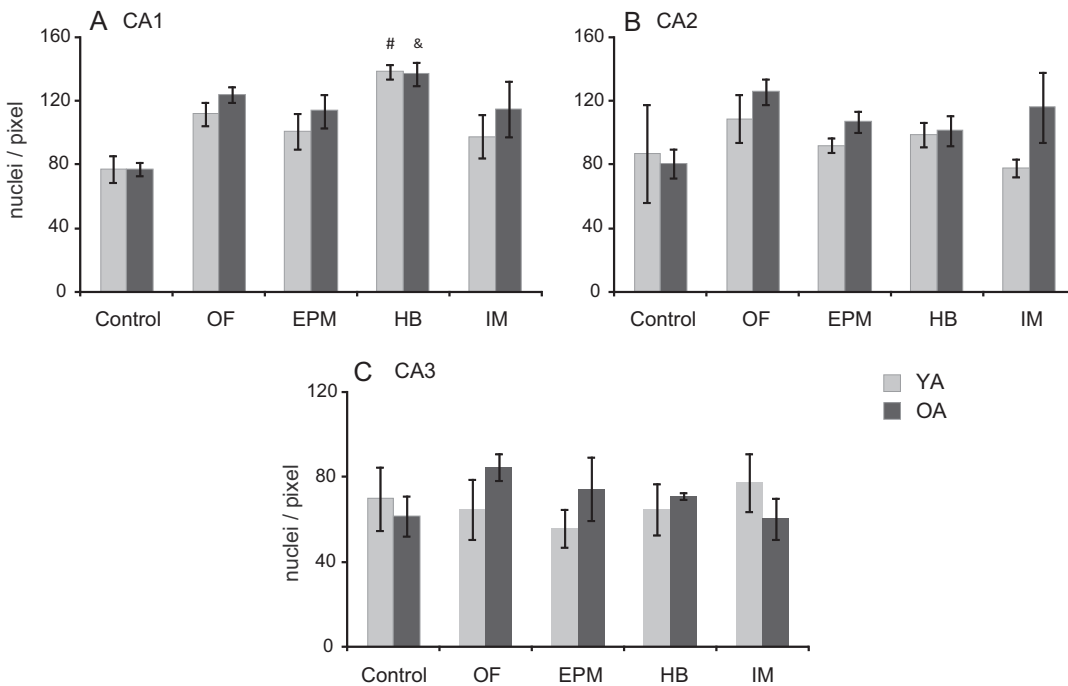


Fig. 5. c-Fos protein expression measured after home cage, Open Field with illuminated center, Elevated Plus Maze and Hole Board exposure as well as after acute restraint (IM) in A. CA 1, B. CA 2 and C. CA 3 field of the hippocampus in YA (light grey bars) and OA (dark grey bars) rats. Values are presented as mean ± SEM. # $p < 0.05$ for comparison of Control and Experimental treatment in YA. * $p < 0.05$ for comparison of Control and Experimental treatment in OA.

hand, none of the behavioral stimuli produced a significant increase in the number c-Fos immunopositive nuclei (as compared with the control group).

The activation pattern of the cortical nucleus of the amygdala resembled that of BLA and MeA, despite the fact that the main effect of age ($F(1,33)=3.94, p<0.06$) was weaker. The effect of test ($F(4,33)=4.666, p<0.01$) and the age \times test interaction ($F(4,33)=6.159, p<0.001$) were nevertheless significant. Higher c-Fos protein expression was observed for the control ($p<0.05$, Fig. 3D) group in OA. In YA HB test evoked significant neuronal activation ($p<0.05$, as compared with the control group), while the activation elicited by Acute Restraint approached significance (IM, $p=0.067$). In aged rats none of the behavioral challenges resulted in significant activation as compared with the baseline.

3.2.2.2. Hypothalamus. The hypothalamus displayed a heterogeneous response to both diverse behavioral stimuli and ageing. While the paraventricular and dorsomedial nuclei displayed an increase in reactivity towards mild stressors in OA, no such effect was observed for the arcuate nucleus (Fig. 4).

Apart from the significant effect of age ($F(1,33)=4.79, p<0.05$) and test ($F(4,33)=35.69, p<0.001$) no significant interaction between the two variables was found in the neuronal activation of the PVN. In OA a significant neuronal activation (as compared with baseline) was observed after exposure to EPM ($p<0.05$) and IM ($p<0.001$), while in YA only the strongest stimulation (IM) gave rise to a strong neuronal response ($p<0.001$). No significant difference between age groups was observed for any of the tests used ($p>0.05$, Fig. 4A).

ANOVA for DMH yielded significant effects of age ($F(1,33)=12.51, p<0.001$), test ($F(4,33)=6.93, p<0.001$) and no significant interaction between the two variables. The activation of the DMH resembles that of the PVN. In OA both EPM and IM challenges resulted in a significant activation ($p<0.05$ and $p<0.01$, respectively). In YA on the other hand none of the tests produced a c-Fos response significantly higher than the baseline. Also there was no age-dependent significant difference in the activation in any of the tests.

For the arcuate nucleus ANOVA yielded only one significant effect, the effect of age ($F(1,33)=10.88, p<0.01$). No significant activation in response to behavioral challenges was observed in neither YA nor OA ($p>0.05$, Fig. 4C). The activation rates were also similar between YA and OA.

3.2.2.3. Hippocampus. In general no effect of age was observed in the activation of CA 1, CA 2 and CA 3 fields of the hippocampus after exposure to behavioral tests used in our study (Fig. 5). An effect of test was observed only for CA 1 field ($F(4,33)=8.40, p<0.001$).

In the CA 1 field a very similar response to all behavioral tests was observed in YA and OA animals. The only significant (as compared to baseline) activation was observed in response to HB test ($p<0.05$ in both YA and OA, Fig. 5A). No age-specific changes in the levels of neuronal activation upon behavioral stimulation were noted. In both YA and OA, Acute Restraint did not elicit the most robust response. The highest response was observed after exposure to HB. In the case of YA it was significantly higher than after OF, EPM and IM ($p<0.05$, not indicated).

In the case of the CA 2 and CA 3 fields of the hippocampus no age-dependent differences in neuronal activation were observed. Also no significant increase from baseline was observed upon behavioral stimulation in either of the structures ($p>0.05$, Fig. 5B and C).

4. Discussion

In the current study we found that age substantially affects the behavior of Lewis rats in a novel environment, as well as the under-

lying neuronal activation of the key structures of the fear/anxiety circuit. The results further confirm our previously reported finding [29], that distinct parts of this circuit are differentially susceptible to age-related changes in activation. These findings also confirm that the set of tests used in the current study in conjunction with the application of Principal Component Analysis constitute a sensitive and useful tool for assessing the effect of age on rat behavior. This study can also be compared to studies that have sought the neurobiological correlates of discrete factors such as strain differences in emotionality and ageing.

Our data clearly indicate that Lewis rats, with age, show increased anxiety levels and a decrease in locomotor activity in a novel environment. In our study, aged animals spent less time in the brightly illuminated central part of the Open Field arena, as well as open arms and the central platform of the Elevated Plus Maze and the central part of the Hole Board Arena. Instead, they kept to "safe" zones such as the border zones of the OF and HB arenas and the closed arms of the EPM. A substantial body of data indicates, that such preference is a function of an age-dependent increase in anxiety [13,14,17,19,39,40] while at the same time showing that it is independent of age-related cognitive and locomotor impairment. The overall decline in locomotor activity observed in our study is a commonly observed ageing deficit [17,19,35,41]. However, given that in our study YA and OA rats showed a similar movement duration in the "safe" zones (e.g. closed arms of the EPM) and that the PCA analysis has clearly separated Anxiety and Locomotor activity as two distinct factors, we can assume that age-dependent changes in both of these factors are not correlated.

The age-related change in the role of anxiety was clearly confirmed by the results of the Principal Component Analysis for EPM and OF tests. It showed that the Anxiety factor explains a higher percentage of variability of behavioral parameters in OA than YA (e.g. 74% vs. 54% for EPM). Similar results were obtained in our previous study [14], in which young (3 month) and aged (24 month) Wistar rats were used. A comparison of the two studies shows strain differences in the initial anxiety level. Wistar rats are known to be less anxious than Lewis rats in the Open Field and Elevated Plus Maze [33]. While the behavior of young Wistar rats in the OF test was driven only by one factor (Locomotor activity), the behavior of Lewis rats was dependent on both Locomotor activity and Anxiety. We found this difference to be consistent between behavioral tests. The PCA performed on parameters describing the behavior in the EPM showed that, in Lewis rats, Anxiety explained the most variability in the behavior of YA, followed by Locomotor activity and Risk assessment factors. In Wistar YA, the variability in the behavior was mainly explained by Locomotor activity and to lesser extent by Anxiety and Risk assessment. This effect was further magnified with age.

In the current study PCA showed similar changes in the assignment of total distance travelled in the whole arenas of the OF and EPM tests with age, which further supports the hypothesis that age-related changes in the factorial structure revealed by PCA are indicative of a general change in emotionality of Lewis rats.

The behavior of Lewis rats in the Hole Board test was driven by two factors: Exploration and Locomotor activity (or Motor activity as named by Fernandes) [31]. Our labeling of the two factors was confirmed by the fact that a cross-correlation of HB Locomotor activity with OF Anxiety yielded much weaker Pearson coefficients than a cross-correlation between HB Exploration and OF Anxiety. In YA this correlation was not significant. In OA, on the other hand, we found that the HB Exploration factor in OA was also correlated with OF Locomotor activity. This shows that (although no distinct Anxiety factor was extracted in HB PCA Analysis) we cannot exclude the possibility that the behavior of OA in the HB arena was to some extent anxiety-dependent. The exploratory activity (number of nose-pokes, activity in the central part of the area) in

the OA Lewis rats decreased as compared with YA Lewis rats, a finding in line with data obtained by Li and co-workers [18] on Sprague-Dawley rats and Miyagawa [19] on Kbl Wistar rats. What was striking was that the number of nose pokes assigned to different factors in YA (Locomotor activity) and OA (Exploration). This shows that the HB arena may represent a different challenge for young and aged animals.

With such clear and marked age-related behavioral changes, we expected the underlying neuronal circuits to react differently to environmental novelty in YA and OA. Indeed, aged Lewis rats were found to react with higher neuronal activation (as indicated by expression of c-Fos protein) to a novel behavioral challenge. However, the effect was both structure and test specific.

In the case of the hypothalamus, the origin of the stress-responsive HPA axis, we have observed an increase in the activation rate in OA in the paraventricular (PVN) and dorsomedial nuclei (DMH). The pattern of activation seemed to move from significant reaction to only the strongest stressor (Acute restraint, IM in PVN) in YA to significant activation in response to both EPM and IM in OA (in both PVN and DMH). The increase in reactivity is in line with results obtained by Stöhr [26] who reported a stronger increase in the plasma CORT levels in 18 month old Lewis rats than in YA after restraint. According to de Kloet and co-workers [42] this effect could be associated with an age-related reduction of mineralocorticoid (MR) and glucocorticoid (GR) receptors density in the hippocampus and hypothalamus, which in turn provides a weaker negative feedback loop for the HPA axis. Young Lewis rats were shown to display higher binding affinity to these receptors, as compared with Wistar rats [43] which results in a stronger inhibition of their HPA axis. One could expect that, as a consequence of HPA axis inhibition, young Lewis rats should be less anxious than Wistar rats. Contrary to these expectations, Lewis rats showed more anxiety than Wistar (our studies) and Fischer 344 rats [26]. In the latter study the authors suggest that the higher anxiety of YA Lewis rats may occur due to hyperactivity of other brain regions involved in the regulation of emotion, such as the amygdala.

In the present study we found that in YA rats parts of the amygdalar complex, namely the medial and cortical nuclei showed profound neuronal activation in response to all behavioral challenges used. In the aged rats, on the other hand, the activation rate was small and did not reach statistical significance in comparison with baseline. This is in line with a smaller activation rate of the amygdalar complex observed in elderly humans in response to negative affective images [8,9].

Whether the observed smaller activation in response to behavioral challenge (as compared with YA) reflects a functional decrease in reactivity of the amygdalar complex and/or is an effect of high basal expression of c-Fos protein remains to be studied. The elevated basal level of c-Fos expression displayed by OA may reflect changes in excitability thresholds, which according to Stöhr [26] occur due to chronic hyperstimulation of CRH sensitive amygdalar neurons. The amygdalar complex, as the main CRH-sensitive fear/anxiety circuit structure [44], rich in CRH [45] and GR receptors [46] may be hyperactive throughout the lifespan of Lewis rats due to initial hypofunction of the HPA axis.

The change in amygdalar reactivity resulting with elevated basal expression of c-Fos protein could be responsible for the higher behavioral arousal of aged Lewis rats. We did not control for their home cage activity and therefore cannot exclude the possibility that their behavioral arousal observed in the novel environment tests was also present without exposure to a behavioral challenge. The elevated basal expression of c-Fos protein and concomitant decreased neuronal activation in response to stressors was also seen in aged Lewis rats in our earlier work [29]. In that experiment we observed elevated basal level of c-Fos protein both in the amygdalar complex and the hippocampus. In the current study

the effect was specific for amygdala only. The main difference between the two experiments was that in the earlier study we have kept animals returning from experimental testing (stressed and possibly emitting aversive ultrasound calls and/or pheromones) in the same room as the control group. This could have resulted in inter-individual transfer of emotional information which in observer/listener individuals may produce significant increases in c-Fos protein expression in brain structures involved in processing of potentially dangerous stimuli (e.g., amygdala and hippocampus Knapska et al. [47]). In OA Lewis rats (more anxious than YA) it could have produced arousal resulting with higher basal expression of c-Fos protein in these two structures. In the current study, to avoid the possible transfer of aversive information between the rats, we have sacrificed the control YA and OA prior to any other experimental procedures. This resulted in a lower (and equal with YA) basal c-Fos expression in the hippocampus of OA, which is in line with results obtained by Desjardins [48], Bucci [49], Lee [50] and Salchner [27]. The fact that the basal expression of c-Fos protein in the amygdalar complex was not affected by the procedural change may indicate that this effect is related to the ageing process alone and represents a functional adaptation.

Whether the general small activation rate (as revealed by Bonferroni post-hoc test) observed in our study results from the design of the experiment (repeated testing in three consecutive tests measuring the activity of the animal in different novel environments) and whether lack of such testing experience could influence the neuronal activation in animals from IM group is debatable. The two-way ANOVA run for parameters describing the activity in the most aversive parts of the OF and EPM tests as well as for nose poke activity in the HB test did not show significant within age-group differences related to the order of testing. We can therefore assume that order of testing should not have had an influence on the neuronal activation either. We cannot exclude that previous experience does, but then we would expect that the IM group would show strikingly higher expression of c-Fos protein (not only because the test itself is more aversive, but also because these animals had no previous experience with behavioral challenges). This was only the case for PVN and DMH, which were expected to show strong response to such a strong stressor. The differences in previous experience should have also been at least partially ameliorated due to extensive habituation sessions in all experimental groups.

5. Conclusion

The main conclusion of the current study is that the ageing process influences both the pattern of neuronal activation and the behavior of Lewis rats by turning them more reactive and anxious with age. The process itself seems to affect the HPA axis and other parts of the fear/anxiety circuit to a different extent. While the initially hypoactive HPA axis starts to react to even mild novel stimuli with age, the hippocampus seems to sustain its activity in an unchanged shape. The amygdala, on the other hand, undergoes an age-dependent decrease in the neuronal activation rate in response to mild stressors. How specific this heterogeneous effect is and what proportion of (high anxiety trait) human individuals may suffer from similar ageing-related changes remains to be studied. Further research is also required to estimate whether the age-related increase in anxiety of such individuals can be easily targeted with specific anxiolytic drugs, which could improve the quality of their lives.

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