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ORIGINAL RESEARCH

Diverse Sensitivity of RHA/Verh and RLA/Verh Rats to Emotional and Spatial Aspects of a Novel Environment as a Result of a Distinct Pattern of Neuronal Activation in the Fear/Anxiety Circuit

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Abstract Psychogenetically selected Roman high (RHA/Verh) and Roman low (RLA/Verh) avoidance rats constitute a well-recognized model of diverse emotional reactivity. The two Swiss lines display marked behavioral and endocrine differences in reaction to a novel environment. In our study we found that these differences are accompanied by a distinct, line-specific pattern of neuronal activation within the fear/anxiety circuit. We have compared the c-Fos protein expression in the medial prefrontal cortex (mPFC), basolateral (BLA), central (CeA), medial (MeA), and cortical (CoA) nuclei of amygdala, paraventricular nucleus of the hypothalamus (PVN), and CA1, CA2, and CA3 fields of the hippocampus upon exposure to a novel situation of different stressorgeneity (open field with illuminated center, elevated plus maze, hole board test and acute restraint). Profound between-line differences in the sensitivity to emotional and spatial aspects of the behavioral challenge were observed for tests measuring spontaneous behavior. This effect seems to reflect different motivational factors driving the rat behavior, which clearly suggests that the diverse emotional reactivity of RHA/Verh and RLA/Verh rats is a result of different activation of the fear/anxiety circuit.

Keywords RHA/Verh–RLA/Verh rats · Spontaneous behavior · PCA analysis · Emotional reactivity · c-Fos protein expression · Fear/anxiety circuit

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Introduction

Emotional reactivity in animals as well as in humans determines the capacity to cope with stressful situations. Individual differences in this respect define temperamental or personality traits and affect the susceptibility or resistance to various psychopathologies and somatic (cardiovascular in particular) diseases. Understanding the neurobiological basis of individual differences in emotionality is of key importance to treating these disorders.

It has been widely demonstrated that the character and intensity of stress responses depends, in part, on genetic factors (for e.g., Kendler et al. 1994; Wichers et al. 2007). One of the most promising approaches to investigate neuronal mechanisms underlying individual differences is the use of psychogenetically selected strains of rodents that diverge in their behavioral response to novel and/or stressful situations. A few models of hypo- and hyperemotional lines of rats have been established by this type of approach (see e.g., Broadhurst 1975; Fujita et al. 1994; Wigger et al. 2001). Swiss lines of Roman high (RHA/Verh) and Roman low (RLA/Verh) avoidance rats, employed in our experiment, belong to this group, although they were originally selected according to two-way avoidance acquisition criteria (Bignami 1965). Extensive research conducted over years has proven, however, that emotional reactivity (inversely related to shuttle box

avoidance performance) is the most prominent behavioral characteristic separating the two lines (Driscoll and Bätting 1982; Steimer et al. 1997; Steimer and Driscoll 2003). At the same time, several studies excluded the possibility of a "general learning deficit" in RLA/Verh rats (e.g., Zeier et al. 1978; Driscoll and Bätting 1982). RLA/Verh rats regarded as more anxious or hyperemotional have been shown to defecate and freeze significantly more than RHA/Verh rats when tested in several novel, or otherwise stressful, situations (e.g., Fernández-Teruel et al. 1992; Escorihuela et al. 1995). Likewise, RLA/Verh rats achieve significantly higher scores in many commonly used anxiety tests (Martin et al. 1982; Ferré et al. 1995; Schwegler et al. 1997). They display lower (both quantitatively and qualitatively) exploratory activity (Pisula and Osiński 2000; Pisula 2003). Moreover, RLA/Verh rats show increased neuroendocrine and autonomic reactivity to environmental and/or psychosocial stressors and adopt a passive coping style when exposed to novel situations (in contrast to the active coping strategy characteristic of RHA/Verh rats) (Gentsch et al. 1982; Walker et al. 1989; Steimer et al. 1997; Steimer and Driscoll 2003; Steimer and Driscoll 2005). An interesting feature that also differentiates the behavioral profile of the two lines is markedly higher sensation (novelty/reward) seeking behavior in RHA/Verh rats associated with higher motor activity, exploration, drug and alcohol consumption, and impulsivity (Fernández-Teruel et al. 1992; Siegel et al. 1993; Driscoll et al. 1998; Guitart-Masip et al. 2006). A growing body of data indicate also that Roman rats display line dependent differences in functional properties of brain neurotransmitters such as 5HT, DA, and GABA (Driscoll et al. 1983; Giorgi et al. 1994; Giorgi et al. 1997; Corda et al. 1997; Giorgi et al. 2003a, b; Guitart-Masip et al. 2006, 2008), known to be involved in the control of emotions (Kalueff 2007). Moreover, Guitart-Masip et al. (2006, 2008) have shown that divergent substance-seeking profiles of these rats result from distinct pattern of D1 and D3 receptor binding and different expression profiles of proteins involved in dopaminergic transmission. The Roman lines therefore, as well defined phenotypes that significantly differ behaviorally and neurochemically, offer an extremely advantageous model to explore biological determinants of emotional reactivity to physical and psychological challenges.

The immediate early gene expression (mostly *c-fos*) has been widely used as a functional marker of neuronal activation to identify cells and brain circuits that are responsive to various appetitive (Fleming and Walsh 1994; Pfaus and Heeb 1997; Ryabinin et al. 2000) and aversive stimuli (Pezzone et al. 1992; Kovacs 1998; Savonenko et al. 1999; Singewald et al. 2003; Boguszewski and Zagrodzka 2005; Knapska et al. 2007). A vast amount of data indicate that systemic as well as neurogenic or psychological stressors induce increased c-Fos expression in the brain structures implicated in regulation of emotions and stress responsiveness such as the prefrontal cortex, amygdaloid complex, hippocampus, and hypothalamus (Honkaniemi et al. 1992; Kononen et al. 1992; Silveira et al. 1993; Cullinan et al. 1995; Mulders et al. 1995; Campeau et al. 1997; Kovacs 1998; Kabbaj and Akil 2001).

Here we used c-Fos immunoreactivity to evaluate the pattern of neuronal activation of the main stress and anxiety-related areas in both lines of Roman rats under basal conditions, and after exposure to anxiogenic, novel situations of various stressorgeneity. The behavioral challenge was either based on spontaneous exploration of a novel environment or was an acute stressor (restraint/immobilization). In the spontaneous behavior tests we have used open field with illuminated center (OF), elevated plus maze (EPM), and hole board (HB) tests, which represent a gradient of aversive stimulation, OF being considered the most and HB the least anxiogenic (Kovacs 1998).

Differences in individual behavioral characteristics in relation to differences in the neurocircuits that underlie emotional reactivity has been studied so far in a few other animal models, i.e., Sprague–Dawley rats selected on the basis of locomotor activity in a novel environment—HR and LR (high and low responders, respectively) (Kabbaj et al. 2000; Kabbaj and Akil 2001), behaviorally selected "active" and "passive" Wistar rats (Babai et al. 2001) and Wistar rat lines selectively bred for high (HAB) versus low (LAB) anxiety-related behavior (Landgraf and Wigger 2002).

To identify more precisely the motivational factors that underlie spontaneous behavior after exposure to a novel environment in RLA/Verh and RHA/Verh rats principal component analysis (PCA) with varimax orthogonal rotation has been used. PCA is considered a particularly beneficial statistical tool for the interpretation of behavioral data (Rodgers and Johnson 1995; Courvoisier et al. 1996; Ramos et al. 1997; Fernandes et al. 1999; Boguszewski and Zagrodzka 2002) as it reveals factors driving particular behaviors.

The aim of the present study was to compare c-Fos expression in the fear/anxiety circuit using a rat model of individual differences in emotional reactivity in the context of response to mild, as well as highly stressful stimulus and to investigate whether there is a relationship between the level and topography of c-Fos expression and individual (temperamental) traits.

Materials and methods

Animals

A total of 40 male rats from psychogenetically selected Roman high avoidance (RHA/Verh, $n = 20$) and Roman

low avoidance (RLA/Verh, $n = 20$) lines were transported from the original breeding colony established at the Laboratoire de Recherches Unité de Psychopharmacologie Clinique (APSIC), Hôpitaux Universitaires de Genève at the age of 5–6 weeks. Animals were housed in groups of five rats per cage (in standard Plexiglass cages, 58 × 40 × 20 cm), with unlimited access to water and standard laboratory rat chow, under L:D 12:12 conditions, with lights on at 7:00 am until the age of 3 months, when they were moved to individual cages (standard 40 × 25 × 17 cm Plexiglass cages) to avoid between-individual transfer of aversive information.

Behavioral testing

After a period of habituation to the new housing conditions the rats were assigned randomly to five groups ($n = 4$ RHA/Verh and $n = 4$ RLA/Verh rats per group). First, the control group was subjected to non invasive homecage activity observation and was sacrificed directly from home cages 24 h later. Animals from the second group were subjected to the acute restraint procedure. Rats from groups 3, 4, and 5 were tested in three consecutive behavioral tests—open field with illuminated center, elevated plus maze, hole board test—in three different orders with 7 days interval between the tests (for experimental design see Table 1). The behavioral data was collected (in MPEG-2 format) and analyzed using a video-based, automated Ethovision System (Noldus, Wageningen, NL) for spatio-temporal measures of behavior. Also an observer-based program (BehaView, Boguszewski et al. 2005) was used by two independent viewers to quantify parameters, which could not be assessed automatically.

Homecage activity observation (HAO)

Rats assigned to the control group were subjected to the same handling procedure as animals from other groups. Handling consisted of 15 min daily exposure to the experimental room for two consecutive weeks. The rats were transported and kept in the experimental room in a clear plexiglass cage identical to their home cage, only with fresh sawdust. On the observation day the behavior of the animals was video-recorded for 20 min. The following parameters were analyzed: distance moved, time spent on selfgrooming, number and duration of exploratory episodes, and the number of rearings.

Table 1 Experimental design

Group		Day 1	Day 2	Day 8	Day 15
1	Control	HAO	c-Fos		
2	IM				IM + c-Fos
3	OF	EPM		HB	OF + c-Fos
4	EPM	HB		OF	EPM + c-Fos
5	HB	OF		EPM	HB + c-Fos

Acute restraint (immobilization, IM)

The acute restraint test (immobilization, IM) was performed using a clear Plexiglass ventilated tube, 20 cm long, 6.5 cm inner diameter, with adjustable length according to the size of the animal and tail protruding (as in Meyza et al. 2007). The size of the tube restricted movements in all directions but did not interfere with respiration. The animals were kept in the apparatus for 15 min.

Open field with illuminated center (OF)

The test arena was a black-painted square (90 × 90 cm), enclosed by walls (30 cm high) with a 50 W halogen bulb suspended 30 cm above the center (Boguszewski and Zagrodzka 2002). The animal was placed in the thigmotaxic zone facing one of the corners and the track of its movements was recorded for 10 min. The only illumination came from the halogen bulb, all other lights were off during the time when testing took place.

Three zones of OF were drawn according to previously selected criteria (Boguszewski and Zagrodzka 2002): the illuminated central zone (a circle directly corresponding to the brightly lit part of the arena), semi-illuminated zone (10 cm encircling the illuminated central zone), and the thigmotaxic zone. The following parameters were analyzed: (1) in the illuminated center: number of entries to zone, distance moved, and movement duration; (2) in semi-illuminated part of arena: number of entries; (3) in thigmotaxic zone: distance moved and movement duration; (4) in the whole arena: total distance moved and total movement duration.

Elevated plus maze (EPM)

A black wooden apparatus based on the one described by Pellow et al. (1985) consisting of two enclosed arms (10 × 50 × 30 cm) and two open arms (10 × 50) connected with a central platform (10 × 10 cm) located 70 cm above the floor was used. The rats were introduced to the closed arm of the maze via lifted doors and therefore were given free choice as to whether to explore the open arms and central platform or not for 5 min. The testing was done in a dimly lit area surrounded with non-transparent, gray curtains in order to prevent any additional stimuli other than the ones coming from the maze. The following parameters were calculated: (1) in enclosed arms: number of entries, total time spent, distance moved, and movement duration; (2) in open arms: number of entries, total time

spent, and distance moved; (3) in the central platform: time spent and number of stretch attendant postures (SAP) into open arms; (4) in the whole arena: total distance moved and ratio of entrances to open/enclosed arms.

Hole board (HB)

The HB test was performed in 50 × 50 × 30 cm box with gray walls and four equidistant, 1 cm deep holes in the central part of a black-painted floor. The testing took place in a room lit by two 80 W light bulbs. At the beginning of the test the rat was placed in one of the corners of the arena and then was allowed to explore the arena freely for 10 min. The following parameters were considered: (1) in central part of the apparatus: number of entrances, time spent, distance moved, and movement duration; (2) in the thigmotaxic zone: distance moved and movement duration; (3) in the whole arena: distance moved and number of nose pokes into holes in the arena floor.

Immunocytochemistry

Rats from groups 2–5 were sacrificed 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 (POCh) solution. The control group was sacrificed directly from their home cages 24 h after the home cage observation took place. All 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). A volume of 40 μm thick coronal sections were taken and subjected to standard c-Fos immunocytochemistry according to the procedure described in detail previously by Savonenko et al. (1999).

c-Fos stained brain slices were microphotographed and assessed for c-Fos protein expression using ImageJ software (WCIF, Toronto, Canada) in the medial prefrontal cortex (mPFC, consisting of prelimbic and infralimbic cortices, approx. Bregma 2.70 mm (Paxinos and Watson 1997)), amygdaloid complex (approx. Bregma −2.80 mm), including basolateral (BLA), central (CeA), medial (MeA), and cortical (CoA) nuclei, the dorsal hippocampus (CA1, 2, and 3 fields, approx. Bregma −2.80 mm) and the hypothalamic paraventricular nucleus (PVN, approx. Bregma −1.8 mm). Each structure was assessed on the basis of measures from four 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 on the particular slice (data shown in arbitrary units). Area and the exact shape of the investigated structures on c-Fos stained slides were confirmed using the adjacent, Nissl-stained sections.

Statistical analysis

Behavioral data

Behavioral parameters of the OF, EPM, and HB, respectively, were analyzed separately for RHA/Verh and RLA/Verh rats by means of principal component analysis (PCA, STATISTICA, 5th edition) with normalized varimax orthogonal rotation of the factor matrix. PCA allows the extraction of independent factors reflecting different drives constituting behavior of a given group of rats, which reduces the number of discussed variables. The number of extracted factors was assessed using Kaiser criterion (eigenvalue > 1). The loadings exceeding the value of 0.5 (and -0.5, respectively) were indicated. The parameters which were included in the PCA and received high loadings to extracted factors were subjected to statistical analysis of between-line differences by means of *U* Mann–Whitney non-parametric rank test. Differences were considered significant if $P < 0.05$.

c-Fos protein expression

Statistical analysis of c-Fos protein expression in all groups was performed using mulitvariate analysis of variance (MANOVA) followed by posthoc Fischer/NIR test for mean values representing activation of each structure in a given individual. Differences were considered significant if $P < 0.05$.

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 Animals Research.

Results

Behavior

Homecage activity observation (HAO, control group)

No differences between Roman high avoidance (RHA/Verh) and Roman low avoidance (RLA/Verh) rats were noted as to the distance moved in the cage as well as number and the duration of exploratory episodes ($P > 0.05$). Also the number of rearings and the time spent on grooming were similar in both groups of rats ($P > 0.05$).

Open field with illuminated center (OF)

The behavior of RHA/Verh and RLA/Verh rats was completely different upon being placed in the OF. RHA/Verh rats explored the testing arena in all its zones, including the brightly illuminated center located directly under the halogen bulb. The number of entrances to this part of the arena (Fig. 1a, $P < 0.05$), as well as distance moved within its borders ($P < 0.05$) and movement duration in it were significantly higher for RHA/Verh rats than for RLA/Verh rats ($P < 0.05$). Also the number of entrances to the semi-illuminated part of the arena, directly encircling the borders of the illuminated part was higher in RHA/Verh rats ($P < 0.05$).

Factor analysis allowed the extraction of two distinct factors (with eigenvalue > 1) driving this behavior (Table 2). In both RHA/Verh and RLA/Verh the first factor, responsible for about 65% of total variance seems to represent locomotor activity as it receives high loadings from parameters such as total distance moved, total movement duration as well as distance moved in the (safe) thigmotaxic zone, and movement duration within its borders. The second factor, responsible for 27% (for RHA/Verh) and, respectively, 22% (RLA/Verh) of total variance, has highest loadings on the number of entrances to illuminated part of the arena and therefore can be attributed to as describing anxiety level of the animals. Also the number of entrances to the semi-illuminated part of the arena loads to Factor 2 in both RHA/Verh and RLA/Verh rats.

The main between-line difference was the assignment of the parameter describing distance moved in the illuminated part of the arena. While in RHA/Verh rats it loads heavily to Factor 1 (locomotor activity), in RLA/Verh it does to Factor 2 (anxiety).

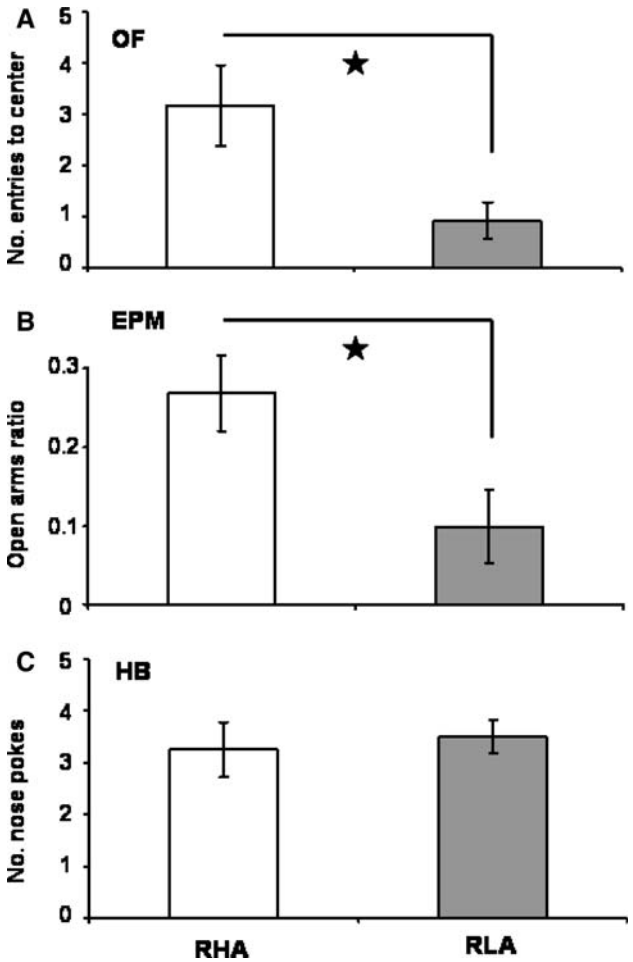

Fig. 1 Selected behavioral parameters from **a** open field with illuminated center test: number of entrances to illuminated part of the arena (10 min); **b** elevated plus maze: ratio of entrances to open/enclosed arms of the maze (5 min); **c** hole board test: number of nose pokes into holes in the arena floor (10 min). Values are presented as mean ± SEM. ★ stands for $P < 0.05$ as revealed by *U* Mann–Whitney test. *White bars* represent RHA/Verh, *gray* RLA/Verh rats

Elevated plus maze (EPM)

The two Roman lines of rats displayed diverse response in the novel environment of elevated plus maze. Given free choice of either exploring it or remaining in the enclosed arm, where they were placed at the beginning of the 5 min test, RHA/Verh rats choose to enter the open arms of the maze much more frequently than RLA/Verh rats (Fig. 1b, $P < 0.01$). The distance moved within the open arms ($P < 0.05$) and in the whole maze ($P < 0.01$) was also higher in RHA/Verh rats, as well as the number of entrances to enclosed arms ($P < 0.05$).

Factor analysis revealed that the behavior of these two lines is differently driven by three distinct factors (Table 3). The main difference between the two lines is the order of extracted factors. In case of RHA/Verh rats Factor 1, representing 53% of total variance, receives high, positive loadings from parameters such as the number of entries to open arms of the maze, time spent, and distance moved in them as well as with ratio of entrances to open arms. It also receives negative loadings from the time spent in enclosed arms of the maze. It seems therefore to be a measure of anxiety level. The same parameters constitute a Factor 2 in the RLA/Verh rats, representing 20% of total variance. In the RHA/Verh rats Factor 2 (26% total variance) is related to locomotor activity as it receives loadings from total distance moved and total movement duration as well as distance moved and movement duration in enclosed arms. The same parameters load heavily to Factor 3 in RLA/Verh rats (15% total variance). The third factor revealed by the analysis for RHA/Verh rats (8% of total variance) represents the risk assessment behavior as it receives loadings from the number of SAP (stretch attend

Table 2 Behavioral parameters and their orthogonal loadings for the open field with illuminated center test

Behavioral parameter	RHA/Verh		RLA/Verh	
	Factor 1 (65%) Locomotor activity	Factor 2 (27%) Anxiety	Factor 1 (65%) Locomotor activity	Factor 2 (22%) Anxiety
Number of entries to illuminated center		0.94		0.95
Distance moved in the illuminated center (cm)	0.83			0.62
Movement duration in the illuminated center (s)		0.76		0.86
Distance moved the thigmotaxic zone (cm)	0.98		0.97	
Movement duration in the thigmotaxic zone (s)	0.98		0.94	
Total distance moved (cm)	0.98		0.97	
Total movement duration (s)	0.98		0.97	
Number of entrances to semi-illuminated part		0.86		0.84

Table 3 Behavioral parameters and their orthogonal loadings for the elevated plus maze test

Behavioral parameter	RHA/Verh			RLA/Verh		
	Factor 1 (53%) Anxiety	Factor 2 (26%) Locomotor activity	Factor 3 (8%) Risk assessment	Factor 1 (61%) Risk assessment	Factor 2 (20%) Anxiety	Factor 3 (15%) Locomotor activity
Number of entries to closed arms			0.80	0.76		
Total time spent in closed arms (s)	−0.62		−0.71	−0.88		
Distance moved in closed arms (cm)		0.95				0.90
Movement duration in closed arms (s)		0.94				0.98
Number of entries to open arms	0.79				0.92	
Total time spent in open arms (%)	0.90				0.98	
Distance moved in open arms (cm)	0.91				0.90	
Total distance moved (cm)		0.81		0.67		0.58
Total movement duration (s)		0.76		0.66		0.67
Ratio open/total entries	0.84				0.97	
Number of SAPs to open arms			0.74	0.98		
Time spent in central platform (s)			0.78	0.95		

postures) into the open arms as well as from the time spent on central platform and number of entries to enclosed arms. It is also negatively correlated with the time spent in enclosed arms. The very same parameters together with loadings from total distance moved and total movement duration form Factor 1 in the RLA/Verh rats (61% of total variance).

Hole board (HB)

The hole board test is designed to investigate exploratory drive of the animal. In this test rats of the two Roman strains did not differ in the number of nose pokes (Fig. 1c, $P > 0.05$). At the same time a difference in a number of entrances to the central part of the arena ($P < 0.01$) as well as in the distance moved within its borders ($P < 0.001$) were observed due to the extensive exploration of this area by RLA/Verh rats. All this time RHA/Verh rats remained more active than RLA/Verh rats in the thigmotaxic zone ($P < 0.001$). Factor analysis (Table 4) revealed that the main difference between the lines was the number of extracted factors. Two main factors drove the behavior of RHA/Verh rats, while for RLA/Verh behavior three factors were identified. The first factor driving the behavior of both rat lines was the exploratory drive. It represents 61% of total variance for RHA/Verh and 43% of total variance for RLA/Verh rats. It receives heavy loadings from parameters describing activity in the central part of the arena and from the number of nose pokes in case of RHA/Verh rats. The second factor (representing, respectively, 25 and 33% of total variance for RHA/Verh and RLA/Verh rats) receives loadings from parameters such as total distance moved and

Table 4 Behavioral parameters and their orthogonal loadings for the hole board test

Behavioral parameter	RHA/Verh		RLA/Verh		
	Factor 1 (61%) Exploration	Factor 2 (25%) Locomotor activity	Factor 1 (43%) Exploration	Factor 2 (33%) Locomotor activity	Factor 3 (15%) Anxiety
Number of entries to the central part	0.70		0.52		0.69
Time spent in central part (s)	0.98		0.94		
Distance moved in the central part (cm)	0.87		0.97		
Movement duration in the central part (s)	0.98		0.97		
Distance moved the thigmotaxic zone (cm)		0.95		0.98	
Movement duration in the thigmotaxic zone (s)		0.94		0.96	
Total distance moved (cm)		0.88		0.82	
Number of nose pokes	0.65				−0.87

distance moved and movement duration in the thigmotaxic zone. It seems therefore to represent general locomotor activity. In case of RLA/Verh rats a third factor (representing 15% of total variance), almost exclusively dedicated to nose poke activity, arose. As nose poke parameter loads negatively and number of entrances to central part loads positively to it, we assume it represents anxiety level of the individual, which in RLA/Verh rats is separated from general exploratory drive.

c-Fos protein expression

Homecage activity observation (HAO, Control group)

The Roman high and low avoidance rats did not differ in basal expression of c-Fos protein in any of the structures taken into the analysis (Figs. 2–5, control bars, $P > 0.05$).

Experimental conditions

In general a distinct pattern of neuronal activation was observed for RHA/Verh and RLA/Verh rats. Mulitvariate analysis of variance (MANOVA) yielded several significant effects: the effect of line ($F(1, 29) = 15.792$, $P < 0.0005$), test ($F(4, 29) = 196.841$, $P < 0.0001$), and structure ($F(8, 232) = 141.434$, $P < 0.0001$). The interaction of line and test was also significant ($F(4, 29) = 4.395$, $P < 0{,}007$), as well as line and structure ($F(8, 232) = 2.036$, $P < 0.05$) and test and structure ($F(32, 232) = 20.763$, $P < 0.0001$). Posthoc Fischer/NIR test showed that all of the observed structures showed significant (as compared with HAO/Control group) and stress-dependent activation in response to behavioral challenge (OF, EPM, HB, and acute restraint–IM). The RLA/Verh rats showed higher rates of activation in most of them, which indicates their higher arousal in response to novel environment. The acute restraint procedure elicited equally very high activation scores in RHA/Verh and RLA/Verh rats.

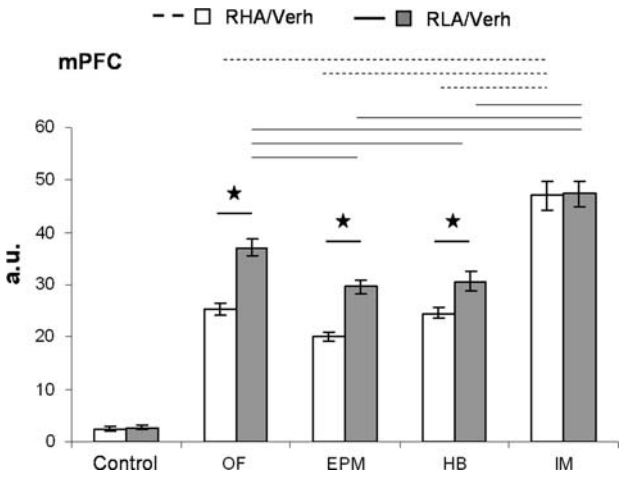

Fig. 2 Neuronal activation pattern of the medial prefrontal cortex (*mPFC*) in control, open field with illuminated center (*OF*), elevated plus maze (*EPM*), hole board (*HB*), and acute restraint (*IM*) conditions. Values are presented as mean ± SEM. ★ stands for $P < 0.05$ as revealed by MANOVA followed by posthoc Fischer/NIR test. *Dashed lines* represent differences ($P < 0.05$) between tests from RHA/Verh rats, *full lines*: for RLA/Verh rats. *White bars* represent RHA/Verh, *gray* RLA/Verh rats

Open field with illuminated center (OF)

The open field with illuminated center elicited significant neuronal activation in all of the observed structures (not indicated). The medial prefrontal cortex (mPFC, Fig. 2), as well as all four measured nuclei of the amygdala, namely the basolateral (BLA, Fig. 3a), central (CeA, Fig. 3b), medial (MeA, Fig. 3c) and cortical (CoA, Fig. 3d) were more activated in RLA/Verh than in the RHA/Verh rats ($P < 0.05$). So were the CA1, CA2, and CA3 fields of the hippocampus (Fig. 4a–c, $P < 0.05$) and the paraventricular nuclei of the hypothalamus (PVN, Fig. 5, $P < 0.05$).

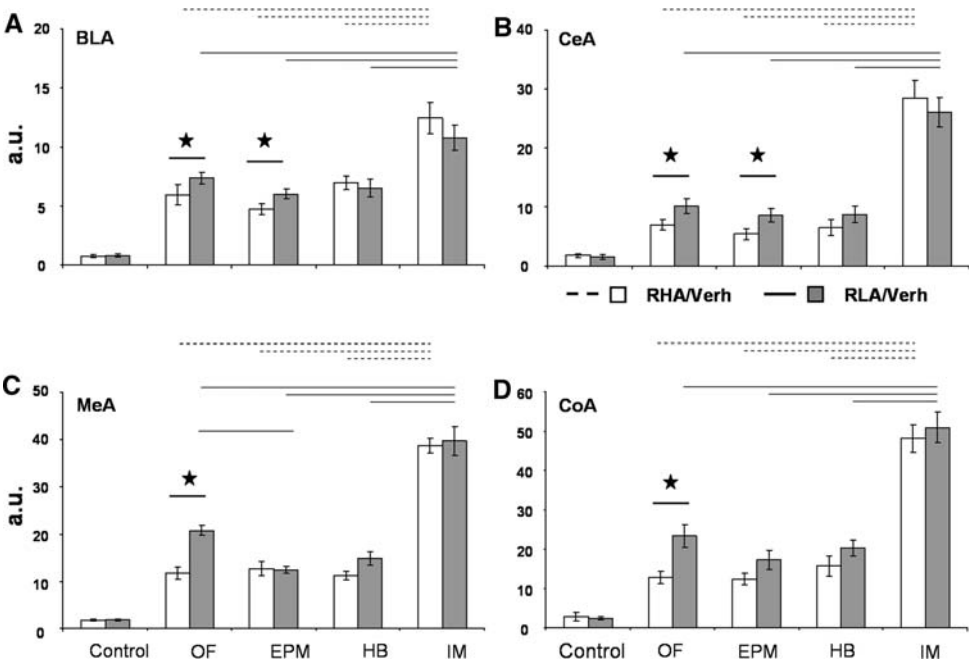

Fig. 3 Neuronal activation pattern of the: **a** basolateral (*BLA*), **b** central (*CeA*), **c** medial (*MeA*), and **d** cortical (*CoA*) nuclei of amygdala in control, open field with illuminated center (*OF*), elevated plus maze (*EPM*), hole board (*HB*), and acute restraint (*IM*) conditions. Values are presented as mean ± SEM. ★ stands for $P < 0.05$ as revealed by MANOVA followed by posthoc Fischer/NIR test. *Dashed lines* represent differences ($P < 0.05$) between tests from RHA/Verh rats, *full lines*: for RLA/Verh rats. *White bars* represent RHA/Verh, *gray* RLA/Verh rats

Elevated plus maze (EPM)

Exposure to the EPM elicited significant neuronal activation in all brain structures observed (not indicated). We found differences between the lines in the activation of the mPFC, BLA, CeA, and CA1 and CA2 fields of hippocampus. In all of these structures RLA/Verh rats showed a higher number of c-Fos positive nuclei activated by this behavioral challenge (Figs. 2, 3a, b, 4a, b, $P < 0.05$).

Hole board test (HB)

The hole board test, as the mildest of stressors used, elicited the smallest, but still significant neuronal activation among the tests measuring spontaneous behavior in the mPFC, amygdala and PVN (Figs. 2, 3, 5). On the contrary the activation observed in the hippocampus after exposure to HB (Fig. 4) was higher than the response to the OF and EPM. RLA/Verh rats showed significantly higher rates of activation for this test in the mPFC and MeA as well as CA1 and CA2 fields of hippocampus (Figs. 2, 3c, 4a, b, $P < 0.05$).

Acute restraint/immobilization (IM)

Acute restraint (proved to be the highest stressor used) elicited the highest numbers of c-Fos positive nuclei in all structures taken into analysis (Figs. 2–5). This activation was moreover significantly higher in both lines, than the one observed for all the other behavioral tests, in mPFC, BLA, CeA, MeA, CoA, PVN as well and CA1 field of hippocampus (Figs. 2, 3a–d, 4a, 5, $P < 0.05$, dashed lines representing differences for RHA/Verh and full lines representing RLA/Verh). In the CA2 field of hippocampus this was also true for RHA/Verh rats (Fig. 3b, $P < 0.05$, dashed lines), but not for results obtained after HB challenge in RLA/Verh rats, where it was not significantly higher than the activation elicited by HB test (Fig. 3b, $P > 0.05$). In the CA3 field of the hippocampus the activation elicited by IM was also higher than after OF and EPM but not HB exposure in RHA/Verh rats (Fig. 3c, $P < 0.05$, dashed lines), while the activation in RLA/Verh rats was not different from the other behavioral challenges. The activation of all structures was similar in RHA/Verh and RLA/Verh rats.

Comparison between the OF, EPM, and HB tests

Apart from between-line differences in reaction to OF, EPM, and HB as novel environments we have observed significant between-test differences in the number of activated c-Fos positive cells within both lines. Moreover, there was a qualitative difference in the neuronal pattern of activation between RHA/Verh and RLA/Verh rats in respect to reaction to diverse stressorgeneity of the tests.

In RLA/Verh rats we observed higher ($P < 0.05$) activation of the mPFC, MeA, and PVN after OF exposure as compared with EPM (Figs. 2, 3c, 5, indicated with full line). In RHA/Verh rats this effect was present only in the PVN (Fig. 5, indicated with dashed line). The activation after OF exposure was also higher than after HB in the mPFC and PVN (Figs. 2, 5, $P < 0.05$, full line). No such difference was observed in RHA/Verh rats.

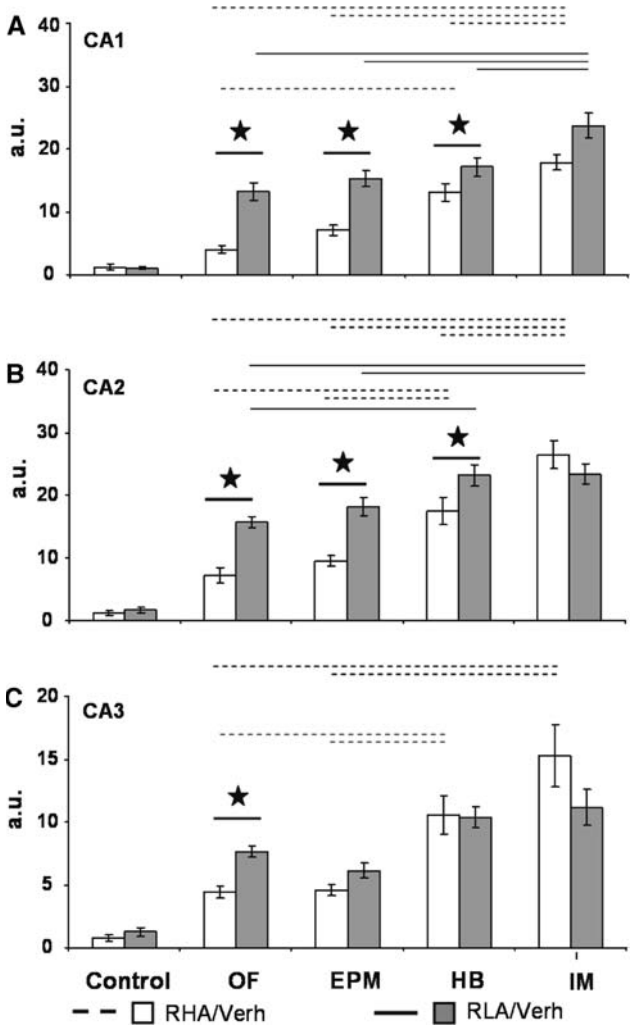

Fig. 4 Neuronal activation pattern of the: **a** CA1, **b** CA2, and **c** CA3 fields of the hippocampus in control, open field with illuminated center (*OF*), elevated plus maze (*EPM*), hole board (*HB*), and acute restraint (*IM*) conditions. Values are presented as mean ± SEM. ★ stands for $P < 0.05$ as revealed by MANOVA followed by posthoc Fischer/NIR test. *Dashed lines* represent differences ($P < 0.05$) between tests from RHA/Verh rats, *full lines*: for RLA/Verh rats. *White bars* represent RHA/Verh, *gray* RLA/Verh rats

In the hippocampus, the situation was opposite. Not only did the HB seem to activate the highest number of cells, but also more differences were observed for RHA/Verh than RLA/Verh rats (Fig. 4a–c, dashed and full lines, respectively). When comparing OF and HB all three parts of the hippocampus are more activated upon exposure to HB in RHA/Verh rats (Fig. 4a–c, dashed lines), while in RLA/Verh this effect can be seen only in the CA2 (Fig. 4b, full line). Activation after OF is also smaller than the one elicited by EPM in the CA1 field of the hippocampus of RHA/Verh rats (Fig. 4a, dashed line), while no such difference was observed for RLA/Verh rats. Also when comparing EPM driven activation with the one evoked by HB, in RHA/Verh the response is significantly smaller after

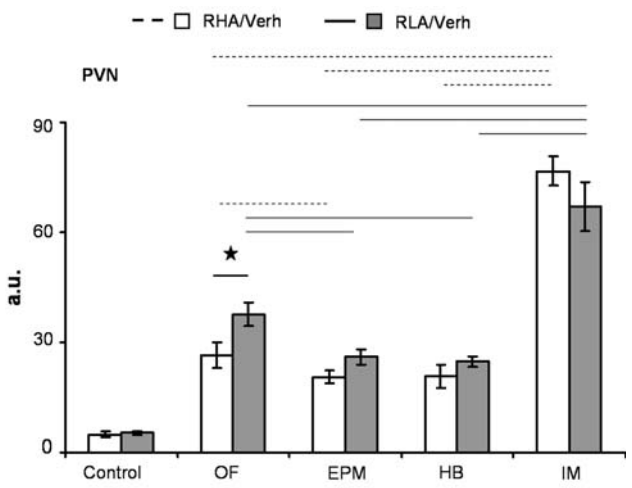

Fig. 5 Neuronal activation pattern of the paraventricular nucleus (*PVN*) of the hypothalamus in control, open field with illuminated center (*OF*), elevated plus maze (*EPM*), hole board (*HB*), and acute restraint (*IM*) conditions. Values are presented as mean ± SEM. ★ stands for $P < 0.05$ as revealed by MANOVA followed by posthoc Fischer/NIR test. *Dashed lines* represent differences ($P < 0.05$) between tests from RHA/Verh rats, *full lines*: for RLA/Verh rats. *White bars* represent RHA/Verh, *gray* RLA/Verh rats

EPM exposure in CA2 and CA3 fields of the hippocampus (Fig. 4a–c, dashed lines), while no difference was observed in RLA/Verh rats.

Discussion

This study is to the best of our knowledge the first one showing that the divergent spontaneous behavioral response of RHA/Verh and RLA/Verh rats to mild anxiogenic stimuli is accompanied by different neuronal activation in restricted parts of the fear/anxiety circuit. The results of principal component analysis (PCA) applied individually for each behavioral test, together with c-Fos protein expression data suggest that RHA/Verh and RLA/Verh rats display distinct sensitivity to stressogenic and spatial aspects of the novel stimuli. This might result from distinct neuronal activation patterns in the medial prefrontal cortex (mPFC), nuclei of amygdala (BLA, CeA, MeA, and CoA), paraventricular nuclei of the hypothalamus (PVN), and hippocampus (CA1, CA2, and CA3 fields), respectively.

The previous studies on Roman rats showed that they react to novel environments in a highly different way, both at the level of behavior (Driscoll and Bätting 1982; Driscoll et al. 1998; Escorihuela et al. 1999; Steimer and Driscoll 2003, 2005) and hormonal changes in the HPA axis (Gentsch et al. 1982; Walker et al. 1989; Steimer et al. 1997; Steimer and Driscoll 2003), amygdala (Roozendaal et al. 1992; Wiersma et al. 1997; Yilmazer-Hanke et al.

2002) and hippocampus (Walker et al. 1989). Our study shows that these differences are also apparent at the level of expression of one of the immediate early genes, c-Fos protein, involved in transcription of stress hormones (for review see Kovacs 1998). Distinct patterns of c-Fos expression were found in other rat models of diverse emotionality, such as the HAB and LAB rats (Salomé et al. 2004), Sprague–Dawley high responders (HR) and low responders (LR, Kabbaj and Akil 2001) as well as animals selected for active and passive response to OF (Babai et al. 2001) in many brain areas involved in regulation of emotions. The data on correlation of c-Fos expression with emotionality are nevertheless often contradictory, as the rat models differ from one another and the tests used by the authors are not the same (for review on that problem see Kovacs 1998). The differences can be observed already at the level of baseline c-Fos protein expression (Kabbaj and Akil 2001; Salomé et al. 2004). In our study no differences were observed between RHA/Verh and RLA/Verh in this respect. The diverse handling procedures between laboratories could be the reason for this discrepancy. In our study, similarly to the one done on HAB and LAB rats (Salomé et al. 2004) we applied sufficient amount of handling prior to behavioral testing, to reduce the difference in initial level of presented anxiety in both hyper- (RLA/Verh) and hyporeactive (RHA/Verh) rats.

In our experiment multivariate analysis of variance (MANOVA) followed by posthoc Fischer/NIR test revealed that the two lines of rats differ in c-Fos protein expression. The hyper-reactive RLA/Verh rats display higher activation rate than RHA/Verh rats following exposure to a novel situation. Moreover, this response is stress-dependent. For mild stress of a novel environment such as the OF, EPM, and HB we have observed a gradient of activation reflecting stressorgeneity of the behavioral challenge. This effect was both structure and line specific. Our results indicate that structures involved in acquisition and processing of emotional aspects of a novel environment, such as medial prefrontal cortex, amygdala, and hypothalamic paraventricular nucleus (LeDoux 2000; Knapska et al. 2007) react with highest activation to the OF, followed by EPM and HB tests. The dorsal hippocampus, known to be involved in assessment of spatial aspects of environment (for review see Moser et al. 2008), on the contrary, showed the highest number of c-Fos positive nuclei following exposure to the HB, followed by the EPM and the smallest activation was found in response to the OF. As to line-specific differences, it should be pointed out that RLA/Verh rats seem to display increased sensitivity to emotional aspects of novel situations, as they better discriminate stressorgeneity of OF from other tests (Figs. 2, 3, 5). On the other hand, the RHA/Verh rats show increased sensitivity to spatial features of novel stimuli, as they differentiate better between the HB, EPM, and OF than RLA/Verh rats (Fig. 4). The fact that the activation rate of the dorsal hippocampus is nevertheless higher in RLA/Verh rats may reflect a generally higher activation of the whole hippocampus, which may be due to strong amygdaloid input to the ventral hippocampus and its role in regulation of emotion (Kjelstrup et al. 2002). The role of the ventral hippocampus in development of distinct emotional traits of RHA/Verh and RLA/Verh rats remains to be investigated.

The difference in the stress-dependent pattern of activation of the hippocampus and the structures involved in regulation of emotions may result from the interplay between these structures. According to Cullinan et al. (1995), the hippocampus actively inhibits the PVN. This would explain why in behavioral tests providing more spatial stimulation (like EPM or HB), the activation of the PVN is smaller than in the OF, which provides little spatial stimuli due to illumination of the central part of the arena only.

In order to see whether the line-specific diversity in stress-dependent activation of brain structures belonging to the fear/anxiety circuit is indeed related to the sensitivity to distinct aspects of the novel environment, we have subjected RHA/Verh and RLA/Verh rats to the acute restraint test (IM). Acute restraint represents a stressor of a totally different nature than spontaneous behavioral tests. The apparatus itself provides mostly tactile sensation of immobilization and little information about the surrounding area, as it is placed in a dimly lit, soundproof box. The immobilization is a source of strong stress, but it is due to physical rather than psychological distress (Dayas et al. 2001; Pace et al. 2005). According to Pace et al. (2005) it should therefore elicit little activation in the hippocampus. This is in agreement with the data presented here since the activation of the CA2 (RLA/Verh) and CA3 (both lines) fields of hippocampus was comparable after exposure to the HB and IM. The activation of the mPFC, amygdala, and PVN by IM on the other hand was significantly higher than the ones evoked by OF, EPM, and HB. At the same time we found no line-specific differences in the number of activated, c-Fos positive cells in these structures, which could indicate the existence of a "ceiling effect", a phenomenon previously observed for corticosterone response to acute restraint in these rats by Gentsch et al. (1982). The "ceiling effect" in this case means, that the lack of difference in activation of the fear/anxiety circuit between RHA/Verh and RLA/Verh rats is a result of a similar sensitivity to physical threat provided by the acute restraint procedure. The answer to that threat is crucial for survival of the individual, and therefore no line-specific or personality-dependent differences in response to it should be expected.

It might be assumed that the different pattern of neuronal activation following mild stress of a novel

environment might underlie different motivational factors driving the behavior of RHA/Verh and RLA/Verh rats. For this reason we applied PCA to a group of parameters characteristic for each of the tests measuring spontaneous behavior.

In the open field with illuminated center PCA allowed the extraction of two independent factors, representing locomotor activity of the animal and its anxiety level. This is in accordance with earlier studies by Whimbey and Denenberg (1967), Boguszewski and Zagrodzka (2002) and Aguilar and co-workers in the study on F2 intercross offspring of RHA/Verh and RLA/Verh rats (2002). Factor analysis revealed that RHA/Verh and RLA/Verh rats despite having a generally similar factor pattern, where 65% of total variance was a result of impact of parameters reflecting locomotor activity (distance moved and movement duration in the thigmotaxic zone and the whole arena), while 27 and 22% (respectively) reflect parameters correlated with anxiety (number of entries to the illuminated center and movement duration in that zone), are likely to "perceive" the aversive features of the test differently. This is well illustrated by the fact that in RHA/Verh rats the distance moved in the illuminated part of the arena loads to Factor 1 (locomotor activity) while in RLA/Verh rats it loads to Factor 2 (anxiety). This could suggest, that while RHA/Verh rats consider it stressful, but worth exploring, the RLA/Verh once they enter this zone, leave it immediately without much exploration.

One could expect that the behavior of highly emotional RLA/Verh rats should be mostly driven by anxiety, which should constitute Factor 1 for these rats. In our experiment it was not so. This might be due to the generally low mobility of these rats (as compared with RHA/Verh rats), which in statistical terms of PCA analysis proved to be a stronger effect than lack of exploration of the central, aversive part of the arena. Whether the low mobility could be entirely explained by enhanced anxiety of RLA/Verh rats is disputable, as the extracted factors are not correlated with one another (but they are not independent either). It could nevertheless point to a different coping strategy of RLA/Verh rats, since the possibility of it being due to movement impairment is excluded by the results obtained for homecage activity observation (HAO, Control group).

In the elevated plus maze three factors were extracted, representing locomotor activity, anxiety and risk assessment behavior. The data obtained for RHA/Verh rats in our study are similar to those presented by Aguilar et al. (2002) for F2 intercross offspring of RHA/Verh and RLA/Verh rats. The first factor in both cases receives high loadings from parameters describing the anxiety level (ratio of entrances to open/enclosed arms, number of entrances to open arms, time spent, and distance moved in them), the second seems to represent locomotor activity (distance moved and movement duration in enclosed arms and the whole maze), and the third shows risk assessment activity (number of SAPs to open arms, time spent on the central platform). The behavior of the RLA/Verh rats on the other hand seems to be driven mostly by risk assessment (responsible for as much as 61% of total variance), followed by anxiety and finally, the least by motor activity. This clearly shows that the two lines of rats "perceive" the novel environment of EPM differently. While RHA/Verh rats explore the maze willingly, due to their inborn sensation-seeking profile (Siegel et al. 1993), the RLA/Verh prefer to keep to confined spaces and only look out into open arms in order to assess the risk. This effect might be furthermore enhanced due to the fact that they are given choice of either exploring the open arms of the maze or not (as they are introduced to the test from the end of the enclosed arm), so unlike HAB and LAB rats exposed to open arm paradigm (Salomé et al. 2004) they can choose to remain in the enclosed arm space, and enter the open arms only driven by their inquisitiveness.

For hole board test a different number of factors were extracted for RHA/Verh and RLA/Verh rats. In case of RHA/Verh rats there were two factors: one showing exploratory drive of the animal, represented by exploration of the holes and central part of the arena, the other representing other locomotor activity of the animal. In RLA/Verh rats a third factor, almost exclusively dedicated to nose poke activity arose. The fact that the nose poke activity gives a negative loading to this factor, and that number of entrances to the central part of the arena positively loads to this factor might indicate that it shows the anxiety level of the animal, which is in this case separated from exploratory drive. The lack of such discrimination in RHA/Verh rats clearly suggests that the two lines of rats "perceive" this challenge differently despite showing no significant differences in hole exploration. This effect can be enhanced by placement of novel objects in the holes (Escorihuela et al. 1999) or modification of the hole board arena (Ohl et al. 2001). Our results were obtained for shallow, 1 cm deep holes with no objects in them and are in accordance with the results obtained for inbred lines of Roman rats (RHA-I/Verh and RLA-I/Verh) by Escorihuela et al. (1999) for a no-object paradigm.

Due to reduction of the number of variables to 2–3 per test, we provide a coherent insight into motivational factors that drive the distinct behavior of the two lines of rats. Taken together with c-Fos expression data, they draw a more detailed picture of what underlies diverse coping styles of RHA/Verh and RLA/Verh rats (Steimer and Driscoll 2005). This diversity is only apparent when animals are performing spontaneous behavior tests. We can therefore speculate about different personality or temperamental traits, as suggested by Steimer and Driscoll (2003).

The active coping style of RHA/Verh rats allows greater exploration of the novel environment compared to RLA/Verh rats. In the OF, it provides more spatial stimulation, which in turn may attenuate the stress response of the PVN (Cullinan et al. 1995) and other structures involved in regulation of emotions and encourage more intense exploratory behavior. The RLA/Verh rats react passively to the same stimulus, which inhibits exploratory drive and leads to robust neuronal activation of the fear/anxiety circuit. In the EPM we observe a similar effect. The active versus passive coping strategies give rise to different motivational conflicts in the two lines. While for RHA/Verh rats the conflict arises between anxiety and locomotor/exploratory activity, and is by and large won by exploratory drive, in RLA/Verh rats the conflict arises between anxiety and risk assessment. Both these drives in RLA/Verh rats limit the amount of exploration and spatial stimulation and allow a robust activation of the fear/anxiety circuit due to emotional aspects of the same environment, which in turn inhibits locomotor and exploratory activity.

The HB test provides little stressful and a lot of spatial information and should be considered the mildest of stressors used in our study. In RLA/Verh rats it elicits anxiety nonetheless. This line-specific difference could be due to diverse activation of the mPFC, as it is the only brain structure, apart from CA1 and CA2 fields of the hippocampus in which we observed line-specific differences in neuronal activation upon HB exposure. This may suggest that the distinct personality traits are a function of subtle differences in functioning of structures involved in higher processing of emotional stimuli.

This points to a conclusion, that perhaps, the main difference between the two lines arises from different interpretation of the nature of stressors. This would explain why for RHA/Verh a novel environment is a challenge they willingly explore, while the RLA/Verh rats represent far more reluctant attitude and respond with higher anxiety level and neophobic reactions.

In general, we conclude that the diverse emotional profiles of RHA/Verh and RLA/Verh rats are not only a result of higher reactivity of RLA/Verh rats, observed in our study as a higher activation rate of all investigated brain structures. Since this effect is observed only after exposure to behavioral challenge involving spontaneous activity, and is not present in control and highly stressful conditions, we believe, that it is based on diverse interpretation of aversive features of presented stimuli. This most likely arises from differences in the sensitivity to distinct aspects of the behavioral challenges.

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