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ORIGINAL ARTICLE

Angiotensin II enhancement during pregnancy influences the emotionality of rat offspring (*Rattus norvegicus*) in adulthood. Potential use of the Rat Grimace Scale

Tomáš Senko, Lucia Olexová, Miroslava Mokošáková, Lucia Kršková

Department of Animal Physiology and Ethology, Faculty of Natural Sciences, Comenius University in Bratislava, Slovakia

Correspondence to: Tomáš Senko, MSc.
Department of Animal Physiology and Ethology
Faculty of Natural Sciences, Comenius University
Ilkovičova 6, 842 15, Bratislava 4, Slovakia.
e-mail: senko5@uniba.sk

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Abstract

OBJECTIVES: One of the systems, which can be prenatally reprogrammed, is the renin–angiotensin–aldosterone system (RAAS). The aim of our experiment was to determine how prenatal activation of RAAS via exposure to elevated levels of angiotensin II (Ang II) influences the rat offspring's emotionality.
METHODS: Pregnant female rats were implanted with osmotic minipumps that continually released Ang II and oval object of the same shape and size was implanted into control dams. The adult offspring (AngII and control groups) were tested in rat grimace scale (RGS), open field test (OF) and elevated plus maze (EPM).
RESULTS: Psychological stress increased the RGS score in both groups of animals. AngII animals had significantly lower RGS score (i.e. less negative emotions) in the home cage but higher index of emotional reactivity in RGS. AngII animals had also significantly lower frequency of defecation in OF and had no effect on changes in anxiety-like behaviour.
CONCLUSION: We concluded that maternal activation of RAAS modified some aspect of emotionality of experimental animals and led to an enhanced emotional response to stress situation.

INTRODUCTION

Over the past decades, it has been increasingly recognized that prenatal environmental and pharmaceutical exposures can adversely influence fetal programming, which plays a role in the risk of many adult health disorders. One of the systems, which can be prenatally reprogrammed, is the renin-angiotensin-aldosterone system (RAAS). The RAAS is an important regulator of the blood pressure and the fluid/electrolyte homeostasis (Zhuo & Li 2011) but the components of the RAAS present in the central nervous system modulate many emotional and behavioural responses (Llorens-Cortes & Mendelsohn 2002; Von Bohlen Und Halbach & Albrecht 2006; Bali *et al.* 2014). RAAS

is important in the pathogenesis of various cardiovascular diseases, accompanied by behavioural changes (Ciobica *et al.* 2011; Saavedra *et al.* 2011; Duchemin *et al.* 2013) *e.g.* increased anxiety behaviour (McLaughlin *et al.* 2003; Johansen *et al.* 2012; Tuovinen *et al.* 2012) that is often considered as part of emotionality (Ramos & Mormède 1997). Also animals with changes in RAAS show changes in emotionality and anxiety-like behaviour (Gentsch *et al.* 1987; Söderpalm 1989; Wilson *et al.* 1996; Voigt *et al.* 1999; Goto *et al.* 1993; Ferguson & Gray 2005; Kršková *et al.* 2009).

Animal models are an invaluable tool to understand the prenatal programing. Emotional states of these animals may be inferred from their behaviour. There are many tests of emotionality including anxiety, e.g. open field test, elevated plus maze test, black/white box (Hall 1934; Archer 1973; Royce 1977; Ramos & Mormède 1997), but different emotional states can be expressed through changes in facial expression, which have been recognized in a variety of animal species with dissimilar facial musculature (Keating *et al.* 2012; Waller 2013, Dalla Costa *et al.* 2014; Wathan 2015), including mice (Langford *et al.* 2010; Defensor *et al.* 2012) and rats (Sotocinal *et al.* 2011; Finlayson *et al.* 2016).

Rat grimace scale (RGS) is a system that was developed to determine the level of pain (Langford *et al.* 2010; Sotocinal *et al.* 2011; Dalla Costa *et al.* 2014) and the emotional facial expressions reflect biological responses to stress (Lerner *et al.* 2007). Changes in facial expressions reflect not only physical stress (*e.g.* application of a substance inducing pain), but also psychological (mental) stress. This statement is supported by a study performed in humans (Madokoro & Sato 2012) that analysed the relationship between facial expressions and intensity of psychological stress. In mice, changes in facial expressions in response to social stimuli were identified (Defensor *et al.* 2012). Moreover, the study of Langford *et al.* (2010) contains a brief reference about restraint stress resulting in a tendency towards increased grimace scales scores.

There is still little knowledge about the relationship between facial expression and the effect of psychological stress in laboratory rodents. Therefore, the first aim of this study was to verify whether the exposure of rats to psychological stress – represented by restraint stress in an immobilisation chamber – affects RGS scores, which reflect the presence of emotions in facial expression. But the major aim of our experiment was to find out whether prenatal activation of RAAS via exposure to elevated levels of Ang II influences an offspring's emotionality including anxiety-like behaviour.

MATERIAL AND METHODS

In our experiment, Wistar rats (VELAZ Praha, Czech Republic) were used. The parental generation consisted of nine females and five males. Animals were housed in groups of two or three animals (separately males and females) in standard light conditions (12:12 h; lights on at 6 am), with an average temperature of 21±2 °C and with relative humidity 55±10%. Water and food (standard laboratory chow) were available *ad libitum*. After an acclimatisation period of 7 days, female rats were divided into two groups: the control group (Ctrl: n=4), and the angiotensin II group (AngII: n=5). The animals were mated overnight. The phase of ovulatory cycle and the zero day of gravidity (the presence of spermatozoa in a vaginal smear) were identified according to the Gleich and Frohberg's method (Gleich & Frohberg 1977). After fertilization, animals were kept under the same conditions as before.

On the sixth day of gravidity, we implanted osmotic minipumps (model 2002, Alzet, Canada) placed subcutaneously on the back into the mothers of the AngII group, through which Ang II (concentration 1.36 mg/ml in physiological solution) was continually released at 2 μg/kg/h for 14 days. In contrast, an oval object of the same shape and size (sham operation) was implanted into Ctrl mothers. Narcosis was carried out using solution of ketamine (Narketan™ 10, Chassot GmbH, Germany) (concentration 135 mg/ml) and xylazine (Rometar 2%, Spofa, Czech Republic) (concentration 18 mg/ml). On the tenth day of gravidity, dams were housed individually. The litters were postpartum culled to eight animals per litter (four males, four females). Every mother with the litter was housed in its own cage until weaning at postnatal day 21. After weaning, rats of each gender were housed separately in groups of four animals per cage.

Body weight did not differ between control and AngII dams before, or at the end of pregnancy. Ang II administration had no effects on litter size or birth weight of the offspring. Sex ratio of pups was similar between both groups and the body weight did not change in male or in female rats during the experiment. Increased Ang II during pregnancy raised blood pressure in the offspring. Treatment also increased aldosterone and decreased plasma renin activity (Svitok *et al.* in press).

In the adulthood, the Ctrl (males [n=6] and females [n=6]) and AngII (males [n=6] and females [n=6]) were tested in three behavioural tests – the rat grimace scale, the open field test and the elevated plus maze.

At 68–69 days of age, the offspring were tested with RGS. To determine the RGS score, we modified the method of Sotocinal *et al.* (2011). Each animal was first observed in the home cage and was subsequently transferred to the immobilisation chamber (adapted to the size of the animal) which is considered as a mildly stressful context characterised by immobilisation and social isolation (Paré & Glavia 1986; Brown *et al.* 2005).

We tested animals in 10-minute intervals in both conditions. The facial expressions of rats were recorded using a camera (Lumix DMC-TZ 7, Panasonic, Japan). We took one photo every minute, and so in total, there were 10 photos of each animal in the home cage and

Tab. 1. The four action units of Rat grimace scale.

Orbital tightening	Negative emotions involve a narrowing of the orbital area, a tightly closed eyelid, or an eye squeeze. An eye squeeze is defined as the orbital muscles around the eyes being contracted.
Nose/Cheek flattening	Negative emotions involve flattening of the nose, which, in turn, changes the length of the snout. This flattening and change in length can be detected by the bump on the bridge of the nose fading as the furrow separating the whiskers from the cheeks deepening
Ear changes	Negative emotions involve folding and curling of the ears. Ear position on the head is changing accompanied by rotation of the ears to the sides and by extending space between auricles.
Whisker changes	Negative emotions involve moving forward of the whiskers (away from the face) from the baseline position and tend to bunch, giving the appearance of whiskers standing on end.

Note. Modified from Sotocinal *et al*. 2011.

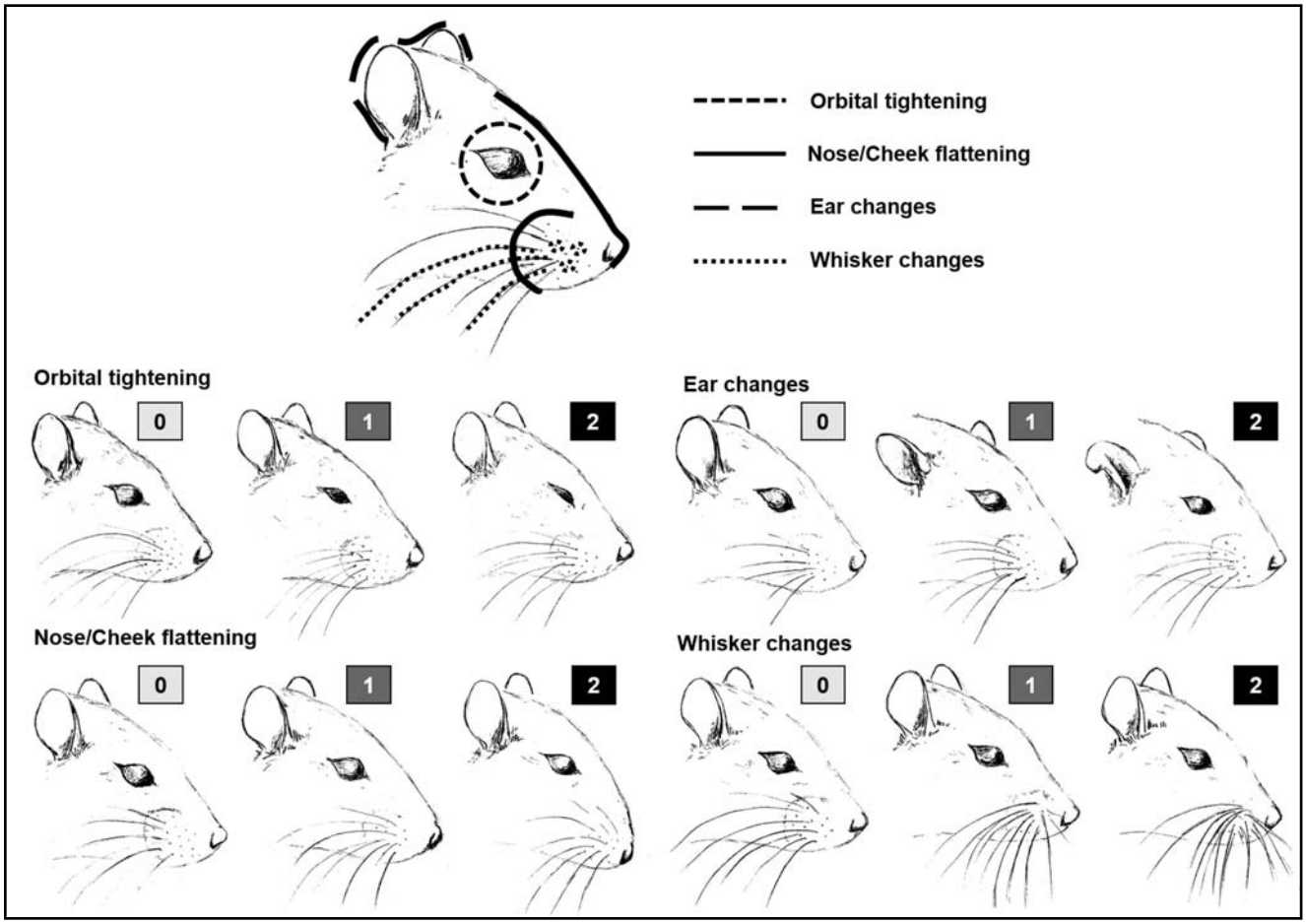

Fig. 1 Illustrative image of Rat grimace scale (RGS) – The four action units: Orbital tightening, Nose/cheek flattening, Ear changes, Whisker changes. Numbers and brightness of colours show the present level of negative emotions: 0 – not present; 1 – moderate; 2 – obvious. (Created on the base of Sotocinal *et al.* 2011).

another 10 photos in the immobilisation chamber. Each photo was analysed by three independent trained observers who rated the photographs by four action units expressing the emotional state of the animal (Table 1).

Each of the action units was scored from 0 to 2, with the score denoting the intensity of emotional expression: (a) "0" indicates high confidence of the scorer that the action unit was absent; (b) "1" indicates either high confidence of a moderate appearance of the action unit or equivocation over its presence or absence; (c) "2" indicated the detection of an obvious appearance of the action unit, with high confidence (Figure 1) (Sotocinal *et al.* 2011) Subsequently, we calculated the RGS score for each animal. The RGS score was obtained from the mean score of the individual ratings of action units. RGS values obtained by each of the observers were averaged. Inter-observer reliability was evaluated using a Cronbach's Alpha, which gave a coefficient of 0.895. Therefore, the scores of the three observers were

averaged and used for statistical analysis. An index of emotional reactivity was calculated as the difference between the RGS score in the stress situation and the RGS score in the home cage.

At 73 days of age, all the animals were tested in the open field test – Conducta system (Experimetria Ltd., Hungary). The testing chamber of this system consisted of a dark plastic box (48×48×40 cm) with its floor divided into 25 squares (5×5) and built-in infrared beam lights (16 diodes at 16 mm distance from each other in the three lines) for recording the animals' movement. During the light phase, each animal was put into the centre of the testing chamber and subsequently tested for 20 minutes. The information about locomotor activity (ambulation distance) expressed as progressive movement in cm was exported from Conducta system. Ambulation distance in inner zone (inner square 3×3) was used as measure of the anxiety level and frequency of defecation was used as measure of emotionality.

At 77 days of age, the offspring were individually tested for 5 minutes in the elevated plus maze. The maze was made of wood and consists of four arms in the shape of cross: two open arms (50×10 cm) and two arms of the same size with an open roof but enclosed by walls (40 cm high). The two open arms are opposite each other and converge into a central platform (10×10 cm). Rats were placed initially in the central platform facing a corner allowing an equal choice of entering an open or closed arms. Time spent in open/closed arms and numbers of arm entries were used as measures of the anxiety level. Frequency of defecation was used as a measure of the emotionality (Walf & Frye 2007). All these parameters were registered manually.

All the data are expressed as mean ± SEM and were analysed using STATISTICA v 7.0 (StatSoft, Inc., Tulsa, USA). RGS score was analysed by general linear model two-way analysis of variance – ANOVA (gender × group) with conditions as a repeated measure. Index of emotional reactivity, behavioural parameters of emotionality/anxiety in open field test and elevated plus maze were analysed with two-way ANOVA (gender × group). If the interaction was significant ($p<0.05$), differences in behavioural parameters between control and AngII rats were estimated by Bonferroni *post hoc* test.

Methods and procedures of the present study were approved by the local Ethics Committee of the Comenius University in Bratislava, Slovak Republic and the Directive of the European Parliament and of the Council on the protection of animals used for scientific purposes (2010/63/EU) was followed.

RESULTS

Information about the relationship between facial expression and the effect of psychological stress in laboratory rodents is lacking. Therefore, we first evaluated the influence of psychological stress – represented by restraint stress in immobilisation chamber – on the RGS score. Two-way ANOVA for repeated measures revealed significant differences in conditions, $F_{(1, 20)}=764.233$, $p<0.001$, gender, $F_{(1,20)}=15.677$, $p<0.001$, and interaction conditions × group, $F_{(1,20)}=21.259$, $p<0.001$.

Restraint stress induced an increase of RGS score ($p<0.001$) in comparison with the home cage. Differences between Ctrl and AngII group were significant only in the home cage. In this condition, the AngII group reached a lower RGS score ($p<0.05$) in comparison with the Ctrl group (Figure 2a). Males achieved a significantly higher score than females ($p<0.001$).

In emotional reactivity, using two-way ANOVA we found significant difference in group, $F_{(1,20)}=21.2587$, $p<0.001$. Animals prenatally exposed to Ang II reached a higher emotional reactivity (Figure 2b).

In the open field test, two-way ANOVA revealed a significant differences in frequency of defecation for group, $F_{(1,20)}=4.734$, $p=0.032$. Animals prenatally

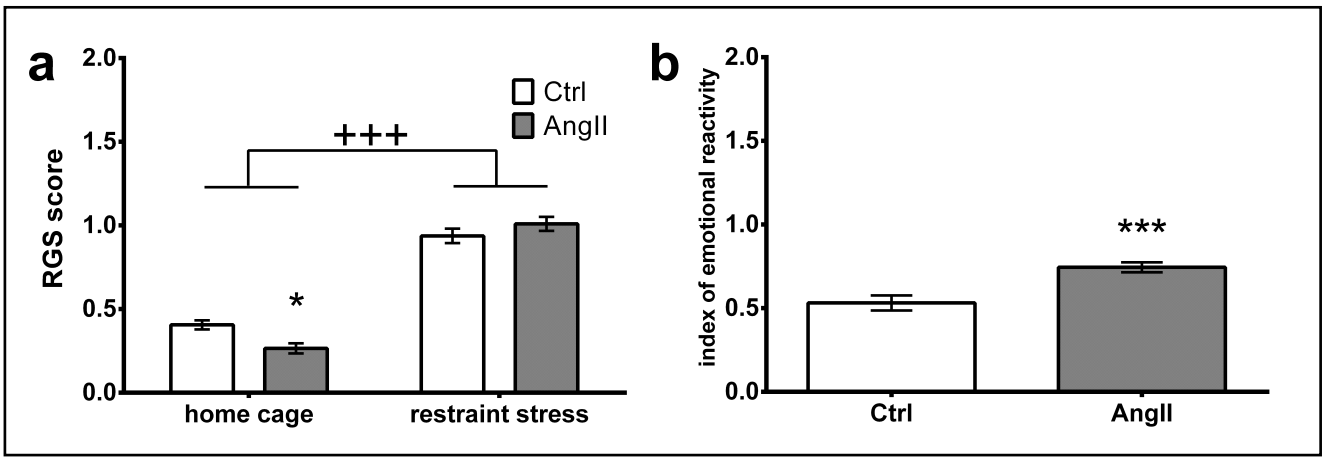

Fig. 2. RGS scores (**a**) and emotional reactivity (**b**) in control (Ctrl n=12) and angiotensin (Ang2 n=12) groups in two conditions - home cage, restraint stress. Data are expressed as means ± SEM. Asterisks indicate significant differences between groups (* $p<0.05$; *** $p<0.001$). Plus symbols indicate significant differences between conditions (+ + + $p<0.001$).

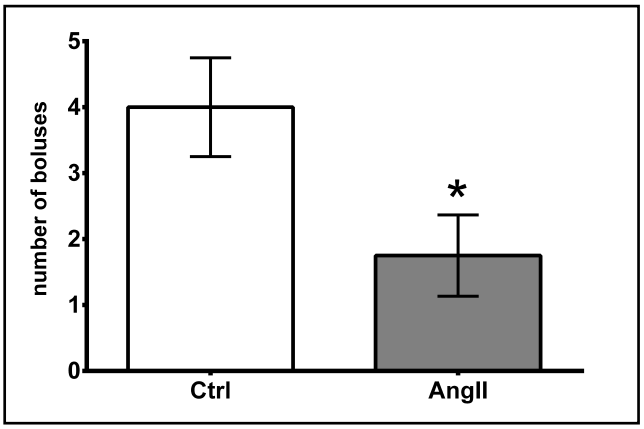

Fig. 3. Frequency of defecation (20 minutes open field test) in control (Ctrl n=12) and angiotensin (Ang2 n=12) group. Data are expressed as means ± SEM. Asterisks indicate significant differences between groups (* $p<0.05$).

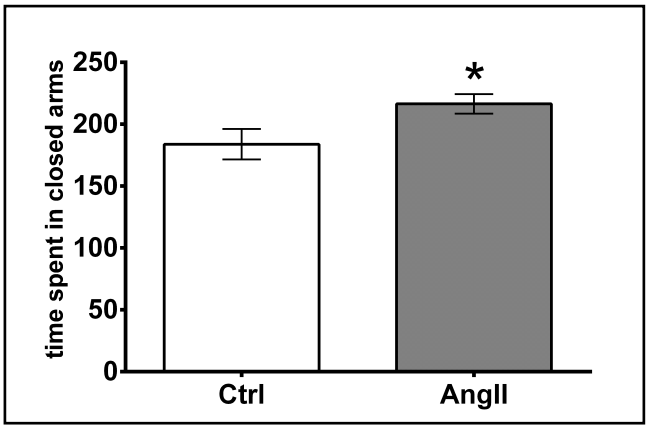

Fig. 4. Time spent in closed arms (elevated plus maze) in control (Ctrl n=12) and angiotensin (Ang2 n=12) group. Data are expressed as means ± SEM. Asterisks indicate significant differences between groups (* $p<0.05$).

exposed to Ang II had reached lower frequency of defecation (Figure 3). There were no significant changes in total ambulation distance and ambulation distance in the inner zone.

In the elevated plus maze, two-way ANOVA revealed a significant differences only in time spent in closed arms for group, $F(_{1, 20})=4.527$, $p=0.046$. Animals prenatally exposed to Ang II spent significantly more time in closed arms (Figure 4). There were no significant changes in numbers of arm entries and frequency of defecation.

DISCUSSION

One of the systems, which can be developmentally reprogrammed, is the RAAS. In our experiment, we were finding out whether prenatal activation of RAAS via exposure to elevated levels of Ang II influences an offspring's emotionality including anxiety-like behaviour in adulthood.

We used classical methods to determine emotionality in laboratory rats – the open field test, the elevated plus maze and we also used a relatively new method – the rat grimace scale.

By employing four action units (Sotocinal *et al.* 2011) we observed changes in emotions in the face of rats after exposure to psychological stress. In our study, psychological stress (restrain stress) resulted in significant changes of the RGS score in both groups. Ctrl and AngII animals had higher RGS scores in the immobilisation chamber in comparison with the home cage. These results confirmed our hypothesis that psychological stress increases the presence of negative emotions. The study of Langford *et al.* (2010), which was performed in mice, found only a trend towards elevated baseline MGS scores (parallel of RGS score) after using a restraining apparatus, but in our study, there was clearly a significant increase of the RGS score. Nevertheless, more extensive information about the relationship between facial expression and the effect of psychological stress in laboratory rodents is absent. Animals prenatally exposed to a higher level of Ang II had low RGS score (*i.e.* less negative emotions) in the home cage.

In the most common behavioural test, the open field test, we observed that AngII group had a lower frequency of defecation in unknown environment of testing chamber. These results suggest a lower level of emotionality in the AngII group. However, higher index of emotional reactivity in RGS suggest a stronger emotional response to stress situation (immobilization stress in restraint chamber) in this group.

We did not find significant differences in anxiety-like behaviours. The only difference we were able to find between groups was that AngII animals spent more time in closed arm in EPM. However, we could not consider AngII animals as more anxious whereas differences in other anxiety-like behaviors (time spent in open arms, number of arm entries in EPM and ambulation in inner zone in OF) were not present.

Our results indicate that prenatal exposure to elevated levels of Ang II, although did not change anxiety-like behaviour, significantly influenced some aspects of emotionality and led to an enhanced emotional response to stress situation.

Studies describing behavioural effects of prenatal exposure to elevated levels of Ang II are lacking. Therefore, we compare our results with animal models with disrupted regulatory mechanisms of blood pressure control, *i.e.* TGR – transgenic (mREN2)27 rats, SHR – spontaneously hypertensive rats. TGR rats with up-regulated RAAS are considered more emotional based on increased self-grooming, frequency of defecation and level of anxiety-like behaviour (Wilson *et al.* 1996; Voigt *et al.* 1999; Kršková *et al.* 2009).

Our findings are in some aspects in line with data of another animal model – SHR rats, with an up-regulated

sympathetic nerve system. SHR animals have a lower frequency of defecation and spent less time with self-grooming in the open field test, are therefore considered less emotional than normotensive controls (Goto *et al.* 1993; Ferguson & Gray 2005). But on the contrary to our present results, SHR rats have reduced anxiety-like behaviour that is represented by more time spent in the inner zone of the open field test (Gentsch *et al.* 1987; Söderpalm 1989; Goto *et al.* 1993).

Nonetheless, based on our results, we can conclude that psychological stress is strong enough to influence the presence of negative emotions in the face of rats and, therefore, the RGS score is an appropriate method for determination of emotionality. In our opinion, this method can supplement the evaluation of emotionality in the open-field test based mainly on ambulation and defecation (Ramos & Mormède 1997). New model based on activation of RAAS via prenatal exposure of elevated levels of Ang II enables better understanding of the development of emotional problems connected with cardiovascular diseases.

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Conflict of interest statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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