Psychiatry Research 271 (2019) 649–657

Contents lists available at ScienceDirect

Psychiatry Research

journal homepage: www.elsevier.com/locate/psychres

Intervention mechanism of repeated oral GABA administration on anxiety-like behaviors induced by emotional stress in rats

He Yongjian[a,b,#], Ouyang Junyan[a,b,#], Hu Zhuoyan[a], Yang Jie[a,b], Chu Yue[a,b], Huang Shaowen[a,b], Yang Yichao[c], Liu Chunhong[a,b,*]

[a] College of Food Science, South China Agricultural University, Guangzhou, GZ, China
[b] Guangdong Provincial Key Laboratory of Food Quality and Safety, South China Agricultural University, Guangzhou 510642, China
[c] School of Public Health, Guangzhou Medical University, Guangzhou, GZ, China

ARTICLE INFO

Keywords:
GABA
Emotional stress
Nitric oxide
Anxiety-like behaviors
OF
EPM

ABSTRACT

The purpose of this study was to investigate the effects and mechanism of repeated oral administration of gamma-aminobutyric acid (GABA) on anxiety-like behaviors induced by emotional stress. Male Sprague-Dawley rats were randomly divided into five groups (8 rats each): control, emotional stress model, three emotional stress + GABA-treated groups (0.5, 1, 2 mg/kg). The rats were given empty water bottles after the training of drinking water to induce emotional stress. Each group was treated with saline or different doses of GABA respectively for 21 consecutive days. Then open field and elevated plus maze were used to assess anxiety-like behaviors. Both frontal cortex and plasma NO metabolites nitrate and nitrite (NO_x) levels were determined spectrophotometrically. Results showed that oral administration of GABA significantly reversed the stress-induced anxiety-like negative responses dose-dependently. The frontal cortex NO_x levels were lower in stressed rats than in control group ($P < 0.05$), but higher in 2 mg/kg GABA-treated group than stress model group ($P < 0.05$). On the other hand, NOx levels in plasma showed a gradual decline trend. Collectively, these results suggest that short repeated oral administration of GABA has an anxiolytic-like effect possibly via preventing NO reduction caused by stress and improving availability of NO in the frontal cortex.

1. Introduction

Gamma-aminobutyric acid (GABA), the most abundant inhibitory neurotransmitter in the central nervous system (CNS) (Defaix et al., 2018) is known to be synthesized from glutamate and acts on $GABA_A$ and $GABA_B$ receptors to mediate synaptic inhibition (White et al., 1998). Central administration of GABA has long been implicated to affect anxiety-like behaviors in animals (Bi et al., 2013; Solati et al., 2013). However, GABA crosses the blood-brain barrier (BBB) poorly owing to its high polarity and flexible structure (Al-Awadi et al., 2006), so there is little research about the effects of oral administration of GABA. Recently, an animal study reported that decreased walking time and urinary cortisol level were observed in dogs after a single oral administration of GABA (Uetake et al., 2012), indicating a calming effect of GABA. In human studies, Abdou et al. (2006) found that a single oral intake of GABA significantly increased alpha waves and decreased beta waves in brain. With these they concluded that GABA could induce relaxation and diminish anxiety. In addition, Nakamura et al. (2009) found that taking GABA chocolate before an arithmetic task could reduce sympathetic nerve activity by estimating the activity of the autonomic nervous system through heart rate variability and no significant change was seen in salivary Chromogranin A (an index for acute psychological stress) levels, suggesting a psychological stress-reducing effect of GABA. These findings are all focused on the acute effects of GABA by a single administration; data about the repeated treatment of GABA is scarce. Accordingly, the mechanism of how the oral intake of GABA mediates anxiety-like behaviors and leads to its anxiolytic-like function has not been fully elucidated.

Nitric oxide (NO), which is synthesized from L-arginine (L-arg) by at least 3 subtypes of nitric oxide synthase (nNOS, iNOS and eNOS), functions as a neurotransmitter or intracellular messenger in the central and peripheral nervous system (Moreno et al., 2013; Vincent, 2010). Extensive evidence has revealed that NO is involved in the modulation of stress-induced anxiety-like behaviors and inhibiting NO production can produce an anxiogenic effect under certain conditions(Czech et al.,

* Corresponding author at: College of Food Science, South China Agricultural University, 483#, Wu-Shan Ave., Tian-He District, Guangzhou 510642, China.
E-mail address: liuch@scau.edu.cn (C. Liu).
# These authors share the first authorship.

https://doi.org/10.1016/j.psychres.2018.12.025
Received 29 April 2018; Received in revised form 3 December 2018; Accepted 5 December 2018
Available online 05 December 2018
0165-1781/

2003; Masood et al., 2003), and the frontal cortex is the main tissue associated with NO in the brain (Reif et al., 2006). Moreover, NOS is colocalized with GABA, and it is expressed in most brain regions by small populations of neurons (mainly GABAergic), as well as in spinal cord and peripheral tissues (Gonchar and Burkhalter, 1997). Additionally, $GABA_A$ receptor agonists such as diazepam have been well documented to enhance both NOS activity and NO synthesis, thereby indicating a functional interaction between GABA and NO in the brain (Paul et al., 2001). Thus it is possible that NO may be involved in the mechanism underlying the modulation of GABA on anxiety-like behaviors.

The purpose of the present study was to investigate the anxiolytic-like effect of short -term repeated oral administration of GABA and to clarify whether the intervention effect of GABA was associated with nitric oxide metabolite levels. An empty water bottle was randomly presented to animals which had been trained to drink water at a set time. Anxiety-like responses to emotional stress and GABA were evaluated by open field (OF) and elevate plus maze test (EPM). According to the various evidence mentioned above, we hypothesized that NO production might be decreased in emotional stressed animals and GABA might prevents the reduction of NO caused by stress for its anxiolytic-like ability. To test this hypothesis, we assessed both the plasma and frontal cortex NO metabolites nitrate and nitrite (NO_x) levels, and performed correlation analysis to determine the dose-dependent responses of GABA between NO_x levels and anxiety-like behaviors (Sun et al., 2003).

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (6 to 7 weeks old) were purchased from Guangdong Medical Laboratory Animal Center (Foshan, China) and kept in an animal house at a constant temperature (22 ± 2)°C under a 12/12 h light/dark cycle (lights on 07:00–19:00). All experiments were approved by the Animal Care Committee of the laboratory Animal Center of South China Agricultural University, Guangzhou. Each rat was housed individually in a single rat cage and allowed free access to food and water. Records of body weight and food consumption were obtained weekly. In addition, Animals were required to adapt to the laboratory conditions for one week before experiments, and were handled once per day for 5 min one week before behavioral testing. All experiments were carried out in accordance with the National Institutes of Health/Institutional Animal Care and Use Committee guidelines.

2.2. Experimental design

After the one week adaption period, animals were randomly distributed into five groups ($n = 8$ each): control (CON), emotional stress (ES), emotional stress and different doses of GABA (0.5, 1 and 2 mg/kg body weight, purchased from Sigma, St. Louis, MO, USA), respectively (Kimura et al., 2002). Animal model of emotional stress was induced by empty water bottles stimulation method (Shao et al., 2003). Each group was trained to drink water at 09:00–09:10 and 21:00–21:10 by allowing them access to water bottles only during these time periods for 7 days. After the training period, all rats were randomly given empty water bottles during one of the two watering periods for 14 days except the control group. Rats in control group had free access to water during the two watering periods. During the 21 consecutive days (including the 7 training days and 14 emotion stress days), the three GABA groups were treated with different doses of GABA by gavage, meanwhile, control group and ES group were administered normal saline. GABA was dissolved in distilled water and was administered at a volume of 0.5 mL/100 g. Every day, the rats were gavaged at 8:00, and received training or emotion stress at 9:00 and 21:00. Detailed training and emotional stress procedure was shown in Table 1.

2.3. Behavioral tests

The open field (OF) and elevated plus maze (EPM) tests were used for behavioral evaluation. The OF is one of the most widely used platforms in animal behavioral studies. A number of important conventional and ethological parameters can be collected and analyzed during the performance of the OF. These data allow the researcher to measure behaviors ranging from overall locomotor activity to anxiety-related emotional behaviors (Carola et al., 2002; Seibenhener and Wooten, 2015). The EPM is a reliable measurement tool to investigate anxiety-related defensive behaviors in rodents, particularly rats and mice. It has been used to assess animal anxiety levels (Okonogi et al., 2018). Fig. 1 showed the overall experimental scheme of the study. On the day of behavioral tests, animals were transported to the test room to acclimate for a minimum of 60 min and each animal was tested only once. All tests were video recorded and analyzed using an video tracking system (Noldus Information Tech. Ethovision XT 8.0, Wageningen, Netherlands).

2.3.1. Open field test

The open field test was performed twice through the experiment: after 7-day-adaption period and after the end of the emotional stress. The purpose of the first assessment was to distinguish the locomotor activity of rats, which was a main confounding factor when estimating the anxiety-like behaviors of rats, and rats whose locomotion at the same level were sifted into the following grouping. The second open field test was to determine the behavioral alterations after emotional stress and GABA administration.

The open-field apparatus consisted of an open-topped plastic box (50 cm × 50 cm × 40 cm). The floor area was divided into two parts: a central zone (30 cm × 30 cm) and a peripheral zone. The test room was dimly illuminated with an incandescent lamp (25 W) located 200 cm directly above the center of the open field. Each rat was placed gently into the center of the arena for 5 min free exploration and then returned to its home cage. The floor and walls of the open field were cleaned with 75% ethanol at the end of each test in order to avoid olfactory cues between animals. The following parameters were recorded: (a) time spent in center (time began to be computed at the moment the center point of the animal body entered the central zone and ended upon exit), (b) distance traveled in center, (c) total distance traveled, (d) maximum speed during the test, number of (e) rearing (standing on hind paws with forepaws lifted, with or without touching the walls of the open field) and (f) grooming (including washing or licking forepaws, hind paws, face, body or genitals), (g) moving time (with a speed faster than 1.5 cm/s). An anxiety index was calculated as described in previous literature (Huynh et al., 2011).

$$AnxietyIndex = 1 - [(timeincenter/5min) + (distancetraveledincenter/totaldistancetraveled)]/2$$

Anxiety index and variable (f) were considered the major indices of anxiety-like behaviors. In general, a higher anxiety index, increased grooming numbers and decreased moving time represented increased anxiety. Variables (c) and (d) and were indicative of locomotion while variable (e) was an evaluation indicator of exploratory behaviors.

2.3.2. Elevated plus maze

The EPM test was performed on the following day of the second open field test. EPM is a widely used animal model to assess anxiety-like behaviors (Cai et al., 2010). The EPM apparatus consisted of two open arms (45 cm × 10 cm) and two closed arms (45 cm × 10 cm) enclosed by 30 cm high walls, extending from the central platform (10 cm × 10 cm). The open arms were surrounded by 1 cm upwards convex edge to prevent the rat from falling to the ground. The maze was made of black-painted wood and elevated 80 cm above the ground. An incandescent lamp (25 W) was hanging 130 cm above the central

Table 1
Emotional stress procedure.

Group	Time	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Control	09:00–09:10	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	21:00–21:10	N	N	N	N	N	N	N	N	N	N	N	N	N	N	–
ES/ ES + GABA	09:00–09:10	N	N	EB	N	EB	EB	N	EB	N	N	EB	N	N	EB	EB
	21:00–21:10	EB	EB	N	EB	N	N	EB	N	EB	EB	N	EB	EB	N	–

ES: emotional stress; N: drink water; EB: empty water bottle.
Note: The numbers at the top represent days.

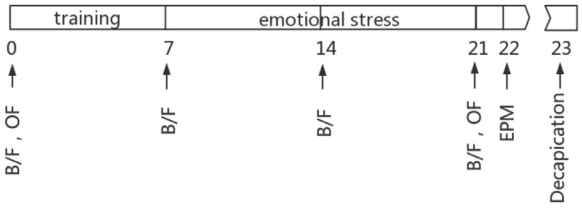

Fig. 1. Experimental timeline of the study. B/F: measurement of body weight and food intake; OF: open field; EPM: elevated plus maze.

platform of the maze, which was monitored by a camera connected to the tracking system. Prior to the EPM, each animal was habituated by handling and placing them in an open field box (50 cm × 50 cm × 40 cm) for 5 min. This procedure could increase animals' locomotion and the likelihood of entering the open arms of the maze when they were exposed to a novel environment immediately before testing in the EPM, which was based on previous studies (Nissen et al., 2012; Zhang et al., 2010), nevertheless, it was independent of EPM exposure (Walf and Frye, 2007). Rat was placed on the central platform facing one of the open arms opposite to the experimenter, for a free exploration of 5 min. Rats were handled in a consistent manner and placed in the same position facing the same open arm. Once the test was over, the rat was returned to its home cage and the floor and walls were cleaned with 75% ethanol and dried thoroughly, prior to the next trial. The following variables were recorded: (a) time spent in open arms, (b) open arm entries, (c) closed arm entries, (d) total distance traveled, number of (e) grooming, (f) time immobile (immobile threshold was set to 5%, the percentage of change in body area below which the subject was considered immobile, and the sample rate was set to 10/s) and number of (g) rearing. An added formula of anxiety index was applied for measuring anxiety in the EPM. Usually, a higher value of anxiety index and longer immobility time were correlative with increased anxiety (Cohen et al., 2008a, 2008b; Lima et al., 2010).

$$AnxietyIndex = 1 - [(timeinopenarm/5min) + (openarmentries/totalentries)]/2$$

2.4. Plasma and frontal cortex NO_x assay

At the day after the behavioral tests, rats were decapitated in an adjoining room and blood samples were collected in vacuum tubes containing EDTA-2 K. Plasma was immediately separated by centrifugation at 1000 × *g* for 10 min at 4°C and then stored at −80°C until analysis. On the other hand, frontal cortex was dissected out, cleaned with ice cold normal saline and stored at −80°C until they were assayed. On the day of analysis frontal cortex samples were thawed on ice and homogenized in a proportion of 1:9 (w/v) ice cold phosphate buffer (0.1 mol/L, pH 7.4) (Chakraborti et al., 2008). Then homogenates were centrifuged at 1000 × *g* for 10 min at 4°C, the supernatants and plasma were both extracted for the determination of nitrate and nitrite (NO_x) levels.

NO_x levels were measured by an enzymatic colorimetric assay based on Griess reaction-dependent method (Tsikas, 2007) according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, China). Briefly, freshly prepared 1 mmol/L NADPH solution and nitrate reductase were mixed with 100 μL plasma or 500 μL frontal cortex homogenates. Then the samples were incubated for 1 h at 37°C for the enzymatic reaction, which allowed nitrate to convert into nitrite. After that, a cofactor preparation and lactate dehydrogenase solution was added to all samples to removed excess NADPH, which had been shown to interfere in the subsequent diazotization of nitrate-derived nitrite. Samples were incubated for another 40 min at room temperature and then centrifuged at 2000 × *g* for 10 min at 4°C. At last, the supernatants were mixed with Griess reagents (1% sulfanilic acid and 0.1% N-(1-naphthyl) ethylenediamine at a ratio of 1:1) to convert the nitrite into an azo derivative. After a 10 min incubation, absorbances of all samples were measured at 550 nm by a spectrophotometer (U 3010, Hitachi, Japan) (Law et al., 2002; Ma et al., 2010). Protein concentration was determined by the method of Bradford using BSA as the standard (Bradford, 1976). NO_x concentration was calculated in the following formula and expressed as μmol/L or μmol per g protein in tissue.

$$NO_x(\mu mol/L) = (OD_{sample} - OD_{blank})/(OD_{standard} - OD_{blank}) \times 20(\mu mol/L)$$

2.5. Statistical analysis

All data are expressed as mean ± SD. The analysis was done by using SAS 9.0 software. Differences between groups in the behavioral test and NO_x levels were analyzed by one-way analysis of variance (ANOVA). If the variances were homogeneous, the least significant difference (LSD) test was used. If not, the Games-Howell test was used. Pearson correlation coefficients (R) were calculated between anxiety-like behaviors and NO_x content. $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. Open field

The results of open field test are depicted in Fig. 2. The time spent in center was significantly lower in the stress model than the control group. The time spent in center was markedly suppressed by emotional stress (by approximately 84%) compared with the control ($t = 2.340$, $df = 15$, $P = 0.025$; Fig. 2a). On the contrary, the time spent in center was significantly higher in the 1 mg/kg and 2 mg/kg GABA groups than the stressed group ($t = -3.384$, $df = 15$, $P = 0.002$ and $t = -5.256$, $df = 15$, $P < 0.001$, respectively).

Emotional stressed rats performed a lower distance traveled in center in the open field than the control group ($t = 2.494$, $df = 15$, $P = 0.017$). However, administration of GABA (1 and 2 mg/kg) significantly increased the distance traveled in center ($t = -2.625$, $df = 15$, $P = 0.013$ and $t = -3.342$, $df = 15$, $P = 0.002$, respectively) in a dose-dependent manner compared with the stressed group (Fig. 2b).

Values of total distance traveled, maximum speed and number of rearing are given in Fig. 2c–e. No significant differences were observed among these three parameters ($P > 0.05$), suggesting that both emotional stress and different doses of GABA (0.5–2 mg/kg) had no

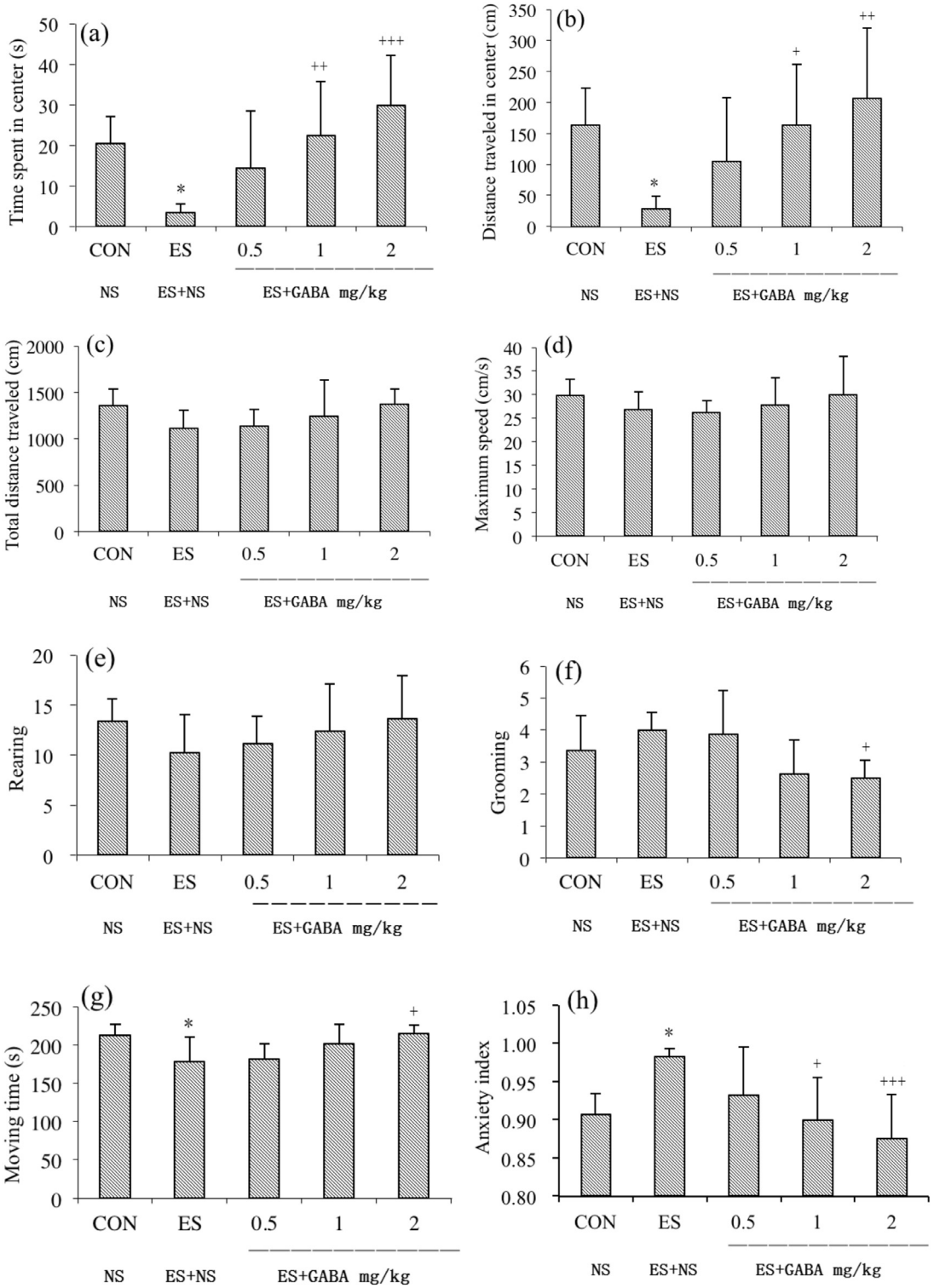

Fig. 2. Effects of emotional stress and GABA administration (0.5, 1, 2 mg/kg Bodyweight/day) in the open field. (a) time spent in center; (b) distance traveled in center; (c) total distance traveled; (d) maximum speed; (e) number of rearing; (f) number of grooming; (g) moving time; (h) anxiety index. $^{*}P < 0.05$ compared with CON group. $^{+}P < 0.05$ compared with ES group; $^{++}P < 0.01$ compared with ES group; $^{+++}P < 0.001$ compared with ES group;. CON: control; ES: emotional stress; NS: normal saline.

substantial modifications on spontaneous locomotor activity (Fig. 2c and d) and exploratory behaviors (Fig. 2e).

All of the GABA groups exhibited reductions of the number of grooming, but only the highest dose reached the statistical significance in comparison to the rats exposed to emotional stress ($t = 2.292$, $df = 15$, $P = 0.028$; Fig. 2f).

A shorter moving time was observed in the stressed group than in the control group ($t = 2.245$, $df = 15$, $P = 0.031$), whereas treatment with GABA at all doses reversed the effect of emotional stress and significantly increased the moving time at 2 mg/kg dose level ($t = -2.695$, $df = 15$, $P = 0.011$; Fig. 2g).

The anxiety index in the open field performance, which combines

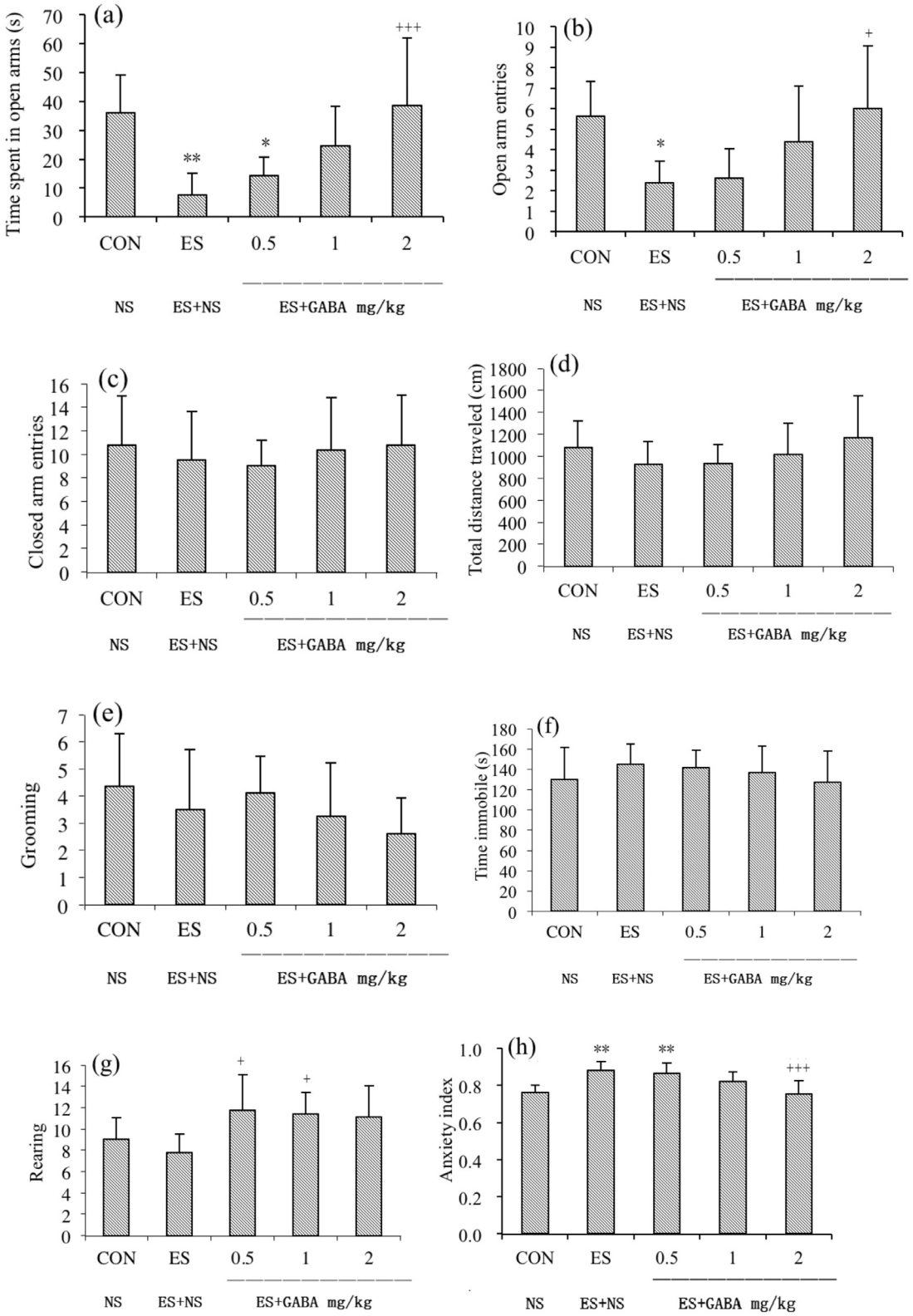

Fig. 3. Effects of emotional stress and GABA administration (0.5, 1, 2 mg/kg Bodyweight/day) in the elevated plus maze. (a) time spent in open arms; (b) open arm entries; (c) closed arm entries; (d) total distance traveled; (e) number of rearing; (f) number of grooming; (g) time immobile; (h) anxiety index. $^{*}P < 0.05$ compared with CON group; $^{**}P < 0.01$ compared with CON group. $^{+}P < 0.05$ compared with ES group; $^{++}P < 0.01$ compared with ES group; $^{+++}P < 0.001$ compared with ES group. CON: control; ES: emotional stress; NS: normal saline.

time spent in center, distance traveled in center and total distance traveled into an index score, was shown in Fig. 2h. Stressed rats displayed a higher anxiety index than the controls did in the arena ($t = -2.333$, $df = 15$, $P = 0.026$). Moreover, GABA had a tendency to decreased anxiety index in stressed rats. With the increasing doses of GABA, the anxiety index was decreased gradually and reached a

significant difference in the 1 mg/kg and 2 mg/kg groups (t = 2.413, df = 15, P = 0.021 and t = 4.265, df = 15, P < 0.001, respectively).

3.2. Elevated plus maze

The results of EPM are depicted in Fig. 3. The time spent in open arms was significantly lower in the stressed group and the 0.5 mg/kg GABA group compared with the control group (t = 3.377, df = 15, P = 0.002 and t = 2.282, df = 15, P = 0.029, respectively), and significantly higher in the 2 mg/kg GABA groups (t = −4.225, df = 15, P < 0.001) than the stressed group.

Frequency of entries to the open arms significantly decreased in the stressed rats compared with the control ones (t = 2.183, df = 15, P = 0.036), but this effect was attenuated by administration of GABA (2 mg/kg) (t = −2.292, df = 15, P = 0.028; Fig. 3b).

The frequency of closed arm entries, total distance traveled, number of grooming and immobility time are all presented in Fig. 3c–f. No significant differences were observed in these four parameters (P > 0.05), suggesting that both emotional stress and different doses of GABA (0.5–2 mg/kg) had no significant influence on spontaneous locomotor activity of rats.

As shown in Fig. 3g, no significant difference was observed between the stressed and control groups (P > 0.05), however, to varying degrees, treatment with GABA at all doses (0.5–2 mg/kg) performed a higher frequency of rearing than the stressed rats in the EPM, 0.5 mg/kg and 1 mg/kg group even reached significant differences (t = −2.505, df = 15, P = 0.017 and t = −2.408, df = 15, P = 0.021, respectively) in the subsequent comparisons.

The anxiety index in the EPM performance, which combines time spent in open arms, number of open arm entries and total entries into an index score, was shown in Fig. 3h. Stressed rats displayed a higher anxiety index than the controls did in the EPM (t = −3.685, df = 15, P = 0.001), so did the 0.5 mg/kg GABA group (t = −2.876, df = 15, P = 0.007). But with the increasing doses of GABA, the anxiety index was decreased slowly and reached a significant difference at the dose of 2 mg/kg (t = 3.912, df = 15, P < 0.001).

3.3. NO_x levels

Fig. 4 illustrated that plasma NO_x levels were significantly higher in stressed rats and 0.5 mg/kg GABA group than that in controls (t = −4.828, df = 15, P < 0.001 and t = −3.219, df = 15, P = 0.003, respectively), but significant 25% and 32% decreases were observed in 1 and 2 mg/kg GABA-treated groups (30.20 ± 5.98 μmol/L and 27.52 ± 5.65 μmol/L) as compared with the stressed rats (40.42 ± 7.56 μmol/L; t = 2.349, df = 15, P = 0.025; t = 3.219, df = 15, P = 0.003), respectively. But on the contrary, frontal cortex NO_x levels showed a significant 21% decrease in rats exposed to emotional stress compared with the controls (4.36 ± 0.37 vs 5.50 ± 0.68 μmol/g protein; t = 2.300, df = 15, P = 0.028) and a dose-dependent manner was found in GABA groups. The frontal cortex NO_x levels were significantly higher in 2 mg/kg GABA group compared with the ES group (t = −2.410, df = 15, P = 0.021).

3.4. Correlations between frontal cortex NO_x levels and OF, EPM behaviors

The above behavioral indicators with statistical differences were picked out for Pearson correlation analysis. The Pearson correlation analysis between frontal cortex NO_x levels and OF behaviors revealed that grooming behavior was correlated negatively with NO_x concentration in GABA-treated rats (P < 0.01; Fig. 5a). In addition, a positive association was also found between moving time and NO_x concentration in the OF (P < 0.01; Fig. 5b). In the EPM behaviors, only anxiety index was found to be correlated negatively with NO_x concentration in GABA-treated rats (P < 0.01; Fig. 5c).

4. Discussion

The major findings of this study were: (i) Repeated oral administration of GABA reduced stress-induced anxiety-like behaviors both in the OF and EPM. (ii) Plasma NO_x levels were markedly increased in stressed rats and the effect were significantly reversed by GABA dose-dependently. Conversely, the NO_x levels in frontal cortex markedly decreased in stressed rats and the effect were significantly reversed by 2 mg/kg GABA. (iii) Frontal cortex NO_x levels in GABA-treated rats were negatively correlated with grooming behavior while positively correlated with moving time in the OF. Likewise, a significant negative coefficient was found between frontal cortex NO_x levels and anxiety index in the EPM.

In our study two important behavioral tests OF and EPM were applied here for evaluation of the effects of GABA against emotional stress. It has been reported that repeated test situation may induce habituation rather than anxiety and rodents would be more likely to stay in a safer zone in the apparatus (Carobrez and Bertoglio, 2005), hence behavioral tests were only performed at the end of the stress procedure in our experiments. OF test is widely used for assessment of anxiety as well as exploration and locomotor activity (Kuniishi et al., 2017). Effects of GABA were measured by time spent in center, distance traveled in center, grooming numbers, moving time and anxiety index in the OF. Anxiety-like behaviors and anxiety index were dose-dependently decreased by GABA in the 0.5–2 mg/kg range. In particular, significant increase in moving time and decrease in number of grooming were observed only at the highest dose (2 mg/kg). These findings obtained with respect to the changes in anxiety-like behaviors were consistent and confirmatory of a fair amount of evidence on many GABA analogues and diazepam (a kind of benzodiazepine drugs which

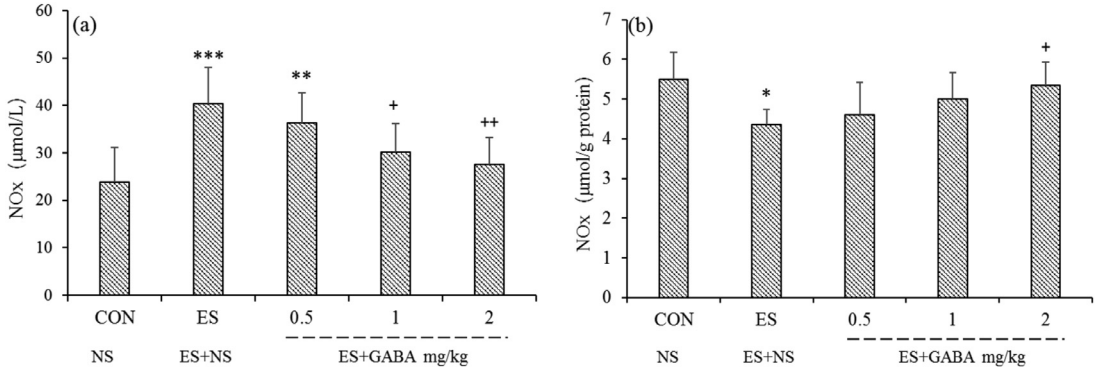

Fig. 4. Effects of emotional stress and GABA administration (0.5, 1, 2 mg/kg Bodyweight/day) on plasma and frontal cortex NO_x levels. (a) plasma; (b) frontal cortex. *P < 0.05 compared with CON group; **P < 0.01 compared with CON group; ***P < 0.001 compared with CON group. ^{+}P < 0.05 compared with ES group; ^{++}P < 0.01 compared with ES group. NO_x: nitrate and nitrite; CON: control; ES: emotional stress; NS: normal saline.

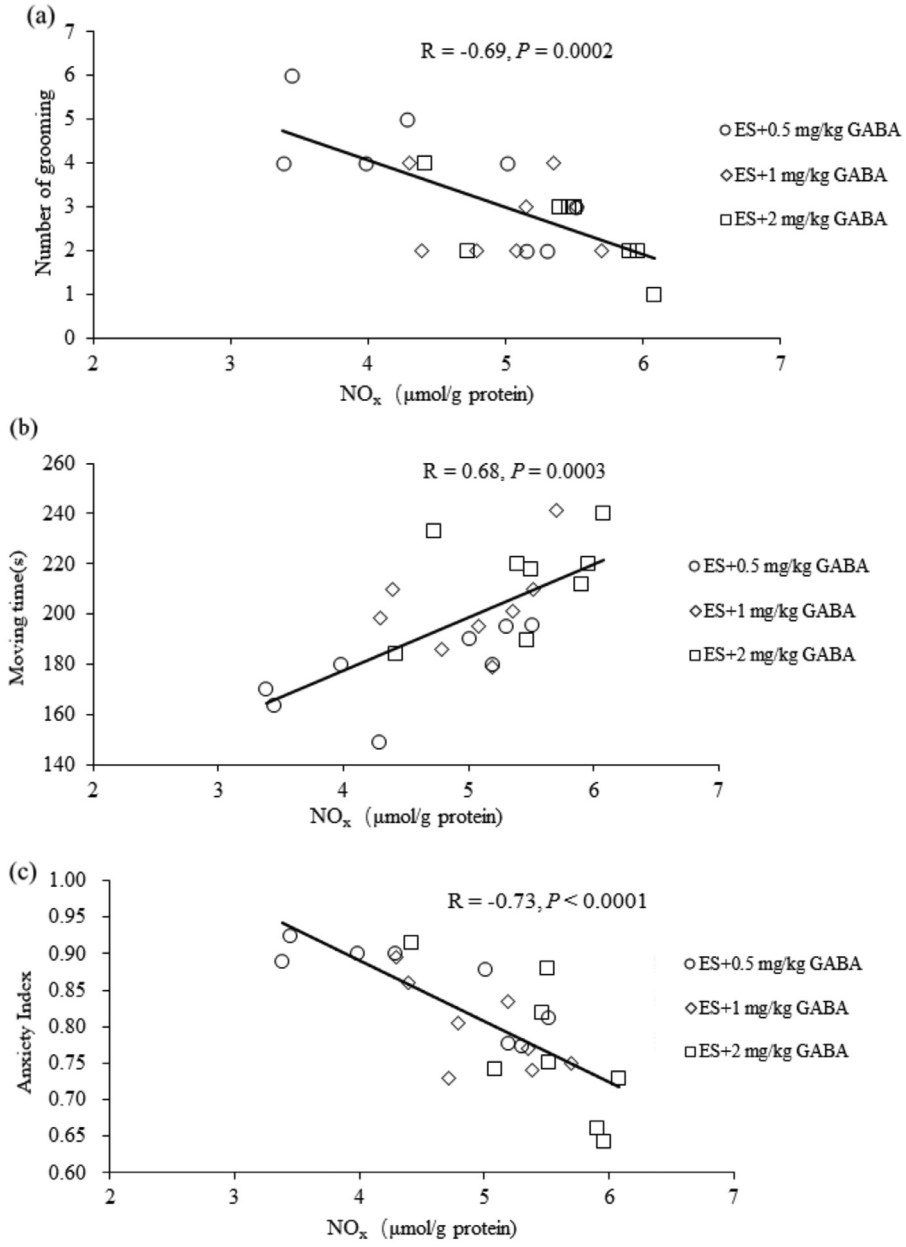

Fig. 5. Pearson correlations between number of grooming and NO_x levels (a), and between moving time and NO_x levels (b) in GABA-treated groups (n = 24) at different doses (0.5, 1, 2 mg/kg Bodyweight/day) in the OF. Pearson correlation between anxiety index and NO_x levels (c) in GABA-treated groups (n = 24) at different doses (0.5, 1, 2 mg/kg Bodyweight/day) in the EPM. NO_x levels were expressed as μmol per g protein in tissue.

may stimulate the release of GABA) (Aracil-Fernández et al., 2013; Barros et al., 1994; Boufleur et al., 2012). Nevertheless, findings with regard to locomotion were less concordant. For instance, some studies in male rats demonstrated that administration of diazepam resulted in a dose-dependent decrease in locomotor activity in the OF while evident increased exploratory behaviors were observed in the hole-board test at the doses ranging from 0.25 to 2 mg/kg (Casarrubea et al., 2009; Dunne et al., 2007). However, in accordance with previous findings of other research about diazepam (Birkett et al., 2011; Gilhotra and Dhingra, 2011), neither locomotor activity nor exploratory behaviors were significantly different between animals submitted to emotional stress and treated with GABA in the present study. Moreover, an earlier study even reported that exploratory behaviors were severely decreased by diazepam at 3 and 10 mg/kg doses in the OF (Consolini et al., 2006). Even though there were methodological differences among these studies, it's still unclear what could account for their discrepancies in essence. One reason for these disparities might be explained by the different doses of drugs. It was suggested that an inverted U-shape dose-response was observed in the central entries to the OF (Ennaceur et al., 2010), in other words, GABA elicits a pronounced effect for stress alleviation at low doses while produces a more potently sedative effect at higher doses (e.g., 5 or 10 mg/kg), leading to the inhibition of locomotor activity, the present results support this assumption.

EPM is also used to assess the anxiolytic effects of pharmacological agents and the mechanisms underlying anxiety-related behaviors (Selakovic et al., 2016). In our study, Effects of GABA were measured by time spent in open arms, entries to open arms and closed arms, grooming numbers, immobility time and anxiety index in the EPM. Both the duration and entries to the open arms were evidently increased and at the same time anxiety index was dose-dependently decreased in all GABA-treated groups in the 0.5–2 mg/kg range. Similarly to the results of OF test, grooming behavior was decreased in a dose-

dependent response though the reduction did not reach statistical significance, probably attributed to a small sample size. Meanwhile, locomotor activity was not affected in the EPM, further corroborating the anxiolytic-like ability of GABA in our range of doses as mentioned above. Moreover, the immobility time, a critical parameter to evaluate the state of sedation of animals (Wesołowska and Nikiforuk, 2007), was not prominently and visibly changed between stressed rats and GABA-treated groups, which was once again in agreement with our hypothesis that the sedative effect of GABA was not conspicuous at low doses. However, it should also be noted that the 0.5 and 1 mg/kg doses stimulated the exploratory activity, and they were quantitatively more remarkable than that seen following a dose of 2 mg/kg. One possibility might be due to a sedative effect at the 2 mg/kg dose, implying that influence of GABA on exploratory activity were more sensitive and evident in the EPM than in the OF. Taken together, GABA ameliorates anxiety-like behaviors without altering locomotor activity in our behavioral tests. Given that results of behavioral tests are mutable according to the durations and types of stress and treatment, ages and strains of rats, illumination of the testing room, housing conditions or other methodological differences (Belviranli et al., 2012; Chiba et al., 2012), a more stable experimental and housing environment is required for further studies in ethology.

Another important finding of this study suggests a close relationship between nitric oxide and anxiety-like behaviors. Initially, we discovered that the concentration of NO metabolites measured in frontal cortex was 21% lower in emotional stress group than in normal controls, then the higher frontal cortex NO_x levels, the lower numbers of grooming in the OF were observed in the following correlation analysis. Our data also showed that NO_x concentration was correlated positively with moving time in the OF and correlated negatively with anxiety index in the EPM, respectively. These observations suggested that anxiety-like behaviors in emotional stressed rats were related to the down regulation of the central NO system. Such a notion was also supported by past studies that inhibition of nNOS either by gene deletion or treatment with 3-Bromo-7-Nitroindazole (a selective nNOS inhibitor) decreased open arm exploration in single-housed mice (Nelson et al., 1995; Workman et al., 2008). GABA treatment at 2 mg/kg dose significantly elevated NO_x levels, indicating an improvement to the central NO system. We presumed that GABA exerted an inhibitory action on the increased sympathetic nerve activity along with NO since past research had proved that emotional stress could increase plasma norepinephrine levels and activate the sympathetic nervous system (SNS), leading to aggressive or anxiety-like behaviors (Lin et al., 2003; Xing et al., 2013). Zhang and Patel (1998) found that blockade of the GABA system could eliminate the decrease in renal nerve discharge after a microinjection of sodium nitroprusside (an NO donor) in the PVN, indicating that the inhibitory effect of endogenous NO on the renal sympathetic nerve activity was mediated by GABA, related studies also supported our hypothesis (Decavel and Van Den Pol, 1990; Sakuma et al., 1992). And most importantly, systemically administered GABA can access some areas of the brain that lack the BBB, for example, surrounding regions of hypothalamus, to trigger changes in autonomic function (Cottrell and Ferguson, 2004). These findings together with our results raised the possibility that orally administered GABA reached the hypothalamus through the site that lacked the BBB, inhibited the excitability of the SNS activated by emotional stress and developed a calming and anxiolytic-like function via a NO pathway. Interestingly, GABA exerted a totally opposite influence on plasma NO_x levels compared with the frontal cortex, in other words, plasma NO_x levels were increased in stressed rats while decreased in GABA-treated groups in a dose-dependent manner. This variability might be explained by the differentiation of NOS between the peripheral nervous system and the CNS (Workman et al., 2008).

Several limitations of our study should be taken into account when interpreting the current results. First, the impacts of repeated oral administration of GABA on NO content in different brain regions (hypothalamus, hippocampus, amygdala and so on) are not determined especially the site lacks the BBB, so the accurate functional activation brain area of GABA remains unknown. Second, the activity and expression of 3 subtypes of NOS (nNOS, iNOS and eNOS) are not measured in our study, thus we cannot ascertain GABA acts on which subtype of NOS to induce the inhibition of the SNS, the controversial results mentioned above suggest that there may be distinct pharmacological mechanisms of GABA that affect NO metabolism in the peripheral nervous system otherwise than in the CNS. Finally, the anxiolytic-like effect of orally administered GABA should be tested in a wider dose range and other neurobiological factors associated with anxiety-like behaviors should be determined simultaneously in further investigations.

In summary, the present study shows that emotional stress induces anxiety-like behaviors in rats, accompanied by down regulation of NO release in frontal cortex of brain. Moreover, repeated oral administration of GABA ameliorates anxiety-like behaviors and raises NO production in the 0.5–2 mg/kg range, implying an effect on improving anxiolytic-like ability. However, NO production is decreased by GABA in an opposite manner in plasma. These findings will enrich our knowledge in understanding the pharmacological mechanisms of GABA and provide us an insight to its dual effect on central and peripheral NO regulation.

Conflict of interest

The authors declare they have no conflict of interest.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 31770376), the Natural Science Foundation of Guangdong Province (No. 2016A030313395) and the Project for Generic Technique Innovation Team Construction of Modern Agriculture of Guangdong Province (No. 2016LM2152).

References

Abdou, A.M., Higashiguchi, S., Horie, K., Kim, M., Hatta, H., Yokogoshi, H., 2006. Relaxation and immunity enhancement effects of γ-Aminobutyric acid (GABA) administration in humans. Biofactors 26 (3), 201.

Al-Awadi, M., Pavlik, A., Al-Sarraf, H., 2006. Increased brain uptake and brain to blood efflux transport of 14 C-GABA in spontaneously hypertensive rats. Life Sci. 79 (9), 847–853.

Aracil-Fernández, A., Almela, P., Manzanares, J., 2013. Pregabalin and topiramate regulate behavioural and brain gene transcription changes induced by spontaneous cannabinoid withdrawal in mice. Addict. Biol. 18 (2), 252–262.

Barros, H., Tannhauser, S., Tannhauser, M., Tannhauser, M., 1994. The effects of GABAergic drugs on grooming behaviour in the open field. Basic Clin. Pharmacol. Toxicol. 74 (4–5), 339–344.

Belviranli, M., Atalik, K., Okudan, N., Gökbel, H., 2012. Age and sex affect spatial and emotional behaviors in rats: the role of repeated elevated plus maze test. Neuroscience 227, 1–9.

Bi, L.L., Wang, J., Luo, Z.Y., Chen, S.P., Geng, F., Chen, Y.H., Li, S.J., Yuan, C.H., Lin, S., Gao, T.M., 2013. Enhanced excitability in the infralimbic cortex produces anxiety-like behaviors. Neuropharmacology 72, 148–156.

Birkett, M.A., Shinday, N.M., Kessler, E.J., Meyer, J.S., Ritchie, S., Rowlett, J.K., 2011. Acute anxiogenic-like effects of selective serotonin reuptake inhibitors are attenuated by the benzodiazepine diazepam in BALB/c mice. Pharmacol. Biochem. Behav. 98 (4), 544–551.

Boufleur, N., Antoniazzi, C.T., Pase, C.S., Benvegnú, D.M., Barcelos, R.C., Dolci, G.S., Dias, V.T., Roversi, K., Roversi, K., Koakoskia, G., 2012. Neonatal tactile stimulation changes anxiety-like behavior and improves responsiveness of rats to diazepam. Brain Res. 1474, 50–59.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72 (1–2), 248–254.

Cai, L., Yan, X.B., Chen, X.N., Meng, Q.Y., Zhou, J.N., 2010. Chronic all-trans retinoic acid administration induced hyperactivity of HPA axis and behavioral changes in young rats. Eur. Neuropsychopharmacol. 20 (12), 839–847.

Carobrez, A., Bertoglio, L., 2005. Ethological and temporal analyses of anxiety-like behavior: the elevated plus-maze model 20 years on. Neurosci. Biobehav. Rev. 29 (8), 1193–1205.

Carola, V., D'Olimpio, F., Brunamonti, E., Mangia, F., Renzi, P., 2002. Evaluation of the

elevated plus-maze and open-field tests for the assessment of anxiety-related behaviour in inbred mice. Behav. Brain Res. 134 (1), 49–57.
Casarrubea, M., Sorbera, F., Crescimanno, G., 2009. Structure of rat behavior in hole-board: I) multivariate analysis of response to anxiety. Physiol. Behav. 96 (1), 174–179.
Chakraborti, A., Gulati, K., Ray, A., 2008. Age related differences in stress-induced neurobehavioral responses in rats: modulation by antioxidants and nitrergic agents. Behav. Brain Res. 194 (1), 86–91.
Chiba, S., Numakawa, T., Ninomiya, M., Richards, M.C., Wakabayashi, C., Kunugi, H., 2012. Chronic restraint stress causes anxiety-and depression-like behaviors, down-regulates glucocorticoid receptor expression, and attenuates glutamate release induced by brain-derived neurotrophic factor in the prefrontal cortex. Prog. Neuropsychopharmacol. Biol. Psychiatry 39 (1), 112–119.
Cohen, H., Geva, A.B., Matar, M.A., Zohar, J., Kaplan, Z., 2008a. Post-traumatic stress behavioural responses in inbred mouse strains: can genetic predisposition explain phenotypic vulnerability? Int. J. Neuropsychopharmacolog. 11 (3), 331–349.
Cohen, H., Matar, M.A., Buskila, D., Kaplan, Z., Zohar, J., 2008b. Early post-stressor intervention with high-dose corticosterone attenuates posttraumatic stress response in an animal model of posttraumatic stress disorder. Biol. Psychiatry 64 (8), 708–717.
Consolini, A.E., Ragone, M.I., Migliori, G.N., Conforti, P., Volonté, M.G., 2006. Cardiotonic and sedative effects of Cecropia pachystachya Mart.(ambay) on isolated rat hearts and conscious mice. J. Ethnopharmacol. 106 (1), 90–96.
Cottrell, G.T., Ferguson, A.V., 2004. Sensory circumventricular organs: central roles in integrated autonomic regulation. Regul. Pept. 117 (1), 11–23.
Czech, D.A., Jacobson, E.B., LeSueur-Reed, K.T., Kazel, M.R., 2003. Putative anxiety-linked effects of the nitric oxide synthase inhibitor L-NAME in three murine exploratory behavior models. Pharmacol. Biochem. Behav. 75 (4), 741–748.
Decavel, C., Van Den Pol, A.N., 1990. GABA: a dominant neurotransmitter in the hypothalamus. J. Comp. Neurol. 302 (4), 1019–1037.
Defaix, C., Solgadi, A., Pham, T.H., Gardier, A.M., Chaminade, P., Tritschler, L., 2018. Rapid analysis of glutamate, glutamine and GABA in mice frontal cortex microdialysis samples using HPLC coupled to electrospray tandem mass spectrometry. J. Pharm. Biomed. Anal. 152, 31.
Dunne, F., O'halloran, A., Kelly, J.P., 2007. Development of a home cage locomotor tracking system capable of detecting the stimulant and sedative properties of drugs in rats. Prog. Neuropsychopharmacol. Biol. Psychiatry 31 (7), 1456–1463.
Ennaceur, A., Michalikova, S., van Rensburg, R., Chazot, P., 2010. Distinguishing anxiolysis and hyperactivity in an open space behavioral test. Behav. Brain Res. 207 (1), 84–98.
Gilhotra, N., Dhingra, D., 2011. Thymoquinone produced antianxiety-like effects in mice through modulation of GABA and NO levels. Pharmacol. Rep. 63 (3), 660–669.
Gonchar, Y., Burkhalter, A., 1997. Three distinct families of GABAergic neurons in rat visual cortex. Cereb. Cortex 7 (4), 347–358.
Huynh, T.N., Krigbaum, A.M., Hanna, J.J., Conrad, C.D., 2011. Sex differences and phase of light cycle modify chronic stress effects on anxiety and depressive-like behavior. Behav. Brain Res. 222 (1), 212–222.
Kimura, M., Hayakawa, K., Sansawa, H., 2002. Involvement of gamma-aminobutyric acid (GABA) B receptors in the hypotensive effect of systemically administered GABA in spontaneously hypertensive rats. Jpn. J. Pharmacol. 89 (4), 388–394.
Kuniishi, H., Ichisaka, S., Yamamoto, M., Ikubo, N., Matsuda, S., Futora, E., Harada, R., Ishihara, K., Hata, Y., 2017. Early deprivation increases high-leaning behavior, a novel anxiety-like behavior, in the open field test in rats. Neurosci. Res. 123, 27–35.
Law, A., O'donnell, J., Gauthier, S., Quirion, R., 2002. Neuronal and inducible nitric oxide synthase expressions and activities in the hippocampi and cortices of young adult, aged cognitively unimpaired, and impaired Long–Evans rats. Neuroscience 112 (2), 267–275.
Lima, D.C., do Vale, T.G., Arganāraz, G.A., Varella, P.P.V., Frussa-Filho, R., Cavalheiro, E.A., da Graça Naffah-Mazzacoratti, M., Amado, D., 2010. Behavioral evaluation of adult rats exposed in utero to maternal epileptic seizures. Epilepsy Behav. 18 (1), 45–49.
Lin, W., Wang, W., Shao, F., 2003. New animal model of emotional stress: behavioral, neuroendocrine and immunological consequences. Chin. Sci. Bull. 48 (15), 1565–1568.
Ma, L., Liu, J., Li, N., Wang, J., Duan, Y., Yan, J., Liu, H., Wang, H., Hong, F., 2010. Oxidative stress in the brain of mice caused by translocated nanoparticulate TiO_2 delivered to the abdominal cavity. Biomaterials 31 (1), 99–105.
Masood, A., Banerjee, B., Vijayan, V., Ray, A., 2003. Modulation of stress-induced neurobehavioral changes by nitric oxide in rats. Eur. J. Pharmacol. 458 (1), 135–139.
Moreno, J., Gaspar, E., López-Bello, G., Juárez, E., Alcázar-Leyva, S., González-Trujano, E., Pavón, L., Alvarado-Vásquez, N., 2013. Increase in nitric oxide levels and mitochondrial membrane potential in platelets of untreated patients with major depression. Psychiatry Res. 209 (3), 447–452.
Nakamura, H., Takishima, T., Kometani, T., Yokogoshi, H., 2009. Psychological stress-reducing effect of chocolate enriched with Î³-aminobutyric acid (GABA) in humans: assessment of stress using heart rate variability and salivary chromogranin A. Int. J. Food Sci. Nutr. 60 (Suppl. 5), 106–113.
Nelson, R.J., Demas, G.E., Huang, P.L., Fishman, M.C., 1995. Behavioural abnormalities in male mice lacking neuronal nitric oxide synthase. Nature 378 (6555), 383.
Nissen, I., Estrada, F.S., Nava-Kopp, A.T., Irles, C., de-la-Peña-Diaz, A., Fernandez-G, J.M., Govezensky, T., Zhang, L., 2012. Prolame ameliorates anxiety and spatial learning and memory impairment induced by ovariectomy in rats. Physiol. Behav. 106 (2), 278–284.
Okonogi, T., Nakayama, R., Sasaki, T., Ikegaya, Y., 2018. Characterization of peripheral activity states and cortical local field potentials of mice in an elevated plus maze test. Front. Behav. Neurosci. 12, 62.
Paul, V., Subramanian, E.H., Rajasekaran, K., 2001. Pharmacological evidence for a role of γ-aminobutyric acid A receptor mechanism in modulating nitric oxide synthase activity in rat brain. Neurochem. Int. 38 (3), 209–211.
Reif, A., Herterich, S., Strobel, A., Ehlis, A.C., Saur, D., Jacob, C.P., Wienker, T., Töpner, T., Fritzen, S., Walter, U., Schmitt, A., Fallgatter, A.J., Lesch, K.P., 2006. A neuronal nitric oxide synthase (NOS-I) haplotype associated with schizophrenia modifies prefrontal cortex function. Mol. Psychiatry 11 (3), 286–300.
Sakuma, I., Togashi, H., Yoshioka, M., Saito, H., Yanagida, M., Tamura, M., Kobayashi, T., Yasuda, H., Gross, S.S., Levi, R., 1992. NG-methyl-L-arginine, an inhibitor of L-arginine-derived nitric oxide synthesis, stimulates renal sympathetic nerve activity in vivo. A role for nitric oxide in the central regulation of sympathetic tone? Circ. Res. 70 (3), 607–611.
Seibenhener, M.L., Wooten, M.C., 2015. Use of the open field maze to measure locomotor and anxiety-like behavior in mice. J. Vis. Exp. Jove 2015 (96), 52434.
Selakovic, D., Joksimovic, J., Obradovic, D., Milovanovic, D., Djuric, M., Rosic, G., 2016. The adverse effects of exercise and supraphysiological dose of testosterone-enanthate (TE) on exploratory activity in elevated plus maze (EPM) test - indications for using total exploratory activity (TEA) as a new parameter for ex. Neuro Endocrinol. Lett. 37 (5), 383.
Shao, F., Lin, W., Wang, W., Washington Jr., W.C, Zheng, L., 2003. The effect of emotional stress on the primary humoral immunity of rats. J. Psychopharmacol. 17 (2), 179–183.
Solati, J., Hajikhani, R., Golub, Y., 2013. Activation of GABA A receptors in the medial prefrontal cortex produces an anxiolytic-like response. Acta Neuropsychiatr. 25 (04), 221–226.
Sun, J., Zhang, X., Broderick, M., Fein, H., 2003. Measurement of nitric oxide production in biological systems by using Griess reaction assay. Sensors 3 (8), 276–284.
Tsikas, D., 2007. Analysis of nitrite and nitrate in biological fluids by assays based on the Griess reaction: appraisal of the Griess reaction in the L-arginine/nitric oxide area of research. J. Chromatogr. B 851 (1), 51–70.
Uetake, K., Okumoto, A., Tani, N., Goto, A., Tanaka, T., 2012. Calming effect of orally administered γ-aminobutyric acid in Shih Tzu dogs. Anim. Sci. J. 83 (12), 796–798.
Vincent, S.R., 2010. Nitric oxide neurons and neurotransmission. Prog. Neurobiol. 90 (2), 246–255.
Walf, A.A., Frye, C.A., 2007. The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. Nat. Protoc. 2 (2), 322–328.
Wesołowska, A., Nikiforuk, A., 2007. Effects of the brain-penetrant and selective 5-HT 6 receptor antagonist SB-399885 in animal models of anxiety and depression. Neuropharmacology 52 (5), 1274–1283.
White, J.H., Wise, A., Main, M.J., Green, A., Fraser, N.J., Disney, G.H., Barnes, A.A., Emson, P., Foord, S.M., Marshall, F.H., 1998. Heterodimerization is required for the formation of a functional GABAB receptor. Nature 396 (6712), 679–682.
Workman, J.L., Trainor, B.C., Finy, M.S., Nelson, R.J., 2008. Inhibition of neuronal nitric oxide reduces anxiety-like responses to pair housing. Behav. Brain Res. 187 (1), 109–115.
Xing, B., Liu, P., Jiang, W.H., Liu, F., Zhang, H., Cao, G.F., Chen, T., Dang, Y.H., 2013. Effects of immobilization stress on emotional behaviors in dopamine D3 receptor knockout mice. Behav. Brain Res. 243, 261–266.
Zhang, K., Patel, K.P., 1998. Effect of nitric oxide within the paraventricular nucleus on renal sympathetic nerve discharge: role of GABA. Am. J. Physiol. 275 (2), 728–734.
Zhang, L., Medina, M., Hernández, V., Estrada, F., Vega-Gonzalez, A., 2010. Vasopressinergic network abnormalities potentiate conditioned anxious state of rats subjected to maternal hyperthyroidism. Neuroscience 168 (2), 416–428.