Turkish Journal of Medical Sciences

Volume 44 | Number 6 Article 8

1-1-2014

Behavioural and neurobiological consequences of 2 different chronic stressors in rats

REFİA ASLIHAN DÖNMEZ

FATMA DUYGU KAYA

OKŞAN DERİNÖZ

ÖMER HAKAN EMMEZ

SELÇUK CANDANSAYAR

See next page for additional authors

Behavioural and neurobiological consequences of 2 different chronic stressors in rats

Authors
REFİA ASLIHAN DÖNMEZ, FATMA DUYGU KAYA, OKŞAN DERİNÖZ, ÖMER HAKAN EMMEZ, SELÇUK CANDANSAYAR, and HAYRUNNİSA BOLAY BELEN

TÜBİTAK

Turkish Journal of Medical Sciences

http://journals.tubitak.gov.tr/medical/

Research Article

Turk J Med Sci
(2014) 44: 955-966
© TÜBİTAK
doi:10.3906/sag-1309-24

Behavioural and neurobiological consequences of 2 different chronic stressors in rats

Refia Aslıhan DÖNMEZ[1,*], Fatma Duygu KAYA[2], Okşan DERİNÖZ[3], Ömer Hakan EMMEZ[4], Selçuk CANDANSAYAR[1], Hayrunnisa BOLAY BELEN[5]

[1]Department of Psychiatry, Faculty of Medicine, Gazi University, Ankara, Turkey
[2]Department of Psychiatry, Elmadağ Hospital, Ankara, Turkey
[3]Department of Paediatric Emergency, Faculty of Medicine, Gazi University, Ankara, Turkey
[4]Department of Neurosurgery, Faculty of Medicine, Gazi University, Ankara, Turkey
[5]Department of Neurology, Faculty of Medicine, Gazi University, Ankara, Turkey

Received: 17.09.2013 • **Accepted:** 03.01.2014 • **Published Online:** 24.10.2014 • **Printed:** 21.11.2014

Background/aim: To compare the behavioural and neurobiological consequences of chronic headache and chronic mild stress (CMS) in rats.

Materials and methods: Forty-eight male Wistar rats were divided into 4 groups: 1) control group, 2) chronic headache group, 3) CMS group, and 4) sham group. Their behaviour prior to (D0) and after (D14) chronic stress was analysed. Afterwards, they were exposed to the Elevated Plus Maze (EPM) in order to evaluate anxiety-like behaviour and the Forced Swim Test (FST) for observation of depressive-like behaviour. Ultrasonic vocalisations (USVs) were recorded by a USV detector system at D0 and D14 and during the FST. The c-fos expressions in various brain regions were analysed 2 h after the EPM and FST.

Results: The control group showed significantly more sleeping behaviour at D14 ($\chi^2 = 8.213$, $P = 0.042$), emitted more negative and positive affect USVs at D14 ($\chi^2 = 9.853$, $P = 0.020$) and during FST ($\chi^2 = 4.000$, $P = 0.046$) than the chronic headache and CMS groups, and showed significantly less anxiety-like behaviour in the EPM than the CMS group ($P = 0.021$).

Conclusion: These results suggest that CMS increases anxiety-like behaviour but not depressive-like behaviour, while chronic headache does not have a significant effect on these behaviours in rats.

Key words: Stress, rats, behaviour, ultrasonic vocalisations, Elevated Plus Maze, Forced Swim Test

1. Introduction

Chronic stress is a risk factor for the development of many psychopathological conditions in humans, such as major depression and anxiety disorders. Animal models of chronic stress help us understand the physiological and behavioural outcomes of both physical and psychosocial stressors. One of the most widely used models for psychosocial stressors is the chronic mild stress (CMS) model. In this model, rodents are subjected to different and unpredictable mild psychological stressors for a period of time (1). It has been repeatedly shown that the CMS model causes a significant decrease in preference of sweet solutions for rodents, which is interpreted as anhedonia, a major sign of depression in humans (2,3). Phenotypes of depression such as decreased weight gain (4), altered locomotor activity (5), decrease in sexual behaviour (6,7), altered diurnal rhythms, and sleep disturbances with decreased REM latency and increased number of REM episodes (8,9) have also been reported after CMS. Results about anxiety-like behaviour after chronic mild stress in rodents are inconclusive (10).

Chronic physical pain can also be seen as a form of stress. Pain is a complex experience and is not only dependent on the regulation of nociceptive sensory systems but also causes activation of the mechanisms that control mood in limbic areas. There are many reasons to think that pain and mood have a reciprocal interaction. The affective-motivational component of pain is an important determinant of the overall pain experience, and negative affect may cause an increase in the manifestation and expression of pain-related disorders, both in humans (11) and in rodents (12). Although the primary drugs used for the management of chronic pain have traditionally been opioid receptor agonists and nonsteroidal antiinflammatory drugs, antidepressants (such as serotonin noradrenaline reuptake inhibitors and

* Correspondence: aslihandonmez@gmail.com

tricyclic antidepressants) are also used to treat chronic pain. In addition, the relationship between pain and mood has been further supported by numerous clinical studies indicating comorbidity of chronic pain and major depression (13,14).

Migraine is a type of chronic pain. Comorbidity between migraine and psychiatric disorders is high (15) and they are reported to have similar neurobiological abnormalities in the same neural networks (16). However, the basis of this relationship is not known. Understanding the complex relationship between chronic stress and psychiatric disorders would help clinicians to prevent and treat disorders related to chronic stress. In this study, we aim to compare the neurobiological and behavioural consequences of 2 different chronic stressors. In order to fulfil this aim, we used the CMS model and chronic headache model in rats. Our hypothesis is that both stressors might have similar neurobiological and behavioural consequences.

2. Materials and Methods

2.1. Animals

This study was performed in the Neuroscience Laboratory of the Neuropsychiatry Centre at Gazi University. Ethical approval was obtained from the Gazi University Animal Studies Ethical Committee. The study used male Wistar rats aged between 70 and 90 days and weighing 200 to 300 g. Animals were housed in a controlled environment with a room temperature of 22 ± 2 °C and a 12-h light/dark cycle. Rats received food and water ad libitum.

The animals were divided into 4 groups: 1) control group (n = 12), 2) chronic headache group (n = 12), 3) CMS group (n = 12), and 4) sham group (n = 12).

Control group: These animals were housed under the previously described conditions without the application of physical or psychological stress.

Chronic headache group: The chronic headache model described previously (17) was applied to this group. Rats were anaesthetised with ketamine (50 mg/kg) and xylazine (8 mg/kg). The degree of anaesthesia was adjusted to the heart rate and hind leg pinch reaction. During surgery, body temperature was kept constant at 37 ± 0.5 °C with a heating pad. The animals were placed in a stereotaxic frame (Stoelting, USA). An incision was made on the 2–3 mm medial of the right orbital hole. Nasociliary fibres of trigeminal nerve were localised and tied. For the sham group, the same incision was made on the left side; nasociliary fibres were localised and left untied.

CMS group: We used the unpredictable CMS model described in other research (18,19) with some modifications, including the following:

- Loud music: Moderately loud white noise at 10 kHz was played continuously from a house radio within 2 m of the caged rats.
- Tilted cages: Cages were placed on a rack titled at a 45° angle.
- Wet bedding: About 300 mL of tap water was poured onto the bedding of the rat cages.
- Water deprivation: Water bottles were removed from 1700 to 0900 hours.
- Photoperiod reversal: Rats were kept in a separate room where photoperiods were reversed (light from 1800 to 0600 hours, dark from 0600 to 1800 hours).

These stressors were applied singly and in random order for 14 days. No stressors were applied during the weekend. The study design is presented in Table 1.

2.2. Behavioural analysis

Behavioural analyses of all animals were conducted both by direct observation and a behavioural analysis system on the 1st (D0) and 14th (D14) days. For the chronic headache and sham groups, D0 was the day prior to surgery. For the chronic stress group, D0 was the day before the application of chronic mild stress. Direct observation was made for 30 min by one of the authors, who recorded time and duration of behaviours such as freezing, bilateral head grooming, right head grooming, body grooming, drinking, eating, and sleeping. At the same time, rat behaviour was automatically recorded by a noninvasive behavioural analysis system. This system is composed of a standard rat cage fixed on a platform with several force-displacement transducers, connected to a personal computer (Laboras, Metris, the Netherlands). The platform detects and classifies the behaviours by using the vibrations created by the movement of the animal (such as immobility, locomotor activity, and rearing) (20,21). Rats were free to access food or water located in standard rat cages and needed to rear to eat or drink.

All experiments were simultaneously recorded by a video-camera system in order to confirm the data obtained from the automated analysis system and to differentiate freezing periods from immobility.

2.3. Ultrasonic vocalisation calls

Many vertebrates use species-specific vocalisations to communicate information regarding mother–offspring interactions, mating, mood (fear, pain, distress, aggression, joy, etc.), behavioural intentions (approach, avoid, groom), and environmental conditions (presence of predators or the location of food). This information is important in order to understand the behaviour of animals in laboratory conditions (22).

Adult rats primarily emit 2 types of ultrasonic vocalisations (USVs) that are distinguished on the basis of frequency with peak energy. The vocalisations typically referred to as '22-kHz vocalisations' have frequencies between 18 to 32 kHz and duration of 300 to 4000 ms, and they are emitted at a sound pressure level of 65 to 85 dB (23). Rats emit 22-kHz vocalisations in a number of aversive

Table 1. Study design

Day	Control	Chronic stress	Chronic headache	Sham
D0	Behavioural analysis and USV call recordings	Behavioural analysis and USV call recordings	Behavioural analysis and USV call recordings	Behavioural analysis and USV call recordings
D1	Standard care	Loud static for 8 h (0900–1700 hours)	Surgery	Surgery
D2	Standard care	Wet bedding for 8 h (0900–17:00 hours)	Standard care	Standard care
D3	Standard care	Overnight water deprivation for 16 h (1700–0900 hours)	Standard care	Standard care
D4	Standard care	Tilted cages for 8 h (0900–1700 hours)	Standard care	Standard care
D5	Standard care	Photoperiod reversal (0600–0600 hours)	Standard care	Standard care
D6	Standard care	Standard care	Headache	Standard care
D7	Standard care	Standard care	Headache	Standard care
D8	Standard care	Tilted cages for 8 h (0900–1700 hours)	Headache	Standard care
D9	Standard care	Overnight water deprivation for 16 h (1700–0900 hours)	Headache	Standard care
D10	Standard care	Loud static for 8 h (0900–1700 hours)	Headache	Standard care
D11	Standard care	Wet bedding for 8 h (0900–1700 hours)	Headache	Standard care
D12	Standard care	Photoperiod reversal (0600–0600 hours)	Headache	Standard care
D13	Standard care	Standard care	Headache	Standard care
D14	Behavioural analysis and USV call recordings			
D15	Rats 1–6: EPM[1] Rats 7–12: FST[2] pretest session and USV call recordings			
D16	Rats 7–12: FST test session and USV call recordings			
D17	Standard care			
D18	Standard care			
D19	Standard care			
D20	Standard care			
D21	Rats 1–6: FST pretest session and USV call recordings			
D22	Rats 1–6: FST test session and USV call recordings Rats 7–12: EPM Brain removal for c-fos analysis			

[1]EPM: Elevated Plus Maze, [2]FST: Forced Swim Test.

situations including distressing events, and it is assumed that they reflect a negative affective state of the animal [see the review by Portfors (22) for more information]. The so-called '50-kHz vocalisations' have a frequency at peak energy of 32 to 96 kHz and a much shorter duration (30 to 50 ms). Sometimes 50-kHz vocalisations are referred to as 'chirps' because of their brief duration (24). Rats emit 50-kHz vocalisations in nonaversive conditions, including sexual behaviour, play, and manual tactile stimulation ('tickling'). It has been suggested that these vocalisations are associated with a positive affect of the animal (22).

These vocalisations are inaudible to humans without the use of specialised equipment. The ultrasonic sounds (within a range of 15–100 kHz) of laboratory animals can be monitored and analysed with a USV detector system (Sonotrack, Metris). Sonotrack uses a hardware bandpass filter (10th-order Butterworth filter) with sharp cut-offs at 15 and 100 kHz. This filter prevents aliasing and also removes almost all environmental sounds. The data are presented without further filtering and smoothing. In Sonotrack, the dB scale is relative to a 1 mV (RMS) signal. In the spectrogram, red indicates the strongest signal value

(50 mV or 35.3 V RMS or 31 dB) and black indicates the background noise (approximately 10 mV in Sonotrack or 7 mV RMS or 16 dB). The shift in frequency at the beginning and the end of the vocalisation is characteristic of a biological sound.

In this study, we used Sonotrack to detect the USV calls of all animals at D0 and D14 behavioural analysis, as well as during pretest and the test sessions of the Forced Swim Test (FST). The detected sounds were then divided into 2 groups, Band I (vocalisations between 18 and 32 kHz, related to distress) and Band II (vocalisations between 32 and 50 kHz, related to positive affect).

2.4. Forced Swim Test

The FST, originally reported by Porsolt et al. (25), has developed into the most widely used model for assessing antidepressant-like activity in rodents. For this test, a glass cylinder with a depth of 60 cm and a diameter of 30 cm was used. The cylinder was filled with 30 cm of tap water at 23–25 °C. For the pretest (habituation) session, rats were placed individually into the cylinder, allowed to swim for 15 min, removed from the water, dried under a lamp, and returned to their cages. The same procedure was repeated 24 h later in the test session. On this occasion, the time in the cylinder was 5 min. During the test session, an observer recorded the total time for 3 different types of behaviour: (a) climbing, defined as upward movement of the forepaws, usually against the side of the swim cylinder; (b) swimming, defined as the horizontal movement of the rat throughout the cylinder; (c) immobility, defined as floating in the water without struggling and only making minimum movement necessary to keep the head above water (26). All sessions were simultaneously recorded by a video-camera and USVs were obtained using Sonotrack.

Half of the animals in each group (n = 6) were exposed to the FST on day 15 (the first day after chronic physical and psychological stress), while the other half were exposed to the FST on day 22 (1 week after chronic stress) (see Table 1 for study design).

2.5. Elevated Plus Maze

The Elevated Plus Maze (EPM) was described by Pellow et al. as a simple method of assessing the anxiety responses of rodents (27). The maze used in this study was made of Plexiglas and consisted of 2 open arms (50 cm long and 10 cm wide) and 2 closed arms (50 cm long and 10 cm wide, enclosed by 30-cm walls). Each arm was attached to plastic legs that achieved elevation of 50 cm. Animals were placed individually at the centre of the maze, facing the same closed arm, and were allowed 5 min of free exploration. A video-camera was placed above the system in order to record the behaviour of the rats, and an observer recorded the number of entries to each arms and the time spent on each arm. The maze was thoroughly cleaned with alcohol after each test. Each rat was tested once. The final results were calculated as percentages (28), as shown below.

$$\text{Percentage of closed (open) arm entries} = \frac{\text{number of closed [open] arm entries}}{\text{number of total arm entries}} \times 100$$

$$\text{Percentage of duration spent in closed (open) arms (s)} = \frac{\text{mean duration spent in closed [open] arms}}{\text{total duration spent in all arms}} \times 100$$

Half of the animals in each group (n = 6) were exposed to the EPM on day 15 (first day after chronic physical and psychological stress), while the other half were exposed to the EPM on day 22 (1 week after chronic stress) (see Table 1 for study design).

2.6. Immunohistochemistry

The animals were anaesthetised with a lethal dose of thiopental sodium 2 h after the FST or EPM. They were perfused transcardially by heparinised saline, followed by 4% 0.1 M paraformaldehyde solution. The brains were prepared for c-fos immunohistochemistry as previously described (29). The cerebral cortical sections of the entire cortex, 50-μm-thick coronal sections at every 150 μm, were evaluated for c-fos immunoreactivity.

2.7. Statistical analysis

Statistical analysis was done using SPSS 15.0. Nonparametric tests were used. For comparison between groups, Kruskal–Wallis analysis was used. For comparison within each group, Wilcoxon analysis was used. Mann–Whitney U analysis was conducted in order to find which group caused the significant differences.

3. Results

3.1. Behavioural results

We compared results of behavioural analysis at baseline (D0) and day 14 (D14) among groups and within each group. These results are presented in Table 2 and Figure 1.

3.1.1. Comparison within each group

Control and CMS groups did not show any statistically significant behavioural change between D0 and D14. For the chronic headache group, eating duration was significantly longer at D14 (range: 0–915 s, mean ± standard error: 138.85 ± 83.44) than at D0 (0–499, 53.69 ± 39.73) ($Z = -2.028$, $P = 0.043$). For the sham group, freezing duration was longer at D0 (0–1424, 312.17 ± 114.21) than at D14 (0–531, 134.17 ± 48.60) ($Z = -2.045$, $P = 0.041$), and rearing duration was longer at D0 (65–574, 324.92 ± 112.46) than at D14 (62–434, 215.33 ± 32.49) ($Z = -2.275$, $P = 0.023$).

3.1.2. Comparison between groups

The difference among the 4 groups was statistically significant for sleeping behaviour at D14 ($\chi^2 = 8.213$, $P =$

Table 2. Comparison of behavioural analysis at baseline (D0) and 14th day (D14) of 4 groups.

	Control		Chronic headache		Sham		CMS			
	Mean ± SE	Sig	Mean ± SE	Sig	Mean ± SE	Sig	Mean ± SE	Sig	χ2 (D0/D14)	P (D0/D14)
Freeze D0 Freeze D14	86.17 ± 40.88 118.33 ± 57.54	0.878	396.23 ± 126.72 294.31 ± 105.34	0.333	312.17 ± 114.21 134.17 ± 48.60	0.041*	236.83 ± 104.97 248.58 ± 75.71	0.953	6.145/1.592	0.105/0.661
B. head groom D0 B. head groom D14	64.92 ± 5.10 57.08 ± 6.52	0.530	56.77 ± 8.60 63.08 ± 10.27	0.807	92.08 ± 14.78 76.50 ± 15.83	0.182	82.42 ± 10.59 85.67 ± 11.14	0.875	5.393/3.826	0.145/0.281
R. head groom D0 R. head groom D14	53.92 ± 13.89 57.92 ± 15.96	1.000	35.38 ± 6.18 57.92 ± 15.96	0.279	31.33 ± 12.36 38.00 ± 8.57	0.350	41.67 ± 11.65 65.25 ± 11.87	0.213	2.475/2.412	0.480/0.491
Body groom D0 Body groom D14	117.17 ± 17.64 103.67 ± 15.57	0.388	94.96 ± 21.94 106.77 ± 26.74	0.485	105.50 ± 22.07 70.75 ± 18.01	0.182	87.58 ± 23.52 128.33 ± 22.35	0.158	2.213/3.758	0.529/0.289
Eat D0 Eat D14	9.25 ± 9.25 19.33 ± 13.78	0.465	53.69 ± 39.73 138.85 ± 83.44	0.043*	0 10.83 ± 7.31	0.180	0 0	0.317	5.934/5.855	0.115/0.119
Drink D0 Drink D14	15.33 ± 9.45 21.17 ± 7.78	0.563	17.62 ± 12.86 13.31 ± 5.43	0.398	7.92 ± 3.80 10.83 ± 7.31	0.866	4.83 ± 3.46 11.83 ± 6.05	0.574	1.492/5.800	0.684/0.112
Sleep D0 Sleep D14	0 86.33 ± 54.6	0.068	0 0	1.00	0 84.83 ± 66.78	0.180	0 0	1.00	0/8.213	1.00/0.042*
Locomotion D0 Locomotion D14	102.80 ± 37.32 64.36 ± 11.38	0.790	44.38 ± 7.88 48.92 ± 7.51	0.556	47.33 ± 8.52 38.00 ± 6.05	0.125	73.50 ± 9.12 58.00 ± 7.74	0.117	5.700/4.553	0.127/0.208
Immobility D0	232.17 ± 66.02	0.929	506.38 ± 122.75	0.087	335.25 ± 84.86	0.695	293.17 ± 112.46	0.937	2.800/1.095	0.424/0.778
Immobility D14	282.00 ± 88.11		331.15 ± 94.64		339.25 ± 70.50		312.08 ± 82.66			
Rear D0	364.75 ± 60.72	0.594	280.54 ± 47.74	0.675	324.92 ± 112.46	0.023*	388.33 ± 57.61	0.060	2.707/6.070	0.439/0.108
Rear D14	372.82 ± 50.77		265.46 ± 50.89		215.33 ± 32.49		283.00 ± 37.83			

Sig: Wilcoxon analysis to compare D0 and D14 within each group; P: Kruskal–Wallis analysis to compare D0 and D14 between each group.

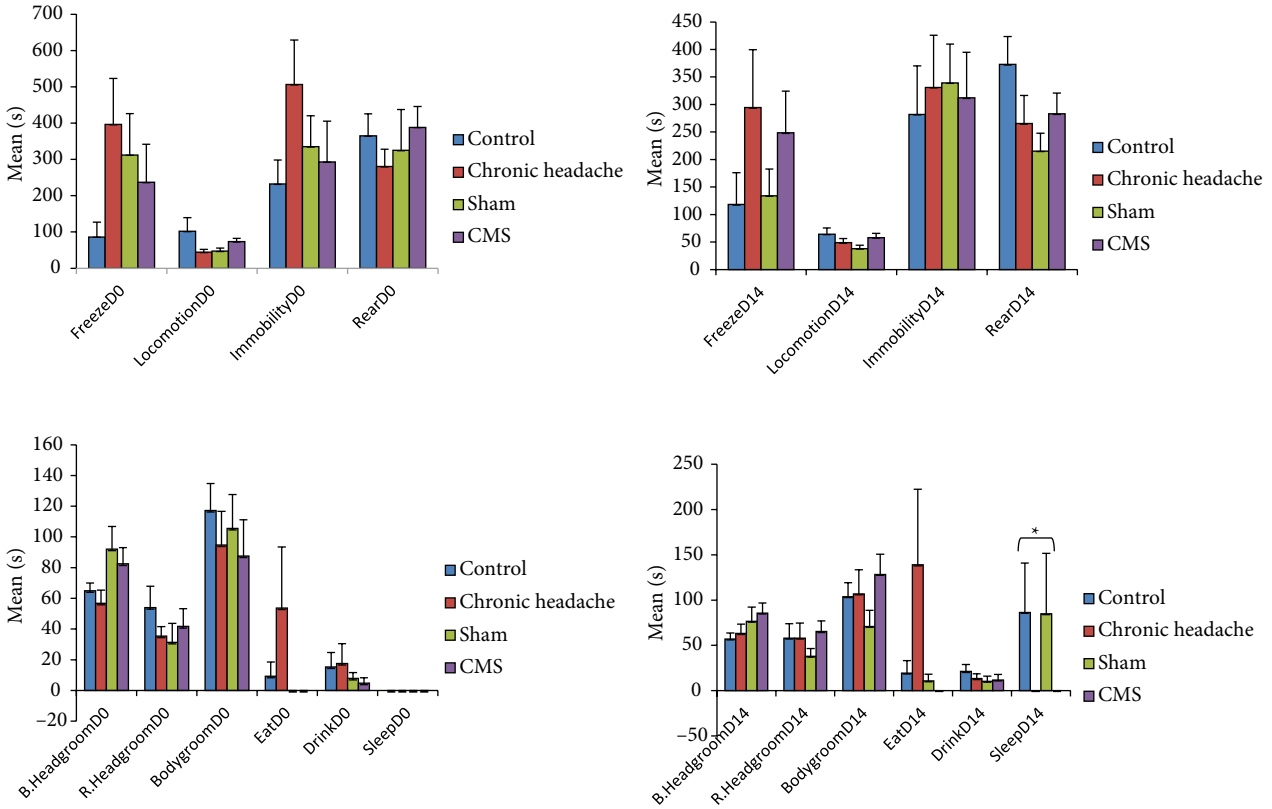

Figure 1. Comparison of behavioural analysis of groups at day 0 (D0) and day 14 (D14).

0.042). After a Mann–Whitney U analysis, it was found that the control group slept longer (0–604, 86.33 ± 54.60) at D14 than the CMS (no animals slept, Z = –2.134, P = 0.033) and chronic headache (no animals slept, Z = –2.215, P = 0.027) groups.

3.2. Ultrasonic vocalisation calls

USV calls were monitored and analysed with the Sonotrack USV detector system at D0 and D14 and during the FST (pretest and test). The results are shown in Tables 3 and 4 and Figure 2.

At D0, none of the animals emitted USVs during behavioural analysis. At D14, 3 animals from the control group (25%) emitted USVs with a frequency of between 20 and 40 kHz (distress class). No animals from the other groups emitted USVs (χ^2 = 9.853, P = 0.020).

During the FST pretest, a total of 29 animals (59.18%) emitted USVs and the majority were distress calls. There was no statistically significant difference among groups with regard to existence of USVs (χ^2 = 1.819, P = 0.611), nature of USVs (χ^2 = 7.431, 0.283), and duration of USVs (for Band I, χ^2 = 6.741 and P = 0.081; for Band II, χ^2 = 2.627 and P = 0.269). During the FST test session, only 3 animals from the control group emitted USVs that fell between a 40 and 60 kHz frequency (mean duration ± SD: 2.20 ± 1.73), and only 1 animal from the sham group emitted USVs that fell between a 20 and 40 kHz frequency (duration: 1.80 s). This caused a statistically significant difference between groups (χ^2 = 4.000 and P = 0.046).

3.3. Elevated Plus Maze

Results of the EPM are presented in Figure 3. Concerning animals that were exposed to the EPM 1 day after the chronic stress regimen, there were statistically significant differences among the 4 groups with regard to percentage of open arm entries (χ^2 = 9.737 and P = 0.021) and open arm duration (χ^2 = 9.473, P = 0.024). This statistically significant difference was caused by the difference between the sham group and the control and CMS groups. The control group (23.82 ± 8.26, P = 0.022) and CMS group (16.00 ± 5.61, P = 0.022) had a significantly higher percentage of open arm entries than the sham group (none of the animals entered the open arms). Regarding percentage of duration spent in open arms, the control group (19.70 ± 8.27, P = 0.022) and CMS group (5.02 ± 2.44, P = 0.022) had significantly higher scores than the sham group.

Concerning animals that were exposed to the EPM 1 week after chronic stress regimen, there were no statistically significant differences among the 4 groups with regard to

Table 3. Ultrasonic vocalisations (USVs) in experimental groups during behavioural analysis.

Day	Groups	USV calls							
		(-)	(+)	χ^2	P	kHz	Onset (min)	Duration (ms)	Concurrent behaviour
D0	Control	12	0	-	-	-	-	-	-
	Chronic headache	12	0						
	Sham	12	0						
	CMS	12	0						
D14	Control	9	3	9.853	0.020*	20–40	2.18 27.11 8.32	42 msn 165 msn 6 msn	Bilateral head grooming Freezing Eating
	Chronic headache	12	0				-	-	-
	Sham	12	0				-	-	-
	CMS	12	0				-	-	-

*: Statistically significant for $P < 0.05$.

Table 4. USVs during Forced Swim Test.

	Group					
	Control N(%)	Chronic headache N(%)	Sham N(%)	CMS N(%)	χ^2	P
FST pretest						
USV (-)	3 (25)	6 (46.2)	5 (41.7)	6 (50)	1.819	0.611
USV (+)	9 (75)	7 (53.8)	7 (53.8)	6 (50)		
FST-test						
USV (-)	9 (75)	13 (100)	11 (91.7)	12 (100)	6.760	0.080
USV (+)	3 (25)	-	1 (8.3)	-		
FST pretest						
Nature of USVs						
Band I[1]	4 (44.4)	6 (85.7)	5 (71.4)	6 (100)		
Band II[2]	1 (11.1)	-	1 (14.3)	-	7.431	0.283
Band I + II	4 (44.4)	1 (14.3)	1 (14.3)	-		
FST test						
Nature of USVs					4.000	0.046*
Band I	-	-	1 (100)	-		
Band II	3 (100)	-	-	-		
Band I + II	-	-	-	-		

[1]: USVs between 18 and 32 kHz, related to distress.
[2]: USVs between 32 and 50 kHz, related to positive affect.
*: Statistically significant for $P < 0.05$.

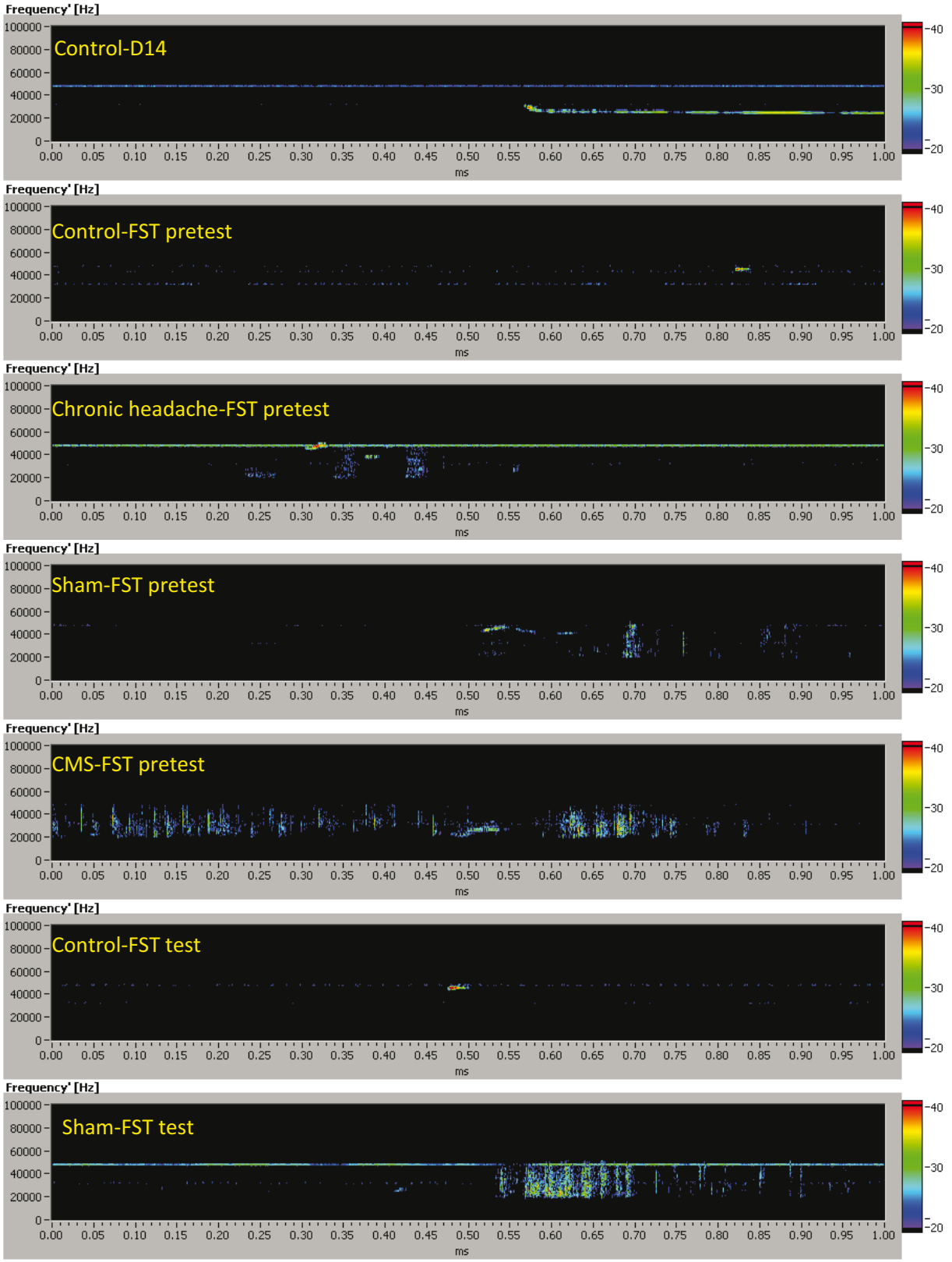

Figure 2. Sonograms of ultrasonic vocalisations (USVs) emitted by the rats.

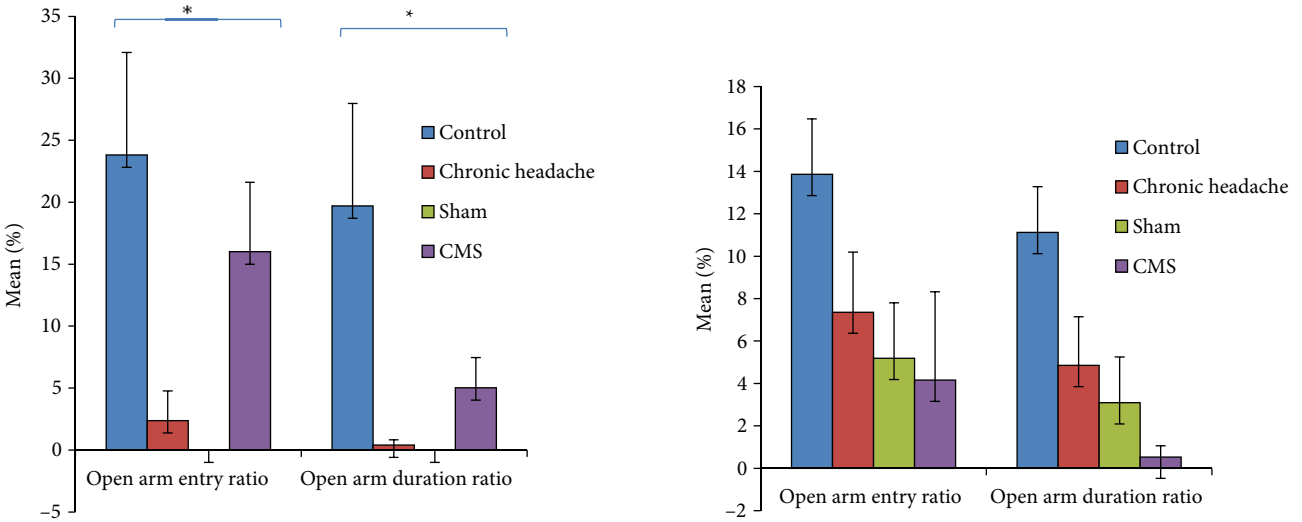

Figure 3. Results of the performance of each group for Elevated Plus Maze.

percentage of open arm entries and duration spent in open arms. Mann–Whitney U analysis was performed in order to detect a statistically significant difference between any 2 groups. According to this analysis, percentage of open arm entries and duration spent in open arms were significantly higher in the control group (13.86 ± 3.28, 11.12 ± 3.93) than the CMS group (4.16 ± 4.16, 0.53 ± 0.53; P = 0.021).

3.4. Forced Swim Test

Results of the FST test session are presented in Figure 4. According to Kruskal–Wallis analysis, there was no statistically significant difference among the 4 groups with regard to their mean duration of climbing, swimming, and immobility behaviour in the FST for both time intervals. In addition, Kruskal–Wallis analysis was performed in order to find whether animals emitting USV calls during the FST pretest and FST test sessions differed from the animals that did not emit USV calls during the FST. The results showed no statistically significant differences (data not shown).

3.5. Immunohistochemistry results

c-fos immunoreactivity of the cortex (insular cortex, agranular cortex, piriform cortex, endopiriform cortex, cingulated cortex), basal ganglia (caudate putamen, claustrum), amygdale (medial nucleus, central nucleus, basolateral nucleus, basomedial nucleus), thalamus (ventral posterolateral nucleus, ventral posteromedial nucleus, paraventricular nuclei, lateral septal nuclei), and hypothalamus (paraventricular nuclei, ventromedial nucleus, ventrolateral nucleus) were evaluated 2 h

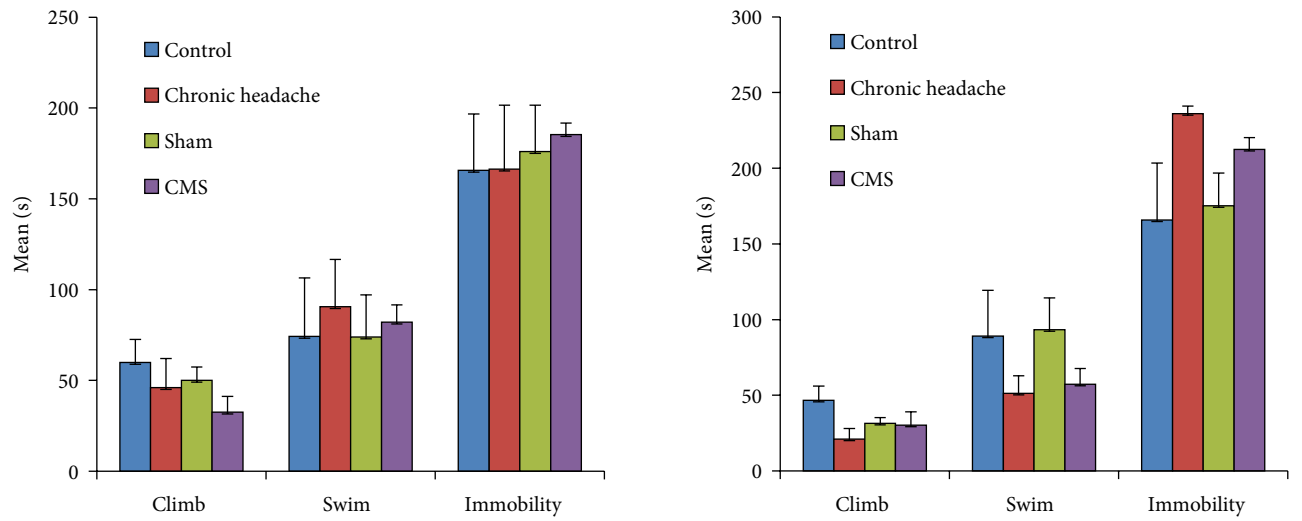

Figure 4. Results of performance of each group for Forced Swim Test.

after the EPM and FST. There were no statistically significant differences among groups with regard to c-fos immunoreactivity in any brain region (data not shown).

4. Discussion

Our results partially confirm our hypothesis. Two different chronic stressors caused mostly similar neurobiological and behavioural consequences; however, difference occurred with regard to anxiety-like behaviour. Rats that were exposed to CMS showed significantly more anxiety-like behaviour than the control group at 1 week (but not 1 day) after stressor regimen with the EPM. The chronic headache group did not show any significant differences compared to the control group in the EPM; although they both showed more anxiety-like behaviour at 1-day and 1-week intervals, the difference was not statistically significant. Although depressive-like phenotypes after a CMS model in rodents have been repeatedly and conclusively reported, results about anxiety-like behaviour after a CMS model are inconclusive (10). These discrepancies appear to be caused by the application of different anxiety models at different time intervals following exposure to CMS. Regarding the EPM, although some studies confirm our results that rats show more anxiety-like behaviour (less time spent in open arms) at 3 days (30) and 5 days (31) after exposure to CMS, there are several other studies that report the opposite (32,33). In addition, several other studies report that CMS does not have a significant effect on performance in the EPM (10,34,35). To our knowledge, there are no previous studies that evaluated the behaviour of rats with chronic headache in the EPM. In one study, KCl-induced cortical spreading depression (CSD, the most likely cause of migraine aura) caused significantly higher anxiety-like behaviour in an open field test for rats (36). Other studies investigating anxiety-like behaviour in rats experiencing physical pain used neuropathic and inflammatory pain models. It was shown that chronic inflammatory pain resulted in anxiety-like behaviour as evidenced by EPM, open field test, and social interaction test (29). There are some conflicting results about neuropathic pain. One study showed that chronic neuropathic pain increased anxiety-like behaviour in the EPM (37), while another study reported that neuropathy increased anxiety-like behaviour in rats in an open field test but not in the EPM (38). Taken together, these previous results about the effect of chronic psychological and physical stressors show that chronic stress may cause anxiety-like behaviour in rats, but the results depend on which model of chronic stress is used. In our study, the results indicate that CMS causes an increase in anxiety-like behaviour, while chronic headache does not appear to affect anxiety in rats.

In this study, we used the FST to evaluate depressive-like behaviour after chronic stress. Rats exposed to CMS and chronic headache did not show significantly different depressive-like behaviours than the control group. Although previous studies repeatedly showed that the CMS model causes a significant increase in depressive-like behaviour in rodents, different models of depression other than the FST were used in those studies (2–9). Several studies used the FST as a model of depression after CMS, and some reported a significant increase in depressive-like behaviour as measured by longer immobility time (32,39,40), while only one study reported that CMS reduced immobility time in the FST (41). To our knowledge, there is no previous study that investigated the effect of chronic headache on FST behaviour. Our results about the FST indicate that physical and psychological stressors induce similar behaviour in the FST, and this behaviour is not significantly different from that of the control group. Behaviour analysis results before (D0) and after (D14) chronic stressors were not conclusive enough to offer specific information about the behavioural consequences of the 2 different kinds of chronic stress in this study. CMS did not cause any significant behavioural change between D0 and D14; however, chronic headache caused a significant increase in eating behaviour. When the 4 groups were compared at D14, both chronic stressors caused a significant decrease in sleeping behaviour. To our knowledge, there is no previous study to which we can compare our results about the behavioural consequences of chronic stress in rats. We expected to find that both chronic stressors would increase pain and anxiety-related behaviours such as freezing and grooming. However, our results did not meet our expectations. We had previously reported that single CSD induction by N-methyl-D-aspartate receptor (NMDA) caused a significant increase in freezing behaviour in rats (42). However, this study used a chronic headache model rather than an acute headache model induced by NMDA administration. In this study, there was no significant difference between chronic stress groups and controls with regard to freezing behaviour.

Our results also show that chronic stressors do not cause an increase in USV, neither during normal behavioural analysis nor during the FST. In this study, the control group emitted significantly more USVs than the other 3 groups. At D14, these vocalisations were also called '22-kHz vocalisations' (23) and are assumed to reflect a negative state of the animal (22). During the FST pretest, approximately half of the animals in each group emitted these '22-kHz vocalisations', which may show that these animals were stressed when they were forced to swim for the first time. During the FST test session (second day of forced swim), none of the animals from the chronic stress groups emitted any USVs, although 3 animals emitted '50-kHz vocalisations', which have been associated with positive affect of the animal (22). This caused a significant difference between groups. These results show that control animals

showed a 'desensitisation' to the aversive stimulus (forced swim), while animals exposed to chronic stress did not.

We also analysed the c-fos expression in various brain regions 2 h after the EPM and FST. We did not find any significant differences among the 4 groups with regard to c-fos immunoreactivity. Previous studies showed that exposure to only 1 trial in the EPM (as done in this study) caused an increase in expression of c-fos in the limbic cortical regions, paraventricular nucleus of the hypothalamus, septal region, medial nucleus of the amygdale, and dorsomedial nucleus of the hypothalamus (43–45), while repeated exposures to the EPM increased c-fos expression in the piriform cortex, septal nucleus, thalamic and hypothalamic paraventricular nuclei (46), and medial prefrontal cortex and amygdala (47).

In summation, our results indicate that chronic psychological and physical stress cause similar behavioural effects. Chronic psychological stress, not chronic physical stress, increases anxiety-like behaviour, but it does not seem to affect depressive-like behaviour. Both kinds of chronic stress cause nonspecific but significant changes in c-fos neural activity after the EPM and FST. These results may advance our general knowledge about the complex relationship between chronic pain and psychiatric disorders.

Acknowledgement

This work was supported by a grant from the Scientific Research Project Department of Gazi University, Ankara, Turkey (01/2009–02).

References

1. Katz RJ. Animal model of depression: pharmacological sensitivity of a hedonic deficit. Pharmacol Biochem Behav 1982; 16: 965–968.
2. Willner P. Validity, reliability and utility of the chronic mild stress model of depression: a 10-year review and evaluation. Psychopharmacology (Berl) 1997; 134: 319–329.
3. Vollmayr B, Henn FA. Stress models of depression. Clin Neurosci Res 2003; 3: 245–251.
4. Bielajew C, Konkle AT, Merali Z. The effects of chronic mild stress on male Sprague Dawley and Long Evans rats: biochemical and physiological analyses. Behav Brain Res 2002; 136: 583–592.
5. Dang H, Chen Y, Liu X, Wang Q, Wang L, Jia W, Wang Y. Antidepressant effects of ginseng total saponins in the forced swim test and chronic mild stress models of depression. Prog Neuropsychopharmacol Biol Psyhciatry 2009; 33: 1417–1424.
6. Willner P, Muscat R, Papp M. Chronic mild stress-induced anhedonia: a realistic animal model of depression. Neurosci Biobehav Rev 1992; 16: 525–534.
7. D'Aquila PS, Brain P, Willner P. Effects of chronic mild stress on performance in behavioural tests relevant to anxiety and depression. Physiol Behav 1994; 56: 861–867.
8. Moreau JL, Scherschlicht R, Jenck F, Martin JR. Chronic mild stress induced anhedonia model of depression; sleep abnormalities and curative effects of electroshock treatment. Behav Pharmacol 1995; 6: 682–687.
9. D'Aquila PS, Newton J, Willner P. Diurnal variation in the effect of chronic mild stress on sucrose intake and preference. Physiol Behav 1997; 62: 421–426.
10. Cox BM, Alsawah F, McNeill PC, Galloway MP, Perrine SA. Neurochemical, hormonal, and behavioural effects of chronic unpredictable stress in the rat. Behav Brain Res 2011; 220: 106–111.
11. Leo RJ. Chronic pain and comorbid depression. Curr Treat Options Neurol 2005; 7: 403–412.
12. Hummel M, Lu P, Cummons TA, Whiteside GT. The persistence of a long-term negative affect state following the induction of either acute or chronic pain. Pain 2008; 140: 436–445.
13. Ruoff GE. Depression in the patient with chronic pain. J Fam Pract 1996; 43: S25–S34.
14. Fishbain DA, Cutler R, Rosomoff HL, Rosomoff RS. Evidence-based data from animal and human experimental studies on pain relief with antidepressants: a structured review. Pain Med 2000; 1: 310–316.
15. Baskin SM, Smitherman TA. Migraine and psychiatric disorders: comorbidities, mechanisms, and clinical applications. Neurolog Sci 2009; 30 (Suppl. 1): S61–S65.
16. Casucci G, Villani V, Finocchi C. Therapeutic strategies in migraine patients with mood and anxiety disorders: physiopathological basis. Neurolog Sci 2010; 31: S99–S101.
17. Bolay H, Moskowitz MA. Mechanisms of pain modulation in chronic syndromes. Neurology 2002; 59: 2–7.
18. Lin YH, Liu AH, Xu Y, Tie L, Yu HM, Li XJ. Effects of chronic unpredictable mild stress on brain-pancreas relative protein in rat brain and pancreas. Behav Brain Res 2005; 165: 63–71.
19. Liang S, Byers DM, Irwin LN. Sex and diet affect the behavioural response of rats to chronic mild stressors. Physiol Behav 2008; 93: 27–36.
20. Quinn LP, Stean TO, Trail B, Duxon MS, Stratton SC, Bilinton A, Upton N. LABORAS: Initial pharmacological validation of a system allowing continuous monitoring of laboratory rodent behavior. J Neurosci Methods 2003; 130: 83–92.
21. Quinn LP, Stean TO, Chapman H, Brown M, Vidgeon-Hart M, Upton N, Billinton A, Virley DJ. Further validation of LABORAS using various dopaminergic manipulations in mice including MPTP-induced nigro-striatal degeneration. J Neurosci Methods 2006; 156: 218–227.

22. Portfors CV. Types and functions of ultrasonic vocalizations in laboratory rats and mice. J Am Assoc Lab Anim Sci 2007; 46: 28–34.

23. Wohr M, Borta A, Schwarting RKW. Overt behavior and ultrasonic vocalization in a fear conditioning paradigm: a dose-response study in the rat. Neurobio Learn Mem 2005; 84: 228–240.

24. Panksepp J, Burgdorf J. 50-kHz chirping (laughter?) in response to conditioned and unconditioned tickle-induced reward in rats: effects of social housing and genetic variables. Behav Brain Res 2000; 115: 25–38.

25. Porsolt RD, Le Pichon M, Jalfre M. Depression: a new animal model sensitive to antidepressant treatments. Nature 1977; 266: 730–732.

26. Slattery DA, Cryan JF. Using the rat forced swim test to assess antidepressant-like activity in rodents. Nat Protoc 2012; 7: 1009–1014.

27. Pellow S, Chopin P, File SE, Briley M. Validation of open: closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J Neurosci Met 1985; 14: 149–167.

28. Walf AA, Frye CA. The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. Nat Protoc 2007; 2: 322–328.

29. Parent AJ, Berube P, Beaudry H, Bergeron J, Berube P, Drolet G, Sarret P, Gendron L. Increased anxiety-like behaviors in rats experiencing chronic inflammatory pain. Behav Brain Res 2012; 229: 160–167.

30. Bondi CO, Rodriguez G, Gould GG, Frazer A, Morilak DA. Chronic unpredictable stress induces a cognitive deficit and anxiety-like behavior in rats that is prevented by chronic antidepressant drug treatment. Neuropsychopharmacology 2008; 33: 320–331.

31. Maslova LN, Bulygina VV, Markel AL. Chronic stress during prepubertal development: immediate and long-lasting effects on arterial blood pressure and anxiety-related behavior. Psychoneuroendocrinology 2002; 27: 549–561.

32. Kompagne H, Bardos G, Szenasi G, Gacsalyi I, Harsing LG, Levay G. Chronic mild stress generates clear depressive but ambiguous anxiety-like behavior in rats. Behav Brain Res 2008; 193: 311–314.

33. Li Y, Zheng X, Liang J, Peng Y. Coexistence of anhedonia and anxiety-independent increased novelty seeking behavior in the chronic mild stress model of depression. Behav Proc 2010; 83: 331–339.

34. Mitra R, Vyas A, Chatterjee G, Chattarji S. Chronic-stress induced modulation of different states of anxiety-like behavior in female rats. Neurosci Lett 2005; 383: 278–283.

35. Matuszewich L, Karney JJ, Carter SR, Janasik SP, O'Brien JL, Friedman RD. The delayed effects of chronic unpredictable stress on anxiety measures. Physiol Behav 2007; 90: 674–681.

36. Bogdanov VB, Bogdanova OV, Koulchitsky SV, Chauvel V, Multon S, Makarchuk MY, Brennan KC. Behavior in the open field predicts the number of KCl-induced cortical spreading depressions in rats. Behav Brain Res 2013; 236: 90–93.

37. Matsuzawa-Yanagida K, Narita M, Nakajima M, Kuzumaki N, Niikura K, Nozaki H, Takagi T, Tamai E, Hareyamo N, Terada M et al. Usefulness of antidepressants for improving the neuropathic pain-lie state and pain-induced anxiety through actions at different brain sites. Neuropsychopharmacology 2008; 33: 1952–1965.

38. Gregoire S, Michaud V, Chapuy E, Eschalier A, Ardid D. Study of emotional and cognitive impairments in mono-neuropathic rats: effect of duloxetine and gabapentin. Pain 2012; 153: 1657–1663.

39. Bielajew C, Konkle ATM, Kentner AC, Stewart A, Hutchins AA, Santa-Maria Barbagallo L, Fouriezos G. Strain and gender specific effects in the forced swim test: effects of previous stress exposure. Stress 2003; 6: 269–280.

40. Fujisaki C, Utsuyama M, Kuroda Y, Watanabe A, Seidler H, Watanabe S, Kitagawa M, Hirokawa K. An immunosuppressive drug, cyclosporine-A acts like antidepressant for rats under unpredictable chronic stress. J Med Dent Sci 2003; 50: 93–100.

41. Harro J, Haidkind R, Harro M, Modiri AR, Gillberg PG, Pahkla R, Matto V, Orelland L. Chronic mild unpredictable stress after noradrenergic denervation: attenuation of behavioural and biochemical effects of DSP-4 treatment. Eur Neuropsychopharmacol 1999; 10: 5–16.

42. Akcali D, Sayin A, Sara Y, Bolay H. Does single cortical spreading depression elicit pain behavior in freely moving rats? Cephalalgia 2010; 30: 1195–1206.

43. Silveira MCL, Sandner G, Graeff F. Induction of Fos immunoreactivity in brain by exposure to the elevated plus-maze. Behav Brain Res 1993; 56: 115–118.

44. Duncan GE, Knapp DJ, Breese GR. Neuroanatomical characterization of fos induction in rat behavioural models of anxiety. Brain Res 1996; 713: 79–91.

45. Benjamini V, Guimarães FS. Activation of neurons containing the enzyme nitric oxide synthase following exposure to an elevated plus-maze. Brain Res Bull 2006; 69: 347–355.

46. Galvis-Alonso OY, Garcia AMB, Orejarena MJ, Lamprea MR, Botelho S, Conde CA, Morato S, Garcia-Cairasco N. A combined study of behavior and Fos expression in limbic structures after re-testing Wistar rats in the elevated-plus maze. Brain Res Bull 2010; 81: 595–599.

47. Albrechet-Souza L, Borelli KG, Brandao ML. Activity of the medial prefrontal cortex and amygdala underlies one-trial tolerance of rats in the elevated plus-maze. J Neurosci Met 2008; 169: 109–118.