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Chronic unpredictable mild stress paradigm in male Wistar rats: effect on anxiety- and depressive-like behavior

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Abstract

OBJECTIVES: There are several models of depression. Chronic unpredictable mild stress (CMS) appears to have the greatest validity, although it is often being criticized for low reliability. **METHODS:** Male Wistar/DV rats were used in this study to assess our modified 2-week model of CMS as a combination of psychosocial, physical and metabolic stressors and to compare the effect of acute administration of venlafaxine (VFX) and diazepam (DZP), either in stress or no stress conditions. The animals were exposed to one particular stressor each day. The time of day and duration of the stressor differed across the procedure to avoid animals to adapt to the stress stimulus. After cessation of stress, the animals underwent the following behavioral tests to assess motor activity, cognition, anxiety- and depression-like behavior: Open field test, Elevated plus maze, Forced swim test, Stress-iduced hyperthermia, Light/dark test and Y maze. To assess hypothalamic-pituitary-adrenal axis (HPA) reactivity in our CMS model, plasma corticosterone levels were measured 24 h after termination of stress. **RESULTS:** Corticosterone levels were significantly increased compared to control values ($p<0.05$) in our experimental schedule of CMS. Our paradigm produced delayed anxiety-like behavior observed in Open field (decreased time spent in central zone 3 weeks after CMS, $p<0.05$), with anxiolytic effect of CMS shortly after its cessation. Stressed animals spent more time in the open arms of Elevated plus maze ($p<0.05$) and travelled longer distance in the light zone of the Light/dark box ($p<0.01$). CMS did not increase the behavioral despair analyzed in Forced swim test yet it disrupted the capacity of the Stress-induced hyperthermia test (CMS rats failed to react to the stress by increasing the core temperature). **CONCLUSIONS:** Based on our results, we can conclude that our CMS protocol leads to increased corticosterone levels as a result of HPA axis hyperactivity and produces delayed onset of anxiogenic behavior. Moreover, CMS exerted a substantial effect on the behavioral outputs, interfering with drug testing.

ORIGINAL ARTICLE

INTRODUCTION

Depression and anxiety are the global commonest mental disorders. Unipolar depressive disorder is the third leading health concern, accounting for 4.3% of the global burden of disease. The World Health Organization (WHO) has predicted that by 2030 depression will be the leading cause of disease burden globally (WHO 2011). In spite of the fact that depression and anxiety are different syndromes, they share genetic and neurobiologic qualities, which leads to overlapping symptomatology between these two disorders in many cases. Similarities in the etiology of the two disorders has been suggested as a result of their high comorbidity (Hirschfeld, 2001). Another explanation of the comorbidity between anxiety and depression is the hypothesis that high anxiety is a risk factor for the incidence of other mood disorders (Martin 2003; Dabkowska & Dabkowska-Mika 2014).

Stress is one of the main factors in the development of anxiety and depression. This connection was discovered by Board *et al.* (1956), but the first detailed 24-h study was described in 1973 by Sachar *et al.* (1973), showing elevation of plasma cortisol concentrations in depressed patients and an altered circadian pattern of cortisol secretion. Several weeks of exposure to stressors can lead to an increased reactivity of the hypothalamic-pituitary-adrenal axis (HPA) and cause an increased level of plasma corticosterone in rats (Bielajew *et al.* 2002; Grippo *et al.* 2005). This is consistent with the finding of dysregulation of the HPA axis in humans affected by major depression (Parker *et al.* 2003; Romer *et al.* 2009).

Chronic mild unpredictable stress (CMS) is a widely used model of depressive- and anxiety-like behavior with good predictive face as well as construct validity (Willner, 1997). The CMS model was first described by Katz and colleagues using severe stressors (Katz, 1981, 1982) and further developed and refined by Willner, whose model differs in the intensity of the stressor used. The idea is that by mixing different types of stressors, the animals cannot predict what will happen next and therefore it is harder if not impossible to adapt to the situation (in line with behavioral despair). A vast majority of publications have confirmed that CMS causes behavioral changes that parallel symptoms of depression and therefore produce the „depressive" behavioral profile (Willner, 2005). The capability of CMS to produce an anxiety-like phenotype has not been sufficiently addressed. Nevertheless, anhedonic behavior after CMS could be reversed by chronic treatment with antidepressants, including atypical antidepressants (Muscat *et al.* 1992), selective serotonin reuptake inhibitors (SSRIs) (Montgomery *et al.* 2001; Rygula *et al.* 2006), along with tricyclics (Stamford *et al.* 1991; Bondi *et al.* 2008) and monoamine oxidase inhibitors in rats (Willner *et al.* 1987; Willner, 2005). The chronic reversal effects of serotonin-norepinephrine reuptake inhibitors (SNRIs) such venlafaxine was also described to be effective in several studies (Briones *et al.* 2012; Darwish *et al.* 2013).

The aim of our study was to validate our 2-week model of CMS as a combination of psychosocial, physical and metabolic stressors in order to test newly synthesized substances with potential antidepressant and/or anxiolytic properties. The acute administration of venlafaxine (VFX) and diazepam (DZP) was chosen to assess the behavioral profile in healthy and CMS rats. The aim was to assess whether acute VFX or DZP administration could reverse the effect of CMS which, to the best of our knowledge, has not been described in the literature. Another aim was to evaluate the potential of the two-week model of CMS to induce anxiety-like behavior in rats.

MATERIALS AND METHODS

Animals

Male Wistar/DV rats (220–240g, n=60) were used in this study. All animals were housed under standard laboratory conditions (temperature: 22±2°C, humidity: 55±10%) with a 12 h light/12 h dark cycle (lights on at 7 a.m.). Pelleted food and tap water was available *ad libitum*. All performed experiments were in compliance with the Principles of Laboratory Animal Care issued by the Ethical Committee of the Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences and the experimental protocol was approved by the State Veterinary and Food Administration of the Slovak Republic.

Experimental groups

The rats were acclimatized to the animal housing facility for 2 weeks prior to experimental procedures. The animals were assigned to groups according to their basal motor activity measured by using an Open field test (OF). To avoid between-test interaction, at least four days without testing were applied. The following groups were used: Venlafaxine (VFX) + physiological condition (no stress), n=10; Diazepam (DZP) + physiological condition (no stress), n=10; Control + physiological condition (no stress), n=10; VFX + chronic unpredictable mild stress condition (CMS), n=10; DZP + CMS, n=10; Control + CMS, n=10.

Chronic unpredictable mild stress (CMS) procedure

The animals were exposed to one particular stressor each day. The time of day and duration of the stressor differed across the procedure to avoid animals to adapt to the stress stimulus. The following stressors were used: (A) 24-h predator´s odor stress – wooden block (5×5×10 cm) covered by a fabric well soiled by cat odor; (B) 5-min Forced swim test – rats were placed in a glass cylinder (45 cm tall and 25 cm in diameter) filled with water (18±1 °C) for 5 min; (C) 24-h overcrowding – animals were taken from their home cage and placed in a cage in which the total number of animals reached 6;

(D) 24-hour food deprivation; (E) 24-hour soiled cage – 500 ml water was poured into the home cage; (F) 24-hour air-puff stress in home cage (divided into forty-five 1-minute periods, spread randomly within 24 hours and separated with the average resting period of 30 minutes (13–44 min); (G) rest day. CMS was delivered for 14 days.

Drug administration

Rats were injected intraperitoneally with a single dose of either saline (control group – C), diazepam (APAURIN sol inj – 2.5 mg/kg) or venlafaxine hydrochloride (Chemoz, Czech Republic, purity, 98.5%), which was dissolved in saline (10 mg/kg). The volume of 1 ml/kg body weight was injected 30 minutes before each behavioral test except Stress-induced hyperthermia, in which a 60-minute interval was used due to the different character of the test.

Behavioral analysis

In all tests, the movements of the rat were tracked with a digital camera and analyzed by computer software ANYMAZE™ (Stoelting Europe, Ireland). All experiments were conducted between 8 a.m. and 1 p.m. during the light phase. Assignment of animals into experimental groups was conducted prior to experimental procedures based on their activity in basal Open field test, which lasted 5 min and is decribed below. All the mazes were cleaned after each animal with 60% ethanol to remove odor cues.

Elevated plus maze (EPM)

A cross-shaped elevated maze with two open arms and two closed arms was used to assess anxiety-like behavior. All parts of the apparatus were made of dark polyvinyl plastic. The arms of the maze were 50 cm above the floor, 50 cm long and 10 cm wide. Each session started by placing the rat in the central area (10×10 cm) facing the open arms of the maze and lasted 5 min. The test was conducted 1 day after cessation of CMS.

Stress-induced hyperthermia (SIH)

Rectal temperature measurement along with handling was used as a stressor resulting in an increase in rectal temperature, an indicator of anxiety-like behavior. SIH was expressed as $\Delta T = T_1 - T_0$, where T_0 was rectal temperature (rT) at the beginning and T_1 was rT 30 minutes after stress stimulus (first measurement of rT). Rectal body temperature of the animals was measured using a digital rectal thermometer BAT-12 (Physitemp Clifton, NJ, USA) with rectal probe RET-2. The test was conducted 2 days after cessation of CMS.

Y maze

The Y maze apparatus consisted of three identical arms (50×16×32 cm) made of black plastic joined to form a "Y" shape with the rat bedding covering the bottom. Large extramazal cues were placed on the nearby walls. The rats were placed in the start arm. After 15 min of the first trial, in which the novel arm was enclosed, the rat was returned for 1 min to the home cage, the bedding was mixed to reduce odors and cues and during the second trial, which lasted 5 min, the novel arm was uncovered.

The entries made into each arm of the Y-maze during the 5-min test were converted into percentages of total entries made into all three arms. The dependent variable for spatial memory was a difference score, which was calculated by subtracting the percentage of entries into the other arm from the percentage of entries into the novel arm. The test was conducted 1 week after cessation of CMS.

Light/dark test (L/D)

The L/D apparatus for evaluation of anxiety-like behavior consisted of a white plastic arena, size 60×40 cm, with a dark compartment (40×20×40 cm) and an opening (10×8 cm). At the beginning of the experiment, the rat was placed in the light part of the box, facing the opening to the dark part. Test duration was 5 min and was conducted 1 week after cessation of CMS.

Forced swim test (FST)

The rats were placed in the glass cylinder (45 cm tall and 25 cm in diameter) filled with water (24±1 °C) for a 15-min pre-test period to induce depression-like behavior. The following 5-min test started 24 hours later. The depth was sufficient to ensure that the animals could not touch the bottom of the container with their hind paws. The animals were returned to their home cage after resting under a heating lamp until dry. The predominant behavior (swimming, climbing, and immobility) was recorded and evaluated manually every 5 seconds. This provided an overall total of 60 scores. The test was conducted 2 weeks after cessation of CMS.

Open field (OF)

Open field is a dark polyvinyl plastic arena measuring 60×60 cm surrounded by 25 cm high walls. Each session started by placing the rat in the central area of the maze. After 10 min of basal activity measurement, the substances were administered, followed by a 60-min test session. After each session, the number of fecal boli (emotional reactivity) was noted. The test was conducted 3 weeks after cessation of CMS.

Plasma corticosterone analysis

To examine whether CMS affected hypothalamo-pituitary-adrenal (HPA) axis reactivity, plasma corticosterone level was measured. The animals were divided into two experimental groups. Undisturbed controls (n=5) were compared to controls who underwent CMS (n=5). The animals were euthanized 24 hours after termination of CMS. The rats were rapidly removed and decapitated. Trunk blood was collected into 15 ml hep-

arinized (HEPARIN LÉČIVA sol inj 50 K) cold tubes. Samples were centrifuged (2000×*g* for 10 min at 4 °C) and plasma supernatant was stored at –80 °C until analysis. Plasma corticosterone concentrations were measured using a Corticosterone Rat/Mouse ^{125}I-labelled RIA kit (DRG Instruments GmbH, Marburg, Germany) according to the manufacturer's instructions. All samples were measured in a single assay with the intra-assay variation coefficient of 3.7%. The assay sensitivity was 1.6 ng/mL.

Statistical analysis

Data analyses were performed using STATISTICA 7.0 (Statsoft, Inc. Tulsa, OK) software. The data are represented by mean ± S.E.M and were analyzed by factorial analyses of variance (ANOVA) with treatment (C vs. VFX and DZP) and condition (stress/no stress) as main factor followed by Fisher´s *LSD post-hoc* test, if applicable. The $p<0.05$ value was considered statistically significant. To investigate the differences between intervals in a one-hour running Open field test, a two-way repeated-measures ANOVA was applied with treatment (C vs. VFX and DZP) and condition (stress/no stress) as main factors and a 20 min interval (interval 1–3) as repeated-measures factor.

RESULTS AND DISCUSSION

Chronic stress represents a major risk factor for the development of many psychopathological conditions, such as major depression and/or anxiety disorders. Animal models of chronic stress help to understand the physiological and behavioral outcomes of different types of stressors. However these models have also limitations, such as the difficulty in establishing a model that perfectly recapitulates the symptoms of depression in human patients. In addition, not all rodents successfully reach the state of stress when administrated chronic mild stress. Several weeks of exposure to stressors can lead to an increased reactivity of the hypothalamo-pituitary-adrenal axis (HPA) and triggers pathological pathways of mental disorders (Bielajew *et al.* 2002; Grippo *et al.* 2005). Previous studies reported that chronic mild stress induced hypothalamo-pituitary adrenal axis hyperactivity with subsequent increased secretion of cortisol and corticosterone from the adrenal cortex. Park *et al.* (2016) found that the duration of the chronic unpredictable stress was crucial for the long-term effect of stress. Although 2-week chronic mild unpredictable stress increased corticosterone levels, the hyperactivity of HPA did not last after the recovery period, as seen after 4-week CMS (Park *et al.* 2016). HPA-axis dysfunction is regarded as the neurobiological basis of affective disorders characterized by anxiety disorders, major depressive disorder, psychotic depression, and bipolar disorder (Moreau *et al.* 1994). Others have shown that humans affected by major depression have dysregulated HPA axis (Parker *et al.* 2003; Romer *et al.* 2009). We saw a significant increase of corticosterone levels compared to control ($p<0.05$, Figure 1) in our experimental schedule of CMS. This HPA hyperactivity was observed 24h after cessation of stress, which is in concordance with other studies (Park *et al.* 2016; Ayuob *et al.* 2016).

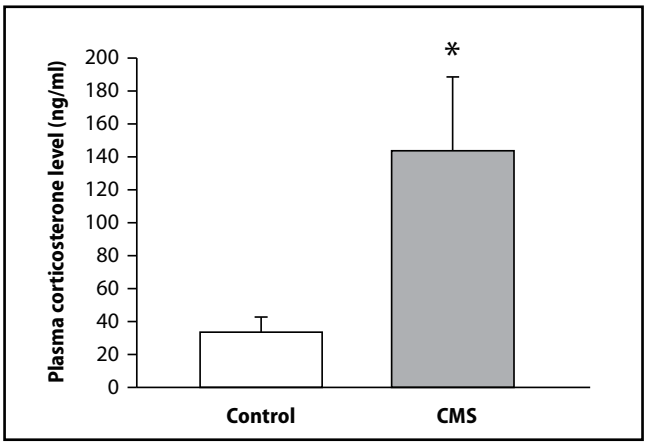

Fig. 1. Effect of chronic unpredictable mild stress (CMS) on plasma corticosterone level in comparison with control, *$p<0.05$ compared to control group

While decreased immobility in FST is widely accepted as a measure for antidepressant activity, and despite the fact that most of the studies described higher immobility level in FST after CMS (Bielajew *et al.* 2003; Kompagne *et al.* 2008), not all studies found significant increase in immobility (Harro *et al.* 1999; Liang *et al.* 2008; Wainwright *et al.* 2011; Donmez *et al.* 2014). Neither did we find significant changes caused by CMS in FST [$F(1,54)=0.454$, $p=0.503$; Figure 2]. Thus, CMS did not increase behavioral despair, which is in concordance with the study of Jaime and colleagues (Jose Jaime *et al.* 2016) who suggested that young male rats might be resilient to CMS. Thirty percent of animals exposed to CMS are resistant to the development of anhedonia, whereas the remaining animals are responsive (Bergstrom *et al.* 2008). Stress resilience could be intrastrain dependent. The age and different intensity, duration and nature of stressors provide further partial explanation of these discrepancies.

The model of CMS used also affects anxiety-like behavior in rats. It is well known from clinical studies that the level of comorbidity between depression and anxiety is high (Ballenger, 1999). Nevertheless, results from studies testing anxiety-related behavior induced by CMS are rather ambiguous. Some groups of researchers found a decreased (D'Aquila *et al.* 1994; Kompagne *et al.* 2008) while others an increased (Griebel *et al.* 2002; Maslova *et al.* 2002) level of anxiety in rodents. The interval between termination of the CMS procedure and behavioral analysis along with the length of CMS by itself seems to be important for evaluation of locomotor activity and depressive- as well as anxiety-like behavior. Some authors reported increased anxiety when tested one day after CMS (Jose Jaime *et al.* 2016),

whereas no effects on anxiety behavior were observed by several other authors (Cox *et al.* 2011).

We thus suggest that anxiety-like behavior after CMS could be time-dependent. In the present study we found a significant decrease in anxiety-like behavior, which manifested as increased time spent in open arms of EPM [$F(1,54)=5.460$, $p<0.05$, Figure 3]. Alhough an increased time spent in open arms between diazepam and control group was observed during physiological conditions as well as during CMS, these changes failed to be significant in the following *post-hoc* analysis. A comparable behavioral pattern was seen also in the L/D box, where shortly after CMS termination the rats travelled a longer distance in the light zone of the box. The statistical analysis revealed the main effect of the condition in the distance travelled in the light zone [$F(1,54)=9.366$, $p<0.01$, Figure 4] and an interaction between treatment and condition [$F(2,54)=4.644$, $p<0.05$], indicating increased locomotor activity in the light zone after undergoing chronic mild stress.

Although anxiety-like behavior was not seen in our paradigm shortly after termination of CMS, the tests performed later confirmed anxiety-like behavior of stressed rats. CMS did not affect the locomotor (distance travelled) activity in the Open field test [$F(1,51)=1.216$, $p=0.28$, Figure 5] but it significantly affected performance in the central zone [$F(1,51)=5.472$, $p<0.05$, Figure 6]. In other words, rats spent less time in the central zone of the OF. The test was conducted with delay of three weeks after cessation of CMS and might be a result of higher anxiety in the experimental subjects. This behavior resembles signs of post-traumatic stress disorder, which is characterized by delayed onset of anxiety and a hyperactive autonomic nervous system. In a previous study, Donmez *et al.* (2014) reported that rats exposed to CMS showed significantly more anxiety-like behavior than did controls one week yet not one day after stress regimen in EPM. Matuszewich and colleagues (2007) investigated the effect of CMS on unconditioned as well as conditioned response task. Although the authors did not reach significant results in unconditioned tests (EPM, LD), the animals tested 14 days after stress were less likely to enter the open arm and spent more time in the open arm than rats tested immediately. A lasting anxiogenic effect of predator stress (which was used in our paradigm) was reported also in several unconditioned response tasks (Adamec & Shallow, 1993; Calvo-Torrent *et al.* 1999). An anxiogenic effect lasting 7 days after CMS was reported also by Cuadra and colleagues (2001). Based on these data we can conclude that the CMS model produces delayed changes in the anxiety-like phenotype in rats.

SIH has been particularly useful as a screening approach to evaluate the efficacy of novel anxiolytic drug candidates (Vinkers *et al.* 2009). Drug classes with clinically effective anxiolytic properties such as $GABA_A$ and 5-HT_{1A} receptor agonists were identified to atten-

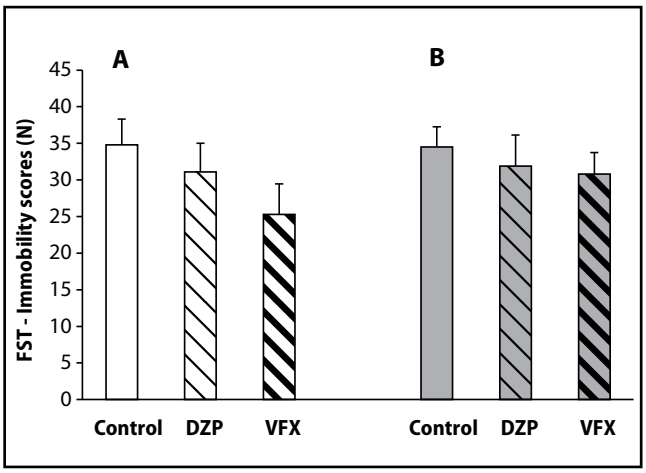

Fig. 2. Effect of chronic unpredictable mild stress (CMS) on rat performance in Forced swim test. A – physiological conditions without CMS, B – CMS conditions.

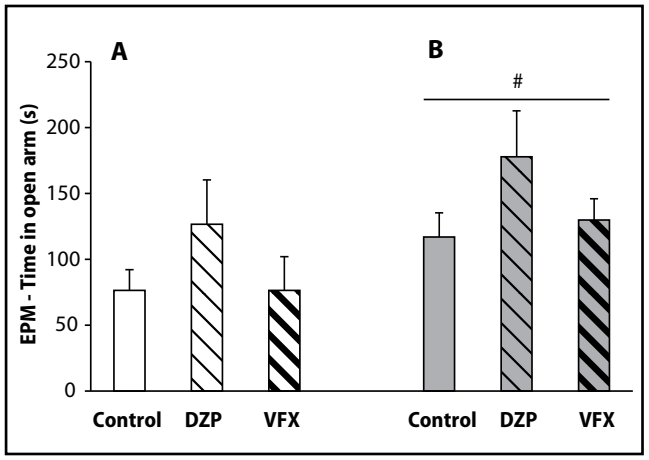

Fig. 3. Effect of chronic unpredictable mild stress (CMS) on rat performance in Elevated plus maze Test. # $p<0.05$ compared to group without stress. A – physiological conditions without CMS, B – CMS conditions.

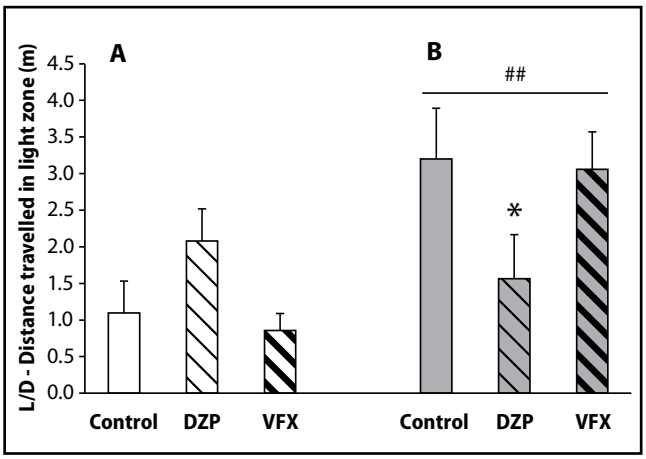

Fig. 4. Effect of chronic unpredictable mild stress (CMS) on rat performance in Light/Dark Box. *$p<0.05$ compared to control group, ##$p<0.01$ compared to group without stress. A – physiological conditions without CMS, B – CMS conditions.

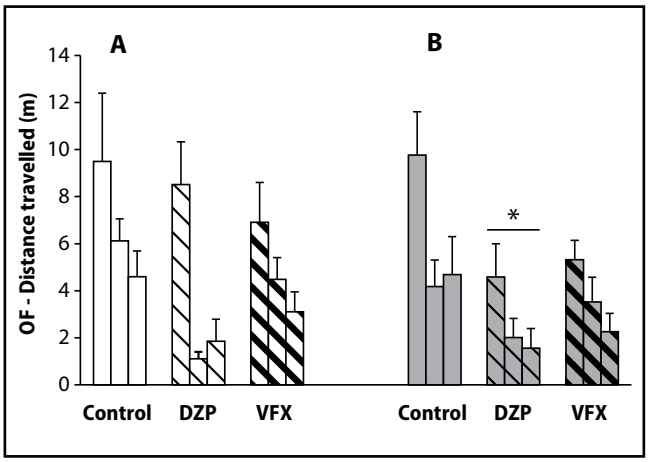

Fig. 5. Effect of chronic unpredictable mild stress (CMS) on rat performance in Open field test. *p<0.05 compared to control group. A – physiological conditions without CMS, B – CMS conditions.

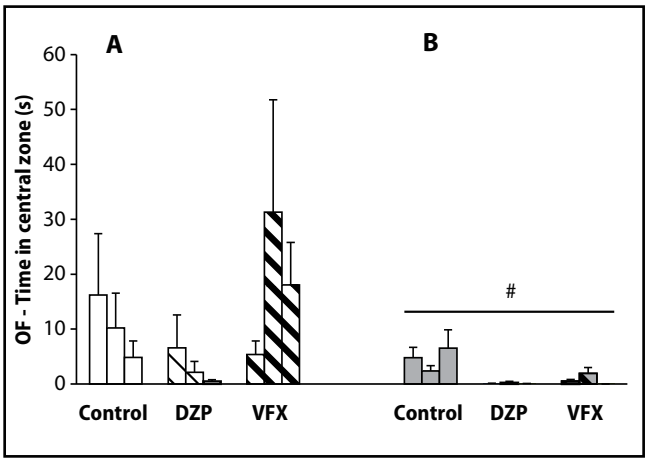

Fig. 6. Effect of chronic unpredictable mild stress (CMS) on rat performance in Open field test. #p<0.05 compared to group without stress. A – physiological conditions without CMS, B – CMS conditions.

uate the SIH response in animals (Olivier *et al.* 2002; Spooren *et al.* 2002). In the present study, the factorial ANOVA showed an interaction between treatment and condition [F(2,54)=7.612, p<0.01]. The following *post-hoc* analysis revealed significant differences between control and DZP group (p<0.05, Figure 7A) as well as between control and VFX group (p<0.001, Figure 7A). However, after CMS our results showed no response to the stress stimulus in SIH (Figure 7B). Since we were unable to reach a critical increase of body temperature in the control group, the tested VFX and DZP did not differ from controls in this setup. We assume that the low stress response after CMS in SIH paradigm may at least be partially explained by habituation of stress responses. This is a confounding factor when studying anxiety in animal models (Holmes *et al.* 2001; McIlwain *et al.* 2001). Generally, repeated daily stress exposure results in stress response habituation in the SIH paradigm (Van der Heyden *et al.* 1997) and decreases SIH amplitude (Bhatnagar *et al.* 2006; Barnum *et al.* 2007). One might speculate that our CMS caused stress-induced fever, as seen in humans with psychological stress (Oka 2015). However our data do not support this thesis. Basal body temperature did not differ between non-stressed and stressed groups (Control: 37.04±0.20 vs. 37.36±0.16; DZP: 37.33±0.10 vs. 36.79±0.17 and VFX: 37.31±0.14 vs. 36.82±0.06). The SIH response generally constitutes a normal and healthy stress response and our model of CMS probably disrupted the SIH capacity.

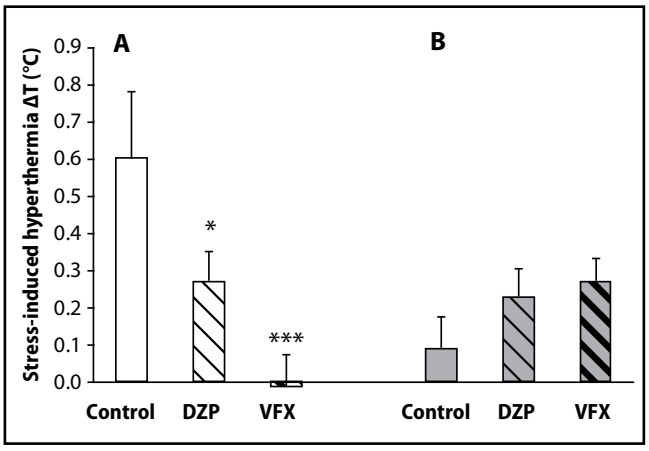

Fig. 7. Effect of chronic unpredictable mild stress (CMS) on rat performance in Stress-induced hyperthermia test. *p<0.05 compared to control group, ***p<0.001 compared to control group. A – physiological conditions without CMS, B – CMS conditions.

Chronic stress in rats generally results in impaired learning and memory when tasks requiring the use of spatial information are tested (Wright & Conrad 2005; Conrad, 2010). However, in the present study we did not observe significant differences in spatial memory tested in Y-maze (data not shown). These discrepancies might be due to stress duration. Bowman *et al.* (2003) proposed a biphasic effect of chronic stress on the central and peripheral nervous system – shorter exposure to stress being beneficial, whereas longer stress exposures would lead to detrimental consequences (Bowman *et al.* 2003). Our model represented a rather mild unpredictable paradigm for 2 weeks of duration, whereas the authors Wright and Conrad (2005) used in their study a 3-week model of restrainment stress for 6 h per day. This difference appears to be crucial in spatial recognition impairment.

CONCLUSION

The aim of our study was to assess a modified 2-week model of CMS as a combination of psychosocial, physical and metabolic stressors in order to subsequently test newly synthesized substances with potential anti-

depressant and/or anxiolytic properties. Based on our results, we can conclude that our CMS protocol leads to increased corticosterone levels as a result of HPA axis hyperactivity and produces delayed onset of anxiogenic behavior. CMS has a substantial effect on the behavioral output interfering with drug testing.

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