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Effects of *Rhodiola rosea* L. extract on behavioural and physiological alterations induced by chronic mild stress in female rats

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Abstract

Rhodiola rosea L. is one of the most popular adaptogen and an antistress plant in European and Asiatic traditional medicine. Our previous studies have confirmed the adaptogenic and antistress properties of a single administration of *R. rosea* L. extract in rats exposed to acute stress. There is increasing evidence that prolonged exposure to stressful life events and depression are both related to significant behavioural, endocrinological and neurobiological changes in human and animal subjects. The aim of this study was to determine whether chronic treatment with a hydroalcoholic *R. rosea* extract (RHO) standardized in 3% rosavin and 1% salidroside can prevent alterations induced in female rats following 6 weeks of a chronic mild stress (CMS) procedure. This was analysed through the behavioural and physiological parameters of consumption of 1% sucrose solution, locomotor and exploratory activities, body weight gain and oestrous cycle length. After the first 3 weeks of stress, RHO was administered daily by gavage at doses of 10, 15 and 20 mg/kg for the remaining 3 weeks. In addition, the antidepressant drug fluoxetine (10 mg/kg os), which has been shown to reverse CMS-induced disruptions, was used as the reference treatment. Rats subjected to the CMS procedure demonstrated decreased sucrose intake, reduced moving behaviour, minimized weight gain and dysregulation of their oestrous cycle. Treatment with RHO completely reverted all of these changes. The effects of RHO were comparable to those of fluoxetine. Interestingly, neither RHO nor fluoxetine influence the behavioural and physiological parameters tested in non-stressed animals. These findings strongly showed that chronic administration of RHO results in potent inhibition of the behavioural and physiological changes induced by chronic exposure to mild stressors.

Key words

adaptogens; anhedonia; antistress; CMS; fluoxetine; oestrous cycle; *Rhodiola rosea*

Introduction

It is well known that stress conditions caused by physiological and environmental factors can induce marked behavioural alterations due to a release of glucocorticoids and activation of a brain stress network, which involve activation of both the pituitary adrenal axis and the autonomic sympathetic system (Koob and Heinrichs, 1999; Dallman, *et al*., 2003). Continuous exposure to unpredictable environmental stressors leads to continuous hypersecretion of neurotransmitters [i.e. serotonin, dopamine, norepinephrine and corticotropin-releasing factor (CRF)], which can contribute to the development of neuropsychiatric disorders in humans (Ressler and Nemeroff, 2000; Dalla, *et al*., 2005). Recent epidemiological studies have highlighted a close relationship between stressful life events and depression, which often manifests as symptoms at the psychological, behavioural and physiological levels (Mitchell, *et al*., 2003; Kessing, 2007). Several studies have also shown that prolonged exposure to a variety of mild and unpredictable environmental stressors is related to significant behavioural, endocrinological and neurobiological changes in rodents (Willner, *et al*., 1992; Kioukia-Fougia, *et al*., 2002; Bekris, *et al*., 2005; Xia, *et al*., 2006).

The chronic mild stress (CMS) procedure in a rodent model has been shown to have a high degree of validity and utility for the study of behaviours associated with stressors. In fact, the CMS procedure uses the administration of unpredictable, mild stressors that are designed to mimic the daily problems

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that have been reported to contribute to the onset of depression in some humans (Willner, 1997; Solberg, *et al*., 1999). It has been showed that stress alters the response to reward behaviour in male rodents (Willner, 1997). Here, the CMS paradigm results in the induction of anhedonic behaviour, which is defined as a loss of interest in normally rewarding stimuli and is generally accepted as belonging to the spectrum of the human depressive symptomatologies (Willner, 1997). Decrease in the responsiveness to rewards is typically reported as a decrease in the consumption of palatable, dilute sucrose solutions, which is the behavioural measure most extensively used to gauge the anhedonic effects of CMS (Willner, *et al*., 1987; Willner, 1997).

Some pharmacologically active plants that have been defined as 'adaptogens' can increase the ability of an organism to adapt to environmental stress factors and to avoid damage from such, mainly by regulating various elements of the stress system and by modulating stimulus-response coupling (Wagner, *et al*., 1994; Panossian, 2003; Panossian and Wagner, 2005). *Rhodiola rosea* L. (Crassulaceae), which is also known as 'golden root' or 'rose root', is one of the most important adaptogen, and it is a popular plant in traditional medicine in Eastern Europe and Asia, with a reputation for alleviating emotional, mental and physical disorders (German, *et al*., 1999; Spasov, *et al*., 2000; Shevtsov, *et al*., 2003; Panossian and Wagner, 2005). In traditional folk medicine, *R. rosea* is used to stimulate the nervous system, to decrease depression, to enhance work performance, longevity and resistance to high altitude sickness, and to treat fatigue and symptoms of asthenia subsequent to intense physical and psychological stress (Kelly, 2001; Brown, *et al*., 2002; Zhu, *et al*., 2003). Recently, we confirmed the adaptogenic, antistress and antidepressive properties of *R. rosea* L in mice following a single administration (Perfumi and Mattioli, 2007). Moreover, we have also demonstrated that a single administration of a *R. rosea* L. extract can reduce anorexia induced in rats by acute exposure to different stressful conditions (Mattioli and Perfumi, 2007).

Therefore, in the present study, we used the same *R. rosea* L. extract (RHO; 3% rosavins and 1% salidroside) as in our previous studies and in several preclinical and clinical investigations (Darbinyan, *et al*., 2000; Kelly, 2001; Brown, *et al*., 2002; Abidov, *et al*., 2003; Panossian and Wagner, 2005). This was tested on different parameters to determine whether chronic treatment with this extract can prevent behavioural and physiological alterations induced by CMS exposure in female rats.

We believe that it is very important to study the effects of RHO on stress-related changes in female rats as it is known that women are more susceptible than men to stress-related mental illness and twice as likely to experience depression (American Psychiatric Association, 1994; Kendler, 1998; Kendler, *et al*., 2000). For this, the following behavioural and physiological parameters were analysed: consumption of 1% sucrose solution, locomotor and exploratory activities, body weight gain and oestrous cycle length.

It is known that CMS exposure is related to the induction of anhedonic behaviour, and therefore the ability of *R. rosea* extract to revert a decrease on the consumption of 1% sucrose solution was tested.

Because an overall decrease in moving behaviour was seen, which was more evident in female than male stressed rats, we also evaluated the effects of RHO on the CMS-induced locomotor and exploratory activity changes using the open-field (OF) test (Dalla, *et al*., 2005).

Some experimental studies have reported a disruption, usually expressed as a lengthening, of the oestrous cycle of the female rats exposed to CMS (Konkle, *et al*., 2003; Dalla, *et al*., 2005; Grippo, *et al*., 2005; Baker, *et al*., 2006). There is also an extensive overlap of depressive disorders with menstrual-related phenomena in women (e.g. premenstrual, postpartum, perimenopausal and postmenopausal syndromes) (American Psychiatric Association, 1994; Halbreich, 2003). Therefore, to confirm the influence of stress on the reproductive hormones and to explore the effects of RHO on this physiological alteration, the oestrous cycle was monitored before and during the administration of stressors, via an analysis of the cell types present in vaginal fluid.

Finally, as chronic treatments with all the major classes of antidepressant drugs are effective in preventing or reversing the behavioural effects of CMS, we also used fluoxetine (FLU) as the reference drug (Moreau, *et al*., 1992; Monleon, *et al*., 1995; Willner, 1997; Li, *et al*., 2003; Grippo, *et al*., 2006; Rygula, *et al*., 2006).

Experimental procedures

Animals

Female Wistar rats (Harlan SRC, Milan, Italy), weighing 175–225 g at the beginning of the experiments, were used throughout this study. The animals were housed individually in plastic cages (40 × 25 × 15 cm) in a temperature (22 °C)- and humidity (45–55%)-controlled environment, with a 12 h:12 h light/dark cycle (lights on at 7.00 a.m.) unless otherwise indicated. Food and tap water were available *ad libitum* for the duration of the experiments, except when otherwise detailed. The rats were allowed 1 week to acclimatize to the surroundings before the start of any experimentation.

The oestrous cycles of these female rats were monitored every morning throughout the study to track the oestrous cycle during the baseline and stress and treatment conditions. All experiments were conducted in accordance with the European Community Council Directive for the Care and Use of Laboratory Animals (86/609/EEC), and every effort was made to minimize the pain and discomfort to the rats throughout the study.

Drugs

A dry hydroalcoholic extract from the roots of *R. rosea* L. (RHO) was used (provided by EPO S.r.l., Milan, Italy). The High performance liquid chromatography (HPLC) analysis report showed a content of 3% total rosavins, expressed as rosavin and 1% salidroside. The ratio of rosavin to salidroside (3:1) is in line with published data (Kurkin and Zapesochnaya, 1986; Abidov, *et al*., 2003). The extract was administered by intragastric administration (i.g.) at doses of 10, 15 and 20 mg/kg/10 ml.

Fluoxetine HCl (FLU) was purchased from SIGMA (Milan, Italy) and administered orally (i.g.) at a dose of 10 mg/kg/10 ml. Both RHO and FLU were dissolved in ethanol absolute and diluted in tap water to obtain a final ethanol concentration of 1% v/v in all treatment conditions. The same vehicle was administered to vehicle group.

Chronic mild stress

The animals were first trained to consume a 1% (w/v) sucrose solution. The training took place before the beginning of any stressful procedures and consisted of eight 1-h baseline tests performed twice weekly (with an interval of 3 days), in which sucrose was presented in the home cage, following 14 h of food and water deprivation. The sucrose intake was measured by weighing pre-weighed bottles containing the sucrose solution at the end of the test. Subsequently, sucrose consumption was monitored from week 0 to week 6, under similar conditions and at weekly intervals (every Tuesday, between 10.00 a.m. and 11.00 a.m.) throughout the whole experimental period. Moreover, body weight measurements were recorded on the same day, 2 h before the sucrose preference test. As body weight changes could be a confounding factor for interpreting findings related to sucrose consumption following the CMS procedure (Matthews, *et al*., 1995), sucrose intake was expressed as a function of body weight (g/kg).

On the basis of their sucrose intake in the final baseline test, the animals were distributed into two matched groups: stressed (CMS; $n = 36$) and non-stressed (CTRL; $n = 31$). The CMS group was subjected to the CMS procedure for a period of six consecutive weeks. Each week of this stress regimen consisted of two periods of food or water deprivation; two periods of 45° cage tilt; two periods of intermittent illumination (lights on and off every 2 h); one period of soiled cage (250 ml water in sawdust bedding); one period of continuous overnight illumination; two periods of paired housing; two periods of low intensity stroboscopic illumination (150 flashes/min) and two periods of no stress (as described by Willner, *et al*., 1987 and modified by Papp, *et al*., 2002). All stressors were applied for 10–14 h duration, individually and continuously, day and night. The non-stressed animals that were used as the controls were housed in separate rooms and had no contact with the stressed animals. They were deprived of food and water for 14 h preceding each sucrose test, but food and water were freely available in the home cage.

On the basis of their sucrose intake following the initial 3 weeks of stress, both the stressed and control groups were each further divided into five matched subgroups ($n = 6$–8), and for the subsequent 3 weeks, they received once daily oral administrations of vehicle (10 ml/kg) or RHO (10, 15, 20 mg/kg/10 ml) or FLU (10 mg/kg/10 ml) as the reference drug. All of the drugs were orally administered at 1.00 p.m., following the weekly sucrose intake test (approximately 2 h later) so as not to interfere with the application of the stressor.

Open-field test

Spontaneous OF activities were measured before the start of the CMS (baseline) and at the beginning (Monday mornings) of weeks 3 and 6 of the CMS procedure. The rats were transferred to the test room 1 h before testing, for acclimatization. One rat at a time was introduced into the transparent plastic OF cage (40 × 40 × 40 cm) and their behaviour was recorded during a 5-min observation period. A number of conventional and ethological parameters were collected during these sessions. In particular, the horizontal activity (i.e. ambulation time) and the vertical activity (i.e. rearing) were recorded automatically (Dalla, *et al*., 2005). The OF chamber was cleaned prior to testing each animal.

Determination of oestrous cycle phases

The phases of the oestrous cycle were monitored daily in all of the rats (CMS and CTRL groups) for the 2 weeks preceding the onset of CMS and throughout the CMS period. Each morning, the phases of the oestrous cycle were determined by vaginal swabs. The samples were placed onto microscope slides, and dipped into diluted methylene blue (1 mg/ml in distilled water) for 30 s to aid in the visualization of the cells. The slides were then washed in distilled water and examined under a microscope at 20× magnification. The phases of oestrous cycle were determined based on the predominant cell type present on each day, according to standard criteria (Long and Evans, 1922). These were as follows: pro-oestrus (large clumps of round nucleated epithelial cells, occasional cornified cells, few or no leucocytes), oestrus (clumps of cornified cells, few or no round nucleated epithelial cells, no leucocytes), metoestrus (some cornified cells, some round nucleated epithelial cells, some leucocytes) and dioestrus (mostly leucocytes, some round nucleated epithelial cells).

Statistical analysis

Statistical analysis of sucrose intake, body weight, oestrous cycle and the behavioural measurements from the OF analysis were made using repeated analysis of variance (ANOVA), with group (stress/control) and treatment (vehicle/drug) as the between-subject factors and time (weeks) as the within-subject factor. When necessary, two-way ANOVAs (stress × time) were performed. The Newman–Keuls test was used for *post hoc* comparisons of mean values. Statistical significance was set at $P < 0.05$.

Results

Sucrose intake

Weeks: baseline test-3 Figure 1 shows the sucrose intake in the control (CTRL) and stressed (CMS) groups at baseline and through the first half of the CMS period. In this final baseline test, no difference was seen for the sucrose intake between the two groups ($P > 0.05$). Exposure to 3 weeks of stress produced a marked decrease in the consumption of 1% sucrose solution in the CMS group, while it showed a weak tendency to increase in the control animals. A repeated ANOVA with stress as the independent factor and time as the repeated factor revealed a statistically significant stress and time effect [$F(1,65) = 145.238$, $P < 0.001$; $F(4,260) = 2.613$, $P < 0.05$ respectively], and interaction time × stress effects were seen on sucrose intake [$F(4,260) = 25.498$, $P < 0.001$]. During the 3 weeks of CMS, the sucrose intake was significantly lower in the stressed rats than in non-stressed group in all of the weekly tests (weeks 0–3), a finding indicating the establishment of the CMS protocol. In particular, a marked reduction was already seen after 1 day of stress (week 0) [$F(1,65) = 67.400$, $P < 0.001$], and over the further 3 weeks [$F(1,65) = 70.653$, $P < 0.001$; $F(1,65) = 134.804$, $P < 0.001$; $F(1,65) = 212.489$, $P < 0.001$, for weeks 1–3 respectively] (Figure 1). In addition, the CMS group consumed significantly less sucrose in all of the weekly tests (weeks 0–3) compared with its respective baseline intake [$F(4,35) = 25.491$, $P < 0.001$]. Conversely, the sucrose intake in the control group tended to increase significantly each week compared with its baseline value [$F(4,30) = 5.789$, $P < 0.001$].

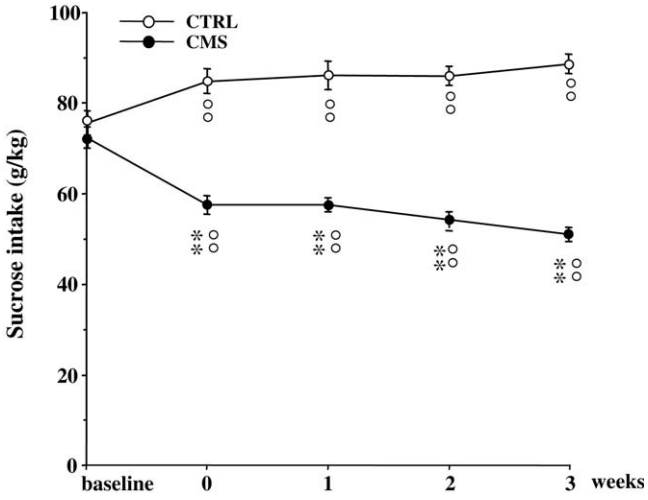


Figure 1 Consumption of 1% sucrose solution (g/kg body weight) in non-stressed (CTRL; $n = 31$) and stressed (CMS; $n = 36$) female rats from baseline test to week 3 of the CMS exposure. Data were mean ± SEM values. Significant differences: $^{**}P < 0.01$ compared with related CTRL group; $^{\circ\circ}P < 0.01$ compared with own baseline value.

Weeks: 4–6 Figure 2 shows the effects of RHO and FLU administration on sucrose intake over the last 3 weeks of the stress procedure in the non-stressed (CTRL) and stressed (CMS) groups (Figure 2A,B). A repeated two-way ANOVA with stress and treatment as the between factors and time as the within factor revealed that the sucrose intake was significantly decreased in the CMS group [$F(1,57) = 78.185$, $P < 0.001$], in comparison with the control group. Moreover, a treatment effect was also seen [$F(4,57) = 3.448$, $P < 0.01$], although there was no a treatment × stress interaction [$F(4,57) = 1.401$, $P > 0.05$]. A further separate repeated ANOVA revealed a gradual decrease in sucrose intake in the stressed group treated with the vehicle (CMS-VEH) compared with the vehicle of the non-stressed group (CTRL-VEH) [$F(1,11) = 18.598$, $P < 0.01$]. This decrease was seen at weeks 4 ($P < 0.01$), 5 ($P < 0.001$) and 6 ($P < 0.001$).

As shown in Figure 2A, chronic treatment with RHO or FLU had no significant effects on sucrose intake in the control animals, which showed no marked variations in their preference for the sucrose solution [$F(4,26) = 1.149$, $P > 0.05$]. Conversely, chronic treatment with both drugs gradually increased the sucrose consumption in the stressed animals, resulting in a significant effect of treatment that developed over the 3 weeks of treatment [$F(4,31) = 4.632$, $P < 0.01$] (Figure 2B). Moreover, a statistically significant treatment × time interaction was seen [$F(4,62) = 2.431$, $P < 0.05$].

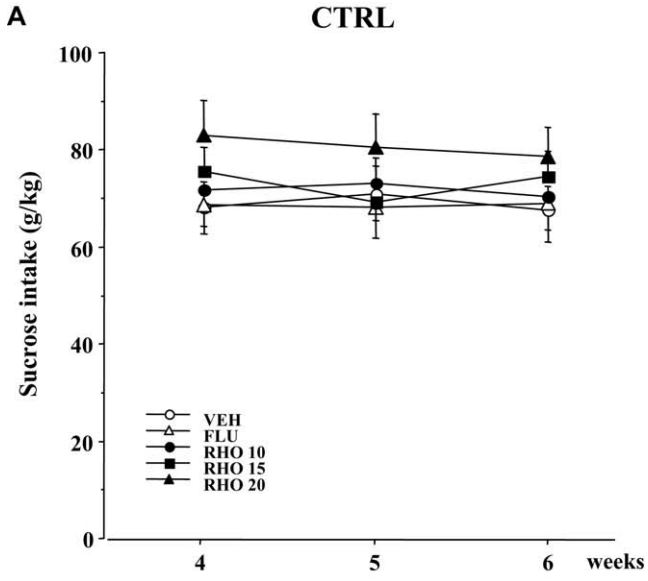
Chronic treatment with RHO reversed the CMS-reduced sucrose solution consumption in a dose-dependent manner. RHO at 20 mg/kg quickly enhanced the sucrose intake, with an effect already seen after 1 week of treatment (at week 4) ($P < 0.05$); this persisted at the same level until the end of the experimental period (week 5: $P < 0.01$; week 6: $P < 0.001$) (Figure 2B). Of note, the sucrose intake in these treated stressed rats was comparable to that of the vehicle-treated controls at all weekly tests ($P > 0.05$ for each week).

Similar sucrose intake changes in the stressed animals that were administered with RHO at doses of 10 and 15 mg/kg/10 ml reached statistical significance only after 3 weeks of treatment (week 6: $P < 0.01$, $P < 0.05$ respectively) (Figure 2B).

In contrast to the ready onset of action of RHO, the increase in sucrose intake in the stressed animals administered with FLU reached statistical significance after 2 weeks of treatment (week 5: $P < 0.05$). This effect was further enhanced by one further week of treatment, with the amount of sucrose solution drunk by these animals at week 6 significantly higher than that of the vehicle-treated stressed animals ($P < 0.001$) and comparable both to the sucrose intake of the vehicle-treated controls ($P > 0.05$) and to that of the controls treated with the same drug ($P > 0.05$) (Figure 2B).

Body weight

Weeks: baseline test-3 Figure 3 shows the body weight gains with respect to the baseline values in the control (CTRL) and



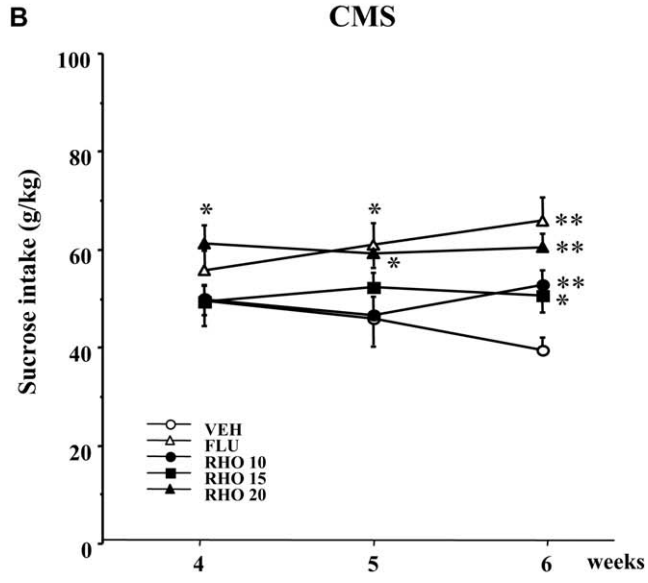


Figure 2 Effects of chronic oral treatment with vehicle (VEH), *Rhodiola rosea* L. extract (RHO: 10, 15, 20 mg/kg, as indicated) or fluoxetine (FLU: 10 mg/kg) on the consumption of 1% sucrose solution (g/kg body weight). (A) Non-stressed (CTRL) female rats. (B) Stressed (CMS) female rats. The treatments were started immediately after the sucrose intake test on week 3. Data were mean ± SEM values (n = 6–8). Significant differences: $^{*}P < 0.05$, $^{**}P < 0.01$, compared with related vehicle-treated group; where not indicated, the differences were not statistically significant.

CMS groups throughout the first 3 weeks of the CMS period. A repeated ANOVA with stress as the independent factor and time as the repeated factor revealed that there was a statistically significant time and CMS effect on body weight gain [$F(4,260) = 633.813$, $P < 0.001$; $F(1,65) = 55.721$, $P < 0.001$ respectively], and a significant time × stress interaction [$F(4,260) = 24.810$, $P < 0.001$].

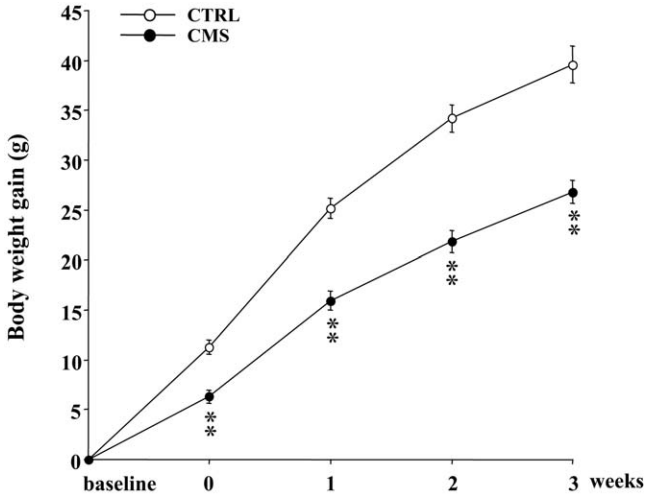


Figure 3 Body weight gains in non-stressed (CTRL; n = 31) and stressed (CMS; n = 36) female rats from baseline test to week 3 of the CMS exposure. Data were mean ± SEM values. Significant differences: $^{**}P < 0.01$ compared with related non-stressed group.

Post hoc analysis confirmed that the body weight gains were significantly lower in the stressed animals than in the control group at all of the weekly measurements (weeks 0–3; $P < 0.001$ for each week).

Weeks: 4–6 Figure 4 shows the effects of RHO and FLU on body weight gain in control (CTRL) and CMS rats. A repeated two-way ANOVA with stress and treatment as the between factors and time as the within factor revealed significant CMS effects, where the body weight gain of the stressed rats was significantly lower [$F(1,57) = 11.013$, $P < 0.001$] than the treatment effect [$F(4,57) = 36.700$, $P < 0.001$]. Moreover, there was a statistically significant time effect [$F(2,114) = 114.602$, $P < 0.001$], but no time × stress interaction ($P > 0.05$).

Post hoc analysis revealed that the body weight gain of the CTRL vehicle [$F(2,5) = 23.560$, $P < 0.01$] and CTRL-RHO rats significantly increased over time [RHO 10: $F(2,5) = 8.986$, $P < 0.01$; RHO 15: $F(2,5) = 21.164$, $P < 0.001$; RHO 20: $F(2,6) = 20.600$, $P < 0.001$] (Figure 4A), as well as in the CMS vehicle [$F(2,6) = 10.960$, $P < 0.01$] and CMS RHO rats [RHO 10: $F(2,6) = 9.070$, $P < 0.01$; RHO 15: $F(2,6) = 5.315$, $P < 0.05$; RHO 20: $F(2,7) = 8.616$, $P < 0.01$] (Figure 4B). However, the body weight gain was significantly lower in the stressed animals compared with their controls at all weeks [$F(1,57) = 11.193$, $P < 0.01$; $F(1,57) = 5.689$, $P < 0.05$; $F(1,57) = 8.915$, $P < 0.01$ for weeks 4, 5 and 6 respectively].

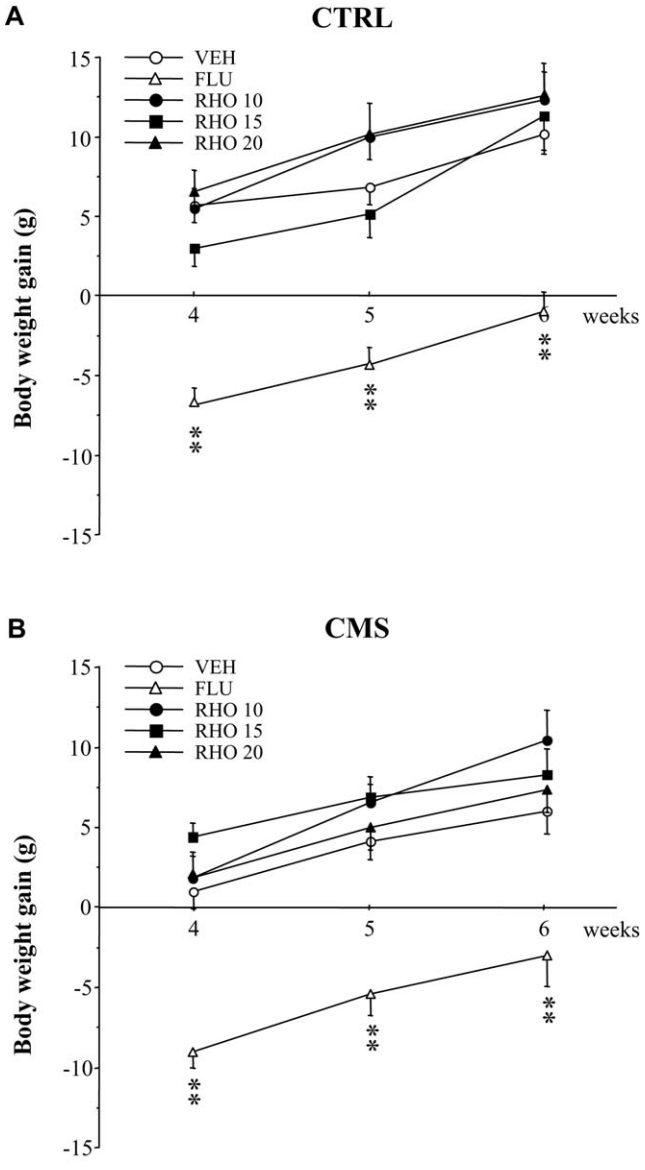


Figure 4 Effects of chronic oral treatment with vehicle (VEH), *Rhodiola rosea* L. extract (RHO: 10, 15, 20 mg/kg, as indicated) or fluoxetine (FLU: 10 mg/kg) on the body weight gains. (A) Non-stressed (CTRL) female rats. (B) Stressed (CMS) female rats. The treatments were started immediately after the sucrose intake test on week 3. Data were mean ± SEM values (n = 6–8). Significant differences: $^{**}P < 0.01$ compared with related vehicle-treated group; where not indicated, the differences were not statistically significant.

Of note, for the FLU treatment, rather than a body weight gain, there was a marked waste in body weight at week 4 that was seen in both the non-stressed (Figure 4A) and stressed (Figure 4B) rats. However, this waste tended to be re-gained over time in both non-stressed and stressed treated rats [$F(2,6) = 44.438$, $P < 0.001$; $F(2,6) = 10.960$, $P < 0.01$ respectively].

Open-field test

Figure 5 shows the behavioural movements, as ambulation time (Figure 5A) and rearing (Figure 5B), in the control (CTRL) and CMS groups before (baseline) and after 3 weeks of CMS (week 3). As shown, the 3 weeks of chronic stress induced a marked decrease in the behavioural movements in the stressed rats compared with both their movements at baseline and to the non-stressed group. A two-way repeated ANOVA with stress as the independent factor and time as the repeated factor revealed statistically significant time and CMS effects on both ambulation time [$F(1,65) = 12.011$, $P < 0.001$; $F(1,65) = 9.536$, $P < 0.001$ respectively] and amount of rearing [$F(1,65) = 25.005$, $P < 0.001$; $F(1,65) = 11.750$, $P < 0.05$ respectively]. Moreover, a significant time × stress interaction was seen in both parameters [$F(1,65) = 15.705$, $P < 0.001$; $F(1,65) = 15.615$, $P < 0.001$, for ambulation time and rearing respectively].

As shown in Figure 6, chronic treatments with RHO and FLU markedly increased the ambulation time (Figure 6B) in the stressed rats, whereas they did not have any effects on the control group (Figure 6A,C). In addition, the amount of rearing was significantly reduced in CMS group by FLU treatment (Figure 6D). A two-way repeated ANOVA with stress and treatment as the independent factors confirmed a statistically significant CMS effect both on ambulation time [$F(1,57) = 5.871$, $P < 0.01$] and amount of rearing [$F(1,57) = 57.510$, $P < 0.001$]. Moreover, a significant treatment effect for ambulation time [$F(4,57) = 3.396$, $P < 0.01$] and a significant time × stress interaction for amount of rearing [$F(4,57) = 3.243$, $P < 0.01$] were seen. Indeed, the rats treated with 20 mg/kg RHO showed a greater ambulation time compared with the vehicle-stressed group ($P < 0.01$). Additionally, the ambulation time in these RHO-treated stressed rats was similar both to that of the vehicle-treated controls ($P > 0.05$) and to that of the controls treated with the same drug ($P > 0.05$). On the contrary, the lower doses of 10 and 15 mg/kg RHO did not have any effects (Figure 6B).

Moreover, a *post hoc* analysis revealed that both ambulation time and rearing (Figure 6B,D) were significantly increased in the stressed animals that were chronically treated with FLU ($P < 0.01$, $P < 0.05$ respectively), compared with the vehicle-stressed group (Figure 6B). Of note, the behavioural movements in the vehicle-stressed rats were lower than that of the vehicle-control rats [$F(1,11) = 14.096$, $P < 0.01$; $F(1,11) = 12.336$, $P < 0.01$ for ambulation time and rearing respectively].

Oestrous cycle

Weeks: baseline test-3 The baseline oestrous cycle was 4.23 ± 0.08 days in the control group and 4.08 ± 0.05 days in

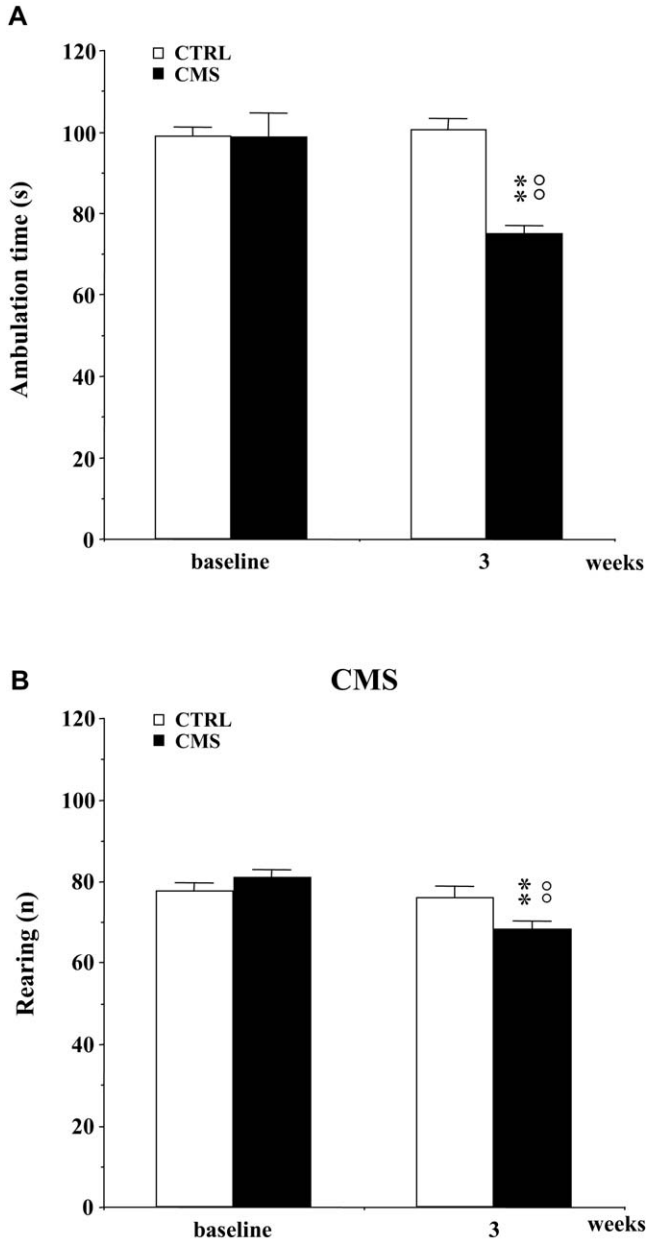


Figure 5 Moving behaviours in non-stressed (CTRL; $n = 31$) and stressed (CMS; $n = 36$) female rats from baseline test to week 3 of the CMS exposure. (A) Ambulation time. (B) Amount of rearing. Data were mean ± SEM values. Significant differences: $^{**}P < 0.01$ compared with related non-stressed group, $^{\circ\circ}P < 0.01$ compared with own baseline value; where not indicated, the differences were not statistically significant.

the CMS group; these values were not significantly different ($P > 0.05$) (Figure 7). However, the rats exposed to CMS showed a progressive increase in oestrous cycle length during the first 3 weeks of the CMS period (Figure 7). The ANOVA for the weekly cycle length with stress as the independent factor and time as the repeated factor yielded a significant main effect of time [$F(3,195) = 30.476$, $P < 0.001$] and CMS [$F(1,65) = 20.412$, $P < 0.001$], and a significant time × stress interaction [$F(3,195) = 19.458$, $P < 0.001$].

Post hoc analysis revealed that the weekly cycle length in the CMS group was already significantly greater than that of the control group following 1 week of CMS [$F(1,65) = 4.882$, $P < 0.05$], and it gradually increased after 2 [$F(1,65) = 9.829$, $P < 0.01$] and 3 [$F(1,65) = 62.451$, $P < 0.001$] weeks of CMS. Moreover, following 3 weeks of CMS, the cycle length of the CMS group was significantly greater than its respective baseline length [$F(3,105) = 36.865$, $P < 0.001$]. The majority of the animals ($n = 33$ out of 36) that displayed irregular cycles in this study (cycle lengths longer than 5 days) remained mostly in the oestrous phases of the cycle ($n = 26$ out of 36).

The cycle length of the control group did not differ from its baseline following the 3 weeks of CMS ($P > 0.05$).

Weeks: 4–6 Figure 8 shows the effects of RHO and FLU on the oestrous cycle length in the control (CTRL) and CMS rats. A repeated two-way ANOVA with stress and treatment as the between factors and time as the within factor revealed a significant CMS effect on oestrous cycle length [$F(1,57) = 34.857$, $P < 0.001$]. Although there was no treatment effect ($P > 0.05$), statistically significant time effect [$F(2,114) = 7.438$, $P < 0.001$] and a time × stress interaction were seen [$F(2,114) = 6.678$, $P < 0.01$]. Further separate repeated ANOVA revealed that the oestrous cycle length was significantly increased in the stressed rats treated with vehicle (CMS-VEH) compared with the control group (CTRL-VEH) [$F(1,11) = 22.225$, $P < 0.001$].

As shown in Figure 8A, chronic treatment with RHO or FLU had no significant effects on oestrous cycle length in the non-stressed animals, which showed no marked variations in the frequency of their oestrous cycles [$F(4,26) = 1.802$, $P > 0.05$]. Conversely, chronic treatment with both RHO and FLU gradually normalized the oestrous cycle in the stressed animals, an effect that developed after the 3 weeks of treatment (by week 6) [$F(4,31) = 3.128$, $P < 0.05$] (Figure 8B). In particular, at week 6, the treatment with all doses of RHO (10, 15, 20 mg/kg) ($P < 0.05$, for each doses) and with FLU ($P < 0.001$) induced full recoveries of the frequencies of the oestrous cycles, in comparison with the vehicle-treated stressed animals. Of note, at the end of each drug-treatment (week 6), the oestrous cycle lengths were comparable both to that of the vehicle-treated controls ($P > 0.05$) and to that of the controls treated with the same pharmacological agents ($P > 0.05$). Moreover, stressed animals treated with the highest dose of RHO showed a cycle lengths comparable to that of the same treated controls also at week 5 ($P > 0.05$).

Discussion

The present study was undertaken to investigate whether a hydroalcoholic extract from roots of *R. rosea* L. (RHO) reduced behavioural and physiological changes that are

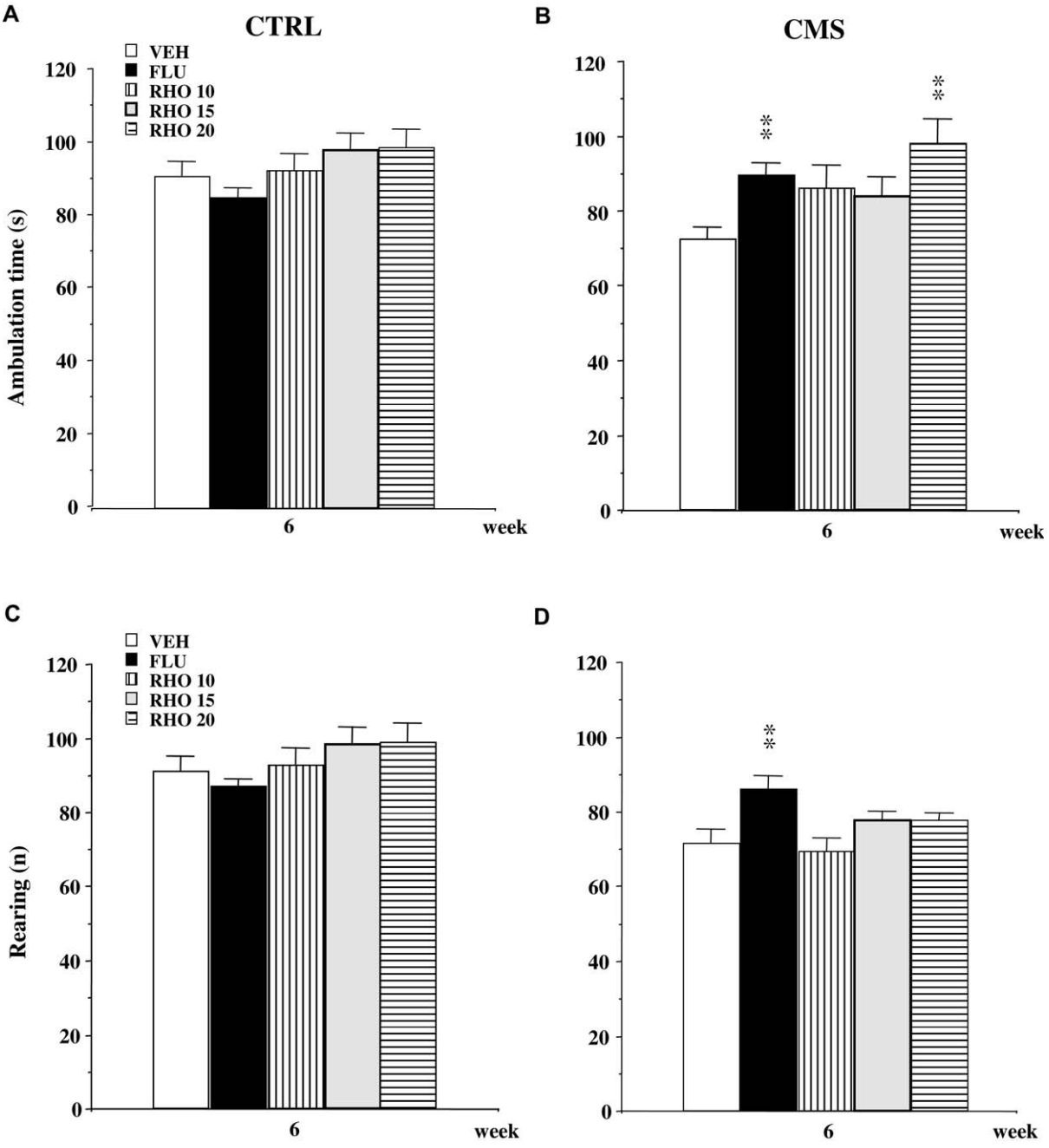


Figure 6 Effects of chronic oral treatment with vehicle (VEH), *Rhodiola rosea* L. extract (RHO: 10, 15, 20 mg/kg, as indicated) or fluoxetine (FLU: 10 mg/kg) on moving behaviours. (A,B) Ambulation time. (C,D) Amount of rearing. (A,C) Non-stressed (CTRL) female rats. (B,D) Stressed (CMS) female rats. The treatments were started immediately after the sucrose intake test on week 3. Data were mean ± SEM values (n = 6–8). Significant differences: $^{**}P < 0.01$ compared with related vehicle-treated group; where not indicated, the differences were not statistically significant.

induced by chronic exposure to mild, unpredictable stress (CMS) in female rats. Stress is known to suppress the subsequent performance of reward behaviours. In rats, chronic sequential exposure to unpredictable stressors, although neither life-threatening nor painful, has been seen to reduce the consumption of and preference for highly palatable sweet solutions, to impair conditioned place preference acquisition, and to increase reward thresholds in a brain stimulation paradigm

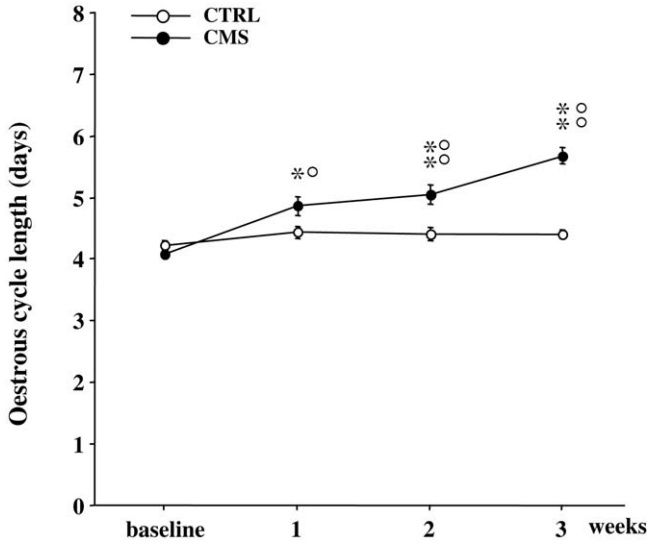


Figure 7 Oestrous cycle length in non-stressed (CTRL; $n = 31$) and stressed (CMS; $n = 36$) female rats from baseline test to week 3 of the CMS exposure. Data were mean ± SEM values. Significant differences: $^{*}P < 0.05$, $^{**}P < 0.01$ compared with related non-stressed group; $^{\circ}P < 0.05$, $^{\circ\circ}P < 0.01$ compared with own baseline value; where not indicated, the differences were not statistically significant.

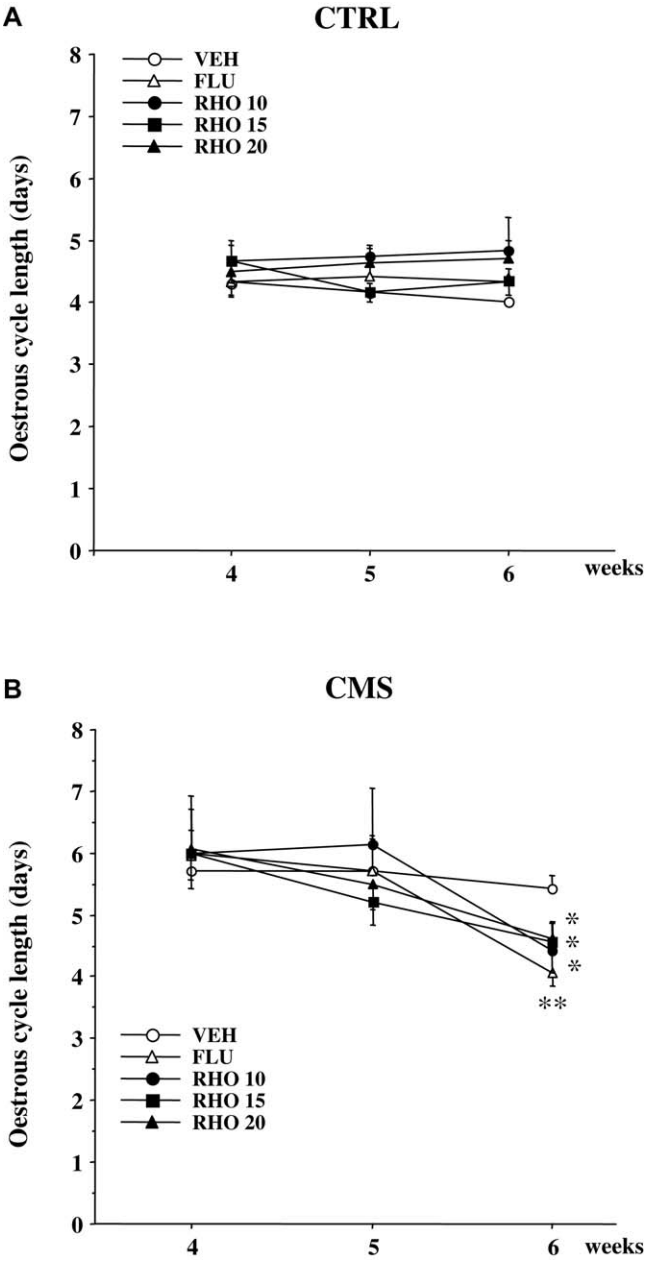


Figure 8 Effects of chronic oral treatment with vehicle (VEH), *Rhodiola rosea* L. extract (RHO: 10, 15, 20 mg/kg, as indicated) or fluoxetine (FLU: 10 mg/kg) on oestrous cycle length. (A) Non-stressed (CTRL) female rats. (B) Stressed (CMS) female rats. The treatments were started immediately after the sucrose intake test on week 3. Data were mean ± SEM values ($n = 6$–8). Significant differences: $^{*}P < 0.05$, $^{**}P < 0.01$ compared with related vehicle-treated group; where not indicated, the differences were not statistically significant.

(Moreau, *et al*., 1992; Willner, 1997). The induction of anhedonic behaviour, which is considered as an index of an established stressful status, generally reflects the core symptom of human depression (Willner, 1997).

In this regard, the results of this study confirm earlier reports (Bekris, *et al*., 2005; Dalla, *et al*., 2005) that chronic sequential exposure to a variety of mild stressors causes a decrease in the consumption of 1% sucrose solution in stressed animals, which was noted even after 1 day of stress. Conversely, the non-stressed control group increased its consumption of the sucrose solution over time. Of note, the changes in fluid consumption in rats exposed to CMS represent a specific hedonic deficit, as the decreased sucrose intake was not a result of body weight changes.

The main finding of the present study is certainly that the CMS-induced reductions in sucrose consumption can be reverted by chronic administration of the *R. rosea* extract. Indeed, in the rats exposed to the CMS procedure that had the consequent low sucrose intake levels, there was an increase back to the normal control values after 3 weeks of chronic treatment. This antianhedonic effect of RHO was dose dependent, and in particular, the pretreatment with the highest dose of 20 mg/kg RHO extract reversed the CMS-induced decrease in sucrose intake already after the first week of treatment; pretreatment with the lower doses of RHO (10 and 15 mg/kg) had significant effects on the sucrose intake only by the end of the 3 weeks of administration.

Several studies have shown that the anhedonic behaviour induced by CMS can be effectively reversed by chronic treatment with the traditional antidepressant drug FLU (Muscat and Willner, 1992; Rygula, *et al*., 2006). Moreover, as in most previous studies with the CMS model (Willner, 1997), also in

this study the action of this antidepressant had several parallels with that of its clinical activity, both in terms of its efficacy (full recovery at the end of the treatment period), specificity (lack of significant effects in control animals) and time course (at least 2 weeks of treatment required to reverse the deficit in the sucrose consumption, and 3 weeks of treatment to fully recover). Of note, the magnitude of the effect of RHO was comparable to that of the traditional antidepressant FLU. However, the time required to trigger this onset in behavioural changes was faster than that usually seen following chronic administration of synthetic drugs, as the sucrose intake in the stressed animals receiving the highest dose of RHO extract (20 mg/kg) was increased within the first week of treatment, compared with the 2 weeks required by FLU. Additionally, according to the results obtained with the traditional antidepressant treatment, this RHO extract did not alter the sucrose intake in the non-stressed animals at any of the doses tested.

Decreases in motor and exploratory activities as a consequence of stress have been widely reported in animal studies (Harro, *et al*., 1999; Dalla, *et al*., 2005; Rygula, *et al*., 2006). In accordance with the literature, in the present study, the exposure of these female rats to CMS resulted in an overall decrease in their rearing behaviour, indicating a CMS-induced decrease in exploratory activity. This decreased rearing behaviour appeared early in these female rats (3 weeks) and was maintained at low levels until the end of the CMS (see VEH-CMS group). Meanwhile, CMS also affected the moving behaviour of the rats, as seen by the decreased horizontal activity over time. Therefore, our results are in line with previous studies (Kennett, *et al*., 1986; Dalla, *et al*., 2005) have suggested that females are strongly vulnerable in tests modelling depression, because they maintain reduced activities in the OF test after exposure to repeated stressful conditions.

Three weeks of 20 mg/kg RHO treatment completely reversed the effects on locomotor activity in the stressed animals, which showed a significant increase in the ambulation time compared with the stressed but untreated rats. Moreover, RHO-treated rats showed a higher amount of rearing when compared with their VEH group, even if this increased exploratory activity was not statistically significant. Instead, the lower doses of RHO did not modify these motor and exploratory parameters.

On the other hand, FLU treatment provided an almost complete reversal of the stress-induced reductions in locomotor activity and rearing behaviour. The stressed animals that were chronically treated with FLU showed a significant increase in their locomotor activity and rearing behaviour. Interestingly, neither RHO nor FLU influenced the locomotor and exploratory activities of the non-stressed animals.

The influence of stress on the reproductive hormones has been well documented. In the clinical literature, stress has been reported to induce amenorrhoea and menstrual cycle disruptions in female patients (Genazzani, *et al*., 1991). In animal studies, chronic exposure to physical stressors has been reported to induce a disruption of regular cycle activity, which typically becomes stalled in one phase of the oestrous cycle (Konkle, *et al*., 2003; Baker, *et al*., 2006). Under our conditions, 6 weeks of CMS produced a disruption in the oestrous cycle in these female rats, such that they suffered a lengthening relative to the control conditions. Moreover, the majority of the animals that displayed irregular cycles in this study remained mostly in the oestrous phases of the cycle, in agreement with previous observations that animals exposed to CMS tend to remain preferentially stalled in a particular phase of the oestrous cycle (Baker, *et al*., 2006).

This desynchronization of the oestrous cycling was completely reversed by chronic treatment with RHO and FLU. Indeed, both RHO-treated and FLU-treated rats returned to regular cycling after 3 weeks of treatment. In particular, all doses of RHO tested normalized the oestrous cycles of these rats, suggesting an influence of RHO use also on neuroendocrine functions that are affected by stressful conditions.

Finally, exposure to stressors has also been shown to influence body weight. Indeed chronic application of CMS typically alters the rate of weight gain, particularly in male rats (Faraday, 2002; Dalla, *et al*., 2005). Similarly, in the present study, the data obtained showed a stressor-related reduced gain in body weight in these female rats following the first week of stress application. This reduction in body weight gain is a reliable index of the stress experience (Muscat and Willner, 1992; Konkle, *et al*., 2003). Moreover, it is important to note that the chronic administration of RHO did not interfere with body weight gain either in the non-stressed or stressed rats at any of the doses tested. Conversely, in agreement with clinical studies, chronic treatment with FLU lead to an initial marked weight loss after the first week of treatment, followed by a recovery of body weight over time (Harvey and Bouwer, 2000; Gobshtis, *et al*., 2007). This reflects a potentially undesirable side effect of the traditional antidepressant drugs, because depression and/or its recovery may be associated with either weight gain or weight loss (Harvey and Bouwer, 2000; Maina, *et al*., 2004).

Thus, the behavioural and physiological data from the present study provides evidence that repeated administration of *R. rosea* extracts has antistress properties for prolonged exposure to a variety of mild and unpredictable stress conditions. Furthermore, there is increasing evidence that stressful life events are related to depressive manifestations (Mitchell, *et al*., 2003; Kessing, 2007). At the same time, the CMS procedure is also argued to be particularly valid for the study of depressive disorders, because it induces anhedonia, a core symptom of human depression (Willner, 1997). For this reason, the data obtained allow us to state that the repeated administration of *R. rosea* extract also produces antidepressant effects in rats, which are comparable to those of the classical antidepressant drugs.

Therefore, this study confirms and extends our previous findings, in which we have demonstrated that *R. rosea* extract has antistress properties in a rat acute-stress model (Mattioli and Perfumi, 2007), and that this *R. rosea* extract is active in preclinical mouse models of behavioural depression (Perfumi and Mattioli, 2007).

The exact mechanisms underlying the antistress and antidepressant effects of this *R. rosea* extract remain unknown. However, it is well accepted that stressor exposure can induce alterations in some crucial neuronal pathways. Indeed, alterations in serotoninergic and dopaminergic activities of stressed rats have been seen in the brain regions that make up the key structures for the effects of the neurobiological substrates for stress and depression (Graeff, *et al*., 1996).

In line with these findings the clear decrease in sucrose consumption seen in these stressed rats could be directly associated with an increase in the dopaminergic activity of the hypothalamus and depletion in serotonin. Moreover, a dysregulation of the hypothalamic–pituitary–adrenal (HPA) axis is also present after exposure to chronic stress (Bekris, *et al*., 2005; Dalla, *et al*., 2005; Grippo, *et al*., 2005). At the same time, the activation of the HPA axis by stress exerts inhibitory effects on the hypothalamic–pituitary–gonadal axis, which could explain disruptions in the reproductive cycle in depression (Knobil, 1990; Grippo, *et al*., 2005).

On the other hand, the decreased serotoninergic activity in the CMS-stressed females could result from changes in serotoninergic function mediated by altered corticosteroid (Grippo, *et al*., 2005) and/or sex steroid (Zhou, *et al*., 2002) availability. Several reports have shown elevated CRF concentrations, increased corticosterone levels and altered regulation of adenocorticotropin hormone in female rats (Konkle, *et al*., 2003; Dalla, *et al*., 2005).

Therefore we would speculate that the reversal of the CMS-induced behavioural changes provided by the *R. rosea* extract could be the result of its effects on the central serotoninergic, dopaminergic and norepinephrinergic systems. In fact, extracts of *R. rosea* have been reported to influence the levels and activities of the biogenic monoamines, such as serotonin, dopamine and norepinephrine, in the cerebral cortex, brain stem and hypothalamus (Kurkin and Zapesochnaya, 1986). Furthermore, the effect of RHO administration on acute stress could be attributed to its claimed ability to modulate the activation of several components of the stress-response system, such as the sympatho-adrenal system, which mainly controls the rapid response of an organism to an acute stressor (Mattioli and Perfumi, 2007). Additionally, the ability of a *R. rosea* extract to revert stress-induced behavioural and physiological alterations could also be related to its ability to reduce the secretion of CRF, by acting as a CRF antagonist (Mattioli and Perfumi, 2007). Obviously, further investigations are necessary to confirm this view.

It is well known that chronic FLU administration can restore the neurochemical changes seen following CMS application to normal levels by the enhancement of brain serotonin, norepinephrine and dopamine levels. Moreover, FLU treatment induces a normalization of the HPA axis activity by its action on CRF and cortisol levels (Di Chiara, *et al*., 1999; Li, *et al*., 2003).

Several groups of biologically active substances have been identified in extracts of *R. rosea*, including organic acids, flavonoids, tannins and high amounts of phenolic compounds. In particular, the phenolic compounds include phenylpropane derivatives, such as the rosavins (rosavin, rosine, rosarin), which are specific components of *R. rosea*, and phenylethane derivatives, such as salidroside, which is present in all species of the *Rhodiola* genus and in a wide variety of species outside this genus (Kurkin and Zapesochnaya, 1986; Wang, *et al*., 1992; Yoshikawa, *et al*., 1996; Linh, *et al*., 2000). Therefore, it is far from established which compound(s) are actually responsible for the effects of RHO. However, several studies have proposed that the phenolic compounds, particularly the phenylpropane and phenylethane derivatives, represents the major active agents for the therapeutic activity of this plant (Kelly, 2001; Brown, *et al*., 2002). Because these compounds, which include rosavin, triandrin and salidroside, are structurally related to the catecholamines, they could have important roles in coordinating and integrating the behavioural responses to stress (Kurkin and Zapesochnaya, 1986; Wang, *et al*., 1992; Yoshikawa, *et al*., 1996; Panossian and Wagner, 2005). However, further comparative studies using the main active components of this extract are necessary.

In conclusion, the present study provides original evidence that chronic administration of *R. rosea* extract results in a potent inhibition of the behavioural and physiological changes induced by chronic exposure to mild stressors that is comparable to that of classical antidepressant drugs. This effect could have important clinical implications, as it is well known that continuous exposure to unpredictable environmental stressors contributes in the development of neuropsychiatric disorders, such as depression in humans (Ressler and Nemeroff, 2000; Dalla, *et al*., 2005). Therefore, *R. rosea* L. might represent a promising pharmacological approach with important modulatory functions for mediating or regulating specific behavioural and physical responses that are typical of psychopathologies in which exposure to chronic stressors is a major contributing factor. Moreover, as women are more susceptible than men to stress-related mental illnesses (Kendler, *et al*., 2000) and there is an extensive overlap of depressive disorders with menstrual-related phenomena in women (e.g. premenstrual, postpartum, perimenopausal and postmenopausal syndromes) (American Psychiatric Association 1994; Halbreich, 2003), *R. rosea* L. extracts might prove to be effective for the reduction of hormone-related neurodisorders that are induced by different stressors.

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