Original Article

Anxiety and Hyperlocomotion Induced by Chronic Unpredictable Mild Stress Can Be Moderated with Melatonin Treatment

(anxiety / CUMS / dopamine / hippocampus / locomotion / melatonin)

N. SPASOJEVIC, B. STEFANOVIC, P. JOVANOVIC, S. DRONJAK

Laboratory of Molecular Biology and Endocrinology, Institute of Nuclear Sciences "Vinca", University of Belgrade, Serbia

Abstract. Preclinical studies have shown that melatonin exercised antidepressant-like and anxiolytic-like effects in animal models of anxiety. The aim of the present study was to correlate the changes in behaviour induced by melatonin treatment with the activity of the dopaminergic system in the hippocampus of Wistar rats exposed to chronic, unpredictable, mild stress (CUMS). Male Wistar rats, 11 weeks old, were subjected to chronic stress for 28 successive days. Separate groups of control and stressed rats were intraperitoneally injected daily either with melatonin (10 mg/kg/day, i.p.) or placebo (5% ethanol). The open-field and elevated plus-maze tests were used to assess locomotor activities and anxiety levels. The content of dopamine (DA) in the hippocampal tissues was determined using radioenzymatic assay, while changes in tyrosine hydroxylase (TH) mRNA and protein levels in the hippocampus were determined using real-time RT-PCR and Western immunoblotting. Chronic stress led to reduction in the hippocampal dopaminergic content without affecting the levels of TH protein. These changes were accompanied by increased locomotor activity and higher anxiety levels in the open-field test. Administration of melatonin for 28 days resulted in an increase in the hippocampal DA content as a result of elevated TH protein levels. Melatonin showed an improvement in anxiety-like behaviour along with significantly reduced exploration. We could conclude that melatonin may stimulate dopaminergic synthesis in the hippocampus in order to suppress stress-induced behaviour.

Introduction

Stressful life events are associated with the onset of psychiatric disorders, including major depression (Kendler et al., 1999). The hippocampus is a brain region with a long established role in various cognitive processes, including locomotion, learning and memory (Buzsáki and Moser, 2013), but also regulates behaviours related to anxiety (Bannerman et al., 2004). Moreover, it is proposed that anxiolytic-like drugs induce their effects by acting on a behavioural inhibition system that includes the hippocampus (McNaughton and Gray, 2000). Chronic, unpredictable, mild stress (CUMS) was initially developed in rats using multiple stressors as a behavioural model of anhedonia that could be reversed by treatment with antidepressants (Katz, 1982; Dhingra and Bansal, 2015). Chronic, mild stress leads to altered synaptic function in the hippocampus (Jayatissa et al., 2006). These impairments may be compatible with altered emotionality, cognitive impairment, social isolation and disturbed memory (Bartesaghi, 2004). Dopamine (DA) is an excitatory monoamine neurotransmitter, widely distributed in the mammalian brain, including the hippocampus (Goodman and Gilman, 2005). The major DA pathways in the brain are involved in movement and locomotion (Cenci, 2007). In addition to movement control, DA also plays an important role in the pathophysiology of depression. Previously, diminished DA neurotransmission in depressive disorders has been reported (Dunlop and Nemeroff, 2007). Extracellular DA levels are tightly coupled to the activity of brain tyrosine hydroxylase (TH), the initial and rate-limiting enzyme in catecholamine synthesis (Nagatsu et al., 1964).

Received March 23, 2016. Accepted July 22, 2016.

This work was supported by the Ministry of Education and Science of the Republic of Serbia, Contract No. 173044.

Corresponding author: Natasa Spasojevic, Laboratory for Molecular Biology and Endocrinology, Institute of Nuclear Sciences "Vinca", P.O.B. 522-090, 11000 Belgrade, Serbia. Phone/Fax: (0038) 1113408688; e-mail: snatally@vinca.rs

Abbreviations: COMT – catechol-O-methyl-transferase, CUMS – chronic unpredictable mild stress, DA – dopamine, MEL – melatonin, Nac – nucleus accumbens, TBST – Tris-buffered saline-tween 20, TH – tyrosine hydroxylase, vHPC – ventral hippocampus.

Antidepressant drugs are effective in treating major depression, but many have unpleasant side-effects. Alternative pharmacological mechanisms of action that may provide a useful alternative to existing antidepressant medications are constantly being examined (Dording et al., 2002). Melatonin (MEL) is an endogenous indoleamine produced and secreted by the pineal gland. The physiological, pharmacological and behavioural properties of MEL have been widely studied. In fact, MEL has several important functions, including regulation of the circadian rhythms, modulation of seasonal changes, in reproduction as well as in antioxidant, anti-inflammatory and anticonvulsant effects (Gitto et al., 2013). Preclinical studies have shown that MEL exercised antidepressant-like and anxiolytic-like effects in rodent models of anxiety (Kopp et al., 1999). With CUMS, repeated administration of agomelatine, the MEL receptor agonist, reverses CUMS-induced reduction in sucrose consumption, suggesting reduction of anhedonia in the stressed animals (Papp et al., 2003). Renewed attention has been given to the role of melatonin in modulating behaviour and stress response.

In the present study, the effects of MEL were investigated when applied during the CUMS procedure (consisting of prolonged repetition of low-intensity stressors) on two different anxiety-like related behaviours in rats. In addition, the aim was to determine whether any effect on behaviour is associated with the levels of dopamine and *TH* gene expression in the hippocampus.

Material and Methods

Animals

Adult Wistar rat males (11 weeks old, 250–310 g) maintained in a temperature-controlled room (21 ± 1.0 °C) and 12h/12h light/dark cycle were used. The rats were given *ad libitum* access to standard laboratory food and water. The care was taken to minimize the pain and discomfort of the animals according to the recommendations of the Ethical Committee for the use of laboratory animals of the "Vinca" Institute based on Directive 2010/63/EU. All procedures with animals were approved by the Ethical Committee for the use of laboratory animals of the "Vinca" Institute and Ministry of Agriculture and Environmental Protection, Authority for Veterinary permission No. 323-07-04657/2015-05 /02.

Experimental design

In the experiment we used 32 animals, which were allocated into control (unstressed) and chronic, unpredictable, mildly stressed (CUMS) groups. These groups were further divided into two subgroups each, receiving daily injection of vehicle (5% ethanol) or MEL in a dose of 10 mg/kg body weight by intraperitoneal (i.p.) route, one hour before the dark phase to avoid disruption of circadian rhythms that occur if melatonin is administered throughout the day. Melatonin (Q-1300, Bachem, Bubendorf, Switzerland) was dissolved in NaCl (0.9%) containing 5% ethanol. Exposure to CUMS and the vehicle, i.e. drug administration, started on the same day and was continued for four weeks. To consider body weight evolution, the injected volumes were adjusted twice a week to the body growth of animals.

Chronic unpredictable mild stress

The CUMS procedure was designed to maximize the unpredictable nature of the stressors. The CUMS groups were exposed to the following stressors in random order: forced running (15 min, 10 m/min); soiled cage (500 ml 22 °C water spilled into bedding); 45° cage tilt along the vertical axis; 17-h food deprivation, 5-h cold room (4 °C) and water deprivation (water bottles were removed from the cage during this time), 5-h paired housing (animals were placed in the cage of another rat also in the stress group) and individual housing (48 h). Immediately after the CUMS procedure, all animals were decapitated, whole hippocampal tissue was quickly removed and placed on ice, frozen in liquid nitrogen and stored at -70 °C until analysed.

Open-field test

The open-field test provides simultaneous measurement of locomotion, exploration, and anxiety. The open-field arena consisted of a 100 cm × 100 cm × 40 cm square arena and divided into 16 equal squares (dimensions of each square were 25 cm × 25 cm). Rats were carried to the test room in their home cages and were handled by the base of their tails at all times. All rats were placed in the testing room 1 h before the test took place in order to allow them to acclimate. A single rat was placed gently in the centre of the arena, and its activity was measured manually during a 5-min period. The open field was cleaned between each rat using 10% alcohol solution to eliminate possible odours left by other animals. Rats were then removed from the arena and returned to their home cages. The following variables were recorded for each minute of the test session: 1) ambulation in the board area: number of entries into the outer squares; 2) ambulation in the central area: number of entries into the inner squares; 3) time spent in the central area; 4) number of centre crossings; 5) grooming time: duration of time the animal spent licking or scratching itself while stationary; 6) resting time: duration with which the rat was completely stationary.

Elevated plus maze

The elevated plus maze contains four arms (two open without walls and two enclosed by 15 cm high walls). The arms (50 cm long and 10 cm wide) are arranged to form a plus shape. The elevated plus maze was raised half a meter from the floor. Subjects were initially placed on the central platform of the maze facing an enclosed arm and allowed to explore freely for 5 min. The elevated plus maze was cleaned with 10% alcohol solution and dried with paper towels before testing another rat. The behaviour of each individual was analysed, taking

into account the standard measurement: 1) total number of entries into the arms; 2) frequency of entry into the open arms (= number of entries in the open arm/total number of entries) * 100; 3) frequency of time spent in the open arms (= number of seconds spent in open arms/300 s) * 100. The entry of animals into the arms means that all four paws were located in that arm and release with two paws out of the arm.

Radioenzymatic assay

Hippocampi were immersed into cold (4 °C) perchloric acid (0.3 µg of tissue per 30 µl of 0.1 N HClO4), homogenized in a motor-driven homogenizer, the homogenates centrifuged (20 000 rpm, 20 min, 4 °C) in centrifuge K70D (MLW, Engelsdorf, Germany) and the supernatants (30 µl) used for determination of catecholamines. The content of catecholamines in the tissues was determined using the modified chromatographic method of Peuler and Johnson (1977) based on the conversion of catecholamines into the corresponding O-methylated derivatives by purified catechol-O-methyl-transferase (COMT) in the presence of S-adenosyl-l-(3H-methyl)-methionine. The O-methylated derivatives were extracted and oxidized into 3H-vaniline. Radioactivity was measured using a toluene-based scintillation cocktail in an LKB-Wallac model 1219 scintillation counter at an efficiency of 40 % for tritium.

RNA isolation and real-time RT-PCR

Total RNA from hippocampal tissue was extracted using TRIzol® Reagent (Thermo Fisher Scientific, Waltham, MA). Hippocampal tissue was homogenized in 1 ml TRIzol® Reagent per 100 mg of tissue using an electrical homogenizer (IKA-WERKE, GmbH & Co, Staufen, Germany). Reverse transcription was performed using Ready-To-Go You-Prime First-Strand Bead (GE Healthcare Life Sciences, Pittsburgh, PA) and pd $(N)_6$ primer according to the manufacturer's protocol. PCR reaction was performed in the ABI Prism 7000 Sequence Detection System (Thermo Fisher Scientific) at 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. TaqMan PCR reaction was carried out using Assay-on-Demand Gene Expression Products (Thermo Fisher Scientific) for TH (ID: Rn00562500_m1). A reference endogenous control was included in each analysis to correct the differences in the inter-assay amplification efficiency, and all transcripts were normalized to cyclophylin A (ID: Rn00690933) expression.

Western blot analysis

Hippocampal tissue was homogenized in RIPA Lysis Buffer System (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, sc-24948). Centrifugation was carried out for 20 min in a Hettich Mikro 200R centrifuge (Andreas Hettich GmbH & Co., Tuttlingen, Germany) at 12 000 rpm, 20 min at 4 °C. After centrifugation the supernatant was taken and protein concentration determined by the method of Lowry et al. (1951). Thirty µg of hippocampal protein extract separated by 10% SDS-poly-acrylamide gel electrophoresis was transferred to a supported PVDF membrane (Immobilon-P membrane, Merck Millipore, Billerica, MA). The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline-tween 20 (TBST) for 1 h. All following washes (three times for 15 min) and antibody incubation (overnight at 4 °C for primary antibody and 1h at 4 °C for secondary antibody) were also performed in TBST at ambient temperature in a shaker. For measuring TH, protein levels, a polyclonal anti-TH primary antibody, rabbit (ab51191, dilution 1 : 1000, Abcam, Cambridge, UK), was used. The washed membrane was further incubated with the horseradish peroxidase-conjugated secondary anti-rabbit antibody for luminol-based detection (ab6721, dilution 1 : 5000, Abcam). The secondary antibody was then visualized by Immobilon Western Chemiluminescent HPR Substrate (Merck Millipore).

Statistical analysis

The results are reported as means ± S.E.M. The significance of differences in the dopamine content and gene expression levels of the examined catecholamine biosynthetic enzyme in the hippocampus and differences in performance in the open field and elevated plus maze between rats subjected to CUMS and/or melatonin treatment were estimated by two-way ANOVA test. The Tukey post hoc test was used to evaluate the differences between the groups. Statistical significance was accepted at $P < 0.05$.

Results

Open-field test

Ambulation in the board area (Fig. 1a)

The two-way ANOVA analysis showed a significant effect of MEL on ambulation in the board area ($F_{(1,31)} = 6.17$, $P < 0.05$). Statistical analysis showed that the CUMS rats explored the outer squares significantly more than the control animals (32 %, $P < 0.01$). Furthermore, MEL reduced locomotion over the outer squares in the CUMS rats (16 %, $P < 0.01$). However, no significant difference between the control group treated with MEL and the group treated with a placebo was noted.

Ambulation in the central area (Fig. 1b)

In the open field, the CUMS and control rats entered a similar number of inner squares during the 5-min observation period. However, the stressed animals treated with MEL entered 38 % more inner squares than the CUMS rats treated with a placebo ($P < 0.05$).

Time spent in the central area (Fig. 1c)

Chronic stress had a highly significant effect ($F_{(1,31)} = 10.08$, $P < 0.01$). The rats exposed to CUMS spent less time in the central area than the controls (54 %, $P < 0.01$). Comparison of the CUMS groups revealed a significant difference between the placebo group and the treated group (72 %, $P < 0.01$). However, there was no significant difference between the controls.

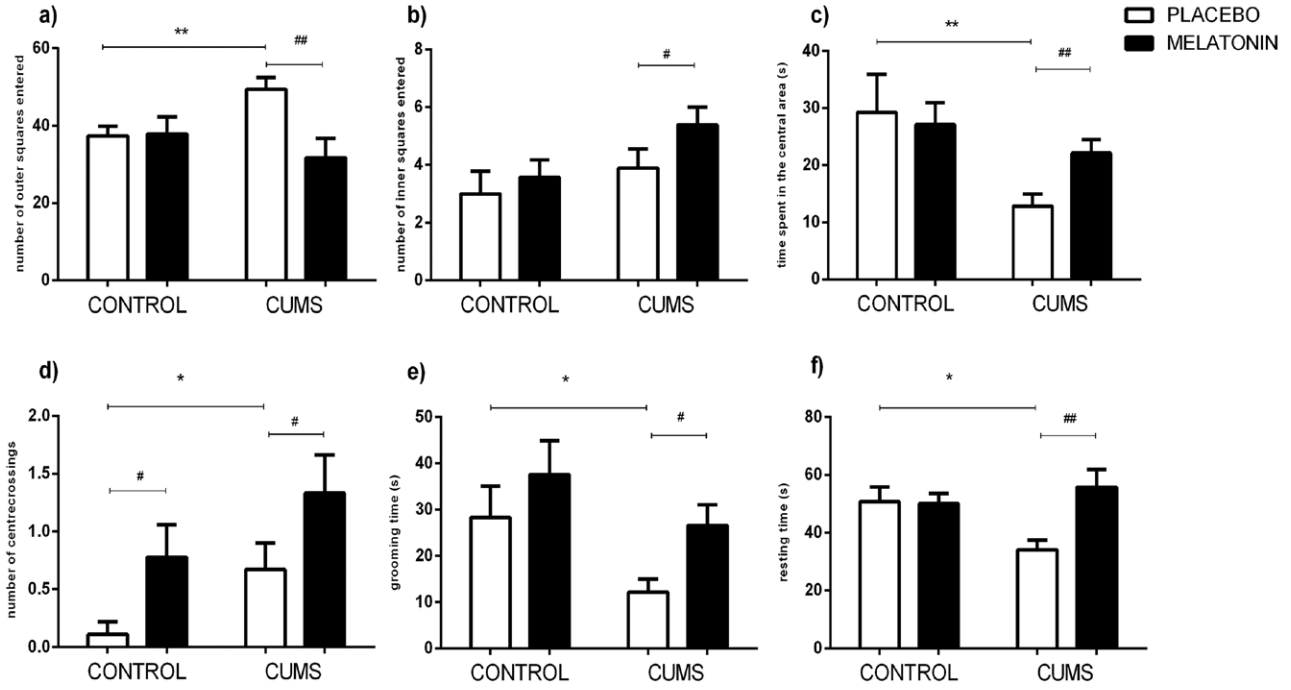

Fig. 1. Effects of chronic melatonin treatment on open-field test behaviours of animals exposed to CUMS for 28 days: (**a**) ambulation in the board area; (**b**) ambulation in the central area; (**c**) time spent in the central area (s); (**d**) number of centre crossings; (**e**) grooming time (s); (**f**) resting time (s). The values are means ± S.E.M. of eight rats. Statistical significance: * $P < 0.05$; ** $P < 0.01$ CUMS *vs.* unstressed control; # $P < 0.05$; ## $P < 0.01$ placebo *vs.* melatonin (Tukey test).

Number of centre crossings (Fig. 1d)

Two-way ANOVA demonstrated a significant effect of both the 4-week CUMS exposure ($F_{(1,31)} = 4.82$, $P < 0.05$) and the MEL treatment ($F_{(1,31)} = 6.94$, $P < 0.05$) on the number of crossings. Post-hoc testing showed that CUMS provoked more frequent crossings (500 %, $P < 0.05$). Treatment with MEL significantly enhanced the number of crossings both in the control (600 %, $P < 0.05$) and the CUMS (98 %, $P < 0.01$) group of animals.

Grooming time (Fig. 1e)

The duration of grooming was significantly influenced by stress ($F_{(1,31)} = 5.80$, $P < 0.05$) and treatment ($F_{(1,31)} = 4.43$, $P < 0.05$). The Tukey test showed that the stressed rats groomed less (57 %, $P < 0.05$) than the controls in the open field. MEL treatment prevented the CUMS-induced decrease in grooming (118 %, $P < 0.05$).

Resting time (Fig. 1f)

Two-way ANOVA displayed a significant effect of MEL on the resting time ($F_{(1,31)} = 4.89$, $P < 0.05$) as well as an interaction between treatment and stress ($F_{(1,31)} = 5.62$, $P < 0.05$). The CUMS rats spent less time resting in the open field than the controls (33 %, $P < 0.05$). Melatonin caused the stressed animals to spend more time resting (63 %, $P < 0.01$).

Elevated plus maze

Total arm entries (Fig. 2a)

Locomotor activity was unaffected by stress, but a significant effect of MEL ($F_{(1,31)} = 17{,}88$, $P < 0.001$) was noted. Melatonin produced a significant decrease in locomotion activity in both the control (38 %, $P < 0.05$) and the CUMS (48 %, $P < 0.01$) rats.

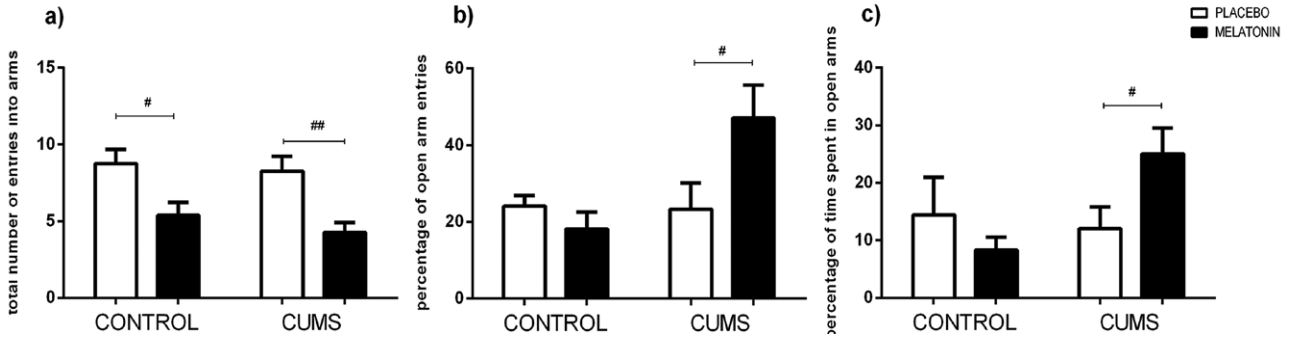

Fig. 2. Effect of chronic melatonin treatment on the elevated plus-maze test behaviours of animals exposed to CUMS for 28 days: (**a**) total arm entries; (**b**) percentage of open arm entries; (**c**) percentage of time spent in the open arms made in the elevated plus maze. The values are means ± S.E.M. of eight rats. Statistical significance: # $P < 0.05$; ## $P < 0.01$ placebo *vs.* melatonin (Tukey test).

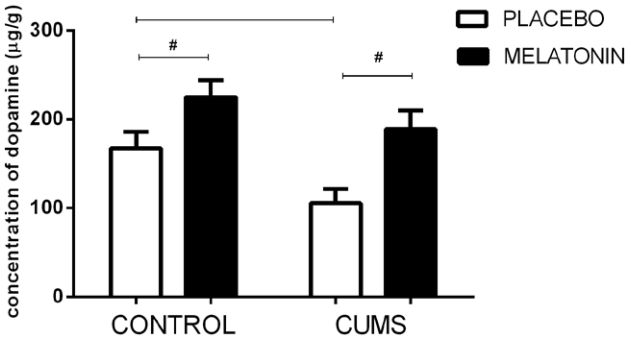

Fig. 3. Effect of chronic melatonin treatment on dopamine (DA) concentration in the hippocampus of rats exposed to CUMS for 28 days. The values are means ± S.E.M. of eight rats. Statistical significance: * $P < 0.05$ CUMS *vs.* unstressed control; # $P < 0.05$ placebo *vs.* melatonin (Tukey test).

Frequency of open arm entries (Fig. 2b)

An interaction between melatonin and stress was observed ($F_{(1,31)} = 6.25$, $P < 0.05$). Statistical analyses revealed a significant effect of melatonin on the CUMS rats (102 %, $P < 0.05$), whereas no difference was found between the control groups.

Frequency of time spent in open arms (Fig. 2c)

Rats treated with MEL spent a highly significantly greater amount of time in the open arms than the animals treated with a placebo (108 %, $P < 0.05$). A significant difference was not noted between the control and the stressed rats. Moreover, melatonin failed to reach statistical significance in the control animals.

Content of dopamine in the hippocampus

Under the examined stress conditions, significant variations of DA content in the hippocampal tissue ($F_{(1,31)} = 4.93$, $P < 0.05$) were observed. MEL also significantly affected the DA content ($F_{(1,31)} = 10.11$, $P < 0.01$). Post-hoc analysis revealed that the content of DA in the tissue was lower in the hippocampus of the CUMS group compared to the control group (by 37 %, $P < 0.05$) (Fig. 3). However, MEL treatment increased the hippocampal DA content both in the control (by 34 %, $P < 0.05$) and the stressed (by 78 %, $P < 0.05$) animals.

Gene expression of TH enzyme

The results presented in Fig. 4a indicate a major influence of CUMS ($F_{(1,31)} = 28.87$, $P < 0.001$) and MEL ($F_{(1,31)} = 33.48$, $P < 0.001$) on TH mRNA levels in the hippocampus. Post-hoc analysis demonstrated a significant decrease in stressed rats (by 53 %, $P < 0.001$), compared to those found in the control animals (Fig. 4a). MEL further decreased TH mRNA expression both in the control and the stressed group of animals (by 56 %, $P < 0.01$ and 42 %, $P < 0.05$, respectively) compared to the vehicle group.

Chronic MEL treatment also affected the TH protein content ($F_{(1,31)} = 12.43$, $P < 0.01$) in the hippocampus (Fig. 4b). Stress did not change the levels of this protein. Animals treated with MEL, however, had higher TH protein levels (controls by 56 %, $P < 0.05$ and CUMS rats by 42 %, $P < 0.05$).

Discussion

We assessed the MEL effects on anxiety-induced behaviours and locomotor activity in chronically stressed rats. The test results in this study showed that mild unpredictable stress influenced locomotion, exploratory behaviour and emotionality in the rats. Exposing animals to a new environment in the form of an open-field arena induced hyperlocomotion. Hariss et al. (1998) have shown that rats exposed to CUMS from 3 to 8 weeks exhibit enhanced locomotor and exploratory activity. The animals in this study also showed signs of anxiety in the open field by spending less time in the centre. Grooming is a very important and evolutionary form of behaviour that is typical of a large number of species. Neglected hygiene is one of the signs of depression and can be changed by treatment with antidepressants (Yalcin et al., 2008). In this paper, the stressed animals spent less time grooming, analogous to the results of Wang et al. (2013). Similar results were obtained

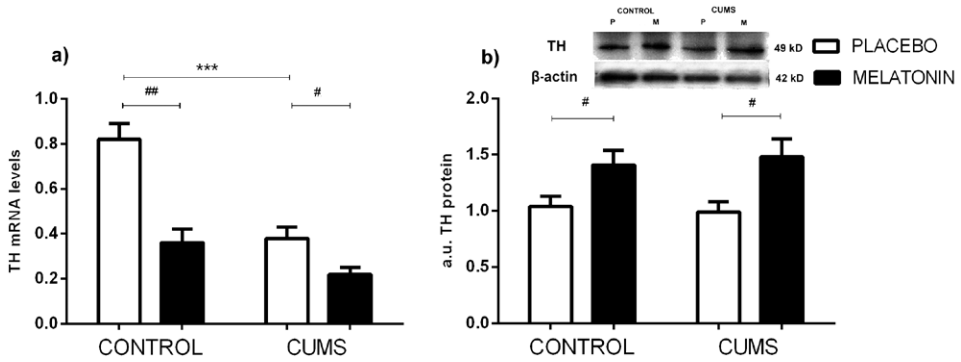

Fig. 4. Effect of chronic melatonin treatment on tyrosine hydroxylase (TH) gene expression in the hippocampus of rats exposed to CUMS for 28 days: (**a**) TH mRNA levels. The final result was expressed as fold change relative to the calibrator and normalized to cyclophylin A. (**b**) TH protein levels. The final result was expressed in arbitrary units and normalized in relation to ß-actin. The values are means ± S.E.M. of eight rats. Statistical significance: *** $P < 0.001$ CUMS *vs.* unstressed control; # $P < 0.05$; ## $P < 0.01$ placebo *vs.* melatonin (Tukey test).

by Rosa et al. (2014) when the animals were chronically exposed to corticosterone. It has previously been confirmed that animals exposed to this form of stress have increased levels of corticosterone (Dronjak et al., 2007).

The elevated plus maze is one of the most widely used tests to measure anxiety-like behaviours (Dawson and Tricklebank, 1995). In this maze, stressed, anxious animals should display reduction in percent entries into the open arms and in percent time spent in the open arms. The total number of entries into all arms provides a measure of general hyperactivity or sedation. However, our findings reflected the fact that the chronic mild stress procedure did not affect anxiety-like behaviours or horizontal locomotion in this test. Strekalova and al. (2011) also have shown that this procedure causes an anxiolytic-like profile in the elevated plus maze. Interestingly, these results are contrary to the detected stress-induced hyperlocomotion in an open-field test. These paradoxical results could be a non-specific consequence of chronic stress or a result of differences in the stressfulness of these tests.

Ouhaz et al. (2013) reported that lesions in the ventral hippocampus (vHPC) induced behavioural changes: hyperactivity and anxiety. The present study showed that CUMS decreased the hippocampal dopaminergic content. Several authors have previously noted similar observations (Rasheed et al., 2010; Su et al., 2014). Interestingly, the amount of TH protein remained unaltered, indicating that stress did not lead to changes in the synthesis of dopamine. The vesicular monoamine transporter type 2 gene (*VMAT2*) has a crucial role in the storage and synaptic release of all monoamines. Depletion of DA could be the result of VMAT2 protein reduction in the hippocampus (Spasojevic et al., 2012). The dopamine transporter KO mice display novelty-induced hyperactivity, which is likely to be due to higher levels of brain DA, caused by the absence of clearance of DA from the synaptic cleft (Gainetdinov et al., 1999). An early preclinical study demonstrated that vHPC activation induces an increase in DA efflux in forebrain targets, such as the nucleus accumbens (NAc), suggesting that this brain region can augment DA neuron activity (Legault and Wise, 1999). Rouillon et al. (2007) established the manner in which the hippocampus exerts modulation of locomotion by activation of both D1-like and D2-like postsynaptic dopamine receptors in NAc. Furthermore, dopamine D1 activation shortens the duration of phases in stereotyped grooming sequences, but increases the speed of grooming actions (Matell et al., 2006). D2 receptor agonist also decreases grooming, probably by acting on D2 autoreceptors (Fornaquera et al., 1995). Simultaneous activation of both D1 and D2 receptors at postsynaptic levels can explain the hyperactivity and changes in self-grooming duration.

Musshoff et al. (2002) suggested that MEL plays an important role in modulating the neuronal excitability in the hippocampus. In this study, treatment with MEL for a period of 28 days affected locomotion in the open-field test, reducing the number of crossed squares and extending the time the animals spent inactive. In addition, the treated rats spent more time in the centre of the arena, pointing to reduced anxiety. These results are consistent with several studies in distinct models of mood disorder (Mairesse et al., 2013; Boulle et al., 2014). Chronic treatment with MEL also prevented changes in grooming induced by chronic unpredictable stress, similar to the results obtained by Detanico et al. (2009). Decreased locomotor activity in melatonin-treated animals was also observed in the elevated plus-maze test. Additionally, it was found that MEL significantly increased the percentage of time spent in the open arms and the open arms entries, demonstrating anxiolytic-like properties.

Chronic MEL treatment increased DA levels in the hippocampus of both the control and the CUMS rats. *In vivo* findings by Esteban et al. (2010) suggest that MEL exerts a long-term effect on striatal DA content by enhancing monoamine synthesis in aged rats, which might improve cognition and motor coordination. Similarly, TH protein elevation in the hippocampus of treated animals was detected. It has been reported, conversely, that long-term MEL treatment significantly increases TH protein levels *in vitro* (McMillan et al., 2007) and TH enzymatic activity in the striatum of hamsters (Alexiuk and Vriend, 2007). However, our data shows that mRNA levels were further reduced. Boulle et al. (2014) suggest that agomelatine treatment prevents phosphorylation of CREB in the hippocampus. The *TH* gene contains a CRE that has been shown to be critical for activation of gene expression. Changes in mRNA and protein levels do not correlate, probably mainly due to the regulation control at different levels: translation, RNA and protein stability. What is interesting about this work is that MEL promotes dopaminergic synthesis while also triggering hypolocomotion and anxiolysis.

Zisapel et al. (1982) observed an inhibition of DA release by MEL in vHPC, which may lead to reduced stimulation of NAc. Moreover, the decrease of locomotor activity, observed after treatment with dopamine D2/D3 receptor agonists, has been proposed to result from the activation of DA autoreceptors localized presynaptically and/or on dopaminergic cell bodies (Eilam and Szechtman, 1989). Data from Boulay et al. (1999) suggests that the D2 autoreceptors mediate these hypolocomotion effects. The release of DA from axon terminals in the NAc, striatum and prefrontal cortex is inhibited by presynaptic D2 autoreceptor stimulation (Kalivas, 1993). Considering that Hamdi (1998) established melatonin's ability to increase affinity of the D2 receptors, then endogenous DA would act preferentially on these receptors, resulting in greater stimulation. It should also be taken into account that, according to Chenu et al. (2013), an agomelatine-induced increase in extracellular levels of NA and DA is not antagonized by melatonergic receptor antagonist S22153, whereas its positive behavioural effects are. The exact mechanisms underlying the antidepressant and anxiolytic activities of MEL, evidently, are not well established. MEL has been shown

to also act as a 5-HT_{2A} antagonist and regulates both the spontaneous efflux and evoked release of 5-HT in the hippocampus (Monnet, 2002). Several reports have indicated that GABA-mimetic drugs have antidepressant and anxiolytic properties and that administration of a benzodiazepine receptor antagonist blunted MEL activity (Raghavendra et al., 2000).

Our present investigation provided evidence that prolonged exposure of rats to socio-environmental stressors of low intensity can induce marked changes, together with increased locomotor activity and hyperarousal. Melatonin effectively prevented both neurochemical and behavioural alterations triggered by stress. This data indicates that MEL did not act on individual symptoms but corrected all aspects of the behaviour changes triggered by CUMS. Our findings strongly suggest that this drug impacts mechanisms that lie at the core of anxiety/depressive disorders. How MEL exerts these effects on behaviour is still subject to continuous study. We could, however, suggest that the behavioural effects of MEL use could be due to increased DA synthesis in the hippocampus of the stressed rats.

References

Alexiuk, N. A., Vriend, J. (2007) Melatonin: effects on dopaminergic and serotonergic neurons of the caudate nucleus of the striatum of male Syrian hamsters. *J. Neural Transm*. **114**, 549-554.

Bannerman, D. M., Rawlins, J. N. P., McHugh, S. B., Deacon, R. M. J., Yee, B. K., Bast, T., Zhang, W. N., Pothuizen, H. H., Feldon, J. (2004) Regional dissociations within the hippocampus – memory and anxiety. *Neurosci. Biobehav. Rev.* **28**, 273-283.

Bartesaghi, R. (2004) Effect of early isolation on the synaptic function in the dentate gyrus and field CA1 of the guinea pig. *Hippocampus* **14**, 482-498.

Boulay, D., Deportere, R., Perrault, G., Borrelli, E., Sanger, D. J. (1999) Dopamine D2 receptor knock-out mice are insensitive to the hypolocomotor and hypothermic effects of dopamine D2/D3 receptor agonists. *Neuropharmacology* **38**, 1389-1396.

Boulle, F., Massart, R., Stragier, E., Païzanis, E., Zaidan, L., Marday, S., Gabriel, C., Mocaer, E., Mongeau, R., Lanfumey, L. (2014) Hippocampal and behavioural dysfunctions in a mouse model of environmental stress: normalization by agomelatine. *Transl. Psychiatry* **4**, e485.

Buzsáki, G., Moser, E. I. (2013) Memory, navigation and theta rhythm in the hippocampal-entorhinal system. *Nat. Neurosci.* **16**, 130-138.

Cenci, M. A. (2007) Dopamine dysregulation of movement control in L-DOPA-induced dyskinesia. *Trends Neurosci.* **30**, 236-243.

Chenu, F., El Mansari, M., Blier, F. (2013) Electrophysiological effects of repeated administration of agomelatine on the dopamine, norepinephrine, and serotonin systems in the rat brain. *Neuropsychopharmacology* **38**, 275-284.

Dawson, G. R., Tricklebank, M. D. (1995) Use of the elevated plus maze in the search for novel anxiolytic agents. *Trends Pharmacol. Sci.* **16**, 33-36.

Detanico, B. C., Piato, A. L., Freitas, J. J., Lhullier, F. L., Hidalgo, M. P., Caumo, W., Elisabetsky, E. (2009) Antidepressant-like effects of melatonin in the mouse chronic mild stress model. *Eur. J. Pharmacol.* **607**, 121-125.

Dhingra, D., Bansal, S. (2015) Antidepressant-like activity of plumbagin in unstressed and stressed mice**.** *Pharmacol. Rep.* **67**, 1024-1032.

Dording, C. M., Mischoulon, D., Petersen, T. J., Kornbluh, R., Gordon, J., Nierenberg, A. A., Rosebaum, J. E., Fava, M. (2002) The pharmacologic management of SSRI-induced side effects: a survey of psychiatrists. *Ann. Clin. Psychiatry* **14**, 143-147.

Dronjak, S., Spasojevic, N., Gavrilovic, L., Varagic, V. (2007) Effects of noradrenaline and serotonin reuptake inhibitors on pituitary-adrenocortical and sympatho-adrenomedullar system of adult rats. *Neuro Endocrinol. Lett.* **28**, 614-620.

Dunlop, B. W., Nemeroff, C. B. (2007) The role of dopamine in the pathophysiology of depression. *Arch. Gen. Psychiatry* **64**, 327-337.

Eilam, D., Szechtman, H. (1989) Biphasic effect of D-2 agonist quinpirole on locomotion and movements. *Eur. J. Pharmacol.* **161**, 151-157.

Esteban, S., Garau, C., Aparicio, S., Moranta, D., Barceló, P., Fiol, M. A., Rial, R. (2010) Chronic melatonin treatment and its precursor L-tryptophan improve the monoaminergic neurotransmission and related behaviour in the aged rat brain. *J. Pineal. Res.* **48**, 170-177.

Fornaquera, J., Huston J. P., Carey, R. J., Schwarting, R. K. (1995) Stimulation of D1-or D2-receptors in drug-naive rats with different degrees of unilateral nigro-striatal dopamine lesions. *Psychopharmacology (Berl.)* **119**, 145-154.

Gainetdinov, R., Wetsel, W., Jones, S., Levin, E., Jaber, M., Caron, M. (1999) Role of serotonin in the paradoxical calming effect of psychostimulants on hyperactivity. *Science* **283**, 397-401.

Gitto, E., Marseglia, L., Manti, S., D'Angelo, G., Barberi, I., Salpietro, C., Reiter, R. J. (2013) Protective role of melatonin in neonatal diseases. *Oxid. Med. Cell. Longev.* **2013**, 980374.

Goodman, L. S., Gilman, A. G. (2005) 5-Hydroxytryptamine (serotonin) and dopamine. In: *As Bases Farmacológicas da Terapêutica (The Pharmacological Basis of Therapeutics)*, eds. Hardman, J. G., Limbird, L. E., Gilman, A. G., pp. 229-234, McGraw-Hill, Rio de Janeiro. (in Portuguese)

Hamdi, A. (1998) Melatonin administration increases the affinity of D2 dopamine receptors in the rat striatum. *Life Sci.* **63**, 2115-2120.

Harris, R. B., Zhou, J., Youngblood, B. D., Smagin, G. N., Ryan, D. H. (1998) Failure to change exploration or saccharin preference in rats exposed to chronic mild stress. *Physiol. Behav.* **63**, 91-100.

Jayatissa, M. N., Bisgaard, C., Tingstrom, A., Papp, M., Wiborg, O. (2006) Hippocampal cytogenesis correlates to escitalopram-mediated recovery in a chronic mild stress rat model of depression. *Neuropsychopharmacology* **31**, 2395-2404.

Kalivas, P. W. (1993) Neurotransmitter regulation of dopamine neurons in the ventral tegmental area. *Brain Res. Rev.* **18**, 75-113.

Katz, R. J. (1982) Animal model of depression: pharmacological sensitivity of a hedonic deficit. *Pharmacol. Biochem. Behav*. **16**, 965-968.

Kendler, K. S., Karkowski, L. M., Prescott, C. A. (1999) Causal relationship between stressful life events and the onset of major depression. *Am. J. Psychiatry* **156**, 837-841.

Kopp, C., Vogel, E., Rettori, M. C., Delagrange, P., Misslin, R. (1999) The effects of melatonin on the behavioural disturbances induced by chronic mild stress in C3H/He mice. *Behav. Pharmacol.* **10**, 73-83.

Legault, M., Wise, R. A. (1999) Injections of N-methyl-D-aspartate into the ventral hippocampus increase extracellular dopamine in ventral tegmental area and nucleus accumbens. *Synapse* **31**, 241-249.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. (1951) Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**, 265-275.

Mairesse, J., Silletti, V., Laloux, C., Zuena, A. R., Giovine, A., Consolazione, M., van Camp, G., Malagodi, M., Gaetani, S., Cianci, S., Catalani, A., Mennuni, G., Mazzetta, A., van Reeth, O., Gabriel, C., Mocaër, E., Nicoletti, F., Morley-Fletcher, S., Maccari, S. (2013) Chronic agomelatine treatment corrects the abnormalities in the circadian rhythm of motor activity and sleep/wake cycle induced by prenatal restraint stress in adult rats. *Int. J. Neuropsychopharmacol.* **16**, 323-338.

Matell, M. S., Berridge, K. C., Wayne Aldridge, J. (2006) Dopamine D1 activation shortens the duration of phases in stereotyped grooming sequences. *Behav. Processes* **71**, 241-249.

McMillan, C. R., Sharma, R., Ottenhof, T., Niles, L. P. (2007) Modulation of tyrosine hydroxylase expression by melatonin in human SH-SY5Y neuroblastoma cells. *Neurosci. Lett.* **419**, 202-206.

McNaughton, N., Gray, J. A. (2000) Anxiolytic action on the behavioural inhibition system implies multiple types of arousal contribute to anxiety. *J. Affect. Disord.* **61**, 161-176.

Monnet, F.P. (2002) Melatonin modulates [^{3}H]serotonin release in the rat hippocampus: effects of circadian rhythm. *J. Neuroendocrinol.* **14**, 194-199.

Musshoff, U., Riewenherm, D., Berger, E., Fauteck, J. D., Speckmann, E. J. (2002) Melatonin receptors in rat hippocampus: molecular and functional investigations. *Hippocampus* **12**, 165-173.

Nagatsu, T., Levitt, M., Udenfriend, S. (1964) Tyrosine hydroxylase. The initial step in norepinephrine biosynthesis. *J. Biol. Chem.* **239**, 2910-2917.

Ouhaz, Z., Ba-M'hamed, S., Bennis, M. (2013) Haloperidol treatment at pre-exposure reduces the disturbance of latent inhibition in rats with neonatal ventral hippocampus lesions. *C. R. Biol.* **337**, 561-570.

Papp, M., Gruca, P., Boyer, P. A., Mocaër, E. (2003) Effect of agomelatine in the chronic mild stress model of depression in the rat. *Neuropsychopharmacology* **28**, 694-703.

Peuler, J. D, Johnson, G. A. (1977) Simultaneous single isotope radioenzymatic assay of plasma norepinephrine, epinephrine and dopamine. *Life Sci.* **21**, 625-636.

Raghavendra, V., Kaur, G., Kulkarni, S. K. (2000) Anti-depressant action of melatonin in chronic forced swimming-induced behavioural despair in mice, role of peripheral benzodiazepine receptor modulation. *Eur. Neuropsychopharmacol.* **10**, 473-481.

Rasheed, N., Ahmad, A., Pandey, C. P., Chaturvedi, R. K., Lohani, M., Palit, G. (2010) Differential response of central dopaminergic system in acute and chronic unpredictable stress models in rats. *Neurochem. Res.* **35**, 22-32.

Rosa, P. B., Ribeiro, C. M., Bettio, L. E., Colla, A., Lieberknecht, V., Moretti, M., Rodrigues, A. L. (2014) Folic acid prevents depressive-like behaviour induced by chronic corticosterone treatment in mice. *Pharmacol. Biochem. Behav.* **127**, 1-6.

Rouillon, C., Abraini, J. H., David, H. N. (2007) Hippocampal modulation of locomotor activity induced by focal activation of postsynaptic dopamine receptors in the core of the nucleus accumbens. *Hippocampus* **17**, 1028-1036.

Spasojevic, N., Stanisavljevic, D., Gavrilovic, L., Jovanovic, P., Cucakovic, A., Dronjak, S. (2012) Hippocampal asymmetry in expression of catecholamine synthesising enzyme and transporters in socially isolated rats. *Neuro Endocrinol. Lett.* **33**, 631-635.

Strekalova, T., Couch, Y., Kholod, N., Boyks, M., Malm, D., Leprince, P., Steinbusch, H. M. (2011) Update in the methodology of the chronic stress paradigm: internal control matters. *Behav. Brain Funct.* **7**, 9.

Su, G. Y., Yang, J. Y., Wang, F., Ma, J., Zhang, K., Dong, Y. X., Song, S. J., Lu, X. M., Wu, C. F. (2014) Antidepressant-like effects of Xiaochaihutang in a rat model of chronic unpredictable mild stress. *J. Ethnopharmacol.* **152**, 217-226.

Wang, Z., Zhang, D., Hui, S., Zhang, Y., Hu, S. (2013) Effect of tribulus terrestris saponins on behaviour and neuroendocrine in chronic mild stress depression rats. *J. Tradit. Chin. Med.* **33**, 228-32.

Yalcin, I., Belzung, C., Surget, A. (2008) Mouse strain differences in the unpredictable chronic mild stress: a four-antidepressant survey. *Behav. Brain Res*. **193**, 140-143.

Zisapel, N., Egozi, Y., Laudon, M. (1982) Inhibition of dopamine release by melatonin: regional distribution in the rat brain. *Brain Res.* **246**, 161-163.