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Original Article

Neuroprotective effects of melatonin administration against chronic immobilization stress in rats

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Abstract: Chronic stress can impair brain functions and play a well-known role in the development of stress-related disorders such as anxiety. Melatonin (Mel) is a neurohormone which regulate several physiological processes including mood and behavior. This experimental study was designed to evaluate the effect of Mel on chronic immobilization stress (CIS) for 6 weeks in rats and to elucidate its possible underlying mechanisms. Twenty-eight adult male Wistar albino rats were divided into four equal groups: the control group, the Mel-treated group which was injected daily with Mel (10 mg/kg/day; IP) for 6 weeks, the stressed group which was subjected to CIS protocol daily for 6 weeks, and the Mel-treated stressed group which was injected with Mel and concurrently exposed to CIS protocol for 6 weeks. The Mel-treated stressed group showed reduction of both relative adrenal weight and the serum corticosterone levels, suppression of the anxiety-like behavior, increased levels of serotonin, noradrenaline and oxytocin in the frontal cortex, and improved histopathological structure and decreased chromogranin A (CgA) protein expression in the frontal cortex when compared with the chronically stressed group. We concluded that Mel is anxiolytic and this effect was mediated in part by its ability to increase the central release of oxytocin and monoamines and to downregulate CgA protein expression in the frontal cortex.

Keywords: Chronic immobilization stress, noradrenaline, melatonin, oxytocin, serotonin

Introduction

Stress is an unavoidable phenomenon in this modern world and is associated with physiological and psychological alterations [1]. Prolonged or exaggerated responses to chronic stress can impair brain functions and play a well-known role in the development of stress-related disorders such as anxiety [2]. Chronic immobilization stress (CIS) model was widely accepted for studying stress-induced alterations [3]. The well-documented center responsible for the neuroendocrine response to stress is the hypothalamic-pituitary-adrenal (HPA) axis [4].

Multiple areas of the frontal cortex have important roles in modulating the response to stress. The neuronal activities of these areas are modulated by various neurotransmitter systems including the serotonergic and noradrenergic systems [5]. In addition to its known endocrine functions, oxytocin can modulate stress response [6]. Axons of hypothalamic oxytocin-containing neurons reach different brain regions including the frontal cortex and amygdala, which has a high-density of oxytocin receptors [7, 8]. The anxiolytic effects of oxytocin are areas of growing interest. However, the underlying intraneuronal mechanisms of these effects are largely unknown [9].

Chromogranin A (CgA) is a stress-related glycoprotein stored and secreted from many neuroendocrine cells, neurons, and adrenal medulla [10]. Its expression in neuroendocrine cells may provide further information about the stress response [11].

Melatonin (Mel) is synthesized and secreted by the pineal gland in a circadian manner being low during the daytime and high during the night. It is essential for the regulation of different physiological functions of the neuroendocrine system via binding to its receptors in different brain areas [12]. During stress, the plasma level of Mel is altered [13]. Moreover, stress can attenuate the nocturnal secretion of Mel [14] as the pineal gland is a target of gluco-

corticoid damage during stress due to its high density expression of glucocorticoid receptors [15]. Little is known about the relationship among Mel administration, the central release of oxytocin, and the expression of CgA in the frontal cortex of rats exposed to chronic stress.

In light of these considerations, the aim of this experimental study was to evaluate the effect of chronic Mel administration on CIS for 6 weeks in rats and to elucidate its possible underlying mechanisms. Anxiety-like behavior, the serum levels of corticosterone, the frontal cortex tissue homogenate levels of monoamines and oxytocin were estimated, and the histopathological structure and CgA expression in the frontal cortex were examined.

Materials and methods

Animals

A total of 28 adult male Wistar albino rats weighing about 190-250 g were used in this study. The rats were housed in groups of four per cage in the animal house of Faculty of Medicine, Assiut University, Assiut, Egypt. They were maintained at room temperature on a natural 12:12-h light-dark cycle with water and food (standard rat pellets) available *ad libitum*. One week before the start of the experiment, animals were acclimatized to the laboratory conditions. The experimental procedures followed the internationally accepted principles for Guide for the Care and Use of Laboratory Animals and were approved by the Ethics Committee at Faculty of Medicine, Assiut University, Assiut, Egypt (Approval Number: IRB00008718).

Drugs

Melatonin (N-acetyl-5-methoxytryptamine) was purchased from Sigma-Aldrich (St. Louis, Mo., USA). It was dissolved just before use in a vehicle composed of 1% ethanol and 99% distilled water [16] and injected intraperitoneally (IP) in a volume of 1 mL for each rat at a dose of 10 mg/kg/day [17] at 5:00 PM.

Chronic immobilization stress (CIS) protocol

Animals were subjected to CIS protocol at 9:00 AM daily for 90 min as previously described [18]. Briefly, rats were placed with their trunks wrapped on a wooden plate which prevented the movement of their trunks but allowed free movement of its limbs and head. Chronic immobilization stress was considered an easy and convenient method for physiological and psychological stress in rodents [1].

Experimental design and collection of samples

Rats were randomly distributed into four equal groups (n = 7). The control group: rats were not exposed to stress, the Mel-treated group: rats were daily injected with Mel for 6 weeks, the stressed group: rats were subjected to CIS protocol daily for 6 weeks, and Mel-treated stressed group: rats were daily injected with Mel and concurrently subjected to the same stress protocol for 6 weeks. Behavioral tests were carried out after the 6 weeks' period of stress, in the following order: the open field test (OFT) and elevated plus maze test (EPM). Then, body weight (BW) was recorded and fasting blood samples were collected from the retroorbital venous plexus by an experienced laboratory technician. Blood was centrifuged at 3000 g for 15 min and the clear serum was removed and kept at -20°C until use. Animals were sacrificed by cervical dislocation and adrenal glands were quickly removed, weighed and adrenal to BW ratio (relative adrenal weight) was calculated. Brains were quickly removed, immediately washed with ice-cold saline and separated into two hemispheres; one frontal cortex was immediately frozen in liquid nitrogen and stored at -80°C for further analysis and the other was used for the histopathological and immunohistochemical examination.

Behavioral tests

The behavior of the rats was recorded at 9:00 AM by two investigators unaware of the group to which each animal belonged to.

Open field test (OFT): The OFT is one of the most commonly used tests of rodent behavior and is usually used to evaluate the anxiety-like behavior of animals by assessing their movement in an open field [19]. The apparatus is a wooden, open-topped box (100 cm × 100 cm) which is enclosed by 40 cm high walls. Its floor is divided into 25 squares (20 cm × 20 cm), distinguished as 9 central and 16 peripheral squares. The animal was placed in the center of the apparatus and then, time spent in the central area (TCA) and numbers of returns to the

center (NRC) were recorded in 10 min. The central area of a novel environment is considered anxiogenic and aversive. An indicator of the emotional reactivity is the NRC [20]. The number of rearing (standing on hind limbs), grooming (face washing, body, and paw licking) and defecation (by counting the number of fecal pellets) were also counted. Rearing and grooming indicate the anxiety state of the animals [21]. Between each examination, 70% ethyl alcohol was used for cleaning the apparatus.

Elevated plus maze test (EPM): The instrument is composed of two enclosed arms (10 cm × 45 cm × 50 cm) and two open arms (10 cm × 45 cm) extending from a central platform (10 cm × 10 cm) and located 70 cm above the floor. The animal was placed in the center of the maze, facing an open arm and allowed to explore the maze for 5 min then the behavior of the animals was recorded for 10 min. After testing each animal, 70% ethyl alcohol was used for cleaning the arms. The recorded parameters were the number of entries into the open arms and total time spent in the open arms [22]. An arm entry was defined by the entrance of all four legs of the animal into one of the arms [23].

Biochemical measurements

Estimation of serum corticosterone levels: To detect the alterations in the HPA axis, the serum corticosterone levels were measured by corticosterone enzyme-linked immunosorbent assay (ELISA) kit (Assaypro LLC, St. Charles, MO, USA) according to the manufacturer's instructions.

Estimation of serotonin and noradrenaline (NA) levels in the frontal cortex: Serotonin and NE levels were measured by high-performance liquid chromatography (HPLC) with diode array detector (Agilent Technologies 1200 series) as described previously [23]. Briefly, frontal cortical tissues were weighed and then homogenized in 75% aqueous HPLC methanol 1:10 w/v. Thereafter, homogenates were centrifuged at 4000 g for 10 min. Samples were then injected directly into the Zorbax Extend C18 column (150 mm × 4.6 mm, 5 µ). The mobile phase was 97:3 phosphate buffer (20 mM, pH 3)/acetonitrile and the flow rate was 1.5 mL/min. UV detection was carried out at 270 nm. Serotonin and NA were separated after 12 min. The sample concentration of each monoamine was identified by comparing the chromatogram of each sample to that of the standard curve made by Eurochrom HPLC Software, version 1.6.

Estimation of oxytocin levels in the frontal cortex: Equal weights of the frontal cortex were obtained from all animals and homogenized in phosphate buffer saline (1:10 w/v), and then the homogenates were centrifuged at 5000 g for 15 min at 4°C. Oxytocin levels were estimated by ELISA kit for oxytocin (Enzo Life Sciences, NY, USA).

Histopathology and immunohistochemistry

The frontal cortex was fixed in 10% neutral buffered formalin, dehydrated and embedded in paraffin. 5 µm thick paraffin sections were prepared and stained with Hematoxylin & Eosin stain (H&E) for general histological examination according to Bancroft and Gamble [24].

For immunohistochemical detection of chromogranin A (CgA) in the frontal cortex, avidin-biotin immunoperoxidase technique was performed. Briefly, 5 um paraffin sections were cut and mounted on coated slides, then treated with 0.01 M citrate buffer (pH 6.0) for 10 min to unmask antigen. Sections were incubated with 0.3% hydrogen peroxide for 30 min to abolish endogenous peroxidase activity. Slides were incubated with the polyclonal CgA antibody (Thermo Fisher Scientific Fremont, CA, USA) at a dilution of 1:100 at 4°C for 18-20 hours. Then, slides were washed and incubated with the secondary antibodies followed by the avidin-biotin complex. Finally, sections were demonstrated using 0.05% diaminobenzidine chromogen and counterstained with Mayer's hematoxylin. CgA-positive cells appeared with brown granules [25]. Negative control was done by incubating the slides with the omission of the primary antibody. Slides were examined using an Olympus optical microscope (Olympus, Center Valley, PA, USA) equipped with an Olympus U-CMAD3 digital camera interfaced to a computer. Quantification of CgA protein expression was performed by computer-assisted Image J software (NIH, Bethesda, MD, USA).

Statistical analysis

All analyses were done with SPSS version 16 (SPSS Inc., Chicago, Ill., USA). Results were expressed as mean ± standard deviation (SD). The experimental groups were compared using

Table 1. Behavioral performance of rats in response to CIS and Mel treatment in the open field test

Groups	TCA (seconds)	NRC	Grooming	Rearing	Defecation
Control group	10.75 ± 5.23	6.33 ± 2.34	4.00 ± 2.28	4.00 ± 2.37	3.50 ± 1.76
Melatonin-treated group	10.83 ± 3.92	6.67 ± 2.80	4.00 ± 2.00	4.00 ± 2.53	3.3 ± 1.37
Stressed group	4.00 ± 2.26	1.67 ± 0.82	7.67 ± 2.42	7.17 ± 2.40	5.83 ± 1.17
	*P = 0.019	*P = 0.003	*P = 0.029	*P = 0.034	*P = 0.032
	1P = 0.012	1P = 0.004	1P = 0.016	1P = 0.05	1P = 0.014
Melatonin-treated stressed group	16.00 ± 5.69	6.67 ± 5.47	4.00 ± 1.26	3.83 ± 1.47	2.00 ± 1.26
	2P = 0.004	2P = 0.033	2P = 0.009	2P = 0.028	2P = 0.005

Data were expressed as mean ± SD. *P: significant vs. the control group. 1P: significant vs. the melatonin-treated group; 2P: significant vs. the stressed group.

Table 2. Behavioral performance of rats in response to CIS and Mel treatment in elevated plus maze test

Groups	Number of entries in the open arms	Time spent in open arm (seconds)
Control group	4.50 ± 1.87	149.67 ± 48.57
Melatonin-treated group	5.50 ± 1.87	114.67 ± 27.94
Stressed group	1.83 ± 0.75	63.83 ± 17.39
	*P = 0.014	*P = 0.004
	1P = 0.004	1P = 0.004
Melatonin-treated stressed group	3.83 ± 1.47	95.67 ± 31.96
	2P = 0.017	2P = 0.045

Data were expressed as mean ± SD. *P: significant vs. the control group. 1P: significant vs. the melatonin-treated group; 2P: significant vs. the stressed group.

Table 3. Relative adrenal weight and the serum corticosterone levels in the different studied groups

Groups	Relative adrenal weight (%)	Serum corticosterone levels (ng/mL)
Control group	0.01 ± 0.006	2.22 ± 0.231
Melatonin-treated group	0.012 ± 0.009	2.23 ± 0.196
Stressed group	0.06 ± 0.012	9.02 ± 0.699
	*P = 0.004;	*P = 0.004;
	1P = 0.004	1P = 0.004
Melatonin-treated stressed group	0.015 ± 0.008	2.52 ± 0.248
	2P = 0.004	2P = 0.004

Data were expressed as mean ± SD. Relative adrenal weight was calculated by dividing adrenal weight by total body weight × 100. *P: significant vs. the control group. 1P: significant vs. the melatonin-treated group; 2P: significant vs. the stressed group.

the Mann-Whitney U test. A value of $P \leq 0.05$ was considered statistically significant.

Results

Effects of CIS and Mel on the behavior of animals

Recording of the behavior of animals in the OFT revealed insignificant differences between the control and the Mel-treated groups in TCA (10.75 ± 5.23 vs. 10.83 ± 3.92 seconds), NRC (6.33 ± 2.34 vs. 6.67 ± 2.80), and the number of grooming (4.00 ± 2.28 vs. 4.00 ± 2.00), rearing (4.00 ± 2.37 vs. 4.00 ± 2.53) and defecation (3.50 ± 1.76 vs. 3.3 ± 1.37). In OFT, the stressed rats spent more time in the corners and avoided the stressful opened central area, this was evidenced by significant decrease in the TCA (4.00 ± 2.26 seconds) and NRC (1.67 ± 0.82) and they showed also significant increase in the number of grooming (7.67 ± 2.42) and rearing (7.17 ± 2.40) as well as increased autonomic manifestations, namely the number of defecation (5.83 ± 1.17) in comparison to the control group (P = 0.019, P = 0.003, P = 0.029, P = 0.034, and P = 0.032; respectively). Similar findings were found when comparing the stressed group with the Mel-treated group (P = 0.012, P = 0.004, P = 0.016, P = 0.05, and P = 0.014; respectively). In contrast with these findings, administration of Mel along with the application of CIS resulted in significant improvement of the tested parameters evidenced by significant increase in TCA (16.00 ± 5.69 seconds) and NRC (6.67 ± 5.47) and significant decrease in the number of grooming (4.00 ± 1.26), rearing (3.83 ± 1.47) and defecation (2.00 ± 1.26) in comparison to the stressed

group (P = 0.004, P = 0.033, P = 0.009, P = 0.028, and P = 0.005; respectively). Furthermore, the Mel-treated stressed group showed insignificant changes in TCA, NRC, and the number of grooming, rearing and defecation when compared with both the control group (16.00 ± 5.69 vs. 10.75 ± 5.23 seconds, 6.67 ± 5.47 vs. 6.33 ± 2.34, 4.00 ± 1.26 vs. 4.00 ± 2.28, 3.83 ± 1.47 vs. 4.00 ± 2.37, and 2.00 ± 1.26 vs. 3.50 ± 1.76; respectively) and the Mel-treated group (16.00 ± 5.69 vs. 10.83 ± 3.92 seconds, 6.67 ± 5.47 vs. 6.67 ± 2.80, 4.00 ± 1.26 vs. 4.00 ± 2.00, 3.83 ± 1.47 vs. 4.00 ± 2.53, and 2.00 ± 1.26 vs. 3.3 ± 1.37; respectively) (**Table 1**).

Elevated plus maze test showed insignificant differences between the control and the Mel-treated groups in the number of entries (4.50 ± 1.87 vs. 5.50 ± 1.87) and total time spent in open arms (149.67 ± 48.57 vs 114.67 ± 27.94 seconds). Significant decreases in the number of entries (1.83 ± 0.75) and total time spent in open arms (63.83 ± 17.39 seconds) were observed in the stressed rats suggesting an elevated anxiety-like behavior when compared to the control group (P = 0.014 and P = 0.004; respectively) and the Mel-treated group (P = 0.004 and P = 0.004). Co-administration of Mel with CIS application resulted in significant increases in the number of entries to 3.83 ± 1.47 and total time spent in open arms to 95.67 ± 31.96 seconds in comparison to the stressed group (P = 0.017 and P = 0.045; respectively). Furthermore, the number of entries and total time spent in open arms recorded in Mel-treated stressed group were insignificant vs. the control group (3.83 ± 1.47 vs. 4.50 ± 1.87, 95.67 ± 31.96 vs. 149.67 ± 48.57 seconds; respectively) and the Mel-treated group (3.83 ± 1.47 vs. 5.50 ± 1.87, 95.67 ± 31.96 vs. 114.67 ± 27.94 seconds; respectively) (**Table 2**).

Effects of CIS and Mel on relative adrenal weight

Relative adrenal weight revealed insignificant differences between the control and Mel-treated groups (0.01 ± 0.006 vs. 0.012 ± 0.009%). The stressed group showed a significantly increased relative adrenal weight (0.06 ± 0.012%) when compared to the control and Mel-treated groups (P = 0.004 and P = 0.004; respectively). On the other hand, co-administration of Mel to animals exposed to stress reduced relative adrenal weight (0.015 ± 0.008%) significantly in comparison to the stressed group (P = 0.004). The Mel-treated stressed group showed also no significant differences against both the control and Mel-treated groups (0.015 ± 0.008 vs. 0.01 ± 0.006 and 0.012 ± 0.009; respectively) (**Table 3**).

Effects of CIS and Mel on the biochemical measurements

*Serum corticosterone level*s: Insignificant differences in the serum corticosterone levels between the control and Mel-treated groups (2.22 ± 0.231 vs. 2.23 ± 0.196 ng/mL) were noticed. Because of CIS, the serum corticosterone levels were significantly elevated to a value of 9.02 ± 0.699 ng/mL in the stressed group in comparison with both the control and Mel-treated groups (P = 0.004 and P = 0.004; respectively). Concurrent administration of Mel with CIS reduced the serum corticosterone levels (2.52 ± 0.248 ng/mL) significantly when compared with the stressed group (P = 0.004). Furthermore, the Mel-treated stressed group showed insignificant differences in comparison with both the control and Mel-treated groups (2.52 ± 0.248 vs. 2.22 ± 0.231 and 2.23 ± 0.196 ng/mL; respectively) (**Table 3**).

Serotonin, NA and oxytocin levels in the frontal cortex: No significant differences were obtained when comparing the levels of serotonin, NA and oxytocin in the frontal cortex of animals in the control group with the Mel-treated group (30.07 ± 1.03 vs. 31.80 ± 2.18 mg/L, 0.31 ± 0.04 vs. 0.33 ± 0.05 mg/L, and 296.0 ± 12.96 vs 309.17 ± 11.58 pg/mL; respectively). After exposure to CIS, the stressed group showed a significant decrease in the levels of serotonin (23.44 ± 4.74 mg/L) and NA (0.15 ± 0.08 mg/L) in the frontal cortex homogenate when compared to both the control (P = 0.016 and P = 0.013) and Mel-treated (P = 0.010 and P = 0.010) groups. On the other hand, oxytocin level in the stressed group increased slightly to 341.17 ± 56.83 pg/mL but did not reach a significant level when compared to the control and Mel-treated groups. Administration of Mel in association with CIS resulted in normalization of the levels of serotonin (31.87 ± 1.23 mg/L) and NA (0.29 ± 0.04 mg/L) in the frontal cortex homogenate as evidenced by insignificant changes when compared with both the control and the Mel-treated groups. In comparison with

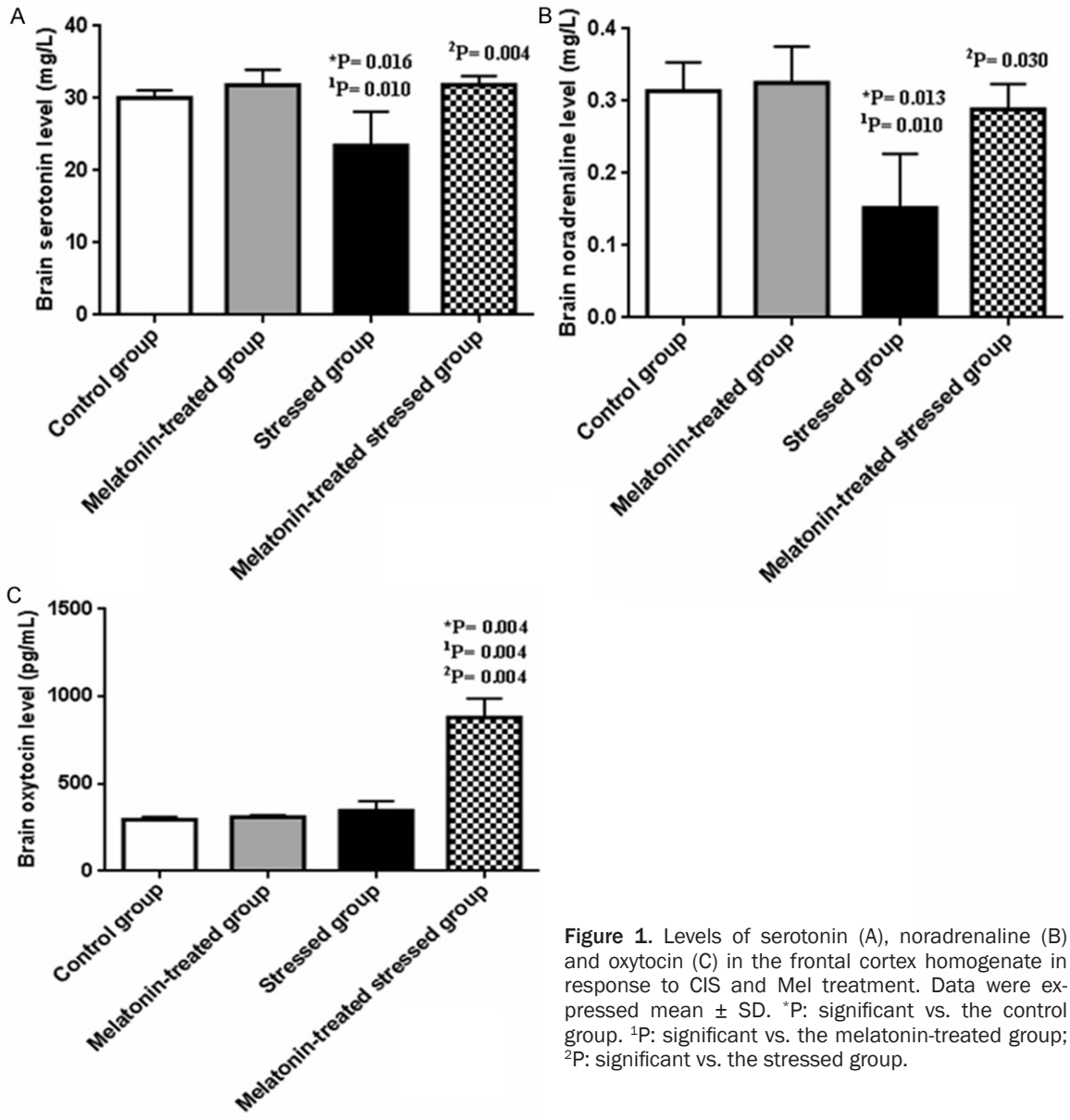

Figure 1. Levels of serotonin (A), noradrenaline (B) and oxytocin (C) in the frontal cortex homogenate in response to CIS and Mel treatment. Data were expressed mean ± SD. *P: significant vs. the control group. 1P: significant vs. the melatonin-treated group; 2P: significant vs. the stressed group.

the stressed group, the levels of serotonin and NA were significantly increased (P = 0.004 and P = 0.030; respectively). Regarding the level of oxytocin in the frontal cortex tissue homogenate of rats in the Mel-treated stressed group, a significant increase was obtained (880.00 ± 107.52 pg/mL) versus the control, Mel-treated and stressed groups (P = 0.004, P = 0.004, and P = 0.004; respectively) (**Figure 1**).

Effects of CIS and Mel on the frontal cortex histopathology and immunohistochemistry

Sections of the frontal cortex from rats in the control and Mel-treated groups stained by H&E show the normal histological appearance of the layers of the cortex. Neuronal cells exhibited large vesicular nuclei with basophilic cytoplasm. The stressed group revealed altered grey matter with disorganization of the layers of the cortex. Some cells appeared with pyknotic nuclei. Co-supplementation of Mel with CIS application resulted in an improvement as the cortical layers appeared normal. However, few cells with pyknotic nuclei were still present (**Figure 2**).

The immunohistochemical assay showed that the control and Mel-treated groups revealed insignificant differences in CgA-cellular protein

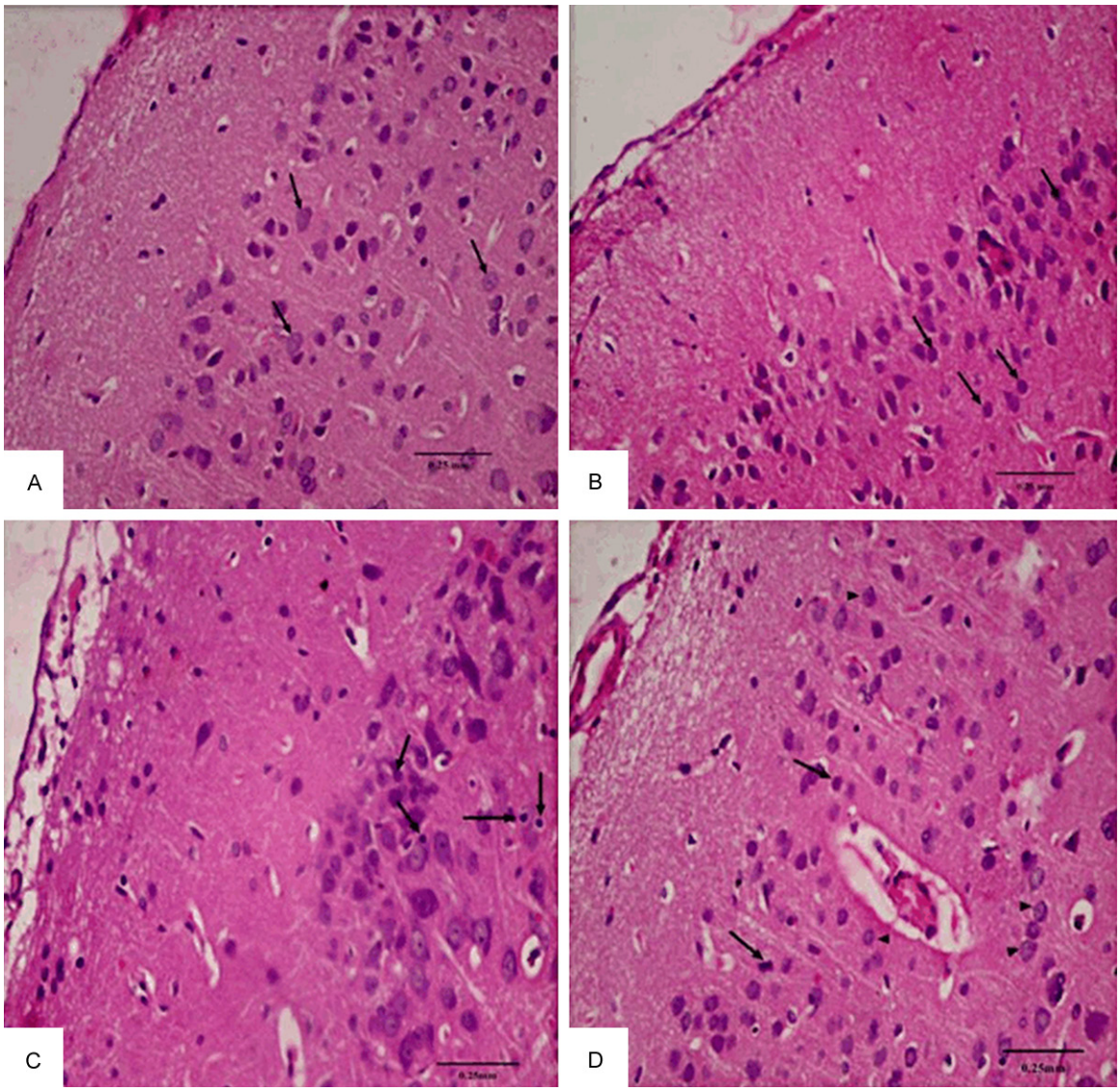

Figure 2. Haematoxylin and eosin staining of the frontal cortex in response to CIS and Mel treatment: A: The control group shows the normal grey matter containing normal neurons with vesicular nuclei and basophilic cytoplasm (arrow); B: The Mel-treated group shows also the normal morphology of grey matter neurons (arrow) with vesicular nuclei; C: The stressed group shows altered gray matter with disorganization of layers of the cortex. Some cells appeared with pyknotic nuclei (arrow); and D: The Mel-treated stressed group shows nearly normal appearance of the cortical neurons. Few cells appeared with pyknotic nuclei were still present (arrow). Magnification: × 200; scale bars: 0.25 mm.

expression (21.17 ± 2.64 vs 22.17 ± 3.06) in grey matter neurons of the frontal cortex. The stressed group revealed a significantly upregulated CgA-cellular expression (41.0 ± 4.60) in most grey matter neurons when compared with both the control and Mel-treated groups (P = 0.004 and P = 0.004; respectively). The Mel-treated stressed group showed a significant downregulation of CgA-cellular expression (30.67 ± 4.32) in comparison with the stressed group (P = 0.008). However, CgA-cellular expression in this group was still significantly higher than both the control and Mel-treated groups (30.67 ± 4.32 vs 21.17 ± 2.64 and 22.17 ± 3.06; P = 0.005 and P = 0.008; respectively) (**Figure 3**).

Discussion

The present findings demonstrated that rats exposed to CIS for 6 weeks showed increased relative adrenal weight, serum corticosterone

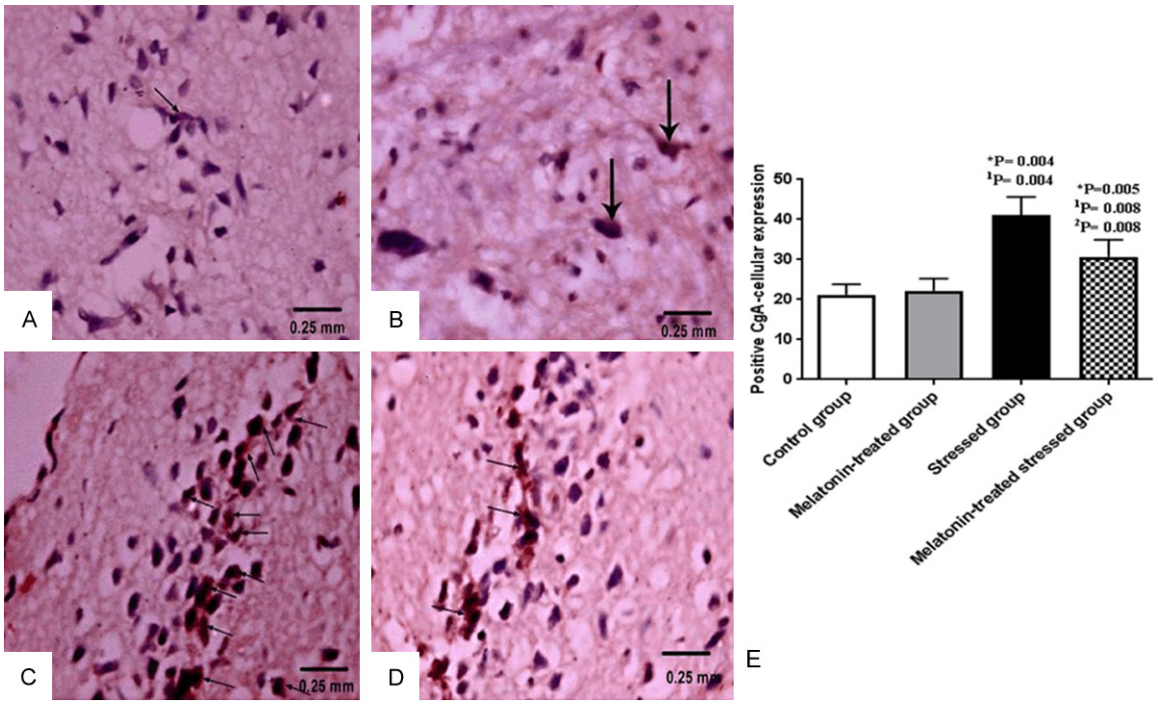

Figure 3. Immunohistochemical staining of the frontal cortex CgA-cellular expression in response to CIS and Mel treatment: A: The control group shows a slight positive CgA-cellular expression (arrow); B: The Mel-treated group shows a slight positive CgA-cellular expression (arrow); C: The stressed group shows an increased positive CgA-cellular expression (arrow) in most of the grey matter neurons; and D: The Mel-treated stressed group shows a decreased positive CgA-cellular expression (arrow) in the grey matter neurons in comparison with the stressed group (arrow). Magnification: × 200; scale bars: 0.25 mm. E: Quantitative analysis of CgA-cellular expression. Data were expressed mean ± SD. *P: significant vs. the control group. 1P: significant vs. the melatonin-treated group; 2P: significant vs. the stressed group.

levels, and anxiety-like behavior. Along with this, the stressed rats exhibited decreased levels of serotonin, noradrenaline, and oxytocin in the frontal cortex homogenates, altered histopathological structure, and increased CgA protein expression in the frontal cortex. Our study revealed also that chronic melatonin administration significantly mitigated these behavioral, biochemical and histopathological alterations elicited by exposure to CIS.

The increased relative adrenal weight and serum corticosterone levels in the stressed rats in our study were indicative of a successive stress protocol induction because both of them are consequent to the stress-induced activation of HPA axis [26]. These findings might explain the obviously elevated anxiety-like behavior in the stressed rats recorded by the OFT and EPM in our study. In the OFT, decreased TCA and NRC revealed that the pathways were concentrated in the corner areas, suggesting a reduction in the locomotor activity of rats induced by stress. The decreased locomotor activity and the increased number of grooming, rearing and defecations were believed to be objective manifestations of the development of a state of emotional stress in rats which promoted the emotional release of the rats [27, 28]. Furthermore, CIS reduced the number of entries and total time spent in open arms in EPM. Similar results were obtained in previous studies using different models of stress [29, 30].

The present study revealed that CIS resulted in significantly decreased levels of both serotonin and NA in the frontal cortex when compared to both the control and Mel-treated groups. This was in agreement with other studies [29, 31]. On the contrary, Ahmed et al. [3] found an increased brain level of serotonin in the stressed rats. Reasons for this discrepancy could be explained by an initial increased level of serotonin to counteract stress but with chronic exposure to stress, neuronal damage

with consequent neurotransmitter depletion occurred because of increased free radicals and blood pressure [32]. Chronic stress was reported to be associated also with atrophy of NA axonal projections [33].

Both central and peripheral levels of oxytocin increased in response to different stressful stimuli [34]. Its release during stressful situations served to dampen stress [35]. Our study revealed that the stressed group had an increased level of oxytocin, however; this increase was insignificant when compared with both control and Mel-treated groups. In agreement, Zheng, et al. [36] reported increased oxytocin mRNA expression and the number of oxytocin-immunoreactive cells in the paraventricular nucleus (PVN) of the hypothalamus following chronic stress.

Histopathological examination of the frontal cortex of rats in the stressed group in the present study showed the presence of structural alterations. Stress-induced structural remodeling with atrophy of neurons in the frontal cortex was previously described [37]. This atrophy might be attributed to the persistent elevation of the circulating corticosteroids [38]. Additionally, a significantly upregulated positive CgA-cellular protein expression in most grey matter neurons in comparison with the control and Mel-treated groups was observed. Chromogranins are considered valuable indicators of sympathoadrenal activity due to their unique distribution in neuroendocrine and nervous system tissues [10]. Furthermore, CgA can indirectly cause neuronal damage by inducing microglial cells to secrete tumor necrosis factor-α [39] which might also explain the observed changes in the frontal cortex. Previous studies reported an increased CgA level with environmental stress [11, 40].

The blood-brain barrier permeability of Mel might suggest a possible role in neuroprotection [21]. Thus, we used Mel to evaluate its neuroprotective effect against CIS. Our dose was chosen based on previous experimental studies [41, 42] in which no toxicity was reported. In the present study, Mel treatment in association with the application of CIS protocol significantly reduced relative adrenal weight and the serum corticosterone levels and improved behavioral alterations induced by CIS. In agreement, a decreased glucocorticoid level with Mel treatment was obtained by Torres-Farfan et al. [43] through inhibition of adrenocorticotropin. The reduction of corticosterone levels could explain the noticed improvements in the anxiety like-behaviors of animals. The improvement of the anxiety-like behavior with Mel was in line with other studies [23, 41, 44]. It could be also mediated by the ability to Mel to alter the neurotransmitter systems [20]. This was evidenced in our study by the observed increased levels of serotonin and NA in the frontal cortex. Increased serotonin with Mel administration was noticed in a previous study using diazinon-induced anxiety animal model [23] and might be caused by changes in the serotonin synthesis and release by Mel [45].

The marked increase in oxytocin level in the frontal cortex homogenate of rats treated with Mel and concurrently exposed to CIS vs. other groups was an interesting finding in our study. Oxytocin can attenuate stress-induced HPA activity and anxiety behavior through its inhibitory effect on the gene encoding CRH mRNA expression [46]. This might explain the decreased adrenal weight, the reduced levels of corticosterone, and hence, the improved anxiety-like behavior observed in the Mel-treated stressed group in our study. Moreover, oxytocin can suppress the anxiety-like behavior of rats via attenuation of glutamatergic transmission in the medial prefrontal cortex and increment of the release of GABA with decreased excitation of the glutamatergic projections from the medial prefrontal cortex to the central nucleus of the amygdala [47]. Also, binding of oxytocin to its receptors in the amygdala inhibited activity of the neural pathways projecting to hypothalamic and brainstem areas involved in stress [35].

The increased level of oxytocin in the Mel-treated stressed group in our study could be produced by the associated increased levels of serotonin and NA because the activated noradrenergic and serotonergic neurons can stimulate oxytocin release from the PVN via α1-adrenergic and serotonergic receptors [8]. Intranasal oxytocin administration was tested as a treatment for anxiety disorders, but previous studies estimated robust treatment responders and non-responders [48]. Treatment that could increase oxytocin release, like Mel in our study, appeared to be a promising pharmacological preventive therapy for patients suffering from chronic stress.

In addition, our study showed that Mel supplementation with CIS application improved the histopathological alterations and reduced the cellular expression of CgA in the rat frontal cortex. Chromogranin A is commonly used as a diagnostic and prognostic marker to monitor the response to pharmacotherapeutic intervention in different diseases such as neuropsychiatric diseases [39]. Hence, its decreased expression indicated the attenuation of the effect of CIS by Mel administration on the frontal cortex. In agreement, improved histopathological alterations in the rat brain by melatonin were previously reported [19] and these effects may be related to its anti-apoptotic and antioxidant activities [42].

In conclusion, the present study concluded that Mel has a stress-attenuating effect in rats subjected to CIS. This effect was mediated in part by its ability to increase the central release of oxytocin and monoamines and to downregulate CgA protein expression in the frontal cortex. However, further experimentation is needed to better understand Mel function within the brain via identification of other molecular markers involved in chronic stress to provide benefits for its effective usage in therapeutic purposes.

Disclosure of conflict of interest

None.

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