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Melatonin treatment affects changes in adrenal gene expression of catecholamine biosynthesizing enzymes and norepinephrine transporter in the rat model of chronic stress-induced depression

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Abstract

This study investigated the effects of melatonin treatment on adrenal catecholamine content, synthesis, uptake and vesicular transport induced by the chronic unpredictable mild stress (CUMS) model of depression in rats. This entailed quantifying the norepinephrine, epinephrine, mRNA and protein levels of tyrosine hydroxylase (TH), dopamine-b-hydroxylase (DBH), phenylethanolamine N-methyltransferase (PNMT), norepinephrine transporter (NET) and vesicular monoamine transporter 2 (VMAT2) in the adrenal medulla. CUMS caused a significant depletion of norepinephrine stores and protein levels of TH, DBH and NET, whereas the gene expression of PNMT was increased. It was observed that melatonin treatment in the CUMS rats prevented the stress-induced decrease in norepinephrine content and the protein expression of TH, DBH and NET in the adrenal medulla of chronically stressed rats. The present study demonstrates the stimulatory effect of melatonin on adreno-medullary synthesis, the uptake and content of catecholamine in the rate model of chronic stress-induced depression.

Key words

depression, melatonin, adrenal-medulla, catecholamines, enzymes, transporters

Introduction

Melatonin is a neurohormone primarily synthesised by the pineal gland during darkness (Pandi-Perumal et al. 2006) with a well established role in regulating seasonal and circadian rhythms. It is essential for the regulation of different physiological functions of the neuroendocrine system. Stress is considered a determinant in the etiology of depression (Pal et al. 2016). The chronic unpredictable mild stress (CUMS) procedure is an animal model that mimics the role of chronic stress in precipitating depression. The sympathoadrenal system plays a key role in the maintenance of homeostasis. Catecholamines, released from chromaffin cells of the adrenal medulla, are implicated in the development of several stress-related disorders such cardiovascular disease and metabolic disorders, which are associated with depressive states (Bradley and Rumsfeld 2018). Preclinical studies have shown that melatonin exhibits antidepressant-like properties (Estrada-Reyes et al. 2018; Satyanarayanan et al. 2018). Studies on melatonin's effect on the adrenal gland, which represents an important endocrine organ controlling essential physiological functions, are still deficient. The present study was designed to further scrutinise the antidepressant-like effects of exogenous melatonin, by examining its effects on catecholamine content and the regulation of the gene expression of catecholamine biosynthesizing enzymes, TH, DBH, phenylethanolamine N-methyltransferase (PNMT) and transporters, norepinephrine transporter (NET) and vesicular monoamine transporter 2 (VMAT2) in the adrenal medulla of rats subjected to chronic unpredictable mild stress.

Materials and methods

The experiments were performed on adult (two-month-old) male Wistar rats. The weight of males at the beginning of the experiments was 250-310g. All animals were housed in a temperature-controlled room (20±2.0 ^{0}C) with 42±5% relative humidity and synchronized to a 12-hour light/dark regime. Rats were kept four per standard plastic cages (width 59.5 cm, length 38 cm, height 20 cm) and given ad libitum access to standard laboratory food (commercial rat pellets) and tap water. Animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals (8th edition, National Academies Press). All procedures with animals were approved by 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.

In this study, we used a total of 24 animals which were allocated to control (unstressed) and chronic unpredictable mildly stressed groups. These groups were further divided into two subgroups each and were receiving daily injection of vehicle (5% ethanol) or melatonin in dose of 10 mg kg -1 body weight by intraperitoneally (i.p.) route

one hour before the dark phase. This time was chosen to avoid disruption of circadian rhythms that occur if melatonin is administered throughout the day. Melatonin (Q-1300, Bachem, Switzerland) was dissolved in NaCl (0.9%) containing 5% ethanol. The dose of 10 mg kg -1 is a supraphysiological dose chosen with the aim of assessing the drug's therapeutic effect and not with replacing or restorative purposes. Exposure to chronic unpredictable mild stress and the vehicle, i.e. drug administration started on the same day and was continued for 4 weeks. To consider body weight evolution, injected volumes are adjusted twice a week on body growth of animals.

The CUMS procedure was carried out according to the methods described by Stefanovic et al. (2016), and it was designed to maximize the unpredictable nature of the stressors. The CUMS groups of animals were exposed to the following stressors in random order: forced running (15 min., 10 m/min); soiled cage (500 ml 22 ^{0}C water spilled into bedding); 45^0 cage tilt along the vertical axis; 17-h food deprivation, 5-h cold room (4 ^{0}C) and water deprivation (water bottles were removed from cage during this time), 5-h grouped housing (8 rats per cage) and individual housing (48-h). Control animals were left undisturbed in the home cage with the exception of general handling (i.e. regular cage cleaning and measuring body weight), which was matched to that of the CUMS group. Immediately after the CUMS procedure, all animals were sacrificed, between 10:00–11:00 a.m., by rapid decapitation with a guillotine (Harvard-Apparatus, USA), adrenal glands excised, adrenal medulla dissected quickly on ice, frozen in liquid nitrogen and stored at -70 ^{0}C until analyzed.

Ethylene glycol tetraacetic acid (EGTA) and DL-Norepinephrine hydrochloride (NA) were purchased from Sigma–Aldrich. Ammonium formate was supplied by Fisher Scientific (Loughborough, UK); formic acid (49–51%) by Fluka analytical (Switzerland); methanol by J.T. Baker (Deventer, The Netherlands), perchloric acid (70%) by Sigma–Aldrich and magnesium chloride ($MgCl_2 \times 5H_2O$) by Sigma–Aldrich. Purified water was obtained via a BlueClearRO600P reverse osmosis water cleaner system with integrated BlueSoft07-MB mixed bed salt remover (Euro-Clear Ltd., Hungary).

The stock standard solution of NA [1 mg/mL] was prepared in methanol and kept at −20 °C. Standard solutions in concentrations of 1, 2, 4, 8, 16, 32, 40 and 50 µg/mL were prepared by dilution of the stock standard solution in DEPROT (2% EGTA; 0,1N $HClO_4$; 0,2% $MgCl_2$). The ammonium formate buffer (100 mM, pH 3,6) was used as one of the mobile phase components. Tissue samples were homogenized in DEPROT (1 mg:10 µL) using an Ultra-turrax homogenizer (T10 basic), sonicated (Branson Sonic Power Company, Danbury, Connecticut; 3×10 s) and centrifuged (Sorvall SuperT21; 30 min, 18.000 rpm, +4 °C). Supernatants were transferred in separate tubes and

placed in the autosampler of the HPLC system. Data were obtained using a Thermo Scientific (DionexUltiMate 3000) HPLC system consisting of degasser unit, binary pump (HPG-3200SD), autosampler (WPS-3000 SplitLoop), column oven (Col. Comp. TCC-3000) and RS electrochemical detector (ECD-3000RS) equipped with the 6041RS ultra Amperometric Analytical Cell and glassy carbon working electrode. A Hibar 125-4 LiCrospher100 RP-18 (5 µm) HPLC column (Merck Millipore, Darmstadt, Germany) was used. Instrument control and data acquisition are carried out by the Chromeleon7 Chromatography Data System (Thermo Scientific). The following chromatographic conditions were obtained after the method optimization. The mobile phase consisting of the ammonium formate buffer (100 mM, pH 3,6) as an A solution and methanol as a B solution was pumped at a flow rate of 500 µL/min with the following step gradient. The run was started with a mobile phase consisting of 98% A and 2% B solution. Starting from 9th minute of run the part of B solution raised reaching the 50% in the 15th min and 80% in the 18th min. Starting from the 20th min until the end of the run (30th min) the column was re-equilibrated with the initial mixture of mobile phase solutions (2% of A and 98% of B solution). The applied potential for electrochemical measurements was +850 mV and the separation temperature was set at 25 °C. The 50 µL of samples and standard solutions were applied to the system.

The total RNAs from adrenal medulla was extracted using TRIzol® Reagent (Invitrogen, CA, U.S.A.). Total RNA was isolated using chloroform-isopropanol extraction and quantification and RNA quality was carried out using spectrophotometer (NanoDrop 1000 Spectrophotometer, Thermo Scientific). Adrenal medulla was homogenized in 1 ml TRIzol® Reagent per 100 mg of tissue using electrical homogenizer (IKA-WERKE, GmbH & Co, Germany). Reverse transcription was performed using Ready-To-Go You-Prime First-Strand Bead (GE Healthcare Life Sciences, PA USA) and pd $(N)_6$ primer according to manufacturer's protocol. Real-Time RT-PCR assay was done exactly as previously described by Jovanovic et al. (2018). PCR reaction was performed in the ABI Prism 7000 Sequence Detection System at 50 ^{0}C for 2 min., 95 ^{0}C for 10 min., followed by 40 cycles at 95 ^{0}C for 15 s and 60 ^{0}C for 1 min. TaqMan PCR reaction were carried out using Assay-on-Demand Gene Expression Products (Thermo Fischer Scientific, MA USA) for TH (ID: Rn00562500_m1), for DBH (ID: Rn00565819_m1), for PNMT (ID: Rn01495589_g1), for NET (ID: Rn00580267_m1) and for VMAT2 (ID: Rn00565488_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 cyclophilin A (ID: Rn00690933) expression. The obtained results were analyzed by RQ Study Add ON software for 7000 v 1.1 SDS instrument (ABI Prism Sequence Detection System) with a

confidence level of 95% ($p<0.05$). Relative expression of the target gene was expressed in relation to the calibrator, i.e., 1 control sample.

Adrenal medulla was homogenized in RIPA Lysis Buffer System (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, sc-24948). After centrifugation (12 000 r.p.m., 20 min at 4 ^{0}C), the supernatant was taken and protein concentration was determined by the method of Lowry et al. (1951). 30µg of adrenal medulla protein extract separated by 12% SDS-poly-acrylamide gel electrophoresis were transferred to a supported PVDF membrane (Immobilon-P membrane, Merck Millipore, Massachusetts, USA). The membranes were blocked in 5% non-fat dry milk in Tris-Buffered Saline-Tween 20 (TBST) for 1h. All following washes (three times for 15 min.) and antibody incubation (overnight at 4 ^{0}C for primary antibody and 1h at 4 ^{0}C for secondary antibody) were also performed in TBST at ambient temperature on a shaker. For measuring TH, DBH, PNMT, NET and VMAT 2 protein levels, a rabbit polyclonal anti-TH primary antibody (ab51191, dilution 1:1000, Abcam, Cambridge, UK), a rabbit polyclonal anti-DBH primary antibody (ab43868, dilution 1:2500, Abcam, Cambridge, UK), a rabbit polyclonal anti-PNMT primary antibody (ab90862, dilution 1:1000, Abcam, Cambridge, UK), a rabbit polyclonal anti-NET primary antibody (ab41559, dilution 1:1000, Abcam, Cambridge, UK), and rabbit polyclonal anti-VMAT 2 primary antibody (ab81855, dilution 1:5000, Abcam, Cambridge, UK) respectively, were used. Washed membrane was further incubated in the horseradish peroxidase-conjugated secondary anti-rabbit antibody for luminol-based detection (ab6721, dilution 1:5000, Abcam, Cambridge, UK). The secondary antibody was then visualized by Immobilon Western Chemiluminescent HPR Substrate (Merck Millipore, Massachusetts, USA). Western blot analysis was performed as previously described Jovanovic et al. (2018).

The results are reported as means ± S.E.M. The significance of the differences in the catecholamine concentration, gene expression and protein levels of the examined TH, DBH, PNMT, NET, and VMAT2 in the adrenal medulla of four groups of rats were estimated by a 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

A two-way ANOVA displayed the significant effects of stress ($F_{(1,23)} = 26.09$, $p < 0.001$), melatonin treatment ($F_{(1,23)} = 21.47$, $p < 0.001$) and the interaction between stress and treatment ($F_{(1,23)} = 6.89$, $p < 0.05$) on the content of norepinephrine in the adrenal medulla. Post-hoc analysis demonstrated that when compared with a naive control, stress produced a significant depletion (by 45%, $p < 0.01$) of norepinephrine stores (**Fig 1a**). Contrary to this,

melatonin treatment increased adrenal medullary norepinephrine content by 60% in stressed rats ($p < 0.05$). Under the examined conditions in this study the levels of epinephrine were not affected by CUMS or melatonin treatment (**Fig 1b**).

The two-way ANOVA analysis showed the significant effect of stress ($F_{(1,23)} = 32.04$, $p < 0.001$) on the TH mRNA level (**Fig: 2 a and b**). Post-hoc analysis revealed that stress leads to an increase in the TH mRNA level (by 70%, $p < 0.01$). Treatment with melatonin failed to alter TH gene expression. The two-way ANOVA also pointed to the significant effect of melatonin on the TH protein level ($F_{(1,23)} = 15.99$, $p < 0.05$) and the interaction between stress and melatonin treatment ($F_{(1,23)} = 6.09$, $p < 0.001$). Exposure to chronic stress results in a decrease in the levels of TH protein (by 24%, $p < 0.05$). On the other hand, levels of this protein in stressed animals were increased after melatonin treatment (by 95%, $p < 0.001$).

The adrenal DBH mRNA levels were also significantly influenced by stress ($F_{(1,23)} = 7.71$, $p < 0.01$). Stressed rats had increased levels of DBH mRNA in comparison to the unstressed animals (by 123%, $p < 0.001$). Melatonin treatment did not affect the levels of the DBH enzyme (**Fig: 2 c and d**). The results indicate the influence of stress ($F_{(1,23)} = 6.71$, $p < 0.5$) and melatonin treatment ($F_{(1,23)} = 14.55$, $p < 0.001$) on DBH protein levels in the adrenal medulla. An examination of DBH protein levels after exposure to CUMS showed that chronic stress decreased levels of this protein (by 22%, $p < 0.05$) when compared to the unstressed group. It was also found that DBH protein levels were increased in stressed groups treated with melatonin (62%, $p < 0.01$).

A major effect of CUMS was observed on adrenal PNMT mRNA levels ($F_{(1,23)} = 7.70$, $p < 0.05$). A Tukey test revealed an increased PNMT mRNA level in stressed animals (by 113%, $p < 0.01$). No changes in PNMT mRNA levels were observed after melatonin treatment. The two-way ANOVA demonstrated a significant effect of both stress exposure ($F_{(1,23)} = 39.59$, $p < 0.001$) and melatonin treatment ($F_{(1,23)} = 7.80$, $p < 0.05$) on PNMT protein in the adrenal medulla. Post-hoc testing showed that stress provoked an enhanced synthesis of the PNMT protein (by 179%, $p < 0.5$). Furthermore, treatment with melatonin increases levels of the PNMT protein in the stressed (by 42%, $p < 0.5$) group of animals (**Fig: 2 e end f**).

The results presented in **Fig: 3a** indicate the significant effect of stress ($F_{(1,23)} = 26.82$, $p < 0.001$) on NET mRNA levels in the adrenal medulla. Chronically stressed animals had increased NET mRNA levels (by 322%, $p < 0.01$)

compared to the unstressed controls, whereas melatonin treatment did not alter the expression of this transporter. With regard to the effects on NET protein in the adrenal medulla, the two-way ANOVA showed a significant influence of both the stress conditions ($F_{(1,23)} = 9.93$, $p < 0.05$) and the melatonin treatment ($F_{(1,23)} = 9.66$, $p < 0.05$). The CUMS procedure leads to a decrease in the amount of NET protein in the adrenal medulla (by 51%, $p < 0.05$). Melatonin treatment caused a significant increase in NET protein levels (by 102%, $p < 0.001$) in chronically stressed rats **(Fig: 3b)**. Our results, on the other hand, demonstrated that neither stress nor treatment significantly changed the VMAT2 gene expression (**Fig 3 c and d**).

Discussion

We have shown previously that the CUMS procedure induces depression-like behaviours in male rats, mimicking some symptoms present in human depression, such as a prolonged immobility in the forced swimming test (Stefanovic et al. 2016). The results of the present study show that CUMS caused a significant depletion of norepinephrine stores in the adrenal medulla, whereas epinephrine stores remained unchanged. The unchanged levels of epinephrine may reflect enhanced synthesis. Our results show that CUMS caused a significant increase in adrenal mRNA and the protein level of PNMT, enzyme that catalyzes the conversion of norepinephrine to epinephrine. Under stressful situations, PNMT gene expression is regulated by glucocorticoid mechanisms. Glucocorticoids have been proposed to regulate PNMT in a least two ways, posttranscriptionally and transcriptionally (Wong et al. 1992; Tai et al. 2002). We have found previously that CUMS elevated plasma corticosterone level (Dronjak et al. 2007) and this might be the reason for increased adrenal PNMT and preserved amount of epinephrine during chronic stress. Our data suggests a reduction in norepinephrine synthesis and re-uptake in the adrenal medulla of rats exposed to CUMS. The results of Santana et al. (2015) also suggests that mice exposed to chronic unpredictable stress for a period of 21 days develop depressive-like behaviour accompanied by an impairment of adrenal medullary function. The levels of adrenal TH mRNA, DBH mRNA and NET mRNA were elevated, whilst the protein level of TH, DBH and NET were significantly decreased. It should, however, be noted that the mRNA and protein measurements do exhibit a degree of discordance. The inhibition of protein translation might be achieved through differential regulation of the activity of ribosomal translational machinery or via the recently characterised translational interference from ncRNA, namely miRNA (Biggar et al. 2015). Gai et al. (2017) demonstrate that miR-375 is specifically expressed in the rat medulla chromaffin cells, and that miR-375 acts as a

negative mediator of adrenal medullary catecholamine biosynthesis and release and the consequent transcription of TH and DBH by targeting Sp1 in response to stress.

Our study found that a daily administration of melatonin enhanced norepinephrine content and protein expression of TH, DBH, PNMT and NET in the adrenal medulla of chronically stressed rats. The results of some other authors also suggest that melatonin plays a pivotal role in mediating the adaptation mechanism in animals following chronic stress (Haridas et al. 2016; Noseda et al. 2014). It has been reported that melatonin affects the morphological features of nerve tissue and has a neuroprotective role through involvement in the regeneration of peripheral nerves. Moreover, melatonin exerts a positive effect on axon length and development after peripheral nerve stress (Uyanikgil et al. 2017). Gallol and Mohammed (2018) examined the morphological changes produced by melatonin administration on the sustentacular cells in the adrenal medulla of male viscacha . They reported that melatonin is able to modify the S100 protein expression on sustentacular cells in the adrenal medulla and are in accordance to the observations made during winter, a season in which the viscacha is subject to short-photoperiod and high levels of serum melatonin. Melatonin also ameliorates oxidative and inflammatory stress produced by nicotin in the adrenal gland (Abdel Fattah et al. 2018). Mokhtar et al. (2017) demonstrated a high expression of TH in the chromaffin cells of the adrenal medulla in the melatonin-treated groups, compared with the controls. This neurohormone has also been reported to attenuate methamphetamine-induced reduction of TH in different brain regions (Kaewsuk et al. 2009). Martins et al. (2004) observed a decrease in the expression of TH and DBH in the adrenal glands of obese mice and melatonin deficiency has also been demonstrated to correlate with obesity (Szewczyk-Golec et al. 2017). Melatonin exerts its effects on adipose tissue through sympathetic-induced lipolysis and modulates seasonal adiposity, at least in experimental animals (Bartness et al. 2014). As melatonin modulates several processes involved in obesity and its related metabolic alterations, it could be speculated that melatonin also achieves the anti-obesogen and weight-reducing effects, modulating the gene expression of catecholamine biosynthetic enzymes in the adrenal medulla of obese animals. There are no clear mechanisms through which melatonin affects gene transcription and the translation processes. Several authors have reported that melatonin could regulate miRNA expressions to perform its anticancer function in different cancers (Han et al. 2011; Lee et al. 2011; Mori et al 2016). Further experiments are necessary to confirm our results and hypotheses and to investigate mechanisms of the melatonin effect on gene expression.

In conclusion, our results showed that melatonin enhanced norepinephrine content and protein expression of TH, DBH, PNMT and NET in the adrenal medulla of rats exposed to CUMS, and we suggest that melatonin may possess a more significant role in depression than has thus far been realised. Our data indicates that response of adrenal medulla of stressed animals to melatononin treatment goes to towards the improvement of catecholaminergic function by changes that promote an enhancment of catecholamine synthesis, the uptake and content in chromaffin cells. The mechanism by which melatonin may normalize catecholamine content in the adrenal medulla including a direct stimulation of gene expression for biosynthesizing enzymes and transporter. These findings add new information about the mechanisms through which melatonin physiologically modulates adrenomedullary function during stress.

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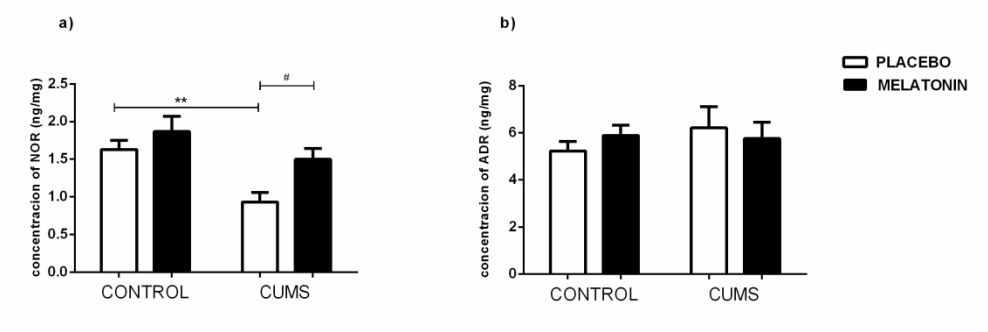
Figure captions

Figure 1: Effects of chronic melatonin treatment on the concentration of noradrenaline (NA) (a) and adrenaline (A) (b) in the adrenal medulla of rats exposed to CUMS for 28 days. The values are means ± S.E.M of 24 rats. Statistical significance: **p < 0.01 control vs. CUMS; #p < 0.05, ##p < 0.01 placebo vs. melatonin (Tukey test).

Figure 2: Effect of chronic melatonin treatment on tyrosine hydroxylase (TH), dopamine β-hydroxylase (DBH) and phenylethanolamine N-methyltransferase (PNMT) gene expression in the adrenal medulla of rats exposed to CUMS for 28 days. The relative mRNA levels of TH, DBH and PNMT were determined by applying RT-PCR. The final result was expressed as fold change relative to the calibrator and normalized to cyclophilin A for variation between samples (a, c and e). TH, DBH and PNMT protein levels were determined by Western immunoblotting. The final result was expressed in arbitrary units and normalized in relation to β-actin (b, d and f). The values are means ± S.E.M of 24 rats. Statistical significance: *p < 0.5; **p < 0.01 conrol vs. CUMS; #p < 0.5; ##p < 0.01; ###p < 0.001 placebo vs. melatonin (Tukey test).

Figure 3: Effect of chronic melatonin treatment on norepinephrine transporter (NET) and vesicular monoamine transporter 2 (VMAT2) gene expression in the adrenal medulla of rats exposed to CUMS for 28 days. The relative mRNA levels of NET and VMAT2 transporter were determined by applying RT-PCR. The final result was expressed as fold change relative to the calibrator and normalized to cyclophilin A for variation between samples (a and c). NET and VMAT2 protein levels were determined by Western immunoblotting. The final result was expressed in arbitrary units and normalized in relation to β-actin (b and d). The values are means ± S.E.M. of 24 rats. Statistical significance: **p < 0.01 conrol vs. CUMS; #p < 0.5; ##p < 0.01; ###p < 0.001 placebo vs. melatonin (Tukey test).



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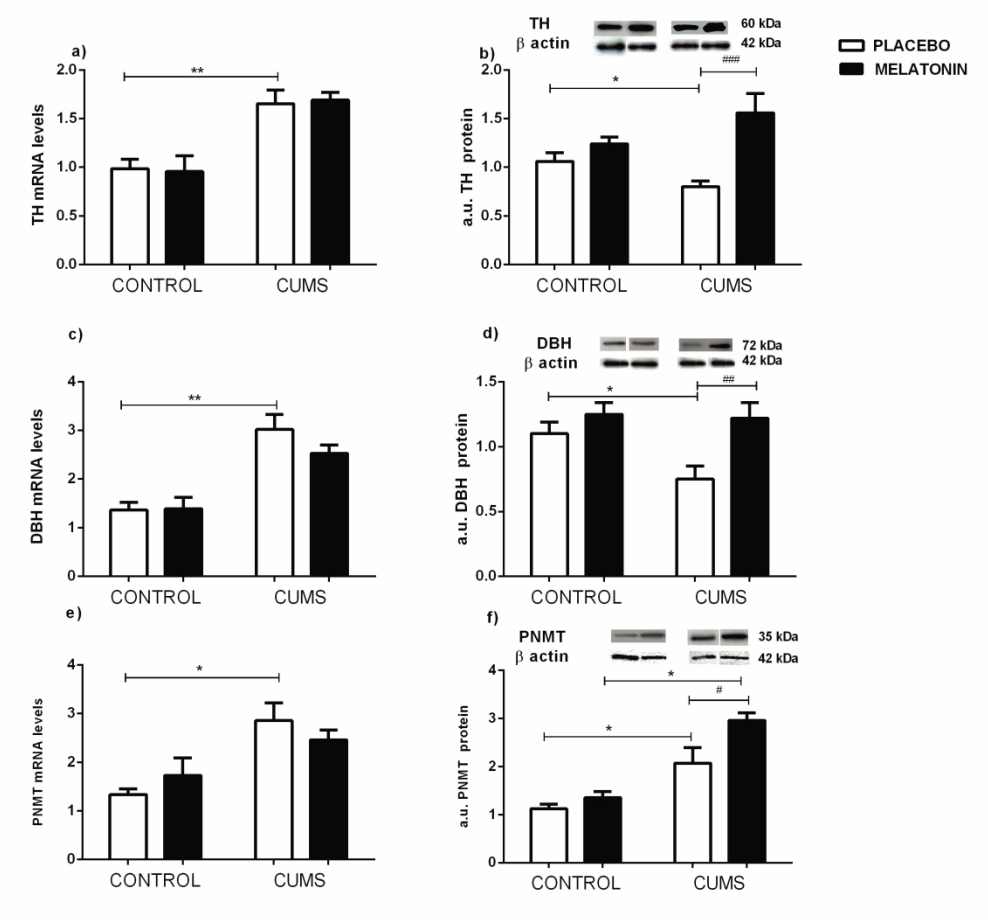

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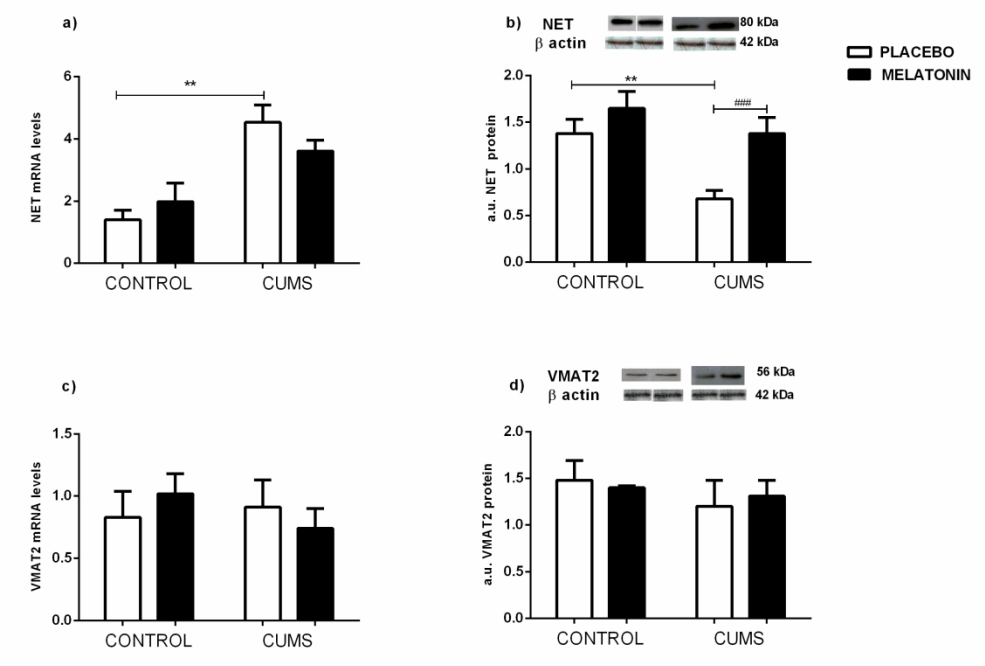

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