




Article

# The Changes of Expression and Methylation of Genes Involved in Oxidative Stress in Course of Chronic Mild Stress and Antidepressant Therapy with Agomelatine

Paulina Wigner <sup>1</sup>, Ewelina Synowiec <sup>1</sup> , Paweł Józwiak <sup>2</sup>, Piotr Czarny <sup>3</sup>, Michał Bijak <sup>4</sup> , Gabriela Barszczewska <sup>1</sup>, Katarzyna Białek <sup>1</sup>, Janusz Szemraj <sup>3</sup>, Piotr Gruca <sup>5</sup>, Mariusz Papp <sup>5</sup> and Tomasz Śliwiński <sup>1,\*</sup> 

<sup>1</sup> Laboratory of Medical Genetics, Faculty of Biology and Environmental Protection, University of Lodz, 90-236 Lodz, Poland; paulina.wigner@gmail.com (P.W.); ewelina.synowiec@biol.uni.lodz.pl (E.S.); gabriela.barszczewska@unilodz.eu (G.B.); biaalek.k@gmail.com (K.B.)

<sup>2</sup> Department of Cytobiochemistry, Faculty of Biology and Environmental Protection, University of Lodz, 90-236 Lodz, Poland; pawel.jozwiak@biol.uni.lodz.pl

<sup>3</sup> Department of Medical Biochemistry, Medical University of Lodz, 92-216 Lodz, Poland; piotr.czarny@umed.lodz.pl (P.C.); janusz.szemraj@umed.lodz.pl (J.S.)

<sup>4</sup> Department of General Biochemistry, Faculty of Biology and Environmental Protection, University of Lodz, 90-236 Lodz, Poland; michal.bijak@biol.uni.lodz.pl

<sup>5</sup> Polish Academy of Sciences, Institute of Pharmacology, 31-343 Krakow, Poland; gruca@if-pan.krakow.pl (P.G.); nfpapp@cyfronet.pl (M.P.)

\* Correspondence: tomliw@biol.uni.lodz.pl

Received: 8 May 2020; Accepted: 9 June 2020; Published: 11 June 2020



**Abstract:** Preclinical studies conducted so far suggest that oxidative stress processes may be associated with the mechanism of depression development. This study shows the effects of chronic administration of agomelatine on expression and the methylation status of *Sod1*, *Sod2*, *Gpx1*, *Gpx4*, *Cat*, *Nos1*, and *Nos2* in the brain structure and blood in the chronic mild stress (CMS) animal model of depression. The animals were exposed to the CMS procedure and treatment with agomelatine (10 mg/kg/day, IP) for five weeks and then were sacrificed. TaqMan Gene Expression Assay, Western blot, and methylation-sensitive high-resolution melting techniques were used to evaluate mRNA and protein expression of the genes, and the methylation status of their promoters. *Gpx1*, *Gpx4*, and *Sod2* expression in the PBMCs and *Sod1* and *Sod2* expression in the brain were reduced in the stressed group after agomelatine administration. CMS caused an increase in the methylation of the third *Gpx4* promoter in peripheral blood mononuclear cells and *Gpx1* promoter in the cerebral cortex. Additionally, stressed rats treated with agomelatine displayed a significantly lower *Gpx4* level in the hypothalamus. The results confirm the hypothesis that the CMS procedure and agomelatine administration change the expression level and methylation status of the promoter region of genes involved in oxidative and nitrosative stress.

**Keywords:** chronic mild stress model; agomelatine; oxidative stress; gene expression and methylation

## 1. Introduction

Depression is a common and serious mental disorder. According to a report by the World Health Organization, 350 million people suffer from depression globally. Additionally, 800,000 people in the world commit suicide every year [1,2]. Depression is a multifactorial disease with a complex development mechanism. Previous studies have shown that antioxidant disturbances play an important role in the

pathogenesis of neurological and psychiatric diseases, particularly depression [3]. This may be due to the fact that brain has a lower antioxidant capacity than other organs and thus is more sensitive to oxidative disorders [3]. More specific, the reduced levels of enzymatic and non-enzymatic antioxidants, including glutathione, zinc, uric acid, vitamins A, E, C, coenzyme Q10, glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT) were observed in the course of depression. Moreover, the low activity of GPx and SOD may be associated with severity of the disease. However, in the case of the enzymes, the obtained results are contradictory [4–17]. Additionally, a post-mortem study had shown an increased level of prooxidative enzyme, xanthine oxidase, in the serum and thalamus [18,19]. The overproduction of the reactive oxygen species observed in the course of depression may lead to damage to the important biomolecules. Thus, patients with depression were characterized by an elevated level of markers of peroxidation damage to lipid and DNA, including 8-F2-isoprostane (8-iso-PGF2 $\alpha$ ), malondialdehyde (MDA), and 8-hydroxy-2-deoxyguanosine [4–17]. Unfortunately, an excessive oxidative stress and poor antioxidant defense may lead to the induction of neuronal damage. The overproduction of reactive oxygen species may lead to the induction of apoptosis of neural cells [20]. Thus, patients with depression were characterized by a reduced volume of the hippocampus [21].

Studying the antioxidant properties of antidepressants is a worthwhile approach and can result in the development of promising new antidepressive drugs [22]. This is of particular importance, as one-third of patients do not respond to antidepressant treatment at all [23]. Oxidative stress intensification, which is observed in the course of depression, may influence the therapeutic action and side effects of antidepressants [24]. However, the mechanism of drug metabolism and action is not fully clear. Agomelatine is a melatonin analogue and a potentially strong antioxidant [25]. Melatonin is produced by the pineal gland and regulates the circadian rhythm as well as wakefulness and sleep by the duration of nocturnal secretion [26]. It plays an important role in the central nervous system as agonist at the melatonergic MT1 and MT2 receptors and as antagonist of the 5-HT2c receptors [27,28]. In addition to the MT1 and MT2 receptors, agomelatine is characterized by low affinity with other receptors, including adrenoceptors, dopamine, GABA (gamma-aminobutyric acid), muscarinic, histamine, benzodiazepine, and ion channels [29]. Therefore, agomelatine may regulate sleep disorders in patients suffering from depression and reduce severity of the disease [30]. However, the previous studies showed that agomelatine therapy is the most effective for patients with seasonal affective disorder, including seasonal depression. Animal and human studies confirmed that agomelatine showed beneficial effects on the regulation of disturbances of sleep and circadian rhythms [31–34]. Previously conducted studies confirmed the antioxidant nature of agomelatine in many different cells, although the results are inconsistent [35–37]. Therefore, the aim of the study presented herein was to investigate the effect of chronic mild stress (CMS; animal model of depression) and treatment with agomelatine in rats on mRNA and protein expression, and the promoter methylation of genes involved in oxidative stress. All of the genes analyzed in our study are characterized in Table S1.

## 2. Materials and Methods

### 2.1. Animals

Male Wistar Han rats (Charles River, Germany), weighing approximately 200–220 g, were housed as previously described by Wigner et al. (2020) [38]. All tests in the study were approved by the Bioethical Committee at the Institute of Pharmacology of the Polish Academy of Sciences in Krakow (Poland) and were conducted in compliance with the rules and principles of the 86/609/EEC Directive.

### 2.2. Chronic Mild Stress Procedure

The CMS experiment was conducted according to the method previously described [39]. The CMS procedure has been used because it is a well-validated and widely used animal model of depression, and therefore, much of the current understanding of the mechanisms of antidepressant drug action has been derived using this model.

First, the studied rats (30 animals) were apprenticed to for the weekly sucrose-intake test, which was carried out lasted 1 h following 14 h deprivation of water and food. Subsequently, the animals were divided into two groups matched for their sucrose intakes—control and stressed animals—as described by Wigner et al. (2020) [38]. Then, control and stressed animals were divided into subgroups based on their sucrose consumption values. Each subgroup ( $n = 6$ ) received a vehicle (daily 10 mL/kg, IP) or agomelatine (daily 10 mg/kg, IP) for five weeks. The dose was selected on the basis of data in the literature and our own previous studies showing its efficacy in the CMS model of depression [40]. Then, weekly sucrose-intake tests were performed 24 h after the last dose. Finally, 24 h after the last sucrose intake test, the studied animals were decapitated, and samples of brain structures and blood were collected. Anesthesia was not used before decapitation.

### 2.3. Specimen Collection of Peripheral Blood Mononuclear Cells and Homogenates of Animal Brain Tissue

After decapitation of the studied animals, samples of blood were collected into vacutainer tube containing anticoagulants. Then, Gradisol L (Aqua-Med, Lodz, Poland) and differential migration of cells during centrifugation ( $400\times g$ , 30 min,  $4\text{ }^{\circ}\text{C}$ ) was used to isolate peripheral blood mononuclear cells (PBMCs). After centrifugation ( $400\times g$ , 30 min,  $4\text{ }^{\circ}\text{C}$ ), a pellet of PBMCs was stored at  $-20\text{ }^{\circ}\text{C}$  until required.

Additionally, after removal, the studied animal brain structures were placed in liquid nitrogen and stored at  $-80\text{ }^{\circ}\text{C}$  until required. Then, they were manually homogenized by FastGene<sup>®</sup> Tissue Grinder (Nippon Genetics Europe, Düren, Germany) and sonicated. Finally, the prepared specimens were used to isolate DNA and RNA.

### 2.4. RNA Isolation, cDNA Synthesis, and Real-Time Analysis of the Expression of Genes Encoding Antioxidant Enzymes

Total RNA was isolated from PBMCs and frozen brain structures using the commercial spin column methods (GenElute Mammalian Total RNA Miniprep Kit, Sigma-Aldrich, Saint Louis, MO, USA; ISOLATE II RNA/DNA/Protein Kit, Bioline, London, UK, respectively) according to the manufacturer's instructions. Then, spectrophotometric verification was used to measure the concentration and quality of the RNA samples, which were stored at  $-20\text{ }^{\circ}\text{C}$  until use. The reaction of reverse transcription was performed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. The expression levels of all studied genes were detected and identified by real-time PCR using a TaqMan Universal Master Mix, no UNG, and species-specific TaqMan Gene Expression Assay—assay ID: Rn00566938\_m1 (*Sod1*), Rn00690588\_g1 (*Sod2*), Rn00577994\_g1 (*Gpx1*), Rn00820818\_g1 (*Gpx4*), Rn00560930\_m1 (*Cat*), Rn00583793\_m1 (*Nos1*), and Rn00561646\_m1 (*Nos2*) (Thermo Fisher Scientific, Waltham, Massachusetts, USA). However, before starting the real-time PCR analysis, the authors carried out a review of the available literature and selected the 18S (*18S ribosomal RNA*) gene as the best reference gene. The reaction mixture consisted of total cDNA samples, TaqMan Universal Master Mix, no UNG (Applied Biosystems, Foster City, CA, USA), TaqMan Probe (Thermo Fisher Scientific, Waltham, MA, USA), and RNase-free water. The conditions of PCR included 10 min at  $95\text{ }^{\circ}\text{C}$  (enzyme activation), followed by 60 cycles of 30 s at  $95\text{ }^{\circ}\text{C}$  (denaturation) and 1 min at  $60\text{ }^{\circ}\text{C}$  (for annealing/extension). Real-time PCR runs were performed using a CFX96<sup>TM</sup> Real-Time PCR Detection System Thermal Cycler (Bio Rad Laboratories Inc., Hercules, CA, USA). The experiments were performed in duplicate for each sample. Gene expression was calculated in relation to that of the reference gene ( $\Delta\text{Ct}$  sample =  $\text{Ct target gene} - \text{Ct reference gene}$ ). Next, the relative mRNA expression were calculated as fold =  $2^{-\Delta\text{Ct}}$  sample. Moreover, the fold change in expression caused by antidepressant administration was assessed using the  $2^{-\Delta\Delta\text{Ct}}$  method [41].

### 2.5. DNA Isolation, Methylation, and HRM Analysis

Total DNA was extracted from PBMCs and frozen brain structures by using commercial spin column methods (QIAamp DNA Mini Kit, Qiagen, Hilden, Germany; ISOLATE II RNA/DNA/Protein Kit, Bioline, respectively) following the manufacturer's instructions. Then, spectrophotometric

verification used for the measurement of the total concentration and quality of the isolated RNA samples, which were stored at  $-20\text{ }^{\circ}\text{C}$  until use. Methylation of the gene promoter region was assessed using methylation-sensitive high-resolution melting (MS-HRM) [42,43].

CpG islands (regions of the genome that contain a large number of CpG dinucleotide repeats) in the promoters of the studied genes were predicted by the EMBOSS CpGplot bioinformatics tool ([https://www.ebi.ac.uk/Tools/seqstats/emboss\\_cpgplot/](https://www.ebi.ac.uk/Tools/seqstats/emboss_cpgplot/), Settings: Window: 100, Shift: 1, Obs./Exp.: 0.6, GC content: 50%). The primers were designed according to the instructions of Wojdacz et al. (2009) in MethPrimer (<http://www.urogene.org/methprimer2/>) [44]. Such a procedure necessitates DNA samples to be subjected to sodium bisulfite modification using a CiTi Converter DNA Methylation Kit (A&A Biotechnology, Gdynia, Poland), following the commercial instructions. A Bio-Rad CFX96 Real-Time PCR Detection System (BioRad Laboratories Inc., Hercules, CA, USA) was used for methylation analyses. Each PCR reaction consisted of 5x HOT FIREPol<sup>®</sup> EvaGreen<sup>®</sup> HRM Mix (no ROX) (Solis BioDyne, Tartu, Estonia), 500 nM of forward and reverse primers, and DNA samples (10 ng/ $\mu\text{L}$ ) after bisulfite modification. The reaction conditions included initial activation, annealing, and elongation according to manufacturer's instruction. However, the temperature of annealing was determined experimentally (see Table S2). The HRM analysis involved a step of denaturation at  $95\text{ }^{\circ}\text{C}$  for 15 s, reannealing at  $60\text{ }^{\circ}\text{C}$  for 1 min, and melting from 60 to  $95\text{ }^{\circ}\text{C}$  at a ramp rate of  $0.2\text{ }^{\circ}\text{C}$  every 2 s. The analysis of the obtained data was made using Bio-Rad Precision Melt Analysis Software (BioRad Laboratories Inc., Hercules, CA, USA). Thus, the methylation status was assessed by comparison of the HRM profiles obtained from the amplification of methylated DNA (CpGenome<sup>™</sup> Rat Methylated Genomic DNA Standard, Merck Millipore Burlington, MA, USA) and unmethylated DNA (CpGenome<sup>™</sup> Rat Unmethylated Genomic DNA Standard, Merck Millipore Burlington, MA, USA). To this end, serial dilutions were prepared: 0%, 10%, 25%, 50%, 75%, and 100% methylated DNA.

## 2.6. Western Blot Analysis

The protein levels of all the studied genes in the rat brain tissue were estimated using Western blotting analyses (Laemmli, 1970). Frozen brain structures samples were homogenized using a FastGene<sup>®</sup> Tissue Grinder (Nippon Genetics Europe, Düren, Germany) in RIPA buffer (Radioimmunoprecipitation assay buffer) with 1 mM of phenylmethylsulfonyl fluoride as serine protease inhibitor. Then, the samples were sonicated twice and centrifuged, and supernatant was terminally collected. The protein concentration of received samples was estimated by the Lowry procedure [45]. Homogenate samples were run under reducing conditions on 10% SDS polyacrylamide gels and transferred onto Immobilon-P (Millipore, Bedford, MA, USA). Then, the blots were blocked with 5% non-fat dry milk in 0.1% TBST buffer (Tris-buffered saline with Tween-20) and after the block, the membranes incubated with the primary antibodies and the opportune secondary antibodies were conjugated with horseradish peroxidase (see Table S3). Finally, the blots were incubated with peroxidase substrate solution (Thermo Fisher Scientific, Waltham, MA, USA), and the proteins were visualized on X-ray film by enhanced chemiluminescence. Densitometry analysis of protein bands was using by Gel-Pro<sup>®</sup> Analyzer Software (Media Cybernetics Inc., Silver Spring, MD, USA). The integrated optical density (IOD) of the immunoreactivity bands was measured based on digital images. Results were normalized using the reference protein ( $\beta$ -actin) resolved by electrophoresis.

## 2.7. Statistical Analysis

The results were presented as the mean value  $\pm$  standard error of the mean. Data of the effect of 2 weeks of CMS on sucrose consumption were analyzed by using *test t*. The data of gene expression and methylation were analyzed using a two-way analysis of variance (two-way ANOVA) with *post hoc* Tukey's test *p* values  $< 0.05$  were considered as statistically significant. All the analyses were completed using Statistica 12 (Statsoft, Tulsa, OK, USA), SigmaPlot 11.0 (Systat Software Inc., San Jose, CA, USA) and GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA).

### 3. Results

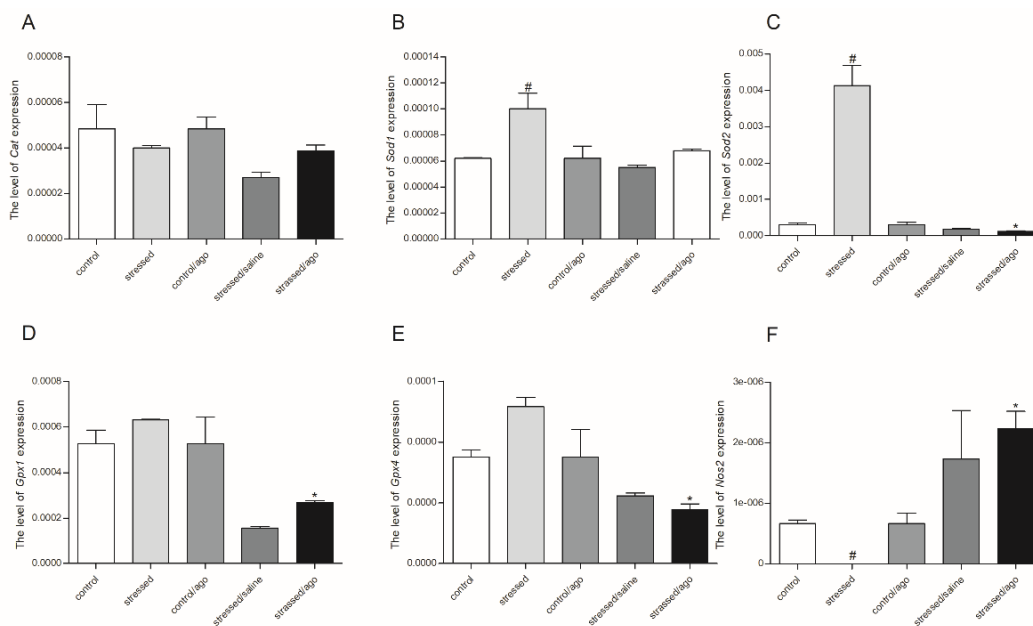
#### 3.1. Correlation between Differences in Sucrose Intake of Rats Subjected to Stress and Agomelatine Treatment

As shown in Table S4, before the stress was initiated (Week 0), the consumption of 1% sucrose solution was comparable in all groups. The intake decreased by approximately 50% ( $p < 0.01$ ) after two weeks of the CMS procedure. A similar effect was observed in Stressed/Saline and Stressed/Ago groups ( $p < 0.05$ ,  $p < 0.01$ , respectively). Chronic (five-week) administration of agomelatine had no effect in the case of control animals but normalized the intake in the stressed rats ( $p < 0.001$ ).

#### 3.2. mRNA Expression Level of the Studied Genes

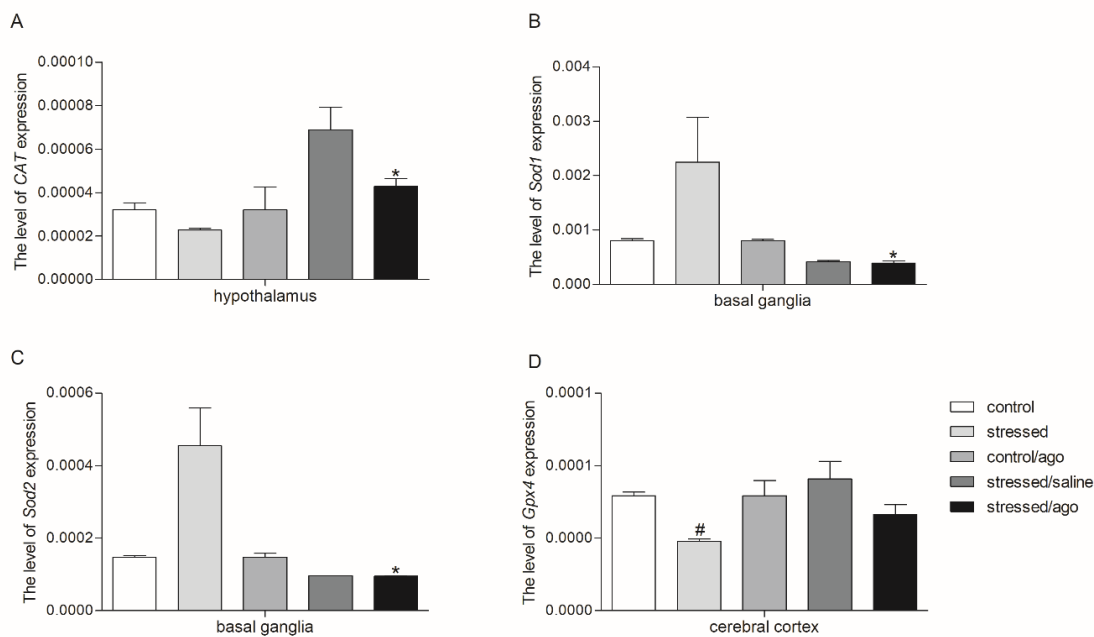
##### 3.2.1. Gene Expression in PBMCs and Brain Structures

As shown in Figure 1, Figure 2, and Figure S1, the effect of CMS and agomelatine on *Sod1*, *Sod2*, *Gpx1*, *Gpx4*, *Cat*, and *Nos1* mRNA expression depended on the tissue and brain structure. Agomelatine reduced the expression of *Sod1* and *Sod2* in the basal ganglia ( $p < 0.05$ ). Similarly, in the case of PBMCs, reduced expression of *Sod2*, *Gpx1*, *Gpx4* ( $p < 0.05$ ) was observed after agomelatine. Additionally, the CMS procedure increased the expression of *Sod2* in PBMCs ( $p < 0.05$ ). On the other hand, the expression of *CAT* in the hypothalamus ( $p < 0.05$ ) and *Nos2* in PBMCs ( $p < 0.05$ ) was significantly increased as an effect of the antidepressant therapy in the stressed rats. Moreover, the cerebral cortex of the stressed group was characterized by reduced expression of *Gpx4* ( $p < 0.05$ ), whereas *Sod1* expression in PBMCs was elevated in the stressed rats ( $p < 0.05$ ), but *Nos2* expression was decreased in the PBMCs of stressed rats.



**Figure 1.** CAT - catalase (A) SOD1 – superoxide dismutase 1 (B), SOD2 – superoxide dismutase 2 (C), Gpx1 – glutathione peroxidase 1 (D), Gpx4 - glutathione peroxidase 4 (E), and NOS2 nitric oxide synthetase 2 (F) mRNA expression in peripheral blood mononuclear cells (PBMCs) of rats exposed to chronic mild stress (CMS) procedure for two weeks (Control, Stressed) and in animals exposed to CMS for seven weeks and administered vehicle (1 mL/kg) or agomelatine (10 mg/kg) for five weeks (Control/Ago, Stressed/Saline, Stressed/Ago). Relative mRNA expression was calculated using a  $2^{-\Delta\text{Ct}}$  ( $\text{Ct}_{\text{gene}} - \text{Ct}_{18\text{S}}$ ) method. Data were presented as means  $\pm$  SEM. N = 6. \*  $p < 0.05$  for differences between stressed and stressed/ago groups, and #  $p < 0.05$  for differences between controls and stressed groups.





**Figure 2.** CAT (A) SOD1 (B), SOD2 (C), Gpx1 (D), Gpx4 (E), and NOS1 (F) mRNA expression in the hippocampus, amygdala, hypothalamus, midbrain, cerebral cortex and basal ganglia of animals exposed to CMS procedure for two weeks (Control, Stressed) and in animals exposed to CMS procedure for seven weeks and administered vehicle (1 mL/kg) or agomelatine (10 mg/kg) for five weeks (Control/Ago, Stressed/Saline, Stressed/Ago). Relative mRNA expression was estimated using a  $2^{-\Delta\Delta Ct}$  ( $Ct_{\text{gene}} - Ct_{18S}$ ) method. Data were presented as means  $\pm$  SEM. N = 6. \*  $p < 0.05$  for differences between stressed and stressed/ago groups, and #  $p < 0.05$  for differences between controls and stressed groups.

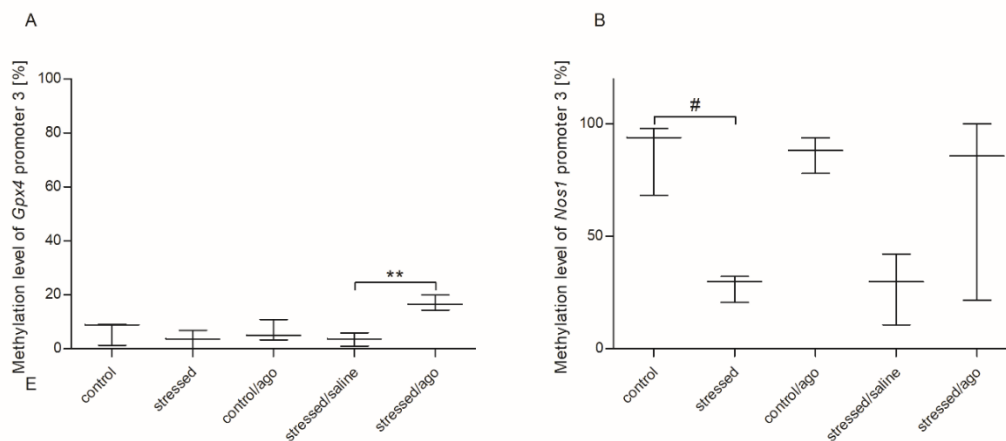
### 3.2.2. Comparison of Agomelatine Impact on Gene Expressions in PMBCs and Brain Structure

As shown in Supplementary Figure S2, agomelatine caused a drop of *Gpx4* expression in the hypothalamus and cerebral cortex and increased the gene expression in PMBCs ( $p < 0.001$ ). No significant differences were detected in the case of other studied genes.

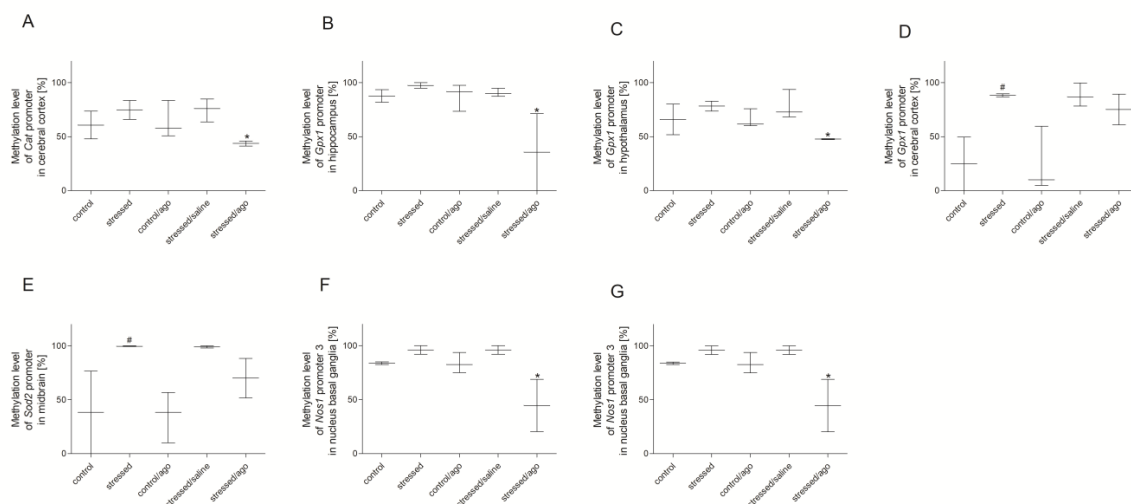
### 3.3. Methylation Changes in the Studied Gene Promoters

#### 3.3.1. Methylation Status of Gene Promoters in Peripheral Blood Mononuclear Cells and Brain Structures

As shown as Figure 3, the methylation status of the third *Nos1* promoter in PMBCs was significantly decreased in the stress group ( $p < 0.05$ ) and the methylation of the third *Gpx4* promoter was increased in the stressed animals after agomelatine administration ( $p < 0.05$ ). As shown in Figure 4, the CMS procedure significantly increased the methylation level of promoter 2 of *Gpx1* in the cerebral cortex ( $p < 0.05$ ). Agomelatine significantly reduced the methylation status of *Nos1* promoter 3 in the amygdala and basal ganglia (test  $p < 0.05$ ), *Gpx1* promoter in the hippocampus ( $p < 0.05$ ) and hypothalamus ( $p < 0.05$ ), as well as *Cat* promoter in the cerebral cortex ( $p < 0.05$ ). Moreover, the methylation status of the *Sod2* promoter region in the midbrain was elevated in the stressed rats compared to the controls ( $p < 0.05$ ). No significant differences were detected in the case of other promoter regions of the studied genes (Figure S3 and Table S4).



**Figure 3.** The methylation status *Gpx4* promoter 3 (A), *NOS1* promoter 3 (B), in PBMCs of animals exposed to CMS procedure for two weeks (Control, Stressed) and in animals exposed to CMS procedure for seven weeks and administered vehicle (1 mL/kg) or agomelatine (10 mg/kg) for five weeks (Control/Ago, Stressed/Saline, Stressed/Ago). Data presented as means  $\pm$  SEM. N = 6. \*\*  $p < 0.01$  for differences between stressed and stressed/ago groups, and #  $p < 0.05$  for differences between controls and stressed groups.



**Figure 4.** The methylation status of *CAT* promoter in cerebral cortex (A), *Gpx1* promoter in hippocampus (B), hypothalamus (C), and cerebral cortex (D), *SOD2* promoter in midbrain (E), *NOS1* promoter 3 in (F) and amygdala (G) of animals exposed to CMS procedure for two weeks (Control, Stressed) and in animals exposed to CMS procedure for seven weeks and administered vehicle (1 mL/kg) or agomelatine (10 mg/kg) for five weeks (Control/Ago, Stressed/Saline, Stressed/Ago). Data presented as means  $\pm$  SEM. N = 6. \*  $p < 0.05$  for differences between stressed and stressed/ago groups, and #  $p < 0.05$  for differences between controls and stressed groups.

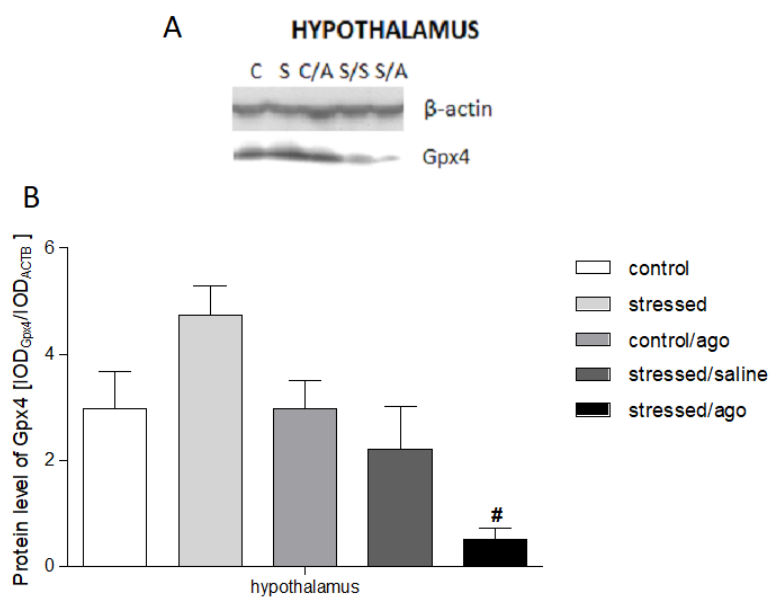
### 3.3.2. Comparison of Agomelatine Impact on the Methylation Status of Gene Promoters in PBMCs and Brain Structure

As shown in Figure S4, significant differences were found in the effect of agomelatine on the methylation status of *Gpx4* promoter 3, *Sod2* promoter, and *Nos1* promoter 3 between blood and brain structures. In particular, agomelatine increased the methylation level of *Sod2* promoter in the hypothalamus and cerebral cortex compared to blood ( $p < 0.05$ ,  $p < 0.01$ , respectively). Furthermore, an antidepressant therapy decreased the methylation of the *Nos1* promoter 3 region in the midbrain and basal ganglia compared to blood ( $p < 0.05$ ). Additionally, the hippocampus, hypothalamus,

midbrain, and cerebral cortex showed lower methylation levels of the *Gpx4* promoter 3 region than blood ( $p < 0.001$ ).

### 3.4. Gene Expression at Protein Level

The protein expression level of Sod1, Nos1, Nos2, *Gpx4*, and Cat did not differ between the studied groups (Figure S5). As shown in Figure 5, agomelatine significantly reduced only the level of *Gpx4* protein in the hypothalamus ( $p < 0.05$ ).



**Figure 5.** *Gpx4* protein expression in the brain structures—hippocampus, amygdala, hypothalamus, midbrain, cerebral cortex and basal ganglia—of animals exposed to CMS procedure for two weeks (Control, Stressed) and in animals exposed to CMS procedure for seven weeks and administered vehicle (1 mL/kg) or agomelatine (10 mg/kg) for five weeks (Control/Ago, Stressed/Saline, Stressed/Ago). (A) Representative Western blot analysis in midbrain and cerebral cortex. C—controls, S—stressed for two weeks, C/A—Control/Agomelatine, S/S—Stressed/Saline, S/A—Stressed/Agomelatine. (B) *Gpx4* protein level measured in all studied brain structures. The graphs show the mean integrated optical density (IODs) of the bands from all analyzed samples. The relative protein expression levels was calculated using the IOD<sub>gene</sub>/IOD<sub>ACTB</sub> method. Data are presented as means  $\pm$  SEM. N = 6. #  $p < 0.05$  for the difference between stressed and stressed/ago groups.

## 4. Discussion

Our study presents effects of antidepressant treatment with agomelatine on the disorders of the selected genes encoded antioxidant enzymes in the PBMCs and various brain structures (hippocampus, amygdala, hypothalamus, midbrain, cerebral cortex, basal ganglia) of the rats exposed to the chronic mild stress procedure. As in previous studies, the CMS procedure caused a significant (approximately 50%) reduction of the consumption of 1% sucrose solution, indicating a generalized deficit in sensitivity to reward in the stressed animals, i.e., a characteristic symptom of depression [46,47]. This effect was normalized by a chronic (5 weeks) administration of agomelatine, which did not affect the behavior of control, non-stressed animals. The CMS procedure and agomelatine treatment also influenced mRNA and protein levels and the status of promoter region methylation of selected genes encoding enzymes involved in oxidative stress in the PBMCs and in various brain structures. Overall, the gene changes seem to confirm earlier suggestions that the overproduction of reactive oxygen species leads to development of depression-like symptoms in the CMS rats [4,5,48] as well as to increases in the level of thiobarbituric acid-reactive substances (TBARS), lipid peroxidation, protein oxidation, and oxidative DNA damage in several brain structures [49–51]. These findings are consistent with clinical reports



showing that elevated plasma levels of reactive oxygen species (ROS) and decreased activity of antioxidant enzymes are observed in patients suffering from depression [52].

Demirdaş et al. (2016) showed that the activity of Gpx and glutathione level in the brain and other organs were lower in the CMS animals compared to non-stressed rats, but they were higher in the agomelatine-treated group [53]. Moreover, an in vitro study conducted on a neuronal cell line confirmed that agomelatine caused an increase in glutathione level and glutathione peroxidase activity [54]. Thus, the CMS animal model depression may be associated with the intensification of oxidative stress and reduced antioxidant levels, both effects being normalized by agomelatine. On the other hand, in our study, agomelatine decreased levels of *Gpx1* and *Gpx4* expression in PBMCs and reduced the CMS-induced enhancement of *Gpx4* expression at the protein level in the hippocampus. The decreased level of *Gpx1* and *Gpx4* expression in stressed animals after antidepressant therapy may be a result of reduced reactive oxygen species level caused by chronic agomelatine administration. The reduced ROS concentration after therapy may cause a decreased demand for antioxidative defense enzymes [55]. Other results showed that melatonin receptor agonists are characterized by ROS scavenging activity and antioxidant system modulation [56] and that the elevated expression of antioxidant enzymes is associated with the activation of melatonin receptors [34]. Differences between the analyzed studies may result from the dualistic model of the agomelatine action. Agomelatine may act as a free radical scavenger by acting directly on the ROS level or it may indirectly affect the activity of antioxidant enzymes. In the first case, the enzyme activity decreases as a result of lowering the production level of ROS. In turn, in the second case, increasing the activity of antioxidant enzymes caused by agomelatine leads to a decrease of ROS level. Similarly, in the case of human study, the obtained results are divergent. On the one hand, the depression may be associated with the decreased activity of GPx and SOD [15–17]. On the other hand, Gałecki et al. and Kodydková et al. reported the opposite effect [7,8].

The next antioxidant enzyme studied in this study was catalase, which is an enzyme that was shown to be substantially increased in the serum of depressed patients [57]. In addition, in CMS animals, the activity of Cat was elevated in various brain structures [51]. The increased Cat activity in stressed animals and depressed patients is a consequence of increased hydrogen peroxide production. The previous studies showed that depression is associated with an increased activity of xanthine oxidase, which generated hydrogen peroxide. Then, hydrogen peroxide may be neutralized by catalase into water and oxygen [16,18,19]. The recent study showed that agomelatine administration caused an increase in catalase activity in the prefrontal cortex, hippocampus, and striatum of mice after chemically induced seizures [55]. In this study, we also found an increased expression of *Cat* in the hypothalamus and reduced methylation status of the *Cat* promoter region in the cerebral cortex in the agomelatine-treated CMS rats. Mello et al. (2016) demonstrated that agomelatine increased Sod activity in the striatum, whereas Cat activity was reduced in the cerebellum and elevated in the posterior cortex [58]. Thus, the results suggest that agomelatine may modulate the expression and promoter methylation of gene-encoding *Cat*. The chronic administration of agomelatine may cause an increase of *Cat* expression and decrease methylation in the *Cat* promoter region, consequently contributing to the increase in the activity of this enzyme and reducing the concentration of hydrogen peroxide. An interesting relationship has been observed in the case of Sod expression. Sod catalyzes the dismutation of the superoxide radical into ordinary molecular oxygen and hydrogen peroxide [56]. In the PBMCs of stressed rats, the *Sod1* and *Sod2* mRNA expression were increased, whereas the chronic administration of agomelatine normalized this effect. Moreover, agomelatine treatment reduced *Sod1* and *Sod2* expression in the basal ganglia of the stressed rats. Similarly, in depressed patients, the serum activity of SOD was higher than in control subjects [56]. In this connection, that superoxide radical excess is neutralized by Sod, while the increased expression level and activity of Sod were observed in stressed rats and depressed patient. However in animal models, the activity of this enzyme was decreased both in serum and in brain structures [51,59].

The next enzyme associated with mechanisms of depression and nitrosative stress is Nos. Depressed patients are characterized by elevated serum levels of NO and its metabolites [60–62], and these effects were normalized by antidepressant therapy [60]. In addition, in mice exposed to the CMS model, the production of NO was increased [59]. In this study, we found that the CMS caused reduction of the methylation status of the third *NOS1* promoter region in PBMCs, whereas the methylation level of the third and seventh *Nos1* promoters in the basal ganglia and the expression of *Nos2* in PBMCs after agomelatine therapy were lower than in the saline-treated stressed animals. There are reports suggesting that melatonin plays an important role in NO generation; the compound was found to inhibit constitutive NOS (NOS1) and to control the mitochondrial isoform of NOS [63,64]. Consequently, it can be assumed that agomelatine exhibits antioxidant activity by modulating nitric oxide levels by the stimulation of melatonin MT1 receptors [60,65].

These findings seem to confirm the idea that insufficient antioxidant defense may be associated with depression-like symptoms observed in animals after the CMS procedure. Moreover, it may be suggested that the antioxidant imbalance causes an overproduction of toxic free radicals, leading to serious damage to biomolecules, including lipids, proteins, and DNA, contributing to various diseases. Long-term exposure to free radicals may consequently cause cell death as well as neuronal and glia atrophy, leading to a decreased volume of gray matter and reduced number of cells observed in depressed patients [66,67]. All these effects are attenuated by antidepressant drugs, which may recover the oxidative and antioxidative balance by normalizing the expression level of antioxidant enzymes.

Together, these studies indicate that agomelatine may prevent the ROS overproduction in animals exposed to the CMS procedure. The results also suggest that agomelatine has antioxidant activity, and this may be involved in the mechanisms of its relief of depression-like symptoms. Agomelatine reduces the mRNA expression of *Sod2*, *Gpx1*, *Gpx4*, and *Nod2* in PBMCs, and *Sod1* and *Sod2* in the basal ganglia. The reduced expression level of the antioxidant enzyme may be associated with a decreased generation of free radicals after agomelatine therapy, and therefore, the expression level of genes involved in oxidative stress could be an indicator of its antidepressant effectiveness. On the other hand, the presented study has some limitations. First, only the effects of CMS and agomelatine treatment were analyzed without assessing the effects of other biochemical pathways, including inflammation or the tryptophan catabolites (TRYCATs) pathway. Previous studies confirmed that depression is a multifactorial disease. Both pro-inflammatory factors and toxic metabolites of the TRYCATs pathway can additionally increase the production of reactive oxygen species. Therefore, in the future, other biochemical pathways that may have an impact on the development of oxidative stress should also be analyzed. On the other hand, elements of the pathways that are affected by the overproduction of reactive oxygen species, including pathways for repairing oxidative DNA damage, should also be investigated [20,68,69]. Another limitation is the limited possibility to extrapolate animal broadcast results to changes observed in humans. Therefore, the next step of the study should be the analysis of changes in the studied genes in patients with depression.

## 5. Conclusions

The presented results confirm the hypothesis that the oxidative and nitrosative stress is involved in the pro-depressive effects of the CMS procedure and in the mechanisms of therapeutic action of agomelatine in this model of depression: (1) CMS causes the modulation of *Sod1* expression in PBMCs and *Gpx4* in the brain; (2) the administration of agomelatine modifies the expression of *Sod2*, *Gpx1*, *Gpx4*, and *Nos2* in PBMCs, and *Sod1* and *Sod2* in the brain; (3) CMS and agomelatine change the methylation status of *Gpx1*, *Gpx4*, and *Nos1* promoter in PBMCs and in the brain. Interestingly, the presented results suggested that agomelatine may have various mechanisms of action. The treatment with agomelatine may directly impact of ROS concentration or may indirectly modulate the gene expression, methylation, and activity of antioxidant enzymes. Thus, further research is needed to explain the cause of this phenomenon. It is possible that other biochemical pathways that affect the intensification of oxidative stress have a decisive impact on the antidepressant action of agomelatine.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/2073-4425/11/6/644/s1>. Table S1: Characteristics of the genes studied (All data contained in the table were compiled with the help of Genomatix Software Suite, Intrexon Bioinformatics Germany GmbH, Munich, Germany, 2019), Table S2: Conditions of the antibodies used in the Western blot analysis, Table S3: The characteristics of primers used for analysis of methylation levels in the promoter regions of the studied genes, Table S4: The effects of chronic mild stress and treatment on sucrose intake in controls, depressed rats and rats after treatment with venlafaxine (N = 6), Table S5: The methylation status of CAT promoter (A) Gpx1 promoter (B), Gpx4 promoter 2 (C) Gpx4 promoter 3 (D) SOD1 (E), SOD2 (F) NOS1 promoter 3 (G), NOS1 promoter 7 (H) in hippocampus, amygdala, hypothalamus, midbrain, cortex and basal ganglia of animals exposed to CMS procedure for two weeks (Control, Stressed) and in animals exposed to CMS procedure for seven weeks and administered vehicle (1 ml/kg) or agomelatine (10 mg/kg) for five weeks (Control/Ago, Stressed/Saline, Stressed/Ago), Figure S1: Differences in CAT (A) SOD1 (B), SOD2 (C), Gpx1 (D), Gpx4 (E) gene expression between hippocampus, amygdala, hypothalamus, midbrain, cortex and basal ganglia and PBMCs of animals exposed to CMS procedure for two weeks (Control, Stressed) and in animals exposed to CMS procedure for seven weeks and administered vehicle (1 ml/kg) or agomelatine (10 mg/kg) for five weeks (Control/Ago, Stressed/Saline, Stressed/Ago), Figure S2: CAT (A), SOD1 (B), SOD2 (C), Gpx1 (D), Gpx4 (E) mRNA expression in PBMCs and in the hippocampus, amygdala, hypothalamus, midbrain, cortex and basal ganglia of animals exposed to CMS procedure for two weeks (Control, Stressed) and in animals exposed to CMS procedure for seven weeks and administered vehicle (1 ml/kg) or agomelatine (10 mg/kg) for five weeks (Control/Ago, Stressed/Saline, Stressed/Ago), Figure S3: The methylation status of Cat promoter (A) Sod1 (B), Gpx1 (C), NOS1 promoter 7 (D) in PBMCs of animals exposed to CMS procedure for two weeks (Control, Stressed) and in animals exposed to CMS procedure for seven weeks and administered vehicle (1 ml/kg) or agomelatine (10 mg/kg) for five weeks (Control/Ago, Stressed/Saline, Stressed/Ago), Figure S4: Differences in the methylation status of CAT (A), Gpx1 (B) Gpx4 promoter 2 (C) promoter 3 (D), SOD1 (E), SOD2 (F) and NOS1 promoter 3 (G), promoter 7 (H) between hippocampus, amygdala, hypothalamus, midbrain, cortex and basal ganglia and PBMCs of animals exposed to CMS procedure for two weeks (Control, Stressed) and in animals exposed to CMS procedure for seven weeks and administered vehicle (1 ml/kg) or agomelatine (10 mg/kg) for five weeks (Control/Ago, Stressed/Saline, Stressed/Ago), Figure S5: SOD1 (A) and NOS1 (B) protein expression in animals exposed to CMS procedure for two weeks (Control, Stressed) and in animals exposed to CMS procedure for seven weeks and administered vehicle (1 ml/kg) or agomelatine (10 mg/kg) for five weeks (Control/Ago, Stressed/Saline, Stressed/Ago).

**Author Contributions:** Conceptualization, T.Ś. and M.P.; Methodology, P.W., E.S., P.J., P.C., P. G. and M.P.; Validation, P.W., E.S., P.J., P.G. and M.P.; Formal Analysis, P.W., E.S.; Investigation, P.W., E.S., P.J., P.C., K.B., M.B., G.B., P. G. and M.P.; Data Curation, T.Ś.; Writing – Original Draft Preparation, P.W.; Writing – Review & Editing, J.S., M.P., T.Ś.; Supervision, T.Ś.; Project Administration, T.Ś.; Funding Acquisition, T.Ś. All authors have read and agreed to the published version of the manuscript.

**Funding:** Source of support: This study was funded by the National Science Centre of Poland grant (2015/19/BNZ7/00410).

**Conflicts of Interest:** The authors declare no conflict of interest.

**Data Availability Statement:** The data that support the findings of this study are available from the corresponding author upon reasonable request.

## References

1. WHO. Available online: <https://www.who.int/news-room/fact-sheets/detail/depression> (accessed on 1 January 2020).
2. Greden, J.F. The burden of recurrent depression: Causes, consequences, and future prospects. *J. Clin. Psychiatry* **2001**, *62*, 5–9. [[PubMed](#)]
3. Halliwell, B. Oxidative stress and neurodegeneration: Where are we now? *J. Neurochem.* **2006**, *97*, 1634–1658. [[CrossRef](#)] [[PubMed](#)]
4. Eren, I.; Nazıroğlu, M.; Demirdağ, A. Protective effects of lamotrigine, aripiprazole and escitalopram on depression-induced oxidative stress in rat brain. *Neurochem. Res.* **2007**, *32*, 1188–1195. [[CrossRef](#)] [[PubMed](#)]
5. Eren, I.; Nazıroğlu, M.; Demirdağ, A.; Celik, O.; Uğuz, A.C.; Altunbaşak, A.; Ozmen, I.; Uz, E. Venlafaxine modulates depression-induced oxidative stress in brain and medulla of rat. *Neurochem. Res.* **2007**, *32*, 497–505. [[CrossRef](#)] [[PubMed](#)]
6. Kumar, H.; Sharma, B.M.; Sharma, B. Benefits of agomelatine in behavioral, neurochemical and blood brain barrier alterations in prenatal valproic acid induced autism spectrum disorder. *Neurochem. Int.* **2015**, *91*, 34–45. [[CrossRef](#)]
7. Maes, M.; Galecki, P.; Chang, Y.S.; Berk, M. A review on the oxidative and nitrosative stress (O&NS) pathways in major depression and their possible contribution to the (neuro) degenerative processes in that illness. *Prog. Neuro Psychopharmacol. Biol. Psychiatry* **2011**, *35*, 676–692.

8. Kodydková, J.; Vavrova, L.; Zeman, M.; Jiráček, R.; Macáček, J.; Stankova, B.; Tvrzicka, E.; Žák, A.; Rychlikova, J. Antioxidative enzymes and increased oxidative stress in depressive women. *Clin. Biochem.* **2009**, *42*, 1368–1374. [[CrossRef](#)]
9. Maes, M.; De Vos, N.; Pioli, R.; Demedts, P.; Wauters, A.; Neels, H.; Christophe, A. Lower serum vitamin E concentrations in major depression: Another marker of lowered antioxidant defenses in that illness. *J. Affect. Disord.* **2000**, *58*, 240–246. [[CrossRef](#)]
10. Maes, M.; Mihaylova, I.; Kubera, M.; Uytterhoeven, M.; Vrydags, N.; Bosmans, E. Coenzyme Q10 deficiency in myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is related to fatigue, autonomic and neurocognitive symptoms and is another risk factor explaining the early mortality in ME/CFS due to cardiovascular disorder. *Neuro Endocrinol. Lett.* **2009**, *30*, 470–476.
11. Samuelsson, M.; Gerdin, G.; Ollinger, K.; Vrethem, M. Taurine and glutathione levels in plasma before and after ECT treatment. *Psychiatry Res.* **2012**, *198*, 53–57. [[CrossRef](#)]
12. Suh, S.W.; Won, S.J.; Hamby, A.M.; Yoo, B.H.; Fan, Y.; Sheline, C.T.; Tamano, H.; Takeda, A.; Liu, J. Decreased brain zinc availability reduces hippocampal neurogenesis in mice and rats. *J. Cereb. Blood Flow Metab.* **2009**, *29*, 1579–1588. [[CrossRef](#)] [[PubMed](#)]
13. Liu, T.; Zhong, S.; Liao, X.; Chen, J.; He, T.; Lai, S.; Jia, Y. A meta-analysis of oxidative stress markers in depression. *PLoS ONE* **2015**, *10*, e0138904. [[CrossRef](#)] [[PubMed](#)]
14. Maes, M.; Mihaylova, I.; Kubera, M.; Uytterhoeven, M.; Vrydags, N.; Bosmans, E. Lower whole blood glutathione peroxidase (GPX) activity in depression, but not in myalgic encephalomyelitis / chronic fatigue syndrome: Another pathway that may be associated with coronary artery disease and neuroprogression in depression. *Neuro Endocrinol. Lett.* **2011**, *32*, 133–140. [[PubMed](#)]
15. Rybka, J.; Kedziora-Kornatowska, K.; Banas-Lezanska, P.; Majsterek, I.; Carvalho, L.A.; Cattaneo, A.; Anacker, C.; Kędziora, J. Interplay between the pro-oxidant and antioxidant systems and proinflammatory cytokine levels, in relation to iron metabolism and the erythron in depression. *Free Radic. Biol. Med.* **2013**, *63*, 187–194. [[CrossRef](#)]
16. Herken, H.; Gurel, A.; Selek, S.; Armutcu, F.; Ozen, M.E.; Bulut, M.; Kap, O.; Yumru, M.; Savas, H.A.; Akyol, O. Adenosine deaminase, nitric oxide, superoxide dismutase, and xanthine oxidase in patients with major depression: Impact of antidepressant treatment. *Arch. Med. Res.* **2007**, *38*, 247–252. [[CrossRef](#)] [[PubMed](#)]
17. Stefanescu, C.; Ciobica, A. The relevance of oxidative stress status in first episode and recurrent depression. *J. Affect. Disord.* **2012**, *143*, 34–38. [[CrossRef](#)]
18. Herken, H.; Akyol, O.; Yilmaz, H.R.; Tutkun, H.; Savas, H.A.; Ozen, M.E.; Kalenderoglu, A.; Gulec, M. Nitric oxide, adenosine deaminase, xanthine oxidase and superoxide dismutase in patients with panic disorder: Alterations by antidepressant treatment. *Hum. Psychopharmacol. Clin. Exp.* **2006**, *21*, 53–59. [[CrossRef](#)]
19. Michel, T.M.; Camara, S.; Tatschner, T.; Frangou, S.; Sheldrick, A.J.; Riederer, P.; Grünblatt, E. Increased xanthine oxidase in the thalamus and putamen in depression. *World J. Biol. Psychiatry* **2010**, *11*, 314–320. [[CrossRef](#)]
20. Bakunina, N.; Pariante, C.M.; Zunszain, P.A. Immune mechanisms linked to depression via oxidative stress and neuroprogression. *Immunology* **2015**, *144*, 365–373. [[CrossRef](#)]
21. Lange, C.; Irle, E. Enlarged amygdala volume and reduced hippocampal volume in young women with major depression. *Psychol. Med.* **2004**, *34*, 1059–1064. [[CrossRef](#)]
22. Pandi-Perumal, S.R.; Srinivasan, V.; Cardinali, D.P.; Monti, M.J. Could agomelatine be the ideal antidepressant? *Expert Rev. Neurother.* **2006**, *6*, 1595–1608. [[CrossRef](#)]
23. Joffe, R.T.; Levitt, A.J.; Sokolov, S.T. Augmentation strategies: Focus on anxiolytics. *J. Clin. Psychiatry* **1996**, *57*, 25–31. [[PubMed](#)]
24. Michel, T.M.; Pülschen, D.; Thome, J. The role of oxidative stress in depressive disorders. *Curr. Pharm. Des.* **2012**, *18*, 5890–5899. [[CrossRef](#)] [[PubMed](#)]
25. Espino, J.; Bejarano, I.; Paredes, S.D.; Barriga, C.; Rodríguez, A.B.; Pariente, J.A. Protective effect of melatonin against human leukocyte apoptosis induced by intracellular calcium overload: Relation with its antioxidant actions. *J. Pineal Res.* **2011**, *51*, 195–206. [[CrossRef](#)] [[PubMed](#)]
26. Weaver, D.R. Melatonin and Circadian Rhythmicity in Vertebrates. In *Neurobiology of Sleep and Circadian Rhythms*; Turek, F.W., Zee, P., Eds.; Dekker: New York, NY, USA, 1999; pp. 197–262.



27. Yous, S.; Andrieux, J.; Howell, H.E.; Morgan, P.J.; Renard, P.; Pfeiffer, B.; Lesieur, D.; Guardiola-Lemaître, B. Novel naphthalenic ligands with high affinity for the melatonin receptor. *J. Med. Chem.* **1992**, *35*, 1484–1486. [[CrossRef](#)] [[PubMed](#)]
28. Millan, M.J.; Gobert, A.; Lejeune, F.; Dekeyne, A.; Newman-Tancredi, A.; Pasteau, V.; Rivet, J.M.; Cussac, D. The novel melatonin agonist agomelatine (S20098) is an antagonist at 5-hydroxytryptamine<sub>2C</sub> receptors, blockade of which enhances the activity of frontocortical dopaminergic and adrenergic pathways. *J. Pharmacol. Exp. Ther.* **2003**, *306*, 954–964. [[CrossRef](#)] [[PubMed](#)]
29. de Bodinat, C.; Guardiola-Lemaitre, B.; Mocaer, E.; Renard, P.; Munoz, C.; Millan, M.J. Agomelatine, the first melatonergic antidepressant: Discovery, characterization and development. *Nat. Rev. Drug Discov.* **2010**, *9*, 628–642. [[CrossRef](#)]
30. Krauchi, K.; Cajochen, C.; Mori, D.; Graw, P.; Wirz-Justice, A. Early evening melatonin and S-20098 advance circadian phase and nocturnal regulation of core body temperature. *Am. J. Physiol.* **1997**, *272*, R1178–R1188. [[CrossRef](#)]
31. Grassi-Zucconi, G.; Semprevivo, M.; Mocaer, E.; Kristensson, K.; Bentivoglio, M. Melatonin and its new agonist S-20098 restore synchronized sleep fragmented by experimental trypanosome infection in the rat. *Brain Res. Bull.* **1996**, *39*, 63–68. [[CrossRef](#)]
32. Redman, J.R.; Guardiola-Lemaitre, B.; Brown, M.; Delagrange, P.; Armstrong, S.M. Dose dependent effects of S-20098, a melatonin agonist, on direction of re-entrainment of rat circadian activity rhythms. *Psychopharmacology* **1995**, *118*, 385–390. [[CrossRef](#)]
33. Van Reeth, O.; Weibel, L.; Olivares, E.; Maccari, S.; Mocaer, E.; Turek, F.W. Melatonin or a melatonin agonist corrects age-related changes in circadian response to environmental stimulus. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **2001**, *280*, R1582–R1591. [[CrossRef](#)] [[PubMed](#)]
34. Leproult, R.; Van Onderbergen, A.; L’Hermite-Baleriaux, M.; Van Cauter, E.; Copinschi, G. Phase-shifts of 24-h rhythms of hormonal release and body temperature following early evening administration of the melatonin agonist agomelatine in healthy older men. *Clin. Endocrinol.* **2005**, *63*, 298–304. [[CrossRef](#)] [[PubMed](#)]
35. Gupta, S.; Sharma, B. Pharmacological benefits of agomelatine and vanillin in experimental model of Huntington’s disease. *Pharmacol. Biochem. Behav.* **2014**, *122*, 122–135. [[CrossRef](#)]
36. Inanir, S.; Copoglu, U.S.; Kokacya, H.; Dokuyucu, R.; Erbas, O.; Inanir, A. Agomelatine protection in an LPS-induced psychosis-relevant behavior model. *Med. Sci. Monit.* **2015**, *21*, 3834–3839. [[CrossRef](#)] [[PubMed](#)]
37. Karakus, E.; Halici, Z.; Albayrak, A.; Polat, B.; Bayir, Y.; Kiki, I.; Cadirci, E.; Topcu, A.; Aksak, S. Agomelatine: An antidepressant with new potent hepatoprotective effects on paracetamol-induced liver damage in rats. *Hum. Exp. Toxicol.* **2013**, *32*, 846–857. [[CrossRef](#)]
38. Wigner, P.; Synowiec, E.; Czarny, P.; Bijak, M.; Jóźwiak, P.; Szemraj, J.; Gruca, P.; Papp, M.; Śliwiński, T. Effects of venlafaxine on the expression level and methylation status of genes involved in oxidative stress in rats exposed to a chronic mild stress. *J. Cell Mol. Med.* **2020**, *24*. [[CrossRef](#)]
39. Papp, M. Models of affective illness: Chronic mild stress in the rat. *Curr. Protoc. Pharmacol.* **2012**, *57*. [[CrossRef](#)]
40. Papp, M.; Gruca, P.; Boyer, P.-A.; Mocaer, E. Effect of agomelatine in the chronic mild stress model of depression in the rat. *Neuropsychopharmacol* **2003**, *28*, 694–703. [[CrossRef](#)]
41. Schmittgen, T.D.; Livak, K.J. Analyzing real-time PCR data by the comparative C(T) method. *Nat. Protoc.* **2008**, *3*, 1101–1110. [[CrossRef](#)]
42. Wojdacz, T.K.; Dobrovic, A. Methylation-sensitive high resolution melting (MS-HRM): A new approach for sensitive and high-throughput assessment of methylation. *Nucleic Acids Res.* **2007**, *35*, e41. [[CrossRef](#)]
43. Wojdacz, T.K.; Dobrovic, A.; Hansen, L.L. Methylation-sensitive high-resolution melting. *Nat. Protoc.* **2008**, *3*, 1903–1908. [[CrossRef](#)]
44. Wojdacz, T.K.; Borgbo, T.; Hansen, L.L. Primer design versus PCR bias in methylation independent PCR amplifications. *Epigenetics* **2009**, *4*, 231–234. [[CrossRef](#)]
45. Lowry, O.H.; Rosebrough, N.J.; Farr, A.L.; Randall, R.J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275.
46. Gamaro, G.D.; Manoli, L.P.; Torres, I.L.; Silveira, R.; Dalmaz, C. Effects of chronic variate stress on feeding behavior and on monoamine levels in different rat brain structures. *Neurochem. Int.* **2003**, *42*, 107–114. [[CrossRef](#)]

47. Bekris, S.; Antoniou, K.; Daskas, S.; Papadopoulou-Daifoti, Z. Behavioural and neurochemical effects induced by chronic mild stress applied to two different rat strains. *Behav. Brain Res.* **2005**, *161*, 45–59. [[CrossRef](#)]
48. Demyttenaere, K. Agomelatine: A narrative review. *Eur. Neuro Psychopharmacol.* **2011**, *21*, S703–S709. [[CrossRef](#)]
49. Liu, J.; Wang, X.; Shigenaga, M.K.; Yeo, H.C.; Mori, A.; Ames, B.N. Immobilization stress causes oxidative damage to lipid, protein, and DNA in the brain of rats. *FASEB J.* **1996**, *10*, 1532–1538. [[CrossRef](#)]
50. Fontella, F.U.; Siqueira, I.R.; Vasconcellos, A.P.; Tabajara, A.S.; Netto, C.A.; Dalmaz, C. Repeated restraint stress induces oxidative damage in rat hippocampus. *Neurochem. Res.* **2005**, *30*, 105–111. [[CrossRef](#)]
51. Lucca, G.; Comim, C.M.; Valvassori, S.S.; Réus, G.Z.; Vuolo, F.; Petronilho, F.; Dal-Pizzol, F.; Gavioli, E.C.; Quevedo, J. Effects of chronic mild stress on the oxidative parameters in the rat brain. *Neurochem. Int.* **2009**, *54*, 358–362. [[CrossRef](#)]
52. Bilici, M.; Efe, H.; Koroğlu, M.; Uydu, H.A.; Bekaroğlu, M.; Değer, O. Antioxidative enzyme activities and lipid peroxidation in major depression: Alterations by antidepressant treatments. *J. Affect. Disord.* **2001**, *64*, 43–51. [[CrossRef](#)]
53. Demirdaş, A.; Nazıroğlu, M.; Ünal, G.Ö. Agomelatine reduces brain, kidney and liver oxidative stress but increases plasma cytokine production in the rats with chronic mild stress-induced depression. *Metab. Brain Dis.* **2016**, *31*, 1445–1453. [[CrossRef](#)]
54. Akpınar, A.; Uğuz, A.C.; Nazıroğlu, M. Agomelatine and duloxetine synergistically modulates apoptotic pathway by inhibiting oxidative stress triggered intracellular calcium entry in neuronal PC12 cells: Role of TRPM2 and voltage-gated calcium channels. *J. Membr. Biol.* **2014**, *247*, 451–459. [[CrossRef](#)]
55. Aguiar, C.C.T.; Almeida, A.B.; Araújo, P.V.P.; Vasconcelos, G.S.; Chaves, E.M.C.; Vale, O.C.D.; Macedo, D.S.; Leal, L.K.A.M.; Viana, G.S.D.B.; Vasconcelos, S. Effects of agomelatine on oxidative stress in the brain of mice after chemically induced seizures. *Cell Mol. Neurobiol.* **2013**, *33*, 825–835. [[CrossRef](#)]
56. Kharwar, R.K.; Haldar, C. Daily variation in antioxidant enzymes and lipid peroxidation in lungs of a tropical bird *Perdica asiatica*: Role of melatonin and nuclear receptor ROR $\alpha$ . *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **2012**, *162*, 296–302. [[CrossRef](#)]
57. Tsai, M.C.; Huang, T.L. Increased activities of both superoxide dismutase and catalase were indicators of acute depressive episodes in patients with major depressive disorder. *Psychiatry Res.* **2016**, *235*, 38–42. [[CrossRef](#)]
58. De Mello, A.H.; Souza, L.D.R.; Cereja, A.C.M.; Schraiber, R.D.B.; Florentino, D.; Martins, M.M.; Rezin, G.T.; Petronilho, F.; Quevedo, J. Effect of subchronic administration of agomelatine on brain energy metabolism and oxidative stress parameters in rats. *Psychiatry Clin. Neurosci.* **2016**, *70*, 159–166. [[CrossRef](#)]
59. Wang, L.; Li, X.X.; Chen, X.; Qin, X.Y.; Kardami, E.; Cheng, Y. Antidepressant-like effects of low- and high-molecular weight FGF-2 on chronic unpredictable mild stress mice. *Front. Mol. Neurosci.* **2018**, *11*, 377. [[CrossRef](#)]
60. Selek, S.; Savas, H.A.; Gergerlioglu, H.S.; Bulbul, F.; Uz, E.; Yumru, M. The course of nitric oxide and superoxide dismutase during treatment of bipolar depressive episode. *J. Affect. Disord.* **2008**, *107*, 89–94. [[CrossRef](#)]
61. Suzuki, E.; Yagi, G.; Nakaki, T.; Kanba, S.; Asai, M. Elevated plasma nitrate levels in depressive states. *J. Affect. Disord.* **2001**, *63*, 221–224. [[CrossRef](#)]
62. Scapagnini, G.; Davinelli, S.; Drago, F.; De Lorenzo, A.; Oriani, G. Antioxidants as antidepressants: Fact or fiction? *CNS Drugs* **2012**, *26*, 477–490. [[CrossRef](#)]
63. Pozo, D.; Reiter, R.J.; Calvo, J.R.; Guerrero, J.M. Inhibition of cerebellar nitric oxide synthase and cyclic GMP production by melatonin via complex formation with calmodulin. *J. Cell Biochem.* **1997**, *65*, 430–442. [[CrossRef](#)]
64. Acuña-Castroviejo, D.; Escames, G.; López, L.C.; Hitos, A.B.; León, J. Melatonin and nitric oxide. *Endocrine* **2005**, *27*, 159–168. [[CrossRef](#)]
65. Storr, M.; Koppitz, P.; Sibae, A.; Saur, D.; Kurjak, M.; Franck, H.; Schusdzarra, V.; Allescher, H.-D. Melatonin reduces non-adrenergic, non-cholinergic relaxant neurotransmission by inhibition of nitric oxide synthase activity in the gastrointestinal tract of rodents in vitro. *J. Pineal Res.* **2002**, *33*, 101–108. [[CrossRef](#)]
66. Drevets, W.C.; Price, J.L.; Furey, M.L. Brain structural and functional abnormalities in mood disorders: Implications for neurocircuitry models of depression. *Brain Struct. Funct.* **2008**, *213*, 93–118. [[CrossRef](#)]



67. Duman, R.S.; Aghajanian, G.K. Synaptic dysfunction in depression: Potential therapeutic targets. *Science* **2012**, *338*, 68–72. [[CrossRef](#)]
68. Czarny, P.; Wigner, P.; Galecki, P.; Sliwinski, T. The interplay between inflammation, oxidative stress, DNA damage, DNA repair and mitochondrial dysfunction in depression. *Prog. Neuro Psychopharmacol. Biol. Psychiatry* **2018**, *80*, 309–321. [[CrossRef](#)]
69. Wigner, P.; Czarny, P.; Galecki, P.; Su, K.P.; Sliwinski, T. The molecular aspects of oxidative & nitrosative stress and the tryptophan catabolites pathway (TRYCATs) as potential causes of depression. *Psychiatry Res.* **2018**, *262*, 566–574.



© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).