Chapter

Melatonin Modulates Hypophyseal-Thyroid Function through Differential Activation of MT1 and MT2 Receptors in Hypothyroid Mice

Shiv Shankar Singh, Prashanjit Laskar, Anindita Deb and Sangita Sutradhar

Abstract

Hypothyroidism is characterized by the low level of thyroid hormones in circulation, which affects the normal metabolic activities of organisms. Propylthiouracil (PTU) induced hypothyroid condition impairs the antioxidant defense system and therefore normal physiology alters. Melatonin influences most physiological activities and is also known for its antioxidative properties. Melatonin modulates physiological activities through receptor-mediated as well as non-receptor-mediated pathways. In this study, we evaluated the involvement of melatonin MT1 and MT2 receptors in the modulation of hypophyseal-thyroid function in PTU-induced hypothyroid mice. We have noted the decreased level of T3 and T4 and increased level of TSH hormone in PTU-treated mice. Melatonin treatment counteracted the PTU-caused changes in circulatory T3, T4, and TSH hormones. PTU treatment caused increased MT1 receptor protein expression in the thyroid as well as the pituitary gland while increased MT2 receptor protein in the pituitary gland. Melatonin treatment caused increased TSH receptor protein in the thyroid gland. Melatonin induced MT2 receptor protein expression in both the thyroid and pituitary glands whereas MT1 receptor proteins in the pituitary gland. This study may suggest that melatonin regulates hypophyseal-thyroid function through differential sensitization of MT1 and MT2 receptors on the pituitary and thyroid glands in hypothyroid mice.

Keywords: melatonin, propylthiouracil, pituitary, thyroid, hypothyroid, melatonin receptors

1. Introduction

Thyroid hormones play a vital role in the physiology of the organism by influencing almost all tissues to grow and it maintains normal cognition, cardiovascular function, bone health, metabolism, and energy balance. Pathological disorders in the thyroid gland bring about functional changes in different organs of the body. The cardiovascular derangements were observed after the altered action of thyroid hormone on certain molecular pathways in the heart and relevant vasculature [1, 2].

Hypothyroidism is a clinical syndrome caused by decreased thyroid activity. Hypothyroidism has been associated with a sub-metabolic state with lowered energy and oxygen metabolism [3]. PTU-induced hypothyroidism impairs the antioxidant defense system as well as the physiological system of gonads during development and maturation in Wistar rats [4]. Perinatal disruption of thyroid function leads to disorders in physiological networks, including the central nervous system [5] and the immune system [6]. Development of hypothyroidism through the ingestion of methimazole or propylthiouracil in maternal rats resulted in the transfer of these drugs to the offspring and induction of several immunological changes, including a relative increase in the proportion of Treg cells in the spleen [7].

Hypothyroidism also led to changes in oxidant and antioxidant systems [8–10]. Further, neuronal developmental pattern related to oxidative stress and the antioxidant system was also affected in rat offspring by maternal hypothyroidism [11]. Melatonin hormone has antioxidative properties and protects membrane lipids, cytoplasmic proteins, and nuclear DNA [12]. Moreover, melatonin stimulates gene expression and the activity of the antioxidant enzymes glutathione peroxidase, superoxide dismutase, and catalase [13–15]. According to Thakkar et al. [16], melatonin performs the synergistic, cumulative, or antagonistic effects through which it institutes the effects of thyroid deficiency in the neonatal period of a rat.

However, melatonin mediates most of its physiological effects including modulation of immune function through activation of G-protein coupled MT1 and MT2 cell surface receptors. Further, melatonin receptors are also localized on various tissues and cells including the thyroid follicular and parafollicular cells. But how melatonin receptors are responding to the modulation of pituitary-thyroid function in the hypothyroid condition has not been studied. Therefore, in this study, we made an attempt to explore the effect of melatonin on modulation of MT1 and MT2 receptor protein expression pattern and hypophyseal-thyroid function in experimentally induced hypothyroid mice.

2. Materials and methods

All of the experiments with animals and their maintenance have been done according to the institutional practice and with the framework of CPCSEA (Committee for the Purpose of Control and Supervision of Experimental Animals) and the Act of Government of India (2007) for the animal welfare.

2.1 Experimental design

Healthy mice colony was housed at ambient laboratories conditions (under 12L:12D cycles and 25 ± 2°C temperature). Mice were kept in groups of five in polycarbonate cages (43 cm × 27 cm × 14 cm) to avoid the population stress and were fed regularly with mice feed and water *ad libitum*. Healthy male Swiss-albino mice were selected from the housed colony and were divided into five groups having five mice in each.

Group I	Control (Con)
Group II	Melatonin (Mel)
Group III	Propylthiouracil (PTU)
Group IV	(Mel + PTU)

The control group of mice received ethanolic saline (0.01% ethanol), 0.1 mL/day for consecutive 30 days. The second group of mice received subcutaneous injections of melatonin (Sigma-Aldrich Chemicals, St. Louis, USA), 25 μg/100 g BW/day for consecutive 30 days during evening (4:30–5:00 pm) hours. The third group of mice received 5-propyl-2-thiouracil, PTU (Sigma-Aldrich Chemicals, St. Louis, USA) in the drinking water, 60 μg/g BW/day for consecutive 18 days [17]. The fourth group of mice received melatonin for consecutive 30 days and also received PTU for the last 18 days of the experimental period of melatonin treatment.

After 24 h of last administration, experimental mice were sacrificed under anesthesia (pentobarbital, 15 mg/kg, intraperitoneal injection) and trunk blood was collected. Serum was separated and stored at −20°C until analyzed. Experimental tissues (pituitary and thyroid) were dissected out on the ice. One part of the thyroid gland was fixed in Bouin's for histological analysis. Thyroid and pituitary glands were stored at −20°C for Western blot analysis.

2.2 Hormonal analysis

The levels of T3, T4, and TSH hormones in the blood were tested using commercial ELISA kits (Diagnostic Automation Inc., USA) as directed by the manufacturer. T3 has a detection range of 0–10 ng/mL, with a specificity of 96.30% and a sensitivity of 0.2 ng/mL. T4 has a detection range of 0–30 μg/dL, with a sensitivity of 0.05 g/mL and a specificity of 96.30 percent. TSH had a detection range of 0–40 IU/mL, a 100% specificity, and a sensitivity of 0.20 IU/mL.

2.3 Histology

Thyroid glands were fixed overnight in Bouin's fixative and processed for paraffin block preparation and sectioning. Mayer's albumin-coated slide was used to stretch sections of 5 μm thickness. Routine hematoxylin–eosin double staining procedures were used to stain thyroid sections. Stained sections of the thyroid gland were examined under the 40X objective of Olympus BX-41 Microscope and micrographs were taken.

2.4 Western blot analysis

Tissues were homogenized in RIPA buffer (NP-40 (1%, v/v), sodium dodecyl sulfate (SDS) (1%, v/v) in PBS supplemented with phenylmethyl sulphonyl fluoride (PMSF), sodium orthovanadate and aprotinin). The total protein content of the sample was determined using the Lowry method [18]. Protein aliquots (100 μg) were resolved on a 10% (w/v) SDS polyacrylamide gel and electrotransferred to nitrocellulose membrane (Santa Cruz Biotech, USA). Primary antibodies (sc-13186, Mel 1AR (MT1); sc-13177, Mel 1BR (MT2); sc-7818, TSH-R; goat IgG, diluted 1:200; and sc-130656, β-actin antibody, rabbit IgG, Santacruz Biotech, USA, diluted 1:500) were used for immune detection. Primary antibodies were diluted in 5% skimmed milk in PBS containing 0.01% Tween-20. Secondary antibodies (goat anti-rabbit IgG-HRP and rabbit anti-goat IgG-HRP, diluted 1:1000) were used. Super Signal West Pico Chemiluminescent Substrate (#34080, Thermo Scientific, Rockford, USA) was used to identify immunological interactions. Scion Image Analysis Software (Scion Corporation, MD, USA) was used to determine the optical density measurement of the band intensity. The ratio of the specific signal to β-actin signal density was determined and presented as the % control value [19].

2.5 Statistical analysis

Statistical analysis of the data was performed using SPSS 17.0 (SPSS Corp., USA) program with one-way ANOVA followed by Tukey's honest significant difference (HSD) multiple range test. The differences were considered significant when $p < 0.05$.

3. Results

3.1 Effect of melatonin on serum level of T_3 and T_4 hormones

In this study, 5-propylthiouracil (PTU) was used to induce hypothyroid condition in experimental mice. 5-propylthiouracil interacts with the thyroid peroxidase enzyme and inhibits its activity. Thyroid peroxidase is an important enzyme involved in the iodination of tyrosine amino acids present in thyroglobulin at the luminal surface of follicular cells. Further, random coupling of iodinated tyrosine produces triiodothyronine (T3) and tetraiodothyronine (T4) on thyroglobulin on the luminal surface of follicular cells. Treatment of PTU caused significant ($p < 0.01$) suppression of circulatory T3 and T4 levels in mice (**Figures 1** and **2**). The persistent low level of T3 and T4 caused hypothyroid pathology in mice. PTU is a known antithyroid drug and is used for the treatment of hyperthyroidism in human beings. Melatonin treatment to healthy mice caused significant ($p < 0.01$) suppression of circulatory T3 and T4 levels, whereas melatonin treatment to hypothyroid groups of mice caused a significant ($p < 0.01$) increase of both T3 and T4 hormone levels.

3.2 Effect of melatonin on anatomical changes in the thyroid gland

Thyroid gland is a bilobed structure present on the trachea at apposition below the cricoid cartilage. In mammals, both lobes of the thyroid gland are joined by a

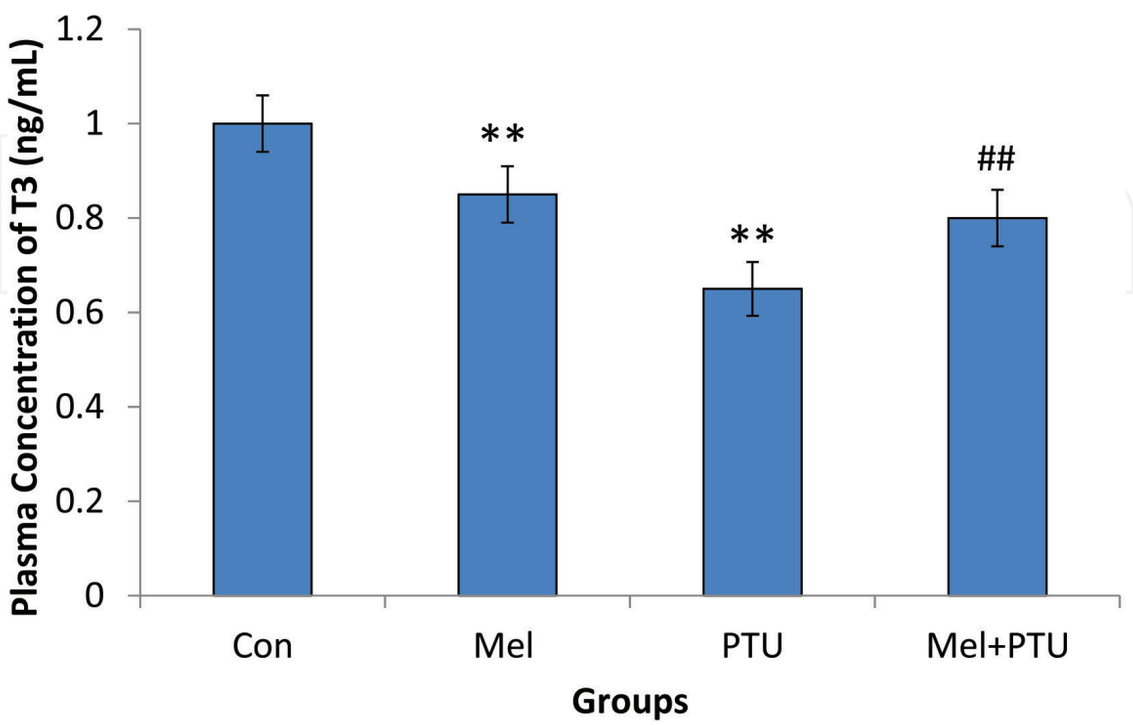

Figure 1.
Plasma T_3 concentration of experimental groups of mice. Histogram represents mean ± SEM. The mean differences were considered significant when $p < 0.01$. $^{**}p < 0.01$: Con vs. Mel; Con vs. PTU; $^{\#\#}p < 0.01$: PTU vs. Mel + PTU.

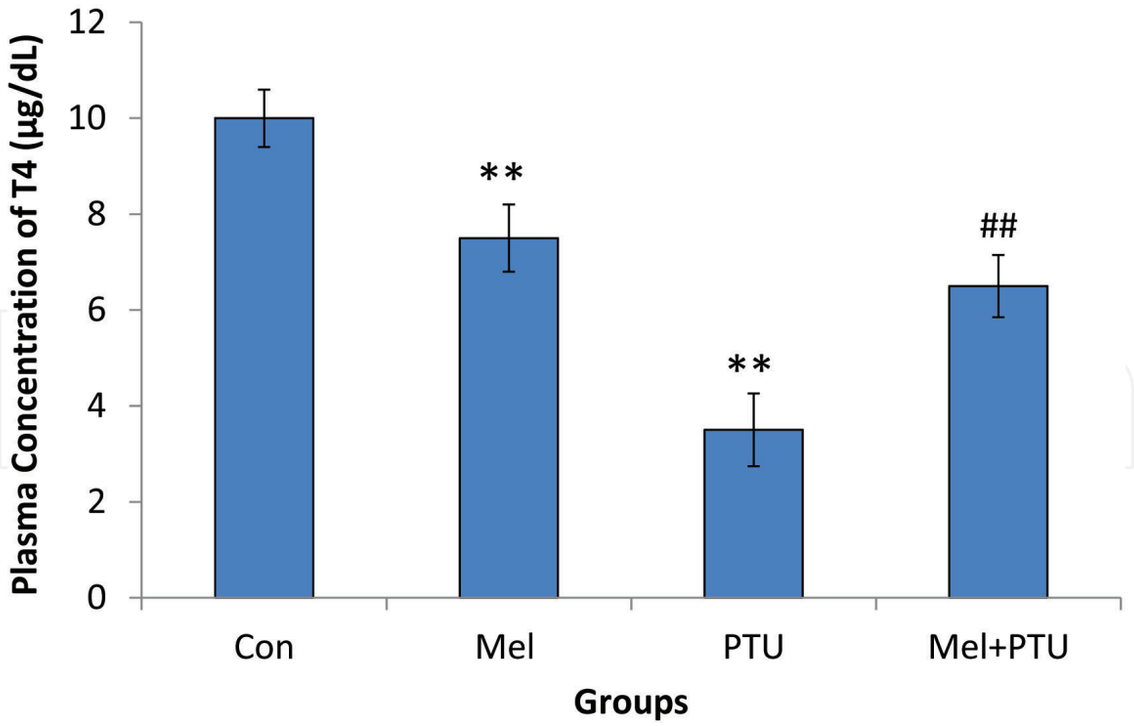

Figure 2.
Serum T_4 concentration of experimental groups of mice. Histogram represents mean ± SEM. The mean differences were considered significant when $p < 0.01$. $^{**}p < 0.01$: Con vs. Mel; Con vs. PTU; $^{\#\#}p < 0.01$: PTU vs. Mel + PTU.

narrow isthmus of tissue. Anatomically, the thyroid gland consists of follicles surrounded by a single layer of cuboidal epithelium. These thyroid follicles are a functional unit of the thyroid gland. These follicles contain lumens, which are filled with colloid materials. This colloid contains iodinated thyronine (i.e., 3,5,3′5′-tetraiodothyronine, T_4 and 3,5,3′-triiodothyronine, T_3) and iodinated tyrosine (3-monoiodotyrosine, MIT and 3,5-diiodotyrosine, DIT) on thyroglobulin molecule. In the control mice, normal size of follicles filled with colloid was observed. PTU-induced hypothyroid mice showed the abnormal shape of thyroid follicles with enlarged follicular cells (**Figure 3**). Follicles were observed with the small size of the luminal area. Melatonin-treated mice showed a normal size of thyroidal follicles. Melatonin-treated hypothyroid mice showed restoration of thyroidal follicles.

3.3 Effect of melatonin on serum TSH level and TSH receptor protein in the thyroid gland

Melatonin regulates the circadian rhythmicity of the neuroendocrine secretion. Melatonin also affects the secretory activity of the pituitary-thyroid axis. Serum TSH hormone level remains unaltered, whereas TSH receptor expression in the thyroid gland increased after melatonin treatment (**Figures 4** and **5**). PTU treatment increased the circulatory TSH hormone but TSH receptor expression in the thyroid gland remained unaffected. In melatonin-supplemented hypothyroid mice, TSH hormone level increased, whereas TSH receptor expression on the thyroid gland was unchanged in comparison with the hypothyroid group.

3.4 Effect of melatonin on MT1 and MT2 receptor proteins in the thyroid gland

Melatonin treatment significantly decreased the MT1 receptor protein in the thyroid gland whereas it significantly increased the MT2 receptor protein in the

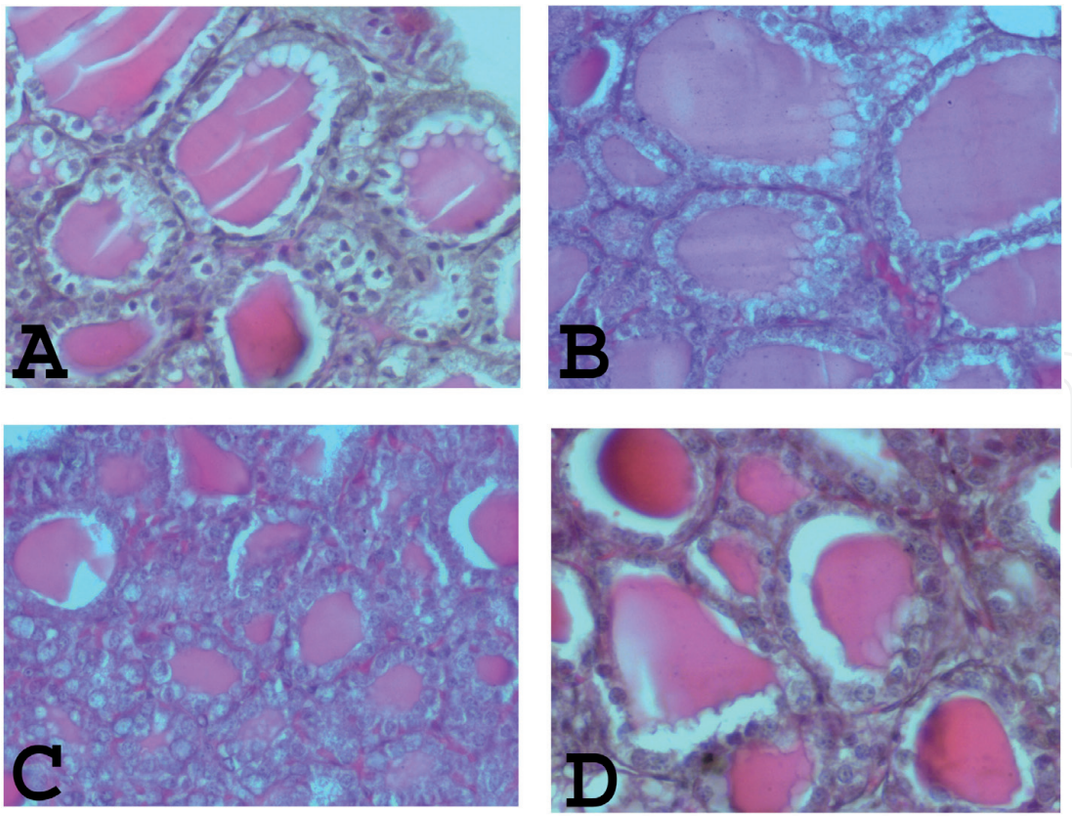

Figure 3.
Micrographs showing effects of melatonin on histology of thyroid in hypothyroid mice. Micrographs were taken at 40× objective of Olympus microscope BX-40. (A) control, (B) melatonin treated, (C) PTU treated, and (D) both melatonin and PTU treated.

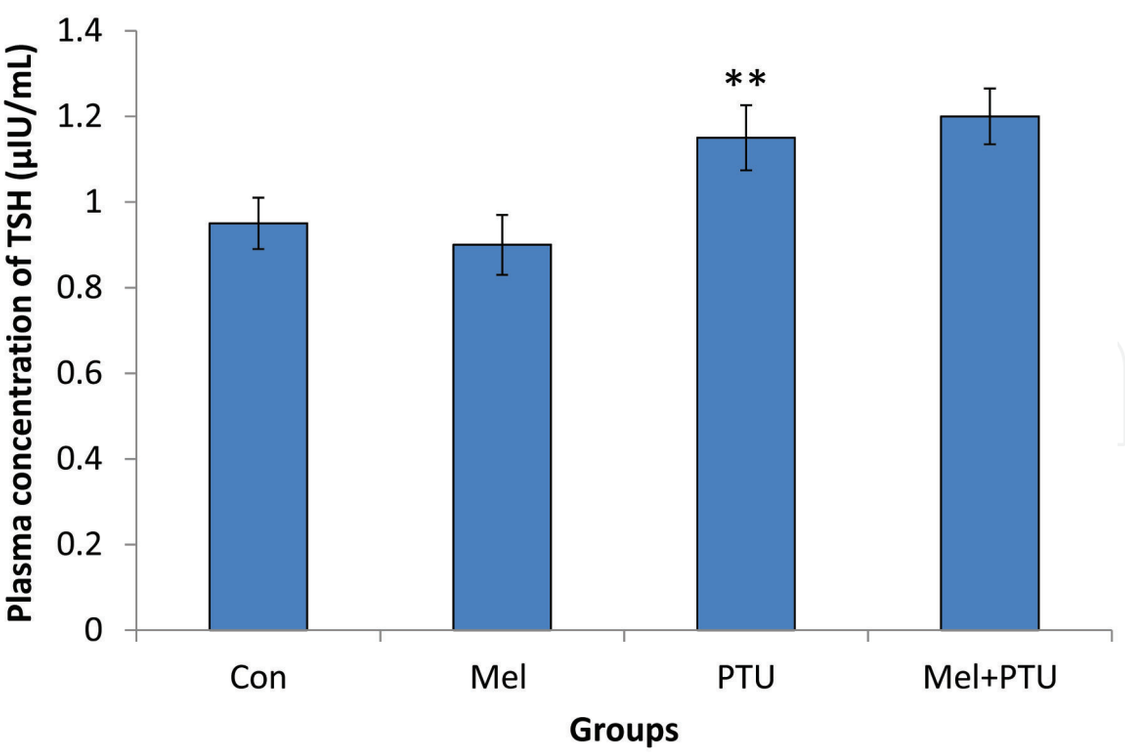

Figure 4.
Serum TSH concentration of experimental groups of mice. Histogram represents mean ± SEM. The mean differences were considered significant when $p < 0.01$. $^{**}p < 0.01$: Con vs. PTU.

thyroid gland (**Figures 6** and 7). PTU treatment caused a significant increase of MT1 and MT2 receptor proteins in the thyroid gland of mice in comparison with control mice. PTU caused stress in hypothyroid mice and induced the expression of

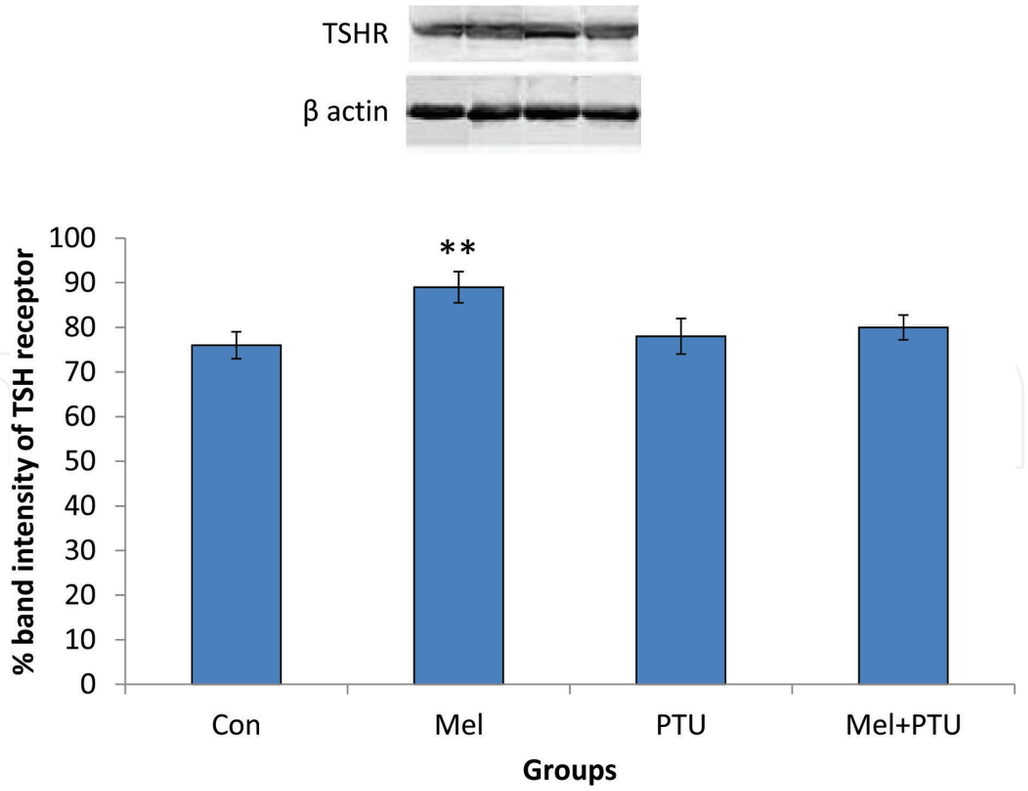

Figure 5.
Western blot analysis of TSH receptor protein expression in thyroid gland. β-actin was used as loading control. Lower panel shows percent expression of protein following Scion image analysis. Histogram represents mean ± SEM. The mean differences were considered significant when $p < 0.01$. $^{**}p < 0.01$: Con vs. Mel.

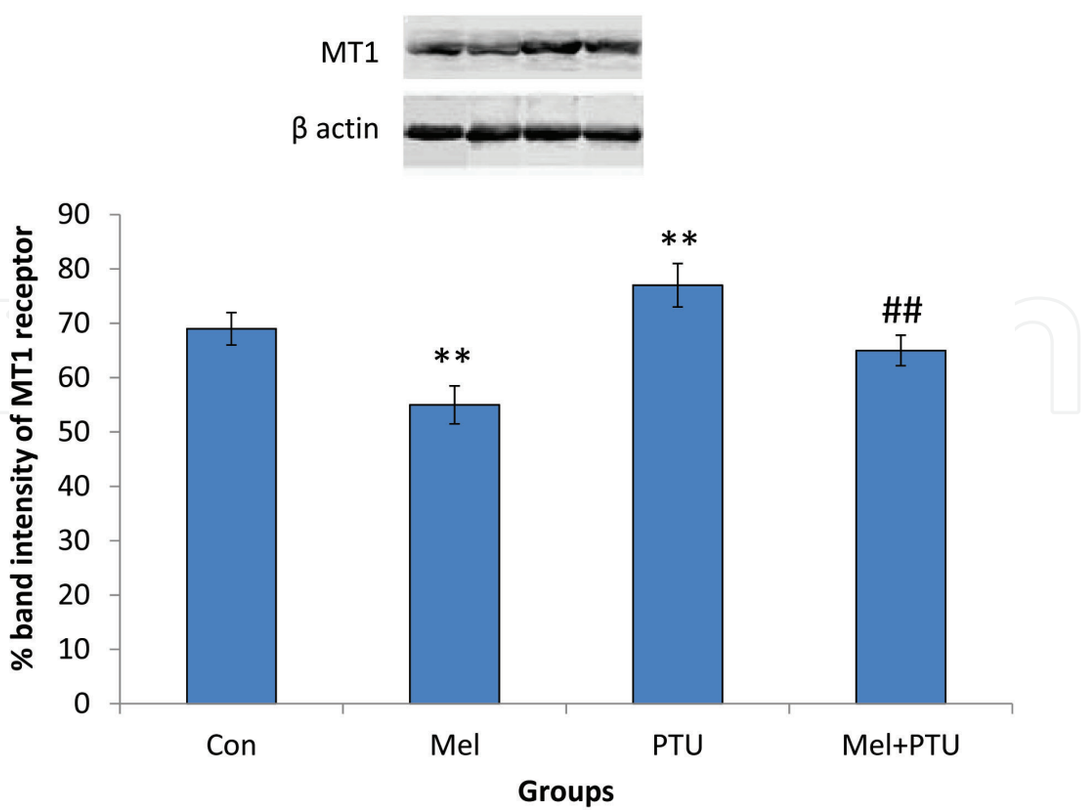

Figure 6.
Western blot analysis of MT1 receptor protein expression in thyroid gland. β-actin was used as loading control. Lower panel shows percent expression of protein following Scion image analysis. Histogram represents mean ± SEM. The mean differences were considered significant when $p < 0.01$. $^{**}p < 0.01$: Con vs. Mel; Con vs. PTU; $^{\#\#}p < 0.01$: PTU vs. Mel + PTU.

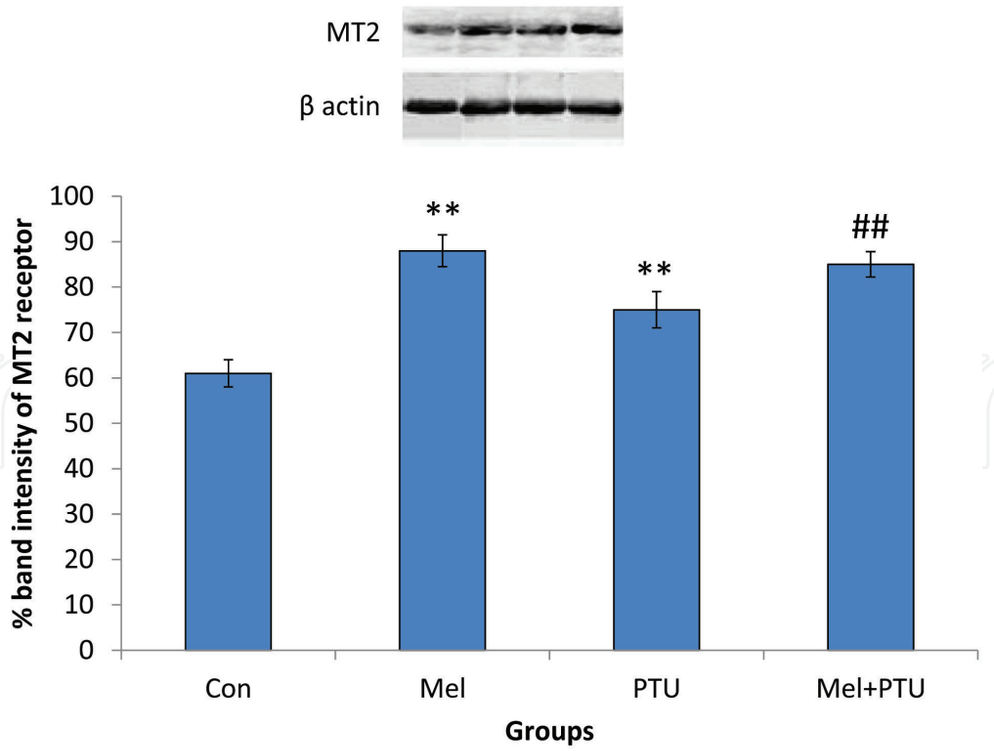

Figure 7.
Western blot analysis of MT2 receptor protein expression in thyroid gland. β-actin was used as loading control. Lower panel shows percent expression of protein following Scion image analysis. Histogram represents mean ± SEM. The mean differences were considered significant when $p < 0.01$. $^{**}p < 0.01$: Con vs. Mel; Con vs. PTU; $^{\#\#}p<0.01$: PTU vs Mel+PTU.

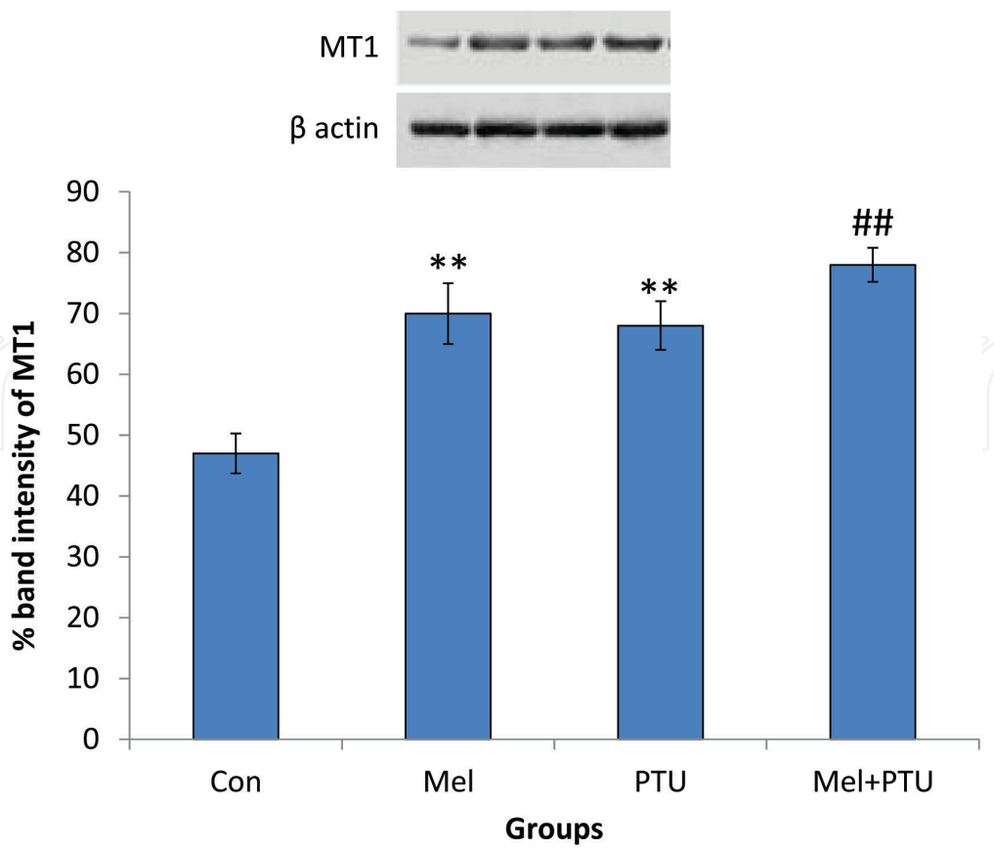

Figure 8.
Western blot analysis of MT1 receptor protein expression in pituitary gland. β-actin was used as loading control. Lower panel shows percent expression of protein following Scion image analysis. Histogram represents mean ± SEM. The mean differences were considered significant when $p < 0.01$. $^{**}p < 0.01$: Con vs. Mel; Con vs PTU; $^{\#\#}p<0.01$: PTU vs Mel+PTU.

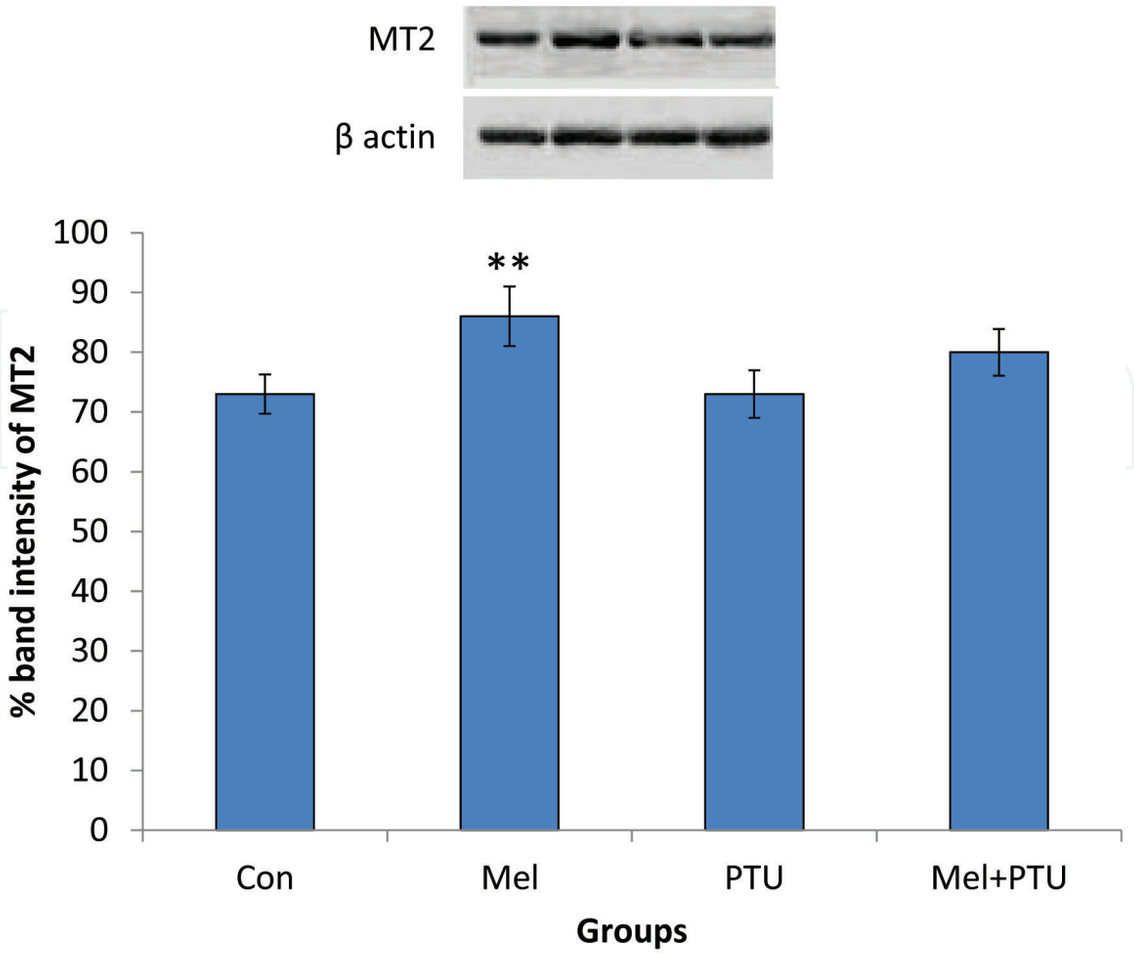

Figure 9.
Western blot analysis of MT2 receptor protein expression in pituitary gland. β-actin was used as loading control. Lower panel shows percent expression of protein following Scion image analysis. Histogram represents mean ± SEM. The mean differences were considered significant when $p < 0.01$. $^{**}p < 0.01$: Con vs. Mel.

MT1 and MT2 receptors in the thyroid of mice. Further, melatonin supplementation to hypothyroid mice caused increased expression of MT2 receptor proteins in comparison with hypothyroid mice.

3.5 Effect of melatonin on MT1 and MT2 receptor proteins in the pituitary gland

Melatonin supplementation increased the MT1 and MT2 receptor expression in the pituitary gland (**Figures 8** and **9**). Hypothyroid mice showed increased MT1 receptor protein expression, while MT2 receptor protein remained unaffected in comparison with control mice. Melatonin supplementation to hypothyroid mice caused a significant increase in MT1 receptor expression in the pituitary gland in comparison with the hypothyroid group. This result suggested that in the pituitary gland MT1 receptor is more responsive to melatonin in minimizing the PTU caused stress in hypothyroid mice.

4. Discussion

Metabolic suppression, lower respiration rate, and reduction in free radical formation reflect the hypothyroid state of an organism. In this study, we have noted a significant decrease in thyroid hormones level after melatonin treatment. Earlier workers reported that melatonin administration causes the impairment of thyroid function [20–22]. *In vitro* treatment of melatonin decreased the thyroid activity in a

dose-dependent manner [23, 24]. Melatonin administration suppresses mitotic activity and so, strong inhibition of thyroid gland function was reported [8, 25, 26]. Treatment of 5-propyl-2-thiouracil (PTU) decreased the thyroid hormone levels as it is a known fact that antithyroid drugs cause a hypothyroid condition in mice. Melatonin-treated hypothyroid group showed an increased level of both T3 and T4 hormones. Melatonin treatment to hypothyroid mice counteracted the PTU caused suppression of both T3 and T4 hormone levels in the mice. Thakkar et al. [16] suggested that melatonin treatment reversed neonatal hypothyroidism-induced negative impacts on thyroid function.

Healthy mice showed a normal level of circulatory T_3 and T_4 levels and normal architecture of follicles in the thyroid gland. PTU treatment caused inhibition of thyroid hormonogenesis and thus reduced the production of T_4 and T_3 hormones. To fulfill the physiological demand, follicular colloid was excessively consumed and follicular size was reduced. Follicular cells enlarge in size with a small luminal area. The report suggested that to fulfill the hormonal demand follicular cells increase in size in PTU-treated rat [27]. Melatonin treatment minimized the PTU-induced harmful effects on hormonogenesis in thyroidal follicles and restored the follicular architecture in hypothyroid mice.

Serum TSH hormone level was unchanged, whereas TSH receptor expression in the thyroid gland increased after melatonin treatment. Reports suggested that the TSH hormone level was unaltered after melatonin administration [28]. PTU treatment increased the circulatory TSH hormone but TSH receptor expression on the thyroid gland was unaffected. Klecha et al. [29] documented the significant increase of TSH hormone level in experimentally induced hypothyroid mice. Melatonin treatment of hypothyroid mice increased the TSH hormone level, while TSH receptor proteins on the thyroid gland remained unaffected. Prolonged administration of melatonin to hypothyroid mice might be promoting thyroid function *via* increasing hypophysial TSH hormone secretion.

Administration of melatonin caused decreased MT1 melatonin receptor expression in the thyroid gland, whereas MT2 receptor expression in the thyroid gland increased. The report suggested that exogenous melatonin differentially influences the MT1 and MT2 receptor expression in the thyroid gland in an age-dependent manner [30]. PTU-induced hypothyroid condition caused increased expression of both MT1 and MT2 receptor proteins in thyroid gland. Melatonin treatment of hypothyroid mice caused increased MT2 receptor protein expression in thyroid gland. Prolonged treatment of melatonin in PTU-induced hypothyroid mice might be influencing thyroid function through activation of MT2 receptors in the thyroid gland.

Melatonin receptors are localized in most regions of the brain and also in the pituitary gland. (I^{125}) iodomelatonin binding assay suggested melatonin binding sites in the pituitary [31]. The melatonin receptor mRNA study also suggested that melatonin mediates its effects through MT1 and MT2 receptors in the pituitary gland. Exogenous melatonin caused an increase in MT1 and MT2 receptor protein expression in the pituitary gland. PTU caused the hypothyroid condition and induced the MT1 receptor protein expression in the pituitary gland of mice. Melatonin supplementation to hypothyroid mice caused a significant increase in MT1 receptor proteins expressed in the pituitary gland. This result suggested that the MT1 receptor of melatonin is more responsive to melatonin in the pituitary of hypothyroid mice. The report suggested the MT1 and MT2 receptor-mediated modulation of the pituitary function in laboratory mice [32].

5. Conclusion

The hypothalamohypophyseal and epithalamo-epiphyseal axes are the two important components of the integrated central neuroendocrine mechanism

underlying the control of functional activity of the thyroid gland. In this study, melatonin treatment along with PTU treatment was effective in improving the thyroid function. Melatonin treatment of hypothyroid mice might improve the thyroid hormones *via* regulating the neuroendocrine axis and upregulation of hypophyseal TSH hormone. Exogenous melatonin differentially modulated the MT1 and MT2 receptor proteins expression in the pituitary and thyroid gland in hypothyroid experimental conditions. Therefore, the above findings may suggest that melatonin regulates the hypophyseal-thyroid function in hypothyroid mice through differential activation of MT1 and MT2 receptors in the pituitary and thyroid gland.

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Conflict of interest

The authors declare that they have no conflict of interest that would prejudice the impartiality of this scientific work.

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