Melatonin Supports CYP2D-Mediated Serotonin Synthesis in the Brain

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ABSTRACT

Melatonin is used in the therapy of sleep and mood disorders and as a neuroprotective agent. The aim of our study was to demonstrate that melatonin supported (via its deacetylation to 5-methoxytryptamine) CYP2D-mediated synthesis of serotonin from 5-methoxytryptamine. We measured serotonin tissue content in some brain regions (the cortex, hippocampus, nucleus accumbens, striatum, thalamus, hypothalamus, brain stem, medulla oblongata, and cerebellum) (model A), as well as its extracellular concentration in the striatum using an in vivo microdialysis (model B) after melatonin injection (100 mg/kg i.p.) to male Wistar rats. Melatonin increased the tissue concentration of serotonin in the brain structures studied of naive, sham-operated, or serotoninergic neurotoxin (5,7-dihydroxytryptamine)-lesioned rats (model A). Intracerebroventricular quinine (a CYP2D inhibitor) prevented the melatonin-induced increase in serotonin concentration. In the presence of pargyline (a monoaminoxidase inhibitor), the effect of melatonin was not visible in the majority of the brain structures studied but could be seen in all of them in 5,7-dihydroxytryptamine–lesioned animals when serotonin storage and synthesis via a classic tryptophan pathway was diminished. Melatonin alone did not significantly increase extracellular serotonin concentration in the striatum of naïve rats but raised its content in pargyline-pretreated animals (model B). The CYP2D inhibitor propafenone given intrastructurally prevented the melatonin-induced increase in striatal serotonin in those animals. The obtained results indicate that melatonin supports CYP2D-catalyzed serotonin synthesis from 5-methoxytryptamine in the brain in vivo, which closes the serotonin-melatonin-serotonin biochemical cycle. The metabolism of exogenous melatonin to the neurotransmitter serotonin may be regarded as a newly recognized additional component of its pharmacological action.

Introduction

Melatonin is used in the treatment of some sleep disorders, including concurrent sleep disturbances in the course of different psychiatric diseases such as schizophrenia and major depressive and seasonal affective disorders (Dolberg et al., 1998; Dalton et al., 2000; Shamir et al., 2000; Singh and Jadhav, 2014). Moreover, high doses of melatonin are recommended for neuroprotection (Venegas et al., 2012; Acuña-Castroviejo et al., 2014).

The synthesis of endogenous melatonin in the pineal gland is governed by the light/dark cycle, which controls circadian and circannual rhythms. However, melatonin can also be produced in a substantial amount in extrapineal organs, including the gastrointestinal tract (in enterochromaffin cells), where it is not controlled by the photoperiod (Pandi-Perumal et al., 2008; Venegas et al., 2012; Acuña-Castroviejo et al., 2014). The synthesis of melatonin from tryptophan via serotonin is catalyzed by N-acetyltransferase and hydroxyindole-O-methyltransferase, these two enzymes being present in a variety of organs/tissues such as the heart, liver, leukocytes, or the brain (e.g., the cerebral cortex and striatum), which also suggests the occurrence of melatonin synthesis in them. Extrapineal melatonin seems not to be engaged in the regulation of the photoperiod (Acuña-Castroviejo et al., 2014); however, together with pineal melatonin, extrapineal melatonin protects cells against damage from oxidative stress due to its antioxidant and anti-inflammatory activity. Moreover, melatonin exerts an antiexcitotoxic effect in the brain by reducing glutamate activity and increasing that of GABA and it stimulates neurogenesis (Pandi-Perumal et al., 2008; Acuña-Castroviejo et al., 2014; Singh and Jadhav, 2014). Being an amphiphilic substance, peripheral melatonin easily crosses the blood–brain barrier (Green et al., 1975). Circulating melatonin is metabolized mainly in the liver by cytochrome P450 isoforms of the CYP1A subfamily (CYP1A1/A2/B1) to form 6-hydroxymelatonin and then 6-sulfatoxymelatonin (Ma et al., 2005; Hardeland, 2010), but it can also be deacetylated to 5-methoxytryptamine (5-MT) (Rogawski et al., 1979; Beck and Jonsson, 1981).

In the brain, melatonin deacetylation to 5-MT seems to be of minor importance; both melatonin and 5-MT are formed mainly in the pineal gland from which they are released to the circulation or to the third ventricle. However, 5-MT formed from gut-derived melatonin in the liver crosses the blood–brain barrier (Beck and Jonsson, 1981; Acuña-Castroviejo et al., 2014) and, together with the brain-derived 5-MT, provides a direct substrate for CYP2D to form serotonin. Thus, the gut-derived or the exogenously supplied melatonin that provides most of the 5-MT for the organism may support serotonin formation by a CYP2D-mediated alternative pathway in vivo.

ABBREVIATIONS: 5,7-DHT, 5,7-dihydroxytryptamine; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, serotonin; 5-MT, 5-methoxytryptamine; aCSF, artificial cerebrospinal fluid; DA, dopamine; DMSO, dimethylsulfoxide; DRN, dorsal raphe nuclei; HPLC, high-performance liquid chromatography; MAO, monoaminoxidase; MRN, median raphe nuclei; NA, noradrenaline.
Recent studies have shown that, apart from the classic pathway of serotonin formation from L-5-hydroxytryptophan, serotonin may also be synthesized via CYP2D-catalyzed O-demethylation of 5-MT. Such a way of serotonin synthesis has been demonstrated for human and rat cDNA-expressed CYP2D isoforms, as well as for brain microsomes (Yu et al., 2003; Haduch et al., 2013). Moreover, using a microdialysis model, the formation of serotonin via this alternative pathway has recently been shown to function in the brain in vivo (Haduch et al., 2015).

The aim of this study was to demonstrate that exogenous melatonin supported (via deacetylation to 5-MT) CYP2D-mediated serotonin synthesis from 5-MT in the rat brain in vivo (Fig. 1) by measuring tissue or extracellular serotonin concentration after intraperitoneal melatonin injection. Serotonin tissue content was measured in brain regions containing CYP2D and serotonergic innervation (model A). The experiment was carried out with naïve and 5,7-dihydroxytryptamine (5,7-DHT)–pretreated animals in the absence/presence of the monoamine oxidase (MAO) inhibitor pargyline. Intraperitoneal pargyline was applied to prevent 5-MT (successively formed from melatonin in the liver) and serotonin (formed from 5-MT in the brain) from the oxidation by MAO and thus to potentiate the effect of melatonin on the serotonin level in the brain (Prozialek and Vogel, 1978; Suzuki et al., 1981; Galzin and Langer, 1986; Raynaud and Pévet, 1991). Intracerebral 5,7-DHT (a neurotoxin specific to serotonergic neurons) was used to decrease the production of serotonin via a classic pathway from tryptophan and serotonin storage in neuronal terminals of the brain (Rogawski et al., 1979; Beck and Jonsson, 1981) and, consequently, to enhance serotonin derived from the peripherally formed 5-MT (from melatonin), as well as to demonstrate the role of cytochrome P450 present in neurons/terminals in serotonin formation from 5-MT in the brain via an alternative pathway. The extracellular, functional concentration of serotonin in the striatum (involved in motor functions) was measured in the absence/presence of pargyline using an in vivo microdialysis (model B). The animals also received a CYP2D inhibitor to ascertain whether CYP2D was engaged in the synthesis of serotonin from melatonin via 5-MT O-demethylation.

**Fig. 1.** Metabolism of exogenous melatonin via deacetylation to 5-MT in the liver and subsequent O-demethylation to serotonin in the brain (the investigated pathway).

### Materials and Methods

#### Chemicals

Melatonin (hydrochloride), pargyline (hydrochloride), quinine (hydrochloride), propafenone (hydrochloride), serotonin (5-hydroxytryptophan) or 5-HT; hydrochloride) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA), dopamine (DA), noradrenaline (NA), 5,7-DHT (a creatinine sulfate salt), and ascorbic acid were purchased from Sigma-Aldrich (St. Louis, MO). Ketamine hydrochloride (Ketamine) and xylazine hydrochloride (Sedazin) came from Biowet (Pulawy, Poland). All organic reagents were of high-performance liquid chromatography (HPLC) grade and were supplied by Merck (Darmstadt, Germany).

#### Animals

All experimental procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Bioethics Commission at the Polish Academy of Sciences Institute of Pharmacology, as compliant with the Polish law. The study was conducted on male Wistar-Han rats (Charles River Laboratories, Sulzfeld, Germany) weighing 300–325 g. The animals were housed in rooms with a controlled temperature and humidity on a 12-hour light/dark cycle and had free access to tap water and standard laboratory food during the study.

#### The Tissue Levels of Serotonin in the Brain after Intrapерitoneal Melatonin Administration (Ex Vivo Measurement, Model A)

To study the supportive effect of exogenous melatonin on the alternative pathway of serotonin synthesis from 5-MT in a brain tissue, melatonin was injected intraperitoneally (100 mg/kg i.p.) to naïve and 5,7-DHT–lesioned rats. The rats were anesthetized with ketamine HCl (75 mg/kg i.p.) and xylazine HCl (10 mg/kg i.p.) and were then placed in stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). All solutions were freshly prepared on the day of experimentation. 5,7-DHT was dissolved in 0.9% NaCl with 0.05% ascorbic acid and was injected into the dorsal raphe nuclei (DRN) and median raphe nuclei (MRN) at a concentration of 10 μg/μl (1 μl, infused at a rate of 1 μl/min) into both raphe nuclei. The following coordinates were used (Paxinos and Watson, 2007): AP (anterior-posterior), −7.9; L (lateral), 0.0 from the bregma; and V (ventral), −7.9 (MRN), −5.9 (DRN) from the surface of the dura. The needle stayed in place for 5 minutes after injection before it was slowly withdrawn. Sham-operated animals were subjected to the same procedure as 5,7-DHT–treated animals, but they received a vehicle (a 0.9% NaCl plus 0.05% ascorbic acid) instead of 5,7-DHT. Ten days after injection of 5,7-DHT (or vehicle), the rats received melatonin (100 mg/kg i.p.), an indirect exogenous substrate for serotonin synthesis and/or pargyline (75 mg/kg i.p., 30 minutes before melatonin) to prevent the melatonin-produced 5-MT and serotonin (formed from 5-MT) from MAO oxidation. Because of its insolubility in water, melatonin was dissolved in a 30% dimethylsulfoxide (DMSO).

All drugs were given according to the following schedule: 5,7-DHT or vehicle (raphe nuclei) → after 10 days, pargyline i.p. → after 30 minutes, melatonin i.p. or 30% DMSO → after 1 hour, decapitation → tissue serotonin (DA, NA).

The rats were divided into 12 experimental groups (Table 1). Groups of nonoperated rats were as follows: control, 30% DMSO (2 ml/kg i.p.); melatonin (100 mg/kg i.p.); pargyline (75 mg/kg i.p.) plus 30% DMSO (2 ml/kg i.p.); and pargyline (75 mg/kg i.p.) plus melatonin (100 mg/kg i.p.). Groups of sham-operated rats were as follows: vehicle (1 μl, into the raphe nuclei) plus 30% DMSO (2 ml/kg i.p.); vehicle (1 μl; into the raphe nuclei) plus melatonin (100 mg/kg i.p.); vehicle (1 μl into the raphe nuclei) plus pargyline (75 mg/kg i.p.); and vehicle (1 μl, into the raphe nuclei) plus pargyline (75 mg/kg i.p.) plus melatonin (100 mg/kg i.p.). Groups of operated (5,7-DHT–lesioned) rats were as follows: 5,7-DHT (1 μl, into the raphe nuclei) plus 30% DMSO (2 ml/kg i.p.); 5,7-DHT (1 μl, into the raphe nuclei) plus melatonin (100 mg/kg i.p.); 5,7-DHT (1 μl, into the raphe nuclei) plus pargyline (75 mg/kg i.p.); 5,7-DHT (1 μl, into the raphe nuclei) plus pargyline (75 mg/kg i.p.) plus 30% DMSO (2 ml/kg i.p.); 5,7-DHT (1 μl, into the raphe nuclei) plus pargyline (75 mg/kg i.p.) plus melatonin (100 mg/kg i.p.).

Moreover, an additional group of naïve rats received bilateral intracerebroventricular injections of the CYP2D inhibitor quinine (150 μg/5 μl i.c.v.), melatonin (100 mg/kg i.p.), or quinine (30 minutes before melatonin) plus melatonin. The CYP2D competitive inhibitor quinine (Kobayashi et al., 1989)
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The data are expressed as the mean ± S.E.M. (n = 5–6). Statistical significance was assessed by a two-way (nonoperated rats) or three-way (operated rats) analysis of variance and Fisher’s LSD test. "P < 0.05 (versus control); **P < 0.01 (versus control); ***P < 0.001 (versus control); #P < 0.05 (versus lesioned animals); $$P < 0.001 (versus lesion plus PARG group)."

<table>
<thead>
<tr>
<th>Brain Structure</th>
<th>Sham Operated Rate</th>
<th>Operated Rate</th>
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<tr>
<td></td>
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<td>Frontal cortex</td>
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<td>Rest of cortex</td>
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<td>581.7 ± 55.9</td>
</tr>
<tr>
<td>Brain stem</td>
<td>950.9 ± 42.6</td>
<td>1124.0 ± 28.5</td>
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Intraperitoneal Administration of Melatonin: An In Vivo Microdialysis

Microdialysis guides were implanted 7 days before melatonin (100 mg/kg i.p.) administration. The rats were anesthetized with ketamine (75 mg/kg i.m.) and xylazine (10 mg/kg i.m.) and were then placed in stereotactic apparatus (David Kopf Instruments). Vertical microdialysis guides (Bioanalytical Systems Inc., West Lafayette, IN) were implanted in the striatum using the following coordinates (Paxinos and Watson, 2007): AP, +1.2; L, +3.0 from the internal capsule, 3.2 from the surface of the dura. The rats were divided into four experimental groups (Figs. 2) as follows:

- control group
- lesioned group (5,7-DHT lesion)
- lesion plus melatonin (5,7-DHT lesion + melatonin)
- lesion plus PARG (5,7-DHT lesion + PARG)

Lowering the pH of aCSF to 7.4, rather than quinine (30 mM), was used in an appropriate intracerebral dose to produce an enzyme-specific micromolar concentration of the inhibitor in the brain (Bromek et al., 2010; Haduch et al., 2015). Such an application of quinine allowed us to ascertain whether CYP2D was engaged in the indirect synthesis of serotonin from melatonin (via O-demethylation of the melatonin-formed 5-MT). The rats were anesthetized with ketamine HCl (75 mg/kg i.p.) and xylazine HCl (10 mg/kg i.p.) and were then placed in the Kopf stereotactic apparatus. The coordinates based on the Paxinos and Watson (2007) atlas were as follows: AP, –8.5; L, ±1.5 from the bregma; and V, –3.5 from the dura. The freshly prepared quinine at a concentration of 30 μM/l was infused at a rate of 1 μl/min into both lateral ventricles (150 μg per ventricle). The needle was left in place for 5 minutes after injection before its slow withdrawal. Control rats (lesion-operated animals) were subjected to the same procedure as the quinine-treated group, except that they received a vehicle (0.9% NaCl) instead of quinine. The placement of the needle was histologically verified in 10 rats in a preliminary experiment and was then checked in two animals from each experimental group.

All drugs were given according to the following schedule: quinine i.c.v. or vehicle → after 30 minutes, melatonin i.p. or 30% DMSO → after 1 hour, decapitation → tissue serotonin.

One hour after melatonin injection, the rats were decapitated and their brains were removed. The brains were cut into the cerebellum, hypothalamus, thalamus, nucleus accumbens, striatum, hippocampus, frontal cortex, the rest of cortex, brain stem, and medulla oblongata. Brain tissues were frozen on dry ice and stored at –80°C until they were further analyzed.

Melatonin and the other pharmacological substances applied were administered at the same time of a day to rats coming from all of the experimental groups. Consequently, all experimental procedures involving control groups and groups treated with melatonin were carried out at the same time of a day (between 10:00 AM and 4:00 PM).

Extracellular Concentrations of Serotonin in the Striatum after Intraperitoneal Administration of Melatonin: An In Vivo Microdialysis

(Model B)

Four baseline samples were collected from freely moving rats at 20-minute intervals after a 165-minute washout period. Then, the appropriate drugs, propafenone (50 μM given using microdialysis probes) and/or melatonin (100 mg/kg i.p.), were administered to naïve or pargyline-treated rats (pargyline 75 mg/kg i.p., 60 minutes before melatonin). The applied effective doses of melatonin and the other pharmacological substances were chosen on the basis of the results of our preliminary experiments (data not shown). In that experimental set, intracerebral propafenone (added to the aCSF) was used instead of quinine as a specific and competitive CYP2D inhibitor (Xu et al., 1995; Zhou et al., 2013) to show the efficacy of another enzyme inhibitor in preventing melatonin effect. Moreover, in our preliminary experiment, propafenone was effective at a concentration 10 times lower than quinine (50 μM versus 500 μM), being thus also easier to dissolve in the aCSF. Dialysate fractions after melatonin injection were collected throughout 180 minutes. All drugs were given according to the following schedule: microdialysis guide (in the striatum) → after 7 days, pargyline i.p. → after 60
minutes, propafenone into the striatum and/or melatonin i.p. → in vivo extracellular serotonin (dopamine).

The rats were divided into six experimental groups (Figs. 3 and 4): control, 30% DMSO (2 ml/kg i.p.); pargyline (75 mg/kg i.p.) plus 30% DMSO (2 ml/kg i.p.); saline (2 mg/kg i.p.) plus melatonin (100 mg/kg i.p.); pargyline (75 mg/kg i.p.) plus propafenone (50 μM) plus 30% DMSO (2 ml/kg i.p.); pargyline (75 mg/kg i.p.) plus melatonin (100 mg/kg i.p.); and pargyline (75 mg/kg i.p.) plus propafenone (50 μM) plus melatonin (100 mg/kg i.p.).

At the end of the experiment, the rats were euthanized and their brains were histologically examined to validate probe placement.

Brain microdialysis procedures were carried out in rats from all of the experimental groups at the same time of a day (between 10:00 AM and 6:00 PM).

Fig. 2. Model A. The effect of intracerebral quinine administration on the melatonin-induced (100 mg/kg i.p.) increase in 5-HT tissue content in the following brain structures: frontal cortex (A), rest of the cortex (B), hippocampus (C), nucleus accumbens (D), striatum (E), thalamus (F), hypothalamus (G), brain stem (H), medulla oblongata (I), and cerebellum (J). The data are expressed as the mean ± S.E.M. (n = 5–8). Statistical significance was assessed by a two-way analysis of variance and Fisher’s test. *P < 0.05 (versus control); **P < 0.01 (versus control); ***P < 0.001 (versus control); *P < 0.05 (versus melatonin); **P < 0.01 (versus melatonin); ***P < 0.001 (versus melatonin). The control values (in picograms per milligram of tissue) were as follows: 465.50 ± 20.39 (frontal cortex), 352.08 ± 17.42 (rest of the cortex), 270.26 ± 7.94 (hippocampus), 424.01 ± 24.26 (nucleus accumbens), 247.19 ± 23.29 (striatum), 577.54 ± 30.39 (thalamus), 459.33 ± 34.76 (hypothalamus), 704.81 ± 25.53 (brain stem), 560.21 ± 18.68 (medulla oblongata), and 45.83 ± 3.59 (cerebellum). MEL, melatonin; QUIN, quinine.
Melatonin Supports Serotonin Formation by Brain CYP2D

Determination of Brain Neurotransmitters

The tissue concentrations of 5-HT, DA, NA, and 5-HIAA were measured using HPLC with electrochemical detection, according to the method of Haduch et al. (2015). Briefly, brain structures were homogenized in 20 volumes (v/v) of ice-cold 0.1 M HClO₂ and were centrifuged at 15,000 × g for 15 minutes at 4°C. The obtained supernatant (5 µl) was injected into the HPLC system. An external standard containing NA, DA, 5-HT, and 5-HIAA at concentrations of 50 ng/ml (or 2.5 ng/ml for the dialysate) was used. The chromatography system comprised an LC-4C amperometric detector with a cross-flow detector cell (Bioanalytical Systems Inc.) as well as a 626 Alltech pump and a Hypersil Gold analytical column (3 µm, 100 × 3 mm; Thermo Scientific, Waltham, MA). The mobile phase contained 0.1 M KH₂PO₄, 0.5 mM Na₂EDTA, 80 mg/l sodium 1-octanesulfonate, and 4% methanol and was adjusted to pH 3.7 with 85% H₃PO₄. The flow rate of the eluent was 0.6 ml/min. The potential of a 3-mm glassy carbon electrode was set at 0.7 V and a sensitivity of 5 nA/V. The column temperature was maintained at 30°C. The Chromax 2007 program (Pol-Laboratory, Warsaw, Poland) was applied for the collection and analysis of data. Neurotransmitter concentrations in the dialysate were measured using a composition of the mobile phase: 0.1 M KH₂PO₄, 0.5 mM Na₂EDTA, 16 mg/l sodium 1-octanesulfonate, and 2% methanol, adjusted to pH 3.6 with 85% H₃PO₄. The flow rate of the eluent was 0.7 ml/min.

Data Analysis

An average neurotransmitter concentration of four stable samples of the dialysate fraction prior to drug administration was regarded as a basal value (100%). The data were statistically analyzed using two-way (nonoperated rats) or three-way (operated rats) analysis of variance (model A), followed by Fisher’s least significant differences post hoc test, or by a repeated-measures analysis of variance (model B), followed by Tukey’s post hoc test (Program Origin 7.5 or Statistica 9, respectively; StatSoft Inc., Tulsa, OK). The results were considered to be statistically significant when \( P < 0.05 \).

Results

The Tissue Levels of Serotonin in the Brain after Intraperitoneal Administration of Melatonin (Ex Vivo Measurement, Model A)

Intact Rats. Melatonin and the applied pharmacological substances (the MAO inhibitor pargyline and the serotonergic neurotoxin 5,7-DHT) affected the tissue concentration of serotonin in the brain; this effect was structure dependent (Table 1). Melatonin (100 mg/kg i.p.) significantly increased serotonin content in the rest of the cortex (131%), hippocampus (154%), nucleus accumbens (191%), thalamus (146%), hypothalamus (161%), and medulla oblongata (135%) compared with the control (Table 1). A similar tendency was observed in other structures. The MAO inhibitor pargyline (75 mg/kg i.p.) potently increased serotonin concentration in all of the investigated brain structures, on average up to 207% of the control (frontal cortex, 200%; rest of the cortex, 202%; hippocampus, 290%; nucleus accumbens, 201%; striatum, 157%; thalamus, 261%; hypothalamus, 198%; brain stem, 200%; medulla oblongata, 156%; and cerebellum, 205%). However, melatonin administered to pargyline-pretreated rats did not elevate the serotonin concentration in most of the brain structures, on average down to 54% of the control (Table 1).

Sham-Operated Rats. Melatonin (100 mg/kg i.p.) significantly elevated serotonin content in all of the brain structures studied in sham-operated rats: the frontal cortex (135%), the rest of the cortex (131%), hippocampus (185%), nucleus accumbens (284%), striatum (271%), thalamus (177%), hypothalamus (163%), brain stem (132%), medulla oblongata (155%), and cerebellum (331%) (Table 1). Pargyline potently increased tissue serotonin content in the brain structures of sham-operated rats, on average up to 209% (frontal cortex, 181%; rest of the cortex, 171%; hippocampus, 164%; nucleus accumbens, 344%; striatum, 205%; thalamus, 185%; hypothalamus, 171%; brain stem, 166%; medulla oblongata, 213%; and cerebellum, 270%) (Table 1). Melatonin administered to pargyline-pretreated sham-operated rats significantly elevated serotonin concentration up to approximately 134% in the striatum, 117% in the thalamus, and 124% in the brain stem compared with pargyline-pretreated animals (Table 1).

5,7-DHT–Lesioned Rats. The neurotoxin 5,7-DHT (10 µg/1 µl), injected into the DRN and MRN, decreased tissue serotonin concentration in all of the brain structures studied, on average down to 54% of
the sham-operated animals (frontal cortex, 75%; rest of the cortex, 61%; hippocampus, 13%; nucleus accumbens, 71%; striatum, 53%; thalamus, 73%; hypothalamus, 42%; brain stem, 42%; medulla oblongata, 51%; and cerebellum, 64%) (Table 1). The lesion was specific, since the concentrations of the catecholaminergic neurotransmitters NA and DA were not affected (data not shown). Melatonin (100 mg/kg i.p.) significantly enhanced the serotonin level in the brain structures of 5,7-DHT–lesioned rats: frontal cortex (142%), nucleus accumbens (234%), striatum (340%), thalamus (222%), brain stem (232%), medulla oblongata (181%), and cerebellum (375%) (Table 1). A similar tendency was observed in the hippocampus (174%), hypothalamus (136%), and rest of the cortex (130%). Pargyline markedly increased serotonin level in the brain structures of 5,7-DHT–lesioned rats, on average up to 227% of the neurotoxin-lesioned animals (frontal cortex, 187%; rest of the cortex, 151%; hippocampus, 231%; nucleus accumbens, 194%; striatum, 355%; thalamus, 211%; hypothalamus, 159%; brain stem, 259%; medulla oblongata, 313%; and cerebellum, 214%). Melatonin administered to the lesions pretreated with pargyline elevated the serotonin concentration in all of the brain structures tested, on average up to 150% of the pargyline-pretreated lesioned rats (frontal cortex, 132%; rest of the cortex, 144%; hippocampus, 228%; nucleus accumbens, 169%; striatum, 156%; thalamus, 112%; hypothalamus, 144%; brain stem, 143%; medulla oblongata, 117%; and cerebellum, 146%).

Effect of Quinine. In another experimental set, the specific CYP2D inhibitor quinine, given to both lateral ventricles of the brain (150 μg/5 μl i.c.v.) 30 minutes before melatonin (100 mg/kg i.p.), prevented the melatonin-evoked elevation in tissue serotonin content in the following brain structures of naïve rats: the rest of the cortex, hippocampus, striatum, thalamus, hypothalamus, brain stem, medulla oblongata, and cerebellum (Fig. 2). Such an effect of quinine was not observed in the frontal cortex and nucleus accumbens. Quinine alone did not affect the tissue content of serotonin.

Extracellular Concentrations of Serotonin and DA in the Striatum after Intraperitoneal Administration of Melatonin: An In Vivo Microdialysis (Model B)

Melatonin (100 mg/kg i.p.) did not significantly affect the extracellular concentration of serotonin in the striatum of naïve rats (control rats). For that reason, the animals were pretreated with pargyline to inhibit the oxidation of melatonin-produced 5-MT in the liver and of serotonin in the brain and, consequently, to enhance the effect of melatonin on the intraneuronal and, possibly, also extraneuronal, serotonin level. As expected, when administered to animals pretreated with pargyline (75 mg/kg i.p.), melatonin increased serotonin concentration up to approximately 100% of the pargyline-treated animals (Fig. 3). The CYP2D inhibitor propafenone (50 μM), given locally through a microdialysis probe, prevented the melatonin-induced increase in serotonin concentration, having decreased it to approximately 25% of the pargyline- plus melatonin-treated animals. Pargyline alone raised the extracellular serotonin concentration up to approximately 325% of the basal level; when single points were compared, the pargyline-treated group was significantly different from the control group (P < 0.0001, t test). Propafenone did not affect the extracellular concentration of serotonin in pargyline-pretreated animals.

Like in the case of serotonin, melatonin did not change the extracellular concentration of dopamine in the striatum of naïve rats. However, when given to animals pretreated with pargyline, melatonin increased dopamine concentration up to approximately 500% of the pargyline-pretreated animals. (Fig. 4). The CYP2D inhibitor propafenone prevented the melatonin-evoked increase in the dopamine concentration in pargyline-pretreated rats. Pargyline alone raised the extracellular dopamine concentration up to approximately 450% of the basal level. Propafenone did not significantly affect the extracellular concentration of dopamine in pargyline-pretreated animals (Fig. 4).

The basal extracellular level of serotonin in the striatum of naïve rats was 0.938 ± 0.069 pg/10 μl, whereas that of dopamine equaled 7.5 ± 0.7 pg/10 μl and did not differ between the experimental groups.

Discussion

Serotonergic projections from the raphe nuclei of the brain stem innervate almost all of the brain structures that control important physiologic functions, including the hypothalamus (food intake, circadian rhythm, and thermoregulation), basal ganglia (motor functions), thalamus (sleep and epilepsy), hippocampus (stress, learning and memory), and cortex (sleep and mood) (Törk, 1990; Di Giovanni et al., 2008). Recent studies revealed that, apart from the classic pathway of serotonin formation from 5-hydroxytryptophan, serotonin may also be synthesized in the brain via CYP2D-catalyzed O-demethylation of 5-MT. Serotonin that was formed in that alternative way was shown in vitro in microsomes derived from different brain structures (Haduch et al., 2013) and was found to function in vivo when we measured its tissue and extracellular concentrations in selected structures of the brain after intracerebral administration of 5-MT (Haduch et al., 2015).

Our study provides further evidence for the role of cytochrome P450 (CYP2D) in serotonin synthesis in the brain in vivo by showing that exogenous melatonin administered intraperitoneally supports (via deacetylation to 5-MT) serotonin formation by cytochrome P450. To observe serotonin formation from melatonin in vivo, in our experiment we used its relatively high dose (100 mg/kg i.p.), which is pharmacologically acceptable. Such high doses of melatonin (up to 200 mg/kg a day) were shown to have low toxicity (Barchas et al., 1967; Jahnke et al., 1999; Acuña-Castroviejo et al., 2014) and were used for psychopharmacological behavioral tests in animals (Papp et al., 2003, 2006). Furthermore, high doses of melatonin are recommended because of its protective properties as an antioxidant in saturating its intracellular therapeutic targets (Venegas et al., 2012; Acuña-Castroviejo et al., 2014). On the other hand, the rate of drug metabolism is much faster in rats (rodents) than in humans, so higher doses are necessary to reach similar plasma or tissue concentration in the two species. The dose of melatonin used in our experiment was found to be effective in elevating tissue and extracellular concentrations of serotonin in the rat brain.

Melatonin increases the tissue content of serotonin in the brain structures of naïve, sham-operated, or 5,7-DHT–lesioned rats (model A). In the case of the brain stem (containing serotonergic neurons), melatonin is considerably more effective in 5,7-DHT–lesioned rats, which suggests the presence of a relatively larger pool of serotonin formed from tryptophan via a classic pathway than from 5-MT (formed from melatonin) via a CYP2D pathway, as well as its considerable reduction by the neurotoxins. As shown in naïve rats, the CYP2D inhibitor quinine (Boobis et al., 1990; Bromek et al., 2010), given to lateral ventricles of the brain, prevents this effect in some brain structures (e.g., cortex, hippocampus, striatum, thalamus, hypothalamus, brain stem, medulla oblongata, and cerebellum), which indicates contribution of CYP2D to the melatonin-produced elevation in serotonin concentration.

As mentioned above, pargyline was applied to prevent 5-MT (formed from melatonin in the liver) and serotonin (formed from 5-MT in the brain) from the MAO oxidation to reinforce the effect of melatonin on the serotonin level in the brain. In contrast with melatonin, both 5-MT and serotonin are rapidly metabolized MAO substrates, the enzyme...
being present in a large amount in the liver and brain (Suzuki et al., 1981; Galzin and Langer, 1986; Raynaud and Pävet, 1991; Hardeland, 2010). However, the effect of melatonin on serotonin level is not visible in the majority of structures of the pargylene-pretreated, nonlesioned animals (both controls and sham-operated), since the amount of the tissue serotonin synthesized physiologically from endogenous substrates (mainly from tryptophan, but also from endogenous 5-MT) and protected from MAO probably largely dominates the serotonin formed indirectly from exogenous melatonin (via melatonin deacetylation to 5-MT and subsequent O-demethylation by CYP2D). However, the melatonin-induced increase in the tissue level of serotonin can be seen in all of the brain structures studied of pargylene-treated rats with a partial lesion of the serotonergic system. Under these experimental conditions, the physiologic synthesis and storage of serotonin is diminished by the neurotoxin 5,7-DHT specific to the serotonergic system, and the amount of serotonin formed by an alternative pathway (via 5-MT O-demethylation by CYP2D) is raised due to a supply of an exogenous substrate (i.e., 5-MT formed from exogenous melatonin in the liver) (Rogawski et al., 1979; Beck and Jonsson, 1981), which crosses the blood–brain barrier (Acuta-Castroviejo et al., 2014). Moreover, both peripheral and brain 5-MT and serotonin formed from it are protected by intraperitoneal pargylene against MAO oxidation.

Melatonin also increases the functional, extracellular concentration of serotonin under specific conditions (model B). In fact, the extracellular level of the neurotransmitter depends not only on its amount synthesized in the neuron but also on its release into the synaptic cleft and reuptake into the neuron. The effect of melatonin on these presynaptic processes has not yet been well recognized, but the available data suggest such a possibility (Pandi-Perumal et al., 2008). Thus, exogenous melatonin does not significantly increase extracellular serotonin in naïve rats but raises it in pargylene-pretreated animals. In the striatum of pargylene-pretreated rats, melatonin potently elevates serotonin concentration, yet such a strong effect does not appear when the tissue content of serotonin is measured (model A). However, in this experimental model and in these conditions (model B), the vesicle-stored neurotransmitter does not mask the amount of serotonin synthesized from exogenous melatonin. In addition, as mentioned above, the 5-MT freshly synthesized from melatonin in the liver and the serotonin formed from it in the brain are protected from MAO. The CYP2D inhibitor propafenone (Xu et al., 1995; Zhou et al., 2013) prevents the melatonin-induced increase in extracellular serotonin, which testifies to engagement of this cytochrome P450 isoform in the synthesis of serotonin from the melatonin-derived 5-MT.

Melatonin simultaneously increases extracellular dopamine concentration in the striatum, this effect being prevented by the CYP2D inhibitor propafenone. The above results are in line with our previous findings obtained after local injection of 5-MT to the striatum (Haduch et al., 2015); moreover, they also support our earlier findings obtained after local injection of 5-MT to the striatum. Thus, exogenous melatonin does not significantly increase extracellular serotonin and dopamine in naïve rats but raises it in pargylene-pretreated animals. In the striatum of pargylene-pretreated rats, melatonin potently elevates serotonin concentration, yet such a strong effect does not appear when the tissue content of serotonin is measured (model A). However, in this experimental model and in these conditions (model B), the vesicle-stored neurotransmitter does not mask the amount of serotonin synthesized from exogenous melatonin. In addition, as mentioned above, the 5-MT freshly synthesized from melatonin in the liver and the serotonin formed from it in the brain are protected from MAO. The CYP2D inhibitor propafenone (Xu et al., 1995; Zhou et al., 2013) prevents the melatonin-induced increase in extracellular serotonin, which testifies to engagement of this cytochrome P450 isoform in the synthesis of serotonin from the melatonin-derived 5-MT.

Melatonin simultaneously increases extracellular dopamine concentration in the striatum, this effect being prevented by the CYP2D inhibitor propafenone. The above results are in line with our previous findings obtained after local injection of 5-MT to the striatum (Haduch et al., 2015); moreover, they also support our earlier hypothesis that increases in 5-MT and serotonin (evoked by the injection of 5-MT or melatonin) stimulate 5-HT2 heteroreceptors located on dopaminergic terminals and indirectly enhance the release of dopamine in this structure (Di Giovanni et al., 2008; Navailles and De Deurwaerdère, 2011).

The obtained results indicate that exogenous melatonin administered peripherally supports CYP2D-catalyzed serotonin synthesis from 5-MT in the brain in vivo, as shown by the elevation in tissue and extracellular serotonin concentrations in the brain after melatonin administration and its prevention by the two CYP2D inhibitors quinine and propafenone. However, it cannot be excluded that in spite of providing the substrate (5-MT) for an alternative pathway of serotonin synthesis, melatonin may also stimulate the endogenous synthesis of serotonin or affect other regulatory processes of the neurotransmitter (i.e., its release or uptake; Miguez et al., 1995), since its low doses (<1 mg/kg i.p.) were found to increase serotonin concentration in some brain structures (Anton-Tay et al., 1968; Miguez et al., 1994).

The results presented herein are in agreement with some previous findings suggesting that the CYP2D-mediated formation of serotonin from 5-MT may take place in vivo. Thus humanized CYP2D6 transgenic mice show a higher concentration of serotonin and its metabolite 5-HIAA in blood plasma (Yu et al., 2003) and the brain (Cheng et al., 2013), and intracerebral administration of 5-MT increases the extracellular concentration of serotonin in the brain, as has been shown using a brain microdialysis in living rats (Haduch et al., 2015). Moreover, individuals with no or a defective CYP2D6 gene (expressing a poor metabolizer phenotype) are more anxiety prone (Bertilsson et al., 1989; González et al., 2008; Cheng et al., 2013), such a behavior being provoked by a low serotonin level in the limbic system of the brain (Hensler, 2006).

In conclusion, our study indicates that exogenous melatonin supports CYP2D-catalyzed serotonin synthesis from 5-MT in vivo. CYP2D-catalyzed serotonin synthesis from the melatonin-derived 5-MT closes the serotonin-melatonin-serotonin biochemical cycle. Hence, the therapeutic effect of melatonin may stem not only from its action on melatonin receptors and from its radical scavenger properties (Singh and Jadhav, 2014) but also from its metabolism to the monoaminergic neurotransmitter serotonin. The latter mechanism may be regarded as a newly recognized additional component of the pharmacological action of melatonin. These findings are of both physiologic and pharmacological importance, since melatonin can be formed endogenously and administered as a drug in the therapy of sleep and mood disorders, as well as a neuroprotective agent.

Authorship Contributions

Participated in research design: Haduch, Daniel.
Conducted experiments: Haduch, Broniek, Wójcikowski, Golebiowska.
Performed data analysis: Haduch, Golebiowska, Daniel.
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