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The role of melatonin membrane receptors in melatonin-dependent oxytocin secretion from the rat hypothalamo–neurohypophysial system — an *in vitro* and *in vivo* approach

Rola błonowych receptorów melatoniny w zależnym od melatoniny uwalnianiu oksytocyny z układu podwzgórze–część nerwowa przysadki szczura — badania *in vitro* oraz *in vivo*

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Abstract

Introduction: Melatonin exerts its biological role acting mainly *via* G protein-coupled membrane MT_1 and MT_2 receptors. To determine whether a response of oxytocinergic neurons to different concentrations of melatonin is mediated through membrane MT_1 and/or MT_2 receptors, the effect of melatonin receptors antagonists, i.e. luzindole (a non-selective antagonist of both MT_1 and MT_2 receptors) and 4-phenyl-2-propionamidotetralin (4-P-PDOT — a selective antagonist of MT_2 receptor), on melatonin-dependent oxytocin (OT) secretion from the rat hypothalamo-neurohypophysial (H-N) system, has been studied both *in vitro* and *in vivo*.

Materials and methods: For *in vitro* experiment, male rats served as donors of the H-N explants, which were placed in 1 ml of normal Krebs-Ringer fluid (nKRF) heated to 37°C. The H-N explants were incubated successively in nKRF {fluid B1} and incubation fluid as B1 enriched with appropriate concentration of melatonin, i.e. 10^{-9} M, 10^{-7} M, or 10^{-3} M and luzindole or 4-P-PDOT, or their vehicles (0.1% ethanol or DMSO) {fluid B2}. After 20 minutes of incubation in fluid B1 and then B2, the media were collected and immediately frozen before OT estimation by the RIA. The OT secretion was determined by using the B2/B1 ratio for each H-N explant. During *in vivo* experiment, rats were given an intracerebroventricular (*i.c.v.*) infusion of 5 µL luzindole or 4-P-PDOT, or their solvent (0.1% DMSO) and 10 minutes later the next *i.c.v.* infusion of 5 µL of either melatonin solution (10^{-7} M) or its vehicle (0.1 % ethanol in 0.9% sodium chloride).

Results: Melatonin at a concentration of 10^{-3} M significantly stimulated, while at a concentration of 10^{-9} M had no effect on, oxytocin secretion from the rat H-N system *in vitro*, also when luzindole or 4-P-PDOT was present in a medium. On the other hand, melatonin at a concentration of 10^{-7} M diminished this neurohormone output from an isolated H-N system and into the blood. Luzindole significantly suppressed such melatonin action, while 4-P-PDOT did not change the inhibitory influence of 10^{-7} M melatonin on oxytocin release, both *in vitro* and *in vivo*.

Conclusions: The present study demonstrates that an inhibitory effect of 10^{-7} M melatonin on oxytocin secretion from the rat H-N system is mediated through a subtype MT_1 membrane receptor and its action is independent of subtype MT_2 receptor. However, for the stimulatory effect of pharmacological concentration (10^{-3} M) of the pineal hormone on oxytocin release, probably mechanisms other than membrane MT_1/MT_2 receptor(s)-dependent are involved. **(Endokrynol Pol 2016; 67 (5): 507–514)**

Key words: *oxytocin; melatonin; luzindole; 4-P-PDOT; melatonin receptors*

Streszczenie

Wstęp: Melatonina wywiera biologiczny efekt, działając głównie za pośrednictwem związanych z białkami G błonowych receptorów MT_1 oraz MT_2. Aby określić czy w odpowiedzi neuronów oksytocynergicznych na różne stężenia melatoniny uczestniczą błonowe receptory MT_1 i/lub MT_2, wpływ antagonistów, tj. luzindolu (nieselektywnego antagonisty obydwu receptorów MT_1 i MT_2) oraz 4-P-PDOT (selektywnego antagonisty receptora MT_2), na zależne od melatoniny uwalnianie oksytocyny (OT) z układu podwzgórze–część nerwowa przysadki (H-N) szczura, badano zarówno *in vitro*, jak i *in vivo*.

Materiał i metody: Po wyosobnieniu z mózgu, układ H-N umieszczano w probówkach zawierających 1 ml płynu Krebsa-Ringera (K-R) ogrzanego do temperatury 37°C. Po okresie równoważenia do probówek dodawano normalny płyn K-R {płyn B1}, a następnie płyn B1 zawierający dodatkowo rozpuszczalnik melatoniny (0,1% etanol) lub jej roztwór w odpowiednim stężeniu, tj. 10^{-9} M, 10^{-7} M lub 10^{-3} M i/lub luzindol, lub 4-P-PDOT, bądź ich rozpuszczalnik (0,1% DMSO) {płyn B2}. Po inkubacji układu H-N w każdym z roztworów (B1 i B2) przez 20 min, płyn inkubacyjny pobierano i natychmiast zamrażano do czasu oznaczenia w zebranych próbkach zawartości OT metodą RIA. Stopień uwalniania OT z układu H-N *in vitro* wyrażano jako stosunek B2/B1. Podczas eksperymentu *in vivo*, szczurom infundowano do komory bocznej mózgu (*i.c.v.*) 5 µl roztworu luzindolu lub 4-P-PDOT bądź ich rozpuszczalnika (DMSO); 10 min później, również *i.c.v.*, wykonano iniekcję 5 µl roztworu melatoniny (w stężeniu 10^{-7} M) lub jej rozpuszczalnika (0,1% etanol w 0,9% NaCl).

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Wyniki: Wykazano, że melatonina w stężeniu 10^{-3} M istotnie nasila, natomiast w stężeniu 10^{-9} M pozostaje bez wpływu na wydzielanie OT z układu H-N *in vitro*, zarówno w obecności luzindolu, jak i 4-P-PDOT w medium inkubacyjnym. Natomiast, w stężeniu 10^{-7} M melatonina istotnie ogranicza wydzielanie oksytocyny, zarówno *in vitro*, jak i *in vivo*. Luzindol znosi hamujący wpływ melatoniny na wyrzut oksytocyny do płynu inkubacyjnego oraz do osocza krwi, natomiast 4-P-PDOT nie zmienia jej uwalniania hamowanego stosowaniem 10^{-7} M melatoniny.

Wnioski: Wyniki tych badań sugerują, że melatonina, w stężeniu 10^{-7} M hamuje wydzielanie oksytocyny z układu H-N szczura przy udziale błonowego receptora MT_1 i to działanie hormonu jest raczej niezależne od receptora MT_2. Pobudzający wpływ farmakologicznych (10^{-3} M) stężeń melatoniny na wyrzut oksytocyny do płynu inkubacyjnego z układu H-N zachodzi, najprawdopodobniej, przy udziale mechanizmów niezależnych od błonowych receptorów MT_1 i/lub MT_2. **(Endokrynol Pol 2016; 67 (5): 507–514)**

Słowa kluczowe: *oksytocyna; melatonina; luzindol; 4-P-PDOT; receptory melatoniny*

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Introduction

Oxytocin (OT) is a neurohormone synthesised by magnocellular neurons of the hypothalamic paraventricular (PVN) and supraoptic (SON) nuclei and is mainly secreted from the neurohypophysis into the blood. Several factors, which play a role as neuromediators and/or neuromodulators in the central nervous system, were found to influence this hormone secretion [1–4]. One of these factors is melatonin, which is able to modify OT secretion under different physiological and pathological conditions both *in vivo* [5–6] and *in vitro* [7–11]. The results, however, have shown that melatonin has either a stimulatory or an inhibitory effect, or is without influence on OT secretion, according to its concentration, the time of the day, animal species, and tissue sample (hypothalamus or neurohypophysis, or hypothalamo-neurohypophysial system) incubated *in vitro*. The strongest inhibitory effect on basal release of OT was exerted by melatonin at a concentration of 10^{-7} M (so-called supraphysiological concentration) when rat hypothalamic tissue [10] or the hypothalamo-neurohypophysial system [12] were incubated *in vitro*, but it was ineffective when rat neurointermediate lobe was used for the *in vitro* studies [8]. Also at a concentration of 10^{-9} M, i.e. a concentration that is close to the physiological level of the hormone in the rat blood, melatonin diminished basal release of OT from the rat hypothalamus [10] and hypothalamo-neurohypophysial system [7, 13], as well as Syrian hamster neurointermediate lobe [9]. On the other hand, a very high concentration of melatonin, i.e. 10^{-3} M (so-called pharmacological concentration), has been found to stimulate basal release of OT from isolated rat neurointermediate lobe [8] and hypothalamo-neurohypophysial system [12]. Recently, we also found that melatonin, at concentrations of 10^{-11}, 10^{-9}, 10^{-7}, and 10^{-5} M, was able to reduce forskolin-induced OT output, with the strongest effect exerting at a concentration of 10^{-7} M, while at a concentration of 10^{-3} M this pineal hormone did not further modify such forskolin-stimulated OT secretion from the rat hypothalamo-neurohypophysial system *in vitro* [12]. When melatonin was applied intracerebroventricularly (*i.c.v.)*, it was able to diminish significantly, at a concentration of 1 ng/mL, suckling-stimulated OT secretion into the blood (30 minutes after *i.c.v.* injection) in lactating female rats, while lower (0.01 ng/mL) and higher (100 ng/mL and 10 μg/mL) concentrations of the hormone remained inactive in this process [6].

Melatonin is known to exert its biological role in the central nervous system acting mainly through protein G-coupled membrane receptors (MT_1 and MT_2), as well as interaction with cytosolic enzyme quinone reductase 2 (QR2) and other intracellular mechanisms [14–16]. In the rat brain, melatonin membrane MT_1 and MT_2 receptors are situated mainly in the anterior part of the hypothalamus, especially the suprachiasmatic nucleus (SCN), as well as in the pars tuberalis of the pituitary [16–20]. What is more, in the rat SCN and pars tuberalis the membrane MT_1 receptor mRNA expression and melatonin binding have been found to exhibit daily variations, with elevated levels occurring during the daytime [19]. In the human brain, the expression of MT_1 receptor has been found in various parts of the hypothalamus, among them in the SCN, PVN, and SON nuclei, and in the pituitary (pars tuberalis as well as anterior and posterior part of the gland), while the expression of MT_2 receptor has been demonstrated to be limited to SCN, SON, and PVN hypothalamic nuclei [21–22]. Although colocalisation of MT_1 receptor with some magnocellular oxytocinergic neurons in the PVN and SON has been discovered [21], and the involvement of melatonin membrane receptors in the mediation of melatonin action on OT release has already been suggested by several authors, the functional importance of MT_1 and/or MT_2 receptors for OT secretion has not been studied yet.

It is known that melatonin has a high affinity for both membrane receptors; therefore, for the study of MT_1 and/or MT_2 receptor-mediated actions of melatonin, more often than melatonin itself, specific ligands for these receptors are employed [15, 23]. Several selective and non-selective agonists and antagonists of melatonin membrane receptors have been identified [23].

Two of them, i.e. luzindole — a non-selective antagonist of both MT_1 and MT_2 receptors and 4-phenyl-2-propionamidotetralin (4-P-PDOT) — a selective MT_2 receptor antagonist, extensively used for the studies, have been shown to be effective in different experimental projects [23–25].

The aim of the present study was to determine (by using luzindole and 4-P-PDOT) whether melatonin membrane receptors (MT_1 and/or MT_2) play a role in melatonin-dependent changes (i.e. inhibition or stimulation) in OT output from the rat hypothalamo-neurohypophysial system, both *in vitro* and *in vivo*.

Material and methods

Animals

Three-month-old male Wistar rats (weighing 250–350 g) were housed (four animals per cage) under conditions of constant temperature (+ 22°C), humidity, and lighting (a 12/12 hour light/dark schedule; lights on from 06.00 a.m.). The animals received commercial pelleted food (LSM, Bacutil, Poland) and had free access to tap water.

Compounds and reagents

Melatonin (N-acetyl-5-methoxytryptamine) and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich Chemie GmbH. N-Acetyl-2-benzyltryptamine (luzindole) and 4-phenyl-2-propionamidotetralin (4-P-PDOT) were purchased from Tocris Bioscience. The OT (Oxytocin synth.) for standard curve preparation as well as for iodination with ^{125}I was from Peninsula Laboratories Europe Ltd.

Experimental protocols

Experiment *in vitro*

Animals were decapitated between 9:30 and 10:30 a.m., the brain and the pituitary with intact pituitary stalk were carefully removed from the skull and a block of hypothalamic tissue was dissected to obtain a hypothalamo-neurohypophysial (H-N) system as previously described [7]. After dissection, the H-N explant was immediately placed in a polypropylene tube with 1 mL of normal Krebs-Ringer fluid (nKRF). The nKRF contained: 120 mM NaCl, 5 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM KH_2PO_4, 0.7 mM $MgSO_4$, 22.5 mM $NaHCO_3$, 10 mM glucose, 1.0 g/L bovine serum albumin, and 0.1 g/L ascorbic acid (pH = 7,4–7,5; osmolality within the range 285–295 mOsm/kg H_2O). Tubes were heated in a water bath to 37°C and constantly gassed with a mixture of 95% O_2 and 5% CO_2 (carbogen). At the beginning of the experiment, the H-N explants were equilibrated in nKRF, which was aspirated twice and replaced with 1 ml of fresh buffer. After 80 minutes of such preincubation, necessary for OT release stabilisation [10], the media were discarded and explants were subsequently incubated for 20 minutes in 1 mL of nKRF {fluid B1} and then, for the next 20 minutes, in 1 mL of nKRF supplemented with the appropriate substances {fluid B2}. The fluid B2 contained: melatonin vehicle (VEH - 0.1 % ethanol) or an appropriate concentration of melatonin, i.e. 10^{-9} M, 10^{-7} M, or 10^{-3} M (these concentrations of melatonin were chosen on the basis of the results of previous *in vitro* experiments [8–10, 12]) and an antagonists solvent — 0.1% DMSO (groups 1-4) or luzindole (groups 5–8), or 4-P-PDOT (groups 9–12) (n: number of samples per group, n = 7). Both luzindole and 4-P-PDOT were at a concentration of 10^{-6} M. Directly after each incubation period, the media (i.e. fluids B1 and B2) were aspirated, immediately frozen, and stored at -20°C until OT estimation by radioimmunoassay (RIA). To determine the *in vitro* secretion of OT, a B2/B1 ratio was calculated for each H-N explant. Because the amount of neurohormone released into the medium varies from one H-N explant to the other and the total values of OT content in the medium usually show a great differentiation within the group, the results are expressed as a B2/B1 ratio.

Experiment *in vivo*

On the day of the experiment, the animals were anaesthetised by an intraperitoneal (*i.p.*) injection of 10% urethane (ethyl carbonate; 1.4 mL/100 g. b.wt.) and a cannula was inserted into the lateral cerebral ventricle (*i.c.v.*) as described previously [6]. Namely, the animals were immobilised in a simple stereotaxic apparatus and a small hole was drilled in the skull 1.5–2.0 mm laterally and 1.5-2.0 mm posteriorly to the crossing of the sagittal and coronal sutures [26]. A simple stainless steel cannula (its tip was 4.0 mm below the dorsal skull surface) was fixed to the skull with dental cement. After the end of *i.c.v.* cannulation, the animals were given the *i.c.v.* infusions *via* a polyethylene tube connected with the cannula and attached to a 10-µL Hamilton syringe filled with an appropriate solution. At the beginning, rats were given an *i.c.v.* infusion of 5 µL 0.1% DMSO (group 1) or luzindole (group 2), or 4-P-PDOT (group 3) solutions (both antagonists at a concentration of 10^{-5} M). Ten minutes later, through the same cannula, the next *i.c.v.* infusion of 5 µL of either melatonin solution (at a concentration of 10^{-7} M) or its vehicle (0.1% ethanol in 0.9% sodium chloride) was given to the animals of each group. Such a concentration of melatonin was chosen because in our previous [12] and present *in vitro* experiments it was found to inhibit significantly OT output from the rat H-N explants. Ten minutes after *i.c.v.* injection of melatonin or its vehicle, the animals were decapitated. Immediately thereafter the blood was col-

lected in heparinised tubes, centrifuged for 20 minutes at 4°C, and plasma samples were frozen for further OT estimation by RIA, as previously described [5–6].

All the experiments (both *in vitro* and *in vivo*) were done between 09.30 and 11.00 a.m. because during the daytime the MT_1 receptor mRNA expression and melatonin binding in the rat SCN and pars tuberalis are elevated [19] and because it is the time when the H-N system is responsive to exogenous melatonin [11].

The experiments were performed with the consent (No. 8/ŁB 535/2011, 19/ŁB 604/2012) of the Local Committee for Animal Care.

Radioimmunoassay of OT

The OT concentration in all samples was determined in duplicate by a specific RIA method described previously [5]. Anti-OT antibodies were raised in rabbits in the Department of Experimental Physiology of the Medical University of Lodz, and their description has been given earlier [6, 27]. The final dilution of anti-OT antibodies was 1:80,000. Cross-reactivity for anti-OT antibodies was with vasopressin 1.12%, with gonadotropin-releasing hormone (Gn-RH), thyrotropin-releasing hormone (TRH), leucine enkephalin (Leu-Enk), angiotensin II, and substance P less than < 0.002%. Iodination of OT with ^{125}I, was performed by the chloramine-T method. The lower limit of detection for the assay was 2.55 pg OT per tube. The intra- and inter-assay coefficients of variation were less than 5.0% and 8.5%, respectively. For the determination of blood plasma hormonal level, OT was extracted from plasma using "Sep-Pak Plus" C18 cartridges (Waters Corporation, Milford, Massachusetts; Made in Ireland); the recoveries of OT during extraction procedure were > 80% and, therefore, the findings were not corrected for procedural losses.

Statistical evaluation of the results

Oxytocin release *in vitro* is finally expressed as a B2/B1 ratio, while blood OT concentration is expressed in picograms per 1 mL of blood plasma. The results are reported as mean ± standard error of the mean (S.E.M.). Significance of the differences between means was evaluated by one-way analysis of variance (ANOVA), followed by *post-hoc* Fisher (NIR) test, using STATISTICA (version 10) software (StatSoft, Poland). $P < 0.05$ was considered as the minimal level of significance.

Results

Experiment in vitro

Melatonin, at a concentration and 10^{-7} M, significantly inhibited (in comparison with the control - VEH value; $p < 0.05$) OT secretion from an isolated rat H-N explants when antagonist solvent, i.e. DMSO, was present in a medium, but at a concentration of 10^{-9} M melatonin remained inactive ($p > 0.05$) in this process (Fig. 1). Luzindole and 4-P-PDOT, applied without melatonin, did not modify OT output *in vitro*. Incubation of H-N explants in a medium containing both 4-P-PDOT and melatonin at a concentration of 10^{-7} M, but not at a concentration of 10^{-9} M, resulted in significant inhibition of OT secretion *in vitro* ($p < 0.05$). However, when explants were incubated in the presence of both luzindole and melatonin (at the concentrations of 10^{-9} M and 10^{-7} M), the pineal hormone did not affect OT release ($p > 0.05$) *in vitro* (Fig. 1). Melatonin at a concentration of 10^{-3} M strongly increased (in comparison with the VEH; $p < 0.00005$) OT secretion from the rat H-N explants in all studied groups, i.e. when DMSO or luzindole, or 4-P-PDOT was present in a buffer (Fig. 1).

Experiment in vivo

Under present experimental conditions, infused *i.c.v.* melatonin at a concentration of 10^{-7} M was able to inhibit (in comparison with the VEH) OT secretion into the blood when animals were pretreated, also *i.c.v.*, with DMSO ($p < 0.005$) or 4-P-PDOT ($p < 0.01$). Blood plasma OT level was not changed by *i.c.v.* infusion of melatonin when animals were previously injected with luzindole ($p > 0.05$) (Fig. 2).

Discussion

The opposite effects of so-called physiological (below 1 nM) or supra-physiological (1 nM – 1 μM), and pharmacological (above 1 μM) concentrations of melatonin have already been reported [15], and the present results showing both inhibitory (at a concentration of 10^{-7} M) and stimulatory (at a concentration of 10^{-3} M) effects of melatonin on OT secretion from isolated rat hypothalamo-neurohypophysial system are compatible with the previous findings. However, this is the first report showing the role of melatonin MT_1 receptor in the regulation of oxytocinergic neurons function.

It is known that melatonin plays its biological role in the central nervous system acting through membrane receptor (MT_1 and/or MT_2)-dependent and membrane receptor-independent mechanisms [14–15]. We assumed, therefore, that melatonin influences OT release from the rat hypothalamo-neurohypophysial system by using these mechanisms according to the concentration applied. To verify such a hypothesis, we evaluated the influence of melatonin membrane receptor antagonists, i.e. luzindole and 4-P-PDOT, on melatonin-dependent OT secretion from the rat H-N system incubated *in vitro*. Three concentrations (10^{-9} M, 10^{-7} M, and 10^{-3} M) of the hormone were employed for the study because previous *in vitro* experiments have

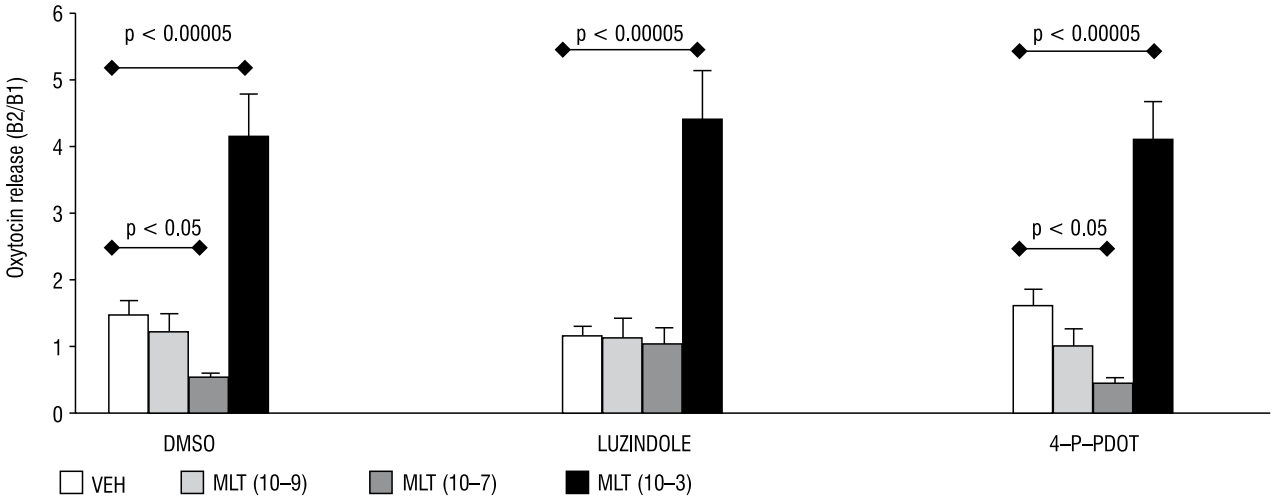

Figure 1. *The effect of melatonin (MLT), at the concentrations of 10^{-9}, 10^{-7}, and 10^{-3} M, on oxytocin release from the rat hypothalamo-neurohypophysial system incubated in vitro in the presence of DMSO (an antagonists solvent) or luzindole (a nonselective antagonist of both MT_1 and MT_2 receptors), or 4-P-PDOT (a selective MT_2 receptor antagonist). Each bar represents mean ± S.E.M.; number of samples per group (n) = 7*

Rycina 1. *Wpływ melatoniny (MLT), w stężeniach 10^{-9}, 10^{-7} i 10^{-3} M, na wydzielanie oksytocyny z układu podwzgórze-część nerwowa przysadki szczura inkubowanego in vitro w obecności DMSO (rozpuszczalnik antagonistów) lub luzindolu (nieselektywny antagonista receptorów MT_1 i MT_2), lub 4-P-PDOT (selektywny antagonista receptora MT_2). Wyniki przedstawiają średnią ± S.E.M.; liczba próbek w grupie (n) = 7*

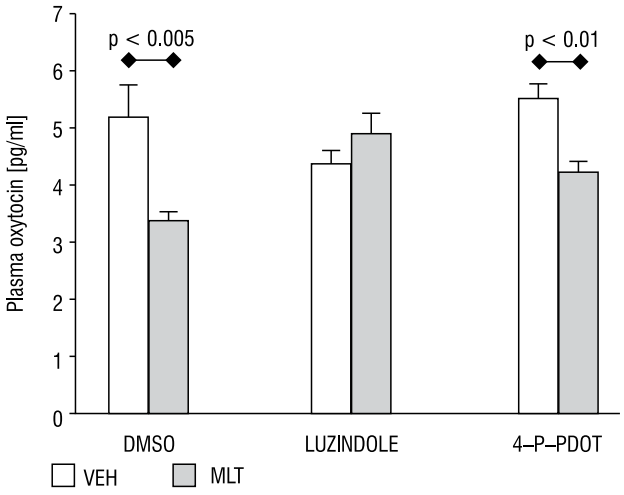

Figure 2. *The effect of i.c.v. — infused melatonin (MLT), at a concentration of 10^{-7} M, or its vehicle (VEH) on blood plasma oxytocin concentration in rats previously i.c.v.-injected with DMSO or luzindole, or 4-P-PDOT. Each bar represents mean ± S.E.M.; number of animals per subgroup (n) = 7*

Rycina 2. *Wpływ dokomorowej (i.c.v.) infuzji melatoniny (MLT), w stężeniu 10^{-7} M, lub jej rozpuszczalnika (VEH) na wydzielanie oksytocyny do krwi szczurów, którym wcześniej, również i.c.v., wstrzyknięto roztwór DMSO lub luzindolu, lub 4-P-PDOT. Wyniki przedstawiają średnią ± S.E.M.; liczba zwierząt w grupie (n) = 7*

shown that the strongest inhibitory effect on basal and forskolin-stimulated release of OT was exerted by melatonin at supraphysiological (10^{-7} M) and physiological (10^{-9} M) concentrations, while at a pharmacological (10^{-3} M) concentration melatonin had the opposite effect on the studied process [8–12]. On the other hand, the concentrations of both antagonists (10^{-6} M and 10^{-5} M) were chosen for our present experiments because they were demonstrated to be the most effective in action [24–25, 28–31]. Several studies have shown that luzindole antagonises the effects of melatonin. Namely, luzindole (at the concentrations of 10^{-6} M–10^{-4} M) applied together with melatonin (at the concentrations of 10^{-6} M–10^{-9} M) significantly suppressed melatonin-dependent actions *in vitro*, while 4-P-PDOT did not change the inhibitory effect of melatonin under different experimental projects [24–25, 29–30, 32–33]. Also, when applied intraperitoneally (*i.p.*), luzindole almost completely inhibited the antinociceptive effect of melatonin [34]. The results presented herein are in agreement with the above cited studies and show that luzindole is able to antagonise the inhibitory effect of 10^{-7} M melatonin, while 4-P-PDOT does not eliminate such an effect of the pineal hormone on OT secretion. This observation suggests, therefore, that an inhibitory effect of 10^{-7} M melatonin on OT secretion is mediated through a subtype MT_1 and is independent of subtype MT_2 membrane receptor. On the other hand, since 10^{-3} M melatonin was able to stimulate significantly OT secretion from the H-N system, also when 4-P-PDOT or luzindole were present in a medium (which implies blockade of melatonin membrane receptors), our next conclusion is that for the stimulatory effect of pharmacological concentration of the pineal hormone on OT

release *in vitro*, mechanisms different from membrane MT_1 and/or MT_2 receptors are probably involved.

The absence of any significant effect of melatonin at a concentration that is close to its physiological level in the blood (i.e. 10^{-9} M) on OT release *in vitro* could be due to several reasons. It is known that melatonin is released from the pineal gland directly into the cerebrospinal fluid of the third ventricle, where its concentration is much higher than in the blood, and it enters the brain from the ventricles [35]. It is, therefore, possible that a concentration of the hormone in the incubatory medium has to be higher than in the blood, to sufficiently penetrate from the medium into the hypothalamo-neurohypophysial tissue and produce a significant effect on OT secretion *in vitro*. On the other hand, the explant employed for our *in vitro* studies was disconnected from other brain regions, which normally deliver either stimulatory or inhibitory afferent signals to the magnocellular nuclei, making the secretion of OT a net result of the action of several factors that could cover or modify the effect of melatonin.

Significant effects of exogenous melatonin were shown to be displayed shortly after systemic or *i.c.v.* injection. For example, in the male rats, vasopressin secretion into the blood was reduced 10 minutes after a single intravenous (*i.v.)* injection of 5 ng/mL melatonin [36], as well as at five minutes after *i.c.v.* infusion of the hormone (at the concentrations of 1 ng/mL and 10 ng/mL) [37]. In lactating female rats, melatonin (at a concentration of 1 ng/mL) diminished significantly suckling-stimulated OT secretion into the blood 30 minutes after *i.c.v.* injection [6]. Moreover, a significant antinociceptive effect of melatonin was observed 10 minutes after *i.p.* injection [34]. Therefore, a 10-minute interval between *i.c.v.* infusion of melatonin and decapitation should be enough to display an effect of exogenous melatonin on OT output from the neurohypophysis into the blood. Indeed, the results from the present *in vivo* studies have shown that infused *i.c.v.* melatonin (at a concentration of 10^{-7} M) significantly diminished OT concentration in the blood when animals were previously injected with DMSO or 4-P-PDOT. However, pre-treatment with luzindole eliminated the inhibitory effect of melatonin, which provides further evidence in favour of the hypothesis that this pineal hormone inhibits OT secretion *via* MT_1 receptor-dependent mechanism and its inhibitory action is independent of subtype MT_2 receptor. Such a suggestion could be true for our *in vivo* and *in vitro* experiments. For the present *in vitro* experiment we used the explants which contained, apart from SON and PVN with intact neuronal projections to the neurohypophysis (i.e. intact axons of the oxytocinergic neurons), also the SCN, where strong MT_1 receptor expression has been discovered in many species [17–18, 20]. Thanks to direct neuronal projection from the SCN to the PVN [38] and SON [39], some of the SCN neurons could, therefore, integrate the afferent signals derived from melatonin *via* its MT_1 receptor and thereafter transmit them directly to oxytocinergic neurons in the SON and/or PVN.

Melatonin membrane receptors are coupled to a variety of G-proteins, so acting through these receptors, melatonin can produce multiple cellular responses [40–41]. Activation of MT_1 receptor may, therefore, result in inhibition of the cyclic adenosine monophosphate (cAMP)-dependent signal transduction cascade, including decreases in protein kinase A (PKA) activity and nuclear factor CREB (cAMP responsive element-binding protein) phosphorylation. It may also induce a phospholipase C (PLC)-dependent signal transduction cascade with protein kinase C (PKC) activation and elevation of cytosolic free calcium ions accumulation [15, 18, 41–43]. Acting through MT_1 receptor, melatonin can also elicit other tissue-dependent signalling responses, including modulation of different specific ion channels (e.g. potassium channels) and/or regulation of a variety of kinases activity [15–16, 41]. As mentioned above, our results indicate that the stimulatory effect of pharmacological dose of melatonin on OT secretion from the rat H-N system is, probably, independent of both subtypes MT_1 and MT_2 receptors and involves other, i.e. membrane receptor-independent, mechanism(s). Specifically, melatonin enters the cell easily where it may bind to transcription factors belonging to the retinoic acid receptor superfamily, especially splice variants of ROR orphan receptors [16], and directly influence the genes expression [44]. This pineal hormone is also able to affect the reactive oxygen species production [45], and/or it may interact with cytosolic proteins including calmodulin and calreticulin; it may also antagonise the binding of calcium ions to calmodulin [14, 40]. Such mechanisms could be responsible for the stimulatory effect of melatonin on OT secretion from the H-N system *in vitro* because this hormone is released from the oxytocinergic neurons axons endings located in the neurohypophysis by exocytosis, which is dependent on calcium ions [1, 3–4].

Melatonin may, therefore, affect the oxytocinergic neurons activity and secretion of OT by acting directly on specific membrane receptor(s) and/or intracellular pathways, or it may act indirectly via modification of certain neuromediators/neuromodulators metabolism in the hypothalamus and/or in the neurohypophysis [3–4]. Indeed, melatonin was found to enhance GABA-ergic inhibitory transmission [46] and to affect the activity of tyrosine hydroxylase in different brain regions [47], whereas acetylcholine, dopamine, and prostaglandins have been found to participate in an in-

hibitory influence of melatonin on neurohypophysial hormone release from the rat hypothalamus *in vitro* [48]. The above-mentioned neurotransmitters/neuromodulators and other agents (e.g. biogenic amines, excitatory and inhibitory amino acids, neurosteroids, endogenous opiates, nitric oxide, carbon monoxide, etc.) have been shown to influence the activity of hypothalamic SON and PVN nuclei and modify the secretion of OT [1–4, 49], and certain combinations of these agents may be crucial for the mechanisms by which oxytocinergic neurons are influenced by melatonin.

In conclusion, we believe that the results of the present studies have thrown some light on the matter of the mechanisms controlling the activity of oxytocinergic neurons in the rat. Namely, they have shown that the pineal hormone melatonin inhibits OT release acting via membrane MT_1 and not through MT_2 receptor, while the stimulatory effect of the hormone on OT secretion from the rat hypothalamo-neurohypophysial system is mediated through membrane receptor-independent intracellular mechanism(s).

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