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THE INFLUENCE OF MELATONIN RECEPTORS ANTAGONISTS, LUZINDOLE AND 4-PHENYL-2-PROPIONAMIDOTETRALIN (4-P-PDOT), ON MELATONIN-DEPENDENT VASOPRESSIN AND ADRENOCORTICOTROPIC HORMONE (ACTH) RELEASE FROM THE RAT HYPOTHALAMO-HYPOPHYSIAL SYSTEM. *IN VITRO* AND *IN VIVO* STUDIES

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Melatonin exerts its biological role acting *via* G protein-coupled membrane receptors - MT_1 and MT_2, as well as through cytoplasmic and/or nuclear receptors. Melatonin has previously been shown to change vasopressin (AVP) and adrenocorticotropic hormone (ACTH) secretion dependently on its concentration. To determine whether the response of vasopressinergic neurones to different concentrations of melatonin is mediated through the membrane MT_1 and/or MT_2 receptors, the influence of luzindole - an antagonist of both MT_1 and MT_2 receptors, and 4-phenyl-2-propionamidotetralin (4-P-PDOT) - a selective MT_2 receptor antagonist, on melatonin-dependent AVP release from the rat hypothalamo-neurohypophysial (H-NH) system was studied *in vitro* (melatonin at the concentrations of 10^{-9}, 10^{-7} and 10^{-3} M) and *in vivo* (melatonin at the concentrations of 10^{-9} and 10^{-7} M). Moreover, the second goal of this study was to find out whether melatonin receptors MT_1 and/or MT_2 are involved in the regulation of ACTH and corticosterone secretion into the blood. We have demonstrated that melatonin, at the concentrations of 10^{-9} and 10^{-7} M, significantly inhibited AVP secretion from isolated rat H-NH explants when antagonists solvent (i.e. 0.1% DMSO) was present in the medium. Neither luzindole, nor 4-P-PDOT, applied without melatonin, did influence AVP release *in vitro*. Luzindole applied together with melatonin (10^{-7} M and 10^{-9} M) significantly suppressed melatonin-dependent effect, while 4-P-PDOT did not eliminate the inhibitory influence of 10^{-7} M and 10^{-9} M melatonin on AVP secretion from isolated rat H-NH explants. Melatonin at a concentration of 10^{-3} M significantly increased AVP release when the H-NH explants were incubated in the medium containing luzindole or 4-P-PDOT. Under present experimental *in vivo* conditions, infused intracerebroventricularly (i.c.v.) melatonin, at a concentration close to its physiological level in the blood, significantly diminished AVP secretion into the blood, however, at higher concentration (10^{-7} M) it remained inactive in this process. Moreover, melatonin at both concentrations of 10^{-9} M and 10^{-7} M, was able to inhibit AVP secretion into the blood (and increase its neurohypophysial content) when animals were previously i.c.v. injected with 4-P-PDOT, but not with luzindole. Blood plasma concentration of ACTH was diminished significantly by 10^{-7} M melatonin in DMSO-infused, but not in luzindole- or 4-P-PDOT-injected rats, however, it remained inactive in modifying the corticosterone blood plasma concentrations in any of the studied subgroups. The present study demonstrates that subtype MT_1 membrane receptor may contribute to the inhibitory effect of physiological concentration of melatonin on functional regulation of vasopressinergic neurones in the rat. However, for the stimulatory effect of pharmacological dose of the hormone on AVP secretion *in vitro*, mechanisms different from membrane MT_1/MT_2 receptors are involved. The present experiment do not determines whether MT_1 and/or MT_2 receptors affect the function of the rat pituitary-adrenal cortex axis.

Key words: *vasopressin, melatonin, melatonin receptors, corticotrophin-releasing hormone, adrenocorticotropic hormone, luzindole, 4-phenyl-2-propionamidotetralin*

INTRODUCTION

Melatonin has been shown to influence the activity of hypothalamic supraoptic (SON) and paraventricular (PVN) nuclei and modify vasopressin (AVP) release under different experimental conditions, both *in vivo* and *in vitro*. However, the results were not consistent and have shown that it has either a stimulatory or an inhibitory effect, or is without impact on AVP secretion from isolated hypothalamus or neurointermediate lobe, or hypothalamo-neurohypophysial system, depending on a dose applied. In general, pharmacological dose of melatonin (10^{-3} M) has been described to have a stimulatory impact (1-3), while lower concentrations of the hormone (10^{-7} M and 10^{-9} M) have an inhibitory (4-7) influence on AVP release *in vitro*. Moreover, we

have found recently that melatonin at the concentrations of 10^{-9}, 10^{-7} and 10^{-5} M significantly reduced the forskolin-stimulated AVP output from the rat H-NH system *in vitro*, the strongest effect exerting at a concentration of 10^{-7} M (3). Similar observations have been done under *in vivo* conditions. Namely, a significant reduction in circulating plasma AVP level has been found at 5 min after intracerebroventricular (i.c.v.) infusion of melatonin at the concentrations of 1 ng/mL and 10 ng/mL, while higher dose of the hormone (10 µg/mL) stimulated significantly AVP release into the blood (8). When melatonin was applied intravenously (i.v.), a dose of 1 ng per animal caused a significant decrease in AVP secretion into the blood 10 min after i.v. injection, whereas a dose of 5 ng melatonin resulted in an increase in plasma AVP concentration (9). In another *in vivo* experiment, a rise of blood plasma AVP concentration, which resulted from hyperthyroidism, was prevented by long term administration of melatonin, injected intraperitoneally (i.p.) at a dose of 3 mg/kg/day for four weeks (10).

Melatonin exerts its biological role acting *via* G-protein-coupled membrane receptors superfamily (MT_1 and MT_2) and cytosolic quinone reductase enzyme family (MT3), as well as through interaction with calmodulin or the nuclear orphan RZR/ROR receptors (11-13). Melatonin membrane receptors (both MT_1 and MT_2), involved in regulation of the central nervous system functions and pituitary hormones' synthesis and secretion, are situated in rodents mainly in the pars tuberalis of the pituitary (14-15) and in the hypothalamus, especially suprachiasmatic (SCN) nuclei (14, 16-20). In the human hypothalamus, the MT_2 receptor expression is limited to SCN, PVN and SON, while the MT_1 receptor is present in several hypothalamic nuclei (in addition to the SCN, PVN and SON) as well as in the anterior and posterior pituitary (21-22).

Melatonin has equally high affinity for both membrane receptors. After discovery of ligands selective for melatonin receptors, for the study of MT_1 and MT_2 receptor-mediated actions of melatonin specific agonists and/or antagonists of these receptors are used. No selective MT_1 melatonin receptor agonist has been described so far, but several antagonists and nonselective agonists have been identified (11-12). Two antagonists are used extensively to study melatonin receptors actions, e.g. luzindole - a nonselective antagonist of both MT_1 and MT_2 receptors, and 4-phenyl-2-propionamidotetralin (4-P-PDOT) - a selective MT_2 receptor antagonist.

Both MT_1 (17) and MT_2 melatonin receptors are expressed on the AVP-containing cells in the SCN and melatonin has been found to inhibit AVP release from cultured SCN neurones (17, 19). The SCN neurones send their axons, among others, to the PVN (23) and SON (24) in the hypothalamus where AVP is synthesised. The parvocellular neurones of the PVN contain, in the same secretory vesicles, AVP and corticotrophin-releasing hormone (CRH) and the co-secretion of AVP and CRH from the nerve terminals has been described (25-27). Wu *et al.* (21) have reported that MT_1 receptor is colocalized with some parvocellular AVP/CRH neurones in the PVN, which suggests that melatonin might directly affect the secretion of not only AVP, but also CRH, and this way could influence the function of the pituitary-adrenal cortex axis. Indeed, melatonin has been found to inhibit significantly stimulated by physical exercise AVP release (28), as well as to diminish enhanced by immobilization stress adrenocorticotropic hormone (ACTH) secretion into the blood (29); both physical exercise and immobilization are well known activators of the stress system (27). Melatonin was also shown to exert direct inhibitory action on several ACTH-dependent responses (e.g. melatonin inhibits glucocorticoid response to ACTH; 30). Recently, the inhibitory effect of melatonin and an MT_1/MT_2 receptors agonist - ramelteon on basal and CRH-induced ACTH production by corticotrope cells has been described (31). Moreover, Richter *et al.* (32) have found that inhibition by melatonin (1-100 nM) of ACTH-stimulated corticosterone synthesis was reversed by luzindole.

The primary aim of the present study was, therefore, to determine the possible role of membrane MT_1 and/or MT_2 receptors in melatonin-dependent modification of vasopressinergic neurones function in the rat. For this purpose, both luzindole and 4-P-PDOT have been employed to study the melatonin-dependent AVP output from isolated rat hypothalamo-neurohypophysial system (H-NH) *in vitro*, as well as the secretion of the hormone from the neurohypophysis into the blood, which has never been studied, yet. The secondary goal of this study was to investigate whether, under present experimental *in vivo* conditions, melatonin and its membrane MT_1 and/or MT_2 receptors are involved in the regulation of ACTH and corticosterone secretion into the blood.

MATERIALS AND METHODS

The experiments were performed with the consent (No. 8/£B 535/2011, 9/£B536/2011, 19£B604/2012, 83/£B604/DO/2012) of the Local Committee for Animal Care.

Animals

Three-months old male Wistar rats (weighing about 250–350 g) were used for the experiments. They were maintained in a light:dark cycle 12L:12D (lights on from 6 a.m.), at a constant temperature (+22°C), and had food and water available *ad libitum*.

Compounds and reagents

Melatonin (N-acetyl-5-methoxytryptamine) and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich Chemie GmbH. N-Acetyl-2-benzyltryptamine (luzindole; a non-selective antagonist of MT_1 and MT_2 membrane receptors) and 4-phenyl-2-propionamidotetralin (4-P-PDOT; a selective antagonist of MT_2 receptor) were purchased from Tocris Bioscience. The AVP (Vasopressin synth.), for standard curve preparation as well as for iodination with ^{125}I, was from Peninsula Laboratories Europe Ltd. The anti-AVP antibodies were raised by Monika Orlowska-Majdak, Ph.D., D.Sc. (Department of Experimental Physiology, Medical University of Lodz). ELISA kits were provided by USCN Life Science Inc., Houston, TX 77082, USA.

Experimental procedure in vitro

Rats were decapitated between 9:30 and 10:20 a.m. The brain together with the pituitary was carefully removed from the skull, and a block of tissue containing the hypothalamus was isolated as previously described (33-34). After dissection, the hypothalamo-neurohypophysial (H-NH) explant was placed in a polypropylene tube with 1 mL of normal Krebs-Ringer fluid (nKRF) heated in a water bath to 37°C and constantly gassed with carbogen (95% O_2 and 5% CO_2). 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). At the beginning of the experiment, the H-NH explants were incubated in 1 mL of nKRF for 80 minutes and these fluids were aspirated and removed - such equilibration is necessary for stabilization of AVP release (4). After this preincubation period, explants were incubated for 20 minutes in 1 mL of nKRF (fluid B1) and then, for next 20 minutes, in 1 mL of KRF supplemented with the studied substance(s) or their vehicle(s) (fluid B2). Directly after each incubation period, the media (i.e. fluids B1 and B2) were aspirated, immediately frozen

and stored at –20°C until AVP estimation by radioimmunoassay (RIA). To determine the AVP secretion *in vitro,* the B2/B1 ratio was calculated for each H-NH explant. The results are expressed as B2/B1 ratio, because the amount of the neurohormone released into the medium varies from one H-NH explant to the other.

Experimental procedure in vivo

On the day of the experiment, the animals were anaesthetized by an intraperitoneal (i.p.) injection of 10% urethane (ethyl carbonate; 1.4 mL/100 g. b.wt.) and a stainless steel cannula was inserted into the lateral cerebral ventricle (i.c.v.) as recommended by Noble *et al.* (35). The i.c.v. infusions were given *via* polyethylene tube 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 luzindole or 4-P-PDOT, or their solvent (0.1% DMSO). Ten min later, through the same cannula, the i.c.v. infusion of 5 µL melatonin solution (at a concentration chosen on the basis of the results of experiment *in vitro*) or its vehicle (0.1 % ethanol in 0.9% sodium chloride) was given. Ten min after i.c.v. administration of melatonin or its vehicle, the animals were decapitated. The neurohypophysis was separated from the brain and homogenized in 0.25% acetic acid, immediately frozen and stored at –20°C for further AVP RIA. Blood plasma samples were collected and frozen. For the determination of blood plasma concentrations of AVP, ACTH and corticosterone, the ELISA assays were applied.

Series I (experiment in vitro)

The aim of series first was to examine the effect of melatonin receptors antagonists (luzindole and 4-P-PDOT, both at a concentration of 10^{-6} M) on melatonin-dependent (the concentrations of melatonin were chosen on the basis of the results of previous *in vitro* experiments; 3, 5) AVP release from isolated rat hypothalamo-neurohypophysial system. Explants were therefore incubated successively in: (1) nKRF (fluid B1) and (2) nKRF alone or KRF enriched with melatonin vehicle (0.1% ethanol) or with an appropriate concentration of melatonin, i.e. 10^{-9} M, 10^{-7} M or 10^{-3} M, and antagonists solvent - 0.1% DMSO (groups 1-4) or luzindole (groups 5-8), or 4-P-PDOT (groups 9-12) (fluid B2) (n: number of samples per group, n=6).

Series II (1st experiment in vivo)

In series II, animals were divided into two groups - A: i.c.v. injected with melatonin vehicle (VEH) and B: i.c.v.-injected with melatonin solution (MLT) at a concentration of 10^{-9} M (this concentration of melatonin was chosen because it is within the physiological range of the hormone in the blood and in the previous (5, 6) and present (series I) *in vitro* experiments was found to inhibit significantly AVP release from the rat H-NH system). In both groups, the influence of pre-treatment (i.e. i.c.v. infusion) with 0.1% DMSO (subgroup 1) or luzindole (subgroup 2), or 4-P-PDOT (subgroup 3), both antagonists at a concentration of 10^{-6} M, on melatonin-dependent changes in the neurohypophysial AVP content and its blood plasma concentration, has been studied (n: number of animals per subgroup, n=6-7).

Series III (2nd experiment in vivo)

In series III, animals were divided into two groups as in series II - A: i.c.v. injected with VEH and B: i.c.v. injected with MLT at a concentration of 10^{-7} M (this concentration of melatonin, although higher than its physiological level in the blood, was used for the study because a concentration of melatonin in the cerebrospinal fluid is much higher than in the blood (36), and in the previous (3) and present (series I) *in vitro* experiments a concentration of 10^{-7} M melatonin inhibited significantly AVP output from the rat H-NH explant). In both groups, similar subgroups as in series II were chosen: pre-treatment with the respective solution of DMSO (subgroup 4) or luzindole (subgroup 5), or 4-P-PDOT (subgroup 6). The AVP content in the neurohypophysis and blood plasma concentrations of AVP, ACTH and corticosterone were tested in all subgroups (n=6 in each subgroup).

All the experiments were done at the time when the H-NH system was found to be responsive to melatonin (10, 37), i.e. between 09.30 and 11.00 a.m.

Radioimmunoassay of AVP

The AVP concentration in studied samples was assayed in duplicate by a specific RIA described previously (6, 34). Arginine vasopressin was iodinated with ^{125}I using the chloramine-T method. The final dilution of anti-AVP antibodies was 1:24,000. Cross reactivity for these antibodies with oxytocin was 0.016%, with lysine vasopressin - 2.7%, with GnRH, TRH, leucine enkephalin, angiotensin II and substance P - less than 0.002% (6). The lower limit of detection for the assay was 1.56 pg AVP per tube. The intra- and inter-assay coefficients of variation were less than 5.0% and 8.5%, respectively.

ELISA assays

Plasma AVP concentration was determined in duplicate by Enzyme-Linked Immunosorbent Assay (ELISA), using ELISA Kit for Rat Antidiuretic Hormone (ADH) (CEB139Ra; Lot: L131030692). The sensitivity of this assay (lower limit of detection) is less than 4.42 pg/mL and the intra-assay precision is less than 10%.

Plasma ACTH concentration was determined in duplicate by Enzyme-Linked Immunosorbent Assay, using ELISA Kit for Rat Adrenocorticotropic Hormone (ACTH) (CEA836Ra; Lot: L130701040). The sensitivity of this assay is less than 4,76 pg/mL and the intra-assay precision is less than 10%.

Plasma corticosterone concentration was determined in duplicate by ELISA kit for corticosterone (CORT) (CEA540Ge; Lot: L130701042). The sensitivity of this assay is less than 2.62 ng/mL and the intra-assay precision is less than 10%.

To avoid inter-assay variability, all samples within the experiment were tested in the same ELISA-assay for AVP, ACTH and CORT, respectively.

Statistical analysis

AVP release *in vitro* is finally expressed as B2/B1 ratio, while AVP content is expressed in nanograms for whole neurointermediate lobe and in picograms per 1 mL of plasma (all shown as a percentage of the control value). Blood plasma ACTH and corticosterone concentrations are expressed in picograms or nanograms per 1 mL of plasma, respectively. All results are reported as mean ± standard error of the mean (S.E.M.). Significance of the differences between means was evaluated by 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

Series I

Melatonin, at the concentrations of 10^{-9} M and 10^{-7} M, inhibited significantly (in comparison with the control value -

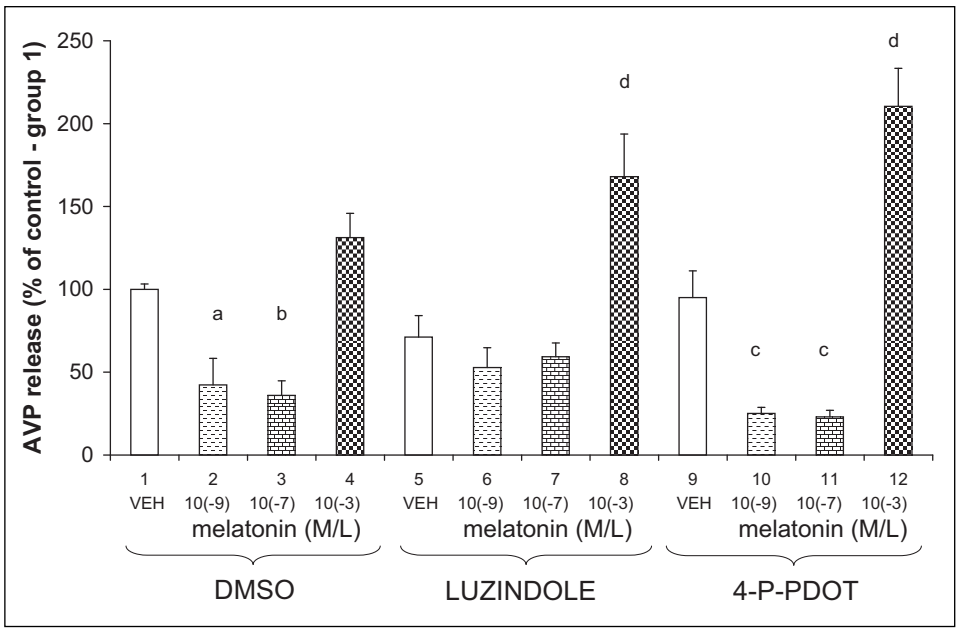

Fig. 1. The effect of melatonin, at the concentrations of 10^{-9}, 10^{-7} and 10^{-3} M, on vasopressin (AVP) release from the rat hypothalamo-neurohypophysial complex incubated *in vitro* in the presence of DMSO (an antagonists solvent) or luzindole (a nonselective antagonist of both MT_1 and MT_2 receptors), or 4-P-PDOT (a selective MT_2 receptor antagonist). Each bar represents mean ±S.E.M. (expressed as a percentage of the control, i.e. DMSO-VEH, value); 1–12 - experimental groups; number of samples per group (n) = 6; [a] P<0.05, [b] P<0.01, [c] P<0.001, [d] P<0.00001 - significantly different versus melatonin vehicle (VEH).

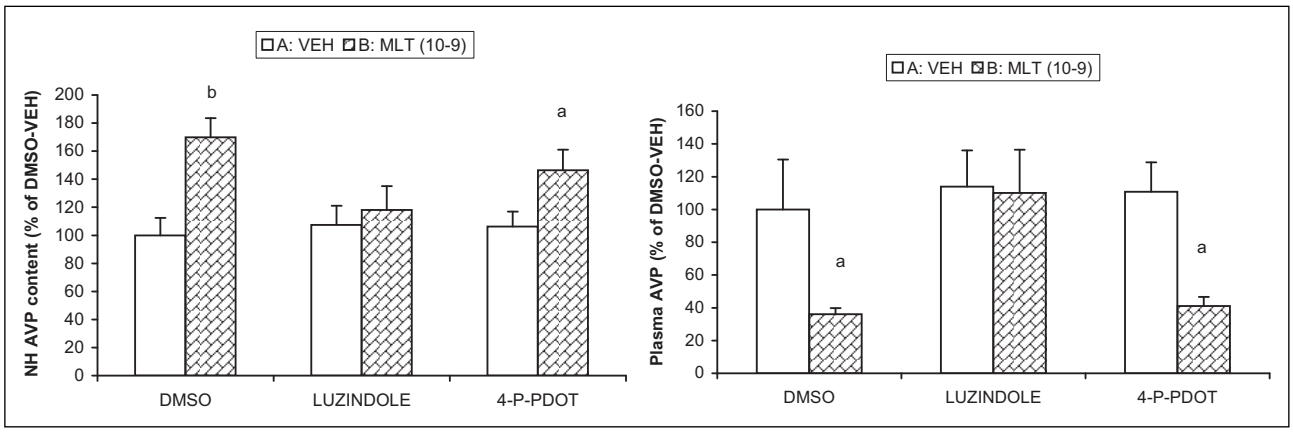

Fig. 2. The effect of i.c.v.-infused melatonin (MLT), at a concentration of 10^{-9} M, or its vehicle (VEH) on the neurohypophysial (NH) vasopressin (AVP) content (left panel) and blood plasma AVP concentration (right panel) in rats previously i.c.v. injected with DMSO (subgroup 1) or luzindole (subgroup 2), or 4-P-PDOT (subgroup 3). Each bar represents mean ±S.E.M. (expressed as a percentage of the control, i.e. DMSO-VEH, value); number of animals per subgroup (n) = 6-7; [a] P<0.05, [b] P<0.005 - significantly different versus VEH in each subgroup.

VEH) AVP secretion from isolated rat hypothalamo-neurohypophysial (H-NH) explants when antagonists solvent, i.e. DMSO, was present in the medium. Luzindole and 4-P-PDOT, applied without melatonin, did not influence AVP release *in vitro*. Incubation of the H-NH explants in the medium containing both 4-P-PDOT and melatonin (at the concentrations of 10^{-9} M and 10^{-7} M) resulted in strong inhibition of AVP secretion *in vitro*. However, when the H-NH explants were incubated in the presence of luzindole and melatonin, the two concentrations of the hormone, i.e. 10^{-9} M and 10^{-7} M, were ineffective in this process (*Fig. 1*). Melatonin at a concentration of 10^{-3} M significantly increased (in comparison with the VEH) AVP output from the H-NH explants when luzindole or 4-P-PDOT, but not DMSO alone, were present in the buffer (*Fig. 1*).

Series II and III

Under present experimental conditions, infused i.c.v. melatonin (group B), at both concentrations of 10^{-9} M and 10^{-7} M, was able to inhibit (in comparison with the VEH, i.e. group A) AVP secretion into the blood when animals were pretreated (also i.c.v.) with 4-P-PDOT (*Figs. 2* and *3*). When animals were previously injected i.c.v. with luzindole melatonin, at both concentrations studied, remained inactive in this process (*Figs. 2* and *3*). However, when animals were i.c.v. pretreated with DMSO melatonin, at a concentration of 10^{-9} M, significantly diminished AVP release (*Fig. 2*), but at a concentration of 10^{-7} M it remained inactive (*Fig. 3*) in modifying AVP output into the blood. The neurohypophysial content of AVP was found to be significantly increased by i.c.v. infusion of melatonin (at both concentrations studied) in DMSO- and 4-P-PDOT-injected, but not in luzindole-infused animals (*Figs. 2* and *3*).

Blood plasma concentration of ACTH was diminished significantly by 10^{-7} M melatonin in DMSO- but not in luzindole- or 4-P-PDOT-injected rats (*Table 1*). Under present experimental conditions, melatonin was found to have no significant effect on corticosterone blood plasma concentrations in any of the studied subgroups (*Table 1*).

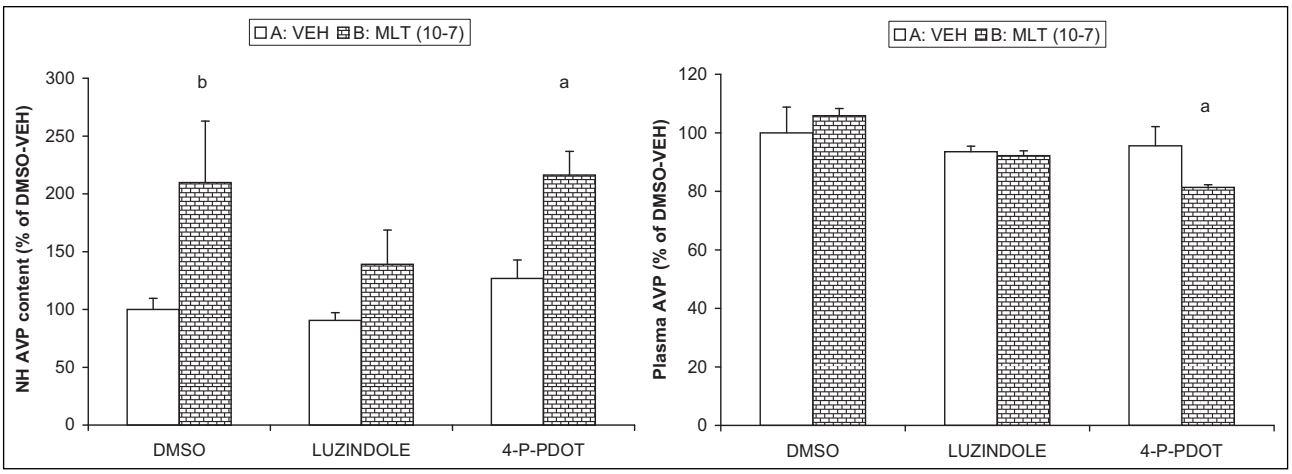

Fig. 3. The effect of i.c.v.-infused melatonin (MLT), at a concentration of 10^{-7} M, or its vehicle (VEH) on the neurohypophysial (NH) vasopressin (AVP) content (left panel) and blood plasma AVP concentration (right panel) in rats previously i.c.v. injected with DMSO (subgroup 4) or luzindole (subgroup 5), or 4-P-PDOT (subgroup 6). Each bar represents mean ±S.E.M. (expressed as a percentage of the control, i.e. DMSO-VEH, value); number of animals per subgroup (n) = 6; [a] $P<0.05$, [b] $P<0.01$ - significantly different versus VEH in each subgroup.

Table 1. The influence of i.c.v. infusion of melatonin (MLT), at a concentration of 10^{-7} M, or its vehicle (VEH) on blood plasma ACTH and corticosterone (CORT) concentration in DMSO- (subgroup 4) or luzindole- (subgroup 5), or 4-P-PDOT-pretreated (subgroup 6) male rats (mean±S.E.M.; number of animals per each subgroup n=6).

Groups Subgroups	**A: VEH-infused**		**B: MLT-infused**		Significance of the difference	
	a - ACTH (pg/mL)	**b - CORT** (ng/mL)	**a - ACTH** (pg/mL)	**b - CORT** (ng/mL)	Aa vs. Ba	Ab vs. Bb
4. DMSO	83.9 ± 7.2	31.7 ± 2.7	39.8 ± 9.6	27.1 ± 1.7	P<0.001	NS
5. Luzindole	30.2 ± 4.7	20.1 ± 1.7	21.8 ± 8.9	22.4 ± 1.3	NS	NS
6. 4-P-PDOT	20.6 ± 4.7	17.8 ± 0.4	27.4 ± 4.4	19.5 ± 1.7	NS	NS

DISCUSSION

Obtained results confirm our previous findings that melatonin, at a concentration which is within the physiological range of the hormone in the blood and at higher than physiological concentration, is able to inhibit AVP output from isolated rat hypothalamo-neurohypophysial (H-NH) explants (3, 5-7), while pharmacological dose of the hormone stimulates AVP secretion *in vitro* (2-3). Our present results are also in agreement with other studies on the actions of melatonin, which have reported opposite effects of so-called pharmacological (above 1 μM) and/or supraphysiological (1 nM – 1 μM) and physiological (below 1 nM) concentrations of the hormone (12). However, it has not yet been elucidated whether melatonin exerts its inhibitory/stimulatory effects on AVP release through G protein-coupled melatonin receptors or/and through membrane receptors-independent mechanisms.

To verify the hypothesis that melatonin influences AVP release from the rat H-NH system through both melatonin membrane receptor(s)-dependent and membrane receptors-independent mechanisms, according to a dose applied, we evaluated the effects of three concentrations of melatonin, used alone or together with melatonin receptors antagonists, i.e. luzindole and 4-P-PDOT, which are frequently employed to study melatonin membrane receptors action (11-12). Several studies have shown that luzindole antagonizes the effects of melatonin, both *in vivo* and *in vitro*. Namely, applied i.p. luzindole (at a dose of 10 mg/kg) almost completely inhibited the antinociceptive effect of melatonin (at a dose of 100 mg/kg) injected also i.p. ten minutes later (38). Moreover, luzindole (at the concentrations of 10^{-6} M – 10^{-4} M) applied together with melatonin (at the concentrations of 10^{-7} M and/or 10^{-9} M) significantly suppressed melatonin-dependent actions *in vitro* (32, 39-41), while 4-P-PDOT (at the concentrations of 10^{-6} M-10^{-9} M) did not change the inhibitory effect of 10^{-7} M and 10^{-9} M melatonin (39). In agreement with the above cited studies, results from our present experiments have shown that luzindole is able to antagonize the inhibitory effect of melatonin on AVP secretion, while 4-P-PDOT does not eliminate such an effect of the hormone, which suggests that an effect of so-called physiological and supraphysiological concentrations of melatonin is, most probably, mediated through a subtype MT_1 membrane receptor. Moreover, our present results may imply that for the stimulatory effect of pharmacological concentration of the hormone on AVP release other than membrane MT_1 and/or MT_2 receptors-dependent mechanisms are involved.

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 (36). It is known that exogenous melatonin crosses the blood-brain barrier easily. After systemic administration, ^{14}C-melatonin reaches at 5 min a maximum level in the brain, and 30

min later it is still present in most of the brain regions, among others in the hypothalamic PVN (42). It could be, therefore, expected that several effects of exogenous melatonin should be displayed shortly after systemic or i.c.v. injection. Previous *in vivo* results have shown that AVP secretion into the blood was significantly reduced 10 min after single i.v. injection of 5 ng/mL melatonin (9), as well as at 5 min after i.c.v. infusion of the hormone at the concentrations of 1 ng/mL and 10 ng/mL (8). Moreover, significant antinociceptive effect of melatonin (at a dose of 100 mg/kg) was observed 10 min after i.p. injection (38). Therefore, under present experimental *in vivo* conditions, a 10 min interval between i.c.v. infusion of melatonin and decapitation should be enough to display an effect of exogenous melatonin on AVP output from the neurohypophysis into the blood. Indeed, the results from the present *in vivo* studies provide (additionally to the *in vitro* experiment) further evidence in favour of the idea that melatonin (at a physiological concentration) inhibits AVP secretion *via* subtype MT_1 receptor-dependent mechanism and its action is rather independent of subtype MT_2 receptor. Our data indicate additionally that the stimulatory effect of pharmacological dose of melatonin on AVP secretion from the rat hypothalamo-neurohypophysial system is, most probably, independent of both subtypes MT_1 and MT_2 receptors, and involves other mechanism(s). Namely, apart from cAMP, which is the main intracellular second messenger for melatonin action through MT_1 and/or MT_2 receptors (17, 43-45), melatonin can also modify intracellular concentration of cGMP, activity of phospholipase C and intracellular free calcium concentration (43-45). Melatonin enters the cell easily and in cytosol it may interact with calmodulin (46) and/or it may directly influence the genes expression through brain-specific nuclear RZR/ROR receptors (13, 47).

Melatonin may also affect the release of AVP by acting directly on vasopressinergic neuron endings located in the neurohypophysis or indirectly *via* modification of metabolism (in the hypothalamus and/or in the neurointermediate lobe) of certain neuromediators and/or neuromodulators, which are known to modify the neurohypophysial hormones release (48-50). Indeed, acetylcholine, dopamine, prostaglandins and endogenous opiates have been found to participate in an inhibitory influence of melatonin on AVP secretion under different experimental conditions (9, 28, 51). A certain combination of several agents (excitatory and inhibitory) may be crucial for the mechanisms by which vasopressinergic neurones are influenced by melatonin, which may activate or inactivate (dependently on its concentration) both G protein-coupled melatonin receptors-dependent intracellular second messengers and membrane receptors-independent ways of its action.

Recently, Kusek *et al.* (52) have shown that repeated stress enhances excitatory input to parvocellular part of the hypothalamic PVN, which are known to synthesise AVP and CRH in the same AVP/CRH neurones and to release both neurohormones from the same secretory vesicles under different stress conditions (25-27, 53). It could be, therefore, suspected that melatonin modifies the secretion of AVP and CRH-dependent ACTH synthesis and secretion through similar mechanisms. The theoretical basis for such an idea is presence of MT_1 receptor, colocalized with some parvocellular AVP/CRH neurones, in the hypothalamic PVN (21), as well as the results of Tsukamoto *et al.* (31) who have found that both melatonin and an MT_1/MT_2 receptors agonist - ramelteon suppressed CRH-induced ACTH production by pituitary corticotrope cells incubated *in vitro*. However, the suggestion that melatonin is an inhibitory regulator of the pituitary-adrenal cortex axis in the rat and suppresses the ACTH and/or corticosterone release through melatonin membrane receptor(s)-dependent mechanisms, has not been confirmed by our present *in vivo* experiment. Luck of the influence of melatonin on corticosterone secretion, under present experimental conditions, may result from several reasons. Namely, it has been shown that MT_1 melatonin receptor mRNA expression could be detected only in the rat adrenal glands collected at 18.00–22.00 h, but not at 10.00–14.00 h (32). Our present experiments were done at the time when the H-NH system was found to be responsive to melatonin (10, 37), i.e. between 09.30 and 11.00 a.m., which could partly explain obtained result, i.e. no effect of melatonin on corticosterone secretion. The other reason for such a result could be too short time period from melatonin infusion and decapitation of the animal. It is the most probable that a 10 min interval between the i.c.v. infusion of melatonin and decapitation was not enough time to display an effect of a single dose of exogenous melatonin on corticosterone output from the adrenal glands into the blood. Indeed, inhibition by melatonin (1–100 nM) of ACTH-stimulated corticosterone synthesis by the adrenal glands *in vitro*, has been notified after 12 hours of incubation (32). Similarly, a diminution of corticosterone secretion, which was a consequence of melatonin administration, has been found after 5 days of treatment (54). On the other hand, however, i.p. injections of melatonin at late afternoon under long-day conditions for 3 weeks did not influence plasma corticosterone rhythms in Fisher 344 rats (55). It could be therefore concluded, that the possible effect of melatonin on the function of the pituitary-adrenal cortex axis in the rat depends on experimental conditions and so far has not been fully elucidated.

In conclusion, the findings of the present study suggest that, in the rat, the inhibitory influence of melatonin (at a concentration which is close to physiological range of the hormone in the blood) on AVP secretion is mediated through subtype MT_1 membrane receptor, and is rather independent of subtype MT_2 receptor, while stimulatory effect of pharmacological doses of the hormone is mediated through membrane receptors-independent intracellular mechanisms. Our present experiment, however, do not determines whether melatonin exerts its possible effect on the function of the rat pituitary-adrenal cortex axis through membrane MT_1/MT_2 receptors or some other mechanisms.

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