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VASOPRESSIN RELEASE FROM THE RAT HYPOTHALAMO-NEUROHYPOPHYSIAL SYSTEM: EFFECTS OF GONADOTROPHIN-RELEASING HORMONE (GNRH), ITS ANALOGUES AND MELATONIN

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The influence of gonadotrophin-releasing hormone (GnRH) and its analogues (*i.e.*, agonist and antagonist) on vasopressin (VP) release from the rat hypothalamo-neurohypophysial (H-N) system was studied both *in vitro* and *in vivo*. Additionally, it was determined whether the possible response of vasopressinergic neurones to these peptides could be modified by melatonin through a cAMP-dependent mechanism. In this study we demonstrate, for the first time, that the highly selective GnRH agonist (*i.e.*, [Des-Gly^{10},D-His(Bzl)^{6},Pro-NHEt^{9}]-LHRH; histrelin) stimulates the release of VP from the rat H-N system, while native GnRH and its antagonist remain inactive in modifying this process *in vitro*. Melatonin significantly inhibited basal and histrelin-induced release of VP *in vitro*, but displayed no significant influence on VP secretion when GnRH or its antagonist were present in a medium. Melatonin fully suppressed forskolin-stimulated VP release from the rat H-N system. On the other hand, addition of forskolin to a medium containing both histrelin and melatonin did not further alter the inhibitory influence of melatonin on the histrelin-dependent release of VP *in vitro*. After intracerebroventricular (i.c.v.) infusion of native GnRH or its agonist, blood plasma VP concentration was significantly higher than in control animals, which was accompanied by decreased content of the hormone in the neurohypophysis. Intravenous (i.v.) injection of melatonin did not change, in any subgroup, blood plasma VP concentration, when compared to the vehicle-injected rats. However, the neurohypophysial levels of the hormone were significantly higher after melatonin injection in control, GnRH- and histrelin-infused animals. Our present results suggest that activation of the GnRH receptor in the hypothalamus is involved in stimulation of VP secretion from the rat H-N system. We have also shown that melatonin, at a concentration close to its physiological level in the blood, significantly reduces the *in vitro* response of vasopressinergic neurones to a GnRH agonist - histrelin; this effect of melatonin could be mediated through intracellular processes that involve, among others, the cAMP-dependent mechanism.

Key words: *vasopressin, gonadotrophin-releasing hormone (GnRH), histrelin, neurohypophysis, melatonin*

INTRODUCTION

Various peptides present in the central nervous system, such as thyrotrophin-releasing hormone, corticotrophin-releasing hormone, prolactin, somatostatin, neuropeptide Y, galanin, substance P, neurokinin A and endothelins (1-8) were shown to influence the activity of hypothalamic supraoptic (SON) and paraventricular (PVN) nuclei and modify vasopressin (VP) synthesis and release. Few experimental data suggested that also gonadotrophin-releasing hormone (GnRH) can alter the secretion of VP (9-10). In mammals more than 20 forms of GnRH have been described (11). Four forms of GnRH such as: m-LHRH (GnRH-I), c-GnRH (GnRH-II), l-GnRH (GnRH-III) and s-GnRH have been found in rats and human central nervous system (11-13). In rats, majority of the neurones producing GnRH-I were found in the hypothalamic preoptic area, several of them being localized close to the SON (12). Also in men, neurones containing GnRH were observed in the SON and PVN of the hypothalamus (14) as well as in the neurohypophysis (15), and anatomical synapses between GnRH- and VP-immunoreactive neurones have been demonstrated in primates (16). However, very few studies have been performed to investigate the effect of GnRH on VP secretion from the hypothalamo-neurohypophysial system (9-10) and the results did not provide clear solution of the problem. On the other hand, although in medical practice more often than GnRH itself, its agonists and antagonists are used (17), the possible relationship between GnRH analogues and VP release have not been studied, yet. The first aim of the present study was, therefore, to investigate the effect of GnRH and its analogues (*i.e.*, agonist and antagonist) on VP secretion from the rat hypothalamo-neurohypophysial system, both *in vivo* and *in vitro*.

Previous results show that melatonin is involved in the pineal-neurohypophysial interrelationship; it modifies VP release under physiological and pathological conditions, both *in vivo* and *in vitro* (18). Moreover, melatonin decreases GnRH release from the rat hypothalamic neurones (19); it inhibits the GnRH gene expression and GnRH secretion as well as regulates melatonin MT_1 receptors present on the GnRH neurones (20-21).

Activation of these receptors by melatonin leads to diminution of cAMP production and attenuation of GnRH-induced gonadotrophin secretion (22). The intracellular mechanism of melatonin action involves inhibition of calcium influx, calcium mobilization from intracellular stores and an adenylyl cyclase (AC)-dependent rise in cyclic adenosine monophosphate (cAMP) production (22-24). Melatonin was shown to inhibit the GnRH- as well as forskolin-induced (forskolin is a strong AC activator) increase in cAMP accumulation in the rat pituitary (23-24). The second purpose of the present study was to investigate whether the GnRH- (and/or its analogues)-dependent secretion of VP may be influenced by melatonin, and whether it may act through cAMP-dependent mechanism.

MATERIALS AND METHODS

Animals

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

Drugs

Peptides: gonadotrophin-releasing hormone [gonadorelin; m-LHRH], its agonist {[Des-Gly^{10},D-His(Bzl)^{6},Pro-NHEt^{9}]-LHRH; histrelin} and antagonist {[Ac-D-2-Nal-p-chloro-D-Phe-β-(3-pyridyl)-D-Ala-Gly-Arg-Pro-D-Ala-NH_2] trifluoroacetate salt; LH-RH Antagonist} were purchased from BACHEM AG, Bubendorf, Switzerland. Melatonin (N-acetyl-5-methoxytryptamine), forskolin and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich Chemie GmbH. The VP (Vasopressin synth.), for standard curve preparation as well as for iodination with ^{125}I, was from Peninsula Laboratories Europe Ltd. The anti-VP antibodies were raised by Dr Monika Orłowska-Majdak (Department of Experimental Physiology, Chair of Experimental and Clinical Physiology, Medical University of Lodz).

Experimental procedure in vitro

On the day of the experiment, the animals were decapitated between 9:30 and 10:30 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 (3). After dissection, the hypothalamo-neurohypophysial (H-N) explant was placed in 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 =285-295 mOsm/kg). At the beginning of the experiment, after incubation of H-N explants in nKRF for 40 minutes the media were aspirated and replaced with 1 ml of fresh nKRF for next 40 minutes incubation. After 80 minutes of such preincubation, which is necessary for stabilization of VP release (25), explants were incubated for 20 minutes in 1 ml of nKRF (fluid B1) and than in 1 ml of KRF supplemented with the studied substance(s) or their vehicle (fluid B2).

Series I

The aim of series first was to examine the effect of GnRH and its analogues on VP release from isolated rat hypothalamo-neurohypophysial system. Explants were therefore incubated successively in: nKRF (fluid B1) and next KRF alone (control - group a; n: number of samples per group, n=8) or enriched with native GnRH (group b) at a concentration of 10^{-8} M (n=7) or 10^{-7} M (n=6), the GnRH agonist (group c) at a concentration of 10^{-8} M (n=8) or 10^{-7} M (n=8), the GnRH antagonist (group d) at a concentration of 10^{-8} M (n=7) or 10^{-7} M (n=6) (fluid B2).

Series II

In the second series of experiments, the influence of melatonin on peptide-dependent VP secretion from the rat hypothalamo-neurohypophysial system was studied *in vitro*. Explants were similarly incubated in nKRF (fluid B1) and next in KRF alone (control - subgroup a, n=7) or supplemented with one of the studied peptides: GnRH (subgroup b, n=6), its agonist (subgroup c, n=7) or antagonist (subgroup d, n=7); all peptides at a concentration of 10^{-7} M (fluid B2). The B2 fluids were additionally enriched with either melatonin vehicle (*i.e.*, 0.1% ethanol; group A) or melatonin solution at a concentration of 10^{-9} M (group B).

Series III

In series III, the influence of melatonin on forskolin- and/or GnRH-induced VP release from isolated rat hypothalamo-neurohypophysial system was tested *in vitro*. The experimental protocol was similar to that of series I and II. Therefore, after incubation in nKRF (fluid B1) explants were next incubated in one of the following media: KRF enriched with melatonin vehicle (0.1% ethanol; VEH; group 1, n=6) or melatonin solution at a concentration of 10^{-9} M (MLT; group 2, n=6), KRF supplemented with forskolin vehicle (0.1% DMSO; group 3, n=7) or forskolin solution at a concentration of 10^{-5} M (Forsk; group 4, n=7) or Forsk and MLT (group 5, n=7), KRF containing GnRH agonist at a concentration of 10^{-7} M and VEH (group 6, n=6) or GnRH agonist and MLT (group 7, n=6) or GnRH agonist and MLT and Forsk (group 8, n=8) (fluid B2).

In all three series, directly after each incubation period, the media (*i.e.*, fluids B1 and B2) were aspirated, immediately frozen and stored at -20°C until VP estimation by radioimmunoassay (RIA).

To determine the VP secretion *in vitro*, the B2/B1 ratio was calculated for each H-N explant in all series. The results are expressed as B2/B1 ratio, because the amount of VP released into the medium varies from one H-N explant to the other.

Experiment in vivo

On the day of the experiment, the rats were anaesthetized by an intraperitoneal (i.p.) injection of 10% urethane (ethyl carbonate; 1.4 ml/100 g. bwt) and a stainless steel cannula was inserted into the lateral cerebral ventricle (i.c.v.) as recommended by Noble *et al*. (26). Immediately after the end of i.c.v. cannulation, a polyethylene cannula (rinsed with 25 U heparin/ml saline) was introduced into the left femoral vein (i.v.). During experiments *in vivo* rats were given an i.c.v. infusion of 5 µl 0.9% NaCl alone or supplemented with either GnRH, GnRH agonist or GnRH antagonist at a concentration of 10^{-7} M (the concentration of the peptides was chosen on the basis of the results of series I). The i.c.v. infusions were given *via* polyethylene tube attached to a 10 µl Hamilton syringe filled with the appropriate solution. Ten minutes later, animals were injected i.v. with 0.2 ml of melatonin solution (at a dose of 5 ng/ml) or with 0.2 ml of melatonin vehicle, *i.e*., 0.1% ethanol in 0.9% NaCl. Ten minutes after i.v. administration of melatonin (MLT) or its vehicle (VEH), the animals were decapitated. The neurohypophysis was separated from the brain

and homogenized in 0.25% acetic acid. The trunk blood was collected and VP was extracted from blood plasma using C18 Sep-Pak cartridges (Waters Corp., Milford, Massachusetts). All samples were immediately frozen and stored at -20°C for VP RIAs.

Series IV

In this series of the experiment, the effect of melatonin on blood plasma VP concentration and neurohypophysial VP content in GnRH- or its analogues-infused rats was studied. The following experimental groups were chosen: rats treated i.c.v. with saline (control; subgroup a, n: number of animals per group, n=8), GnRH (subgroup b, n=8), GnRH agonist (subgroup c, n=8) or GnRH antagonist (subgroup d, n=8) and i.v. with either VEH (group A) or MLT (group B).

All the experiments (both *in vitro* and *in vivo*) were done at a time when the H-N system was found to be responsive to melatonin (27), *i.e*., between 09.30 and 11.30 a.m. The experimental procedures were done with the consent (No. Ł/BD/265 and 16/ŁB322/2006) of the Local Committee for Animal Care.

Radioimmunoassay of VP

The VP concentrations in all samples were assayed in duplicate by a specific RIA described previously (3-5). Arginine vasopressin was iodinated with ^{125}I using the chloramine-T method. The final dilution of anti-VP antibodies was 1:24000. Cross reactivity with oxytocin for anti-VP antibodies was 0.016%, with lysine vasopressin - 2.7%, with gonadotrophin-releasing hormone, tyrotrophin-releasing hormone, leucine enkephalin, angiotensin II and substance P - less than 0.002% (5). The lower limit of detection for the assay was 1.56 pg VP per tube. The intra- and inter-assay coefficient of variation was 3.3% and 6.3%, respectively.

Statistical evaluation of the results

All results are reported as mean±standard error of the mean (S.E.M.). The statistical significances of differences between means were estimated by nonparametric Kruskal-Wallis analysis of variance (ANOVA), when more than two subgroups were compared. Thereafter, the statistical significance of differences between the means of two compared subgroups was evaluated by Mann-Whitney "U" test (Statistica, StatSoft, Krakow, Poland). P-values less than 0.05 were considered as significant.

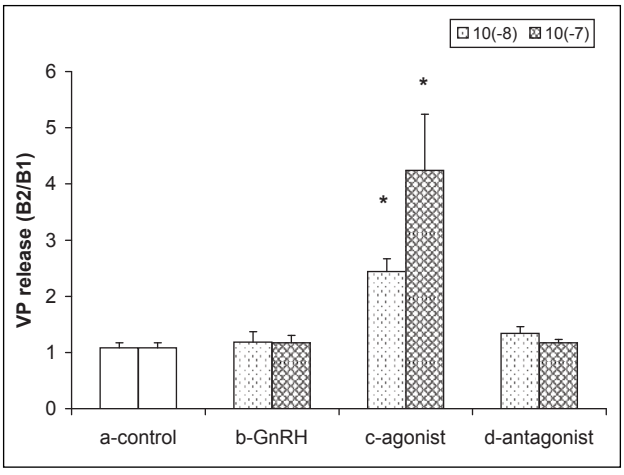

Fig. 1. The effect of gonadotrophin-releasing hormone (GnRH), its agonist and antagonist, at concentrations of 10^{-8} or 10^{-7} M, on vasopressin (VP) release from the rat hypothalamo-neurohypophysial complex *in vitro*. Each bar represents mean±S.E.M.; number of samples per subgroup (n) =6-8; * p<0.05 - significantly different *vs*. control.

RESULTS

Series I

GnRH agonist - histrelin (group c), at concentrations of 10^{-7} M and 10^{-8} M (a concentration of 10^{-7} M was more effective than 10^{-8} M), stimulated significantly VP secretion from isolated rat hypothalamo-neurohypophysial explants (group c *vs*. group a; p<0.05). When native GnRH (group b) or its antagonist (group d) were present in the buffer (independently of peptides' concentration, *i.e.*, 10^{-7} or 10^{-8} M, respectively), basal VP release was not different from the control (group b *vs*. group a; p>0.05 and group d *vs*. group a; p>0.05) (*Fig. 1*).

Series II

Melatonin (at a concentration of 10^{-9} M) significantly diminished basal release of VP (subgroup Ba *vs*. Aa; p<0.05) and also attenuated (subgroup Bc *vs*. Ac; p<0.05) the agonist-induced (subgroup Ac *vs*. Aa; p<0.05) VP secretion from the rat hypothalamo-neurohypophysial explants *in vitro* (*Fig. 2*). Neither GnRH nor its antagonist significantly affected VP secretion from the rat hypothalamo-neurohypophysial system (subgroup Ab *vs*. Aa; p>0.05 and subgroups Ad *vs*. Aa; p>0.05) and melatonin did not modify VP release in the presence of these peptides (at a concentration of 10^{-7} M) in the medium (subgroup Bb *vs*. Ab; p>0.05 and subgroup Bd *vs*. Ad; p>0.05) (*Fig. 2*).

Series III

Forskolin, at a concentration of 10^{-5} M, increased VP secretion into the medium (group 4 *vs*. group 3; p<0.05). When

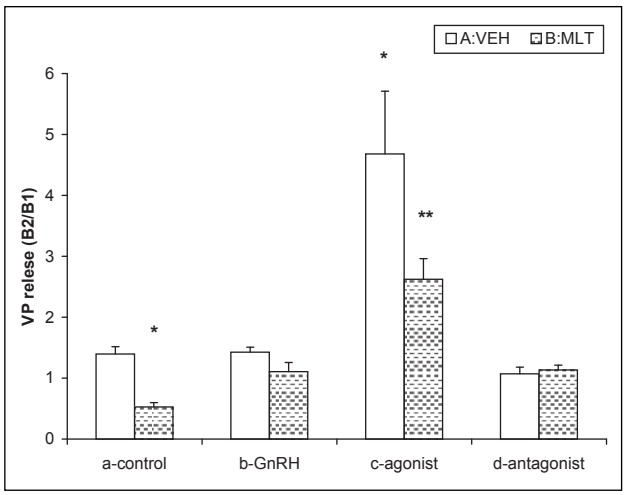

Fig. 2. The effect of melatonin (MLT), at a concentration of 10^{-9} M, on gonadotrophin-releasing hormone (GnRH)-, its agonist- or antagonist-dependent vasopressin (VP) release from the rat hypothalamo-neurohypophysial complex *in vitro* (peptides at a concentration of 10^{-7} M). Each bar represents mean±S.E.M.; number of samples per subgroup (n) =6-7; * p<0.05 - significantly different *vs*. explants incubated in medium containing melatonin vehicle (VEH; subgroup Aa); ** p<0.05 - significantly different *vs*. explants incubated in medium containing GnRH agonist and VEH (subgroup Ac).

melatonin was added to the basal medium (group 2), the medium containing forskolin (group 5) or histrelin (group 7), VP release was significantly inhibited when compared to the VEH containing media (*i.e*., group 2 *vs*. group 1; p<0.05, group 5 *vs*. group 4; p<0.05 and group 7 *vs*. group 6; p<0.05), respectively (*Fig. 3A* and *3B*). Addition of forskolin to the medium containing both GnRH agonist and melatonin (group 8) had no further influence on the inhibitory effect of melatonin on the GnRH agonist-dependent secretion of VP (group 8 *vs*. group 7; p>0.05) into the medium (*Fig. 3B*).

Series IV

During experiment *in vivo*, both GnRH and its agonist were able to diminish the VP content in the neurohypophysis of VEH-injected animals (subgroup Ab *vs*. Aa; p<0.05 and subgroup Ac *vs*. Aa; p<0.05). In melatonin-treated animals, the VP content in the neurohypophysis was significantly higher in control (subgroup Ba *vs*. Aa; p<0.05), GnRH-infused (subgroup Bb *vs*. Ab; p<0.05) and

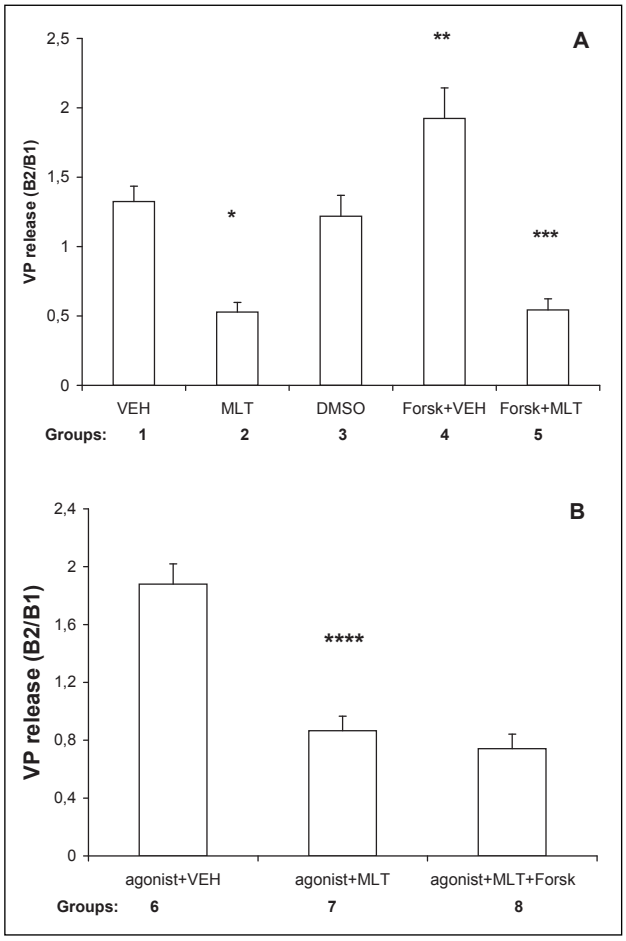

Fig. 3. The effect of melatonin (MLT), its vehicle (VEH), forskolin (Forsk) and its vehicle (DMSO) (A) as well as gonadotrophin-releasing hormone agonist (agonist) and MLT and Forsk (B) on vasopressin (VP) release from the rat hypothalamo-neurohypophysial complex *in vitro*. Each bar represents mean±S.E.M.; n=6-8; * p<0.05 - significantly different *vs* explants incubated in medium containing VEH (group 1); ** p<0.05 - significantly different *vs.* explants incubated in medium containing DMSO (group 3); *** p<0.05 - significantly different *vs*. explants incubated in medium containing Forsk and VEH (group 4); **** p<0.05 - significantly different *vs.* explants incubated in medium containing GnRH agonist and VEH (group 6).

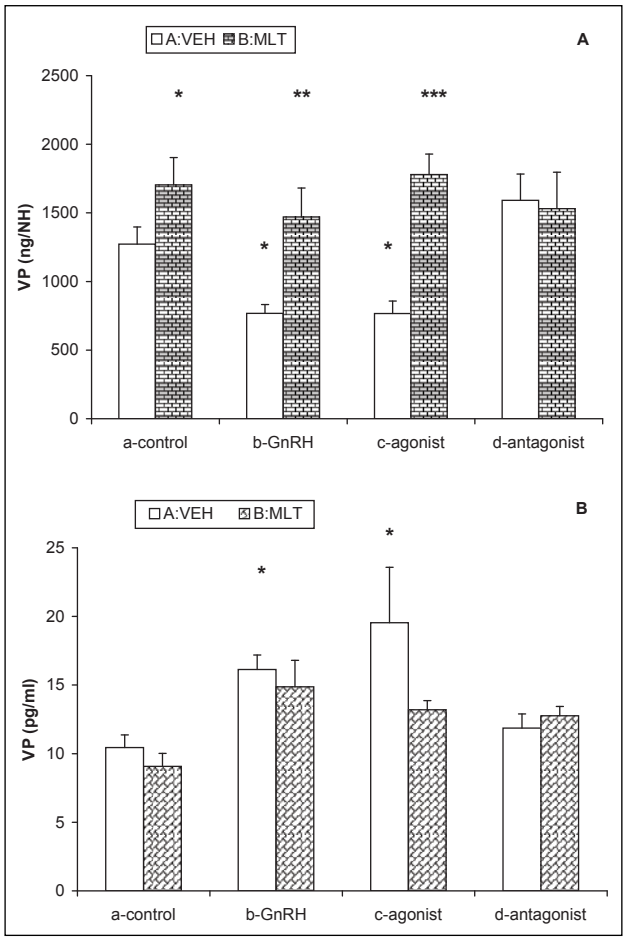

Fig. 4. The effect of i.v. melatonin (MLT; at a dose of 5 ng/ml) injection on vasopressin (VP) content in the neurohypophysis (NH) (A) and blood plasma VP concentration (B) in i.c.v. GnRH-, its agonist- or antagonist-infused male rats (peptides at a concentration of 10^{-7} M). Each bar represents mean±S.E.M.; number of animals per subgroup (n) =8; * p<0.05 - significantly different *vs*. control-VEH (subgroup Aa); ** p<0.05 - significantly different *vs*. GnRH-VEH (subgroup Ab); *** p<0.05 - significantly different *vs*. agonist-VEH (subgroup Ac).

GnRH agonist-infused (subgroup Bc *vs*. Ac; p<0,05), but not in GnRH antagonist-injected (subgroup Bd *vs*. Ad; p>0.05) rats, when compared to VEH-treated animals (*Fig. 4A*). Blood plasma VP concentrations were significantly higher when native GnRH (subgroup Ab *vs*. Aa; p<0.05) or its agonist (subgroup Ac *vs*. Aa; p<0.05) were infused i.c.v., but were not further modified by i.v. injection of melatonin (subgroup Bb *vs*. Ab; p>0.05 and subgroup Bc *vs*. Ac; p>0.05). Melatonin also did not change VP secretion into the blood, when compared to VEH-injected animals, in control (subgroup Ba *vs*. Aa; p<0.05) and GnRH antagonist-infused (subgroup Bd *vs*. Ad; p>0.05) rats (*Fig. 4B*).

DISCUSSION

The influence of GnRH and its analogues on vasopressin release

The synthesis and secretion of VP is known to be stimulated by several factors, *e.g*., haemorrhage, dehydration, hyperosmotic stimulation, angiotensin II or stress (9, 18, 28, 29). The response of magnocellular SON and/or PVN neurones to such stimuli

depends on the presence in the central nervous system of numerous agents, among which peptides play an important role (1, 2, 6, 8). Previously published data demonstrated that an action of GnRH on vasopressinergic neurones activity depends on a peptide dose, experimental conditions (*in vitro* or *in vivo*) and state of water-electrolyte balance (9-10). It was found that infused i.c.v. GnRH attenuated the enhanced VP release from the hypothalamo-neurohypophysial system brought about by haemorrhage or dehydration, but did not significantly affect VP secretion into the blood in euhydrated rats (9). Results from the present study showed that GnRH-I - the natural ligand for GnRH-I receptor, stimulated VP release into the blood, but was ineffective *in vitro*. Additionally, we have demonstrated, for the first time, that highly selective GnRH agonist - histrelin increased VP release from the rat hypothalamo-neurohypophysial system (both *in vivo* and *in vitro*), while GnRH antagonist was ineffective in this process. Dissimilar effects of GnRH and its agonist on VP release into the medium may result from several reasons, *e.g*., from different activity of these peptides. Namely, histrelin belongs to one of the most potent GnRH agonists used in the clinical practice - it is 200 times more active than native GnRH-I (17). It is well known that native GnRH is metabolized very fast (in rats, GnRH mRNA has a rapid rate of decay - $t_{1/2}$, 5-13 min; 30), while its agonists are more resistant to degradation (17). It could be therefore concluded that endogenous GnRH and its agonist - histrelin, acting through specific GnRH receptor in the hypothalamus, play a role in regulation of VP secretion from the rat hypothalamo-neurohypophysial system, and this way may indirectly influence mean arterial pressure (*via* dendritic release of VP from magnocellular PVN neurones; 8), water balance of the organism and brain function (endogenous VP participates in regulation of cerebral blood vessels constriction and water homeostasis in the brain; 31).

Mechanisms by which GnRH could affect VP release are not well understood. In rat and men, only one of the two receptors present in mammals, *i.e*., GnRH-I receptor is active (11). These receptors are localized mainly in the hypothalamic neurones (32). Quite large number of the GnRH-I receptors was found in the rat anterior pituitary (33), but not in the neurohypophysis. Since, for our present *in vitro* experiment we used the whole hypothalamo-neurohypophysial complex, instead of posterior pituitary separated from the brain, it is very probable that GnRH agonist - histrelin increases VP release acting at the hypothalamic level *via* GnRH-I receptor (both *in vitro* and *in vivo*). GnRH could alter the activity of vasopressinergic neurones directly thanks to existence of GnRH/VP contacts (GnRH axon terminals and dendrites were found to contact VP-immunoreactive dendrites and neuroendocrine cell bodies; 16). GnRH and/or its agonist could also modify the SON and/or PVN neurones activity indirectly *via* suprachiasmatic nucleus (SCN); the explant we used for the present *in vitro* experiments contained, apart from SON and PVN also SCN and other anterior hypothalamic nuclei. An anatomical basis for such a hypothesis is existence of synaptic contacts between GnRH-containing axons and neurones in the SCN (34) and direct neuronal projection from the SCN to PVN (35) and SON (36). Moreover, neurones containing GnRH-I were detected within the organum vasculosum of the lamina terminalis (OVLT) (12, 14), which role in regulation of VP secretion is well established (29).

The influence of GnRH and/or its agonist on VP secretion can also be mediated by several neuromediators or neuromodulators (present in the hypothalamus and/or posterior pituitary) such as opioid peptides (37), arachidonic acid and/or its metabolites (38), dopamine (39), glutamate, γ-aminobutyric acid (GABA) and nitric oxide (40-41). The above mentioned neurotransmitters and/or neuromodulators are involved in modifying VP release (1-2, 42) and certain combination of these agents may be of some importance for the mechanisms by which vasopressinergic neurones are influenced by GnRH and its receptor.

In regulation of VP release by GnRH the neuroactive steroids could also be involved. They are synthesized, among others, by astrocytes in the hypothalamus (43) and some interactions between GnRH neurones and astrocytes could participate in the process under discussion. It has been reported that neurosteroids stimulate GnRH release and are involved in regulation of reproduction (44). Neurosteroids, *e.g*., allopregnanolone, were also found to increase calcium influx to the SON neurones (6); the role of calcium in regulation of VP secretion is well documented (2, 45-46). Steroids can affect the vasopressinergic neurones activity not only through specific oestrogen receptor-beta (ER-beta) present in the hypothalamic SON and PVN (47), but also through membrane oestrogen receptors connected with protein G (GPR30). These receptors were detected in the SON and PVN neurones, as well as in the neurohypophysis (48). In male animals, testosterone, after conversion to oestrogen, can also activate these receptors (49) or it may influence VP secretion through glutamate receptors (42), acting at the level of hypothalamic SON (6).

The influence of melatonin on vasopressin release by GnRH and its agonist

Previous experiments *in vitro* showed that action of melatonin on VP release from the rat hypothalamic explants depends on a concentration of the hormone and also on a time of day. Melatonin inhibited VP secretion when the hypothalamic tissue was obtained from animals in light conditions (*i.e*., about 3 hours after lights on), but no effect of the hormone could be seen when tissue samples were obtained during the night, *i.e*., 4-5 hours after lights off (27). Diurnal changes in the melatonin effect on GnRH release from the rat median eminence *in vitro* have also been described. While 1 nM of melatonin significantly inhibited GnRH release at 10:00 h (*i.e.*, 3.5 hours after lights on), it was ineffective at 15:00 h, and treatment with higher doses of the hormone (10 nM and 100 nM) did not significantly affect the GnRH release from the rat median eminence *in vitro* at 10:00 and 15:00 h (19). Present experiments were, therefore, performed during light period of the light/dark cycle, *i.e.*, about 4 hours after lights on, and a concentration of the hormone has been chosen on the basis of previous *in vitro* results, which show that melatonin influences VP release depending on its concentration (18). The concentration of melatonin used for present *in vitro* experiments, *i.e.*, 10^{-9} M, was found to inhibit the *in vitro* release of VP from isolated hypothalamus (27, 50), hypothalamo-neurohypophysial explants (3-5) and neurohypophysis (51, 52). Therefore, the inhibitory effect of melatonin on VP release from the rat hypothalamo-neurohypophysial complex that we observed (*Fig. 2, 3*) is in concordance with previous *in vitro* studies (3-5). Moreover, under present experimental conditions, we have also found that melatonin significantly attenuates the stimulatory effect of GnRH agonist on VP release *in vitro*.

Exogenous melatonin crosses the blood-brain barrier easily and in the brain ^{14}C-melatonin reaches a maximum level at 5 minutes; 30 minutes after systemic administration it is still present in most of the brain regions, among others in the hypothalamic PVN (53). Previous *in vivo* results show that the effect of exogenous melatonin on VP release depends on a dose of the hormone and a way of treatment (18). The *in vivo* release of VP was inhibited 10 min after i.v. injection of melatonin in a single dose of 5 ng/ml, while higher (25 ng/ml) or lover (2.5 ng/ml) doses were ineffective in this process (54). A significant

reduction in circulating plasma VP level was also observed at 5 min after i.c.v. infusion of melatonin (1 ng/ml and 10 ng/ml), while higher dose of the hormone, *i.e*., 10 μg/ml stimulated VP release (55). Therefore, under present experimental *in vivo* conditions, a 10 min interval between the i.v. injection of 5 ng/ml melatonin and decapitation should be enough to display an effect of exogenous melatonin on VP release. Indeed, the neurohypophysial VP content was significantly higher in melatonin-injected rats, when compared to VEH-treated animals (*Fig. 4A*), but there was no significant effect of melatonin on plasma VP concentration (*Fig. 4B*). Such a result could be due to several reasons, for example, an inhibited output of VP from the neurohypophysis and/or augmented transport of the hormone from the hypothalamic SON and/or PVN to the neurohypophysis.

The possible mechanisms by which melatonin can modify vasopressinergic neurones activity (*in vivo* and/or *in vitro*) may include specific membrane melatonin receptors (MT_1 and MT_2) situated mainly in the pars tuberalis of the pituitary (56) and in the hypothalamus, both in the SCN (24, 57-59) and magnocellular SON and PVN nuclei (60). It has been found that VP-containing cells in the SCN express both MT_1 (58) and MT_2 (59) melatonin receptors and melatonin inhibits VP release from cultured SCN neurones (24, 59). Thanks to the presence of MT_1 and MT_2 receptors, the SCN neurones could respond to melatonin signal, and than transmit it to the SON and/or PVN, *via* excitatory (glutamate) or inhibitory (GABA) amino acids (35-36), which are known to modify VP secretion in the rat (1). The SCN neurones can also send their axons to preoptic area of the hypothalamus, which contains the majority of the GnRH-synthesizing neurones in the rat (61), and *via* this way, melatonin can modify the GnRH- and/or GnRH agonist-induced VP release.

Melatonin can modify intracellular concentration of cGMP and activity of phospholipase C, but cAMP seems to be main intracellular second messenger for melatonin (22-24, 62). Melatonin inhibits the rise of cAMP concentration evoked by increased activity of adenylyl cyclase (AC) in the rat pars tuberalis of the pituitary (23). In cultured pituitary cells from neonatal rats melatonin inhibits GnRH-induced luteinizing hormone release, GnRH- and forskolin-induced cAMP accumulation as well as GnRH-induced intracellular free calcium concentration (23, 24, 63). The concentration of forskolin (10^{-5} M) used in the present study, was chosen because it was able to increase the cAMP accumulation in the pituitary cells after 30 min of incubation; this effect of forskolin was inhibited by melatonin in a dose-dependent (10^{-10}-10^{-7} M) manner (24). The present results show that forskolin stimulates VP release into the medium, and melatonin inhibits forskolin-induced VP secretion from isolated rat hypothalamo-neurohypophysial explants (*Fig. 3*). On the other hand, addition of forskolin to the medium containing both melatonin and GnRH agonist - histrelin did not further alter the inhibitory influence of melatonin on histrelin-induced VP secretion into the medium, which could implicate a certain role for other intracellular mechanisms (*e.g.*, calcium ions) (22) responsible for melatonin-dependent VP secretion from the rat hypothalamo-neurohypophysial system.

Melatonin may also act directly on the genome through brain-specific nuclear RZR receptors (64). It may also affect the release of VP by acting directly on vasopressinergic neuron endings located in the neurohypophysis or indirectly *via* modification of the metabolism of certain neuromediators/neuromodulators in the hypothalamus and/or in the neurointermediate lobe (2, 6). Indeed, melatonin was found to influence the activity of tyrosine hydroxylase in different brain regions (65), whereas acetylcholine, dopamine and prostaglandins were found to participate in an inhibitory influence of melatonin on VP secretion (50, 54).

In summary, this paper demonstrates that endogenous GnRH and GnRH agonist - histrelin, acting through specific GnRH receptor in the hypothalamus, play an important role in regulation of VP secretion from the rat hypothalamo-neurohypophysial system and, *via* this way, may indirectly influence mean arterial pressure, water balance of the organism and brain function. We have also shown that melatonin, at a concentration close to its physiological level in the blood, significantly reduces the *in vitro* response of vasopressinergic neurones to a GnRH agonist; such effect of melatonin could be mediated through intracellular processes that involve, among others, the cAMP-dependent mechanism.

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