JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 1998, 49, 1, 151—163

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MELATONIN AFFECTS THE OXYTOCIN AND PROLACTIN RESPONSES TO STRESS IN MALE RATS [1]

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Since the pineal-neurohypophysial interactions are now well established and oxytocin secretion is known to be a component of the neuroendocrine response to the majority of stressful stimuli, the present experiments were undertaken to estimate whether melatonin modifies the response of oxytocinergic neurons to the immobilization stress. Oxytocin (OT) content in the hypothalamus and neurohypophysis as well as plasma level of OT, prolactin (PRL) and adrenocorticotropin (ACTH) were studied after melatonin treatment in sham-operated or pinealectomized male rats.

In sham-operated rats, melatonin diminished the hypothalamic OT content as well as plasma OT and PRL concentrations, but was without effect on neurohypophysial OT and plasma ACTH levels in otherwise not treated rats. In both wehicle- or melatonin-treated rats, food and water deprivation did not affect the OT, PRL and ACTH secretion. Under stress conditions, however, pituitary OT storage was diminished in vehicle-treated rats and melatonin augmented this response of OT to stress. Melatonin also diminished the PRL and ACTH secretion into the blood in stressed rats.

In pinealectomized animals neither hypothalamo-neurohypophysial OT content nor plasma OT, PRL or ACTH concentrations were modified by melatonin treatment in animals otherwise not treated or in those deprived of food and water for 24 hrs. However, melatonin increased the pituitary oxytocin content as well as plasma OT and ACTH concentrations in immobilized animals. Plasma PRL concentration was diminished after melatonin treatment in stressed rats.

The results suggest that the response of oxytocinergic neurons to immobilization stress is augmented by melatonin. The effect of melatonin on the OT, PRL and ACTH secretion is modified by pinealectomy.

Key words: *melatonin, oxytocin, prolactin, ACTH, stress*

INTRODUCTION

The pineal gland exerts a number of actions in the central nervous system (1) including regulation of the neurohypophysial function (2). Indeed, pinealectomy results in a diminution of oxytocin (OT) content in the

[1] Preliminary reports, covering a part of the present findings, were presented at the Third International Congress of the Polish Neuroscience Society, September, 7—10, Łódź, *Acta Neurobiol Exp* 1997; 57, 61, and at the 8th Meeting of the European Neuroendocrine Association, September, 11—13, 1997, Marseille, France, *Ann D'Endocrinol* 1997; 58 (P4—115), 1S 129.

hypothalamo-neurohypophysial system (3—5) and in an increase in plasma OT levels in euhydrated (6) or dehydrated rats (7). The pineal hormone, melatonin, has also been found to modify OT release from the hypothalamo--neurohypophysial system both *in vivo* (3, 4, 8, 9) and *in vitro* (10—12), the action be inhibitory or stimulatory depending on a dose applied.

Data concerning melatonin influence on the regulation of OT secretion under some pathological conditions are not consistent as well. Indeed, melatonin did not affect the OT neurohypophysial content as decreased following bleeding (8) or pinealectomy (3). Similarly, it did not change the decreased OT neurohypophysial content in rats deprived of water for two days (4). In rats dehydrated for eight days, however, the depletion of OT storage was more marked under treatment with melatonin (4). Melatonin was also reported to facilitate the OT release as brought about by intraperitoneal (i.p.) hypertonic saline administration (13).

Oxytocin secretion is known to be a component of neuroendocrine response to several stressful stimuli (14—16). Yet, immobilization as well as intraperitoneal hypertonic saline administration are potent stressful stimuli; they result in a marked activation of the hypothalamo-pituitary-adrenal axis (17—20) and also in an increased OT secretion (13—16, 21). There is strong evidence that prolactin (PRL) is also released in response to a number of stressful stimuli (18, 21—23).

Magnocellular neurons of the hypothalamic paraventricular (PVN) and supraoptic (SON) nuclei produce mainly OT and vasopressin (AVP). There is some evidence, however, for the co-localization of corticotropin-releasing hormone (CRH) and OT in the same secretory vesicles in both magnocellular and parvocellular neurons of the PVN (24, 25) as well as for the co-secretion of OT and CRH from the nerve terminals in the posterior pituitary (25, 26). Oxytocinergic PVN neurons, which produce both oxytocin and CRH, seem to play an essential role for the stress-induced OT release in response to immobilization stress (15). Moreover, brain stem oxytocinergic pathways, originating in the PVN, are thought to be involved in mediating the stress-induced tachycardia in rats (27).

Melatonin influences the neurosecretory activity of neurons in the PVN (28) and modifies both OT and PRL secretion under physiological (9) and pathological (13) conditions. Moreover, melatonin (29) as well as pinealectomy (30) are supposed to be of some importance for the coordination of the endocrine response to chronic stress. What is more, melatonin was reported to prevent the gastric ulceration as brought about by restraint stress (31).

The present experiments were, therefore, undertaken to estimate whether melatonin modifies the response of oxytocinergic neurons to immobilization stress in sham-operated or pinealectomized rats. Since the posterior lobe of the pituitary seems to be important link in the PRL response to stress (23) and

a concomitant release of OT and PRL in response to some stimuli has been described (9, 13, 21), the other goal of this study was to determine whether the effect of melatonin on OT secretion is accompanied by modified PRL secretion. In addition, plasma adrenocorticotropin (ACTH) concentrations have been measured as an index of activation of the hypothalamo-pituitary axis during stress response.

MATERIALS AND METHODS

Animals

Male rats of the Wistar strain were kept at a room temperature of about +22°C and in regulated light-dark cycle (lights on from 0600 to 1800 h) throughout the experiment. All animals had free access to standard pelleted food and tap water before the experiment. In subgroups 2—3 and 5—6, the animals were not allowed to drink and eat for 24 hours before decapitation. All animals were killed by decapitation during the light period between 0900—0930 h.

Experimental design

Table 1: Experimental design and number of animals (n) per group

First series	A: vehicle injected	B: melatonin injected	Second series	C: vehicle injected	D: melatonin injected
Groups / Subgroups			Groups / Subgroups		
1 — respective control	n = 8	n = 9	4 — respective control	n = 8	n = 8
2 — food and water deprived rats	n = 9	n = 9	5 — food and water deprived rats	n = 7	n = 7
3 — stressed rats	n = 9	n = 10	6 — stressed rats	n = 8	n = 8

In the first series 54 animals were subjected to sham operation at eight weeks of age and decapitated after survival period of another eight weeks. In the second series 46 animals pinealectomized at eight weeks of age and decapitated after survival period of another eight weeks were used. In each series, the following groups were chosen: — animals injected, once daily for 14 days, with vehicle solution (1% ethanol in 0.9% sodium chloride; 100 µl per 100 g.b.w.; groups A and C, respectively); — animals injected with melatonin (N-Acetyl-5-methoxytryptamine; Sigma Chemical Co., St. Louis, USA) dissolved in the vehicle (50 µg Mel in 100 µl of solution per 100 g.b.w.; groups B and D, respectively). All injections were made i.p., approximately one hour before lights off, i.e. about 1700 h. Melatonin was injected at the end of light phase of the light: dark cycle because the endocrine response to such injections was found to be the most pronounced (1).

The day before decapitation animals of the groups A—D were randomly divided into following subgroups: — respective controls, i.e., animals with free access to food and tap water during whole experiment (subgroups 1 and 4); — animals deprived of food and water for 24 hours before decapitation (subgroups 2 and 5); — animals deprived of food and water and additionally immobilized, i.e., transferred singly into small cages where they could not move freely, for 24 hours before decapitation (subgroups 3 and 6).

After decapitation the OT content in the hypothalamus and neurohypophysis as well as plasma concentrations of OT, PRL and ACTH were radioimmunoassayed.

Removal of the pineal gland

The animals of second series were pinealectomized under light hexobarbital anaesthesia following the procedure described by Kuszak and Rodin (32). Sham operation (first series) consisted of an identical surgical trauma, including ligature and resection of the superior sagittal sinus, but without removing the pineal gland

Experimental procedure.

On the next day after last injection of vehicle or melatonin solution rats were killed by decapitation and the blood from the trunk was collected in heparinized tubes for OT, PRL and ACTH estimation. The brain with intact pituitary was removed, the infundibular stalk was cut up and the neurointermediate lobe was separated from the anterior lobe. From the brain, rapidly frozen in the freezer, the hypothalamic block was dissected as previously described (4). The neurointermediate lobe was homogenized in 1 ml of 0.25% acetic acid dissolved in 0.9% sodium chloride; tissue suspension was transferred into a centrifuge tube and the homogenizer washed twice with 0.5 ml of the same solution. The pooled sample was heated for 5 minutes in a boiling water bath (in order to inactivate the proteolytic enzymes contained in the homogenized tissue), and centrifuged at 2,500 rpm at 4°C for 20 min. The supernatant was removed and made up to a constant volume with the same solution of acetic acid. The extracts from hypothalamic samples were prepared in a similar manner except that they were homogenized in 0.5 ml of 0.5% acetic acid dissolved in 0.9% sodium chloride. The final extracts, made up to a constant volume with the same solution (i.e., 0.25% or 0.5%, respectively, acetic acid in 0.9% sodium chloride), were frozen and stored at −20°C until radioimmunoassayed for OT.

Radioimmunoassays

The OT content of the neurohypophysial and hypothalamic extracts as well as plasma OT level was determined by double-antibody specific radioimmunoassay as previously described by Juszczak *et al.* (5). Anti-OT antibodies were raised by Dr. Monika Orłowska-Majdak (Department of Physiology, Institute of Physiology and Biochemistry, Medical University of Łódź). The antibody titer was 1 : 80,000 for anti-OT (final dilution). 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 (Ang II) and substance P less than 0.002%. The sensitivity of anti-OT serum was 3.56 pg OT per tube. Oxytocin (Oxytocin synth; Peninsula Laboratories Europe Ltd.) was used for standard curve preparation as well as for iodination with ^{125}I using the chloramine-T method. Intra-assay coefficient of variation (cv) for the OT assay was 4.3%. For the determination of blood plasma hormonal level, OT was extracted from plasma using C18 "Sep-pak" columnes (Water Associates Ltd, Nortwick, UK). The recoveries of hormone during extraction procedure were > 80% and therefore the findings were not corrected for the procedural losses.

Plasma PRL concentrations were determined in duplicate by RIA kit provided by Amersham, using Rat PRL RIA-kit (RPA 553). The sensitivity of RIA was 0.07 ng/tube, with intra-assay cv 3.2%.

Plasma ACTH concentrations were determined in duplicate by RIA kit provided by Peninsula Laboratories Europe, Ltd using Rat ACTH RIA-kit (RIK-8502). The sensitivity of RIA was 1.0 pg/tube, with intra-assay cv 1.7%.

To avoid inter-assay variability, all samples within the experiment were tested in the same RIA for OT, PRL and ACTH, respectively.

Statistical evaluation of the results

The oxytocin content was finally expressed in nanograms for whole hypothalamus or neurointermediate lobe and in picograms per 1 ml of plasma. The plasma PRL and ACTH concentrations were expressed in nanograms or picograms 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 assessed using non-parametric analysis of variance (ANOVA) followed by Mann-Whithney "U" test. $P < 0.05$ was used as the minimal level of significance.

RESULTS

First series

In animals with intact pineal gland, immobilization stress diminished pituitary OT storage both in vehicle- and melatonin-treated rats (subgroups A3, B3), melatonin intensified the effect of immobilization on OT content in the neurohypophysis (*Fig. 1A*). In control animals (subgroup B1) melatonin diminished the hypothalamic OT content (*Fig. 1B*) as well as plasma OT

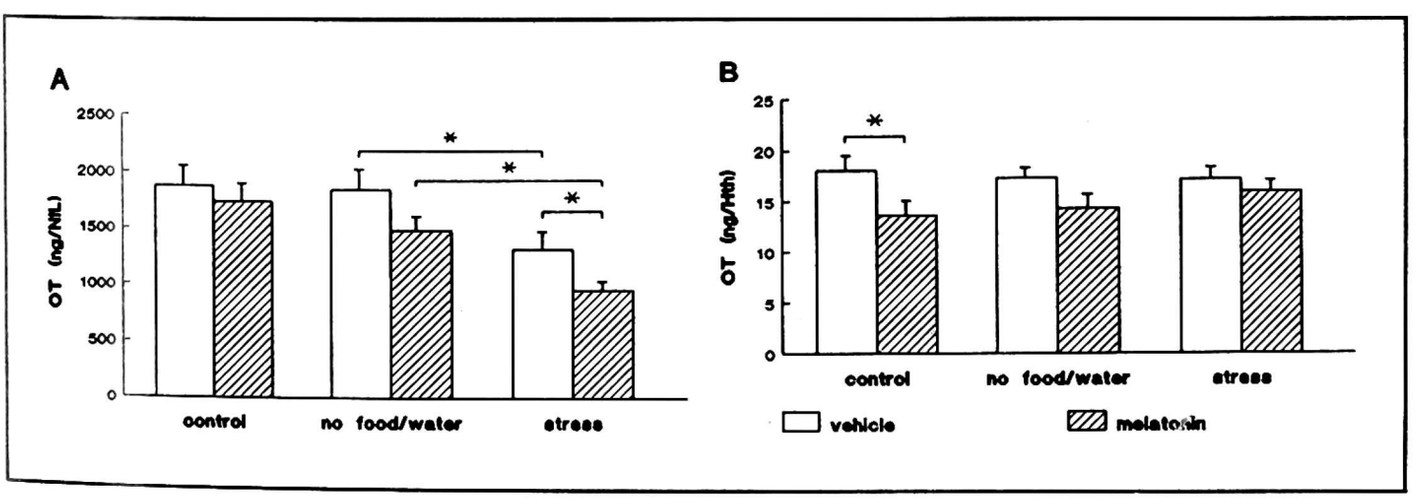

Fig. 1. The effect of melatonin on the neurohypophysial (NIL; A) and hypothalamic (Hth; B) oxytocin (OT) content in sham operated male rats under immobilization stress (mean ±S.E.M.; number of animals: n = 8—10; * — $p<0.05$).

(*Fig. 2A*) and PRL (*Fig. 2B*) concentrations, but was without effect on plasma ACTH levels (*Fig. 2C*). On the other hand, melatonin inhibited the ACTH and PRL release as brought about by immobilization (*Fig. 2B, C*).

Deprivation of food and water for 24 hours (subgroups A2, B2) did modify neither hypothalamic nor neurohypophysial oxytocin contents (*Fig. 1A, B*); under such conditions, plasma OT, PRL and ACTH levels were not altered when compared to respective controls (*Fig. 2A, B, C*).

Second series

In pinealectomized animals, melatonin increased the pituitary OT content (*Fig. 3A*) as well as plasma OT (*Fig. 4A*) concentration in immobilized animals (subgroups C6, D6); plasma PRL concentration was diminished after

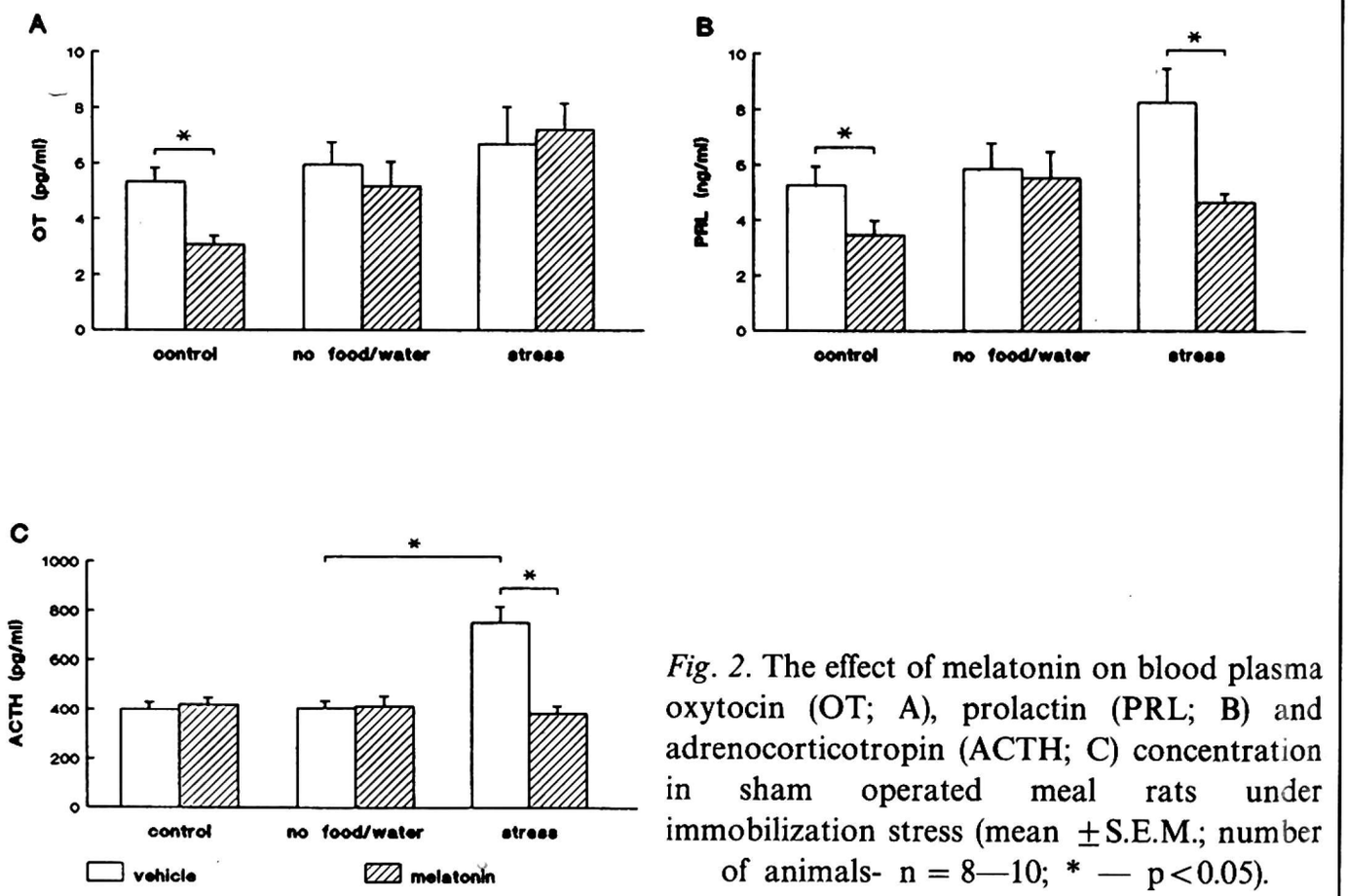

Fig. 2. The effect of melatonin on blood plasma oxytocin (OT; A), prolactin (PRL; B) and adrenocorticotropin (ACTH; C) concentration in sham operated meal rats under immobilization stress (mean ±S.E.M.; number of animals- n = 8—10; * — $p<0.05$).

melatonin treatment in stressed rats (*Fig. 4B*). After immobilization the ACTH secretion (*Fig. 4C*) has been increased both in vehicle- and melatonin-treated rats when compared to respective controls.

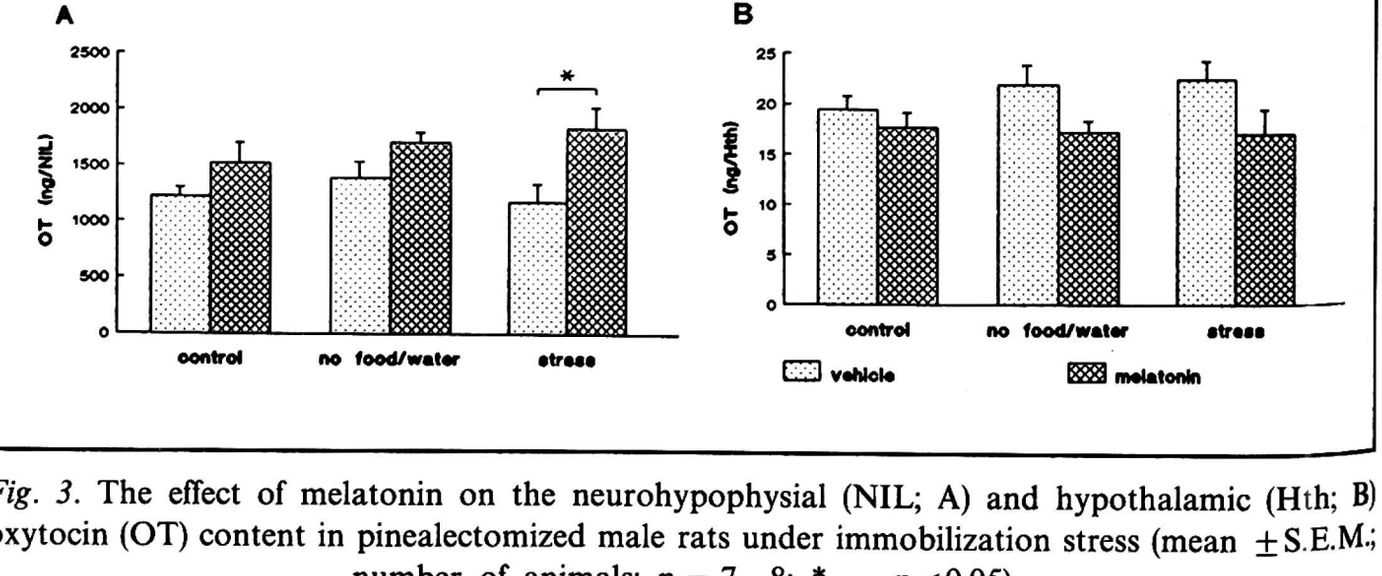

Fig. 3. The effect of melatonin on the neurohypophysial (NIL; A) and hypothalamic (Hth; B) oxytocin (OT) content in pinealectomized male rats under immobilization stress (mean ±S.E.M.; number of animals: n = 7—8; * — $p<0.05$).

After pineal removal, neither neurohypophysial and hypothalamic OT content (*Fig. 3A, 3B*) nor OT (*Fig. 4A*), PRL (*Fig. 4B*) and ACTH (*Fig. 4C*) levels in plasma were modified by melatonin treatment in respective controls (subgroups C4, D4) or deprived of food and water animals (subgroups C5, D5).

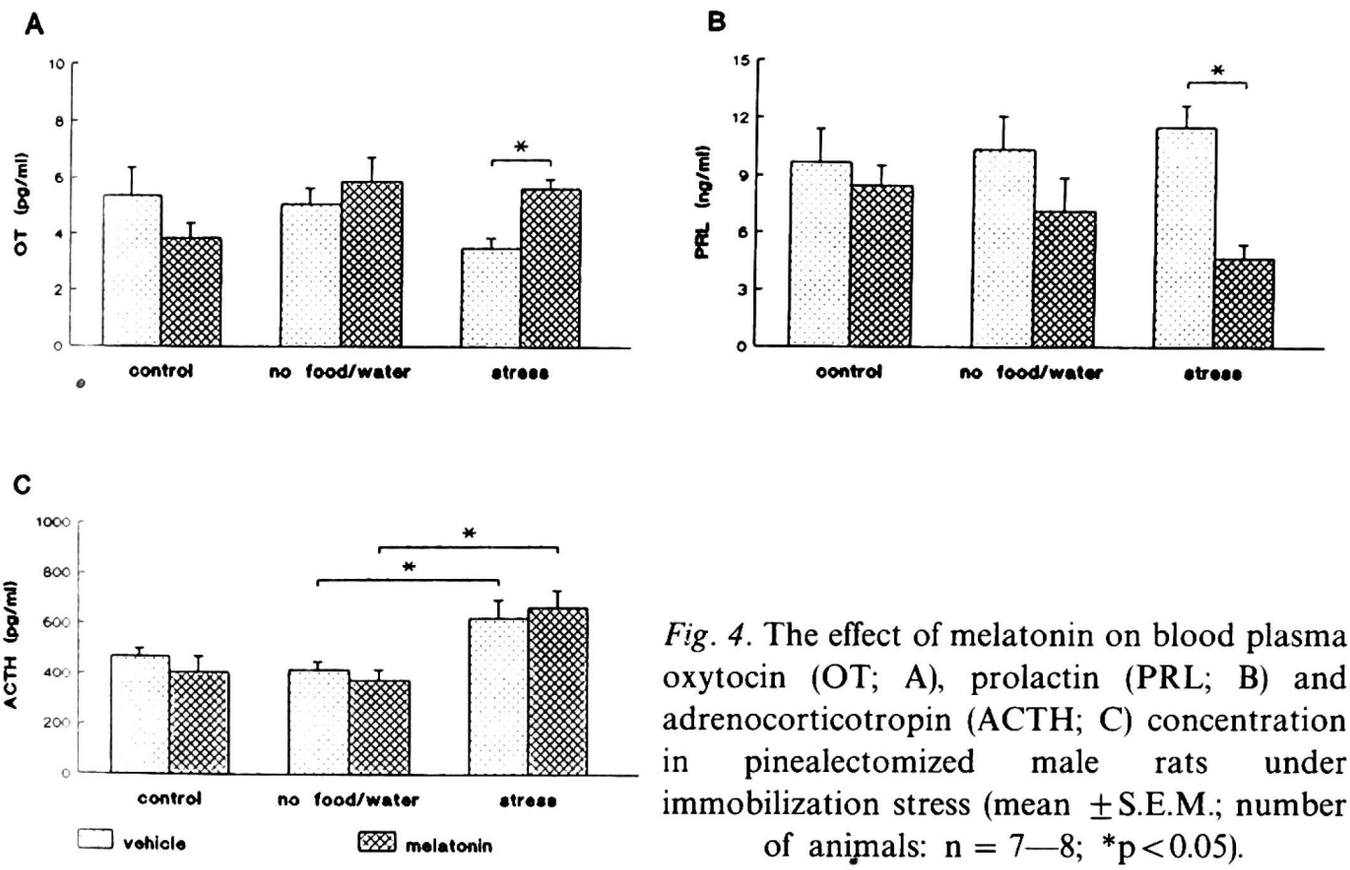

Fig. 4. The effect of melatonin on blood plasma oxytocin (OT; A), prolactin (PRL; B) and adrenocorticotropin (ACTH; C) concentration in pinealectomized male rats under immobilization stress (mean ±S.E.M.; number of animals: n = 7—8; *$p<0.05$).

DISCUSSION

The effect of immobilization and/or deprivation of food and water on OT, PRL and ACTH secretion

A variety of somatic and emotional stressors may induce integrated acute or chronic stress responses (20) which, in general, include an increasing stimulation of the hypothalamo-pituitary-adrenal (HPA) axis, i.e., c-fos mRNA (19) and CRH mRNA (17, 18) induction in hypothalamic PVN as well as increase in circulating ACTH (17, 21) and corticosterone (17, 18, 33). In this study, the experimental model used (i.e., immobilization accompanied by deprivation of food and water for 24 hrs) proved to be an effective stressor as verified by increased ACTH blood level.

Immobilization has been observed to result in an increase of OT (14—16, 21), PRL (18, 21—23) and ACTH (18, 21, 30, 34) secretion. The stimulatory effect of immobilization stress on OT and ACTH release, as observed in this study, is therefore in agreement with a number of previous observations. The present results also confirm previous findings from this laboratory that immobilization decreases the neurohypophysial OT content in the rat (35, 36). In addition, it was shown that in pinealectomized rats no changes in hypothalamic, neurohypophysial or plasma OT levels were noted in vehicle-treated stressed rats (present study).

In the present study, deprivation of food and water for 24 h was without effect on OT content in the hypothalamus and neurohypophysis, which is in agreement with previous data from this laboratory (35, 36). Moreover, such a treatment did modify OT, PRL or ACTH secretion neither in sham-operated nor in pinealectomized rats. These effects are in agreement with previous observation that deprivation of food and water for 60 h did not change the rat plasma PRL and ACTH concentrations as well as the OT mRNA level in the PVN of the rat (34).

The effect of melatonin on stress-induced OT, PRL and ACTH secretion

The main hypothesis underlying this study was that melatonin possibly modifies the stress-induced secretion of OT and/or PRL in the rat. This supposition was based on the observation that after a stressful stimulus (i.e., hypertonic saline i.p. injection) melatonin facilitated the release of OT, but prevented that of PRL (13). The present experiment showed that the immobilization stress, similarly to short-term restraint stress (14—16) enhanced the OT secretion in parallel to increased ACTH concentration in the blood. In addition, we found that melatonin intensified the stress-induced decrease in the neurohypophysial OT content, but somewhat attenuated the release of PRL and ACTH in stressed animals.

In the present study, neither hypothalamic OT content nor plasma OT concentration did reflect the changes in OT neurohypophysial content after melatonin treatment in stressed rats. This observation suggests that in stressed rats melatonin affects the OT transport towards the neurohypophysis without any alteration of the OT release into the blood. Oxytocin synthesis in the hypothalamus also seems to be not influenced by melatonin during stress, since the hypothalamic OT content was modified neither in stressed animals nor in those deprived of food and water. Moreover, although melatonin diminished hypothalamic OT content in control animals (this study), neither melatonin nor pinealectomy had significant effect on hypothalamic OT mRNA level (37).

Mechanisms of melatonin action on the oxytocinergic neurons are still not clear. Melatonin receptors have been demonstrated in several brain areas of male rat with high levels of binding over the suprachiasmatic nuclei (SCN), pars tuberalis of the pituitary and area postrema (38). However, melatonin receptors have been described neither over the hypothalamic SON and PVN areas nor in the neurohypophysis (38, 39). After systemic administration, melatonin crosses the blood-brain barrier and accumulates in the hypothalamus both in cytosolic and nuclear fractions (40). Egzogenous melatonin may, therefore, modify OT secretion during stress acting *via* melatonin receptors localized in the SCN; indeed, the SCN neurons project directly to the PVN, and the latter has been shown, in turn, to be essential for stress-induced OT secretion (15). It is therefore

possible that neural input originating in the SCN and reaching the PVN (both magno- and parvocellular neurons) affects, at least in part, the release of OT: such a course of events is conceivable as to both normal and pinealectomized animals.

Egzogenous CRH was shown to activate the PVN neurons during stress (19). Since CRH is known to induce OT secretion (25), it might be hypothesized that melatonin modifies OT secretion *via* alterations in CRH or ACTH production and/or secretion. Such a course of events, however, seems to be rather unlikely since melatonin affected neither the CRH content in the median eminence (41) nor CRH release from the rat hypothalamus *in vitro* (12). No effects of melatonin on ACTH plasma levels have also been described in intact (41) or sham operated (39; this study) rats. In pinealectomized animals, however, the plasma ACTH concentrations were increased after immobilizaton in vehicle-treated (present study) or otherwise not treated (30) rats.

Melatonin is thought to affect the function of the hypothalamo-neurohypophysial system acting possibly *via* mechanisms involving, among others, the prostaglandins secretion in the hypothalamus (42). Melatonin was found to inhibit prostaglandin E release from the hypothalamus (43); on the other hand, prostaglandins mediate the HPA activity stimulated by central noradrenergic system in stressed rats (33). It is therefore possible that exogenous melatonin might modify OT production and/or secretion during stress acting *via* mechanisms involving prostaglandins metabolism in PVN and/or SON.

There is some evidence that melatonin, due to its lipophilicity (and, therefore, its ability to pass through cell membranes) modulates a number of cellular functions and releases the genomic activation without interaction with a specific membrane receptor (40). Since subcutaneous injection of melatonin results in a rapid increase of its content in the nuclear fraction of a number of tissues including hypothalamus (40), another possibility is that melatonin directly modifies oxytocinergic neurons activation during stress by its effects on a genom. Restraint stress induces c-fos expression in the magnocellular oxytocinergic neurons of the SON and PVN (44); on the other hand, neuronal activation and fos protein production in the rat SON have recently been noted to be diminished after pinealectomy (45). Inhibitory influence of melatonin on the Gn-RH stimulated increase of c-fos immunoreactivity in neonatal rat pituitary cells was recently reported (46).

The decrease of OT and PRL secretion into the blood after treatment with melatonin, as seen in the present study in control animals, is consistent with previously described inhibitory influence of melatonin on the OT (11, 12) and PRL (47) release *in vitro*. However, the parallel release of both hormones (OT and PRL) in response to a stressful stimulus (13, 21) has not always been noted (22). Indeed, melatonin augmented the OT response to immobilization stress,

but inhibited that of PRL (present study). Present findings (i.e., opposite effects of melatonin on stress-induced OT and PRL release) are in agreement with previous data from our laboratory as to dissimilar effects of melatonin on osmotically-stimulated (13) or suckling-induced (9) OT and PRL secretion. Mechanisms related to melatonin influence on OT secretion during stress response seem therefore to be different from these involved in the melatonin effects on PRL secretion under similar conditions.

Because median eminence region is known to be essential for the PRL secretion due to immobilization (23), melatonin receptors in the median eminence/pars tuberalis region (38, 39) could be the target area for the melatonin action on PRL secretion during stress. Moreover, possible mechanisms involving tuberoinfundibular (TIDA) and/or tuberohypophyseal (THDA) dopaminergic neurons — whose pericaryons are localized in the arcuate nucleus and whose axons tend towards the median eminence (TIDA system) or the neurointermediate lobe (THDA system), respectively — should be taken into consideration as well: so more that melatonin receptors have been found in the rat arcuate nucleus (39).

In summary, the present findings show that the response of oxytocinergic neurons to immobilization stress is augmented by melatonin. The effect of melatonin on the OT, PRL and ACTH secretion is modified by pinealectomy.

Acknowledgements: This work has been conducted under contract No. 4.PO5A.104.12 (507-11-071) with the State Committee for Scientific Research, Warsaw. I wish to thank Prof. J. W. Guzek (Department of Pathophysiology, Medical University of Łódź) for his advice and helpful comments on the manuscript; Dr. Bożena Stempniak (Department of Pathophysiology, Medical University of Łódź) for her help in hormonal RIA, and Dr. Monika Orłowska-Majdak (Department of Physiology, Institute of Physiology and Biochemistry, Medical University of Łódź) for the gift of anti-OT antiserum.

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Received: September 5, 1997
Accepted: January 13, 1998
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