Neuroendocrinology Letters ISSN 0172–780X
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The effect of morphine on melatonin secretion in the domestic pig.
In vivo and *in vitro* study.

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Submitted: *February 18, 1999*
Accepted: *April 27, 1999*

Key words: **morphine; melatonin; pig; in vivo; in vitro**

Neuroendocrinology Letters 1999; ***20****:171–178* *pii: NEL203499A04* *Copyright © Neuroendocrinology Letters 1999*

Abstract Up till now the results of performed investigations suggest the involvement of opioids in the regulation of the pineal gland activity in mammals. On the other hand, they show the existence of large interspecies differences in the presence of opioid peptides in the pineal gland and in the effects of opiates on the melatonin secretion. The aim of the present work was to study the influence of morphine on the melatonin secretion in the domestic pig.

Morphine (about 2.5 mg/kg) was given intravenously to immature gilts during the day, during the night or during the night with turned on fluorescent illumination (intensity 500 lx at the level of the animal heads) and plasma melatonin level was measured. The effect of various concentrations of morphine on basal or norepinephrine-stimulated melatonin secretion was also investigated using perfusion culture of pineal glands of immature female pigs.

Morphine did not change plasma melatonin concentration in the domestic pig when administered in a single dose at the beginning of the light or the dark phase of the diurnal light-dark cycle. However, morphine administration at the beginning of the night resulted in significantly decreased the plasma melatonin level in animals exposed to light, with intensity 500 lx, which was insufficient to block nocturnal rise in plasma concentration of this pineal hormone in the untreated pigs. Morphine had also no effect on the level of basal and norepinephrine-stimulated melatonin secretion *in vitro*.

The obtained results suggest that in immature pigs morphine does not influence directly the pineal activity, but may modulate the melatonin secretion indirectly, increasing the sensitivity of the system generating melatonin synthesis and secretion to the light.

ORIGINAL ARTICLE

Introduction

Endogenous opioids, widely distributed in mammalian bodies, play an important role in numerous physiological processes. Among others, it has been demonstrated that opioid peptides are involved in mediation of melatonin influence on the immune system [1, 2]. Results of some studies have also showed the existence of a close relationship between the pineal hormone and analgesic [3] as well as some endocrine effects of opiates [4, 5].

Moreover, evidence has been accumulated that the opioid peptides are involved in the control of the pineal gland activity. The hypothesis, that opioids modulate the pineal gland function, is supported by three groups of investigations: 1) studies showing the existence of opioid peptides in the pineal gland; 2) studies of pineal opioid receptors; and 3) studies of the effect of opiates on the pineal activity.

Opioid peptides found in the pineal glands of mammalian species, investigated up until now, are synthesized both inside as well as outside the pineal gland. Sources of opioids produced outside the gland are nerve fibers and blood vessels. The opioid-immunoreactive nerve fibers were demonstrated in the pineals of the guinea pig [6], the bovine [7], the rat [8] and the domestic pig [9]. Opiates from blood may easily reach the structures of the pineal gland, because pineals of many mammalian species are located outside the blood-brain barrier [10]. Cells that may produce opioid peptides in the pineal gland were classified as neurons as well as pinealocytes. Enkephalin-like immunoreactive neurons were observed in the human pineal gland [11]. Met- and leu-enkephalin immunoreactive cells classified based on their ultrastructure as pinealocytes were noted in the European hamster [12]. In the rat pineal expression of preproenkephalin and proopiomelanocortin genes were demonstrated [13, 14]. Expression of the preproenkephalin gene was found in approximately 7 percent of cells in the rat pineal. These cells were not serotonin-immunoreactive, which may suggest that they do not produce melatonin [15]. Opioid-immunoreactive cells were also found in the pineal gland of the guinea pig [6].

Using the radioligand binding method, opioid receptors were found in the pineal gland of the bovine and classified as delta type [16, 17, 18]. In the mouse pineal gland, expression of the delta opioid receptor gene was demonstrated at much higher levels than in the other brain structures except the pituitary gland [19].

The effect of various opioid agonists and antagonists on pineal activity was investigated in the rat [20, 21, 22, 23, 24, 25], the hamster [25], the sheep [25], the bovine [18] as well as in the human [1, 26]. Results of these investigations showed the existence of large interspecies differences in the influence of opiates on the melatonin secretion.

The aim of the present study was to check the effect of morphine on the plasma melatonin level in immature domestic pigs and on the melatonin secretion from the perfused pineal gland of immature pigs *in vitro*.

Material and methods

Animals and housing conditions

Female crossbred pigs, 108±2 days of age, weighing 38.5±2 kg were purchased 7–14 days before experiments started from a commercial barn (with natural length of day) and used in the investigations. Animals were kept in individual pens in the room, in which natural lighting from windows (sunrise 4:20– 4:30 h, sunset 20:30–21:00 h) was supplemented during the day with fluorescent illumination with light intensity of 500 lx at the level of the animal heads. The fluorescent illumination was automatically turned on at 5:45 and turned off at 19:45. Gilts had free access to water and were fed twice daily (9:00, 14:00) with standard food. Handling of animals was performed in agreement with "Principles of laboratory animal care" (NIH publication No. 86–23, revised 1985) and Polish law on the protection of animals.

Chemicals

Chemicals were purchased as follows: Vetbutal from Biowet-Pulawy (Poland), heparin lithium from Polfa-Warsaw (Poland), morphine hydrochloride from Polfa-Kutno (Poland), gelatine from Merck (Germany), ^{3}H-melatonin (87Ci/mM) and 2-^{125}I-iodomelatonin (2200Ci/mM) from Du Pont NEN (USA), anti-melatonin antibody G/S/704-6483 from Stockgrand Ltd (Univ. of Surrey, Great Britain), and precipitation reagent B-60 from INRA Nouzilly (France). Antiserum R/R/19540-16876 was a gift from Dr JP Ravault (INRA Nouzilly, France). All other reagents were purchased from Sigma (USA).

Experimental procedures

In vivo study

One week before morphine or saline administrations, the pigs were anesthetized with a pentobarbital (Vetbutal-Biowet) and the left jugular veins

were cannulated via cephalic veins according to the method described by Kotwica et al. [27]. Plasma samples (prepared with use of heparin as anticoagulant) were stored at -20°C until melatonin assay.

Experiment I

Two groups of pigs (control and experimental), six animals in each, were used. Pigs from the experimental group received intravenously 100 mg morphine hydrochloride in 5 ml saline between 10:05–10:10. Control pigs were treated at the same time with 5 ml saline. Blood sampling was performed at 10:00, 10:15, 10:30, 10:45, 11:00, 11:15, 11:30, 11:45, 12:00, 12:30, 13:00, 13:30, 14:00, 14:30, 15:00, 16:00, 17:00, 18:00, 19:00 and 20:00.

Experiment II

The experiment was performed on twelve pigs divided into two equally-sized groups (experimental and control). Experimental pigs received intravenously 100 mg morphine hydrochloride in 5 ml saline and control pigs 5 ml saline between 21:20–21:40. Blood samples were taken at 12:00, 14:00, 16:00, 18:00, 20:00, 21:00, 22:00, 22:30, 23:00, 23:30, 24:00, 1:00, 2:00, 3:00, 4:00, 5:00, 6:00, 8:00, 10:00. During the night blood sampling was performed with the help of local dim (below 2 lx) red lighting.

Experiment III

The experiment was carried out on two groups of pigs (six individuals in each). Fluorescent illumination was left turned on during the night and experimental pigs were treated with 100 mg morphine hydrochloride in 5 ml saline (*i.v.*) between 20:05–20:10. Control pigs were infused at the same time with 5 ml saline. Blood samples were drawn at 12:00, 14:00, 16:00, 18:00, 20:00, 20:15, 20:30, 20:45, 21:00, 21:15, 21:30, 21:45, 22:00, 22:30, 23:00, 23:30, 24:00, 0:30, 1:00, 2:00, 3:00, 4:00, 5:00 and 6:00.

In vitro study

The pineal glands were removed no later than 3 minutes after slaughter and divided into three or four pieces, which were immediately mounted in separate perfusion chambers (volume 0.5 ml). The chambers were perfused at a flow rate of 0.2 ml/min with medium 199. The medium was gased with an appropriate mixture of O_2 and CO_2. The medium and chambers were maintained at 38°C. Medium fractions were collected every 5 or 10 minutes. The mean amount of melatonin released between 290 and 340 minutes of perfusion was considered as basal level (as 100%).

Experiment A

Animals (n=4) were slaughtered at 9:00. The pineal glands were divided into four pieces. Between 341 and 400 minutes of incubation, tissue pieces were perfused with a culture medium containing morphine hydrochloride 100μM, 10μM, 1μM or with control medium.

Experiment B

Pigs (n=3) were killed at 18:00 and pineals were divided into three pieces. Between 341 and 610 minutes of incubation explants were perfused with culture medium containing morphine hydrochloride 100μM, 10μM or with the control medium.

Experiment C

Animals (n=3) were slaughtered at 18:00. The pineal glands were divided into four pieces. Between 341 and 430 minutes of incubation, tissue pieces were perfused with a medium containing 1) morphine hydrochloride 10μM, 2) norepinephrine hydrochloride 1μM, 3) norepinephrine hydrochloride 1μM and morphine hydrochloride 10μM. The fourth explant from each gland was perfused with the control medium.

Experiment D

Pigs (n=3) were slaughtered at 18:00. The pineal glands were divided into four pieces. Between 341 and 430 minutes of incubation tissue pieces were perfused 1) with a medium containing morphine hydrochloride 100μM, 2) with a medium containing norepinephrine hydrochloride 10μM, 3) with a medium containing norepinephrine hydrochloride 10μM and morphine hydrochloride 100μM, and 4) with a control medium.

Melatonin assay

The concentration of melatonin in blood plasma was measured by direct RIA using R/R/19540-16876 antibody and iodinated tracer according to validated procedure [28, 29]. Melatonin concentration in the medium was measured by modified direct RIA method of Fraser et al. [30] employing G/S/704-6483 antibody and ^{3}H-melatonin. Intra- and interassay coefficients of variation in both radioimmunoassays were below 10%.

Statistical analyses

Concentrations of plasma melatonin were subjected to repeated measures ANOVA followed by LSD test as post-hoc procedure and t-test. The data from the experiments performed *in vitro* were

analyzed by one-way ANOVA or paired t-test. The value of $p \leq 0.05$ was considered as significant.

Results

In vivo study

In all experiments excitation as well as increased movement and vocalizing were observed in the pigs after administration of morphine.

Experiment I

No significant difference in mean concentration of the plasma melatonin was observed between the control group and the experimental group (Fig. 1). In both groups of pigs, the plasma melatonin level was significantly higher at 20:00 than between 10:00 and 19:00.

Experiment II

No significant difference in mean concentration of plasma melatonin was observed between the control group and the experimental group (Fig. 2). In both groups, the plasma melatonin level was significantly higher between 20:00 and 5:00 than at 8:00, 10:00, and 12:00, 14:00, 16:00, 18:00.

Experiment III

The plasma melatonin level was significantly higher in the control group than in the experimental one at 21:00, 22:00 and 23:30 (Fig. 3).

In the control group, the plasma melatonin level was significantly higher between 20:15 and 5:00 than between 12:00 and 18:00. At 20:00 the plasma melatonin level in the control pigs was significantly higher than at 12:00 and significantly lower than between 21:00 and 4:00.

In the experimental group, the plasma melatonin level was significantly higher between

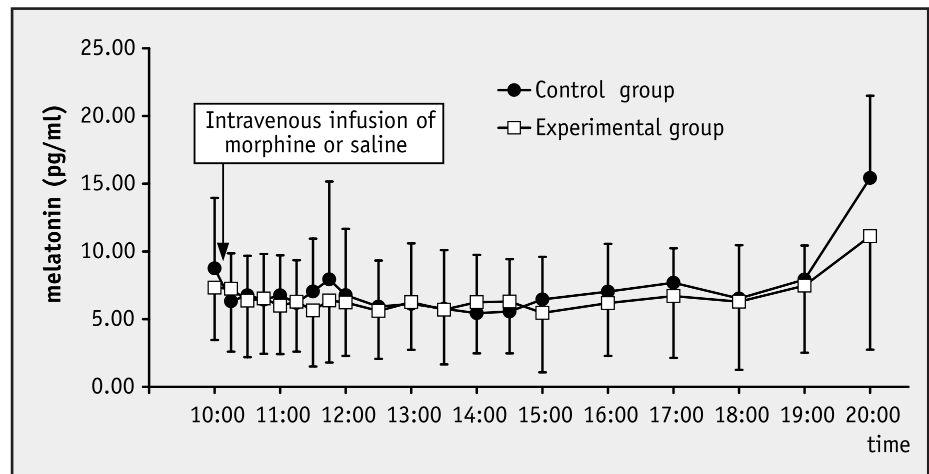

Fig. 1. Mean plasma melatonin concentration (± SD) in the control group and the group of pigs receiving morphine in Experiment I.

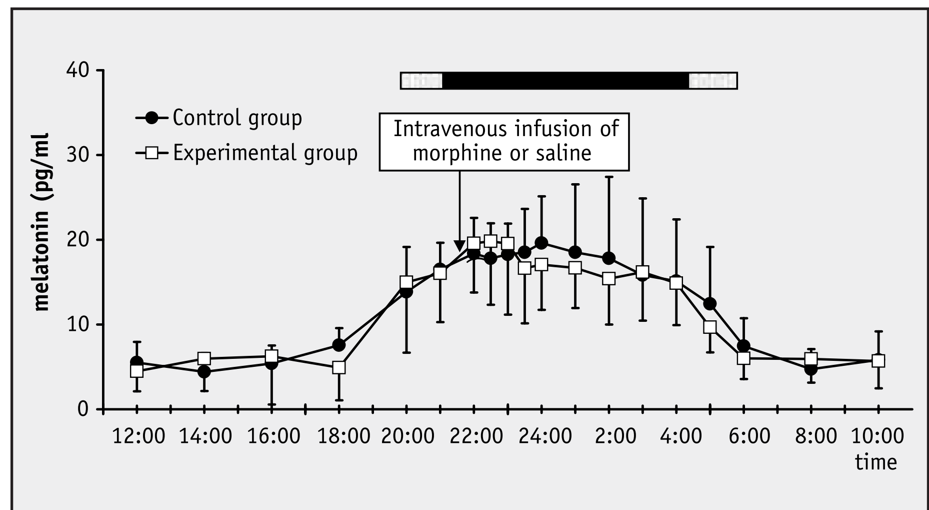

Fig. 2. Mean plasma melatonin concentration (± SD) in the control and the morphine treated pigs in Experiment II.

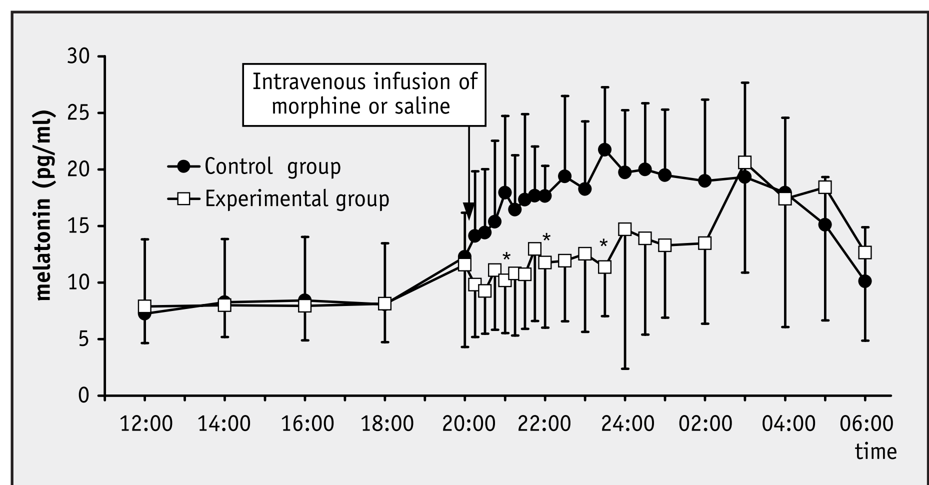

Fig. 3. Mean plasma melatonin concentration (± SD) in the control group and the group of pigs treated with morphine in Experiment III. Horizontal bar indicates period of dusk, darkness and dawn.
*–Values significantly different vs. control at $p \leq 0.05$

24:00 and 5:00 than between 12:00 and 18:00. The plasma melatonin concentration was also higher at 3:00, 4:00 and 5:00 than between 20:00 and 23:30 as well as at 03:00 than at 24:00, 0:30, 1:00 and 2:00.

In vitro study

The melatonin release decreased rapidly during the first two hours of perfusion and then slowly up to 3–4 hours of the culture. After this period melatonin secretion remained relatively stable up to the end of perfusion. The mean release of melatonin between 290 and 340 minutes of perfusion was 24.7±5.1 pg/min/mg of wet tissue.

Experiment A

Treatment with 1μM, 10μM and 100μM of morphine hydrochloride for 60 minutes did not change the level of the melatonin secretion (Fig. 4).

Experiment B

Perfusion with a medium containing 10μM or 100μM of morphine hydrochloride for 4.5 hours did not change the level of the melatonin secretion (Fig. 5).

Experiment C

The melatonin secretion increased to maximal level (about 150–160% of basal secretion) within 20–30 minutes of norepinephrine (1μM) treatment, remained at a stable increased level as long as norepinephrine was present in the medium and decreased immediately after norepinephrine withdrawal (Fig. 6). Morphine at concentration 10μM did not change significantly the level of basal or norepinephrine-stimulated melatonin secretion.

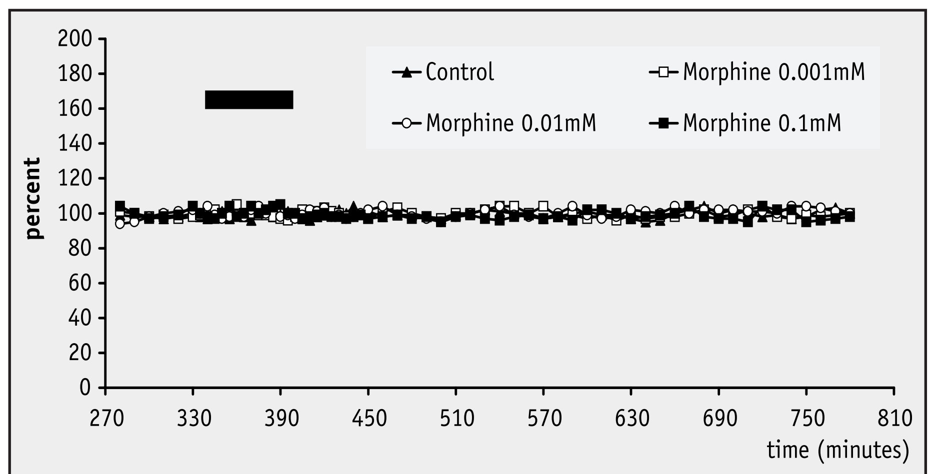

Fig. 4. Mean melatonin secretion from the pineal explants in Experiment A. Dark bar indicates period of morphine treatment.

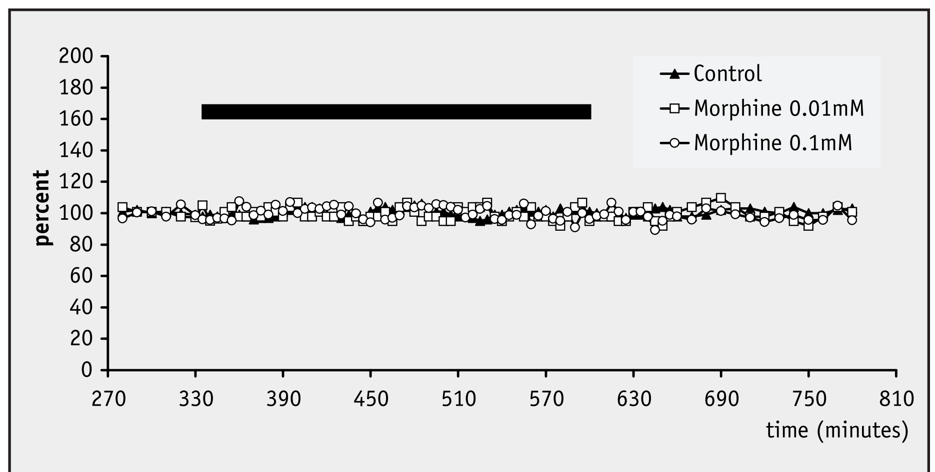

Fig. 5. Mean melatonin secretion from the pineal explants in Experiment B. Dark bar indicates period of morphine treatment.

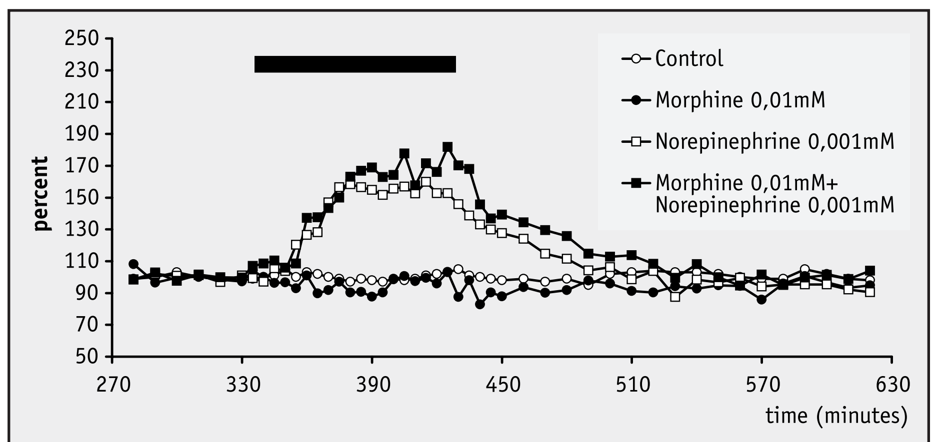

Fig. 6. Mean melatonin secretion from the pineal explants in Experiment C. Dark bar indicates period of morphine and norepinephrine treatment.

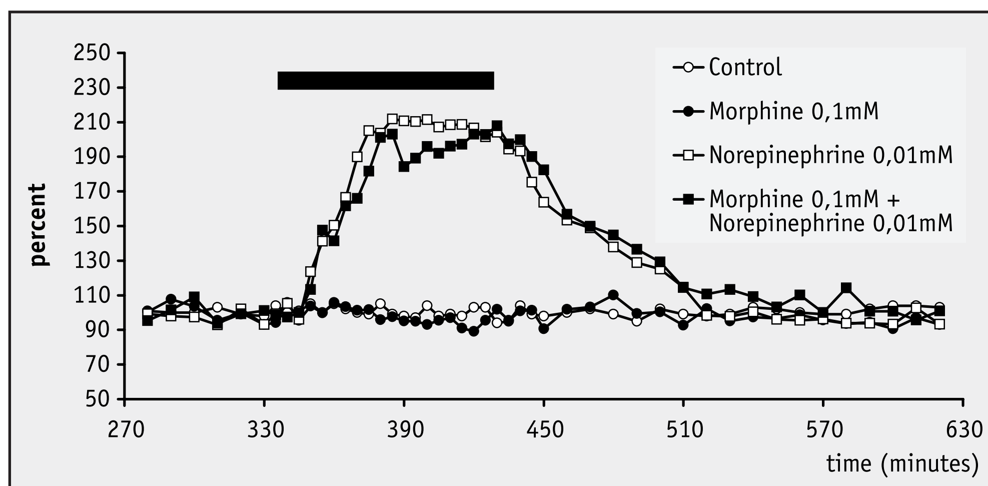

Fig. 7. Mean melatonin secretion from the pineal explants in Experiment D. Dark bar indicates period of morphine and norepinephrine treatment.

Experiment D

Treatment with 10 μM of norepinephrine resulted in about a two-fold increase (over basal level) in melatonin release (Fig. 7). No changes in basal and norepinephrine-stimulated melatonin secretion were observed after treatment with 100μM of morphine.

Discussion

In the present study morphine did not change the plasma melatonin concentration in the domestic pig when it was administered in a single dose at the beginning of the light or the dark phase of diurnal light-dark cycle. Morphine had also no effect on the level of basal and norepinephrine-stimulated melatonin secretion *in vitro*. On the other hand, morphine administration at the beginning of the night resulted in significantly decreased plasma melatonin level in animals exposed to light, with intensity insufficient to block nocturnal rise in plasma concentration of this pineal hormone in the untreated pigs.

The observed lack of the effect of morphine treatment on the plasma melatonin level under natural light-dark cycle in our study cannot be considered as a result of administration of too low a dose of the drug, because it induced clear changes in the behavior of the pigs. Moreover, significant changes in plasma melatonin level in animals exposed to light during the night were observed after treatment with the same dose of morphine. The results of the *in vivo* studies are in agreement with the data obtained in the *in vitro* experiments performed with use of perfusion culture. Independently of morphine concentration in the medium, duration of morphine treatment and time of animal slaughter (at the beginning and at the end of day), no changes in melatonin secretion after treatment with the opioid agonist were observed. Summing up, data from our *in vivo* and *in vitro* studies made a strong base for the conclusion that in immature female pigs morphine administration during daytime and nocturnal darkness does not change the level of plasma melatonin as well as morphine does not influence directly the melatonin secretion from the pineal gland.

The present results obtained using perfusion culture of pineal glands taken from immature pigs are contrary to our previous findings which show that morphine stimulated basal and inhibited isoproterenol-induced melatonin secretion from pineals of adult sows in a static culture [9]. The effects of morphine were blocked by naloxone. The observed differences may suggest that opioidergic regulation of pineal function develops in the domestic pig during or after puberty. It is also possible that it may be a result of the use of different culture systems. Incubation of pineal glands in static culture results in the accumulation of many substances, released by cells, in a culture medium. These substances may influence cell metabolism and may be responsible for indirect actions of morphine, which are not possible or strongly limited in perfusion culture.

The stimulatory effect of opioid agonists on melatonin secretion was observed in the rat. In this species administration of morphine [23] or des-tyrosine-γ-endorphin [21] resulted in the increase in plasma or pineal melatonin levels. Stankov and co-workers [25] described that morphine treatment during the night increased pineal and serum melatonin levels, previously suppressed by light. However, these authors did not find the increase in pineal and serum melatonin levels after administration of morphine during the daytime [25]. Rats treated with naloxone showed results of smaller nocturnal pineal melatonin peaks and slower decrease of melatonin levels at the end of the night [22]. *In vitro* morphine and β-endorphin stimulated the melatonin secretion

from the rat pineal in a static culture, but the mechanism of this action is enigmatic, because it was blocked by naloxone and by propranolol [24, 25]. In the human the increase in the plasma melatonin level was observed after treatment with met-enkephalin analogue FK 33-824 [1] and des-tyrosine-γ-endorphin [26]. In sheep the morphine treatment during the day had no effect on the plasma melatonin level [25]. However, during the night administration of this drug to animals with light suppressed low melatonin level caused significant, but relatively small elevation in the plasma content of this pineal hormone. The effect of morphine was prevented by treatment with naloxone, which did not have any effect on naturally occurring high nocturnal melatonin level in blood [25]. In the hamster, treatment with morphine had no effect on pineal activity [25]. In the bovine, static incubation of pineal explants with morphine resulted in significant, concentration-dependent increase in serotonin N-acetyltransferase activity [18].

An interesting and unexpected finding of the present study was the increase of inhibitory effect of the light on melatonin secretion in pigs after morphine treatment at the beginning of the night. In the control animals fluorescent light with intensity of 500 lx, used in the experiment, did not block nocturnal increase in plasma melatonin level and this result is in agreement with the other study, performed in our laboratory, in which animals were exposed to the same illumination system for one night [31]. However, continuous illumination with the same intensity during nine days completely abolished the diurnal rhythm of melatonin secretion in immature domestic pigs [32]. The increase of the inhibitory effect of the light on melatonin secretion in the domestic pig after morphine treatment may be the result of changes in behavior (greater perception of light in moving than in sleeping animals) as well as may suggest the existence of opioidergic regulation of the sensitivity of melatonin generation system in the pig to the light. The opioidergic regulation of the sensitivity of melatonin generation system to the light is also suggested by the results of our recent studies (unpublished) showing the very slow decrease of plasma melatonin level after day onset in pigs treated with naloxone. Further studies are needed to clarify this problem.

Acknowledgments

The authors wish to express their deep thanks to Dr JP Ravault (INRA, Nouzilly, France) for melatonin antibodies. This work was supported by the State Committee of Scientific Research in Poland (grant KBN 5 P06D 004 08).

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