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EDITORIAL

Mechanism of Melatonin Action

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Summary

Melatonin transduces the effect of photoperiod on the neuroendocrine system. Synthesis of melatonin in the pineal gland is well described, but the location of its target(s) and the mechanism of its action are little known. In attempt to localize melatonin target(s), the presence of high affinity binding sites in rat brain was determined. Such sites were detected in discrete brain areas, including the hypothalamus and anterior pituitary. Subcellular analysis indicated these binding sites were on plasma membranes, which suggests that melatonin modulates cell functions through intracellular second messengers. The effects of melatonin on second messengers were studied using the neonatal anterior pituitary, in which melatonin is known to inhibit the LHRH-induced release of LH. Studies on the effects of melatonin on second messenger is indicated that melatonin inhibits accumulation of cAMP and cGMP as well as synthesis of diacylglycerol and release of arachidonic acid. Time-course analysis indicates that inhibition by melatonin of the LHRH-induced release of LH increases following long preincubation. Since the effect of melatonin on LHRH-induced release of LH is prevented by dibutyryl cAMP, we conclude that melatonin might act by inhibiting production of cAMP.

Key words:

Biorhythms – Pineal – Melatonin – Second messengers – LH release

1. Role of Melatonin in Photoperiodism

Most temperate zone animals undergo seasonal rhythms in reproduction, thermoregulation, weight gain, hibernation, etc. (Glass 1984, Reiter 1980, Ortavant *et al.* 1964). These rhythms are important elements in survival, ensuring that physiological changes are coordinated with seasonal changes in ambient conditions and that births occur during spring time. This allows the most favorable circumstances for young to grow and develop. Seasonal rhythms are driven by changes of photoperiod, the most reliable indicator of the season: decreasing photoperiod lengths indicates that winter is approaching and allows species to

prepare in advance. In hamsters, for example, short photoperiods induce gonadal involution, decrease of gonadal and gonadotropic hormones and inhibition of reproduction (Reiter 1967, 1980). These changes ensure that hamsters breed in the spring and that young hamsters are born in the spring and summer after a short gestation period. In contrast, sheep, which have a longer gestation period, breed during the autumn as a result of lengthening days but still bear young during the spring (Ortavant *et al.* 1964).

Photoperiod length is probably not measured by some hourglass mechanism counting the total duration of light, but rather by a circadian clock-dependent mechanism which generates the rhythm in sensitivity to light (Bünning 1973). On short photoperiods, the light-sensitive phase of the rhythm is hidden in the dark phase, but on long photoperiods it overlaps into the light phase. The existence of the rhythm is documented by the experiment using short photoperiods and additional short light pulse (e.g. 1 min) in the middle of the dark phase (Hoffmann *et al.* 1981). Although the total length of light period would still induce "short day" response if applied at once, the two pulses result in "long day" changes (Fig. 1).

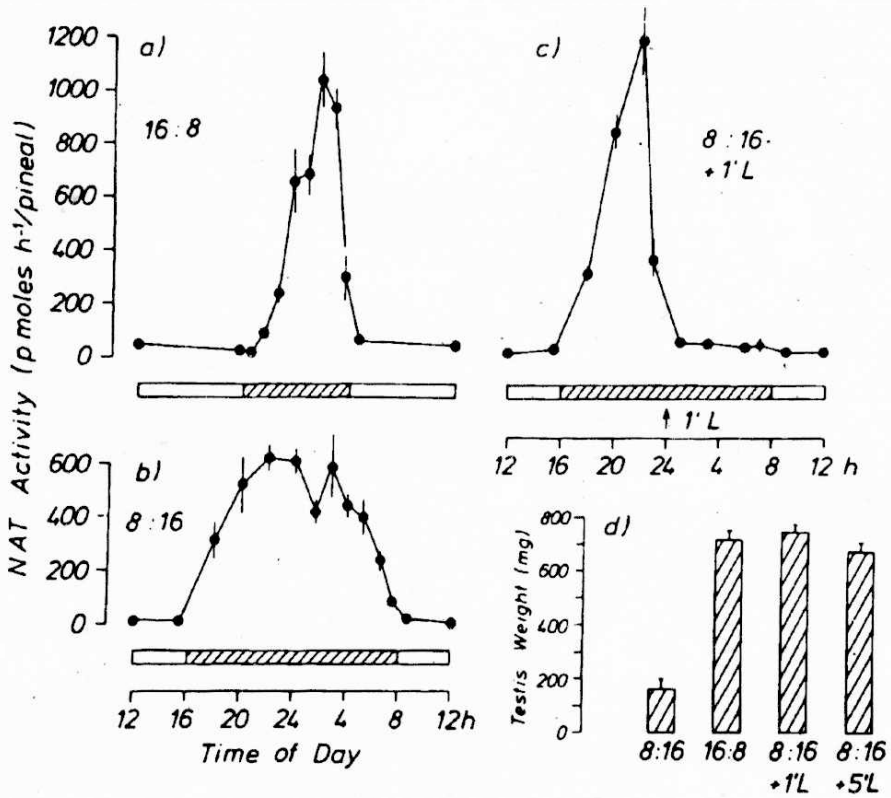

Fig.1
The effect of various photoperiods on daily rhythm in pineal N-acetyltransferase activity and on testicular weight in Djungarian hamsters. a) long photoperiod LD 16:8; b) short photoperiod LD 8:16; c) short photoperiods with 1 min light interruption in the middle of each night; d) testis weight after 45 days in indicated photoperiods. (From Hoffmann *et al.* 1981)

Photoperiodism is thus dependent on endogenous clock and on light perception: it is inhibited after transection of the optic pathways and after lesions of the suprachiasmatic nuclei, the site of putative endogenous pacemaker (Bittman *et al* 1979, Eskes *et al.* 1984). Since lesions of the pineal gland also abolish photoperiodic regulation, the gland is considered as photoperiodic transducer (Reiter 1967, 1980). Melatonin, a product of the pineal gland, has been examined to determine whether it plays a role in photoperiod regulation.

The role of melatonin in photoperiodic regulation was established by a number of experiments. In laboratory conditions with constant light/dark regime throughout the year, melatonin mimics the reproductive changes induced normally by photoperiod and it is effective even in pinealectomized animals (Carter and Goldman 1983, Goldman *et al.* 1979, Tamarkin *et al.* 1976).

2. Melatonin Signal

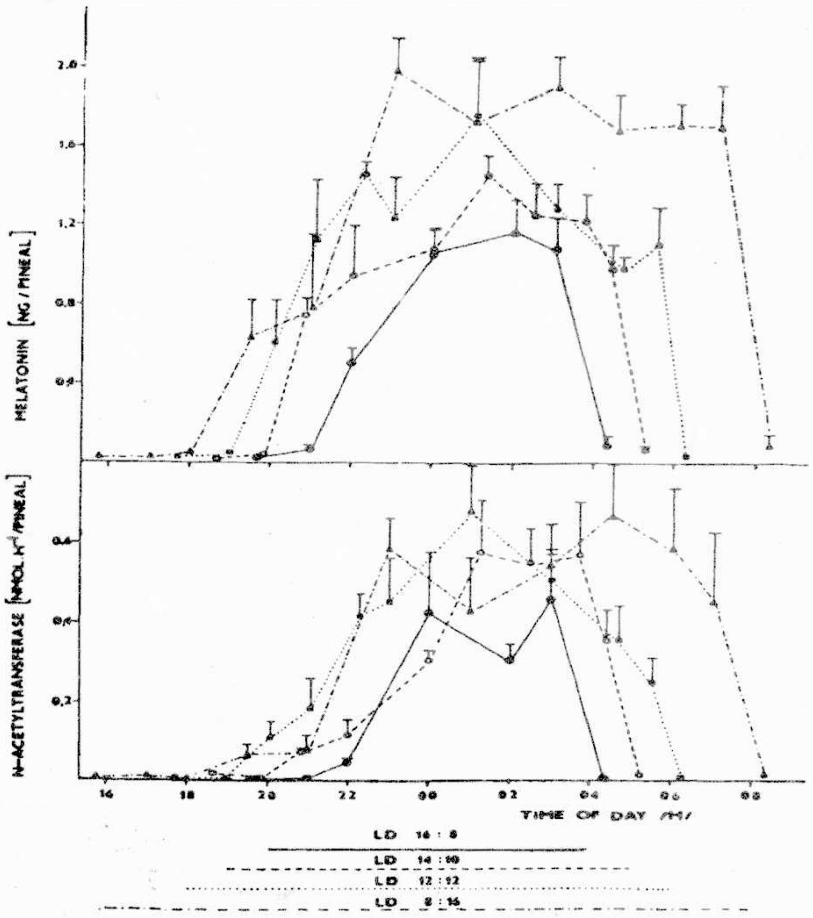

Fig.2
Daily rhythm of melatonin content (above) and N-acetyltransferase activity in pineals of Djungarian hamsters kept under photoperiods LD 16:8 (full circles), LD 14:10 (open circles), LD 12:12 (squares) and LD 8:16 (triangles). Lines below the abscissa denote the periods of darkness on different lighting regimes. (From Illnerová 1986)

The synthesis of melatonin by the pineal gland is characterized by a marked daily rhythm, with low values occurring during the day and 10-fold higher values at night (Klein and Weller 1970, Wilkinson *et al.* 1977). The rhythm is endogenous: it continues in constant darkness or in blind animals with a period close to 24 h. However, the pattern of the rhythm is influenced by photoperiod. On long photoperiods, such as in summer, the period of high melatonin synthesis is short and it is prolonged with decreasing day length in autumn and winter (Fig. 2) (Illnerová and Vaněček 1980, Illnerová *et al.* 1984). The pattern of melatonin rhythm is thus regulated by photoperiod. This photoperiodic regulation of melatonin synthesis makes the rhythm of circulating melatonin a neuroendocrine calendar. Target organs read the season from the length of the elevated levels of melatonin during the dark period.

It is not clear, however, which attribute of the melatonin rhythm is the photoperiodic signal, the phase relative to other rhythms or the duration. On short days, the duration of melatonin pulse is prolonged and concomitantly also the phase relationship among melatonin and other circadian rhythms changes. Two different models of melatonin action are currently discussed, one emphasizing the timing of the melatonin increase and the other the duration of the increase in melatonin. The "phase" model is based on the presumption of the existence of an endogenous rhythm in sensitivity to melatonin (Stetson and Tay 1983). Sensitive phase coincides with melatonin increase only on certain photoperiods (i.e. short in hamster), while on other photoperiods both rhythms are out of phase. The effect follows, of course, only when both rhythms coincide. The hypothesis is based on observations that melatonin injections induce gonadal involution only in hamsters injected in late afternoon or very early in the morning, injections in other times are ineffective (Tamarkin *et al.* 1976, Stetson and Tay 1983). However, since these experiments have been performed on pineal-intact animals, it has been suggested that the efficacy rhythm is due to the rhythm of melatonin synthesis. According to the "duration" model, the length of the melatonin pulse induces the reproductive changes (Carter and Goldman 1983). When melatonin is injected shortly before or immediately after the period of endogenous melatonin increase, the duration of high melatonin levels is prolonged and reproductive changes follow. Melatonin injections in other times are perceived as isolated pulses which do not add to the duration of endogenous melatonin increase and there is thus no effect on reproduction. This model is further supported by the experiments on pinealectomized animals where melatonin infusion or multiple injections are effective in reproductive inhibition around the clock (Carter and Goldman 1983, Goldman *et al.* 1979). The question is, however, not solved yet and there may be species differences in how the melatonin signal is read.

3. Localization of Melatonin Target

As is true for all hormones and transmitters, melatonin must act through specific receptors. To determine where such binding sites are located, the method of choice is autoradiographic analysis of tissue sections incubated *in vitro* with radioactive melatonin. Since high specific radioactivity is required, ^{125}I-melatonin (Vakkuri *et al.* 1984) was chosen as radioactive ligand. Autoradiography of ^{125}I-melatonin binding (Fig. 3, see Plate 1) revealed only few locations in rat brain with significant density of melatonin binding sites: suprachiasmatic nuclei, median

eminence-pars tuberalis complex (ME/PT), anterior pituitary (AP), area postrema, paraventricular and anteroventral thalamic nuclei (Vaněček 1988a, Vaněček *et al.* 1987, Weaver *et al.* 1989, Williams and Morgan 1988, Williams 1989).

In hamster brain, the distribution was similar with additional sites in dorsomedial hypothalamic nuclei, lateral habenular nucleus and preoptic area (Vaněček and Jánský 1989, Weaver *et al.* 1989, Williams *et al.* 1989). More abundant distribution of the melatonin binding sites was described in the chicken (Rivkees *et al.* 1989b) and salmon brain (Ekstrom and Vaněček in preparation). It is not clear which of these sites serves as a target for melatonin regulation of reproduction, but interest has been focused primarily on hypothalamic and pituitary sites.

Melatonin binding sites identified using this technique fulfil the criteria for the receptors. They have very high affinity and specificity for melatonin. The dissociation constant (K_d) for ^{125}I-melatonin is about 30 pM, and their affinity for other indoles is much lower than for melatonin (Fig. 4) (Vaněček 1988a, Rivkees *et al.* 1989a). Moreover, the affinity is decreased after preincubation with GTP analogs, suggesting that the receptor is coupled with GTP-binding protein (Rivkees *et al.* 1989a).

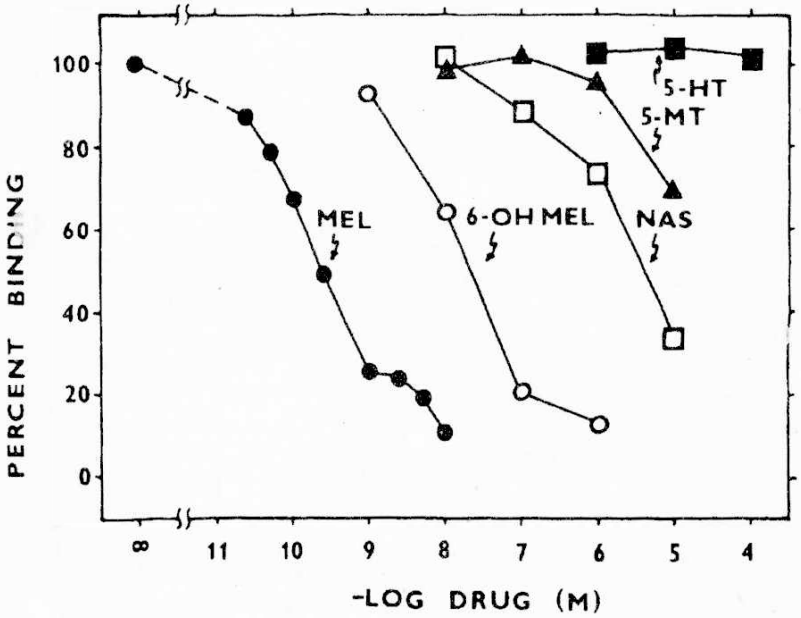

Fig.4
Competition for ^{125}I-melatonin binding sites by various indole amines. Chicken brain membranes were incubated with increasing concentrations of melatonin (full circles), 6-hydroxymelatonin (open circles), N-acetylserotonin (open squares), 5-methoxytryptamine (triangles) or serotonin (full squares) in the presence of 100 pM ^{125}I-melatonin.

The density of melatonin receptors varies with species and location, but in some locations it is affected also by developmental and endocrine changes. In rat anterior pituitary, the melatonin receptor density decreases within 30 postnatal days to about 10 % of neonatal value, while in ME/PT the concentration does not change. (Fig. 5, Vaněček 1988b).

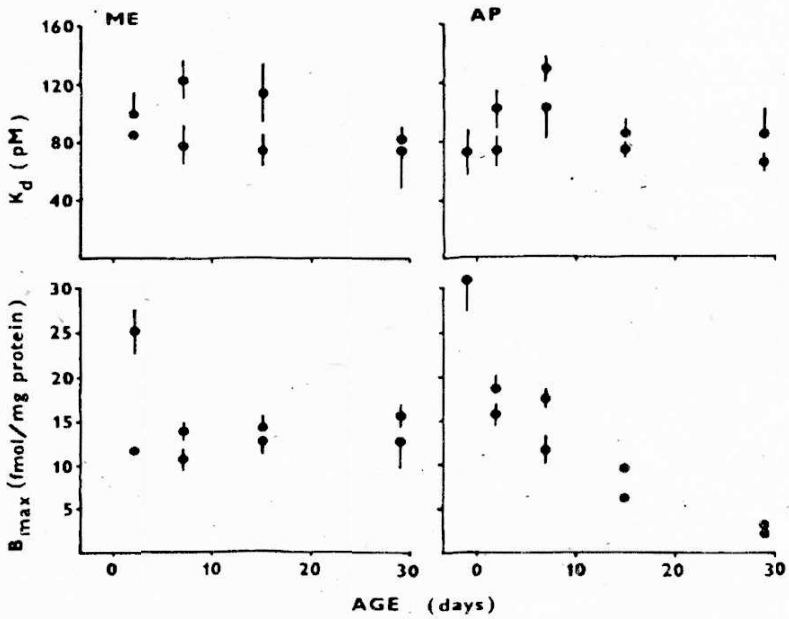

Fig.5
Developmental changes of affinity (K_d) and concentration (B_{max}) of melatonin receptors in median eminence/pars tuberalis (ME) and pituitary (AP) of male rats. (From Vaněček 1988b)

Castration, on the other hand, increases the receptor density in rat AP, which may be due to the postcastrational increase of pituitary gonadotrops. There is also daily rhythm in the receptor density in rat AP and ME/PT with about 50 % increase in the evening (Vaněček and Kosař in preparation). The decreased morning values may be due to the down regulation induced by endogenous melatonin synthesized during night. In contrast, a similar daily change in melatonin receptor number was not found in the hamster (Vaněček and Jánský 1989), chicken and salmon (Ekstrom and Vaněček in preparation).

4. Transduction

Melatonin receptors are located on plasma membranes (Vaněček 1988a, Kosař and Svoboda in preparation). Accordingly, the hormone probably acts through intracellular second messengers to affect cell function. Cyclic AMP was originally suggested to transduce the lightening effect of melatonin in frog skin melanophores (Abe *et al.* 1969, White *et al.* 1987). Later it was shown that melatonin inhibits cAMP accumulation also in other species and other tissues. In rat, hamster and ovine pars tuberalis, melatonin inhibits the forskolin stimulation of cAMP accumulation (Carlson *et al.* 1989, Morgan *et al.* 1989, Vaněček and Vollrath 1989). In the immature rat AP, in which melatonin is known to inhibit LHRH-induced release of LH (Martin and Klein 1976), melatonin has been found to inhibit basal and LHRH-induced cAMP accumulation (Fig. 6, Vaněček and Vollrath 1989, Vaněček and Vollrath 1990a). In adult rat AP, however, melatonin is without effect on either LHRH-induced cAMP accumulation or LH release.

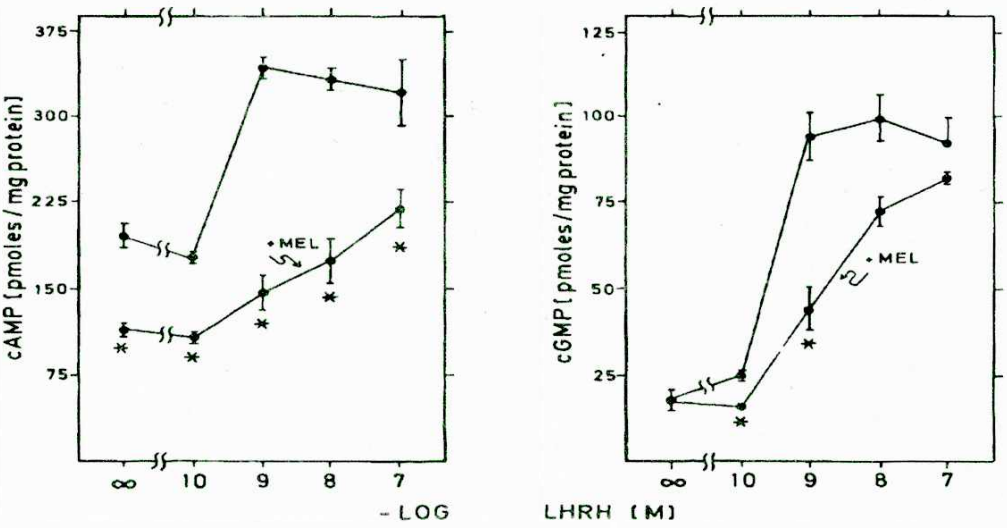

Fig.6
Dose-response curve of LHRH stimulation of cAMP (left) and cGMP (right) accumulation of neonatal rat pituitaries, cultured in the absence or in the presence of melatonin (MEL, 10 nM). * Significantly inhibited by melatonin ($p<0.05$). (From Vaněček and Vollrath 1989)

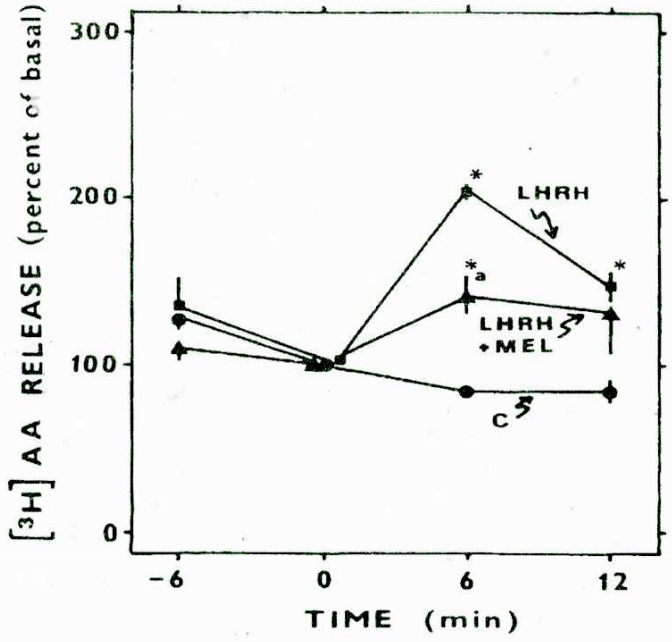

Fig.7
Effect of melatonin on the release of ^{3}H-arachidonic acid stimulated by LHRH. Neonatal pituitaries were prelabeled with ^{3}H-arachidonic acid, washed and transferred through a series of 6-min incubations in medium with or without drug. LHRH (1 μM) was added at time 0, melatonin (10 nM) 6 min earlier. Significantly different ($p<0.05$): * from controls (C), ^{a} from LHRH alone. (From Vaněček and Vollrath 1990b)

Melatonin affects also other potential second messengers: it decreases of cGMP and diacylglycerol accumulation in immature rat AP and inhibits arachidonic acid release from the gland (Fig. 7, Vaněček and Vollrath 1989, 1990a,b). Since all these messengers were shown to affect cell functions, it remains to be which messenger transduces which effect of melatonin. To inhibit metabolism of intracellular messengers, melatonin may act through pertussis toxin-sensitive G-protein. This was indicated by the finding that preincubation with the toxin abolished the melatonin effect on cAMP accumulation as well as its effect on diacylglycerol accumulation (Carlson *et al.* 1989, Vaněček and Vollrath 1989b).

Developmental changes of the melatonin effect on cAMP accumulation in AP correlate with the developmental decrease in the receptor number (Fig. 8). In 5- and 10-day-old rats, melatonin totally inhibited LHRH-stimulation of cAMP, in 15-day-old rats only partially and it had no significant effect in AP of 30-day-old animals (Vaněček and Vollrath 1990a).

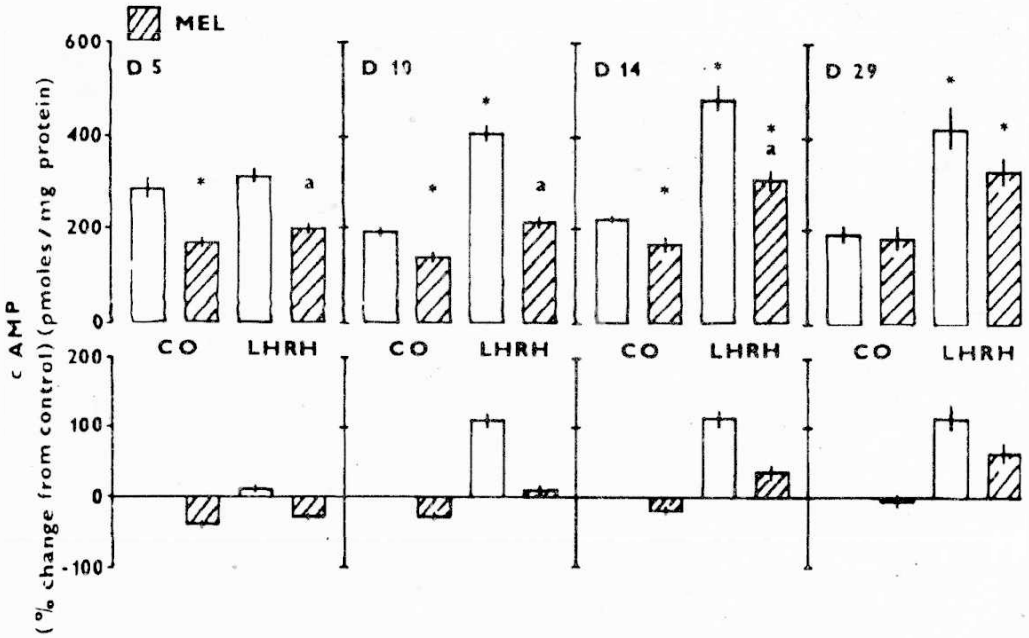

Fig.8
Developmental changes in the inhibitory effect of melatonin on basal (CO) and LHRH-stimulated accumulation of cAMP in cultured hemipituitaries from male rats. Age of rats is shown in days (D) above the bars. * Significantly different from LHRH alone ($p<0.05$). (From Vaněček and Vollrath 1990a)

These changes are in agreement with decreasing effect of melatonin on LH-release (Martin and Sattler 1979) and on gonadal growth in the course of postnatal development (Lang *et al.* 1983, Vaněček and Illnerová 1985). Although melatonin

has significant inhibitory effect on cyclic AMP accumulation regardless of the time, there is a mild daily rhythm in melatonin efficacy. Melatonin had a somewhat greater inhibitory effect on LHRH-induced cAMP accumulation in the evening than in the morning (Vaněček and Vollrath 1990a). These changes may be due to the changes in the receptor number which also increase in the evening.

5. Mechanism of Action

Although the neonatal pituitary may not be the main target of melatonin involved in photoperiodic regulation, it is the best model system to use to analyze how melatonin influences cell physiology. In addition, it is possible that melatonin

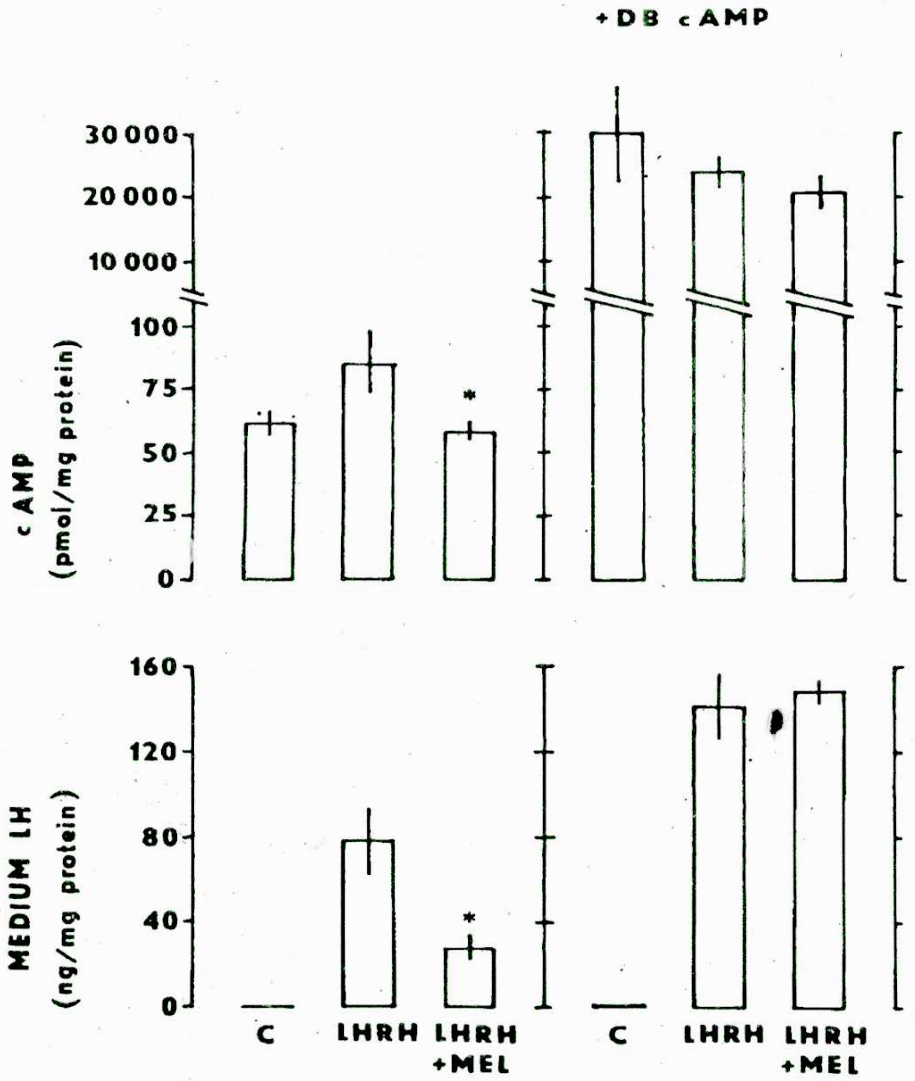

Fig.9
Effect of melatonin on LHRH-induced release of LH from neonatal rat hemipituitaries and on cAMP accumulation in the gland in the absence (left) or in presence (right) of dibutyryl cAMP (1nM). * Significantly different from LHRH alone ($p < 0.05$)

might play a role in the regulation of pituitary function during development. For example, whereas reproductive functions in adult rats are less affected by photoperiod than in hamsters, their sexual development is markedly influenced by photoperiod acting *via* the pineal gland and melatonin (Lang *et al.* 1983, Vaněček and Illnerová 1985). This effect of melatonin might involve the pituitary gland, through melatonin inhibition of the LHRH-induced LH release from the neonatal rat anterior pituitary (Martin and Klein 1976, Martin *et al.* 1977). Melatonin also inhibits cyclic AMP, cyclic GMP and diacylglycerol accumulations in the pituitary, and arachidonic acid release from the gland (Vaněček and Vollrath 1989, 1990a,b,). It is therefore possible to trace processing of the melatonin signal in AP from its binding to the receptor, through the transduction mechanism, to the effect on LH release and to estimate its effects on the reproductive system.

Synthesis of LH in the pituitary and its release from the gland are both regulated by LHRH released from hypothalamic median eminence and reaching the pituitary *via* hypothalamo-portal system (Conn *et al.* 1983, Tang *et al.* 1984, Starzec *et al.* 1988). The LHRH effect on LH release is most probably transduced by Ca^{2+} and/or a phosphatidylinositol metabolite, probably diacylglycerol (Williams 1976, Naor and Catt 1981, Andrews and Conn 1986). In contrast, the synthesis of LH is regulated by cAMP-dependent mechanism (Tang *et al.* 1984, Starzec *et al.* 1988). The inhibitory effect of melatonin on LH release also seems to be transduced by cAMP: preincubation with permeable analog dibutyryl cAMP abolishes the inhibitory effect of melatonin on LH release (Fig. 9). Although some other messengers may be also involved, the results clearly shows that melatonin-induced decrease of cAMP accumulation is the critical signal for inhibition of LH release.

The inhibitory effect of melatonin on LH release does not show daily rhythmicity. There is, however, enhancement of the inhibitory effect after long term pretreatment with melatonin: LHRH stimulation of LH release is markedly reduced in pituitaries treated with melatonin for 6 h as compared to that from glands incubated for only 20 min before addition of LHRH (Fig. 10).

The potentiation by prolonged co-incubation suggests that melatonin may primarily inhibit synthesis of LH rather than its release: cAMP, after a lag phase, stimulates LH synthesis (Tang *et al.* 1984, Starzec *et al.* 1988) and melatonin decreases cAMP levels in AP. The diminished LH release may be then secondary to the decreased intracellular levels of LH. Potentiation of the inhibitory effect of melatonin by long pretreatment with the hormone may explain the mechanism of its action as an endocrine calendar. Short melatonin pulses which are a result of long photoperiods in summer, do not decrease LH release, but the long pulses during autumn and winter inhibit LH release, which may result in gonadal involution and in reproductive collapse. Although this is not necessarily the main mechanism of melatonin action on reproduction, it is the only hypothesis supported by experimental data from investigations of the effects of melatonin on cell physiology.

Generally it is presumed that melatonin inhibits reproduction at the level of LHRH synthesis and release in preoptic and mediobasal hypothalamus. This hypothesis is based on experiments showing that melatonin microimplants in these areas elicit complete gonadal involution in mice while melatonin in other areas is ineffective (Glass and Lynch 1981). However, melatonin from the hypothalamic

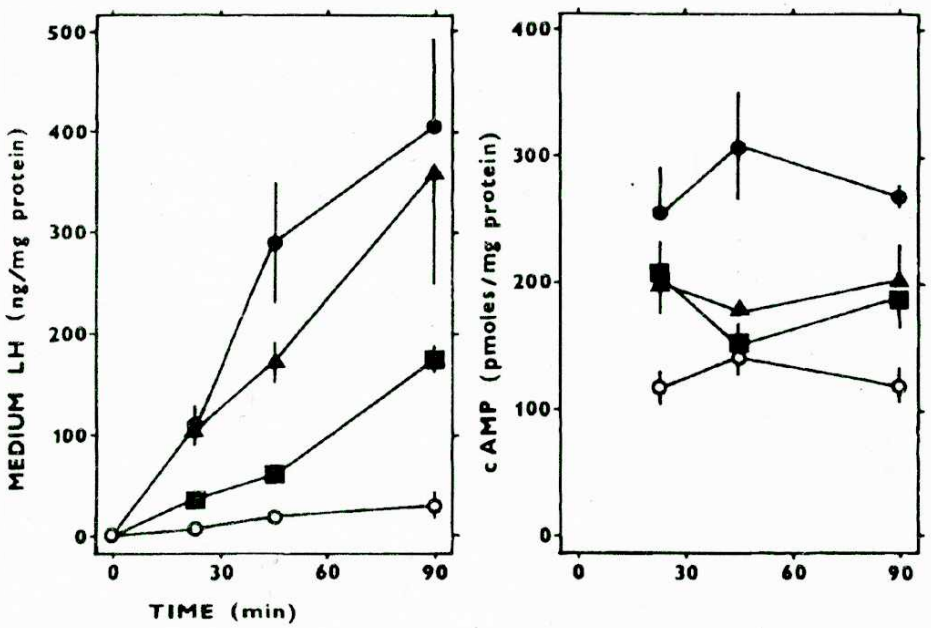

Fig.10
Time course of LHRH stimulation of LH release and cAMP accumulation in untreated pituitaries (full circles), and in those pretreated with melatonin (10 nM) for 10 min (triangles) or for 6 h (squares). Open circles indicate untreated controls

microimplants may reach anterior pituitary *via* hypothalamo-portal circulation and there it could affect LH release. On the other hand, the mechanism of melatonin effect on reproduction may be rather complex and pituitary may be only one of the targets and the proposed model only a minor component of melatonin action.

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Plate 1 – Vaněček

Fig.3
Autoradiography of ^{125}I-melatonin binding in the absence (A) or in the presence (B) of cold melatonin (1 μM) as a displacer. C and D: The corresponding brain sections stained with Toluidine blue. Arrows point to the median eminence/pars tuberalis. (From Vaněček *et al.* 1987)