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Effect of melatonin on the release of prothoracicotropic hormone from the brain of *Periplaneta americana* (Blattodea: Blattidae)

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Abstract. The occurrence of melatonin is known in nearly all organisms, but nothing is known exactly about its function outside of vertebrates. Long-term perifusions as well as short-term batch incubations of brains and moulting glands of the cockroach *Periplaneta americana* were used to identify the effect of melatonin on the release of prothoracicotropic hormone, a glandotropic neuropeptide in the brain, which stimulates the production of the moulting hormone ecdysone in the moulting gland. This is the first experimental evidence of a neurohormonal releasing effect of melatonin in the insect nervous system.

INTRODUCTION

The amino-acid derivative melatonin (N-acetyl-5-methoxytryptamine) is an evolutionary conserved molecule. The presence of melatonin has been described in algae and higher plants (Balzer & Hardeland, 1996), in nearly all invertebrate groups and in vertebrates (Vivien-Roels & Pévet, 1993). In vertebrates, melatonin signals "darkness" and is primarily known as a neuro-hormonal mediator between the environment and physiological functions. As in vertebrates, synthesis and release of melatonin in insects occur as a circadian rhythm, which is entrained by environmental light/dark cycles (Itoh et al., 1995, 1997). Moreover, melatonin is an extremely efficient scavenger of metabolic hydroxyl-radicals, not only in vertebrates but also in insects (Anisimov et al., 1997) .

Insects use photoperiods as a temporal cue to initiate postembryonic developmental processes like moulting, eclosion and diapause. Experiments on the firebug *Pyrrhocoris apterus* indicate that melatonin may participate in photoperiodic time measurement, responsible for transformation of the clock signal to the expression of diapause or non-diapause developmental programmes (Hodková, 1989, 1990).

Moulting is the most characteristic process in postembryonic development of insects which is subjected to rhythmic regulation. This occurs on the level of the moulting gland as well as in the release of the neuropeptide prothoracicotropic hormone (PTTH) from the brain (Vafopoulou & Steel, 1996a). Oscillators in the insect brain are obviously genetically determined (Šauman & Reppert, 1996). However, nothing is known regarding the information transmission between neuronal rhythmic pacemakers and the release of PTTH from the insect brain.

In the cockroach *Periplaneta americana* melatonin and the enzymes implicated in its synthesis have been identified in the brain and in the optic lobes (Binkley, 1990). In the cockroach, melatonin concentrations of 20 to 150 pg per brain were detected (Binkley, 1990). However, nothing is known as yet of the function of melatonin in the insect brain, not even whether it plays a role in moult regulation.

The present investigations of the effects of melatonin and comparatively, that of its precursor serotonin (5-hydroxytryptamine) on the release of PTTH from the brain of the studied cockroach species under in vitro conditions, provided evidence for the first time for the function of melatonin as a releaser of this glandotropic neuropeptide in this insect.

MATERIAL AND METHODS

Long-term perifusions (for explanation of the term see Peschke et al., 1997) and short-term batch-incubations (Richter, 1992) of prothoracic glands (moulting glands) and brains and also of corpora cardiaca-corpora allata of nymphs (last instar larvae) of laboratory-bred *Periplaneta americana* were carried out. To make the determination of their developmental stage as precise as possible, the animals were selected freshly moulted from mass culture and kept in groups in piacryl containers with perforated lids (15 × 25 × 10 cm) under constant conditions (30°C, 50–60% relative humidity, light-dark cycle (12/12 h), water and food (standard rat pellets) ad libitum, with folded paper as hiding facilities). The mean length of the moulting interval under these conditions was 30.5 ± 0.6 days (n = 366).

In the experiments, prothoracic glands of 23-day-old nymphs and brains or retrocerebral complexes (corpora cardiaca-corpora allata) of 18-day-old nymphs were used.

During short-term incubations, ecdysteroid secretion of single prothoracic glands was investigated during two three hour intervals per gland. The first interval served as the control, the second was the experiment. Ecdysteroid secretion in the second interval was expressed as percent change compared to the first interval. Medium 199 (after Parker: Hanks salt and HEPES, pH 7.4) (Life Technologies Karlsruhe, Germany) was used as the incubation medium in all experiments. Throughout the short-term incubations the buffer capacity was elevated (50 mmol/l HEPES) and the incubation tubes were shaken using a vibrator (f = 150/min).

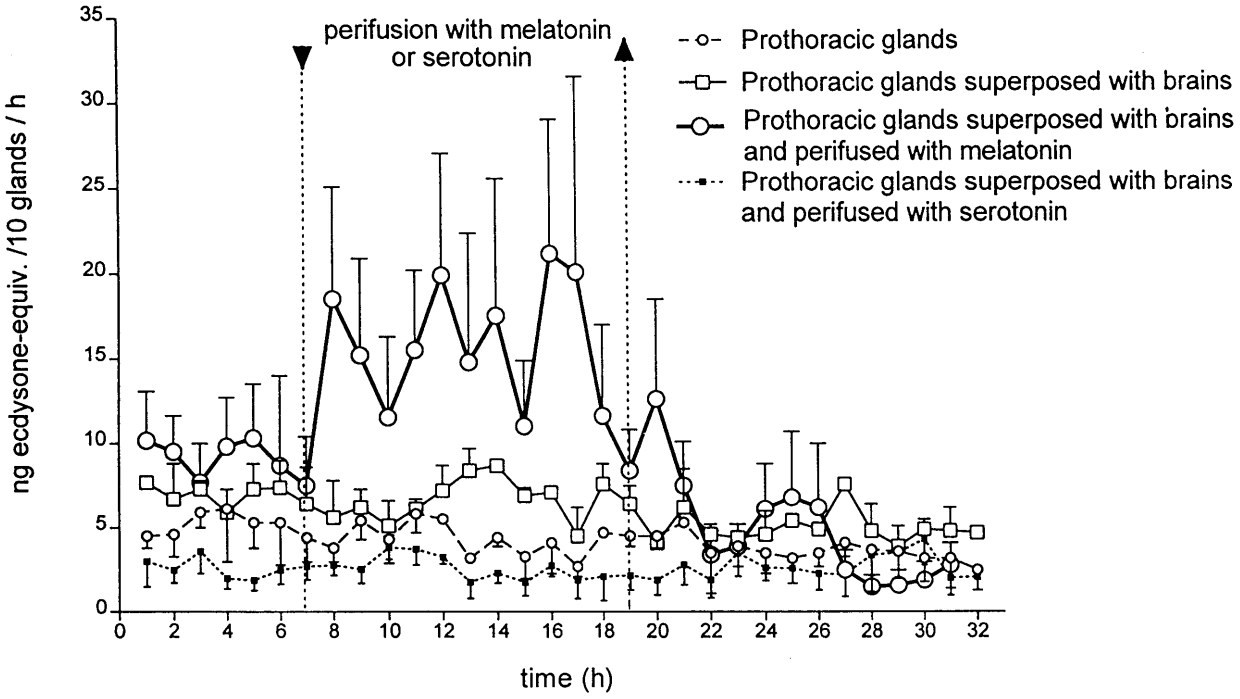

Fig. 1. Release of ecdysteroids by prothoracic glands during 32 h under perifusion conditions. Each data point is the mean ± SEM of 3 samples, each containing 10 glands and 10 brains, in the perifusion cylinder. Arrow heads mark the period of melatonin or serotonin content (10 nmol/l each) in the perifusion medium. Between 8 and 17 h the mean of all data points for prothoracic glands incubated with brains is significantly different from that for prothoracic glands with brains and melatonin ($P < 0.01$).

Perifusion analysis of isolated prothoracic glands and brains was performed in a system described earlier in detail (Csernus & Schally, 1991; Csernus et al., 1998). Briefly, 10 isolated organs were packed into glass columns (6.6 mm diameter) containing Sephadex G-10. A Medium 199 based tissue culture medium, supplemented with 2.22 g/l of sodium hydrogen carbonate, 1.75 g/l of BSA and 80 mg/l of gentamycin was passed through the columns, at a flow rate of 130 µl/min. The medium was kept at 27°C and equilibrated with a mixture of 95% air and 5% carbon dioxide. The test substances were diluted with 5 ml of medium from stock solutions immediately before application and applied to the column through a four-way valve. In the experiments, fractions of 8 ml equivalent to 60 min, were collected.

The releasing effect of melatonin on the prothoracicotropic neurohormone was assayed by radio-immunological determination of ecdysteroids, whose secretion by the prothoracic gland into the incubation or perifusion medium was stimulated by PTTH, released from the brain. Therefore, prothoracic glands were incubated together with brains during the experiments.

Ecdysteroids in the medium were assayed quantitatively by radio-immunoassay as described by Eibisch et al. (1980) on a methanolic extraction (twice with the tenfold amount of 70% methanol/water) by use of an antiserum which recognized ecdysone and 20-OH-ecdysone equally. Results of radio-immunological determinations are expressed as ecdysone equivalents. Tritiated ecdysone (23, 24-^{3}H) (89 Ci/mM) (New England Nuclear Corp., Boston, MA) was used as the radioligand. Melatonin and serotonin were purchased from Sigma, luzindole from ICN Biomedicals.

RESULTS

During long-term perifusions, prothoracic glands showed a steady state low level of ecdysteroid secretion of 4.2 ± 0.2 ng/10 glands/h during a 4 day experimental period (Fig. 1). Periodicity of ecdysteroid secretion by prothoracic glands depending on light-dark cycle was not observed under these conditions. Addition of melatonin to the perifusion medium of prothoracic glands had no effect on ecdysteroid release from the glands. In such experiments, prothoracic glands showed an ecdysteroid secretion of 3.6 ± 0.3 ng/10 glands/h, not significantly different from untreated glands ($P > 0.05$). Incubation of the prothoracic glands with brains (10 glands/ 10 brains) resulted in an increase of ecdysteroid secretion to 6.04 ± 0.24 ng/10 glands/h throughout 3 days (Fig. 1). In these experiments periodicity of ecdysteroid secretion corresponding to light/dark cycle was also not detected.

However, addition of melatonin to the perifusion medium (10 nmol/l) of this combination of organs resulted in an increase of ecdysteroid secretion to 9.86 ± 1.04 ng/10 glands/h (Fig. 1). This is a significant increase of 60% ($P < 0.01$) in comparison to gland/brain combinations without melatonin. Perifusion of gland/brain mounts with serotonin (10 nmol/l) had no statistically significant effect (Fig. 1).

The results of the perifusion experiments were confirmed by the series of experiments with short-term incubations. Under in vitro conditions a prothoracic gland secreted 2.05 ± 0.43 ng ecdysteroids into the incubation medium during a 3-h incubation period. Melatonin had no effect on the prothoracic gland (1.99 ± 0.43 ng/gland/3 h) (Fig. 2a). However, when glands were incubated with brains (one gland/one brain) ecdysteroid secretion of the glands was significantly increased to 2.71 ± 0.67

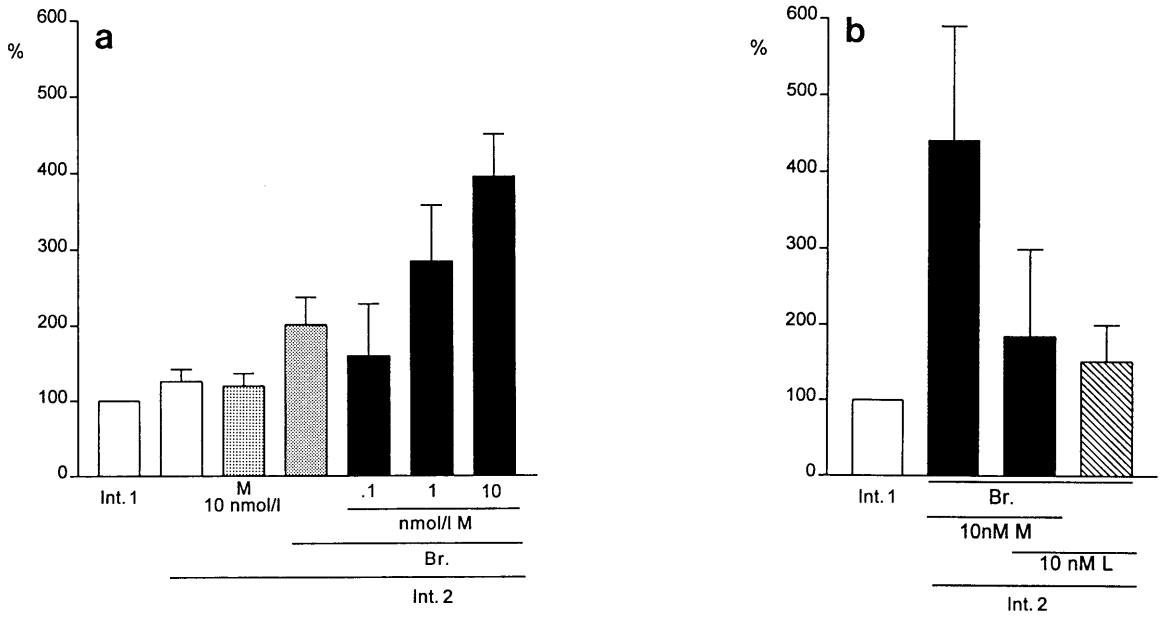

Fig. 2. Effect of melatonin and luzindole on release of ecdysteroids by prothoracic glands in short-term incubations (two intervals of 3 h each). Change of ecdysteroid release in the second incubation interval (test interval, Int. 2) related as a percentage to the first interval (Int. 1). M – administration of melatonin in the incubation medium; Br. – coincubation of one brain with one gland; L – addition of luzindole (10 nmol/l) to the incubation medium. Mean ± SEM (n = 5–12).

ng/gland/3 h ($P < 0.05$). During incubations of glands with brains, ecdysteroid secretion of the glands increased after addition of melatonin into the incubation medium as a result of PTTH release from the brains. The melatonin effect was dose dependent in the tested concentration range from 0.1 to 10 nmol/l (Fig. 2a). Melatonin in the concentration of 10 nmol/l increased the ecdysteroid secretion to 4.94 ± 0.81 ng/gland/3 h, i.e. up to 80% ($P < 0.01$).

The melatonin receptor antagonist luzindole (N-acetyl-2-benzyltryptamine, 10 nmol/l) had no effect on incubations of prothoracic glands with brains (Fig. 2b). Luzindole (10 nmol/l) inhibited the stimulating effect of melatonin on PTTH release from the brain (Fig. 2b). In these experiments ecdysteroid secretion was not significantly different from coincubations of prothoracic glands and brains without melatonin ($P > 0.05$) (Fig. 2b).

During short-term incubations serotonin (10 nmol/l) had no effect on ecdysteroid release by the prothoracic glands (Fig. 3). During incubations of prothoracic glands with brains serotonin in the same concentration significantly decreased ecdysteroid secretion by about 30% ($P < 0.05$) (Fig. 3).

In incubations of prothoracic glands with corpora cardiaca-corpora allata complexes (one gland/one pair)

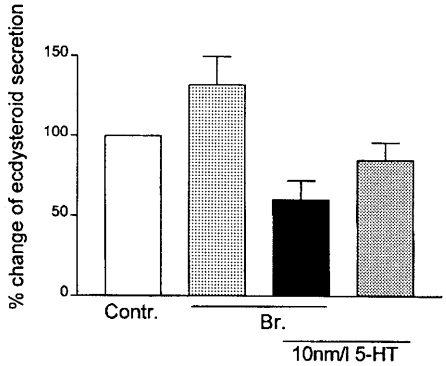

Fig. 3. Effect of serotonin (5HT) on release of ecdysteroids by prothoracic glands in short-term incubations (two intervals of three hours each). Change of ecdysteroid release in the second incubation interval (test interval, Int. 2) related as a percentage to the first interval (Int. 1). Br. – incubation of one brain with one gland. Mean ± SEM (n = 5).

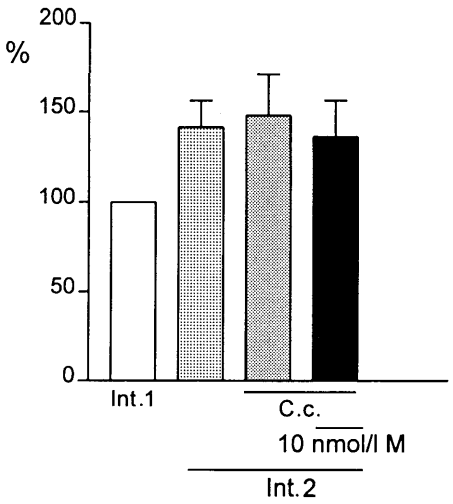

Fig. 4. Release of ecdysteroids by prothoracic glands in short-term incubations (two intervals of 3 h each): Incubation with corpora cardiaca-corpora allata complex (one pair of corpora cardiaca-corpora allata (Cc.)/one gland) and addition of melatonin (10 nmol/l M). Change of ecdysteroid release in the second incubation interval (test interval, Int. 2) related as a percentage to the first interval (Int. 1). Mean ± SEM (n = 5).

there was no effect of melatonin (10 nmol/l) on ecdysteroid secretion from the glands (Fig. 4).

DISCUSSION

The ecdysiotropic hormone PTTH, produced by neurosecretory cells in each brain hemisphere is transported via the corpora cardiaca and released from the corpora allata under the control of photoperiodic cues. In the case of *Manduca sexta*, Lester & Gilbert (1986) found that the cholinergic system is involved in PTTH release. Results of experiments on brains of *Bombyx mori* with immunofluorescence staining indicated that PTTH-producing neurosecretory cells in this species express muscarinic acetylcholine receptors (Aizono et al., 1997). As shown in short-term superfusions of silkworm brains muscarinic, cholinergic transmission might directly regulate PTTH release from neurosecretory cells (Aizono & Shirai, 1995).

In Hemimetabola, like e.g. in *Rhodnius prolixus* (Vafopoulou & Steel, 1996b), as well as in Lepidoptera (Bollenbacher & Granger, 1985), PTTH is released according to a daily rhythm. In the long-term perifusion experiments no daily rhythmicity could be detected, neither in ecdysteroid secretion nor in PTTH release. However, the presence of melatonin and of enzymes involved in its biosynthesis from tryptophan in the brain and in the optic lobes of the cockroach *Periplaneta americana* and daily changes in quantity (Binkley, 1990) suggest a participation of this indoleamine in the control of PTTH release.

Serotonin, a precursor in the biosynthesis of melatonin had no effect on PTTH release in our experiments, although the presence of serotonin in the cockroach brain is well known (Sloley & Downer, 1984; Page, 1987)

The effect of melatonin on PTTH release, shown in both in vitro methods, originates at the level of the PTTH producing neurosecretory cells in the brain and not in the retrocerebral complex. This is apparent from incubations of corpora cardiaca-corpora allata complexes in which melatonin has no effect.

Luzindole is known as a pre-synaptic melatonin receptor antagonist active in man, in several species of mammals, in birds and also in anura (Dubocovich, 1988; Sudgen, 1992). Melatonin receptor sites in cockroaches have not been identified yet. Nevertheless, the antagonistic effect of luzindole on PTTH release in the brain suggests a pre-synaptic effect of melatonin on PTTH producing neurons in the insect brain.

Our results are the first experimental evidence that melatonin plays a role as a releasing factor of the glandotropic neuropeptide PTTH in the brain of *Periplaneta americana*. Besides this, a probably specific receptor-mediated effect of melatonin in an insect brain was shown in the experiments with luzindole.

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