# N-bromoacetyltryptamine strongly and reversibly inhibits in vitro melatonin secretion from mammalian pinealocytes

**Bogdan Lewczuk [1], Weiping Zheng [2,3], Magdalena Prusik [1], Philip A. Cole [2], Barbara Przybylska-Gornowicz [1]**

[1] Division of Histology and Embryology, Department of Functional Morphology, Faculty of Veterinary Medicine, University of Warmia and Mazury in Olsztyn, Poland.
[2] Department of Pharmacology and Molecular Sciences, Johns Hopkins School of Medicine, Baltimore, MD 21205, USA.
[3] Current address: Department of Chemistry, University of Akron, Akron, Ohio 44325, USA.

Correspondence to:    Bogdan Lewczuk
                      Division of Histology and Embryology
                      Faculty of Veterinary Medicine
                      University of Warmia and Mazury
                      Oczapowskiego Str. 13
                      10-713 Olsztyn, POLAND
                      PHONE: +48 89 5233949;  FAX: +48 89 5240408;
                      EMAIL: lewczukb@uwm.edu.pl

ORIGINAL ARTICLE

## Abstract

**OBJECTIVES**: Cell-permeable and specific inhibitors of melatonin secretion are sill lacking among tools of the pineal research. Recently, a large effort has been made in the development of arylalkylamine N-acetyltransferase inhibitors, but in most cases the new drugs were tested exclusively using cell-free assays or non-pineal cells. The aim of the present study was to characterize the effect of N-bromoacetyltryptamine (BAT), the first synthesized cell-permeable inhibitor of arylalkylamine N–acetyltransferase, on melatonin secretion from rat and pig pineal glands.

**METHODS:** The studies were performed in the superfusion cultures of rat and pig pineal explants. Melatonin secretion was determined by radioimmunoassay (RIA).

**RESULTS**: BAT strongly inhibited the non-stimulated and norepinephrine–stimulated melatonin secretion from the pig and rat pineal explants, with $ED_{50} \approx$ 0.3–0.7 μM. The adrenergic stimulation did not modify significantly the inhibitory potency of BAT on the melatonin release. The decline in melatonin secretion induced by the BAT–treatment was biphasic in both rat and pig pinealocytes, with an initial rapid phase followed by a slow one. The half-time of BAT-induced decline in the non–stimulated and norepinephrine-stimulated melatonin secretion was ca. 25–35 minutes. The inhibitory effect of BAT was reversible in pinealocytes of both investigated mammals.

**CONCLUSIONS**: The results show that BAT is a potent and reversible inhibitor of the melatonin secretion in the mammalian pineal gland and open the way for the use of this inhibitor in investigations on the pinealocyte physiology performed *in vitro*.

Bogdan Lewczuk, Weiping Zheng, Magdalena Prusik, Philip A. Cole & Barbara Przybylska-Gornowicz

## Introduction

The pineal gland, via its main hormone – melatonin, plays an important role in regulation of several physiological processes occurring in the diurnal and annual rhythms, like the day-night variations in locomotor activity and sleep as well as the seasonal changes in reproduction [35, 37]. Melatonin also modulates the functions of the immune system and the cardiovascular system as well as influences cancer growth [16, 24, 36]. Numerous experimental data suggest that melatonin is an important component of the antioxidative defense, mainly as a potent free-radical scavenger, which is able to cross all barriers and to enter each cell, but also as a stimulator of various antioxidative enzymes [9, 27, 38]. Mechanisms of melatonin action seem to be very complex and variable. This hormone influences target cells via melatonin membrane receptors [13], nuclear receptors [2] and non-receptor intracellular regulatory proteins [3]. Free-radical scavenging may also be a mechanism of melatonin regulatory action [38]. In vertebrates, melatonin is synthesized outside the pineal gland as well, but a complete and verified list of the organs producing this indoleamine (in most cases for their local purposes) is still a matter of debate [4, 7]. Moreover, melatonin has been detected in some invertebrates and plants [17, 39].

Progress in explaining the role of melatonin in the mammalian physiology and pathology is strongly dependent on the development of pharmacological tools which enable selective influence on the melatonin receptors and artificial modulation of the melatonin secretion level. During the last decade several agonists and antagonists of the melatonin membrane receptors as well as some active ligands of the nuclear receptors have been developed [6, 20, 43]. Exogenous melatonin in a form of various pharmaceutical formulations is also widely used in experimental studies [19, 44]. More recently, the pineal hormone has been introduced into therapy [42]. In contrast, drugs that can specifically reduce melatonin secretion are still lacking among pharmacological tools available for pineal research.

Melatonin is synthesized from tryptophan via hydroxylation, decarboxylation, acetylation and methylation. The first two steps are catalyzed by tryptophan hydroxylase and aromatic aminoacids decarboxylase, the enzymes widely distributed in serotoninergic or catecholaminergic systems of the body. Therefore, their inhibitors, although depressing melatonin synthesis [25, 31], are not suitable for a specific blockade of the pineal hormone secretion. The enzymes involved in the last two steps of melatonin synthesis, arylalkylamine N-acetyltransferase (AA-NAT) and hydroxyindole-O-methyltransferase (HIOMT), are considered to be specific for this biochemical pathway. For this reason, they are the best target points for selective inhibition of melatonin secretion.

The first reports concerning inhibition of AA-NAT were published in the early 1980s [33]. It was demonstrated that AA-NAT is inactivated by cystamine probably via a mechanism of thiol-disulfide exchange and this effect was reversed by dithiothreitol [33]. Similarly,
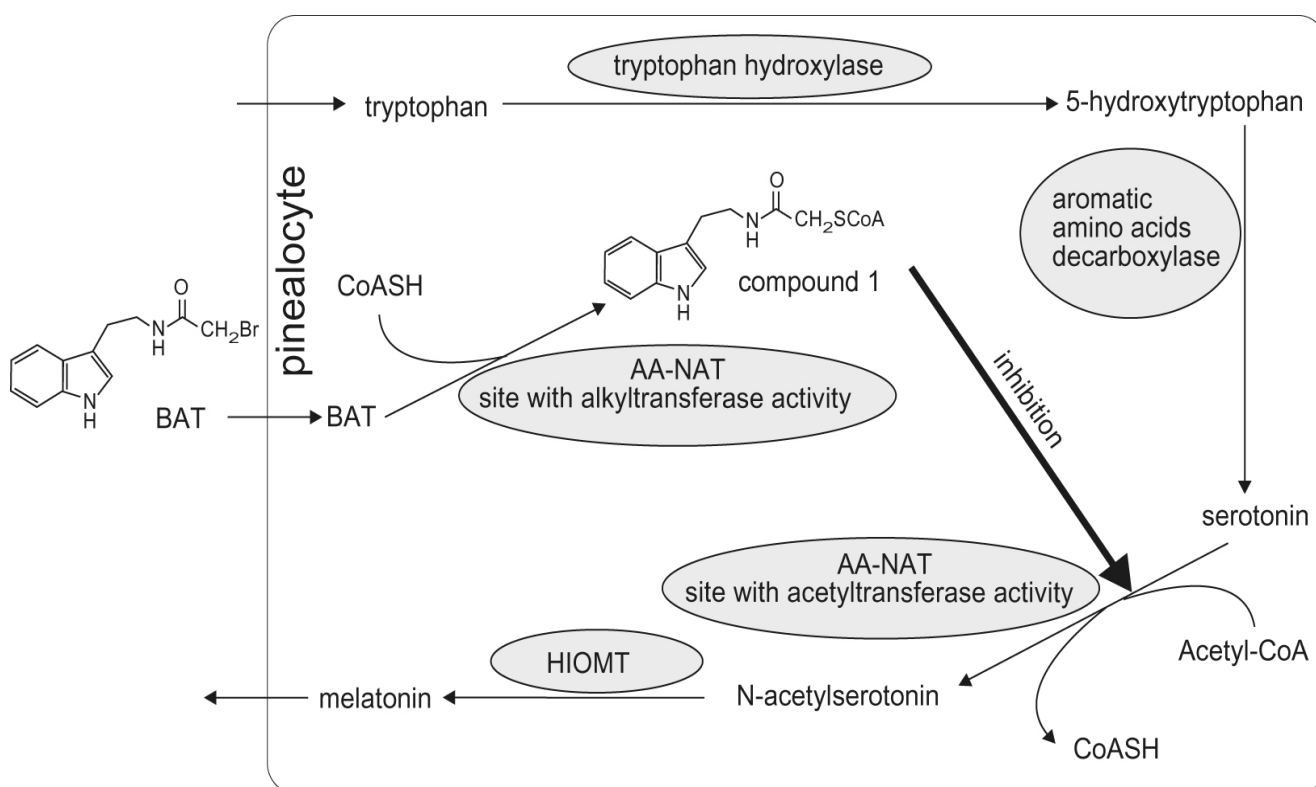


**Figure 1.** The putative mechanism of BAT inhibitory action on the AA-NAT acetyltransferase activity and the melatonin secretion via formation of compound 1.

Sugden and Klein [40] reported inactivation of rat HIOMT by cystamine and selenocystamine. Interestingly, cystamine did not alter bovine HIOMT activity and it increased the activity of ovine HIOMT [40]. No further data concerning inactivation of AA–NAT and HIOMT by disulfide-containing compounds were reported.

The studies on the catalytic mechanism of AA–NAT [18] have led to the design and synthesis of the bisubstrate analog, compound 1, containing tryptamine and coenzyme A linked via an acetyl bridge (*Fig. 1*) as well as for testing this analog as an AA–NAT inhibitor [21]. As a result, it has been found that compound 1 is an extremely potent inhibitor of ovine AA–NAT, with $IC_{50} \approx 150$ nM. It acts as a competitive inhibitor of AA-NAT versus acetyl-CoA and a noncompetitive one versus tryptamine [21]. Compound 1 is ca. 1000-fold more potent in the inhibition of AA-NAT than arylamine N-acetyltransferase, another enzyme present in the pineal gland. Several other bisubstrate analogs inhibiting AA-NAT have also been synthesized [23]. Unfortunately, due to their chemical properties (the presence of the coenzyme A moiety) the bisubstrate analogs including compound 1 are not able to penetrate the cell membrane and to act on living cells.

The limitations in practical use of the bisubstrate inhibitors of AA-NAT caused by their cell-impermeability drew attention to N–bromoacetyltryptamine (BAT), a precursor of compound 1 in the chemical synthesis [22]. BAT is an uncharged, fairly hydrophobic compound and was therefore predicted to cross cell membranes efficiently. The in vitro experiments [22] with ovine AA–NAT have shown that this enzyme catalyzes the alkyl transfer reaction between BAT and reduced coenzyme A, which led to the formation of compound 1 (*Fig. 1*). Due to this phenomenon, incubation of ovine AA-NAT with BAT results in potent inhibition of its acetyltransferase activity, with $IC_{50} \approx 500$ nM [22]. The ability of BAT to reduce melatonin secretion in living cells has been demonstrated on rat pinealocytes in monolayer culture [22]. In this experimental model, BAT decreased, in a concentration-dependent manner, the melatonin secretion induced by the treatment with norepinephrine (NE).

The proposed mechanism of the inhibitory action of BAT on the AA-NAT acetyltransferase activity via formation of the bisubstrate analog has found further support in the studies with other N–haloacetyltryptamines [46]. According to the results, the haloderivatives of N–acetyltryptamine that undergo the alkyl transfer reaction catalyzed by AA-NAT are inhibitors of the arylalkylamine acetyltransferase activity.

In the last two years several other compounds have been designed, synthesized and tested as putative inhibitors of AA-NAT [1, 12, 30]. Some of them have been recognized as potent AA-NAT inhibitors.

Until now, the AA-NAT inhibitors have almost exclusively been tested in cell-free assays [1, 10, 30] or in non-pineal cells expressing AA-NAT [11, 12]. Only in the case of two compounds, BAT and N–chloroacetyltryptamine, their influence on melatonin

secretion from rat pinealocytes was investigated [22, 46]. However, even these studies were limited to the analysis of melatonin accumulation in a culture medium during 6-hour-long stimulation with NE performed in the presence of various concentrations of the inhibitors.

In the present study, the effect of BAT on melatonin secretion was investigated in superfusion cultures of rat and pig pineal glands. Pineals of rats and pigs were chosen due to many significant dissimilarities in the physiological characteristics of both glands, especially completely different mechanisms regulating melatonin synthesis [25, 28, 29, 32]. The purposes of our study were to examine:

1) efficiency of BAT in inhibition of non-stimulated and NE-stimulated melatonin secretion,
2) reversibility of the BAT inhibitory effect on the pineal hormone secretion,
3) dynamics of the changes in melatonin secretion induced by BAT.

## Materials and methods

### Chemicals

Medium 199 containing Earle's salt and HEPES (SIGMA, USA) was prepared from a powdered form (0.85 g/L of $NaHCO_3$ added, pH adjusted to 7.3 with NaOH, final preparation sterilized by filtration). N-bromoacetyltryptamine was synthesized at The Johns Hopkins University School of Medicine in Baltimore as described previously [22, 46] and stored at – 20°C until use. Immediately before the experiments the drug was dissolved in propylene glycol (POCh, Poland) and this stock solution was diluted in the culture medium (the final concentration of propylene glycol did not exceed 0.5% (v/v)). Norepinephrine bitartrate was obtained from Sigma, USA.

Anti–melatonin antibody G/S/704-6483 was purchased from Stockgrand Ltd, University of Surrey, Great Britain, $^3$H-melatonin (87 Ci/mM) – from Amersham Biochemicals, Great Britain; gelatin – from Merck, Germany. All other reagents used in melatonin RIA came from Sigma, USA.
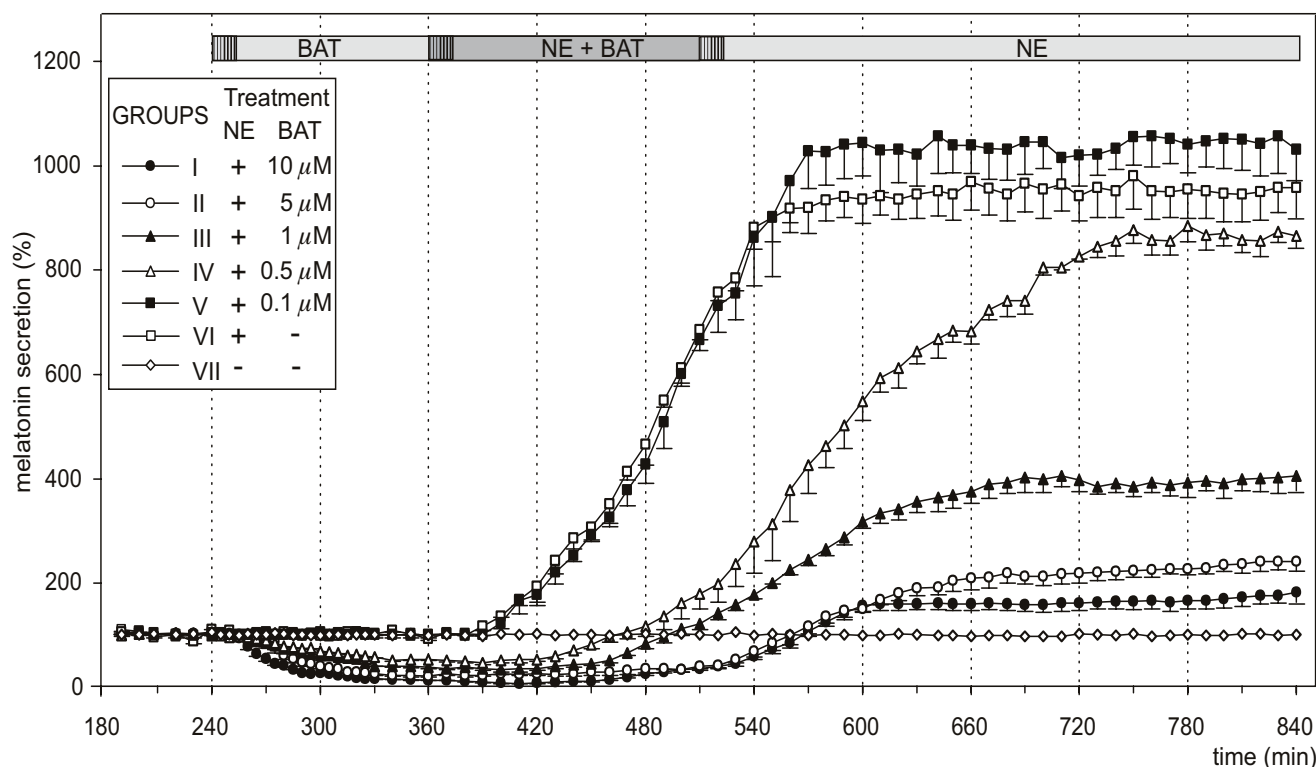
### Animals and the pineal glands

All experimental procedures on animals were performed in accordance with Polish and EU law. They were approved by Local Ethics Committee for Experiments on Animals in Olsztyn.

#### Rats

Female Wistar rats, aged 4 months, were obtained from a local breeding population. They were kept under a cycle of 12 hours light : 12 hours dark (photophase from 7.00 a.m. to 7.00 p.m. with fluorescent lighting of 500 lux intensity at the cage floor level) for at least two weeks. The animals had free access to standard food and water. The rats were anesthetized using halothane vapors and killed by decapitation

Bogdan Lewczuk, Weiping Zheng, Magdalena Prusik, Philip A. Cole & Barbara Przybylska-Gornowicz

Experiment I



| time (min) | 210 | 240 | 270 | 300 | 330 | 360 | 390 | 420 | 450 | 480 | 510 | 540 | 570 | 600 | 630 | 660 | 690 | 720 | 750 | 780 | 810 | 840 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| I | a | a | b | c | d | d | d | c | c | c | d | a | a | a | a | fa | fa | fa | fa | fa | fa | fa |
| II | a | a | a | c | d | d | c | c | c | c | d | a | a | a | a | ef | ef | ef | ef | ef | ef | ef |
| III | a | a | a | b | c | c | b | c | c | a | a | ca | d | d | d | d | d | d | d | d | d | d |
| IV | a | a | a | b | b | b | b | c | a | a | c | c | c | c | c | c | c | c | c | c | c | b |
| V | a | a | a | a | a | a | a | b | b | b | b | b | b | b | b | b | b | b | b | bc | bc | b |
| VI | a | a | a | a | a | a | a | b | b | b | b | b | b | b | b | b | b | b | b | b | b | b |
| VII | a | a | a | a | a | a | a | a | a | a | a | a | a | a | a | a | a | a | a | a | a | a |

(Groups)

**Figure 2**. Melatonin secretion (means ± SEM) from the rat pineal glands incubated between 241 and 510 min of the experiment in the perfusion of medium containing 0.1–10 µM of BAT (groups I – V) or vehicle (groups VI - VII). Between 361 and 840 min of the experiment the pineals from groups I - VI were treated with 10 µM of NE. The explants from group VII were not subjected to the adrenergic stimulation. The mean melatonin secretion between 181 and 240 min of the experiment was taken as 100%. The solid segments of the horizontal bar represent an approximate presence of the nominal concentrations of BAT or NE in medium flowing through the culture chambers; the hatched segments – the periods of the increase in the drug concentration to the nominal value or the decrease to the level below 5% of the nominal concentration. In the table, the means significantly different between groups at $p \le 0.05$ were marked with different letters.

(between 10.00 and 11.00 a.m.). The pineals were removed under sterile conditions and placed in the culture medium.
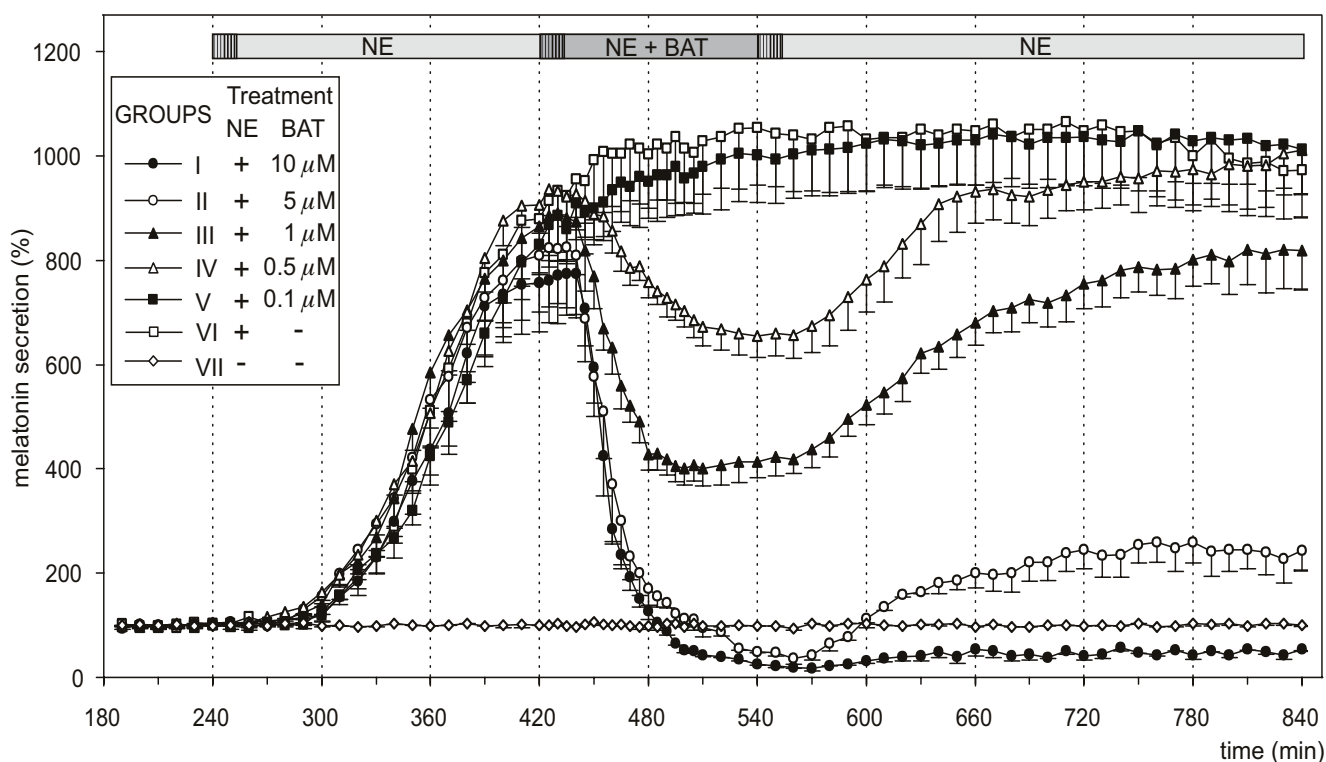
*Pigs*

Female pigs, 4-month-old, were purchased from a commercial piggery with natural light conditions. For at least two weeks before the experiments, the animals were kept in a light-proof animal laboratory room under a cycle of 12 hours light : 12 hours dark, with a photophase from 7.00 a.m. to 7.00 p.m. (fluorescent illumination with intensity of 500 lux at the floor level). The pigs were fed twice daily with standard food and had free access to water.

The gilts were slaughtered between 10.00 and 11.00 a.m. The pineal glands were immediately removed, placed in the culture medium and divided under sterile conditions into three explants.

***Superfusion culture***

Single explants of the pig pineal glands or two rat pineals were covered with a nylon mesh and placed into culture chambers (volume 0.5 ml). The upper pool of each chamber was connected via a system of tubes and valves to four containers with culture media. The lower pool of the culture chamber was attached to a twelve-channel peristaltic pump (COLE PARMER, USA) and a manual fraction collector. The total volume of the superfusion set consisting of the culture chamber,

Experiment II



| time (min) | 210 | 240 | 270 | 300 | 330 | 360 | 390 | 420 | 450 | 480 | 510 | 540 | 570 | 600 | 630 | 660 | 690 | 720 | 750 | 780 | 810 | 840 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| I | a | a | a | b | b | b | b | b | c | a | a | f | f | e | a | a | a | a | a | a | a | a |
| II | a | a | a | b | b | b | b | b | c | a | a | e | e | a | a | d | d | d | d | d | d | c |
| III | a | a | a | b | b | b | b | b | cb | d | d | d | d | d | c | c | c | c | c | c | c | b |
| IV | a | a | a | b | b | b | b | b | b | c | c | c | c | b | b | b | b | bc | bc | bc | b |
| V | a | a | a | b | b | b | b | b | b | b | b | b | b | b | b | b | b | b | b | b | b | b |
| VI | a | a | a | b | b | b | b | b | b | b | b | b | b | b | b | b | b | b | b | b | b | b |
| VII | a | a | a | a | a | a | a | a | a | a | a | a | a | a | a | a | a | a | a | a | a | a |

Between group differences at the selected time-points of the experiment

**Figure 3**. Melatonin secretion (means ± SEM) from the rat pineal glands incubated between 241 and 840 min of the experiment in the perfusion of medium with 10 μM of NE (groups I – VI) or without NE (group VII). Between 421 and 540 min of the experiments the pineals from groups I - V were exposed to BAT at concentrations 0.1 – 10 μM and the glands from groups VI and VII – to vehicle. The mean melatonin secretion between 181 and 240 min of the experiment was taken as 100%. Other explanations see fig 2.

tubes and valves was 1.3–1.4 ml. The superfusion was performed at a flow rate of 0.2 ml/min. The medium was continuously gassed with a mixture of 2% $CO_2$ and 98% $O_2$. The incubation of the rat pineals and pig pineal explants was performed at 37°C and 37.5°C, respectively. The medium fractions were collected every 5 or 10 minutes and frozen at –20°C until melatonin assay.

For precise determination of the time relationship between changes of drug concentration in medium flowing through the culture chambers and changes of melatonin secretion level, the superfusion system was calibrated by addition of radioactive markers to the medium containers and subsequent determination of radioactivity level in the collected medium samples. After switching the medium sources which supplied the culture chamber, the drug concentration in the medium flowing out from the superfusion system increased to 95% of the nominal value or decreased to 5% of the nominal value within 15 (± 1) minutes.
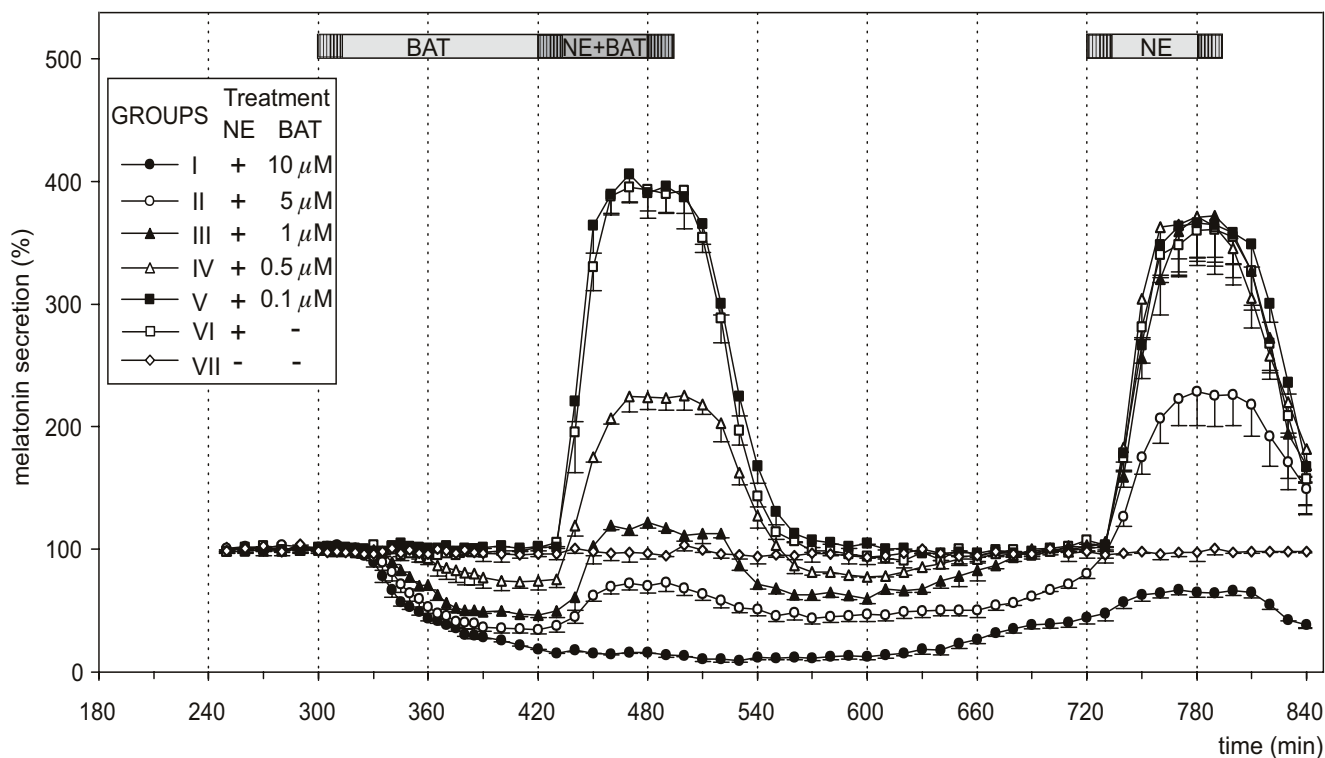
### Experimental procedures

Experiments I and II were performed on the rat pineals, while experiments III and IV were made on explants of the pig pineal gland. Each experiment was carried out on seven groups of explants; each group included three culture chambers.

### Experiment I

The rat pineals from groups I – V were incubated: (1) between 241 and 360 min of the experiment in a medium containing BAT at concentrations 0.1 – 10 μM, (2) between 361 and 510 min of the experiment in a medium containing BAT at concentrations 0.1 – 10 μM and NE at a concentration of 10 μM, and then (3) between

Bogdan Lewczuk, Weiping Zheng, Magdalena Prusik, Philip A. Cole & Barbara Przybylska-Gornowicz

## Experiment III



**Between group differences at the selected time-points of the experiment**

| time (min) | 270 | 300 | 330 | 360 | 390 | 420 | 450 | 480 | 510 | 540 | 570 | 600 | 630 | 660 | 690 | 720 | 750 | 780 | 810 | 840 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| I | a | a | a | c | d | d | f | f | e | e | e | e | e | c | c | c | a | a | a | c |
| II | a | a | a | c | dc | c | e | e | d | d | d | d | d | b | b | b | c | c | c | ab |
| III | a | a | a | b | c | c | d | d | a | da | c | c | c | a | a | a | b | b | b | b |
| IV | a | a | a | b | b | b | c | c | c | c | b | b | b | a | a | a | b | b | b | b |
| V | a | a | a | a | a | a | b | b | b | b | a | a | a | a | a | a | b | b | b | b |
| VI | a | a | a | a | a | a | b | b | b | b | a | a | a | a | a | a | b | b | b | b |
| VII | a | a | a | a | a | a | aed | aed | a | a | a | a | a | a | a | a | a | a | a | a |

Groups

**Figure 4**. Melatonin secretion (means ± SEM) from the explants of the pig pineal gland incubated between 301 and 480 min of the experiments in the perifusion of medium containing 0.1–10 μM of BAT (groups I – V) or vehicle (groups VI and VII). The explants from groups I – VI were treated with 10 μM of NE between 421 and 480 min as well as between 721 and 780 min of the experiment. The explants from group VII were not subjected to the adrenergic stimulation. The mean melatonin secretion between 241 and 300 min of the experiment was taken as 100%. Other explanations see fig 2.

511 and 840 min of the experiment in a medium containing NE at a concentration of 10 μM. The treatment with BAT (between 241 and 510 min of the experiment) was performed as follows: group I – at a concentration of 10 μM, group II – 5 μM, group III – 1 μM, group IV – 0.5 μM, group V – 0.1 μM. The explants from group VI and VII were incubated between 241 and 510 min of the experiment in a medium containing 0.5% (v/v) propylene glycol (vehicle). The explants from group VI were treated with NE between 361 and 840 min of the experiment. The explants from group VII were not subjected to adrenergic stimulation.
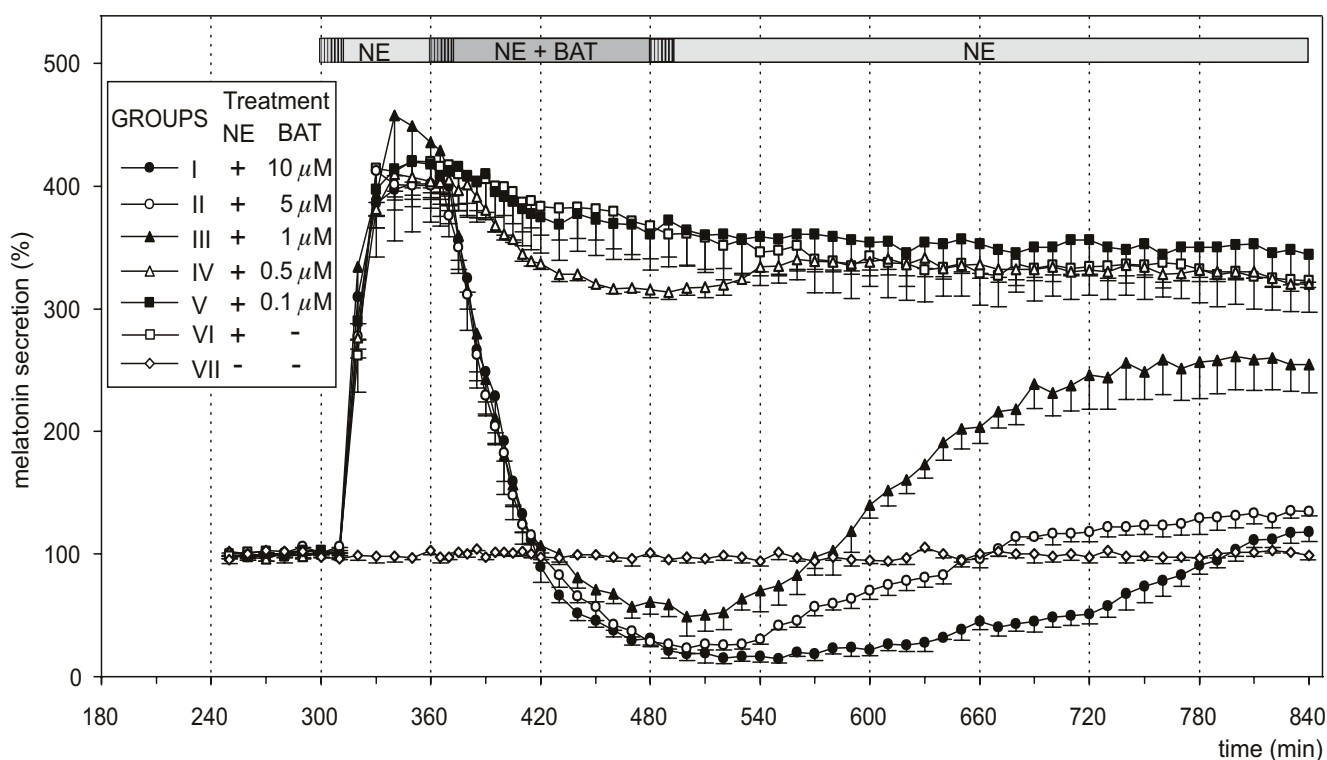
*Experiment II*
The rat pineals from groups I – VI were incubated between 241 and 840 min of the experiment in a medium containing 10 μM of NE. The explants from group VII were not subjected to adrenergic stimulation.

Between 421 and 540 min of the experiments the glands were treated with BAT as follows: group I – at a concentration of 10 μM, group II – 5 μM, group III – 1 μM, group IV – 0.5 μM, group V – 0.1 μM. The explants from groups VI and VII were exposed to 0.5% (v/v) of propylene glycol instead of BAT.

*Experiment III*
The explants of the pig pineals belonging to groups I – VI were subjected to two one–hour-long periods of adrenergic stimulation (NE at a concentration of 10 μM) between 421 and 480 min as well as between 721 and 780 min of the experiment. Two hours before and during the first adrenergic stimulation (between 301 and 480 min of the experiment) the explants were incubated in the flow of a medium containing BAT (the groups I – V) or 0.5% (v/v) of propylene glycol (the group VI). The concentrations of BAT in the

Experiment IV



**Figure 5**. Melatonin secretion (means ± SEM) from the explants of the pig pineal gland incubated between 301 and 840 min of the experiment in the perfusion of medium with 10 µM of NE (groups I – VI) or without NE (group VII). Between 361 and 480 min of the experiment the explants from groups I - V were treated with BAT at concentrations 0.1–10 µM and the explants from groups VI and VII - with vehicle. The mean melatonin secretion between 241 and 300 min of the experiment was taken as 100%. Other explanations see fig 2.

medium were as follows: group I – 10 µM, group II – 5 µM, group III – 1 µM, group IV – 0.5 µM, group V – 0.1 µM. The explants from group VII were treated only with propylene glycol (0.5% (v/v)) between 301 and 480 min of the experiment.

*Experiment IV*

Groups I – VI of the pig pineal explants were incubated between 301 and 840 min of the experiment in a medium containing NE at a concentration of 10 µM. The explants from group VII were not exposed to norepinephrine.

Between 361 and 480 min of the experiments the glands from groups I – V were incubated in a medium containing BAT: group I – at a concentration of 10 µM, group II – 5 µM, group III – 1 µM, group IV – 0.5 µM, group V – 0.1 µM. The explants from groups VI

and VII were incubated at the same time in a medium containing propylene glycol (0.5% (v/v)).

*Melatonin radioimmunoassay*

Melatonin concentration in the medium samples was measured by a direct RIA with the use of G/S/704-6483 antibody and ^{3}H-melatonin according to the previously described and validated procedure [25]. BAT at the highest concentration (10 µM; 1 nmol per assay tube) did not influence the maximum binding of the tracer. Intra- and interassay coefficients of variation were below 10%.

*Data analysis*

The melatonin secretion in each culture chamber was expressed as a percentage of the mean melatonin secretion during one hour preceding the first treatment with drugs (considered as "the basal level"). All data

presented are means (± SEM). Inter-group comparisons at each time-point were performed using one–way ANOVA and Duncan's test (SPSS for Windows, ver. 11, SPSS Inc, USA). The values of $EC_{50}$ were calculated from a sigmoidal dose-response curve using Prism for Windows, ver. 3.00, GraphPad Software, USA. The half-time of melatonin secretion inhibition was determined as duration of the period during which the hormone release reached the middle value between the initial level and the minimal level. The time-point at which the drug concentration reached 95% of the nominal concentration was taken as a starting point in calculations of the inhibition half-time.

## Results

The melatonin secretion from the explants of rat and pig pineals decreased quickly during the first hour of the incubation in medium superfusion and slowly during the subsequent 120–180 minutes of culture (data not shown). After three (the rat pineal explants) or four (the porcine pineal explants) hours the secretion reached a stable level, which did not fluctuate markedly up to the end of the experiments. Previous studies on rat and pig pineals have demonstrated that this constant level of melatonin release does not change upon a treatment with β–adrenoceptors antagonists and therefore may be considered as a non-stimulated or basal secretion [25, 45]. NE induced a significant rise in melatonin secretion from the pineal explants of both species (*Fig. 2, 3, 4, 5*). The incubation in the medium containing 0.5% (v/v) of propylene glycol did not change the non-stimulated and NE-stimulated melatonin secretion (*Fig. 2, 3, 4, 5*).

### Experiment I

The melatonin secretion from the rat pineals decreased significantly during the incubation (between 241 and 360 min of the experiment) in medium containing 0.5–10 μM of BAT (*Fig. 2*). After the two-hour-long treatment with BAT at concentrations of 10 μM, 5 μM, 1 μM, 0.5 μM, the non-stimulated secretion was inhibited by 91%, 80%, 65% and 55%, respectively. BAT at a concentration of 0.1 μM was ineffective. The value of $EC_{50}$ for BAT-inhibition of the non-stimulated melatonin secretion was 0.30 ± 0.1 μM. The half-time of the melatonin secretion decline was ca. 25 minutes.

During the incubation (between 361 and 510 min of the experiment) in the presence of both NE and BAT the melatonin release slightly increased. However, even at the end of this experimental phase it did not exceed 30%, 37%, 130% and 180% of the basal secretion level when BAT was used at concentrations 10 μM, 5 μM, 1 μM and 0.5 μM, respectively. At the same time NE induced more than an eight-fold elevation in the melatonin secretion in the groups of explants treated with vehicle or 0.1 μM of BAT. There were no differences in NE-stimulated melatonin secretion between these two groups.

After withdrawal of BAT from the medium, a subsequent increase in melatonin secretion occurred in groups I – IV. The secretion level similar to that in the explants unexposed to the inhibitor was reached only by the glands treated with 0.5 μM of BAT.

### Experiment II

During the first phase of the experiment, when the glands were exposed solely to NE, the melatonin secretion increased stepwise in all groups of the explants subjected to adrenergic stimulation (*Fig. 3*). In the group of explants incubated in the medium with NE but without BAT the maximal melatonin release, exceeding more than 10-fold the basal secretion level, was reached after five hours of the stimulation.

Introduction of BAT (the second phase of the experiment) resulted in a decline in melatonin secretion: at a concentration of 0.5 μM up to 650%, at 1 μM – up to 400%, at 5 μM – up to 40% and at 10 μM – up to 30% of the basal level. The treatment with BAT at a concentration of 0.1 μM did not change significantly the level of melatonin secretion. The $ED_{50}$ value for the inhibition of the NE-stimulated melatonin secretion was 0.60 ± 0.2 μM. The decrease in melatonin secretion was biphasic: at first rapid, then slow. The half-time of melatonin decline was ca. 25 minutes.

The melatonin secretion increased after removal of BAT from the medium (the third phase of the experiment). The melatonin elevation was very low in the group of glands previously treated with the inhibitor at a concentration of 10 μM. However, in the groups which had been exposed to BAT at concentrations 0.5 μM and 1 μM the melatonin secretion reached the level similar to that in group VI subjected solely to adrenergic stimulation.

### Experiment III

The melatonin secretion declined during the incubation of the pig pineal explants in the presence of BAT (*Fig. 4*). After 120 minutes of the treatment with the inhibitor at concentrations 0.5 μM, 1 μM, 5 μM, 10 μM the melatonin release did not exceed correspondingly: 75%, 50%, 35% and 20% of the basal level. The treatment with 0.1 μM of BAT was ineffective. The $ED_{50}$ value for BAT inhibitory effect was 0.55 ± 0.1 μM. The half-time of the decrease in melatonin secretion was ca. 35 min.

Introduction of NE to the medium between 421 and 480 min of the experiment induced an increase in melatonin secretion in all the groups exposed to BAT except the explants treated with the inhibitor at a concentration of 10 μM. The magnitude of the melatonin rise was closely dependent on the inhibitor concentration. In the case of the explants treated with BAT at concentrations 1 μM and 5 μM, the melatonin secretion did not exceed or only slightly exceeded the basal level. In the group of explants exposed to 0.5 μM of BAT, the NE–induced hormone release was lower by ca. 50% comparing to the groups treated with vehicle or 0.1 μM of BAT. The shape of the NE-induced melatonin peak was similar in all the groups: the secretion increased to

the maximum within 20 minutes, remained stable up to the end of stimulation and then decreased.

The melatonin secretion increased slowly when the treatment with BAT had been finished and the incubation was continued in the medium without drugs. In the case of the explants exposed earlier to 0.5 μM and 1 μM of BAT, the melatonin secretion reached the level noted before the inhibitory treatment.

During the second stimulation with NE the peaks of melatonin secretion were similar in the groups of the explants treated previously with BAT at concentrations from 0.1 μM to 1 μM and with the vehicle. The melatonin increase was lower by 50% in the group pretreated with 5 μM of BAT comparing to the vehicle-treated explants. A very low rise in the melatonin secretion was noted in the group of explants incubated previously with 10 μM of the inhibitor.

### Experiment IV

The melatonin secretion increased quickly in response to stimulation with NE, reaching the maximum within ca. 20 minutes (*Fig. 5*). The treatment with BAT (except for a concentration of 0.1 μM) resulted in a significant, concentration-dependent drop in the melatonin secretion, with $ED_{50} = 0.70 \pm 0.1$ μM. After two hours of incubation in the presence of 5 μM or 10 μM of BAT, the melatonin secretion did not exceed 25% of the non-stimulated level. The decline in melatonin secretion was fast during the first hour of the treatment with BAT and slow during the second hour. The half-time of melatonin secretion decrease was ca. 25 minutes. The elimination of BAT from the culture medium and the continuation of adrenergic stimulation enabled a stepwise increase in the melatonin secretion.

## Discussion

In our study, superfusion cultures of rat and pig pineal glands were employed to characterize the effect of BAT on melatonin secretion. Rat and pig pinealocytes show fundamental differences in the adrenergic regulation of melatonin synthesis, the main mechanism responsible for the diurnal changes in the pineal hormone secretion [25, 29]. In the rat pineal gland, NE released from the sympathetic nerve endings stimulates melatonin secretion via synergistic cooperation of $\beta_1$ and $\alpha_1$-adrenoceptors, in the pig pineal – solely via $\beta_1$-adrenoceptors [8, 25, 29]. In pinealocytes of both species, adenyl cyclase, cAMP and protein kinase A (PKA) are the central points of the adrenergic regulatory cascade [25, 29]. Activation of PKA in rat pinealocytes leads to phosphorylation of the transcription factor CREB, which initiates transcription of the AA-NAT gene and biosynthesis of this melatonin secretion rate-limiting enzyme [28, 29]. Simultaneously, phosphorylation of the arising AA-NAT molecules by PKA protects the enzyme against proteolysis [15]. In contrast to the rat pineal gland, increase in melatonin secretion in pig pinealocytes in response to the short–term adrenergic stimulation is thought to be independent of protein

biosynthesis [25]. The adrenergic stimulation of pig pinealocytes leads via the rise in cAMP level to a rapid increase in the affinity of AA-NAT to arylalkylamines and in this way to the elevation of melatonin secretion [25]. The mechanism of changes in porcine AA-NAT properties has not been explained, but it can be supposed that the increase in AA-NAT affinity to serotonin occurs via PKA-dependent phosphorylation and binding with 14-3-3 proteins as described for ovine AA-NAT [14, 34]. The formation of a complex between AA-NAT and 14–3-3 proteins probably restricts the movement of a free loop of the enzyme molecule in a configuration favoring serotonin binding [34].

The differences in mechanisms regulating serotonin acetylation between rat and pig pinealocytes are reflected in the species-dependent variability in shapes of NE-induced rises in melatonin secretion in vitro [25, 32, 45], which is also clearly observed in the results of the present study. In response to adrenergic stimulation the melatonin secretion from rat pineals peaked after 5 hours, whereas from pig pineals it peaked in 20 min. The rise in melatonin secretion induced by NE treatment was two and half times higher in the rat pineals than in the pig pineal explants. The dynamics and amplitude of the changes in melatonin release during the in vitro adrenergic stimulation are in close agreement with the diurnal profiles of the pineal hormone secretion in the rat and the pig [8, 26, 41].

The mechanisms controlling serotonin acetylation in the rat and pig pineals probably represent the opposite edges of a spread of strategies used by mammalian pinealocytes in the melatonin secretion regulation. There are several reasons to suspect that intermediate systems combining changes in the AA-NAT content and the AA-NAT affinity to serotonin exist in several species including the sheep [14, 34] and the human [5]. Therefore, the present study performed simultaneously on the rat and pig pineals should be considered as a highly representative for mammalian pinealocytes.

The results obtained in our investigations show that BAT inhibits, in a concentration-dependent manner, the non-stimulated and NE–stimulated melatonin secretion from rat and pig pinealocytes. At the lowest concentration (0.1 μM) BAT was ineffective in both species. In the studies with the rat pineals the non-stimulated and NE-stimulated melatonin secretion was decreased by ca. 50% when BAT was used at 0.5 and 1 μM. At the highest concentration of BAT (10 μM) the non-stimulated melatonin release was reduced to ca. 10% of the secretion level before the treatment with BAT and the NE–stimulated release – to ca. 20% of the basal secretion level. The $ED_{50}$ values for the inhibition of the basal and NE–stimulated melatonin secretion from rat pineals were $0.30 \pm 0.1$ μM and $0.60 \pm 0.2$ μM, respectively. These values are in agreement with the previously reported BAT inhibitory effect on the NE–stimulated melatonin secretion in monolayer culture of rat pinealocytes

[22]. The inhibitory effect of BAT on the melatonin secretion from pig pinealocytes was slightly weaker comparing to the rat pineals. The $ED_{50}$ values for the inhibition of the basal and NE-stimulated secretion from porcine pinealocytes were $0.55 \pm 0.1$ μM and $0.70 \pm 0.1$ μM, respectively.

The $ED_{50}$ values for the inhibitory effect of BAT on the melatonin secretion from rat and pig pinealocytes were similar to the $IC_{50}$ value ($\approx 0.5$ μM) determined in an cell-free model for the BAT-inhibition of the ovine AA-NAT acetyltransferase activity [22]. The higher values of $IC_{50}$ were reported for the BAT-inhibition of the human AA–NAT acetyltransferase activity [11]. The values of $IC_{50}$ determined for human AA-NAT using the cell-free assay with phenylethylamine, 5-methoxy-tryptamine and serotonin as substrates were $1.43 \pm 0.7$ μM, $1.1 \pm 0.2$ μM, $1.32 \pm 0.3$ μM, respectively. In the assay based on CHO cells expressing human AA-NAT, the $IC_{50}$ values were as follows: $1.85 \pm 1.1$ μM for the acetylation of phenylethylamine and $3.2 \pm 0.8$ μM for the acetylation of 5–methoxytryptamine.

Compared to inhibitors of enzymes other than AA-NAT involved in the pineal hormone synthesis, BAT is an extremely potent blocker of the melatonin secretion. Para–chlorophenylalanine (pCPA), an inhibitor of tryptophan hydroxylase, at a concentration of 0.1 mM did not change the non-stimulated melatonin secretion and only partially diminished the increase in the pineal hormone release induced by the adrenergic stimulation [rat pinealocytes – 31, pig pinealocytes – 25]. A decrease in the melatonin secretion by 50% or more occurred at 1 mM of pCPA [25, 31]. Similarly, α-methyl-DOPA, an inhibitor of aromatic amino acids decarboxylase, is effective in the melatonin secretion blockade from pig pinealocytes at a concentration as high as 1mM [25].

Negligible differences in the $ED_{50}$ values for the BAT effects on the basal and NE-simulated melatonin secretion, estimated in our experiments with both rat and pig pineal explants, suggest that the adrenergic stimulation does not influence markedly the inhibitory potency of BAT on the serotonin acetylation in mammalian pinealocytes. An opposite situation was described in the studies with CHO cells expressing the recombinant human AA–NAT [11, 12]. The treatment of these cells with forskolin decreased the $IC_{50}$ value for BAT inhibition of the phenylethylamine acetylation from $1.3 \pm 0.5$ to $0.2 \pm 0.04$ [12]. A similar tendency was also observed in the case of other tested AA-NAT inhibitors (especially those with low potency in non-stimulated cells) with the exception of only one compound (S 27481) that showed lower efficiency in forskolin–stimulated cells [12]. The discrepancy between our results and the data obtained in the studies on CHO cells expressing human AA-NAT may have their sources in the differences in the cellular systems employed (rat and pineal pinealocytes vs. non-pineal cells expressing only one melatonin-synthesizing enzyme), the substrates (natural substrate – serotonin vs. artificial one – phenylethylamine) and the measured end-points (melatonin vs. acetylphenylethylamine). Moreover, in that the formation of bisubstrate analog 1 is dependent both on the concentration of coenzyme A and the alkyltransferase activity of the AA-NAT enzyme, these parameters could also contribute to differences with other model systems.

The superfusion culture provided us with the possibility of studying the dynamics of changes in the pinealocyte secretory activity during the incubation with BAT and after withdrawal of the inhibitor. The time-course of the decline in melatonin release induced by the BAT–treatment was biphasic in both the rat and pig pineal explants. During the first hour of the incubation with BAT the decrease was rapid, during the second – slow. The half-time of BAT-induced decline in the non–stimulated and NE-stimulated melatonin secretion ranged from 25 to 35 minutes. In our model the time-course of melatonin decrease reflects the rates of the cell membrane penetration by BAT, transformation of BAT into compound 1 and inhibition of the serotonin acetylation by compound 1. It should be noted that the inhibition of the acetyltransferase active center of AA-NAT does not block the alkyltransferase reaction and does not limit the formation rate of compound 1 [12, 22].

In experiments I and III the explants were treated with BAT for two hours and then subjected to the adrenergic stimulation in the presence of the AA-NAT inhibitor to check the secretory response of pinealocytes. The increase in the melatonin release induced by NE was largely diminished in the rat and pig pineal explants incubated with $0.5 - 10$ μM of BAT. The magnitude of the NE-stimulated increase in melatonin release was dependent on the inhibitor concentration, but in the majority of cases the secretion did not exceed or only slightly exceeded the basal level occurring before the treatment with BAT. It should be noted that BAT did not change the dynamics of NE-induced changes in the melatonin secretion. The increase in the pineal hormone release was slow in the experiment with the rat pineal explants and fast in the experiment with the pig pineal explants. The melatonin secretion from the pig pineal explants treated with $0.5 - 5$ μM of BAT reached the maximum within 20 minutes of the incubation with NE, remained at increased level during the adrenergic stimulation and then rapidly decreased.

The last phases of our experiments, designed to study the recovery of melatonin release after withdrawal of the inhibitor from the medium, clearly show that BAT is a reversible inhibitor of the pineal hormone secretion. The melatonin output from the rat pineals incubated in the presence of NE increased significantly after the removal of BAT from the medium. In the experiments with pig pinealocytes, the recovery of melatonin secretion was demonstrated in the presence as well as in the absence of NE. The melatonin secretion from the pig pineal explants pretreated with $0.5 - 1$ μM of BAT reached the level similar to that before the inhibitory

treatment and recovered the ability to express fully its response to adrenergic stimulation within circa 3 hours of the incubation in a drug-free medium.

In summary, the results show that BAT is a very potent and reversible inhibitor of melatonin secretion in the mammalian pineal gland. The study opens the way for the use of BAT in in vitro investigations on the pinealocyte biochemistry and physiology requiring short-term inhibition of AA-NAT, such as research related to formation of non-acetylated tryptophan derivates. Future investigations will be necessary for the characterization of BAT toxicity and the use of this drug in other kinds of experiments including studies on laboratory animals.

## Acknowledgements

REFERENCES

1 Beaurain N, Mesangeau C, Chavatte P, Ferry G, Audinot V, Boutin JA, et al. Design, synthesis and in vitro evaluation of novel derivatives as serotonin N-acetyltransferase inhibitors. J Enzyme Inhib Med Chem 2002; **17**:409–414.

2 Becker-Andre M, Wiesenberg I, Schaeren-Wiemers N, Andre E, Missbach M, Saurat JH, et al. Pineal gland hormone melatonin binds and activates an orphan of the nuclear receptor superfamily. J Biol Chem 1994; **269**:28531–28534.

3 Benitez-King G, Anton-Tay F. Calmodulin mediates melatonin cytoskeletal effects. Experientia, 1993; **49**:635–641.

4 Bubenik GA. Gastrointestinal melatonin: localization, function, and clinical relevance. Dig Dis Sci 2002; **47**:2336–2348.

5 Coon SL, Weller JL, Korf HW, Namboodiri MAA, Rollag M, Klein DC. cAMP regulation of arylalkylamine N-acetyltransferase (AANAT, EC 2.3.1.87) – A new cell line (1E7) provides evidence of intracellular AANAT activation. J Biol Chem 2001, **276**:24097–24107.

6 Delagrange P, Atkinson J, Boutin JA, Casteilla L, Lesieur D, Misslin R, et al. Therapeutic perspectives for melatonin agonists and antagonists. J Neuroendocrinol 2003; **15**:442–448.

7 Djeridane Y, Vivien-Roels B, Simonneaux V, Miguez JM, Pevet P. Evidence for melatonin synthesis in rodent Harderian gland: a dynamic in vitro study. J Pineal Res 1998; **25**:54–64.

8 Drijfhout WJ, van der Linde AG, Kooi SE, Grol CJ, Westerink BH. Norepinephrine release in the rat pineal gland: the input from the biological clock measured by in vivo microdialysis. J Neurochem 1996; **66**:748–755.

9 Dzięgiel P, Murawska-Ciałowicz E, Jethon Z, Januszewska L Podhorska-Okołów M Surowiak P, et al. Melatonin stimulates the activity of protective antioxidative enzymes in myocardial cells of rats in the course of doxorubicin intoxication. J Pineal Res 2003; **35**:183–187.

10 Ferry G, Boutin JA. High-capacity screening of arylalkylamine N-acetyltransferase inhibitors using a high-performance liquid chromatography system. J Biomol Screen 2000; **5**:361–368

11 Ferry G, Mozo J, Ubeaud C, Berger S, Bertrand M, Try A, et al. Characterization and regulation of a CHO cell line stably expressing human serotonin N-acetyltransferase (EC 2.3.1.87). Cell Mol Life Sci 2002; **59**:1395–1405

12 Ferry G, Ubeaud C, Mozo J, Pean Ch, Hennig P, Rodriguez M, et al. New substrate analogues of human serotonin N-acetyltransferase produce in situ specific and potent inhibitors. Eur J Biochem 2004; **271**:418–428

13 von Gall C, Stehle JH, Weaver DR. Mammalian melatonin receptors: molecular biology and signal transduction. Cell Tissue Res 2002; **309**:151–162.

14 Ganguly S, Gastel J, Weller JL, Schwartz C, Jaffe H, Namboodiri MAA, et al. Role of a pineal cAMP-operated arylalkylamine N-acetyltransferase / 14-3-3-binding switch in melatonin synthesis. PNAS 2001; **98**:8083–8088.

15 Gastel JA, Roseboom PH, Rinaldi PA, Weller JL, Klein DC. Melatonin production: proteasomal proteolysis in serotonin N-acetyltransferase regulation. Science 1998; **279**:1358–1360.

16 Guerrero JM, Reiter RJ. Melatonin-immune system relationships. Curr Top Med Chem 2002; **2**:167–179.

17 Hardeland R, Poeggeler B. Non-vertebrate melatonin. J Pineal Res 2003; **34**:233–241.

18 Hickman AB, Namboodiri MA, Klein DC, Dyda F. The structural basis of ordered substrate binding by serotonin N-acetyltransferase: enzyme complex at 1.8 A resolution with a bisubstrate analog. Cell 1999; **97**:361–369.

19 Hoffmann A, Farker K, Dittgen M, Hoffmann H. A melatonin preparation with a pulsatile liberation pattern: a new form of melatonin in replacement therapy. Biol Signals Recept 1999; **8**:96–104.

20 Karasek M, Pawlikowski M. Antiproliferative effects of melatonin and CGP 52608. Biol Signals Recept 1999; **8**:75–78.

21 Khalil EM, Cole PA. A potent inhibitor of the melatonin rhythm enzyme. J Am Chem Soc 1998; **120**:6195–6196.

22 Khalil EM, De Angelis J, Ishii M, Cole PA. Mechanism-based inhibition of the melatonin rhythm enzyme: pharmacologic exploitation of active site functional plasticity. Proc Natl Acad Sci U S A 1999; **96**:12418–12423.

23 Kim CM, Cole PA. Bisubstrate ketone analogues as serotonin N-acetyltransferase inhibitors. J Med Chem 2001; **44**:2479–2485.

24 Krause DN, Geary GG, Doolen S, Duckles SP. Melatonin and cardiovascular system. In: Olcese J, editor. Melatonin after four decades. New York: Kluwer Academic / Plenum Publishers; 2000. p. 299–310.

25 Lewczuk B. Mechanisms of adrenergic regulation of melatonin secretion in the pig pineal gland – in vitro study. Dissertations and monographs, vol. 60 (in Polish), Olsztyn (Poland): UWM Press; 2002. p. 1–158

26 Lewczuk B, Przybylska-Gornowicz B. The effect of continuous darkness and illumination on the function and the morphology of the pineal gland in the domestic pig. I. The effect on plasma melatonin level. Neuroendocrinol Lett 2000; **21**:283–291.

27 Li G, Li L, Yin D. A novel observation: Melatonin's interaction with malondiadehyde. Neuroendocrinol Lett 2005; **26**:61–66

28 Maronde E, Schomerus C, Stehle JH, Korf HW. Control of CREB phosphorylation and its role for induction of melatonin synthesis in rat pinealocytes. Biol Cell 1997; **89**: 505–511.

29 Maronde E, Pfeffer M, von Gall C, Dehghani F, Schomerus C, Wicht H, et al. Signal transduction in the rodent pineal organ. In: Olcese J, editor. Melatonin after four decades. New York: Kluwer Academic / Plenum Publishers; 2000. p. 109–132.

30 Mesangeau C, Yous S, Chavatte P, Ferry G, Audinot V, Boutin JA, et al. Design, synthesis and in vitro evaluation of novel benzo-β-thiophene derivatives as serotonin N-acetyltransferase (AANAT) inhibitors. J Enzyme Inhib Med Chem 2003; **18**:119–125

31 Miguez JM, Simonneaux V, Pevet P. The role of the intracellular and extracellular serotonin in the regulation of melatonin production in rat pinealocytes. J Pineal Res 1997; **23**:63–71.

32 Naïri-Skandrani L, Djeridane Y, Charbuy H, Touitou Y Isoproterenol-stimulated melatonin production by perifused rat pineal glands: Age- and time-related effects. Neuroendocrinol Lett 2004; **25**:257–261

33 Namboodiri MA, Weller JL, Klein DC. Evidence of inactivation of pineal indoleamine N-acetyltransferase by protein thiol: disulfide exchange. J Biol Chem 1980; **255**:6032–6035.

34 Obsil T, Ghirlando R, Klein DC, Ganguly S, Dyda F. Crystal structure of the 14-3-35 zeta : serotonin N-acetyltransferase complex: A role for scaffolding in enzyme regulation. Cell 2001; **105**:257–267.

35 Paterson AM, Foldes A. Melatonin and farm animals: endogenous rhythms and exogenous applications. J Pineal Res 1994; **16**:167–177.

36 Pawlikowski M, Winczyk K, Karasek M. Oncostatic action of melatonin: facts and question marks. Neuroendocrinol Lett 2002; **23** (Suppl 1):24–29.

37 Pevet P, Bothorel B, Slotten H, Saboureau M. The chronobiotic properties of melatonin. Cell Tissue Res 2002; **309**:183–191.

38 Reiter RJ, Tan DX, Qi W, Manchester LC, Karbownik M, Calvo JR. Pharmacology and physiology of melatonin in the reduction of oxidative stress in vivo. Biol Signals Recept 2000; **9**:160–171.

39 Reiter RJ, Tan DX, Burkhardt S, Manchester LC. Melatonin in plants. Nutr Rev 2001; **59**:286–290.

40 Sugden D, Klein DC. Inactivation of rat pineal hydroxyindole-O-methyltransferase by disulfide-containing compounds. J Biol Chem 1987; **262**:6489–6493.

41 Tast A, Love RJ, Evans G, Telsfer S, Giles R, Nicholls P, et al. The pattern of melatonin secretion is rhythmic in the domestic pig and responds rapidly to changes in daylength. J Pineal Res 2001; **31**: 294–300.

42 Wetterberg L. Melatonin and clinical application. Reprod Nutr Dev 1999; **39**:367–382

43 Witt-Enderby PA, Li PK. Melatonin receptors and ligands. Vitam Horm 2000; **58**:321–354.

44 Yeleswaram K, McLaughlin LG, Knipe JO, Schabdach D. Pharmacokinetics and oral bioavailability of exogenous melatonin in preclinical animal models and clinical implications. J Pineal Res 1997; **22**:45–51.

45 Zhao Z-Y, Touitou Y. Differences in isoproterenol-stimulated melatonin production by perifused rat pineal glands: time dependent effects and role of enantiomeric forms. Life Sci 1994; **54**:497–502.

46 Zheng W, Scheibner KA, Ho AK, Cole PA. Mechanistic studies on the alkyltransferase activity of serotonin N-acetyltransferase. Chem Biol 2001; **8**:379–389.