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Parasympathetic inhibition of pineal indole metabolism by prejunctional modulation of noradrenaline release

Willem Jan Drijfhout *, Cor J. Grol, Ben H.C. Westerink

University Centre for Pharmacy, Department of Medicinal Chemistry, A. Deusinglaan 2, 9713 AW Groningen, Netherlands

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Abstract

The role of the parasympathetic nervous system in rat pineal indole metabolism was investigated by transpineal in vivo microdialysis. On-line coupling to a high performance liquid chromatography system with fluorescence detection (HPLC-FD) allowed simultaneous analysis of three major indolic compounds from the pineal, i.e. serotonin, *N*-acetylserotonin and melatonin. Infusion of the muscarinic receptor agonists, carbachol and oxotremorine, during the dark period resulted in a marked decrease of melatonin release. This effect was suggested to be mediated by a decrease in *N*-acetyltransferase activity, since a similar decrease was seen in *N*-acetylserotonin release, while serotonin levels increased simultaneously. Nicotine did show a very slight effect on the three indoles under these circumstances. Neostigmine failed to influence pineal indole metabolism, indicating that the endogenous tonus of acetylcholine release is either absent or extremely low in the middle of the dark period. The involvement of sympathetic innervation in the muscarinic effects was investigated by measurement of noradrenaline release from the pineal by sensitive off-line HPLC-FD analysis of noradrenaline in the dialysates. Carbachol markedly decreased the noradrenaline input during the infusion. Noradrenaline release returned to baseline values immediately after infusion with carbachol. These data suggest that the in vivo inhibitory effect of muscarinic receptor agonists on pineal melatonin production is mediated by presynaptic muscarinic receptors, located on the sympathetic nerve endings. This prejunctional inhibition of noradrenaline release causes a reduced induction of *N*-acetyltransferase activity, resulting in decreased melatonin release.

Keywords: Parasympathetic; Pineal; Melatonin; Microdialysis; Muscarinic receptor agonist; Carbachol; Noradrenaline release

1. Introduction

In the mammalian pineal gland, the driving force behind melatonin production is noradrenaline release from sympathetic nerves originating in the superior cervical ganglia. Stimulation of postsynaptic β_1- and α_1-adrenoceptors by noradrenaline results in an increased activity of *N*-acetyltransferase, the rate-limiting enzyme in the biosynthesis of melatonin. However, there is accumulating evidence for parasympathetic modulation of melatonin production as well (for a review, see Laitinen et al., 1995).

Evidence for cholinergic transmission can be derived partly from the presence of two important enzymes, choline acetyltransferase, involved in the synthesis of acetylcholine, and acetylcholinesterase, the key enzyme in its degradation. Choline acetyltransferase activity was identified in bovine pineal (LaBella and Shin, 1968; Phansuwan-Pujito et al., 1991a) and rat pineal gland (Rodríguez de Lores Arnaiz and Pellegrino de Iraldi, 1972). In the latter study, choline acetyltransferase activity remained unaltered after superior cervical ganglionectomy, indicating an origin of these nerves other than the ganglia. Schrier and Klein (1974) reported that apparent choline acetyltransferase activity was due to carnitine acetyltransferase. Until now, this was the only negative report in this respect and there seems to be agreement about the presence of choline acetyltransferase in the pineal gland. The presence of acetylcholinesterase in pineal nerve terminals has been reported in rabbit (Romijn, 1973), gerbil (Moller and Korf, 1983), guinea pig (Luo et al., 1990) and rat (Rodríguez de Lores Arnaiz and Pellegrino de Iraldi, 1972). An origin of parasympathetic nerves other than the ganglia was confirmed in some of these studies.

Muscarinic receptors, although generally very low in number, have been described as located in the pineal gland of several species (Taylor et al., 1980; Govitrapong et al., 1989). Data on the functionality of these receptors are not clear cut. Phansuwan-Pujito et al. (1991b) reported an inhibitory action of muscarinic receptor agonists on *N*-

* Corresponding author. Tel.: +31-50 3633313; fax: +31-50 3636908; e-mail: wjd@farm.rug.nl.

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acetyltransferase activity in bovine pineal explants. A lack of effect of pilocarpine on melatonin production in rat pineal slices has been described, whereas the same treatment increased serotonin production and release significantly (Finocchiaro et al., 1989). Regarding the second messengers, muscarinic receptor agonists do not seem to have an effect on cGMP formation. Furthermore carbachol is reported to elicit phosphoinositide hydrolysis (Laitinen et al., 1989), an effect, the sensitivity of which increases with increasing age and which seems to be associated with stimulated melatonin production (Laitinen et al., 1992).

Less information is available about possible nicotinic receptors. Their presence has been demonstrated by microscopic techniques and Western blot analysis (Reuss et al., 1992) and by autoradiographic studies using ^{125}I-α-bungarotoxin (Stankov et al., 1993). In the latter study nicotine treatment resulted in the inhibition of the noradrenaline-stimulated melatonin release from rat pineal explants.

Most studies on the role of either muscarinic or nicotinic receptors in pineal indole metabolism have been carried out in vitro, either in pineal slices or explants. In these tissue preparations innervation, for example by noradrenaline, is absent and is generally replaced by electric field stimulation or the addition of noradrenaline to the culture medium. The actual in vivo innervation is rather complex, involving a number of neurotransmitters such as acetylcholine, γ-amino-butyric acid (GABA), dopamine and various peptides such as vasoactive intestinal peptide and neuropeptide Y (for reviews, see Sugden, 1989; Ebadi, 1993). The development of microdialysis in rat pineal glands (Azekawa et al., 1990; Drijfhout et al., 1993) allows in vivo pharmacological studies in freely moving animals, kept in their normal photoperiod.

We used this technique to further investigate the role of the parasympathetic nervous system in pineal indole metabolism. Carbachol, oxotremorine, nicotine and neostigmine were infused locally and the effects on serotonin, *N*-acetylserotonin and melatonin release were recorded by high performance liquid chromatography with fluorescence detection (HPLC-FD) for analysis of the dialysates. While melatonin is the output of primary interest, the combination of serotonin and *N*-acetylserotonin provides additional information about the *N*-acetyltransferase activity, the rate limiting step in melatonin synthesis. Finally the effect of carbachol on in vivo pineal noradrenaline release was investigated, by analysing the dialysates with a very sensitive off-line HPLC-FD assay for noradrenaline with pre-column derivatization.

2. Materials and methods

2.1. Animals

Male albino rats weighing 250–300 g (Wistar, C.D.L., Groningen, Netherlands) were housed in plastic cages (60 × 40 × 20 cm). Upon arrival the light/dark cycle (12/12) was reversed, with lights on from 17:00 h until 05:00 h. To reverse the circadian rhythm of the animals, they were kept in this new lighting regimen for at least 2 weeks prior to the experiments. All experiments were carried out in the dark period, between 09:00 h and 16:00 h. During daytime, light was provided by a set of fluorescent tubes, resulting in a light intensity of 100–300 lux at cage level. During the experiments in the dark period, dim red light was present throughout the whole experiment. Temperature was maintained at 21 ± 2°C. Water and food were available continuously. The experimental protocols were approved by the Ethical Committee of the Faculty of Mathematics and Natural Sciences, State University of Groningen.

2.2. Surgery and dialysis

One day before the microdialysis experiments, the animals were operated upon in order to implant a transpineal cannula. The implantation was performed as described earlier (Drijfhout et al., 1993). A dialysis tube of saponified cellulose ester (inner diameter 0.22 mm, outer diameter 0.27 mm) bearing a tungsten wire with a sharpened point was fastened in a transverse position to a holder mounted on the right bar of a stereotaxic frame (Kopf). One hole was drilled on each side of the temporal bone (coordinates A 0.8, V 2.5) according to the atlas of Paxinos and Watson (1982). The tube was inserted transversally through the pineal gland and the tungsten wire was removed. A blunt needle was glued to the tube after which both in- and outlet of the tube were fixed on the skull in a vertical position. Surgery was performed under chloral hydrate anaesthesia (400 mg/kg i.p.). The complete surgical procedure lasted about 1.5 h. The rats were allowed to recover from surgery for at least 16 h.

During experiments, the inlet of the probe was connected with a CMA/100 microinjection pump (CMA/Microdialysis, Stockholm, Sweden). The outlet was either on-line connected to an HPLC-FD system in the case of melatonin/*N*-acetylserotonin/serotonin measurements, or connected to a CMA/142 micro-fraction collector (CMA/Microdialysis, Stockholm, Sweden), in which the microdialysates were collected in 300 μl glass vials (Chromacol, Trumbull, USA) prior to analysis. In both situations, samples were collected for a period of 20 min. All connections were made using microbore PEEK tubing (inner diameter 0.13 mm, outer diameter 0.5 mm). The use of a quartz dual channel swivel (Instech, Plymouth, PA, USA) prevented the tubing from getting tangled. Because the animals are very active during the dark period, this is a prerequisite for performing such experiments without a substantial amount of perfusion failures. Perfusion was performed using a Ringer's solution (NaCl, 147 mmol/l; KCl, 4 mmol/l; $CaCl_2$, 1.2 mmol/l; $MgCl_2$, 1.0 mmol/l) at a flow rate of 3.0 μl/min (noradrenaline, 1.8 μl/min).

Drugs were applied locally through the dialysis membrane by dissolving them in the Ringer's solution at the concentrations indicated.

After the experiments, the rats were perfused with a 4% solution of paraformaldehyde under deep chloral hydrate anaesthesia (100 mg/kg i.p.). Then the brain was dissected together with the pineal gland. The location of the probe was determined visually.

2.3. Assay and chromatography

Melatonin, *N*-acetylserotonin and serotonin were assayed with an HPLC-FD system. The basis of this system was primarily as described before (Drijfhout et al., 1993), with minor modifications to enable the detection of *N*-acetylserotonin and serotonin together with melatonin in the same run. A Waters 610 pump was used in conjunction with a Waters M470 fluorescence detector (excitation: 280 nm; emission: 345 nm). Samples were injected into the system through a Valco injection valve with fast electrical actuator (Vici AG, Valco, Switzerland) and subsequently separated on a reversed phase C_{18} column (Supelco, 250 $\times$ 4.6 mm), kept at a constant temperature of 30 ± 1°C using a column heater controlled by a Waters 600 system controller. The mobile phase consisted of a mixture of 10 mmol/l sodium acetate, adjusted to a pH of 4.5 with concentrated acetic acid, 0.01 mmol/l Na_2-EDTA, 500 mg/l heptane-sulfonic acid (Janssen Chimica, Geel, Belgium) and 200 ml/l acetonitrile. The flow rate of the HPLC was programmed, starting at 1.0 ml/min. After 2 min, the flow rate was decreased in 30 s to 0.7 ml/min. 7 min after the start of the run, the flow rate was increased gradually over a period of 1 min to 1.0 ml/min. Standard solutions were used to calibrate the system as a daily routine. Their retention time and peak areas served as a basis for peak identification and quantification. The detection limits of the assay were 5 fmol/injection (melatonin), 2 fmol/injection (*N*-acetylserotonin) and 4 fmol/injection (serotonin), all with a signal to noise ratio of 2/1. The automated control of the HPLC system, the programming of the flow rate, as well as handling and storage of the chromatograms was done with an external computer with Millennium 2010 chromatography software (Millipore, Milford, MA, USA).

For the noradrenaline assay, the method described by Kehr (1994) was used, which involves pre-column derivatization with diphenylethylenediamine (DPE). The reagents used were DPE (0.1 M in 0.1 N HCl), acetonitrile, bicine-buffer (1.75 M, pH = 7) and potassium ferricyanide (5 mM). Acetonitrile and potassium ferricyanide were combined in a ratio of 12.5/1, a mixture referred to as reagent A. DPE and buffer were mixed in a ratio of 2/1 and are referred to as reagent B. Within at least 1 h after the collection of each sample, the vial was transferred to a CMA/200 refrigerated microsampler and cooled to 8°C. Following a pre-programmed derivatization procedure, 28 μl of reagent A and 15 μl of reagent B were then added to the sample, mixed thoroughly and 60 μl of this mixture was aspirated into the loop of the valve. The temperature of this loop was increased with a loop-heater, and kept at a temperature of 80°C, by a BAS LC22-A temperature controller (BAS, West Lafayette, IN, USA). At this high temperature, the derivatization reaction took 6.5 min to complete, after which the sample was injected onto the column. The completely automated derivatization procedure with the CMA/200 refrigerated microsampler was controlled by an external computer with CMA/200-software, version 1.20 (CMA/Microdialysis, Stockholm, Sweden).

The chromatographic system of the noradrenaline assay consisted of a Pharmacia 2248 pump (Pharmacia/LKB, Sweden) in conjunction with a Waters M470 fluorescence detector (excitation: 350 nm; emission: 480 nm). Samples were separated on a reversed phase C_{18} column (Supelco, 250 $\times$ 4.6 mm), kept at a constant temperature of 30 ± 1°C using a water mantle and thermostated waterbath. The mobile phase was essentially the same as described by Kehr (1994) and consisted of 50 mmol/l sodium acetate (adjusted to pH = 7.0 with concentrated acetic acid) (62%), acetonitrile (30%) and methanol (8%). The flow was 1.0 ml/min, resulting in a pressure of about 1400 psi. Signals were recorded using a Kipp and Zonen BD41 flatbed recorder and processed manually. Peak identification and quantification were based on retention time and peak heights by comparison to standard solutions. The detection limit of the assay was 1.8 fmol/injection, the signal to noise ratio, 2/1.

2.4. Experiments

Various cholinergic agents were used to investigate the role of the parasympathetic nervous system in pineal metabolism, especially the production of serotonin, *N*-acetylserotonin and melatonin. The muscarinic receptor agonists, carbachol and oxotremorine, were both used in a concentration of 10^{-5} M and perfused for 2 h. The nicotinic agonist, nicotine, was also perfused in a concentration of 10^{-5} M for 2 h. Finally the effects of a 2 h perfusion with the acetylcholinesterase inhibitor, neostigmine, in a concentration of 10^{-5} M were studied.

In order to get information about the mechanism by which the muscarinic receptor agonists influence pineal metabolism, the effect of a 1.5 h perfusion with carbachol in a concentration of 10^{-5} M on noradrenaline release in the pineal gland was studied.

2.5. Chemicals

Potassium ferricyanide ($K_3Fe(CN)_6 \cdot 6(H_20)$) was obtained from Janssen Chimica (Janssen Chimica, Geel, Belgium). DPE was synthesized as described previously (Irving and Parkins, 1965) and purified by column chro-

matography followed by sublimation and recrystallization from ether. All other chemicals were obtained from Sigma (Sigma Chemical Co., St. Louis, MO, USA).

2.6. Data presentation and statistics

The data are expressed as the relative values compared to basal night-time levels. Therefore in each experiment, the levels from $t = -80$ to $t = 0$ min were averaged and averages were taken as 100%. Statistical analysis was performed by repeated measures analysis of variance (ANOVA), followed by a Dunnet multiple comparison test against controls ($t = -80$–0 min) using Sigmastat statistical software.

3. Results

3.1. Chromatography

Examples of typical chromatograms from dialysates as well as a standard solution are shown in Fig. 1. The standard solution showed baseline separation for all three components of interest, i.e. *N*-acetylserotonin, serotonin and melatonin. During the night all three compounds were present in substantial amounts and could easily be detected. The average (±S.E.M.) night-time values were 962 ± 163 fmol/injection (*N*-acetylserotonin, $n = 19$), 1697 ± 343 fmol/injection (serotonin, $n = 19$) and 497 ± 78 (melatonin, $n = 19$). In the light period, *N*-acetylserotonin levels dropped to below the detection limit. The serotonin levels did not show major fluctuations, whereas melatonin showed a clear decline. However, the daytime values of melatonin stayed well above the minimal detectable quantity. Because microdialysates are relatively clean, identification and quantification were very straightforward.

3.2. Effects of muscarinic receptor agonists

The muscarinic receptor agonist, carbachol, clearly suppressed *N*-acetyltransferase activity when infused at a concentration of 10^{-5} M for 2 h (Fig. 2). Both melatonin and *N*-acetylserotonin levels dropped immediately following the start of infusion. Minimal levels reached were 32 ± 9% (melatonin, $t = 60$ min) and 16 ± 7% (*N*-acetylserotonin, $t = 60$ min). The levels remained low during the period of infusion and increased slowly after the withdrawal of carbachol from the perfusion medium. Basal levels were not reached before the end of the experiment ($t = 200$ min). Serotonin increased gradually following the infusion with carbachol. The highest levels were reached at $t = 100$ min (167 ± 17%).

Fig. 3 shows the results from a 2 h infusion with 10^{-5} M oxotremorine. Qualitatively the results are identical to those of carbachol infusion. *N*-acetylserotonin and melatonin showed a marked decrease directly following the start of the infusion. Minimal levels were 32 ± 7% (melatonin, $t = 60$ min) and 26 ± 6% (*N*-acetylserotonin, $t = 80$ min). In this case also, there was a tendency of the levels to increase to the initial basal values, which were not reached before the end of the experiment ($t = 200$

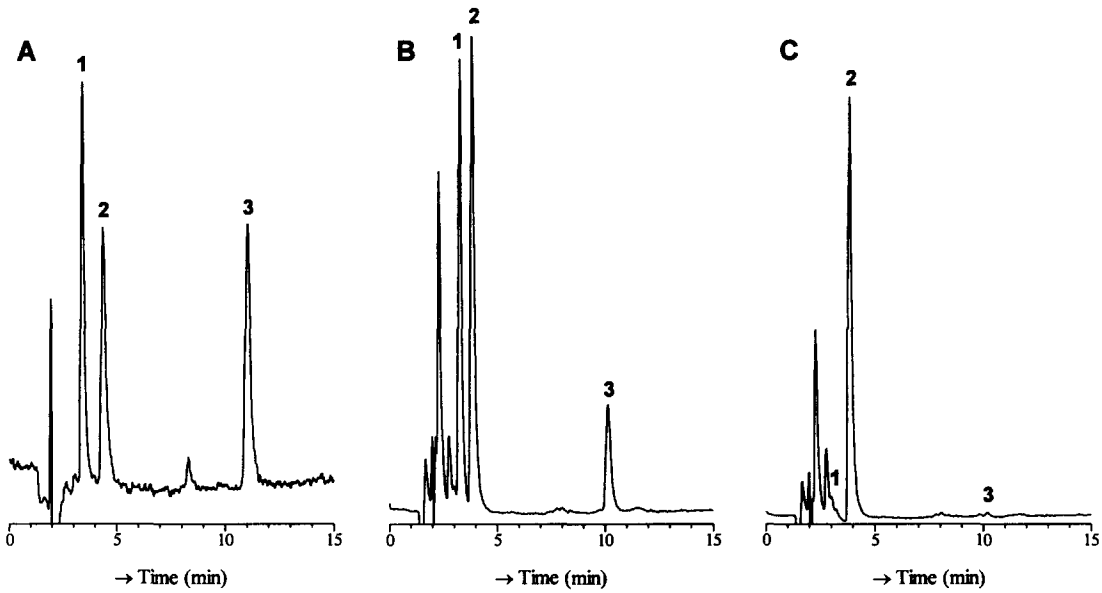

Fig. 1. Typical chromatograms from standard solution and dialysates. A: Standard solution containing 246 fmol *N*-acetylserotonin (1), 289 fmol serotonin (2) and 230 fmol melatonin (3). Retention times are 3.2 min (*N*-acetylserotonin), 3.8 min (serotonin) and 10.1 min (melatonin). B: Pineal dialysate from the dark period, representing 1142 fmol *N*-acetylserotonin (1), 2207 fmol serotonin (2) and 612 fmol melatonin (3). C: Pineal dialysate from the light period, representing 2606 fmol serotonin (2) and 24 fmol melatonin (3). Level of *N*-acetylserotonin (1) has dropped to below the detection limit.

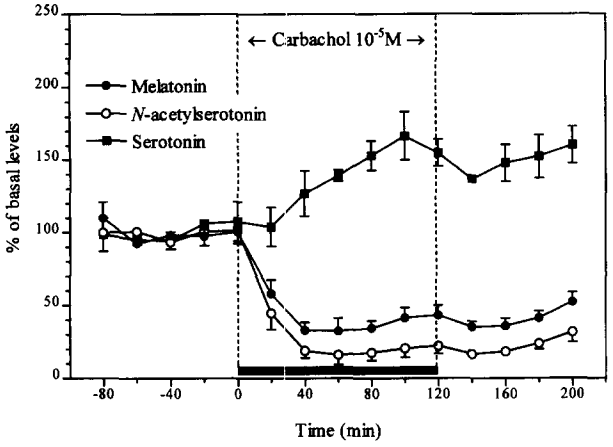

Fig. 2. The effects of infusion with carbachol at a concentration of 10^{-5} M for 2 h ($n = 5$). Plot shows the mean ± S.E.M. values for melatonin (●), *N*-acetylserotonin (○) and serotonin (■). Values are significantly different ($P < 0.05$) from baseline from $t = 20$ min (melatonin and *N*-acetylserotonin) or $t = 80$ min (serotonin). All data are expressed as related to basal night-time values. The black bar at the bottom indicates the duration of infusion. Vertical dotted lines represent start and end of infusion.

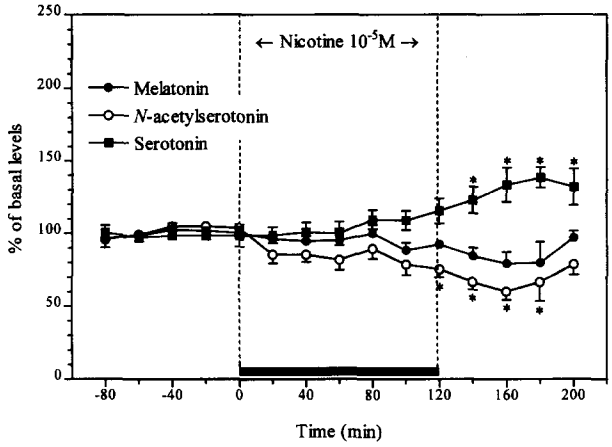

Fig. 4. The effects of infusion with nicotine at a concentration of 10^{-5} M for 2 h ($n = 5$). Plot shows the mean ± S.E.M. values for melatonin (●), *N*-acetylserotonin (○) and serotonin (■). Asterisks (*) indicate significant deviations from baseline ($P < 0.05$). All data are expressed as related to basal night-time values. The black bar at the bottom indicates the duration of infusion. Vertical dotted lines represent start and end of infusion.

min). A gradual increase was seen in serotonin output which reached its maximal value at $t = 180$ min (218 ± 8%).

3.3. Effect of nicotine

Nicotine, however, had no effect in a concentration of 10^{-5} M. During an infusion period of 2 h, no significant effect could be measured on either of the three compounds (Fig. 4). After the infusion period, melatonin, serotonin and *N*-acetylserotonin started to deviate somewhat from baseline values. This deviation was significant at some time points (serotonin: $t = 140$–200 min, *N*-acetylserotonin: $t = 120$–180 min) and indicated a tendency to a gradually decreasing melatonin production in the course of the night. This is a known effect and it appears not to be associated with the infusion of nicotine.

3.4. Cholinergic tonus

The presence of parasympathetic tonus under physiological conditions was tested with the acetylcholinesterase inhibitor, neostigmine. The results of a 2 h infusion period with neostigmine in a concentration of 10^{-5} M are given

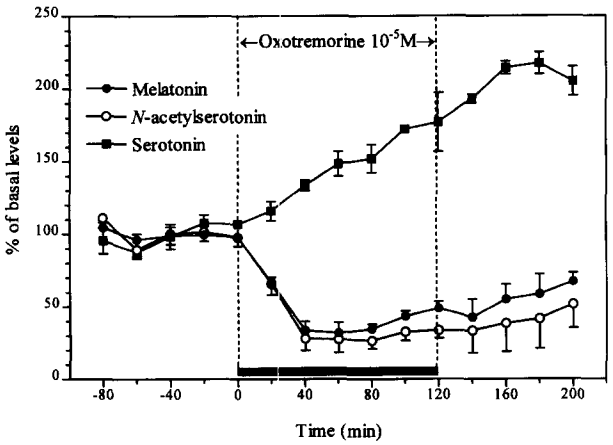

Fig. 3. The effects of infusion with oxotremorine at a concentration of 10^{-5} M for 2 h ($n = 4$). Plot shows the mean ± S.E.M. values for melatonin (●), *N*-acetylserotonin (○) and serotonin (■). Values are significantly different ($P < 0.05$) from baseline from $t = 20$ min (melatonin and *N*-acetylserotonin) or $t = 60$ min (serotonin). All data are expressed as related to basal night-time values. The black bar at the bottom indicates the duration of infusion. Vertical dotted lines represent start and end of infusion.

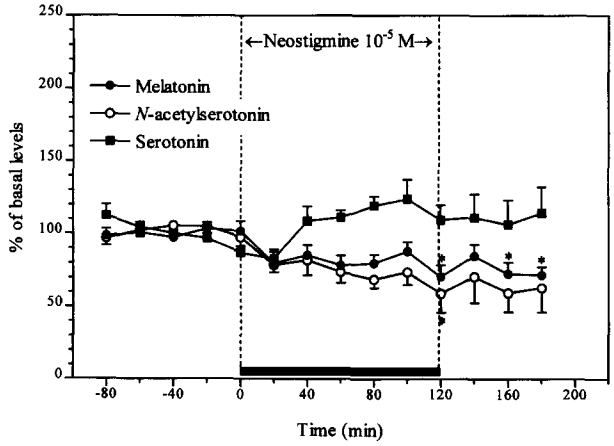

Fig. 5. The effects of infusion with neostigmine at a concentration of 10^{-5} M for 2 h ($n = 5$). Plot shows the mean ± S.E.M. values for melatonin (●), *N*-acetylserotonin (○) and serotonin (■). Asterisks (*) indicate significant deviations from baseline ($P < 0.05$). All data are expressed as related to basal night-time values. The black bar at the bottom indicates the duration of infusion. Vertical dotted lines represent start and end of infusion.

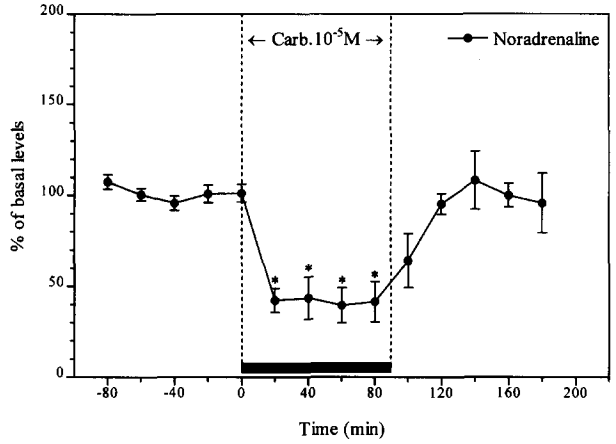

Fig. 6. The effects of infusion with carbachol on noradrenaline release from the pineal gland during night time. Carbachol was infused at a concentration of 10^{-5} M, for a period of 90 min. Asterisks (*) indicate significant deviations from baseline ($P < 0.05$). Data are plotted as the means ± S.E.M. ($n = 5$) and are expressed as related to basal nighttime values. The black bar at the bottom indicates the duration of infusion. Vertical dotted lines represent start and end of infusion.

in Fig. 5. Following the start of the infusion, melatonin and *N*-acetylserotonin levels started to decrease gradually. This decrease in melatonin was significant at $t = 120$, 160 and 180 min with the lowest level at $t = 120$ min (71 ± 8%). *N*-acetylserotonin was significantly below baseline values at $t = 120$ min (59 ± 13%). The levels of serotonin only showed a tendency to increase, an effect which did not reach significance during the experiment. Attempts to affect basal day or night time melatonin levels with the cholinergic antagonist, atropine (data not shown), have failed so far.

3.5. Role of sympathetic innervation

The effect of carbachol on the sympathetic innervation of the gland is shown in Fig. 6. Stable baseline values (10.1 ± 2.0 fmol/injection, $n = 5$) decreased markedly following perfusion with carbachol in a concentration of 10^{-5} M. This decrease was significant from $t = 20$ min to $t = 80$ min) with the lowest value measured at $t = 80$ min (40 ± 10%). When carbachol was withdrawn from the perfusion medium after 90 min, noradrenaline release immediately returned to its baseline levels, reaching 100% at $t = 120$ min.

4. Discussion

The present study provides in vivo evidence for the presence of functional acetylcholine receptors in the pineal gland. The relative potencies of specific nicotinic and muscarinic agents indicate that these acetylcholine receptors belong to the muscarinic subtype receptor family. Activating these receptors results in a fall of melatonin levels, associated with decreases in *N*-acetylserotonin and increases in serotonin, indicating regulation through *N*-acetyltransferase activity. Since sympathetic innervation in terms of noradrenaline release is also blocked by muscarinic agents, the location of these functional receptors is presumably presynaptic. Such a pre-junctional location of muscarinic receptors correlates well with that found for other peripheral tissues where parasympathetic activity modulates sympathetic nerve transmission. The extent to which this modulation exists under physiological conditions remains unclear, since only minor effects are seen in attempts to elevate endogenous levels of acetylcholine.

N-acetyltransferase activity has been widely used as a marker for pineal metabolism. The quantification of *N*-acetyltransferase activity involves in vitro or ex vivo assessment of acetyltryptamine formation from tryptamine using either [1-^{14}C]acetyl-coenzyme A and a radioimmunoassay (Deguchi and Axelrod, 1972) or the non-radioactive enzyme and an HPLC-FD assay (Fajardo et al., 1992; Thomas et al., 1990). The development of pineal microdialysis has enabled the in vivo determination of a variety of pineal indoles, including melatonin. The system described here combines both approaches, by measuring simultaneously the production of melatonin and its precursors, yielding qualitative information about the *N*-acetyltransferase activity responsible for it.

The results indicate that under the present circumstances, changes in melatonin production can be completely attributed to changes in *N*-acetyltransferase activity. Decreases in melatonin are associated with even more pronounced decreases in *N*-acetylserotonin and noradrenaline while serotonin increases are relatively slow. The very rapid decrease of *N*-acetylserotonin following carbachol and oxotremorine treatment, which even exceeds the melatonin response, indicates that *N*-acetyltransferase activity can fluctuate rapidly following pharmacological treatment. Since conventional measurements of *N*-acetyltransferase activity involve destruction of the tissue, information on the time course of changes in activity is limited. Therefore, there is a lack of supporting evidence for such fast reactivity of *N*-acetyltransferase activity to pharmacological inhibition.

The role of muscarinic receptors in the pineal gland is not clear. Their presence has been described in the pineal gland of several species (Taylor et al., 1980; Govitrapong et al., 1989). Various cholinergic agonists were reported to inhibit *N*-acetyltransferase activity in cultured bovine pineal explants (Phansuwan-Pujito et al., 1991b). However, in the bovine pineal, the role of noradrenaline in the stimulation of *N*-acetyltransferase seems very limited (Chan and Ebadi, 1980), whereas dopamine plays a rather important role. It should not be excluded that, in the bovine pineal gland, presynaptic inhibitory muscarinic receptors are involved in the regulation of dopamine release similarly to the regulation of noradrenaline release in the

present study. In rat pineal slices, pilocarpine is reported to increase 5-hydroxytryptophan and serotonin, but not *N*-acetylserotonin or melatonin (Finocchiaro et al., 1989). Gupta et al. (1991) suggested that this increase could account for the increased number of synaptic ribbons they found in cultured pineal glands after treatment with acetylcholine and carbamyl-β-methyl-choline. Based on the blockade of this effect by pirenzepine and its association with increased phosphoinositide hydrolysis they proposed that this effect was mediated by muscarinic M_1 receptors. Support for the presence of a muscarinic receptor of the M_1 subtype has been provided recently from an in situ hybridisation study (Phansuwan-Pujito et al., 1994). A lack of effect on *N*-acetyltransferase activity could be explained by the fact that rats were decapitated either in the light period, or 1 h after the lights had been turned off. At this time, melatonin production as well as *N*-acetyltransferase activity are extremely low and a decrease in either of these parameters will be hard to detect. Therefore the results of these studies do not necessarily contradict our findings. In fact, it is possible that parasympathetic innervation involves both a postsynaptic muscarinic M_1 receptor that promotes daytime serotonin production and synaptic ribbon numbers and a presynaptic muscarinic M_2 or M_3 receptor that down-regulates the nightly sympathetic signal and melatonin production.

Evidence for presynaptic location of the inhibitory muscarinic receptors is derived from the inhibition of noradrenaline release by carbachol. Especially the rapid and pronounced response of noradrenaline to perfusion of carbachol indicates an effective regulatory site in the sympathetic innervation of the pineal. A previous study in which the circadian rhythm of pineal metabolism was studied with this new concept of measuring both innervation and output also indicated a very close time relationship between noradrenaline input and melatonin output.

In the regulation of autonomic neurotransmitter release, negative feedback through autoreceptors is a common mechanism. However, presynaptic heteroceptors also are reportedly involved in the modulation of noradrenaline and acetylcholine release. Muscarinic receptor activation can result in a reduced noradrenaline release in a number of tissues, such as the heart (Manabe et al., 1991), vascular system (Remie et al., 1990; Casado et al., 1994; Ferrer et al., 1992) and airways (Pendry and Maclagan, 1991). Also, presynaptic α_2-adrenoceptors are reported to inhibit acetylcholine release (Zhang et al., 1995). This prejunctional interaction between parasympathetic and sympathetic innervation seems to be quite common in the autonomic nervous system. The present findings suggest that there is at least muscarinic receptor-modulated presynaptic inhibition of noradrenaline release in the pineal gland. In most cases, the muscarinic receptors involved are of the M_2 or M_3 subtype. Suggestions about the subtype present in the pineal gland however, would be completely speculative. Further research on this point would be necessary.

While there is accumulating evidence that muscarinic receptors play a modulatory role in pineal metabolism, the situation regarding nicotinic receptors is unclear. Their presence in the rat pineal gland has been demonstrated by Western blot analysis (Reuss et al., 1992) and autoradiographic studies using [^{3}H]cytisine and [^{125}I]α-bungarotoxin (Stankov et al., 1993). Their functional significance has only been described once (Stankov et al., 1993). Nicotine did not affect the basal outflow of melatonin in pineal explants, but inhibited the noradrenaline-stimulated outflow. The fact that these data were obtained in an in vitro situation, with artificially stimulated melatonin production, makes them difficult to compare with the present data. It is well possible that other regulatory mechanisms mask a small effect of nicotine in vivo. It seems likely, however, that nicotinic receptors only play a modest role, if any, in the regulation of melatonin production.

The presence of choline acetyltransferase and acetylcholinesterase, identification of muscarinic and nicotinic receptors, pharmacological responses to stimulation of these receptors, all provide strong evidence for a parasympathetic innervation of the pineal gland. Though most data indicate that the overall effect is inhibitory on melatonin production, its physiological importance is not yet clear. Our data for neostigmine indicate the presence of a very small cholinergic tonus, but attempts to measure acetylcholine directly have failed thus far. Specific stimulation or dissection of parasympathetic nerve fibers may give more information. However, the exact origin of these fibers is unknown. Moller and Korf (1983) and Romijn (1973) reported parasympathetic innervation that did not degenerate after superior cervical ganglionectomy, indicating that central innervation might be expected. Further research on this point has to be done.

In summary, the in vivo microdialysis system used, coupled to different assays, provides a convenient way to study the pharmacology of the pineal gland. It provides not only information about the effect of various pharmacological agents on the output of the gland, but also about the nature of and the mutual interaction between the various neurochemical and biochemical processes underlying melatonin production.

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