Parasympathetic innervation, prejunctional regulation of noradrenaline release

In peripheral glands, regulation of activity normally involves a combination of sympathetic and parasympathetic innervation. In pineal research the attention is almost entirely focused on the sympathetic one, as described in chapter 4. In the present chapter, the role of the parasympathetic nervous system in rat pineal indole metabolism was investigated.

On-line coupling of the microdialysis to an HPLC system with fluorescence detection allowed simultaneous analysis of three major indolic compounds from the pineal, i.e. serotonin, N-acetylserotonin and melatonin. Since serotonin and N-acetylserotonin are substrate and product respectively of N-acetyltransferase, the rate limiting enzyme in melatonin production, their relation gives information about the enzyme’s activity.

Infusion of the muscarinic receptor agonists, carbachol and oxotremorine, during night-time 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 analysis with fluorescence detection 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.

Data presented in this chapter are published in the following paper:
5.1 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 $\beta_1$- and $\alpha_1$-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 ref. 171).

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 and rat pineal gland. 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 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, gerbil, guinea pig and rat. 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. Data on the functionality of these receptors are not clear cut. Phansuwan Pujito et al. reported an inhibitory action of muscarinic receptor agonists on N-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. 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, an effect the sensitivity of which increases with increasing age and which seems to be associated with stimulated melatonin production.

Less information is available about possible nicotinic receptors. Their presence has been demonstrated by microscopic techniques and Western blot analysis and by autoradiographic studies using $^{[125]}I\alpha$-bungarotoxin. 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, GABA, dopamine and various peptides such as vasoactive intestinal peptide and neuropeptide Y (for reviews, see ref. 93 and 348). The development of microdialysis in rat pineal glands, as described in this thesis, allows in vivo pharmacological studies in freely moving animals, kept in their normal photoperiod.
In this chapter, studies are described in which the technique is used to further investigate the role of the parasympathic nervous system in pineal indole metabolism. Carbachol, oxotremorine, nicotine and neostigmine were infused locally and the effects on serotonin, N-acetylsertotonin and melatonin release were recorded by HPLC analysis with fluorescence detection for analysis of the dialysates. While melatonin is the output of primary interest, the combination of serotonin and N-acetylsertotonin 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 assay for noradrenaline with pre-column derivatization and fluorescence detection.

5.2 Experimental setup

Animals were treated as described on page 58 and kept under a reversed LD cycle, with lights on from 17.00 h until 05.00 h. All experiments were carried out in the dark period, between 09.00 h and 16.00 h. Animals underwent surgery as described on page 59, one or two days before the experiments.

Production and release of serotonin, N-acetylsertotonin and melatonin was measured as described on page 64 in the following experiments: perfusion with carbachol (10^{-5} M, 2 h), oxotremorine (10^{-5} M, 2 h), nicotine (10^{-5} M, 2 h) and neostigmine (10^{-5} M, 2 h). Noradrenaline release was measured in an off-line system, as described on page 67, in the following experiment: perfusion with carbachol (10^{-6} M, 2 h).
5.3 Results

- **Effect of muscarinic receptor agonists**

The muscarinic receptor agonist, carbachol, clearly suppressed N-acetyltransferase activity when perfused in a concentration of $10^{-5}$ M for 2 h (Fig. 5.1). Both melatonin and N-acetylserotonin levels dropped immediately following the start of perfusion. Minimal levels reached were $32 \pm 9\%$ (melatonin, $t = 60$ min) and $16 \pm 7\%$ (N-acetylserotonin, $t = 60$ min). The levels remained low during the period of perfusion 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 perfusion with carbachol. The highest levels were reached at $t = 100$ min ($167 \pm 17\%$).

Fig. 5.2 shows the results from a 2 h perfusion with $10^{-5}$ M oxotremorine, also a muscarinic receptor agonist. Qualitatively the results are identical to those of carbachol perfusion. N-acetylserotonin and melatonin showed a marked decrease directly following the start of the perfusion with oxotremorine. Minimal levels were $32 \pm 7\%$ (melatonin, $t = 60$ min) and $26 \pm 6\%$ (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 ($t = 200$ min). A gradual increase was seen in serotonin output, which reached its maximal value at $t = 180$ min ($218 \pm 8\%$).

![Figure 5.1](image.png)

*Figure 5.1* The effect of carbachol on melatonin (●), N-acetylserotonin (○) and serotonin (■). Carbachol was perfused in a concentration of $10^{-5}$ M. Perfusion lasted for 2 h and started at $t = 0$ min. Changes are significant ($P < 0.05$) from $t = 20$ min (melatonin and N-acetylserotonin) or $t = 80$ min (serotonin). All data are expressed as percentage of average night-time levels and presented as the mean ± S.E.M. ($n = 5$).
Nicotine, however, had no effect in a concentration of $10^{-5}$ M. During a perfusion period of 2 h, no significant effect could be measured on either of the three compounds (Fig. 5.3). After the perfusion with nicotine, 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 perfusion with nicotine.

## 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 in Fig. 5.4. Following the start of the neostigmine perfusion, 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 ($71 \pm 8\%$). N-acetylserotonin was significantly below baseline values at $t = 120$ min ($59 \pm 13\%$). 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.
Figure 5.3 The effect of nicotine on melatonin (●), N-acetylserotonin (○) and serotonin (■). Nicotine was perfused in a concentration of $10^{-5}$ M. Perfusion lasted 2 h and started at $t = 0$ min. Asterisks (*) indicate significant changes ($P < 0.05$). Data are expressed as percentage of average night-time levels and presented as the mean ± S.E.M. (n = 5).

Figure 5.4 The effect of neostigmine on melatonin (●), N-acetylserotonin (○) and serotonin (■). Neostigmine was perfused in a concentration of $10^{-5}$ M. Perfusion lasted for 2 h and started at $t = 0$ min. Asterisks (*) indicate significant changes ($P < 0.05$). Data are expressed as percentage of average night-time levels and presented as the mean ± S.E.M. (n = 5).

5. Parasympathic innervation, prejunctional regulation of noradrenaline release
Role of sympathetic innervation

The effect of the muscarinic receptor agonist carbachol on the sympathetic innervation of the gland is shown in Fig. 5.5. Stable baseline values (10.1 ± 2.0 fmol/injection) 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.

Figure 5.5 The effect of carbachol on noradrenaline release. Carbachol was perfused in a concentration of 10^{-5} M. Perfusion lasted for 1.5 h and started at t = 0 min. Asterisks (*) indicate significant changes (P < 0.05). Data are expressed as percentage of average night-time levels and presented as the mean ± S.E.M.

- Role of sympathetic innervation
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5.4 Discussion

The present study provides 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 prejunctional 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.

- New approach to N-acetyltransferase activity

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 acetyl-1\(^{14}\)C coenzyme A and a radioimmunoassay\(^3\) or the non-radioactive enzyme and an HPLC-FD assay\(^25,367\). 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 simultaneously measuring 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.

- Muscarinic receptors inhibit melatonin

The role of muscarinic receptors in the pineal gland is not clear. Their presence has been described in the pineal gland of several species.\(^107,364\) Various cholinergic agonists were reported to inhibit N-acetyltransferase activity in cultured bovine pineal explants.\(^266\) However, in the bovine pineal, the role of noradrenaline in the stimulation of N-acetyltransferase seems very limited,\(^51\) 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 has been reported to increase 5-hydroxytryptophan and serotonin, but not N-acetylserotonin or...
melatonin.\textsuperscript{98} Gupta et al.\textsuperscript{111} 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-\(\beta\)-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.\textsuperscript{252} 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.

- **Prejunctional interaction sympathetic and parasympathetic innervation**

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 heteroreceptors 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,\textsuperscript{206} vascular system,\textsuperscript{45,97,285} and airways.\textsuperscript{248} Also, presynaptic \(\alpha_2\)-adrenoceptors are reported to inhibit acetylcholine release.\textsuperscript{35} 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 is needed.

- **Nicotinic receptors, presence without function?**

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\textsuperscript{380} and autoradiographic studies using \([^{125}\text{I}]\text{α}-bungarotoxin.}\textsuperscript{338} Their functional significance has only been described once.\textsuperscript{338} 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 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.

- **Physiological importance of parasympathic innervation?**

  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 parasympathic 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. Attempts to measure acetylcholine directly have failed thus far (data not shown). Even the addition of high concentrations of neostigmine in the perfusion fluid did not result in detectable acetylcholine levels. In addition, the release of acetylcholine may be restricted to certain specific circadian time points and therefore difficult to detect. Specific stimulation or dissection of parasympathetic nerve fibers and studies at different stages of the LD cycle may provide more information. However, the exact origin of parasympathetic fibers is unknown. Moller and Korf\(^{226}\) and Romijn\(^{295}\) 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 information, not only 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.
5. Parasympathetic innervation, prejunctional regulation of noradrenaline release