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## Melatonin biosynthesis in the mammalian pineal gland

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*Summary.* Rhythmic production of melatonin by the mammalian pineal occurs in response to noradrenergic stimulation which produces a cascade of biochemical events within the pinealocyte. In the rat, massive changes in NAT activity result from an increase in intracellular c-AMP levels produced by a synergistic interaction whereby an $\alpha_1$ activation amplifies $\beta$-adrenergic stimulation. The intracellular events mediating this effect are described. A major aspect of the temporal control of melatonin production is the programmed down-regulation of responses to noradrenergic stimulation once the initial surge of c-AMP is produced. Noradrenergic activation of the gland also influences other enzymic functions, including tryptophan hydroxylase and HIOMT activities, and produces a dramatic increase in intracellular c-GMP levels. Other neurotransmitters and neuropeptides, e.g. VIP, may also influence pineal function and comparisons are made between the rat, the subject of the bulk of experimental studies, and other species.
*Key words.* Melatonin; adrenergic receptors; second messengers; serotonin N-acetyltransferase; hydroxyindole-O-methyltransferase.

In the last few years considerable evidence has accumulated which firmly implicates melatonin produced by the pineal gland as a regulator of the dramatic changes in reproductive function which occur in seasonally breeding mammals[93] (Bartness and Goldman, this issue). Other seasonal changes in physiology are probably also regulated by melatonin (Ebling and Foster, this issue). As day length changes through the seasons the day/night pattern of melatonin synthesis and secretion is subtly modified. Of the various features of the pattern of melatonin secretion it appears that the duration of the night-time elevation of melatonin is critical[13]. The mechanisms which regulate the seasonal variation in the duration of the melatonin signal are not understood. It seems possible,

however, that changes in the transmembrane and intracellular mechanisms which regulate melatonin synthesis might play an important role. Our understanding of some of the details of these mechanisms has increased in recent years. The central concept of the regulation of pineal melatonin synthesis by noradrenaline (NA) acting on a $\beta$-adrenoceptor to increase the intracellular concentration of cyclic AMP thus inducing the rate-limiting enzyme serotonin N-acetyltransferase (SNAT) is well-established[37,38,63]. However, recent data indicate that other receptor and second messenger mechanisms can play an important modulatory role. In addition some of the other enzymes involved in converting tryptophan to melatonin (tryptophan hydroxylase, hydroxyindole-O-methyltransferase) are also regulated by the sympathetic neural input to the pineal and may have an important role in regulating melatonin synthesis in some species (fig. 1).

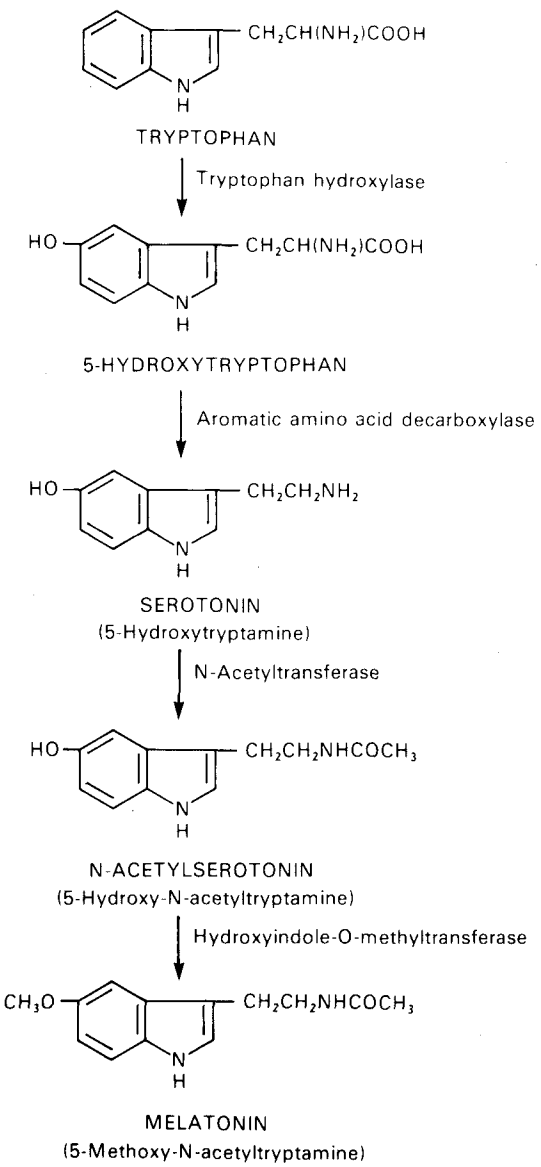


Figure 1. Biosynthesis of melatonin.

*Tryptophan*

5-HT is synthesized from tryptophan by the action of two enzymes. The first, tryptophan hydroxylase transfers a hydroxy group to the 5-position of the indole ring to give 5-hydroxytryptophan (5-HTP). The second, aromatic amino acid decarboxylase, removes the side-chain carboxyl group to give 5-HT. The synthesis of 5-HT is dependent upon the uptake of the essential amino acid tryptophan from the circulation. The concentration of tryptophan in the rat pineal is high[51,105], but, unlike brain tryptophan, it is not correlated with the diurnal rhythm in serum tryptophan which is generated by the diurnal rhythm in feeding[77]. Thus the pineal gland, in contrast to the brain, appears to be isolated from the diurnal changes in the availability of tryptophan in serum. The factors which influence the uptake of tryptophan into pinealocytes have not been investigated. Tryptophan enters the brain through a neutral amino acid transport system; presumably the same or an analogous system carries tryptophan into pinealocytes. The concentration of tryptophan in the pineal available for hydroxylation by tryptophan hydroxylase may be important as the enzyme in vivo does not appear to be saturated with respect to this substrate, at least in the rat. In this species tryptophan loading produces a large increase in pineal 5-HT[21,105]. In contrast, tryptophan loading in the sheep was not an effective means of elevating pineal 5-HT or melatonin synthesis[88], suggesting that in the sheep the hydroxylase is effectively saturated with respect to tryptophan. As the pineal tryptophan concentration and tryptophan hydroxylase activity in the two species are comparable this suggests that the sheep enzyme has a lower $K_m$ for tryptophan than the rat enzyme.

*Tryptophan hydroxylase*

Pineal tryptophan hydroxylase activity is particularly high. Indeed, despite the small size of the gland, the pineal has been used as the source of tissue in attempts to purify and characterize the enzyme[60]. Bovine pineal tryptophan hydroxylase is reported to be a small (30 kDa) protein able to catalyse the hydroxylation of phenylalanine as well as tryptophan. Catalytic activity is sensitive to the oxidation state of sulphydryl groups on the enzyme[31]. Recently a cDNA clone encoding rat tryptophan hydroxylase has been isolated from a rat pineal cDNA expression library[18]. Analysis of the sequence of this clone has revealed extensive homology with phenylalanine hydroxylase and tyrosine hydroxylase suggesting that these enzymes, which share many common characteristics, originate from a common ancestor.

Early reports failed to detect a diurnal rhythm in rat pineal tryptophan hydroxylase activity[19], but in recent years a nocturnal elevation in enzyme activity has been reported by several independent groups[71,73]. Night-time

activity is approximately 2-fold higher than daytime activity. Stimulation of tryptophan hydroxylase activity in vitro by noradrenaline (NA) in rat pineal glands has also been demonstrated[70,74]. Several studies suggest that, unlike the brain enzyme, pineal tryptophan hydroxylase turns over rapidly[20,71,73,79]. The receptor and second messenger mechanisms which regulate the nocturnal increase in enzyme activity have not yet been characterized. However, the availability of a sensitive HPLC assay for the enzyme[79], which allows activity to be measured in pinealocytes in suspension culture, and synthetic oligonucleotide probes able to detect tryptophan hydroxylase mRNA will encourage further studies of the regulation of this enzyme.

In some species the nocturnal elevation in pineal tryptophan hydroxylase activity may be of physiological importance in generating the melatonin signal. In the rat the increase in activity has been suggested to be simply a mechanism for compensating for the marked depletion of 5-HT which occurs in the pineal gland at night as a result of the conversion of 5-HT to N-acetylserotonin, and the release of 5-HT into the extracellular space (see below). However, as not all species show a large nighttime increase in SNAT activity[8] the possibility that a nocturnal increase in tryptophan hydroxylase activity may contribute to the production of a circadian rhythm in pineal melatonin synthesis must be considered. Indeed, in one such species (sheep) the proposal that 5-HT availability may limit the synthesis of melatonin during day-time has received some experimental support[89].

*Serotonin (5-HT)*

The concentration of 5-HT in the pineal gland is very high – higher than any other body tissue[67]. The prevailing view is that pineal 5-HT serves simply as a precursor of melatonin. The possibility that 5-HT itself might be considered a pineal hormone gained some support from a study describing a pineal-dependent circadian rhythm in cerebrospinal fluid 5-HT[27]. However, more recent work has identified the pineal-dependent CSF indole as N-acetylserotonin rather than 5-HT[95]. Nevertheless, it seems likely that a proportion of pinealocyte 5-HT is contained within the vesicles found in the pineal of several mammals[34]. Whether this pool of 5-HT can be made available for acetylation by SNAT at night is not clear. Recently it was shown that stimulation of pineal glands in vitro with NA causes the release of [$^3$H-]5-HT into the incubation medium[1,2]. Efflux triggered by NA also occurred in denervated glands indicating that release of 5-HT from pre-synaptic adrenergic nerve terminals – in which 5-HT and NA can be co-localized – was not involved and that the 5-HT release observed was from pinealocytes per se. The release of 5-HT into the media was related to the dose of NA used, and was stimulated much more readily by l-NA than d-NA. 5-HT release induced by NA was inhibited by an $\alpha_1$-adrenergic antagonist but not by an $\alpha_2$- or $\beta$-adrenergic blocker suggesting a role for pinealocyte $\alpha_1$-adrenoceptors. These interesting results challenge the widely-held view that pineal 5-HT exists simply as a substrate for SNAT. It is interesting to speculate that 5-HT, released into the perivascular space, may act on 5-HT receptors on other pinealocytes and modulate adrenergic responses. As 5-HT release is mediated by an $\alpha_1$-adrenergic action of NA, 5-HT may then be involved in vivo in the potentiating effect of $\alpha_1$-activation on $\beta$-adrenergic stimulation of cyclic AMP, SNAT and melatonin[92]. Taking these speculations a step further, the uptake of 5-HT released by pinealocytes into adrenergic terminals[25] may then be viewed as a mechanism for curtailing the action of 5-HT. Radioligand binding studies have suggested the presence of a serotonin binding site in the bovine pineal gland[22] but much more detailed studies are required to establish whether this site represents a true receptor, and if so, which of the several subtypes of 5-HT receptor it might be. As yet no receptor-mediated effect of 5-HT in pinealocytes has been described.

*Serotonin N-acetyltransferase (SNAT)*

The synthesis of N-acetylserotonin from 5-HT is catalysed by serotonin N-acetyltransferase (SNAT), an enzyme found in the pineal and, to a lesser extent, the retina. This enzyme has resisted efforts at purification largely because the activity is particularly unstable, the tissue is small and SNAT appears to be a relatively minor protein. However, recent work has made notable progress towards purifying and characterizing this enzyme. The pineal N-acetyltransferase activity which acetylates 5-HT is distinct in several respects from other N-acetyltransferase activities found in the liver, blood and also in the pineal itself[101]. The specific enzyme involved in melatonin synthesis is an *arylalkyl*amine N-acetyltransferase (E.C.2.3.1.87) and preferentially acetylates indoleamines (such as tryptamine, 5-HT and 5-methoxytryptamine) rather than phenylethylamines. Activity can be stimulated up to 100-fold in the rat pineal by adrenergic agonists such as NA. Pineal *aryl*amine N-acetyltransferase activity does not acetylate indoleamines, is not activated by adrenergic agonists and may be similar to the N-acetyltransferase found in the liver which metabolises potentially toxic arylamines. The two enzymes can be readily resolved by HPLC using an ion-exchange column, and are differentially labile.

Recent attempts to purify sheep and rat SNAT have utilized the observation that the enzyme is reversibly inactivated by disulphide-containing compounds[58,59]. The enzyme can be bound, then selectively eluted in an active form from a Sepharose-cystamine column. A second, anion-exchange step then further enriches SNAT. It appears that SNAT of both sheep and rat can exist in three molecular forms, depending on the ionic environment, of mol. wt 10 kDa, 30 kDa and 100 kDa. It seems

likely that the low molecular weight form represents SNAT and that the higher forms may be polymers or complexes with other proteins. Further studies should allow the kinetic characteristics of the purified enzyme to be determined. The recent development of these purification methods should allow sufficient SNAT to be purified to permit the production of specific SNAT antibodies and the determination of at least part of the SNAT amino acid sequence: two major goals which despite considerable effort have remained elusive.

### *β-Adrenergic regulation of SNAT*

Our knowledge of the intracellular mechanisms involved in the regulation of SNAT has come almost entirely from studies done on the rat pineal. The pineal of the rat can be cultured easily and after two days in culture when the presynaptic terminals to the gland have degenerated, can be considered to consist entirely of post-synaptic elements. A method for preparing isolated single pinealocytes from the rat[9] has also been valuable in recent years. During daytime in vivo, or in unstimulated glands or pinealocytes in culture, SNAT activity is very low. Activity is increased markedly by NA released into the pineal perivascular space at night from the sympathetic nerve endings which terminate in the gland. The neural pathway passing from the retina to the suprachiasmatic nuclei and to the gland has been described previously and will not be reviewed here[53]. NA acts on the pinealocyte membrane to stimulate β-adrenoceptors. Radioligand binding studies have identified β-adrenoceptors on rat pinealocytes[3] and in sheep and hamster pineal membranes[17,24]. β-Adrenoceptor stimulation activates the enzyme adenylate cyclase via a stimulatory guanine nucleotide binding regulatory protein, Gs, resulting in the synthesis of cyclic AMP. The nocturnal adrenergic stimulation of SNAT is dependent on this increase in cyclic AMP. In rat pinealocytes, cyclic AMP increases rapidly following NA stimulation to reach maximum levels (60-fold control) by 10 min, followed by a gradual decline toward control levels. It is believed that the increase in cyclic AMP mediates the induction of SNAT by activating a cyclic AMP-dependent protein kinase. Subsequent events leading to an increase in SNAT activity are not well-characterized. However, cyclic AMP initiates the transcription of a mRNA required for the increase in SNAT. The simplest hypothesis is that this new mRNA codes for new SNAT molecules, although that is not proven and it could just as well be that the mRNA is required to produce an SNAT activator protein. Cyclic AMP probably also serves to keep SNAT activity high[40] since addition of a β-adrenergic antagonist to cultured glands incubated for several hours with a β-adrenergic agonist to elevate SNAT, results in a rapid decline in SNAT activity. Interestingly, the concentration of pineal cyclic AMP at this time (4–6 h after addition of NA), although higher than in unstimulated glands, is much less than the peak in cyclic AMP observed 10 min after agonist treatment. Perhaps the rapid burst of cyclic AMP formation immediately following NA addition is necessary to trigger the transcription of mRNA, yet much lower concentrations of cyclic AMP are adequate to keep SNAT active.

### *$\alpha_1$-Adrenergic mechanisms*

Recent evidence shows that the changes in pinealocyte cyclic AMP following NA are mediated not only by a β-adrenergic mechanism but also by an $\alpha_1$-adrenergic mechanism. The first indication that α-adrenoceptors were present in the pineal came from studies showing that NA initiated an increase in phosphatidylinositol (PI) turnover as is the case in other tissues[75]. Subsequent ligand binding studies identified and characterized these receptors in the rat[84] and subsequently in the sheep pineal gland[89]. The receptors have the pharmacological characteristics typical of the $\alpha_1$-subtype, have a high affinity for NA, are located on pinealocytes rather than sympathetic nerve endings and are present at a density comparable to the pinealocyte β-adrenoceptor. Activation of rat pinealocyte $\alpha_1$-adrenoceptors does not by itself increase cyclic AMP, induce SNAT or increase melatonin synthesis. However, β-adrenergic stimulation of cyclic AMP and SNAT is markedly potentiated by simultaneous activation of the $\alpha_1$-adrenoceptor[41,98]. The potentiation is evident both in vivo and in vitro, and presumably reflects the physiological situation as NA is a mixed $\alpha_1$- and β-adrenergic agonist which can bind to and activate both $\alpha_1$- and β-adrenoceptors in pinealocytes.

The mechanism underlying this remarkable amplification response appears to involve protein kinase C (PKC), a $Ca^{2+}$-activated, phospholipid-dependent protein kinase. This is suggested by two pieces of evidence; first, low concentrations of various phorbol esters which are known to activate PKC directly, can mimic the action of $\alpha_1$-adrenergic agonists. Alone they do not increase cyclic AMP or SNAT, but when added together with a β-adrenergic agonist such as isoprenaline, a marked (10-fold) amplification of the cyclic AMP[92] and SNAT[108] response is seen. Second, in pinealocytes both the phorbol esters and $\alpha_1$-adrenergic agonists induce a rapid translocation or redistribution of PKC activity from the cytosol to the plasma membrane[30,92]. PKC is thought to be activated in the intact cell only when it is bound to the cell membrane. In addition, $\alpha_1$-adrenergic agonists have been shown to increase intracellular free $[Ca^{2+}]$ in pinealocytes probably by opening a ligand-dependent channel[90]. The increase in intracellular $[Ca^{2+}]$ is entirely dependent on extracellular $Ca^{2+}$ and does not appear to involve a voltage-dependent channel as it is not inhibited by nifedipine. As mentioned above, NA triggers PI hydrolysis by activating pinealocyte $\alpha_1$-adrenoceptors[75]. Interestingly, studies on pineal gland explants and isolated pinealocytes agree that the major product of PI hy-

drolysis is inositol monophosphate with only very small amounts of inositol bisphosphate and essentially no increase in inositol trisphosphate [29, 109]. The significance of this finding is that it seems unlikely that inositol trisphosphate has a role in pinealocytes in releasing $Ca^{2+}$ from intracellular stores as it does in some cells. Perhaps PI hydrolysis in pinealocytes serves to produce diacylglycerol, the presumed endogenous activator of PKC, which is a product of the cleavage of all of the phosphatidylinositols. Synthetic diacylglycerols also mimick $\alpha_1$-potentiation of $\beta$-adrenergic stimulation of cyclic AMP production. These experiments suggest that stimulation of pinealocyte $\alpha_1$-adrenoceptors triggers an increase in intracellular [$Ca^{2+}$] and the hydrolysis of PI generating diacylglycerol, leading to the translocation and activation of PKC. Presumably PKC activation results in the rapid phosphorylation of some component of the system mediating $\beta$-adrenoceptor stimulation of cyclic AMP. Recent evidence has ruled out an effect of PKC on cyclic AMP efflux from the cell and a direct effect on $\beta$-adrenoceptor sensitivity [87]. In addition, the potentiation of $\beta$-adrenergic stimulation by $\alpha_1$-agonists or phorbol esters is still observed in pinealocytes pretreated with phosphodiesterase inhibitors [87] suggesting that an inhibition of cyclic AMP metabolism by cyclic AMP phosphodiesterase is not part of the mechanism of potentiation. It seems likely that PKC activation amplifies $\beta$-adrenergic stimulation of cyclic AMP by phosphorylating Gs or adenylate cyclase itself.

Interestingly, since these observations of an interaction between the cyclic AMP and the $Ca^{2+}$/PI signalling systems were made in pinealocytes, several examples of a similar interaction have been reported in other cells [56, 66, 68]. In some cases it has been possible to demonstrate that PKC activation enhances not only intracellular cyclic AMP accumulation but also adenylate cyclase activity in cell membranes [7, 62]. In others evidence for a role of the G-proteins has been presented [36, 66].

*Negative feedback mechanisms*

In addition to amplifying the $\beta$-adrenergic stimulation of cyclic AMP, PKC also has a negative feedback effect and inhibits further $\alpha_1$-adrenergic stimulation [80], probably by virtue of its ability to phosphorylate and desensitize the $\alpha_1$-adrenoceptor [44]. In physiological terms, this implies that on initial exposure of pinealocytes to NA both $\alpha_1$- and $\beta$-adrenoceptors are activated leading to a massive, exaggerated synthesis of cyclic AMP because of $\alpha_1$-adrenoceptor activation of PKC which amplifies $\beta$-adrenergic adenylate cyclase activation. Almost immediately, however, the activated PKC begins to desensitize the $\alpha_1$-adrenoceptor thus limiting the elevation in intracellular cyclic AMP. Thus both the magnitude and time-course of the cyclic AMP signal generated in response to NA are precisely regulated. These early, large changes in cyclic AMP may determine the extent of the induction of SNAT which is to follow several hours later. By the time newly-induced, active SNAT begins to appear in the pinealocyte the adrenergic cyclic AMP response is relatively small and may consist simply of a $\beta$-adrenergic signal. There are at least two other mechanisms which serve to exaggerate the initial cyclic AMP response and/or reduce the later response. First, there is a diurnal rhythm in the density of pinealocyte $\beta$-adrenoceptors such that at the beginning of the dark period, after adrenergic stimulation has been interrupted for several hours, the density of $\beta$-adrenoceptors is at its greatest [65]. Adrenergic stimulation, i.e., 12 h of darkness or treatment with a $\beta$-adrenergic agonist, reduces the density of $\beta$-adrenoceptors. It is also likely that $\beta$-adrenoceptor stimulation reduces the affinity of the receptor (i.e., desensitizes the receptor) in intact pinealocytes by activating a $\beta$-adrenergic receptor kinase which phosphorylates the occupied $\beta$-adrenoceptor [45]. Second, the increase in cyclic AMP caused by NA induces an increase in cyclic AMP phosphodiesterase activity after a lag-period of several hours which then enhances metabolism of cyclic AMP [52]. Since this increase in phosphodiesterase activity occurs in response to the increase in cyclic AMP it is not surprising to find that it is regulated by a dual ($\alpha_1$- and $\beta$-) receptor mechanism [97].

*Cyclic GMP*

It has been known for some years that in addition to elevating pineal cyclic AMP, NA also produces a large increase in cyclic GMP [61]. Cyclic GMP does not appear to have a role in the regulation of SNAT induction, and its role in the pineal is not known. Elevation of cyclic GMP in the pineal was originally thought to occur in the presynaptic nerve endings and to be mediated exclusively by an $\alpha_1$-adrenoceptor [61]. This is not correct. Subsequent studies have shown that this action of NA occurs on pinealocytes themselves and not presynaptically [39] and is due to a marked $\alpha_1$-adrenergic potentiation of a small $\beta$-adrenergic stimulation of cyclic GMP [98, 99]. As is the case for cyclic AMP, NA elevates cyclic GMP by activating both $\beta$- and $\alpha_1$-adrenoceptors; $\beta$-adrenergic stimulation is a prerequisite and gives a 2–4-fold increase in cyclic GMP; $\alpha_1$-adrenoceptor stimulation enhances $\beta$-adrenoceptor stimulation 100-fold but does not elevate cyclic GMP alone. The small $\beta$-adrenergic stimulation of cyclic GMP seems to involve a guanine nucleotide binding protein, presumably Gs or a closely related protein, as cholera toxin which irreversibly activates the Gs protein, stimulates cyclic GMP [85]. This G-protein may be coupled to guanylate cyclase in an analogous manner to adenylate cyclase, or, may regulate cyclic GMP phosphodiesterase as is the case with G-protein, transducin, in the retina. Denervation of the pineal by superior cervical ganglionectomy or exposure of rats to constant light leads to a gradual decline of the NA-induced cyclic GMP response over several days [39]. The mechanism underlying

this 'desensitization' has not been identified but involves a loss of the large $\alpha_1$-adrenergic component of the response. In contrast, denervation renders cyclic AMP stimulation by NA supersensitive largely by virtue of an enhanced $\beta$-adrenergic component of the response[99].

*Hydroxyindole-O-methyltransferase (HIOMT)*

N-acetylserotonin is converted to melatonin by hydroxyindole-O-methyltransferase (HIOMT), a cytosolic enzyme which, like SNAT, has a very limited tissue distribution being found only in the pineal gland, and in a much lower concentration in the retina[4,5]. HIOMT activity in the Harderian gland[12] appears to represent a different enzyme unrelated to pineal HIOMT. Early measurements of rat pineal HIOMT reported a diurnal rhythm in activity; subsequent measurements using saturating concentrations of substrates in several laboratories have failed to observe a rhythm[78] although clear variations have been reported in lower vertebrates (Underwood, this issue). Some laboratories have attempted to measure HIOMT activity in the absence of added substrates claiming this to be a more realistic measurement of activity in vivo[6]. As the activity observed in such an assay depends not only on the amount of enzyme but also on the concentration of endogenous substrate(s) present, it is not an accurate reflection of HIOMT activity. HIOMT can methylate not only N-acetylserotonin but also 5-hydroxyindoleacetic acid, 5-HT and 5-hydroxytryptophol. However, the affinity of these latter indoles for the enzyme is 10–20-fold less than N-acetylserotonin[5] suggesting that it is the preferred substrate in vivo, certainly at night when the concentration of N-acetylserotonin rises markedly because of the increased activity of SNAT.

HIOMT is one of a family of methyltransferase enzymes which transfer a methyl group from S-adenosylmethionine to acceptor molecules. The enzyme has been purified from bovine, chicken and rat pineal glands[5,43,57,91]. In its native state it has a molecular weight of 76–78 kDa, composed of two identical subunits of 38 kDa. Amino acid analysis of rat, chicken and bovine pineal HIOMT has revealed a broad similarity in amino acid composition, although rat HIOMT appears to be richer in aspartic acid and hydrophobic amino acids[33,43,91]. A cDNA clone of bovine HIOMT has recently been isolated from a bovine pineal cDNA library and the full nucleotide sequence coding for the enzyme has been determined[32]. The activity of HIOMT, like that of tryptophan hydroxylase and SNAT, can be altered by disulphide-containing compounds, probably by the formation of mixed protein thiol: disulphides[86]. Marked differences between rat, sheep and bovine HIOMT are found in the susceptibility of crude enzyme preparations to inactivation by disulphide-containing compounds suggesting specific differences between the species in the amino acid composition of HIOMT or perhaps in the conformation of the enzyme[86]. Also polyclonal antisera raised against bovine or chicken HIOMT recognise HIOMT from other species poorly or not at all[42,57] suggesting distinct species-specific epitopes, perhaps again related to the folding of the protein.

*Adrenergic regulation of HIOMT*

Although a distinct diurnal rhythm in HIOMT activity cannot be detected, the enzyme is regulated by the sympathetic adrenergic neural system. Changes in HIOMT, unlike those in SNAT and tryptophan hydroxylase, occur gradually over a period of days or weeks. Interruption of the daily stimulation of the gland by exposing rats to constant light or by removing the superior cervical ganglia reduces pineal HIOMT activity by 70% after 3 weeks[81,82]. Daily administration of adrenergic agonists such as isoprenaline or NA can prevent or reverse this decline in activity[82]. The changes in activity probably reflect changes in the number of HIOMT molecules rather than activation/inactivation mechanisms[104]. Daily adrenergic stimulation of the pineal by NA released from the sympathetic nerve endings at night serves to maintain HIOMT at a high level. In vivo studies suggest that a $\beta$-adrenergic mechanism is involved[83]. It seems likely that changes in the synthesis and/or degradation of the enzyme are involved but the intracellular mechanisms regulating these changes in HIOMT activity are not known. Conceivably, cyclic AMP regulated by $\alpha_1$- and $\beta$-adrenoceptors may regulate HIOMT as well as SNAT.

Two-dimensional gel electrophoresis of pineal proteins synthesized de novo after NA treatment has revealed that several proteins show a striking increase in the incorporation of radioactive amino acid[102]. Of these, the specific labelling of one protein (adrenergically induced protein, AIP37/6) of 37 kDa and pI 6 increased 10–20-fold, without any apparent change in the total steady-state level of the protein. The amount of AIP37/6 protein does fall slowly after interruption of the daily adrenergic stimulation of the pineal by superior cervical ganglionectomy. The incorporation of [$^{35}$S]-methionine into AIP37/6 is increased by $\beta$-adrenergic stimulation but not by $\alpha_1$-adrenergic agonists, and by agents such as forskolin and cholera toxin which elevate cyclic AMP. AIP37/6 labelling does not require transcription suggesting that NA stimulates the translation of a pool of mRNA which is already available. AIP37/6 and HIOMT have a very similar molecular weight and pI and respond to changes in adrenergic input in a very similar manner. AIP37/6 and HIOMT may in fact be the same protein although proof of this awaits the development of an antibody able to recognize rat HIOMT.

The description of antibodies able to recognize specifically HIOMT and the identification of the cDNA sequence for HIOMT from which specific oligonucleotide probes capable of recognizing HIOMT mRNA can be synthesized are major steps forward[32]. These tools will stimu-

late the study of not only the mechanisms which regulate the adrenergic expression of this enzyme but also the mechanisms which restrict its expression to the pineal gland.

*Vasoactive intestinal polypeptide*

In addition to NA, it has been shown that vasoactive intestinal polypeptide (VIP) can induce SNAT activity in the rat pineal[106]. VIP is present in nerve endings in the pineal gland[96] and VIP receptors have been identified and characterized on pinealocytes[35]. VIP induces a rapid increase in pinealocyte cyclic AMP, which is enhanced by $\alpha_1$-adrenoceptor activation, apparently by the same PKC mechanism which enhances $\beta$-adrenergic stimulation of cyclic AMP[15,16]. VIP stimulation of SNAT activity is also enhanced by $\alpha_1$-adrenoceptor activation[107]. The pinealocyte $\beta$-adrenergic receptor and the VIP receptor are both coupled to a membrane adenylate cyclase presumably through the same stimulatory guanine nucleotide binding protein (Gs). According to one report, the VIP innervation to the pineal does not originate from the superior cervical ganglia but rather from the pterygopalatine ganglion[72]. This raises the possibility that the synthesis of melatonin may be under the control of two neural signals which may interact at the level of the second messenger (cyclic AMP). However, the nature of the stimuli which cause the release of VIP from the nerve endings in the pineal and whether VIP participates in the physiological regulation of melatonin synthesis are not known.

*Role of other transmitters*

Conceivably other neurotransmitters, peptides or hormones may modulate SNAT induction and melatonin synthesis by influencing $\beta$-adrenergic (or VIP-mediated) stimulation of cyclic AMP as $\alpha_1$-adrenoceptor stimulation does. A variety of neurotransmitter candidates and/or their receptors have been reputedly found in the pineal gland. At present there is no convincing evidence that any of these play a role in regulating melatonin synthesis or release. However, in other tissues a variety of transmitters are known to activate PI turnover and/or increase intracellular [$Ca^{2+}$]; the influence of such agents on pineal SNAT or melatonin production may well have been overlooked if, like NA acting at the $\alpha_1$-adrenoceptor, such transmitters merely modulate the response obtained following activation of pinealocytes by the primary transmitter (NA acting through the $\beta$-adrenoceptor). As has been mentioned already, 5-HT released from pinealocytes could play such a modulatory role.

Neuropeptide Y-containing fibres originating from the superior cervical ganglia have been reported in the pineal gland of the gerbil, a species whose pineal is rich in neural elements[72]. Injection of NPY into the rat was found to increase daytime SNAT activity[64] but no in vitro studies of the effect of NPY on cyclic AMP or SNAT have been reported. NPY may be co-localized with NA in the sympathetic nerve endings of the pineal as it is in several sites within the central nervous system[26].

A role for acetylcholine in the pineal is suggested by the finding that a low density of muscarinic cholinergic binding sites are present in the pineal gland of the rat, localized to postsynaptic sites[94]. However, the presence of choline acetyltransferase, the enzyme which synthesizes acetylcholine, is disputed[69], and no effects of cholinergic agonists on the pineal have been reported.

Receptors for GABA have been detected in the bovine pineal gland, where they reportedly mediate an inhibition of noradrenergic stimulation of SNAT[14]. However another study using rat pineal gland explants concluded that GABA does not stimulate or modulate adrenergic regulation of SNAT[49].

Benzodiazepine receptors have been found by several independent groups in the pineal gland of human[48], bovine[46,47] and rat[50]. Both 'peripheral' and 'central' type benzodiazepine binding sites have been described. In the rat the receptors have been shown to be located on pinealocytes and appear to be under adrenergic control as receptor number diminishes following exposure of rats to constant light or removal of the superior cervical ganglia[50,103]. In vitro, benzodiazepines enhance the noradrenergic stimulation of SNAT in the rat[50], but the role of the pinealocyte benzodiazepine receptor in this response has not been critically evaluated and the mechanism of the response awaits thorough investigation. The physiological relevance of these receptors thus remains unclear.

The possibility that prostaglandins synthesized then released by pinealocytes may modulate melatonin synthesis and/or release has received some experimental support[10,11]. $\alpha_1$-Adrenergic stimulation of rat pinealocytes activates phospholipase $A_2$ probably secondary to the increase in intracellular [$Ca^{2+}$] and activation of PKC. Phospholipase $A_2$ activation causes a release of arachidonic acid into the medium[28,29]. Prostaglandins have also been reported to be released by NA from bovine pineal slices[11]. $PGE_2$ at nanomolar concentrations increased pineal melatonin content and release to the culture medium in rat pineal explants in one study[10]. Clearly a role for prostaglandins in regulating melatonin synthesis must be a modulatory one as complete inhibition of prostaglandin synthesis in the same study did not eliminate melatonin release.

*Regulation of melatonin synthesis in other species*

In comparison with the rat, relatively little is known about the mechanisms which regulate melatonin synthesis in other mammals. What is known suggests some interesting differences to the rat. For example, of the other species studied, most do not show such a large nocturnal elevation in SNAT activity as the rat[8], yet the nocturnal elevation in pineal melatonin is broadly similar. The lack of a large change in SNAT in some species

suggests that other mechanisms may also be important in generating the nocturnal elevation in melatonin synthesis. Indeed, several enzyme steps (tryptophan hydroxylase, SNAT, HIOMT) may be subject to regulation as in fact is probably the case in the rat. Further differences at the receptor level are apparent. For example, many investigators have found it difficult if not impossible, to evoke an increase in daytime SNAT activity in the hamster with adrenergic agonists [76], although the nocturnal elevations in SNAT and melatonin are sensitive to inhibition by $\beta$-adrenergic antagonists. In the sheep, in vivo work has indicated a role for $\alpha_1$- but not $\beta$-adrenoceptors in the regulation of melatonin synthesis. Prazosin, a selective $\alpha_1$-adrenergic antagonist, but not propranolol, a $\beta$-adrenergic antagonist, prevented the expected rise in serum melatonin but did not significantly block the rise in pineal SNAT [89]. In contrast, recent data obtained from experiments on a sheep pineal slice preparation indicate that $\beta$-adrenergic stimulation of cyclic AMP increases melatonin synthesis [54, 55].

It is generally thought that the concentration of melatonin in serum is simply a reflection of the concentration of melatonin in the pineal gland; no storage mechanism or exocytotic release of melatonin is envisaged. However, observations of transient peaks of large magnitude in serum melatonin detected by frequent blood sampling in sheep and man [23, 100] are most easily explained by the existence of an active mechanism controlling the release of melatonin.

*Future directions*

Most of our knowledge of the mechanisms regulating melatonin synthesis has come from studies using the rat (fig. 2). The ability to culture rat pineal glands as explants and to dissociate glands into single cells which can be cultured in suspension or attached to cover slips or beads [9] has been a vital technique. Such techniques have generally not been applied to the study of pineal biochemistry in other mammalian species. Some workers have used pineal slices [14, 54, 55] but most studies in species other than the rat have been in vivo. In trying to understand the mechanisms involved in regulating melatonin synthesis such studies have obvious disadvantages. As it appears that there are some interesting differences between species in the mechanisms which regulate melatonin synthesis future studies should aim to develop a reliable culture system for pinealocytes from other species such as the hamster and sheep. After all it is in these species rather than the laboratory rat that a physiological role for melatonin in regulating seasonal changes in reproduction has been well-characterized.

Interest in the possibility that transmitters other than NA can activate pinealocytes seems sure to increase. In this regard, attention should be paid to comparative studies between mammalian and non-mammalian vertebrates (see Falcón and Collin, this issue). As indicated already, there is some evidence to suggest the presence in the mammalian pineal of various peptide-containing nerve fibres, a variety of putative receptor sites on pinealocytes and induction of SNAT by substances other than NA. Further studies are required to establish that a transmitter (or hormone) other than NA has a physiological role as an activator of melatonin synthesis. Such studies should attempt to correlate the presence of a potential activator in pineal nerve endings using immunocytochemical techniques with the identification and characterization of specific binding sites for that activator able

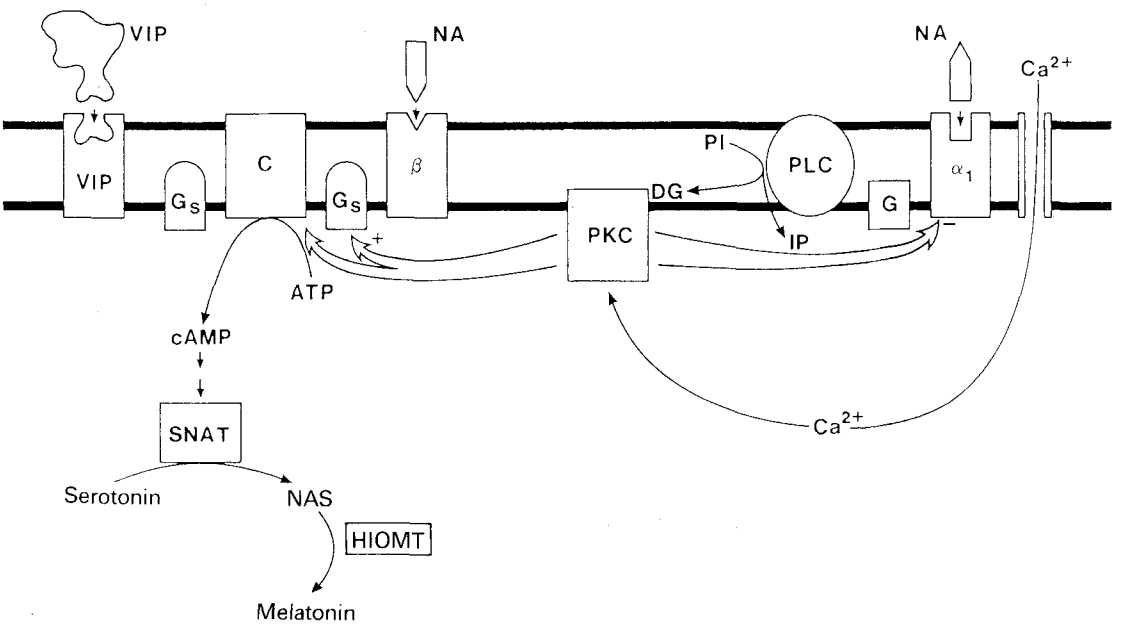


Figure 2. Transduction mechanisms involved in the biosynthesis of melatonin. $\beta$, $\beta$-adrenoceptor; $\alpha_1$, $\alpha$-adrenoceptor; PI, phosphatidylinositol; IP, inositol phosphate; DG, Diacylglycerol; PKC, protein kinase C; $G_s$, stimulatory guanine nucleotide binding protein; C, adenylate cyclase; PLC, phospholipase C; VIP, vasoactive intestinal polypeptide.

to mediate changes in some aspect of pinealocyte biochemistry such as an alteration in cyclic AMP, SNAT or melatonin.

The techniques of molecular biology have only just started to be applied to the study of pineal biochemistry [18, 32]. These techniques promise to allow the development of tools to study the expression of the melatonin-synthesizing enzymes and their mRNA in response to receptor activation. In addition studies with a wider importance in cell biology on the mechanisms which restrict the expression of these genes to this tissue should become feasible.


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## Melatonin and circadian control in mammals

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*Summary.* Although pinealectomy has little influence on the circadian locomotor rhythms of laboratory rats, administration of the pineal hormone melatonin has profound effects. Evidence for this comes from studies in which pharmacological doses of melatonin are administered under conditions of external desynchronization, internal desynchronization, steady state light-dark conditions, and phase shifts of the zeitgeber. Taken together with recent findings on melatonin receptor concentration in the rat hypothalamus, particularly at the level of the suprachiasmatic nuclei, these results suggest that melatonin is a potent synchronizer of rat circadian rhythms and has a direct action on the circadian pacemaker. It is possible, therefore, that the natural role of endogenous melatonin is to act as an internal zeitgeber for the total circadian structure of mammals at the level of cell, tissue, organ, whole organism and interaction of that organism with environmental photoperiod changes.
*Key words.* Melatonin; synchronization; phase adjustment; photoperiod; receptors; phylogeny; ontogeny; circadian rhythms; zeitgeber.

### *Introduction*

As with other vertebrates, investigations into the function of the mammalian pineal body have concentrated primarily on the role played by the chemical melatonin. Melatonin is released into the general circulation during the hours of darkness, irrespective of whether the species is nocturnal or diurnal in its behavioural activity pattern.