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# The contribution of extrapineal sites of melatonin synthesis to circulating melatonin levels in higher vertebrates

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*Abstract.* While the production of melatonin in higher vertebrates occurs in other organs and tissues besides the pineal, the contribution of extrapineal sites of melatonin synthesis such as the retina, the Harderian glands and the gut to circulating melatonin levels is still a matter of debate. The amount of melatonin found in the gastrointestinal tract is much higher than in any other organ including the pineal and the gut appears to make a significant contribution to circulating melatonin at least under certain conditions. The gut has been identified to be the major source of the elevated plasma concentrations of melatonin seen after tryptophan administration and of the changes of circulating melatonin level induced by the feeding regime. Whereas the circadian and circannual fluctuations of the concentration of melatonin in the blood seem to be triggered by changes of the photoenvironment and its effect of pineal melatonin formation, basal daytime melatonin levels and the extent of their elevation at nighttime appear to be additionally controlled by nutritional factors, such as the amount and the composition of ingested food and therefore availability of tryptophan as a rate-limiting precursor of melatonin formation by the enterochromaffin cells of the gastrointestinal tract.

*Key words.* Melatonin; pineal; extrapineal; Harderian glands; retina; gastrointestinal tract; enterochromaffin cells; nutrition; tryptophan; circadian rhythms.

## *Introduction*

Since the isolation and identification of melatonin from ovine pineal glands[38], melatonin research in higher vertebrates has almost exclusively been associated with the pineal gland. This association has become so tight that it is now an almost heretical attempt to explore the possible contribution of extrapineal sites of melatonin formation to circulating melatonin levels. The reason for the pineal gland's exclusive status as the legitimate organ of melatonin secretion and for melatonin's definition as the pineal hormone is primarily the outstanding concentration of melatonin and its synthesizing enzymes in this very little organ weighing less than 1 mg in the rat, and about 100 mg in humans. These high concentrations allowed for reliable measurements in spite of the rather insensitive methods previously employed. The discovery of a circadian rhythm of pineal melatonin content[69], which is triggered by the photoperiod via noradrenaline release and stimulation of N-acetyltransferase activity at darkness[2], confirmed the role of the pineal as a neurochemical transducer of changes in the photoenvironment. The importance of (pineal-derived) melatonin as the humoral messenger of darkness was documented by the finding that either pinealectomy or sympathetic denervation of the gland prevented the nocturnal surge of circulating melatonin[17,31,40,47,66]. This together with the observation that plasma melatonin levels often declined below the detection limit in pinealectomized animals[40] and in humans after removal of pineal tumors[41] led to the conclusion that circulating melatonin derives exclusively from the pineal gland. However, this generally accepted view was blurred by a few reports on the occurrence of melatonin in some extrapineal tissues and by a recently described elevation of circulating melatonin levels in pinealectomized animals after tryptophan administration[28,70]. A critical reassessment of the contribution of the pineal and of extrapineal sites of melatonin synthesis to the circulating melatonin levels in higher vertebrates is therefore warranted.

## *Extrapineal sites of melatonin synthesis*

Apart from the pineal, the retina is the most appreciated independent site of melatonin synthesis in vertebrates[42,44,68]. Because of the morphological and developmental similarities between the retina and the pineal and because of the idea that the pineal gland evolved from a photoreceptor organ, the formation of melatonin by retinal photoreceptors was less surprising and thus readily accepted. The retina exhibits prominent circadian rhythms of NAT activity and melatonin level which are completely in phase with – though independent of – pineal circadian rhythms of these parameters[6,26]. This rhythm is also maintained in vitro in cultured eyecups[13]. The generator of this rhythm has not yet been identified. Either the retina contains a local circadian clock, like that of the frog eye[30] and the photoreceptive avian pineal[62], or the fluctuations in

retinal melatonin synthesis simply reflect the suppression of this process by light during daytime[42,44]. The role of retinal melatonin is unknown. It may act as a modulator of neurotransmission and neuronal excitability in the retina. The amacrine cells of the inner plexiform layer express high-affinity melatonin binding sites[19], and melatonin at picomolar concentrations has been shown to inhibit the release of dopamine in retinal preparations[18]. Even though the trout retina was proposed as an endocrine organ capable of secreting melatonin[24], there is no published evidence in support of a significant contribution of retina-derived melatonin to circulating melatonin levels in higher vertebrates.

The Harderian glands of birds and mammals also contain relatively high levels of melatonin during darkness and presumably are another extrapineal site of melatonin synthesis[49,57,64]. HIOMT activity, indicative of the biosynthesis of melatonin, has been found in the Harderian gland[3,15]. The circadian rhythm of melatonin content in the Harderian gland is not affected by pinealectomy[17]; instead, a compensatory increase of the melatonin content of the Harderian gland several weeks after pinealectomy has been reported[58]. The role of melatonin produced in the Harderian glands is presently unknown. The persistence of the nocturnal rise of circulating melatonin observed by some investigators after pinealectomy has led to speculation that melatonin derived from the retina or the Harderian glands would make a significant contribution to the plasma levels of melatonin[22-24,45,60,63,65,71]. Since more recent studies failed to confirm the persistence of the nighttime surge of plasma melatonin after pinealectomy[17], a significant contribution of these tissues to circulating melatonin levels appears unlikely.

In view of the small amounts of melatonin which may be synthesized in restricted sites like the membraneous cochlea[5], their contribution to circulating melatonin levels also appears insignificant. Even though levels of melatonin were also found to exceed plasma levels in several brain regions of pigeons[64], rats[48], and men[33], no measurable activity of NAT or HIOMT has yet been detected in brain tissue. Since brain melatonin levels fall after pineal removal[48], brain melatonin seems to be concentrated from the circulation.

One extrapineal site of melatonin synthesis, for some unknown reason, became the stepchild of melatonin researchers: the enterochromaffin cells of the gastrointestinal tract. The human appendix was the first site outside the pineal where the occurrence of melatonin could be demonstrated[55]. HIOMT-activity, indicative of the synthesis of melatonin, however, was described earlier in the retina and the Harderian gland[15] than in the gut[54]. Also NAT-activity was only recently described in the gut[36]. Until now, the gut has not seriously been taken into consideration in the discussion of the possible contribution of extrapineal sites of melatonin synthesis to circulating melatonin levels in higher vertebrates[1,34,59].

*The gastrointestinal tract as a source of circulating melatonin*

In higher vertebrates the main site of synthesis of 5-HT, the precursor of melatonin formation, is in the enterochromaffin cells of the gastrointestinal tract[4,20,43,52]. About 90% of the whole serotonin content of the body is localized in the gastrointestinal tract, another almost 10% is found in circulating platelets (which is also derived from the gut) and less than 1% is localized in the brain, mainly in the pineal. The presence and the formation of melatonin by the enterochromaffin cells of the mucosal epithelium was first demonstrated by bioassay and thin layer chromatography[55,56]. Subsequent studies confirmed the presence of melatonin in the gastrointestinal tract by immunocytochemical studies[8,25] and by radioimmunoassay validated by HPLC[64]. HIOMT activity was first described in the rabbit intestine by Quay and Ma[54], and recently Lee and Pang[36] were able to demonstrate the presence of NAT, the suggested rate-limiting enzyme of melatonin synthesis, in the duodenum of quails. They also found a diurnal rhythm of NAT activity in duodenal preparations which paralleled the circadian rhythmicity of this enzyme in the pineal gland and in the retina[36]. The melatonin content of the gut has been shown further to exhibit a pronounced circadian rhythm, similar to that found in plasma, pineal, Harderian glands, retina and hypothalamus, at least in the pigeon[64]. Neither the melatonin level of the gut was decreased by pinealectomy nor was the circadian rhythm of intestinal melatonin content attenuated in pinealectomized animals[8,65]. In rats, immunoreactive melatonin in the gastrointestinal tract was found to exhibit a characteristic regional distribution with peak levels detected in the apical portion of the Lieberkühns crypts and villi of the colon and rectum while lower levels were observed in the jejunum and ileum[9]. This regional distribution is very similar to that of 5HT concentrations and to the density of enterochromaffin cells[11].

Until recently the possibility that melatonin from this pool is released into the circulation has never seriously been considered. Instead, melatonin produced in the gastrointestinal tract has generally been thought to bear paracrine or autocrine activities within the alimentary canal. This concept is supported by the recent identification of high affinity melatonin binding sites in the jejunum of ducks[36]. However, integral functions of melatonin within the gastrointestinal tract have at present been scarcely discovered. Melatonin has been suggested to act as a local regulator of gastrointestinal motility[25]. This is supported by the finding that melatonin in vitro reduced the tone of the gut muscles and counteracted the tonic effect of 5HT[11,53]. Because areas

of the gastrointestinal tract that exhibited the greatest inhibition of spontaneous contractions after melatonin treatment were those containing the highest concentrations of endogenous melatonin, melatonin was thought to inhibit gastrointestinal motility and food transit time in order to allow for a maximal utilization of ingested food[27]. Melatonin was also found to alleviate ethanol-provoked stomach lesions in rats and to partially reverse the 5-HT-induced decrements in gastric glandular mucosal blood flow[16]. In addition, melatonin has been shown to inhibit sodium absorption in the colon of sodium-deficient rats[37] and to inhibit the proliferation of jejunal epithelium cells[39]. Because melatonin appears to be a major inhibitor of cell proliferation[12] affecting the formation and function of microtubules[14,21] – and therefore the mitotic spindle apparatus – melatonin produced by the enterochromaffin cells of the gastrointestinal tract may play a local role as an important modulator of intestinal crypt cell proliferation.

Taken together, there are several lines of evidence in support of the notion that melatonin synthesized and released by the enterochromaffin cells of the gastrointestinal tract exerts a variety of paracrine and autocrine functions in the gut. However, this does not preclude the possibility that it is also released into the portal blood and, if not cleared in the liver, contributes to circulating melatonin levels in higher vertebrates. Actually, there is no reason to believe that melatonin would be handled differently from 5-HT, the other major indoleamine produced in the enterochromaffin cells of the gut. In order to assess the contribution of extrapineal sites of melatonin synthesis to the circulating levels of this hormone, the consequences of the removal of the pineal on the plasma concentrations of melatonin need to be explored. This has been performed in several laboratories with different species and different methods, and maybe therefore, with rather inconsistent and contradicting results. A few authors reported a fall of circulating melatonin levels below the detection limit following pinealectomy in chicks using a sensitive bioassay and mass spectrometry[51] in rats using GCMS[40], and in human subjects after the removal of pineal tumors[41]. This was taken as evidence that all plasma melatonin is exclusively derived from the pineal and that the plasma melatonin level can be used as marker of circadian melatonin secretion by the pineal gland and of its beta-adrenergic regulation[40]. The majority of studies, however, reported only the loss of the melatonin rhythm in the blood as measured by RIA and GCMS in pinealectomized chicks[17], rats[47], sheep[31] and Syrian hamsters[66]. Daytime melatonin levels were found to be almost unaffected by the removal of the pineal (see also[28,70]) only the nighttime rise of plasma melatonin was prevented. This was taken as evidence that the nocturnal surge of blood melatonin is derived from the pineal. Finally, evidence has also been presented that the circadian melatonin rhythm persists (with a less pronounced elevation at nighttime) in the blood of pinealectomized birds[45,63,65] and rats[71]. This observation was taken as evidence for a significant contribution of extrapineal sites to the circadian fluctuations of circulating melatonin, Already one year after the first demonstration of melatonin in the gut, Ozaki and Lynch[47] found that pinealectomy in rats abolished the nighttime surge of plasma melatonin. However, the diurnal rhythm in the urine persisted as long as free access to food was provided. Removal of the food (i.e. elimination of the circadian rhythm of food intake) eliminated the daily pattern of urinary melatonin excretion, refeeding reinstated it. Later studies confirmed the influence of nutritional factors on circulating melatonin levels[61,67,70]. As shown recently, food deprivation significantly increases gastrointestinal tryptophan, serotonin and melatonin levels and it has been suggested that a higher melatonin formation in the digestive tract may be responsible for the increase of circulating melatonin levels seen after food deprivation[10].

Additional support for the possible contribution of melatonin derived from the gastrointestinal tract to circulating melatonin levels in higher vertebrates came from studies on the effect of tryptophan administration on circulating melatonin levels in man. The rapid and large elevation of plasma melatonin seen after the infusion of L-Trp to healthy young men during daytime was suggested to be of extrapineal origin[29]. In subsequent animal experiments this assumption was verified by the demonstration of the persistence of this Trp-induced elevation of circulating melatonin in pinealectomized chicks[28], and rats[70], by the larger elevation of circulating melatonin seen after oral compared to intraperitoneal administration of the same dose of Trp, by the prevention of this elevation in animals with a ligature of the portal vein and by the finding that the Trp-induced increase of melatonin in the portal blood preceded that in the systemic circulation[28]. Evidently, melatonin synthesized and released from the enterochromaffin cells of the gastrointestinal tract can – at least under certain circumstances – contribute significantly to the levels of melatonin found in the systemic circulation. Since melatonin easily penetrates the blood-brain barrier, the enterochromaffin cells of the gut may thus be involved in the mediation of the central effects of melatonin, at least under certain nutritional or pharmacologic influences affecting the synthesis and release of melatonin in the gastrointestinal tract.

### *A critical reassessment of the role of the pineal as the exclusive source of circulating melatonin*

As shown above, melatonin produced by the enterochromaffin cells of the gastrointestinal tract may, under certain pharmacological or nutritional condi-

tions, make a major contribution to circulating melatonin levels. The contribution of this gut-derived melatonin to the basal levels and the circadian fluctuations of plasma melatonin under physiologic conditions in higher vertebrates, however, is difficult to assess. In absolute terms, the amount of melatonin found in the pineal is almost negligible compared to the melatonin content of the gut (0.2–4.5 ng versus 80–2000 ng[28]). But, no data have been published yet on the rate of melatonin synthesis in the gut, and on the fate of the melatonin synthesized and released from the enterochromaffin cells. As shown for serotonin, part of this melatonin may be secreted into the gut lumen. It would therefore be important to measure the concentrations of melatonin in the portal blood under various conditions and to compare these values to those measured in the systemic circulation. The finding that the melatonin level in the portal blood is somewhat higher than in the systemic circulation[28] indicates that melatonin produced in the gastrointestinal tract may indeed contribute to the circulating levels of this hormone.

The possible contribution of extrapineal sites of melatonin synthesis to circulating melatonin levels under normal conditions can also be estimated by some quantitative calculations using urinary excretion rates and the half-life time of melatonin. One important question can be answered on the basis of published data: how much melatonin must be produced in the pineal in order to maintain the level of circulating melatonin during day- and nighttime?

Normal human subjects excrete about 10 µg 6-sulphatoxy-melatonin per 24 h. Excretion during daytime is about 15% of this value, i.e. 1.5 µg/12 h, i.e. 125 ng/h or 2 ng/min (values calculated from the data of ref. 7). The melatonin concentration in the human pineal is about 10 ng/100 mg during daytime[46]. Based on a human pineal weight of 50–100 mg[1], the total melatonin content of the human pineal approximates 10 ng. Consequently, during daytime the human pineal must synthesize and release an amount of melatonin equal to its daytime steady state content every 5 min. The same calculation can be made for the nighttime melatonin production in the pineal. Excretion is 85% of 10 µg, i.e. 8.5 µg/12 h, i.e. 12 ng/min. Nighttime melatonin content is between 50 and 100 ng/pineal. During nighttime the pineal must replace its content every 6 min. The bulk of circulating melatonin in the blood is bound to albumin[50]. Melatonin's half life in the blood is rather short (10–40 min[32,35]), and during a single passage through the liver 90% of the melatonin is cleared[50].

If we consider a half-life of 10 min, a daytime plasma content of 10 pg/ml and a total plasma volume of 5 l, total circulating melatonin amounts to about 50 ng. If half of this amount, 25 ng, will be cleared within 10 min, the pineal would have to release 25 ng/10 min, i.e. its daytime content, 10 ng, in 4 min. During nighttime when 100 pg/ml plasma are circulating and the total melatonin content in plasma is about 500 ng, 250 ng will be cleared and must be restored in 10 min. The pineal would have to produce an amount of melatonin equal to its nighttime content (50–100 ng) every 3 min.

These calculations nicely confirm the view that melatonin produced in the pineal is not stored but rapidly released into the circulation. The more melatonin is produced in the pineal (about 10 times more during nighttime compared to daytime) the more is also released into the circulation. The estimated rate of melatonin synthesis in the human pineal gland in vivo (during daytime about 2 ng/min and during nighttime about 12–25 ng/min) is relatively high, but the synthesis of an amount of melatonin equal to its content within 3–6 min is not unlikely. However, until these estimations are confirmed by actual measurements of the rate of melatonin in the human pineal gland, a contribution of other sites, in particular of the gastrointestinal tract, to circulating melatonin levels must be taken into consideration.

The latter possibility is supported by the majority of studies on the effects of pinealectomy on circulating melatonin levels. According to the estimations made above, plasma melatonin should rapidly decline and disappear within a few minutes after the removal of the pineal. However, many authors found the low daytime levels to be unaffected[17,31,47,66] and in some studies even the nighttime elevation was only found to be attenuated after removal of the pineal[45,63,65,71]. The dynamics of the decline of circulating melatonin after pinealectomy have never been investigated. If melatonin produced by the enterochromaffin cells of the gastrointestinal tract would indeed contribute to circulating melatonin levels in higher vertebrates, this contribution is likely to be affected by the nutritional state. This is indicated by the fact that the synthesis of melatonin in the gut, but not in the pineal, is dependent on the availability of its essential precursor amino acid[28]. This is further supported by the finding that the circadian fluctuation of residual plasma melatonin in pinealectomized rats is triggered by the feeding schedule[47]. It is therefore not unlikely that the extent of the decline of the circulating melatonin levels after removal of the pineal depends on the nutritional state of the experimental animals at the time when blood is obtained for melatonin measurements. Differences in food intake – and therefore in Trp-availability – have never been considered in the discussion of the size of the effects of pinealectomy on residual plasma melatonin concentrations. If indeed a relationship exists between food intake and circulating melatonin levels, the enterochromaffin cells of the gastrointestinal tract are the most likely site of the body where nutritional information, i.e. changes in the availability of the least abundant essential amino acid in proteins, Trp – and therefore

protein content and composition of the food – is translated into a chemical messenger, baseline plasma melatonin levels. This message would then be superimposed by the circadian and the circannual rhythms of pineal melatonin secretion triggered by the changes in the photoenvironment. Melatonin: the chemical expression of darkness and food supply?


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