1 Melatonin, the pineal gland and circadian rhythms; an introduction

Only few endogenous compounds have been identified by as many creative titles as melatonin. Terms like 'hormone of darkness', 'ubiquitously acting hormone', 'dracula of the endocrine system', 'time in a bottle' and 'circadian glue' have been used to express the interesting, but sometimes poorly understood nature of this hormone. Maybe the intruiging names that have been used to identify its main production site, the pineal gland, have contributed to this creativity. Names like 'seat of the soul' and 'third eye' might have stimulated the imagination. Nevertheless, it has become clear that the pineal gland and its hormone melatonin bear interesting characteristics in many physiological processes and the timing system known as the biological clock.

This thesis describes the development of a technique that enables researchers to measure melatonin right there, where it is synthesized, in the pineal gland. In this method, freely moving animals are on-line coupled to an analytical system, which measures melatonin directly. This means that each change in the synthesis of melatonin is recorded within 20 minutes. It may be obvious that such a system offers specific unique opportunities that were not available until now. Insight in the neurochemical and pharmacological mechanisms that are involved in the regulation of mela-

tonin synthesis, profiling of the circadian nature of melatonin release, both in phase and amplitude simultaneously and the responsiveness of this system towards putative melatonin-like drugs are issues that are addressed in the present dissertation.

The reader may not be an expert in all the various research fields that are involved in the experiments described later on. Therefore, this introduction may be helpful in the understanding of some basic principles and specific terminology of pineal research and chronobiology. It is outside the scope of this thesis to present a thorough survey of all literature data on each subject discussed. Therefore, those who are interested are referred to literature references and fine reviews mentioned in the text and listed in chapter 10. Two books that have been published on melatonin are of particular interest.[12,428] They have served as important references for this introductory chapter and are highly recommended to those who want to have a complete coverage of the subject.

The field of pineal research and biological rhythms involves studies on an enormous variety of species, from unicellular bacteria to humans. Variations between species can be substantial and are often important in the interpretation of results. In this chapter, as in most of the thesis, the attention will be mainly focussed on mammalian species, and in certain sections only the human. Because the basic mission underlying most of the experiments is the therapeutic use of melatonin or melatonin-like drugs, this limitation was applied.

With these considerations in mind, the reader is invited to become familiar with some of the basic principles of pineal research and to enter the world of the development of trans pineal microdialysis and its application in pharmacological and chronobiological studies, the world of melatonin on-line.

1.1 History

The history of our knowledge concerning the pineal gland goes back to ancient times when Herophilus (325-280 BC) considered this 'epiphysis cerebri' as the valve regulating the flow of 'spiritus' from the third to the fourth ventricle. Thisspiritus was derived from air that was transformed into 'spiritus animalis' in the ventricular system. The spiritus animalis was the basis for knowledge.

Although probably Herophilus was the first who considered the epiphysis cerebri as the 'seat of the soul', this nomenclature became famous when the French philosopher Descartes used this term in his texts on the pineal. Descartes suggested in 1662 that the pineal controlled the flow of spiritus animalis into motor nerves and that it was an important factor in the movements of the body. He also thought that light input from the eyes was important in its regulation; a remarkable resemblence with todays understanding.

By the end of the nineteenth century, the photosensory role of the pineal in lower vertebrates and its relation with the secretory mammalian pineal gland became known. Also around this time Heubner (1898) and Merburg (1907) discovered the role of the pineal gland in the reproductive system by observations of pineal tumors.

Almost four decades ago crucial advances were made in pineal research. Studies of the dutch scientist Kappers on the innervation of the gland by the sympathetic nervous system via the superior cervical ganglion were really pioneering. Probably the most important discovery in this respect was the identification of the principle pineal hormone melatonin by Aaron Lerner in 1958.[180,181]

From thousands of bovine pineal glands, Lerner and co-workers isolated the compound that was responsible for the skin-lightening effect of pineal extracts on amphibian melanophores. This compound was identified as *N*-acetyl-5-methoxytryptamine. Lerner named this molecule 'melatonin', based on its ability to contract (τονος = contractor) melanin granules in the melanophores.

From this moment on, an increasing scientific interest has resulted in the interdisciplinary field that pineal research is today. The understanding of melatonin's role in many physiological processes, in synchronizing the circadian organization, and the identification of its target sites have made substantial progress over the past years. In the remainder of this chapter an overview will be presented on this present knowledge.

1.2 The pineal gland: production site for melatonin

■ Anatomy

The unpaired pineal gland, in humans resembling the shape of a pine cone, is an appendage of the brain. Its location is either central in the brain, close to the third ventricle (humans), or superficial, mostly close to the cerebellum (rat). In all cases, it has a stalk connection (deep pineal) to the habenular commissure. The gland is a small secretory organ, with a direct photoreceptive (fish and amphibians), mixed photorecep-

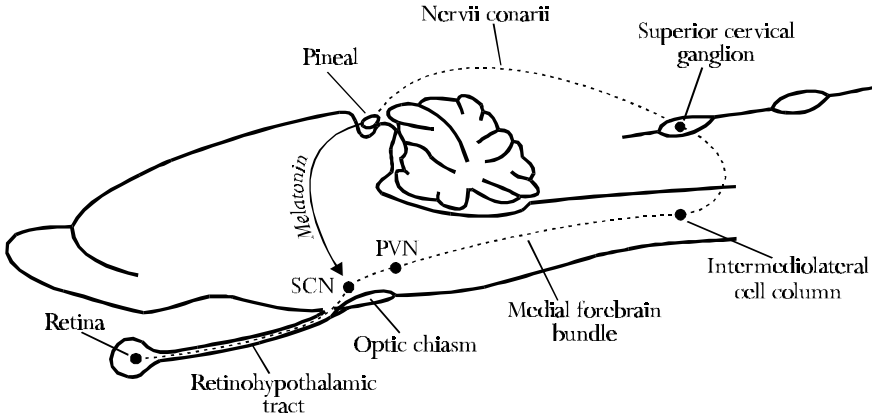


Figure 1.1 Schematic overview of the location of the pineal gland in the rat brain and its major innervation, starting from the retina and including the hormonal projection of melatonin on the SCN. (SCN= suprachiasmatic nucleus, PVN = paraventricular nucleus)

tive and secretory (birds and reptiles) or sole secretory function (mammals). When the gland has a photoreceptive function, photoreceptor cells make a major contribution to the pineal composition. In mammals, the major cellular component of the pineal is the pinealocyte. In rats, this cell type accounts for 80% of the glandular components. The remainder consists mainly of glial cells and nerve fibers. The weight of the pineal is about 1-1.5 mg in rats, whereas in humans the average weight is 100-150 mg.

The pineal contains a dense vascular network and is the second best perfused organ.[125] Arterial input originates from the posterior choroidal arteries and the venous drainage empties into the great cerebral vein. At least in rat and human, the pineal appears to lack a blood-brain barrier. The intensive blood supply assures a rapid transportation of secretory products into the peripheral circulation towards target areas, including the brain. However, a direct secretion into the cerebrospinal fluid must not be excluded.

With age, calcification of the pineal occurs. This process already starts at an early age, when calcium is stored in very fine granules. In humans, largely calcified pineals can act as a reference point in radiological studies. There is no evidence that calcification affects pineal metabolism, i.e. melatonin production.[313]

■ Innervation

By far the most important innervation are sympathetic fibers, originating from the superior cervical ganglion. These fibers are the final part of the neuronal pathway between the suprachiasmatic nuclei (SCN) and the pineal. This pathway (Fig. 1.1) originates in the SCN, which receives light information from the retina through the retinohypothalamic tract. It passes the paraventricular nucleus (PVN), follows the medial forebrain bundle and the reticular formation and ends in the intermediolateral cell column. From there a projection on the superior cervical ganglion exists, from which sympathetic neurons (nervii conarii) innervate the pineal. The main neurotransmitter of the sympa-

thetic innervation is noradrenaline, which is released in the perivascular space, near the pinealocytes. Neuropeptide Y appears to be colocalized in these fibers.

Recent evidence suggests that peripheral innervation is not the only regulatory mechanism in the pineal. Direct central innervation from the lateral and anterior hypothalamic areas and the paraventricular area is reported.[225] Because these areas also receive input from the retina, such pathways may play an important role in the regulation of circadian melatonin production. Neurotransmitters that may be involved in these systems are peptides such as vasoactive intestinal peptide (VIP), peptide histidine isoleucine, arginine vasopressin, arginine vasotocin, oxytocin, neuropeptide Y (NPY) and luteinizing hormone releasing hormone. In addition, the parasympathic innervation may be centrally derived as well, although also peripheral parasympathic innervation via the nervii conarii is found.

■ Biosynthesis

In most species, the pineal gland and more specifically the pinealocytes are the primary sites for melatonin synthesis. Other tissues in which melatonin and its biosynthetic enzymes are reported include the retina,[434] harderian gland, iris-ciliary body and the lacrimal gland. Melatonin synthesis is also reported in other peripheral tissues, such as blood mononuclear leukocytes[99] and the gastro-intestinal tract.[178] Generally it is assumed that in these tissues the melatonin produced has a local function and does not contribute to circulating melatonin to a great extent. This conclusion can be derived from pinealectomy studies, in which rhythmic plasma melatonin levels are abolished.

The biosynthetic pathway, including the enzymes involved has been elucidated by Axelrod and coworkers, described in a classic review in Science.[18] Since then many studies have been reported on pineal metabolism. Several reviews[43,280,282,348] and books[12,428] describe the biosynthesis and metabolism of melatonin in great detail.

In Fig. 1.2 the biosynthetic pathway of melatonin production is presented. The first step involves the uptake of tryptophan from the circulation. This uptake occurs against a concentration gradient, implying an active transport mechanism. Tryptophan is converted into 5-hydroxytryptophan by the mitochondrial enzyme tryptophan hydroxylase. This enzyme is present in high concentrations making the availability of tryptophan rate limiting in this reaction. Tryptophan hydroxylase activity increases during darkness about twofold. The cytoplasmic enzyme aromatic amino acid decarboxylase subsequently catalyzes the decarboxylation of 5-hydroxytryptophan to serotonin. The concentrations of serotonin in the pineal gland are very high, showing a circadian variations with lowest levels during darkness. This drop in serotonin levels during darkness is mainly a result of increased metabolism to *N*-acetylserotonin, but alternate metabolic pathways, such as oxidative deamination to 5-hydroxyindole acetic acid and the methylation to 5-methoxytryptamine may not be excluded. A release from pinealocytes may also attribute to circadian variations in serotonin content.

The next step is crucial in the regulation of melatonin synthesis. It involves the acetylation of serotonin to *N*-acetylserotonin, a reaction that is catalyzed by arylalkylamine *N*-acetyltransferase and uses acetyl co-enzyme A as a cofactor. *N*-acetyltransferase has received most attention as far as the melatonin synthetic pathway is concerned. In the rat its activity shows a remarkable circadian rhythmicity with 20-100 fold increases at

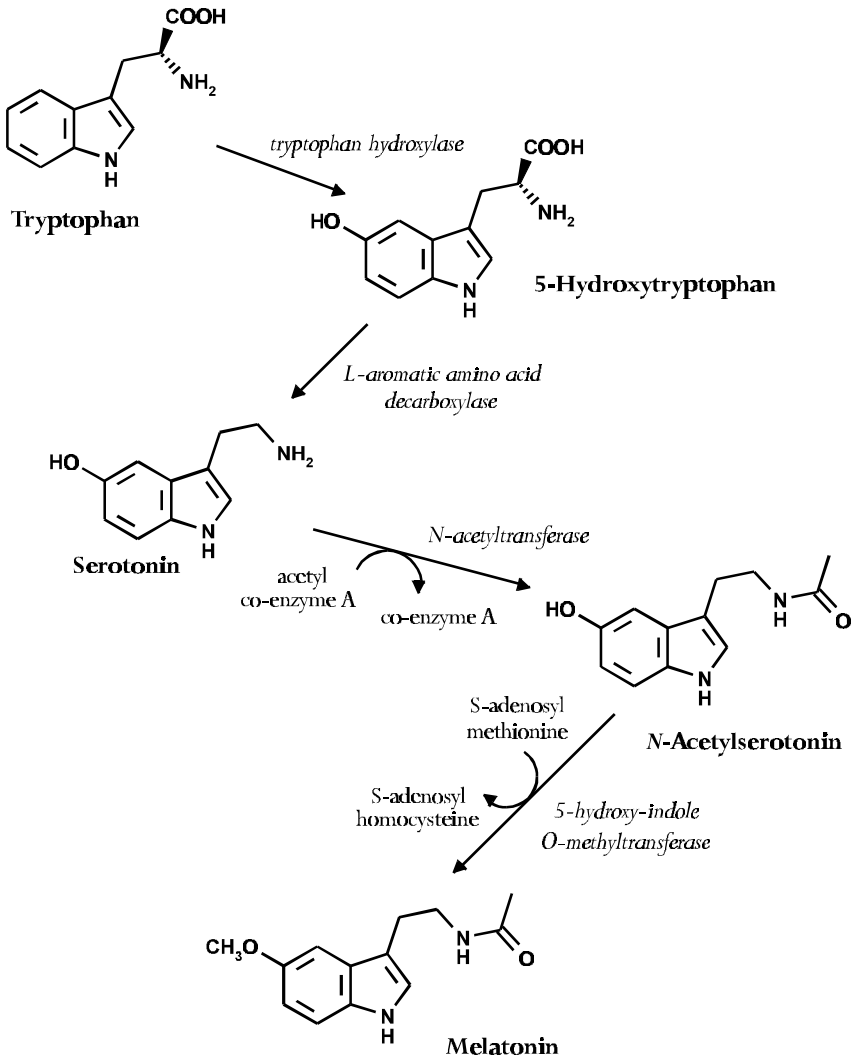


Figure 1.2 Biosynthetic pathway of melatonin and the enzymes involved. Tryptophan is taken up from the blood and converted into serotonin in two steps. The rate-limiting conversion of serotonin to *N*-acetylserotonin is the primary target for regulation of melatonin production. Melatonin is subsequently release into the circulation.

night. This rhythmicity is common among most species, whereas the huge amplitude appears mainly to be associated with the rat pineal gland. Not surprisingly, this enzyme is rate limiting in the melatonin synthesis. The close resemblance between*N*-acetyltransferase activity and melatonin synthesis together with the early development of an assay determining its activity[73] have resulted in a widespread use of*N*-acetyltransferase activity as the primary marker of pineal metabolism.

N-acetylserotonin concentrations closely relate to *N*-acetyltransferase activity. Although data are scarce, circadian variations in its content are reported. A possible secretion of *N*-acetylserotonin from the pinealocytes is not yet confirmed. *N*-acetylserotonin is converted to melatonin by the cytosolic enzyme hydroxyindole-*O*-methyltransferase, using the methyl donor S-adenosyl methionine. Activity of hydroxy indole-*O*-methyltransferase shows no circadian variation and production of melatonin appears to be mainly dependent on the availability of the precursor *N*-acetylserotonin.

The resulting rhythmic melatonin production shows about 2-15 fold increased levels during the dark period, depending on the species. This nocturnal production of melatonin is unrelated to the circadian cycle of locomotor activity. Independent from a species being diurnally or nocturnally active, melatonin is mainly produced during night-time. In contrast to many hormones, melatonin is not stored, but once synthesized, it is assumed to be immediately secreted by passive diffusion into the circulation via a dense vascular system in the pineal gland. Its lipophylic structure enables it to penetrate tissues, membranes, and the blood-brain barrier readily. The hormonal expression of darkness can therefore easily be 'read' by the target organs.

■ Metabolism

Melatonin is primarily metabolized in the liver, to yield 6-hydroxymelatonin (Fig. 1.3). This hydroxylated product can be conjugated with either sulphate or glucuronide. In rodents and humans, the sulphate conjugate predominates and is excreted into the urine. A radioimmunoassay for 6-sulphatoxymelatonin in urine appeared to be a powerful tool in studies on the circadian rhythms of melatonin secretion, because it reflected melatonin production very well, both qualitatively and quantitatively.[40]

In addition to the hydroxylation, there are several other metabolic pathways. One is the formation of *N*-γ-acetyl-*N*-2-formyl-5-methoxykynurenamine, which can be further degraded to *N*-γ-acetyl-5-methoxykynurenamine.[124] These reactions are catalyzed in the brain by oxygenative cleavage of the pyrrole moiety and demethylation by formamidase, respectively. Another metabolite is a cyclic isomer of 2-hydroxymelatonin.[381] Finally also demethylation to *N*-acetylserotonin is described. Especially when high doses of exogenous melatonin are applied, this metabolic route can become almost equally important as the hydroxylation.[179] Physiological activity of these metabolites is not clear yet, but the possibility must not be excluded.

The metabolic pathways result in very fast kinetic properties of exogenously administered melatonin. In humans, the elimination is biphasic, with reported half-lives of 3 and 45 minutes. These appear to be common figures in mammals. Daily production of melatonin is reported to be 28.8 μg in normal adults.[172] Plasma levels following single doses show large individual variations, due to differences in absorption. The fast kinetics of melatonin have triggered the development of oral controlled release delivery systems[176] and transdermal preparations.[175]

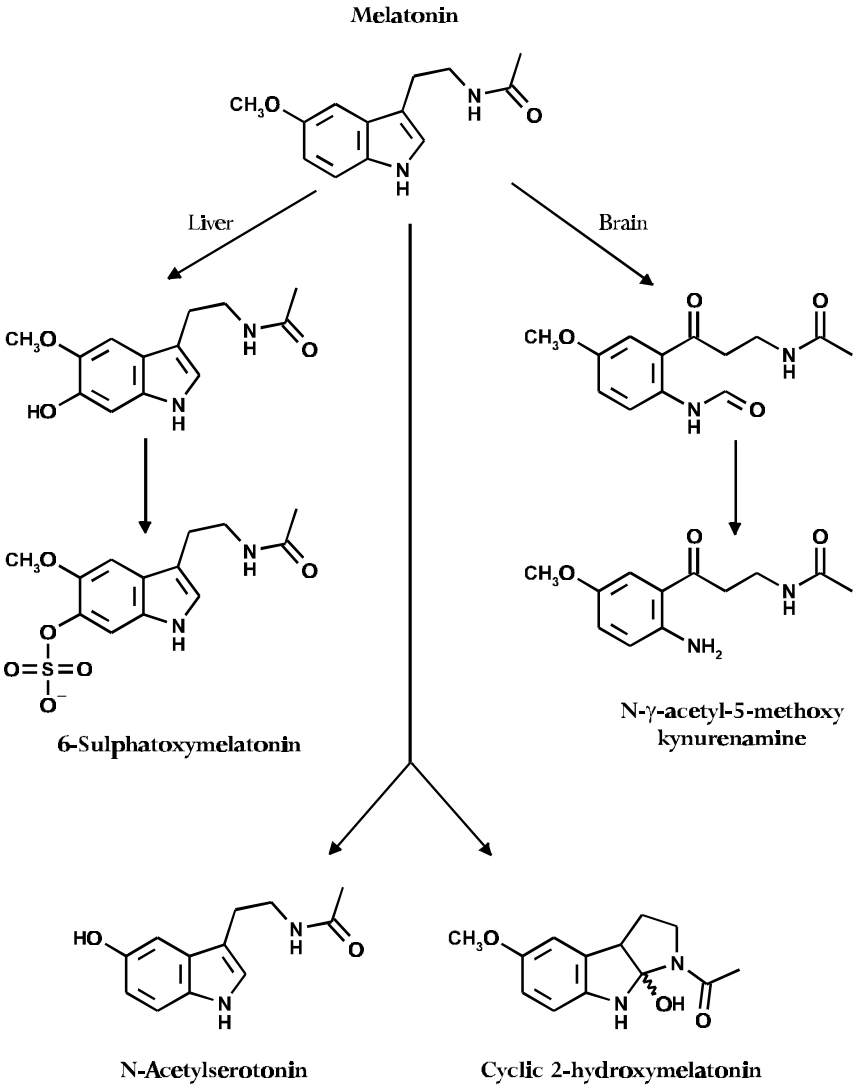


Figure 1.3 Metabolic pathways for melatonin. The two main metabolic routes are hydroxylation at the 6-position in the liver and ring-opening at the 2-position in the brain. Minor metabolic routes result in*N*-acetylserotonin and cyclic 2-hydroxymelatonin.

1.3 Control of melatonin synthesis in the pineal gland

The regulation of *N*-acetyltransferase activity is the main target for most regulatory mechanisms in melatonin production. Over the years, many control mechanisms have been described to be present in the pineal and playing a role in the regulation of *N*-acetyltransferase activity (for reviews, see refs. 280 and 348). Most of these studies have been done in rat pineals. The relative ease with which rat pineals can be cultured, providing a preparation consisting of mainly post-synaptic elements, together with a method for preparing isolated single pinealocytes from the rat[37] have contributed to the popularity of rat pineals as model for mammalian pineals in general. When not specified, data presented in this section involve studies on rat pineal glands. Pineals in other species than mammals, such as birds and reptiles, may vary in their pharmacology to a great extent, but are not considered here.

■ Adrenergic control

By far the major control of melatonin synthesis is exerted by postganglionic sympathetic nerve fibers, releasing their main neurotransmitter noradrenaline near the pinealocytes. As a consequence, adrenergic receptors on the pinealocytes are found to be of crucial importance in the stimulation of *N*-acetyltransferase and hence the melatonin production.

The presence of β-adrenergic receptors has been reported in rat, hamster and sheep pineal glands[17,68,100] and found to be of the β_1-subtype. These receptors are coupled to a G_s-protein. Their stimulation results in enhanced adenylyl cyclase activity and subsequent cAMP levels, necessary for the induction of *N*-acetyltransferase. The density in β-adrenergic receptors shows circadian variations (for review, see ref. 244). In rats, phasing of the rhythms varies among different studies, with peak receptor density either occurring late in the light phase, or during darkness. The variation is rather modest and never exceeds 50% of the mean density. Quite different is the situation in hamsters. β-Adrenergic receptor density is lowest in the second half of the dark phase, exactly in opposite phase compared to melatonin. The amplitude is much greater than in the rat.

Also the responsiveness towards β-adrenergic agonists shows circadian variation. It appeared that rat pineals excised late in the light period showed an enhanced response to adrenergic stimulation compared to pineals removed in the dark period.[433] This can be explained by the continuous endogenous stimulation during the dark period, causing desensitisation. Such dependency of responsiveness to prior exposure is common to many receptor systems. In hamster pinealocytes, the situation is more complicated. In contrast to rat pineals, which respond to β-adrenergic stimulation with an increased *N*-acetyltransferase activity and melatonin production within 1-2 h at any time of day, hamster pineals do not show such an effect when the agonist is applied in the light period. Only in the second half of the dark period, melatonin production can be readily stimulated with β-adrenergic agonists.[278] Also in humans, β-adrenergic agonists are incapable of inducing melatonin production during daytime.[392] Although not tested yet, the human pineal may also be sensitive towards β-adrenergic drugs only during restricted periods of the night. In this respect, the hamster pineal shows more similarity to human than the rat does.

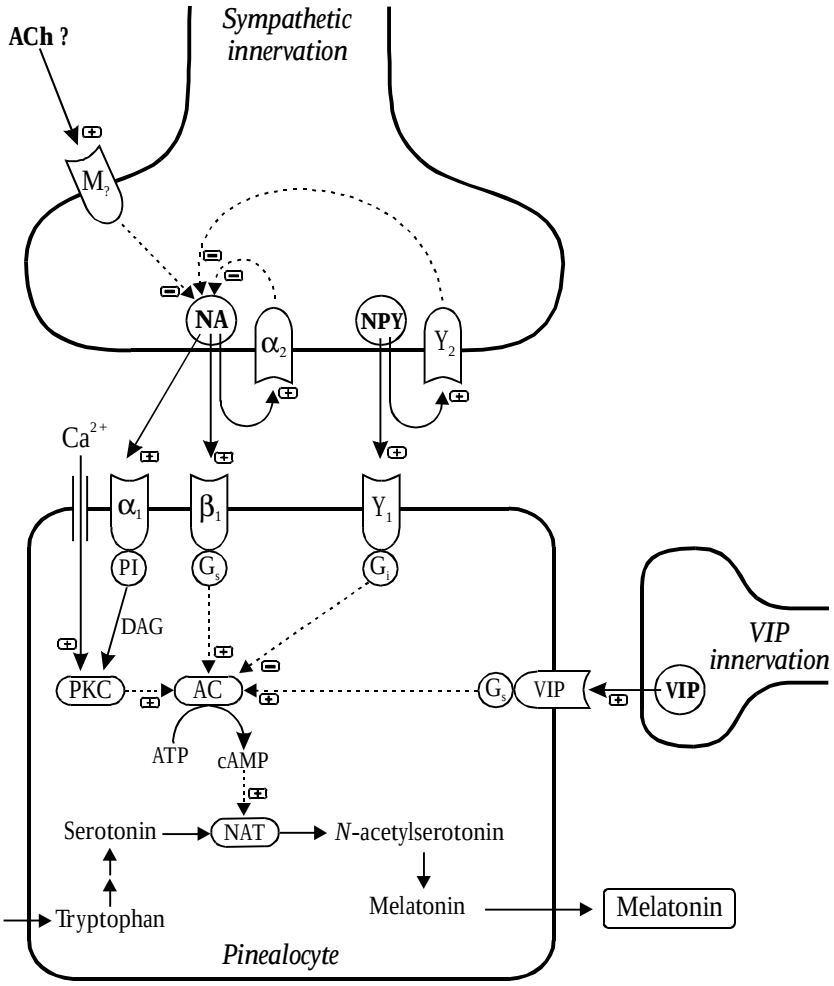


Figure 1.4 Innervation of rat pinealocytes, interactions between receptor systems, also at the level of second messenger systems and the relation with melatonin biosynthesis. See text for extensive explanation. α_1 = α_1-adrenergic receptor, α_2 = α_2-adrenergic receptor, β_1 = β_1-adrenergic receptor, AC = adenylyl cyclase, ACh = acetylcholine, ATP = adenosine triphosphate, cAMP = cyclic adenosine 3',5'-monophosphate, Ca^{2+} = calcium ions, DAG = diacylglycerol, Gs = stimulatory G-protein, Gi = inhibitory G-protein, M = muscarinic receptor, NA = noradrenaline, NAT = *N*-acetyltransferase, NPY = neuropeptide Y, PI = phosphoinositide system, PKC = protein kinase C, VIP = vasoactive intestinal peptide, Y_1 = NPY-Y_1 receptor, Y_2 = NPY-Y_2 receptor

Stimulation of the β-adrenergic receptor accounts for approximately 85% of the nightly increase in melatonin production. The remaining 15% is presumably due to other regulatory mechanisms, in which α-adrenergic receptors may play an important role. The presence of α_1-adrenergic receptors has been reported in rat,[351] hamster[243] and sheep[346]

pineal glands. In hamsters, also a clear circadian rhythm in receptor density has been reported,[243] with lower numbers during night than during daytime. In rats, such rhythmicity is not clear.

Direct stimulation of α_1-adrenergic receptors does not influence pineal metabolism. However, β-adrenergic stimulation of cAMP levels and subsequent *N*-acetyltransferase activity can be markedly potentiated by simultaneous activation of the α_1-adrenergic receptor.[158,387] The mechanism underlying this effect has been elucidated[347] and is schematically drawn in Fig. 1.4. Stimulation of the α_1-adrenergic receptor both increases the intracellular calcium concentration and the phosphatidylinositol turnover, the latter resulting in the formation of diacylglycerol as a second messenger. Both calcium and diacylglycerol activate protein kinase C, which potentiates adenylyl cyclase activity in its production of cAMP. Without adenylyl cyclase activity, i.e. without β-adrenergic stimulation, protein kinase C will be ineffective in increasing intracellular cAMP levels. The increased cAMP levels then stimulate *N*-acetyltransferase activity, resulting in enhanced melatonin production.

The presence of α_2-adrenergic receptors in the rat pineal has been reported as well.[247,269,312] Generally, their function is considered to be regulatory, by a negative feedback on the noradrenaline release. Therefore, their location is considered to be pre-synaptic, although also a post-synaptic location is suggested.[312]

The cAMP response to adrenergic stimulation also appears to have a negative feedback. Recently, the inducible cAMP early repressor (ICER) has been discovered as a product of the cAMP-responsive element modulator (CREM) gene.[341] ICER that is produced at night via cAMP stimulation of CREM, reduces cAMP-induced gene transcription, and shows a marked circadian variation with peak levels corresponding to the declining phase of *N*-acetyltransferase activity. It may therefore play an important role as a negative feedback loop in adrenergic control of gene expression and *N*-acetyltransferase activity in the pineal.

■ Cholinergic control

In contrast to the sympathetic innervation, the parasympathic innervation has received little attention so far. Muscarinic receptors have been described to be present on the pineal gland of rats, sheep and bovine.[107,364] Generally these receptors are very low in number. In bovine, their functionality appears to involve an inhibitory action on *N*-acetyltransferase activity,[266] but in rat pineal slices muscarinic agonists were unable to influence melatonin production, while serotonin release was markedly increased.[98] In cultured pineals, carbachol enhanced phosphoinositide hydrolysis.[168] In chapter 5, evidence is gathered for a pre-synaptic role of muscarinic receptors. Their ability to inhibit noradrenaline release may be an important regulatory mechanism of sympathetic innervation.

Even less is known about nicotinic receptors. Their presence has been described in rat pineals,[290,338] but there is only one report that describes their functionality in inhibiting noradrenaline stimulated melatonin production.[338]

■ GABAergic control

The role of γ-aminobutyric acid (GABA) in the pineal gland has been reviewed in great detail.[298] The presence of GABA as well as the key enzyme in its biosynthesis, glutamic acid decarboxylase, is shown in bovine and rat pineals.[297,314] Its turnover rate shows a marked circadian rhythm with peak levels during darkness.[151] The release of GABA is triggered by noradrenaline, through α_1-adrenergic receptors.[296] The effects of GABA are in general inhibitory to noradrenaline induced *N*-acetyltransferase activity and/or melatonin production. Mediated by postsynaptic type A receptors. GABA may have a feed-forward inhibitory effect that impairs the postsynaptic effect of noradrenaline. Pre-synaptically, type A and type B receptors appear to have opposite functions in facilitating and inhibiting noradrenaline release respectively, from which the inhibitory dominates.

Taken together, the role of GABA is a very complex one, building up a whole circuit of mostly inhibitory projections. This circuit could offer a certain resistance in the passage of information to the pineal, resulting in more balanced responses.

■ Peptidergic control

There is growing interest in the role of several peptides in the regulation of melatonin synthesis. The two most important are vasoactive intestinal peptide (VIP) and neuropeptide Y (NPY). In both cases, their presence and pharmacological significance has been demonstrated.

VIP is mainly present in nerve endings in the pineal gland.[219] Although early reports mention the superior cervical ganglion as a likely origin for VIP nerve fibers, this has been called into question and nowadays either colocalization with parasympathetic fibers or a direct origin in the brain are suggested.[259] Receptors have been identified and characterized in rat[147] and bovine[306] pineal glands. Functionally, VIP receptors are coupled to G_s-proteins and their stimulation results in increased adenylyl cyclase activity and enhanced intracellular cAMP levels, similar to the β-adrenergic receptor.[325,431] Recently, also the involvement of calcium and cGMP has been suggested.[311] By all means, stimulation of VIP receptors results in enhanced *N*-acetyltransferase activity and melatonin production. VIP-stimulated *N*-acetyltransferase activity can be induced by α_1-adrenergic receptor activation. Apparently this occurs by a mechanism via PKC, similar to the α-mediated enhanced β-adrenergic response. The importance of VIP may not be a general characteristic of melatonin regulation, because in hamsters no effect of VIP on either *N*-acetyltransferase or melatonin was observed.[234]

Also the presence of NPY has been described in the pineal, showing a circadian rhythm with peak levels during the dark phase.[323] It is colocalized with noradrenaline in the sympathetic fibers that originate from the superior cervical ganglion. The effects of NPY are exerted via both pre- and postsynaptic receptors. Specific binding of [^{125}I]-NPY was reported in rat pinealocytes.[239] Pharmacological characterization revealed pre-synaptic binding sites as being of the NPY-Y_2 subtype, while postsynaptic sites belonged to the NPY-Y_1 subtype.[326] The pre-synaptic effects involved an inhibition of the noradrenaline release, being independent of the inhibition by α_2-adrenergic receptors. The post-synaptic effects are somewhat controversial. Either small enhancements[326,378] or marked decreases[239] were reported of noradrenaline stimulated melatonin production. Possibly, the

NPY-Y_1 receptor is coupled to two different second messenger systems: inhibition of adenylyl cyclase and elevation of intracellular calcium. Differences in exact experimental conditions could then explain the different findings. Nevertheless, NPY is a good candidate as a neuromodulator of the noradrenergic transmission and could be involved in the circadian and/or seasonal variations of melatonin production.

■ Other control mechanisms

A wide range of other receptor systems is reported to be present in the pineal gland. However, often there is no critical evaluation of the pharmacological properties of these systems.

Benzodiazepine receptors have been found in the pineal several times[99,211] and would enhance the adrenergic stimulated *N*-acetyltransferase activity.[415] Data on dopaminergic melatonin control are mainly derived from the bovine pineal. The presence of both dopamine D_1[324] and D_2-receptors[104] was reported. Their functionality is not fully understood yet, but dopamine exerts inhibitory effects on *N*-acetyltransferase activity in low concentrations, while stimulatory effects at high concentrations.[106] High levels of glutamate are also found in the pineal gland. This prompted the search for a glutamate receptor, which was indeed found.[105] Functionally, glutamate appeared to inhibit the noradrenaline stimulated *N*-acetyltransferase activity, while glutamate antagonists stimulated the basal activity of *N*-acetyltransferase.[108]

Furthermore, the presence of serotonin, substance P, vasopressin, adenosine A2 and σ-receptors has been claimed and the existence of even more receptor systems should not be excluded. However, pharmacological data are generally scarce and need to be completed.

The final conclusion must be that although the adrenergic innervation remains the absolute primary trigger for melatonin synthesis, many other regulatory mechanisms are present. The physiological importance, however still has to be proved in many cases.

1.4 Melatonin receptors

The lipophylic structure of melatonin enables it to penetrate tissues freely. From uptake studies there are no indications that accumulation occurs in specific regions. In order to exert its specific effects, it seems likely that specific melatonin receptors are involved in its physiological activity.

Early studies on putative melatonin receptors have been hampered by the lack of a suitable radioligand. Both tritium and ^{14}C labeled melatonin were used, but possessed poor specific activity. The development of 2-[^{125}I]-iodomelatonin for use in a radioimmunoassay,[379,380] was a real break through. Its high affinity for the putative melatonin receptor and its high specific activity (2000 Ci/mmol) were soon recognized as valuable properties of a useful ligand for melatonin receptors and boosted the search for melatonin binding sites. Binding studies in crude membrane preparations and quantitative autoradiography studies with 2-[^{125}I]-iodomelatonin have largely contributed to the localiza-

tion, characterization and classification of melatonin binding sites and the development of specific melatonin receptor agents. For reviews on characterization and classification, see references 85, 88, 165, 166, 231 and 340.

■ Localization

Because the central nervous system was the presumed main target for melatonin action, initially most binding studies have been performed on brain tissues. Later, also binding sites have been characterized in peripheral tissues.

The presence of 2-[^{125}I]-iodomelatonin binding sites in the brain appeared to be a common characteristic of all species. The distribution however, appeared to be quite different, varying from rather diffuse in lower vertebrates to concentrated in discrete brain areas in most mammalian species.

In rodents, such as the mouse, the rat and the hamster, high affinity 2-[^{125}I]-iodomelatonin binding sites have been identified in suprachiasmatic nuclei (SCN) of the hypothalamus and the paraventricular nucleus (PVN) of the thalamus. Receptors in these areas are likely to be involved in mediating effects on the circadian system, such as the ability to entrain biological rhythms (see page 32). The pituitary, located outside the blood-brain barrier, expresses binding especially in the pars tuberalis, sometimes indicated as median eminence.[328,388,403,414] In the rat, also binding in the area postrema is reported.[169] The neuroendocrine effects of melatonin are likely to be mediated by the action on receptors in the pituitary gland. The retina is reported to contain high concentrations of 2-[^{125}I]-iodomelatonin binding sites.[31,89] Together with the local system of melatonin synthesis, these receptors are thought to trigger dark-adaptive responses in the eye.

In humans, the distribution is quite similar, with one remarkable exception. No binding exists in the pars tuberalis of the pituitary gland.[287,404] In the frontal cortex, some diffuse binding is present. Binding in human brain however, has not been the subject of profound investigation, due to practical problems in obtaining suitable human tissue.

Peripheral tissues have also been investigated for the presence of melatonin binding sites. Tissues in which binding has been found, include the spleen,[429] Harderian gland,[197] adrenal gland,[241] gastro-intestinal tract,[177] human kidney,[333] arteries,[394] human spermatozoa[385] and a human malignant melanoma cell line.[426] Often, these binding sites require better characterization and clarification of their physiological importance.

In addition, specific 2-[^{125}I]-iodomelatonin binding sites have been reported on human T-lymphocytes.[198,204] The immuno enhancing properties of melatonin (page 21) may be mediated by its action on such receptors.

Localization of binding sites for melatonin is not restricted to the cell membrane. Nuclear melatonin binding sites were recently reported in rat liver,[2] human B-lymphocytes[342] and rat brain.[27] In the latter two studies, the binding sites were identified as the Retinoid Z Receptor a (RZRa) and RZRb respectively. These receptors belong to a superfamily of nuclear orphan receptors, that are involved in ligand-induced transcriptional control.

Taken together, the wide distribution of melatonin receptors in the periphery, may indicate a physiological importance of melatonin that exceeds its central role, presumably the photoperiodic and seasonal regulation. A more extensive discussion on the physiological role of melatonin and the possible receptors involved is given in section 1.5.

■ Characterization

Initially, binding sites for 2-[^{125}I]-iodomelatonin have been characterized by kinetic properties and ligand specificity. A high affinity binding site, with affinity in the picomolar range (10-300 pM), was identified with binding being rapid, stable, saturable and reversible. The relative order of potency of several melatonin related compounds is 2-iodomelatonin > melatonin > 6-hydroxymelatonin > > *N*-acetylserotonin > prazosin > serotonin. Besides the high affinity state of this binding site, various reports indicate the presence of a low affinity state (0.3-5 nM) as well. This low affinity state has been demonstrated by saturation studies using high concentrations of radioligand and by the addition of monovalent cations, such as Na^+ and Li^+. Also the addition of guanine nucleotides, such as GTPγS, and the sulphydryl alkylating agent *N*-ethylmaleimide, shift the binding site from high affinity to a low affinity state.

In hamster brain and the RPMI1846 melanoma cell line also a low affinity 2-[^{125}I]-iodomelatonin binding site (0.9-10nM) has been identified.[90,254] Although often considered as the low-affinity state of a high affinity receptor, this binding site shows marked differences. Kinetics of this binding site are extremely fast, so that incubations have to be carried out at 4 °C. Binding is not sensitive to guanine nucleotides or monovalent cations. Most importantly, the relative order of potency of several melatonin related compounds is 2-iodomelatonin > prazosin > *N*-acetylserotonin > 6-hydroxymelatonin > melatonin > > serotonin. The resulting lack of correlation between 2-[^{125}I]-iodomelatonin binding in chicken retina and 2-[^{125}I]-iodomelatonin binding in hamster brain was an important argument to classify this binding site as a subtype of the melatonin receptor. The proposed nomenclature was ML_1 for the high affinity site (chicken and rabbit retina) and ML_2 for the low affinity site (hamster brain).[85]

In order to be characterized as a receptor, a binding site should not merely bind its ligand with high affinity and specificity. Especially a functional response as a consequence of occupation by an agonist should be characterized.

The first such functional response was reported in amphibian skin melanophores (for review, see ref. 293). Melatonin's ability to aggregate pigment granules to the perinuclear region, the basis for its discovery, appeared to be mediated by inhibition of the melanocyte stimulating hormone (MSH) induced increases in intracellular cAMP levels. Similarly, melatonin was capable of counteracting forskolin induced granule dispersion. Forskolin is known to stimulate adenylyl cyclase activity directly. This inhibitory effect on forskolin stimulated accumulation of cAMP could be blocked by pertussis toxin, an agent that selectively inactivates inhibitory guanine nucleotide binding proteins (G_i-proteins). Pharmacological profiling with a series of melatonin analogues subsequently identified the melatonin receptor as an ML_1 type. Later, melatonin appeared to inhibit forskolin stimulated accumulation of cAMP in the pars tuberalis region of the pituitary in a variety of vertebrates.[44,230,237] This functional response was repeatedly used as a functional test for melatonin analogues.[75,127]

In rabbit retina another functional response, correlated to stimulation of the 2-[^{125}I]-iodomelatonin binding site, was identified. Melatonin appeared to be very effective in inhibiting the calcium dependent dopamine release.[83] Large series of melatonin analogues have been tested in this system, including the first melatonin antagonist, luzindole (see page 28).[64,84,86] A marked correlation between IC_{50} values for dopamine release inhibition

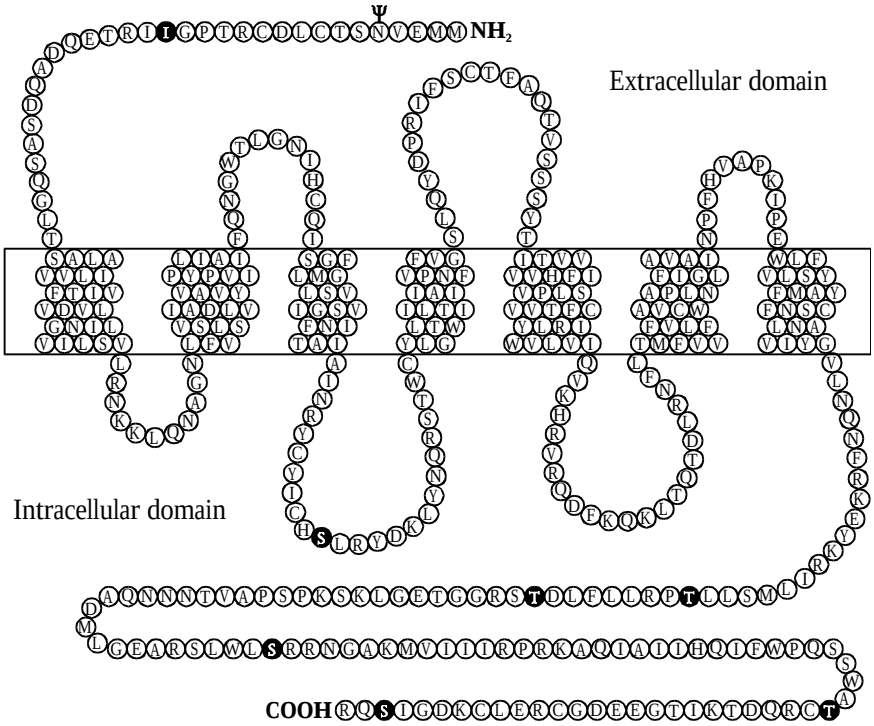


Figure 1.5 Structure of the Xenopus melatonin receptor as proposed by Ebisawa et al.[94] The solid circles are putative phosphorylation sites, the anchor (Ψ) marks a putative N-linked glycosylation site.

in rabbit retina and affinity constants for 2-[^{125}I]-iodomelatonin binding sites in chicken retina strongly suggested the characterization of a melatonin receptor, referred to as the ML_1 subtype.

The sensitivity of 2-[^{125}I]-iodomelatonin binding towards guanine nucleotides and the pertussis toxin sensitive inhibition of forskolin stimulated accumulation of cAMP in amphibian melanophores and several vertebrate tissues, all strongly suggest the ML_1 receptor to be coupled to a G_i-protein. This is extended by a possible coupling to a G_z-protein by very recent studies in cell lines expressing the *Xenopus laevis* recombinant receptor.[430]

■ Cloning the receptor

For many years, molecular biologists have been searching for the gene encoding the melatonin receptor. Recently, cDNA encoding the melatonin receptor was isolated from *Xenopus laevis* melanophores, using expression cloning with mRNA.[94] The receptor appeared to be a protein of 420 amino acids, with seven hydrophobic regions, generally considered as transmembrane domains (Fig.1.5). The little sequence homology to known G-protein coupled receptors explains the difficulties many researchers encountered in

cloning the receptor and characterized the melatonin receptor as a member of a new sub-family of G protein coupled receptors.

The cDNA encoding the receptor was transiently expressed in COS-7 cells. Characteristics of 2-[^{125}I]-iodomelatonin binding were identical to the ML_1 subtype. Also pharmacological characteristics, in terms of rank order in affinity of related melatonin analogues and the inhibition of forskolin-stimulated cAMP accumulation (CHO cells) identified the protein as the well known high affinity melatonin receptor.

By now, also the melatonin receptor in human (350 amino acids) and sheep (366 amino acids) has been cloned, as well as fragments of hamster and rat receptors.[288] The homology between mammalian and *Xenopus* melatonin receptors appeared to be 60%, within the hydrophobic regions 77%. Between sheep and human, the overall identity was 80%, while 87% in the transmembrane domains. Based on pharmacological characteristics, it is suggested that all receptors identified are species homologues of the same receptor. The human chromosome containing the melatonin receptor gene (MTNR1A) is identified as chromosome 8.[330]

The results reported here are extremely important in melatonin receptor research. Knowing the base pair sequence of the gene encoding the receptor, enables *in situ* hybridization with antisense cDNA to localize melatonin receptors. Early results from hamster brains indicate specific signals from pars tuberalis, SCN and PVN. Specific subtypes may be identified in the near future, and may answer the questions about the putative ML_2-receptor, which existence is still matter of debate. Cell lines expressing the receptor will lead to the development of very specific agonists and antagonists (see section 1.6).

1.5 Physiological role of melatonin

At early stages, the pineal gland was considered to be a kind of rudimental organ in mammals. Studies over the past decades have proved the contrary, because its main secretory product, melatonin, appeared to have a modulatory function in a variety of systems. This paragraph summarizes the interaction between this ubiquitously acting hormone and a wide range of physiological processes.

■ Neuroendocrine role

An important issue that has been subject of extensive studies over the years are the effects of melatonin on the neuroendocrine system, especially on the hypothalamic-pituitary axis and the resulting role in reproductive physiology. Many review articles have addressed this issue.[9,43,276,279,281,359]

Most studies on the role of melatonin in the reproductive systems have been carried out in hamster and sheep. Both species are so-called seasonal breeders, i.e. their reproductive status changes with photoperiodic changes in the environment, whereas hamsters

are long day breeders, while sheep are short day breeders. In rats, a more commonly used laboratory animal, the reproductive system appeared to be not or only minimally responsive to manipulations of the pineal gland. The reasons for this are not known, but it might be a result of the highly inbred nature of the laboratory rat, which has been selected for maximal reproductive efficiency.

The reproductive status of hamster appears to be highly dependent on photoperiod. Short photoperiods induce a pronounced regression in testicular weight. The critical length of the daily light period is 12.5 h. When the light period increases, a normal testicular size will result. An important finding was that this dependency on photoperiod had disappeared after pinealectomy. Pinealectomized hamsters in short photoperiods developed normal gonads. Superior cervical ganglionectomy resulted in a similar effect. Because it is known that pinealectomy and superior cervical ganglionectomy completely block the night-time increase in plasma levels of melatonin, these results indicated an antigonadotropic effect of the pineal hormone. Furthermore, it appeared that this reproductive involution was accompanied by marked reductions in plasma concentrations of luteinizing hormone (LH), follicle stimulating hormone (FSH), testosterone and prolactin.

The antigonadotropic action of melatonin was further demonstrated by Tamarkin et al.,[355] who showed that also exogenous melatonin was able to cause an atrophy of the reproductive organs in male and female hamsters under a long photoperiod. Interestingly this effect was evident when melatonin was injected late during the light period, but absent when injections occurred late in the dark period. In pinealectomized hamsters however, melatonin injections late during the light period failed to induce gonadal regression,[355] whereas repeated injections during the course of a day did induce gonadal involution.[356] These findings suggested that especially the duration of elevated melatonin concentrations is important in the control of the reproductive system.

In contrast to hamsters where melatonin appeared to be antigonadotropic, insheep, administration of melatonin in long days could stimulate the reproductive activity, hence a progonadotropic effect was involved.[7,154] Therefore, the action of melatonin cannot be described as pro- or antigonadotropic. More importantly, melatonin must be considered as a crucial factor in the transduction of photoperiodic information.

As far as the mechanism of action of melatonin is concerned, there is strong evidence that the central neuroendocrine control mechanisms are the primary sites of action. Placing micro-implants of melatonin in specific brain areas, revealed the hypothalamus as the central target area, but also the pars tuberalis of the pituitary may be an important site in mediating melatonin's actions.[187] Amplitude and frequency of pulsatile LH secretion may be the most important processes under melatonin control, either by direct interactions, or by effects on the LH releasing hormone.

The neuroendocrine actions of melatonin are not restricted to the reproductive system. Many other processes, regulated by the endocrine system, either seasonal dependent or not, appear to be affected by melatonin, such as coat growth, pelage, body weight, lactation, hibernation, insulin release, gastro-intestinal physiology etc. The wide variety of actions of melatonin led to suggestions that no single organ can escape the influence of the ubiquitously acting hormone melatonin.

Puberty

One particular interaction of melatonin with the neuroendocrine system is its proposed action on the timing of puberty, a subject that is especially interesting from a clinical point of view. In highly photoperiodic animals, such as hamsters, sheep and goats, the circadian profile of melatonin production has unarguable effects on development of the reproductive system. Generally the photoperiod in relation to body weight determines the timing of puberty, but precocious development of the reproductive system can be achieved when time of birth is delayed and autumn-born lambs are kept in short photoperiod.

In species that are much less photoperiodic, such as rats and humans, the effects are less clear cut, although reduction of pituitary gonadotropin releasing hormone (GnRH) receptor content, plasma LH and FSH in prepubertal rats has been described following daily melatonin injections late in the afternoon.[173] This melatonin induced delay of puberty however, is dependent on the strain of rats used.

■ Immune system

The relationship between melatonin and the immune system has been subject of a large number of studies during the past years (for reviews, see refs.110, 204 and 203). It appeared that pinealectomy or the suppression of pineal melatonin production by either propranolol or the inhibitor of serotonin biosynthesis *para*-chlorophenylalanine resulted in reduced immune responses towards T-dependent antigens. This immunosuppressive effect could be counteracted by injections with melatonin in the late afternoon, not in the morning. These immunostimulatory effects of melatonin could be antagonized by the opioid antagonist naltrexone. In addition, pinealectomy appeared to reduce interleukin-2 levels in mice, as well as the associated natural killer cell activity. These findings led to the conclusion that melatonin exerts its effects on the immune system through the release of opioid peptides and interleukin-2 from T-lymphocytes. The presence of 2-[^{125}I]-iodomelatonin binding sites on human blood lymphocytes is in support of a direct action of melatonin on T-cells.

Interestingly, the interaction between the pineal gland and the immune system appeared to involve a two-way mechanism. It was shown that γ-interferon, a substance produced by T-helper lymphocytes following antigen stimulation, could enhance isoprenaline induced melatonin production from cultured rat pineal glands. Remarkably, this activation of melatonin production was accompanied by reduced *N*-acetyltransferase activity rather than increased activity. It was therefore suggested that the stimulatory effect of γ-interferon on pineal melatonin production was mediated by an inhibition of the metabolism of serotonin to 5-hydroxyindole acetic acid, increasing the precursor availability for melatonin synthesis.

Recently, the effect of melatonin on T-helper cells has been specified in more detail. The 2-[^{125}I]-iodomelatonin binding sites appeared to be located on T-helper type 2 cells. From these cells, the release of interleukin-4 was induced by melatonin. Interleukin-4 and/or interleukin-5 then interact with T-helper cells type 1, from which the release of interleukin-2 and γ-interferon is regulated. The proposed role of melatonin is to establish an appropriate balance between T-helper cells type 1 and 2 and is therefore suggested to be a immune balancing agent.

■ Cancer

An action of melatonin that might be related to the immuno enhancing effect, is its inhibitory action on tumor growth. This effect was most strikingly demonstrated by Tamarkin et al.[357] In their studies, rats were treated with 1,17-dimethylbenzanthracene (DMBA), resulting in the development of mammary tumors. Interestingly, the incidence of tumor development was highly increased in pinealectomized rats compared to controls. Moreover, exogenous melatonin administration could reverse the effect of pinealectomy to a great extent. The apparent melatonin induced suppression of prolactin was suggested to be the underlying mechanism, because prolactin is thought to promote mammary cancer. Subsequent studies indicated the suppressive effect of melatonin on various tumors in rats, hamsters and mice.

In addition, tumor growth appeared to be dependent on photoperiod as well.[319] Rats kept in LL conditions were more sensitive to DMBA induced mammary cancer and showed increased proliferative activity of mammary epithelium. This effect could be partially reversed by administration of melatonin. Similarly blinding appeared to suppress the incidence of DMBA induced mammary tumors.

The implications for the use of melatonin in human cancer treatment is discussed on page 46.

■ Circadian rhythms

An important property of melatonin is its ability to influence circadian rhythmicity.[14,46,49D] From early studies it appeared that pinealectomy did not affect free-running activity rhythms. Although it was shown that pinealectomy enhanced the rate of re-entrainment after a phase shift[267] and disrupt circadian rhythmicity in continuous light,[48] the importance of melatonin in the circadian system was underestimated. The fascinating role of melatonin in circadian rhythmicity became apparent when melatonin was applied exogenously. Redman et al.[272] showed that daily melatonin injections could entrain rat free-running activity rhythms in constant darkness (DD) when the time of injection coincided with the onset of activity. Saline injections at the same time appeared to be ineffective.

The entraining properties of melatonin in DD were also envisaged in starlings[112] and lizards,[376] but opposite to the effects in rats, they appeared to respond to melatonin only when it was administered at the beginning of the quiescent period. In contrast to rats, who are nocturnally active animals, starlings and lizards are diurnally active animals, a difference that was supposed to be responsible for the different window of sensitivity towards entrainment with melatonin.

The mechanism behind the entrainment effect of melatonin is considered to be consisting of daily phase advances upon each administration of melatonin. As long as the endogenous tau (τ) period is not too long, this will result in entrainment (see page 32). The window of sensitivity, also called the gate of circadian frequency, is demonstrated in a phase-response curve (Fig. 1.12). Clearly, in rats phase advances only occur when melatonin is applied shortly before the subjective night. In humans, a similar window for phase advances is present, but in addition also a small window for phase delays, when melatonin is administered early in the morning.[185]

The phase advancing effect of melatonin treatment is also the basis for another circadian effect of melatonin, namely the reversal of the direction of re-entrainment after a phase shift.[275] When a phase shift in the LD cycle is applied of 5-8 h, normally rats respond by phase-delaying to the new LD cycle. However, daily injections with melatonin at the new onset of darkness cause all animals to phase advance to the new LD cycle.

Circadian effects of melatonin are not restricted to activity rhythms. Also pineal *N*-acetyltransferase activity[129] and pineal melatonin production[80] can be entrained in DD by daily melatonin administration. Whether all circadian variables are under phase-shifting and entraining control of melatonin is not clear. However, studies demonstrating a direct phase-advancing effect of melatonin on *in vitro* electrical activity of the SCN in slice preparations,[212] indicate that the phase-shifting effects of melatonin are mediated by effects on the SCN. Because the SCN is considered to be the main endogenous pacemaker, by which merely all circadian processes are controlled (see page 35), entraining and phase-shifting effects of melatonin on other circadian variables should not be excluded.

The effects of melatonin on circadian systems are important in the development of new melatonergic agents. The specific actions of melatonin which can be mimicked by melatonin agonists include reversal of the direction of re-entrainment,[273] entrainment in DD[32] and phase shifting effects.[15] In the last case an elegant animal model of delayed sleep phase syndrome (see page 42) was used. When rats were maintained in DD for several months and then transferred to a normal LD cycle, they showed a negative phase angle difference, i.e. the onset of activity was approximately 3-4 h behind the onset of darkness. Melatonin or melatonin agonist treatment at the onset of darkness then clearly reduced this negative phase angle difference to about 0.

The entraining effects of melatonin on endogenous melatonin production is extensively studied in this thesis and applied in an animal model to test melatonin agonists (chapter 7).

■ Radical scavenger

Many functions of melatonin are suggested to be receptor mediated. Since 1993 however, accumulating evidence has been gathered that melatonin can also act as a radical scavenger. As such, its action would be dependent on its chemical structure itself rather than the interaction between its structure and a receptor. The first indications for such a

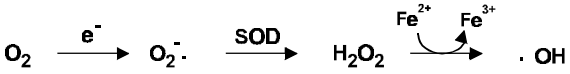


Figure 1.6 The mechanism by which highly toxic and reactive hydroxyl radicals are formed. Oxygen captures an electron to form the superoxide anion ($O_2^{\cdot-}$). This superoxide anion is converted by the enzyme superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2). Hydrogen peroxide can be metabolized in various ways, one of which is the so-called Fenton reaction. In this reaction a transition metal (mostly Fe^{2+}) is oxidized resulting in the formation of the hydroxyl radical.

functionality were derived from observations that Ca^{2+}-pump activity in the heart showed a circadian rhythm with highest activity during the night, which disappeared after pinealectomy.[53] Since it was known that Ca^{2+}-pump (Ca^{2+}-ATPase) activity was suppressed by free radicals,[149] a free radical neutralizing activity of melatonin was suggested. Later studies[54] supported this hypothesis.

Free radicals, bearing one unpaired electron, are extremely reactive and can damage other (macro)molecules. The reactivity and therefore the toxicity of radicals varies. One of the most reactive and dangerous ones is the hydroxyl radical (•OH). It is formed in a cascade of reactions that start with molecular oxygen (Fig. 1.6). By capturing an electron, the superoxide anion (O_2^-) is formed. It is believed that approximately 5% of all O_2 taken in will be converted into this superoxide anion. The relatively harmless O_2^- is catalytically converted by superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2).[213] H_2O_2 is not a radical, it has a relatively large half-life and can be converted in a number of ways. It can be reduced by catalase or glutathion peroxidase to H_2O. However, as a worst case scenario, in the presence of transitions metals such as Fe^{2+}, it can also be reduced to a hydroxyl radical in a process called the Fenton reaction (Fig. 1.6). Besides the hydroxyl radical, also other radicals that are produced *in vivo* can be toxic. The peroxyl radical for example is feared for its self-sustaining effect. One peroxyl radical can abstract hydrogen atoms from other macromolecules which then become radicals themselves. This cascade of reactions is the basis for lipid peroxidation.[102] Furthermore, nitric oxide (NO•), a radical that appears to be involved in a lot of important physiological functions,[227] can be converted to the peroxynitrite anion ($ONOO^-$) which is highly toxic.[28]

The continuous danger of radical formation is generally referred to asoxidative stress. This oxidative stress can be increased by toxins, excessive exercise, radiation, infection, ischemia etc. However, even when all risk factors are kept to a minimum, organisms are continuously exposed to a certain amount of oxidative stress, which is suggested to be related to degenerative diseases and/or aging.[345,363] The defense system of an organism consists of different components. Enzymes, such as superoxide dismutase, glutathion peroxidase and catalase, free radical scavengers such as vitamin C and E, transition metal binders etc. all neutralize free radicals or prevent their formation. The capacity of melatonin to react with free radicals imposes the question whether it is a functional part of the defense mechanism, or an interesting phenomenon of a chemical substance which has no physiological implications.

Most studies thus far have focused on the capacity of melatonin as a scavenger of hydroxyl radicals,[361,264] although it has recently been reported that peroxyl radicals are affected as well.[255,258] Since hydroxyl radicals have an extremely short half-life, it is virtually impossible to detect them directly. The use of a spin-trapping agent, such as 5,5-dimethylpyrroline N-oxide (DMPO) which forms adducts with the hydroxyl radical that are detectable with HPLC and electrochemical detection, is generally used to get an indirect measurement of hydroxyl formation. *In vitro*, melatonin was effective in reducing the formation of DMPO-•OH adducts.[361] By comparing the action of melatonin with *N*-acetylserotonin and serotonin, it could be derived that the structural requirements for its scavenging properties are an indole structure with a methoxy group on the five-position, while an *N*-acetyl group, although not necessary, acts synergistically. Additionally,

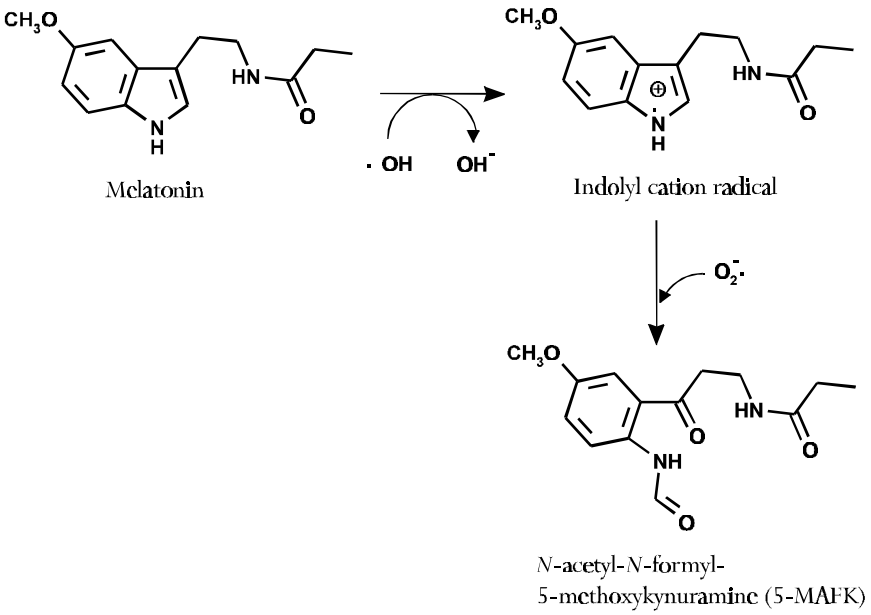


Figure 1.7 Possible mechanism by which melatonin scavenges hydroxyl radicals. Primary target for the hydroxyl radical is the nitrogen of the indole nucleus. One electron is withdrawn from the nitrogen lone pair, resulting in an indolyl cation radical. In the subsequent reaction of this indolyl with a superoxide anion radical, the indole ring structure is broken and *N*-acetyl-*N*-formyl-5-methoxykunuramine (5-MAFK) is the final product.

introduction of a hydroxyl-group as in serotonin and 6-hydroxymelatonin increased the scavenging properties towards peroxyl radicals.[258] The exact mechanism of the neutralization of hydroxyl radicals is not clear yet, but a possible mechanism has been described.[116] The hydroxyl radical interacts with melatonin to yield an indolyl cation (Fig. 1.7). A superoxide anion radical reacts with the indolyl cation to yield 5-methoxy-*N*-acetyl-*N*-formyl-kynuramine (5-MAFK). The overall effect of this mechanism is the neutralization of a hydroxyl radical and a superoxide anion radical, without the production of new radicals or prooxidative compounds.

Several studies have indicated that the antioxidant activity of melatonin may result in therapeutically interesting properties. Cataract formation in newborn rats is inhibited by melatonin,[1] DNA damage following ionizing irradiation of human lymphocytes was reduced in the presence of melatonin,[393] and DNA adduct formation induced by the carcinogen safrole was inhibited by melatonin.[362,360] Based on these and other findings, speculations on the role of melatonin as an anti-aging hormone have been widely discussed and reviewed,[307,264,283] but a definite proof of such an effect has not been described yet.

Apart from direct data that emphasize the role of melatonin as a radical scavenger, some circumstantial evidence has been put forward enabling its role in the antioxidative

defense mechanism. Its high lipophylicity enables it to cross cellular and nuclear membranes easily. Reports actually indicate that melatonin is present both in the cytosol and the nucleus.[218] Especially the ability to penetrate the cell nucleus makes a direct DNA protective function possible. Furthermore, melatonin levels decline with age, which is impaired to degenerative processes that increase with age. In contrast, melatonin levels are low during the first 2-3 months after birth, a period of development in which protective systems against oxidative stress would be beneficial. Melatonin is a well preserved molecule from an evolutionary point of view. The compound is present in organisms varying from the dinoflagellate *Gonyaulax polyedra*[263] to humans and probably in most, if not all organisms. A molecule that is that well preserved, may have an important physiological function.

It is obvious that melatonin has radical scavenging properties. The extent to which this is of physiological importance is not clear yet. Especially further *in vivo* research is needed to prove most of the suggested functions mentioned above. Therefore it is way too early to declare melatonin as an anti-aging drug, but the possibility that it might be that way justifies a close attention to this field of research.

■ Aging

One of the claimed actions of melatonin that probably appealed most to anyone's imagination is its supposed anti-aging effect (for a recent review, see ref.284). Especially the studies of Pierpaoli and co-workers have attributed to speculations on the pineal being an aging clock.[202,256,257] By applying melatonin to the drinking water of different strains of mice, they reported increased life span by about 20% and generally a more youthful state of the animals. In addition, it appeared that implantation of a pineal gland from a young mouse into the thymus of an aged recipient resulted in similar increases in survival and improved morphological structure of the thymus. The authors suggest that the melatonin produced by the grafted pineal gland, through its immunoenhancing effect, is responsible for the increased life span. This would imply a reestablished melatonin production in the transplanted gland, most likely caused by a functional re-innervation. Other studies have indicated that reestablishment of normal pineal functionality after transplantation is very difficult and it has only been described for grafts in the anterior chamber of the eye.[421] Interpretation of these results as effects being mediated by melatonin, therefore requires additional information that melatonin is produced in sufficient amounts following transplantation.

The role of melatonin in the process of aging is suggested more often. It is known that restricted food intake can increase life span by about 30%.[210] Interestingly, restricted food intake also preserves the melatonin rhythm at a high level.[343] As with previous reports, this does not imply a causal relationship, but the effects are interesting enough to speculate about a possible mechanism of the anti-aging properties of melatonin.

In this respect, it is confusing that *N*-(2,4 dinitrophenyl)-5-methoxytryptamine (ML-23), a putative melatonin antagonist, was not able to inhibit the melatonin induced increase of life span in aged rats, but was in fact inducing longevity itself.[238]

As Pierpaoli and co-workers suggested, the longevity induced by melatonin is related to its immunoenhancing effects (see page 21). An alternative explanation is hypothesized by Armstrong and Redman.[16] With increasing age, the amplitude of the melatonin profile

decreases. They suggested that because of these lower melatonin levels, the circadian system becomes destabilized. An unbalanced circadian system then would lead to internal temporal disorder, a possible precursor of disease states. Their conclusion therefore was that the anti-aging effects of melatonin are related to its action as a chronobiotic.

As mentioned in the previous paragraph (page 23), melatonin has antioxidant effects and may be an important endogenous radical scavenger. Because aging processes are generally accompanied by radical reactions, this might be an alternative explanation for the anti-aging effect of melatonin administration.

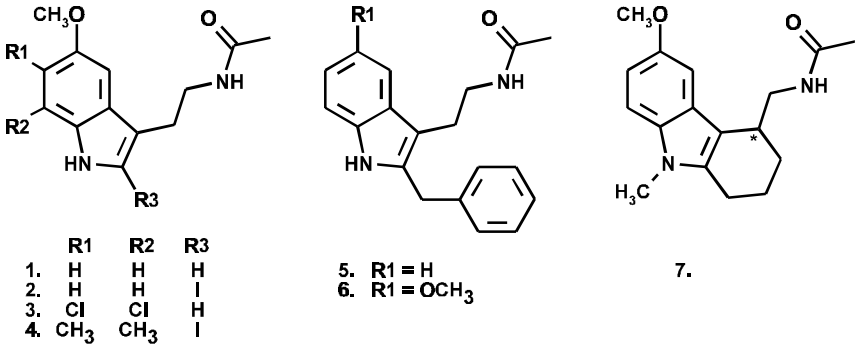
Despite all questions about the complex mechanisms behind aging processes in general and the involvement of melatonin in particular, there are indications that the aging process can be influenced, which makes research in this field a challenging venture.

1.6 Melatonin agents

From the early discovery of melatonin on, there has been a search for compounds that could mimic or counteract the effects of melatonin. The development of such agonists and antagonists is important for several reasons. In pharmacological research, it is important to have both agonists and antagonists. The blockade of an agonist effect by an antagonist is the absolute proof that the agonistic effect is receptor mediated. Secondly agonists can reveal differences between subtypes of the same receptor, for which the endogenous model compound normally has small selectivity. Also from a therapeutical point of view, specific ligands can be advantageous. The short half-life of melatonin may be a serious drawback for its clinical use. Agonists could be modified to increase the bioavailability and the metabolic stability. In addition, newly developed antagonists could have therapeutic applications that melatonin itself is obviously lacking. Finally, because new chemical entities can be patented while melatonin itself can not, the development of synthetic ligands to clinically used drugs can be commercially interesting.

The development of 2-[^{125}I]-iodomelatonin as a high affinity radioligand for the melatonin receptor has enabled receptor binding studies in retina and brain. Furthermore, the many physiological functions as described in section 1.5, have resulted in many test models in which potential melatonin agents could be tested for their pharmacological activity. The most commonly used *in vitro* models include pigment aggregation in *Xenopus Laevis* melanophores, the inhibition of dopamine release from rabbit retina and the inhibition of forskolin stimulated cAMP accumulation in pars tuberalis cell cultures. In the *in vivo* test models, the melatonin induced testicular regression in hamsters, entrainment of locomotor activity under free-running conditions and the reversal of the direction of reentrainment after an 8 h phase advance have provided important pharmacological information of the newly developed melatonin agents.

The thesis of Dr. S. Copinga[65] includes an extensive survey of melatonin (ant)agonist development and the structure-activity relationships of these agents. The present section will only briefly summarize some of the most important issues, supplemented with some recent developments.



	R1	R2	R3
1.	H	H	H
2.	H	H	I
3.	Cl	Cl	H
4.	CH_3	CH_3	I

5. R1 = H
6. R1 = OCH_3

7.

Figure 1.8 Chemical structures of various putative melatonin (ant)agonists based on the indole nucleus.

■ Indole structures

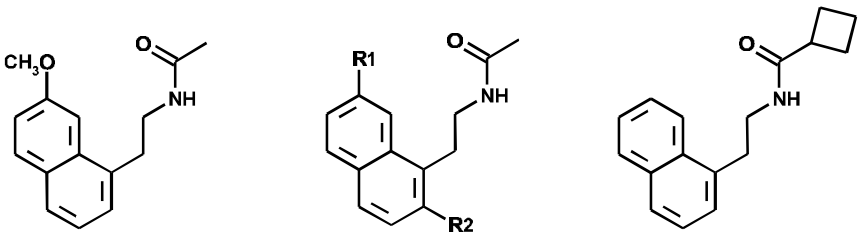
Not surprisingly, the first series of melatonin (**1**) agents were based on the indole nucleus (Fig. 1.8). A wide range of related compounds was synthesized. Structure-activity relationships revealed the presence of a methoxy moiety at the 5-position as well as the amide function in the side chain as essential.[122,166,334] The substitution of halogens and/or a methyl group in the indole nucleus resulted in equipotent or even more potent melatonin agonists (**2**, **3**, **4**), with enhanced metabolic stability. A good example is 2-iodomelatonin (**2**), which has higher affinity than melatonin itself and of which the radioactive analogue has proven to be an extremely useful radioligand.

Also the first well characterized melatonin antagonist luzindole (**5**), was based on the indole structure. The introduction of a benzyl group on the 2-position decreased the affinity for the melatonin receptor, but changed the activity from a full agonist to a competitive antagonist *in vitro*.[84] The data from *in vivo* experiments are somewhat controversial in that luzindole was not able to antagonize the melatonin induced regression of testicular weight in hamsters,[91] but did show anti-depressant activity in a behavioral despair test in mice, an effect that was counteracted by the administration of melatonin.[87] Introduction of a methoxy group on the 5-position, resulting in 5-methoxy-luzindole (**6**), markedly increased the affinity for the melatonin receptor, but changed its pharmacological activity to a partial agonist.

Recently a conformationally restricted analogue of melatonin, *N*-acetyl-4-aminomethyl-6-methoxy-9-methyl-1,2,3,4-tetrahydrocarbazole (AMMTC, **7**) was described.[350] The fixation of the side chain resulted in the introduction of a chiral carbon atom. The enantiomers were separated and the (-)-enantiomer appeared to have a slightly higher affinity than melatonin and was also slightly more effective in the aggregation of pigment granules in *Xenopus laevis* melanophores. In contrast, the (+)-enantiomer was 130-fold and 230-fold less potent in binding and pharmacological activity respectively. The development of such chiral melatonin analogues with conformationally rigid structures may provide valuable information for modelling the melatonin receptor binding site.

■ Naphthalene structures

The replacement of the indole nucleus by the bioisosterically related naphthalene structure resulted in a large series of potent melatonin agents (Fig. 1.9).[75,174] The homologue of melatonin (**8**), *N*-(2-(7-methoxy-1-naphthalenyl)ethyl) acetamide, which has become known as S-20098, appeared to be a selective full melatonin agonist with binding properties and *in vitro* pharmacological activity in the same range as melatonin itself, or even better, depending on the test system. Derivatives of this structure with modifications in the *N*-acyl moiety, included compounds that possessed extremely high affinity for the melatonin receptor, with K_D values in the femtomolar range. When the methoxy group was located at the 2-position instead of the 7-position (**9**), also a high affinity putative melatonin agonist was obtained. Possibly the flexibility of the acyl chain enabled a sufficient distance between the amide function and the methoxy group, a crucial parameter for binding properties. The introduction of a second methoxy group, resulting in a 2,7-dimethoxy substituted compound (**10**), increased binding affinity substantially. It was suggested that in this case the 7-methoxy bound to the same receptor site as the 5-methoxy in melatonin, while the 2-methoxy could enhance affinity similar to halogen substitution at the 2-position in melatonin.



8. **9.** R1 = H, R2 = OCH_3 **10.** R1, R2 = OCH_3 **11.**

Figure 1.9 Chemical structures of various putative melatonin (ant)agonists based on the naphthalene nucleus.

The compound S-20098 has been pharmacologically characterized in a number of pharmacological studies over the past years. S-20098 was effective in entraining the free-running activity rhythms in rats almost as well as melatonin.[32] The compound was equipotent to melatonin in phase advancing the onset of activity towards the onset of darkness in rats with a negative phase angle difference, an animal model of delayed sleep phase syndrome.[15] In addition, S-20098 acted similar to melatonin on EEG spectra of rats.[368] Also S-20098 reversed the direction of re-entrainment after an 8 h phase advance, similar to melatonin.[274]

Taken together, S-20098 is about equally effective as melatonin *in vivo*. Unfortunately, the solubility of the compound is substantially lower than that of melatonin, which may influence the bioavailability in a negative way. Data on the metabolic stability of the

compound may provide additional information concerning the (dis)advantages of the compound compared to melatonin.

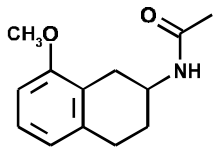
In the series of naphthalenic agents one compound, *N*-(2-(1-naphthyl)ethyl) cyclobutyl carboxamide (S-20928, **11**) is suggested to be a putative melatonin receptor antagonist.[427] There is evidence that S-20928 reverses the melatonin induced pigment aggregation in *Xenopus laevis* melanophores and that it blocks the inhibitory action of melatonin on forskolin stimulated cAMP accumulation in ovine pars tuberalis cells. In addition, S-20928 antagonized the inhibitory action of both melatonin and S-20098 on light induced electrical activity in the SCN.

■ Tetralin structures

The successful development of tetralin based compounds as potent serotonin agents and the structural similarities between serotonin and melatonin have initiated the synthesis of a series of putative melatonin receptor agents, based on the tetralin structure (Fig. 1.10).[64,65] The melatonin analogue 2-acetamido-8-methoxytetralin (AH-001, **12**) is about 100-fold less potent in the competition for 2-[^{125}I]-iodomelatonin binding sites in chicken retina and also about 100-fold less potent in the inhibition of dopamine release from rabbit retina compared to melatonin. The chloroacetyl analogue (AH-017,**13**) is only about 6-fold less potent in binding and about 4-fold less potent in pharmacological activity compared to melatonin. Both AH-001 and AH-017 are tested in the entrainment model described in chapter 7.

The conformationally restricted chemical structure of these compounds will be helpful in the modelling of the active site of the melatonin receptor as has been described recently.[143,144]

The introduction of a phenyl group at the 4-position of the tetralin ring structure and the omission of the 8-methoxy group resulted in a compound (**14**) that competed for the 2-[^{125}I]-iodomelatonin binding site with modest affinity, but showing clear antagonistic properties in the melatonin induced inhibition of dopamine release from the rabbit retina (unpublished data).



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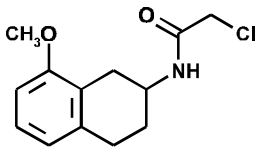

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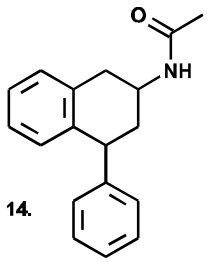

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Figure 1.10 Chemical structures of various putative melatonin (ant)agonists based on the conformationally restricted tetralin structure.

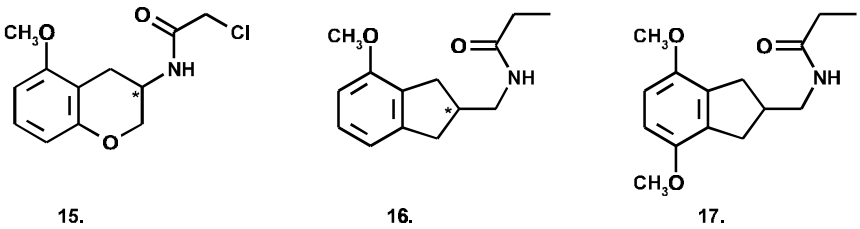

Figure 1.11 Chemical structures of various putative melatonin agonists based on both chroman and indane sturcutres.

■ Other structures

Besides the bioisosteric replacement of the indole nucleus by naphthalene and tetralin structures, some other approaches have resulted in moderately potent melatonin agents. Sugden[349] reported a small series of chroman derivatives (Fig. 1.11). *N*-chloroacetyl-3-amino-5-methoxychroman (**15**) was the most potent compound, with affinity for 2-[^{125}I]-iodomelatonin binding sites in chicken brain in the high nanomolar range. The poor correlation between affinity for 2-[^{125}I]-iodomelatonin binding sites and potency to induce pigment aggregation in *Xenopus laevis* melanophores, led to the conclusion that these compounds may possess some selectivity for melatonin receptor subtypes.

Another series of melatonin analogues is based on the indane structure (Fig. 1.11) (unpublished data). These compounds have increased flexibility in the acyl chain compared to the tetralin structures which could enhance the affinity. Compared to melatonin, these structures may be metabolically more stable. Also, the introduction of a second methoxy group at position 7 (**17**) would induce symmetry of C_2. The most potent analogue of this series of agents is 4-methoxy-2-methylene-propionamide-indane (**16**), which is has about 20-fold less affinity for 2-[^{125}I]-iodomelatonin binding sites in the chicken retina than melatonin. In the dopamine release model, the compound appeared to be a partial agonist. In chapter 7 this compound is tested in an entrainment study.

1.7 Chronobiology

The earth's rotation generates daily changes of the natural environment in terms of light intensity and temperature. The angle of the earth's rotation axis with the ecliptic (23.5°) and its rotation around the sun results in annual changes in day length and seasonality. The necessary accommodation of organisms to this changing environment has resulted in daily and seasonal changes of physiological and behavioral parameters. In fact, life on earth has developed a wide variety of systems that continuously enable the organism to be optimally adjusted to the environment. The discipline that investigates this rhythmicity in life is chronobiology. For an extensive overview of the various aspects of chronobiology, and especially the clinical implications, the reader is referred to ref. 372.

Because terminology and concepts used in chronobiology are not generally familiar, this section will provide the reader with an overview of the most crucial aspects of chronobiology. Because of the scope of this thesis, the main emphasis will be put on mammalian species and its application to melatonin production.

■ Ultradian, circadian and infradian rhythmicity

The repeated changes in physiological and behavioral processes that show periodicity are called biological rhythms. A rough classification of biological rhythms is based on length of period. When the period of rhythms is substantially shorter than one day, they are known as ultradian. Periodicity of roughly 24 h is referred to as circadian, whereas periods that are substantially longer than 24 h are called infradian. Ultradian rhythms are very common and widespread (heart rate, breathing, pulsatile secretion of LHRH from the hypothalamus, intestinal motility etc.). From all circadian rhythms, the sleep/wake cycle may be the best known, but many other processes show circadian rhythmicity. Hormone secretion of cortisol, prolactin and melatonin, temperature, metabolism etc. all show pronounced daily changes and are circadian. Infradian rhythms are often related to reproduction, temperature adjustment and food intake. Estrous cycle, fertility, pelage, coat growth and hibernation are examples of such infradian rhythms.

Irrespective of the period of rhythms, their generation is often a combination of external and internal factors. External factors can be temperature, light, food availability, social cues etc. Internal factors are often oscillators that have an intrinsic oscillating capacity, independent from other time cues. When such oscillators have a distinct anatomical location, they are often referred to as pacemakers. A well known pacemaker in ultradian rhythmicity is the sinus node determining heart rate. Circadian rhythmicity has a central pacemaker located in the hypothalamus, the suprachiasmatic nucleus (see page 35). There is no known oscillator determining infradian rhythmicity. Examples of hibernation in squirrels and migration behavior in birds in complete absence of any external time cue, clearly indicate that also infradian rhythmicity must have an endogenous component.

When rhythmicity is studied in the absence of external factors, the rhythms are indicated as free running. The endogenous period of such free running rhythms is indicated with τ (tau). The value of τ, which determines whether a rhythm is ultra- circa- or infradian, can vary among individuals within the same species. For example, the circadian τ in humans ranges from 22 to 26 h, with an average value of 25 h. Because of the scope of this thesis, the following sections will mainly focus on circadian rhythmicity.

■ Entrainment and phase-response curves

In the interaction of the organism with the environment, the combination of internal and external factors determine the resulting periodicity. Often, endogenous rhythmicity is synchronized by external factors, that act as specific time cues or "Zeitgebers". The result is an overt rhythm with constant periodicity. An important Zeitgeber in everyday life is the light/dark (LD) cycle. In all species, the LD cycle synchronizes the circadian rhythm in activity. Although the behavioral response to the LD cycle can be different, for example some species respond to darkness with increased activity (nocturnal animals) while others sleep during darkness (diurnal animals), the synchronizing effect of the LD

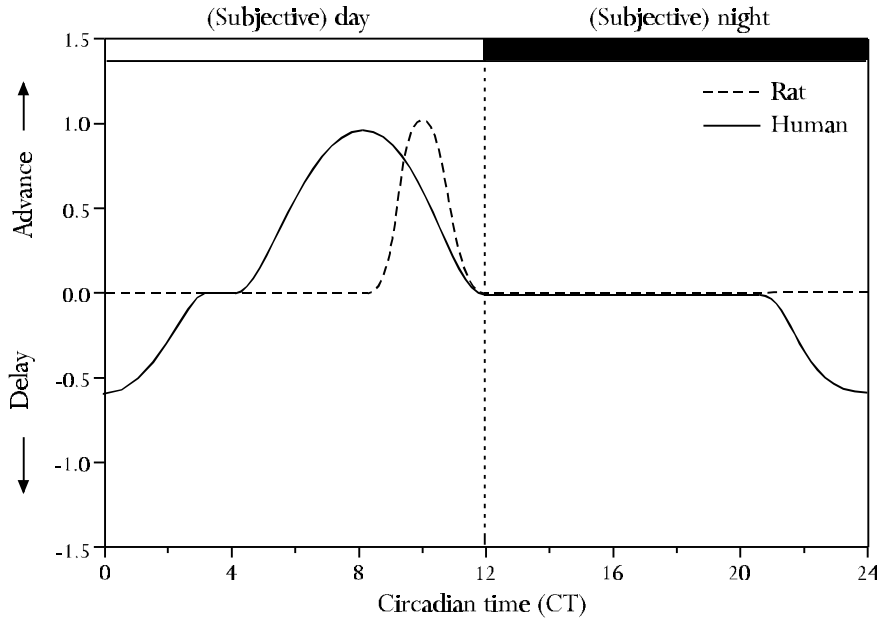

Figure 1.12 A schematic phase-response curve (PRC) to melatonin in rats and humans. The circadian time is based on the subjective day and night.

cycle is similar in all species. When the synchronization of endogenous oscillators by exogenous Zeitgebers is based on a specific underlying mechanism in that oscillator, synchronization is referred to as entrainment.

Also light pulses, or melatonin treatment can act as Zeitgeber and result in entrainment. When a Zeitgeber synchronizes the measured parameter directly, without interacting with the underlying oscillator, this process is called masking. Generally, masking conditions, for example light or stress, are Zeitgebers that are too weak to induce entrainment, but do have acute effects. Because masking can disturb the outcome of circadian experiments radically, they need careful consideration.[207,291] Because the severity of masking depends on the parameter that is investigated, masking insensitive parameters such as circadian production of melatonin are preferable in this respect.

Because entrainment experiments have to be carried out in the absence of other Zeitgebers, such as the LD cycle, the animals are usually maintained in constant darkness (DD). In constant light (LL), animals generally loose circadian rhythmicity. In nocturnal animals kept in DD, the quiescent period is called subjective day, while the active period is indicated with subjective night.

In addition to the period, the phase of a rhythm is an instantaneous state of an oscillation within a period. Phase can be shifted by Zeitgebers. When the phase is shifted to a previous state, it is called a phase advance, while a shift in the opposite direction is called phase delay. Entrainment by a Zeitgeber with period T will force the endogenous cycle with period τ to run with period T. This means that during each cycle, the phase is shifted by τ-T. When τ is smaller than T, this will have to be a phase delay, and in case

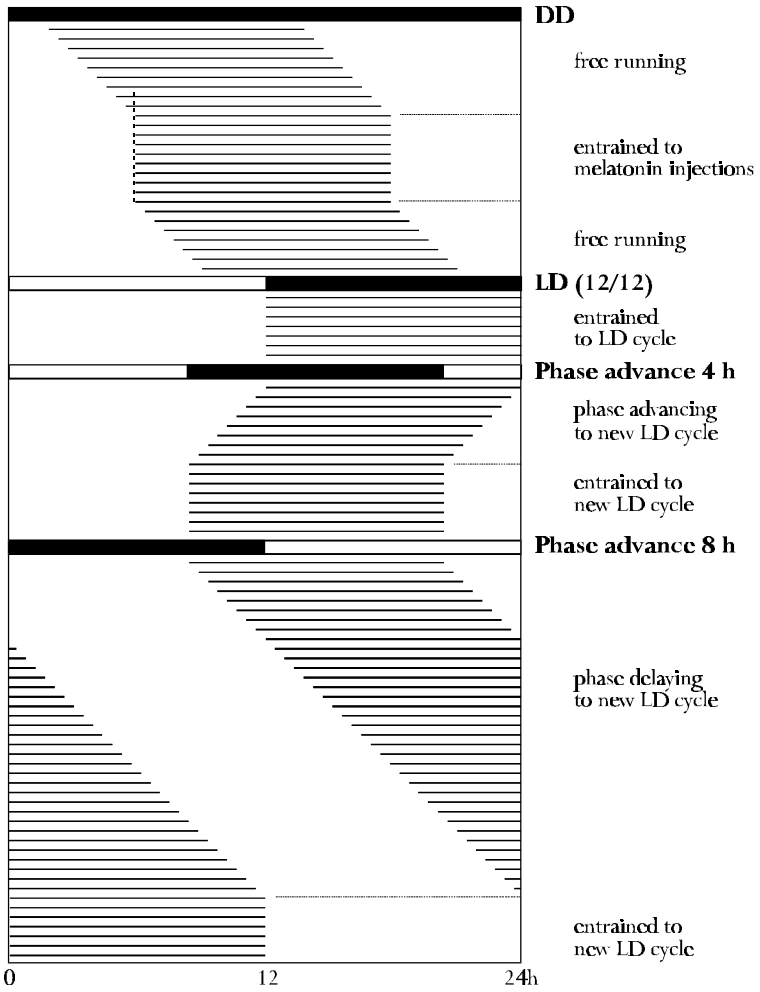

Figure 1.13 An overview of the various conditions and effects in a nocturnal animal, generally considered in circadian research. The horizontal axis represents a 24h cycle, subsequent days are vertically plotted. The horizontal ribbon pattern is a schematic representation of locomotor activity. For further explanation, see text.

of a τ larger T, this has to be a phase advance. Because Zeitgebers will phase delay or advance depending on phase, the effectiveness of a certain Zeitgeber in entrainment will be restricted to certain phases of the cycle. When the phase response (phase advance or phase delay) is plotted as function of the phase at which a Zeitgeber is applied, this results in a phase-response curve (PRC). For each combination of animal and Zeitgeber, a different PRC exists. From this PRC, one could calculate at what phase a certain Zeitgeber has to be applied in order to achieve entrainment.

As an example we consider a rat with a free running period (τ) of 25 h. The intention is to entrain this rat with melatonin as the Zeitgeber to a period (T) of 24 h. Therefore,

the daily phase shift has to be a phase advance of 1 h (25 - 24 = 1). Corresponding to the PRC of rats towards melatonin treatment (Fig.1.12), this would mean that melatonin has to be given late in the subjective day. Such entrainment experiments are described in chapter 7.

Phase shifts can also be achieved with light pulses. In contrast to melatonin, light phase advances at the end of the subjective night and phase delays at the end of the subjective day, resulting in a PRC that is mirrored in the y-axis compared with the PRC for melatonin. Therefore, light pulses have to be applied late in the subjective night, in order to achieve entrainment. An example of such an experiment is described in chapter7.

■ Phase shifts

From the above, it may be obvious that the LD cycle is a major Zeitgeber and therefore of crucial importance to an organism. It is interesting to know what happens when the complete LD cycle is shifted to some extent, a situation that occurs for example in humans during transatlantic flights. In general, the circadian rhythm will show a transient period of several days, during which it will re-entrain to the new LD cycle. After relatively short phase shifts of 5-6 h, the circadian rhythmicity will gradually phase advance or delay similar to the direction of the applied phase shift, so when the LD cycle is phase advanced by 5 h, circadian rhythmicity will also phase advance. When the phase shifts increase, circadian rhythmicity will more likely shift according to the τ-period. This means that animals with a τ-period smaller than 24 h will phase advance and those with a τ-period larger than 24 h will phase delay to the new LD cycle. As a consequence, an 8 h phase advance will have more drastic effects on animals with τ-period > 24 h, while an 8 h phase delay will have the most serious consequences for animals with τ-period < 24 h. In chapter 8 experiments are described that show the effects of an 8 h phase advance on pineal melatonin production.

Free running, entrainment and phase shifts are sometimes complex, but important elements in chronobiology. They reveal special characteristics of underlying pacemakers and provide means to manipulate circadian rhythmicity. In order to make them easier to understand, the various processes are graphically represented in Fig.1.13.

■ The suprachiasmatic nucleus

The major circadian pacemaker in all vertebrates is the suprachiasmatic nucleus (SCN) of the mediobasal hypothalamus. For reviews on the SCN and its role in circadian rhythmicity, see references 138, 232, 303 and 420. The nucleus is a paired structure and is sometimes referred to as a set of two nuclei, both consisting of about 10,000 neurons. The SCN is located just above the optic chiasm, and on opposite sides of the third ventricle (Fig. 1.14). Ablation of the SCN results in complete arrhythmicity, a strong indication for its primary role in the generation of circadian rhythmicity. This is further demonstrated by transplantation and *in vitro* experiments. In these *in vitro* experiments, the SCN can be cultured for several days and shows circadian rhythmicity in terms of metabolism, transmitter release and electrical activity. The period of the SCN in such studies appeared to be temperature compensated, a prerequisite for pacemaker function. In other words, the oscillating nature of this nucleus is the actual clock of the organism, an endogenous property of a set of only 10.000 neurons.

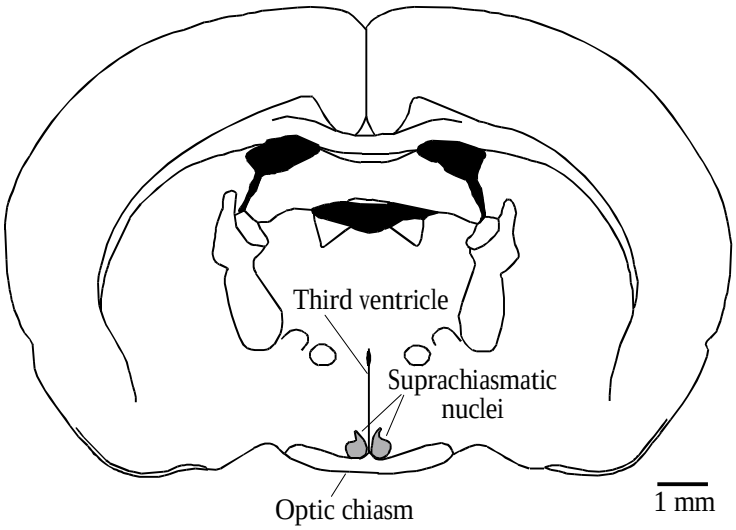

Figure 1.14 The anatomical localization of the suprachiasmatic nuclei in the rat brain. The picture shows a coronal section, bregma -1.3 mm, interaural 7.7 mm, according to the atlas from Paxinos and Watson.

Afferent projections

Circadian rhythms are mainly entrained by the environmental LD cycle. In mammals, the photic information is perceived by the retina and transferred to the SCN through two different pathways. One is a direct projection from the retinal ganglion cells via the optic nerve to the SCN, known as the retinohypothalamic tract. The fibers project on VIP-immunoreactive neurons and contain glutamate as their neurotransmitter. In addition, controversial evidence exists on the role of acetylcholine, substance P and GABA in the hypothalamic tract. The second projection, the geniculohypothalamic tract, arises from cells in the intergeniculate leaflet of the lateral geniculate nucleus, an area densely innervated by retinal ganglion fibers. The most likely candidates for neurotransmitters in this tract are NPY and GABA.

Besides the photic projections, additional afferent projections to the SCN are present from various brain areas, including septum, hypothalamus, hippocampus and midbrain. Of these projections, a prominent one arises from the median and dorsal raphe nuclei in the midbrain. This projection consists of serotonergic fibers, and is probably responsible for the fact that serotonin concentrations in the SCN are high compared to other brain areas. The function of this projection is not well understood, but it seems that the general effect of serotonin is inhibitory to neuronal activity in the SCN. Because lesion experiments have indicated that this projection is not essential for circadian rhythmicity it may have a modulatory role in the non-photic regulation of the pacemaker.

Efferent projections

A major efferent projection from the SCN ends in the paraventricular nucleus of the hypothalamus. Other areas to which the SCN projects include the dorsomedial hypothalamus, the preoptic area, the retrochiasmatic area, the paraventricular nucleus of the thalamus and to the lateral geniculate nucleus, an area that also projects to the SCN. The main neurotransmitters involved in these efferent projections are GABA and peptides, such as arginine-vasopressin, VIP and gastrin releasing peptide. In the projection to the paraventricular nucleus of the hypothalamus, GABA, arginine-vasopressin and VIP are the main neurotransmitters (Fig. 1.15).

Interestingly, all projections terminate in the immediate neighborhood of the SCN. Regarding the wide variety of behavioral and physiological functions regulated by the SCN, it seems likely that most of these functions are controlled via a multisynaptic pathway. One of these pathways that is best studied, is the projection to the pineal gland (Fig. 1.1). Projections from SCN to the paraventricular nucleus of the hypothalamus are continued via the medial forebrain bundle and the mediolateral nuclei of the upper thoracic spinal cord to the superior cervical ganglion. The neurochemical composition of these projections is largely unknown. Sympathetic nerve fibers from this ganglion then finally innervate the pineal gland and stimulate melatonin production by the release of noradrenaline. Besides effects on a variety of neuroendocrine and other processes, melatonin also feeds back to the SCN.

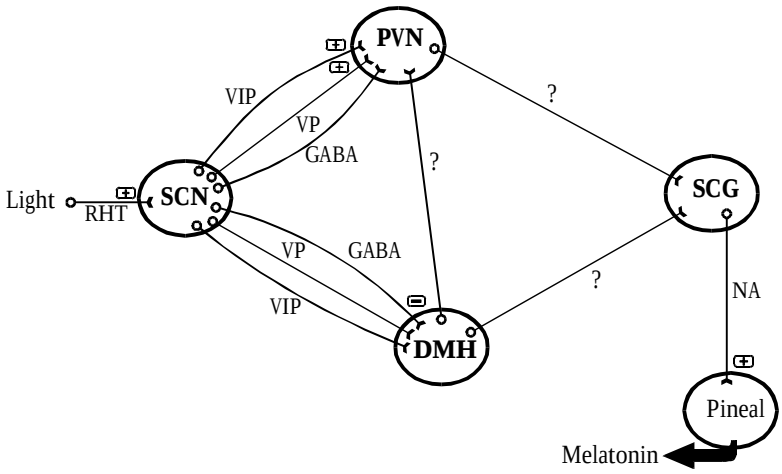

Figure 1.15 Some major efferent projections from the SCN in relation to pineal melatonin production. Most projections have been identified with knife-cut studies and anterograde / retrograde tracing techniques. The neurochemical composition of these projections is not known in many cases. DMH = dorsomedial hypothalamus, GABA = γ-aminobutyric acid, NA = noradrenaline, PVN = paraventricular nucleus of the hypothalamus, RHT = retinohypothalamic tract, SCG = superior cervical ganglion, SCN = suprachiasmatic nucleus, VIP = vasoactive intestinal peptide, VP = vasopressin.

Pacemaker function of the SCN

As mentioned above, the central role of the SCN as a circadian pacemaker has been revealed by SCN lesion and transplantation studies, as well as studies on the intrinsic oscillation properties of the SCN both *in vivo* and *in vitro*. SCN lesions result in complete arrhythmicity of all behavioral and physiological processes, such as locomotor activity, drinking, feeding, hormone release, temperature etc. Also infradian rhythms such as the estrous cycle are perturbed. Apparently, infradian rhythms depend on circadian rhythmicity as well. Arrhythmicity does not mean dysfunctional. Activity, feeding, hormone release etc. remain present, but in a random pattern, not entrainable by the LD cycle. In fact, melatonin production can be elevated compared to daytime production after SCN lesions. This phenomenon is described and experimentally confirmed in chapter 3. The conclusion might be that the SCN is not a rhythmical stimulator, but a rhythmical inhibitor as far as melatonin production is concerned.

The circadian rhythmicity in the SCN is reported many times during *in vivo* experiments. Electrical activity, metabolism and the release of vasopressin in the cerebrospinal fluid all show marked circadian variations with the same phase relationship to the LD cycle in both nocturnal and diurnal animals. The intrinsic nature of this circadian rhythmicity is confirmed by *in vivo* studies in which the SCN was surgically isolated from the surrounding tissue. Further evidence for the intrinsic rhythmicity of the SCN is obtained from similar experiments under *in vitro* conditions, using brain slices or SCN explants. In fact, recent studies have shown that in SCN explants, the release of arginine vasopressin and VIP show circadian rhythmicity with different periods. The consequences of such a dual-oscillator pacemaker function are described in chapter 8.

Another line of evidence for the pacemaker function of the SCN is derived from transplantation studies. Grafting neonatal or fetal SCN tissue into SCN-lesioned animals resulted in restoration of circadian rhythmicity. The development of afferent and efferent projections with the host brain closely resembled those of intact nuclei. In addition, it appeared that host hamsters with a free-running period of about 24 h adapted the smaller free-running period of *tau*-mutated donor hamsters (see page 39), indicating that the circadian rhythmicity is an intrinsic property of the SCN.

Pacemaker properties of the SCN

The molecular mechanism behind the circadian oscillations in the SCN is still unknown. The scientist that will discover it, probably awaits a Nobel-price. Nevertheless, certain properties of the SCN as pacemaker have been described over the years.

Infusions of the reversible neurotoxin TTX in the SCN result in the disappearance of rhythmicity. Washing out the TTX from the tissue restores rhythmicity. Interestingly, phase and period of free-running rhythms after treatment are appropriate to phase and period before treatment. Apparently TTX does inhibit overt rhythms that are dependent on neuronal connections with the SCN, but does not affect the intrinsic oscillator. From this it can be concluded that rhythm generation in the SCN does not involve cellular communication, or at least no communication through sodium-dependent action potentials. Ultradian rhythmicity in intracellular calcium as is present in the SCN, appears

to be TTX resistant. Because calcium can also be involved in cellular communication through gap junctions, it was recently suggested that these calcium oscillations may be involved in the generation of circadian rhythms.[424]

Gene transcription and protein synthesis may be a prerequisite for the generation of circadian rhythms. Protein synthesis inhibitors such as anisomycin and cycloheximide injected near the SCN result in marked phase shifts. In addition, it appeared that light can induce the expression of the immediate-early genes c-*fos* and c-*jun*. There are many similarities in the sensitivity towards light between these genes and entrainment in terms of light intensity and circadian phase. Therefore, it may be that c-*fos* and c-*jun* expression as well as their proteins that regulate expression of other genes, are involved in the generation of circadian rhythms.

It appeared that administration of the cholinergic agonist carbachol to the SCN can induce phase shifts in locomotor activity similar to light pulses. In contrast to light, this phase-shift is not accompanied by enhanced expression of c-*fos*.[62] This may indicate that either acetylcholine is involved in the circadian control of the SCN through a pathway different from the light input, or the light input pathway is not exclusively dependent on c-*fos* induction. In addition, nitric oxide synthase inhibition blocks the light induced phase shifts in locomotor activity, but not the accompanied increased c-*fos* expression.[405] This may again indicate that light input is not solely dependent on c-*fos* expression, but a role of nitric oxide in the photic input pathway downstream from c-*fos* expression may also be an explanation.

■ Genetic aspects

Although the self-sustained rhythmicity of the SCN may be the basis for circadian rhythmicity, the molecular mechanism that is responsible for the oscillations is still not known. Since the early work of Kanopka and Benzer,[162] evidence has accumulated that the generation of rhythmicity has a genetic component (for review, see refs.92 and 354). The isolation of single-gene mutations in *Drosophila* with either shortened, lengthened or abolished periods in circadian rhythmicity of eclosion and locomotor activity has led to the discovery of the *per* (period) gene. Products of *per*, such as *per* RNA and the protein PER are clearly rhythmically expressed. Similar to the *per* gene, from *Neurospora* the frequency (*frq*) gene has been elucidated, mutations of which lead to changes in the period of conidiation. Presumably the products of these genes are mainly localized in the nucleus and play a regulatory role in transcription processes.

In mammals, a spontaneous mutation is discovered in the golden hamster. These *tau* mutations are semidominant and result in a reduction of the period by 2 h in heterozygotes and by 4 h in homozygotes. The *tau* mutant hamsters have proven to be a valuable tool in the SCN transplantation studies as described above. Unfortunately, cloning of the gene and identification of gene products have not been successful until now. In addition, very recently mouse mutants have been selected with an increased period compared to the wild-types, caused by mutations in the *clock* gene.[395]

The homology between the various genes is very poor and clear functional differences have been described. Therefore, it seems unlikely that the genes discovered thus far are closely related and it cannot be excluded that one species contains a number of genes that are involved in the generation of circadian rhythmicity.

Although the molecular background of the biological clock is far from being understood, clear progress is made. The discovery of new mutants and cloning of their clock genes will have to provide new information on the driving force behind circadian rhythmicity.

1.8 Melatonin in humans, its therapeutic potential

The commercially available melatonin preparations have attracted quite some attention from the various media. Many publications provide a well balanced overview of the various actions of melatonin, but several others claim rather recklessly therapeutic use as the cure against jet-lag and sleep disorders or even as a means to increase life-span by up to 25%. In this section, the various actions of melatonin as a possible therapeutic agent will be discussed. An attempt is made to distinguish facts from hypotheses.

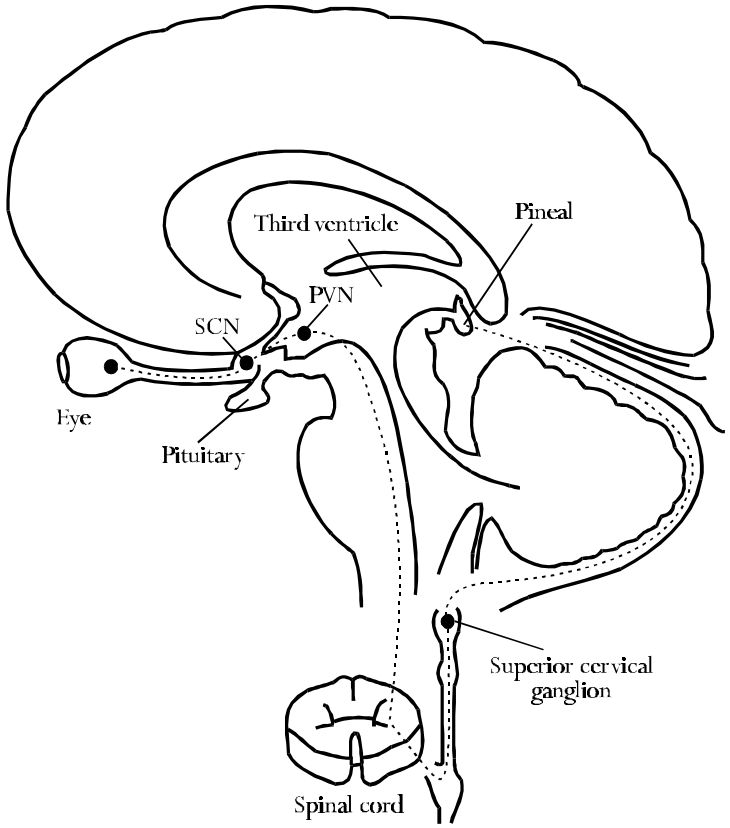

Figure 1.16 Schematic overview of the location of the pineal gland in the human brain, as well as its innervation and some important tissues involved in the various physiological effects of melatonin. (SCN = suprachiasmatic nucleus, PVN = paraventricular nucleus)

A schematic drawing of the location of the pineal gland in human brain as well as the other tissues important in the various actions of melatonin, such as SCN and pituitary and their innervation is presented in Fig. 1.16.

■ Jet-lag

Jet-lag is a pattern of symptoms generally seen after passing several time zones, and is thought to be related to perturbed rhythmicity.[13] Symptoms include disturbed sleep/wake cycle, increased sleep latency, depressed mood and loss of mental efficiency. The occurrence and severity of these symptoms seem to be dependent on the number of time zones passed and the direction of the flight. Eastward flights generally result in more severe jet-lag than westward flights.

The synchronizing role melatonin plays in the rhythmicity of several body rhythms has triggered several studies aimed to restore rhythmicity in jet-lag.[8,11,59,249,250] In doses of 5-8 mg, melatonin was administered in double blind, placebo controlled studies. It appeared that melatonin is capable of improving jet-lag when taken between 20.00 and 24.00 h, destination time, starting on the day of flight until three to four days afterwards. An important parameter in judging the effect on jet-lag is the self-rated global treatment efficiency, measured on the visual analogue scale (VAS) that runs from 0 to 100 (complete ineffective to complete effective). Generally the effects are in the order of 5-20 on the VAS but show substantial variation among individual subjects. These improvements are modest and do not exceed the differences seen between eastward and westward flights (20-40). Other parameters, such as mood, sleepiness, efficiency in work etc. also show moderate, but many times significant improvements. Melatonin taken on a westward flight can improve the situation substantially. A difference in VAS of 60-80 has been reported.[8] Taking melatonin several days before the flight either has no effect, or even worsened the situation.[250]

■ Seasonal affective disorder

Depressions related to seasonal rhythmicity are generally referred to as seasonal affective disorder (SAD). Many times these depressions occur during winter and are called winter depression. The original observation that bright light could alleviate the symptoms of SAD[299] suggested the relationship between reduced environmental lighting and the occurrence of the depression. Light therapy in the morning then became an effective tool against SAD. The fact that light can effectively reduce melatonin production has led to the melatonin hypothesis. An abnormal timing of the melatonin profile could be responsible for the depression. Although there is one report that melatonin is phase delayed in some SAD-patients,[184] this observation is not yet confirmed. A shortening of the melatonin secretion, as suggested by the melatonin hypothesis, was indeed found after light treatment, but the correlation with alleviation of symptoms was very poor.[416] Also suppression of melatonin levels with the β-receptor antagonist atenolol did not improve depressive symptoms.[300] Therefore, the causality of depressed melatonin levels in SAD seems questionable. Treatment of SAD patients with melatonin also did not appear to be effective.[417] Light therapy therefore remains the therapy of choice in the treatment of SAD.

■ Delayed and advanced sleep phase syndromes

Some sleep disorders are clearly phase related. Most striking examples are the delayed and advanced sleep phase syndromes (DSPS and ASPS). Where ASPS is characterized by the difficulty of staying awake in the evening and maintaining sleep after about 03.00 h, DSPS patients experience difficulties in initiating sleep before 01.00 h to 03.00 h, but easily maintain sleep for a normal duration. The terms DSPS and ASPS only refer to patients that have no self-control over their sleep schedule and are phase advanced or delayed compared to the day-night rhythm. Especially social problems, that can be serious, require adequate therapy.

The phase shifting effects of light have triggered its use in the treatment of these patients. Very important was the finding that bright light is more effective in suppressing melatonin than is dim indoor light[182] and similar findings that bright light is more effective in entraining.[411] Over the years, bright light, generally about 2500 lux, has been successfully used in the treatment of DSPS in many patients.[183,301] Typically, patients are exposed to bright light for periods of 2-3 h between 0600 h and 0900 h. Normal sleep patterns, improved sleep and reduced sleepiness early in the day are reported. Although most attention has been paid to DSPS patients, also the light treatment of ASPS, especially in elderly people has been successful.[42,327] Because practical problems reduce compliance with the therapy, alternatives would be useful. Also the often occurring re-establishment of the advanced or delayed phase after stopping the treatment demands for alternatives.

Melatonin, as a hormone of darkness, could be such an alternative. Studies describing the successful use of melatonin in DSPS patients by phase advancing the sleeping period, used a daily dose of 5 mg, given at 22.00 h.[4,70] The positive effect may be at least partly ascribed to a hypnotic effect, additive to a possible circadian effect. Direct hypnotic effects have been described for a wide dosage range.[76] This hypnotic effect makes the use of melatonin for treatment of ASPS more difficult, because melatonin should therefore be administered in the morning. A dose low enough to minimize the hypnotic effect, but still effective as a circadian agent may be preferable.

If neither light nor melatonin by themselves have the desired effect, a combination of both could be considered. Light phase advances when given at dawn, where melatonin phase delays at that time. At dusk, these effects are reversed. Based on these properties, an interaction between light and melatonin may be expected and useful. When administered at antiphase, i.e. morning light/evening melatonin or vice versa, this may be an effective therapy and certainly be an interesting hypothesis for future studies. Because generally the advances or delays seen are not retained on stopping treatment, such therapy may have to be used for long times.

■ Sleep disorders

Apart from sleep disorders that are associated with abnormalities in the circadian rhythm, melatonin has also been used in the treatment of insomnia.[140,201] Although the doses used vary to a great extent (1-75 mg), in general melatonin was moderately beneficial in terms of improved sleep quality, reduced sleep latency and improved daytime alertness.

A remarkable effect of melatonin on the sleep/wake cycle was seen in children that suffered from severe neurological disorders.[141] Often these children have a fragmented sleep pattern and are difficult to handle in a domestic situation. Treatment of 15 children

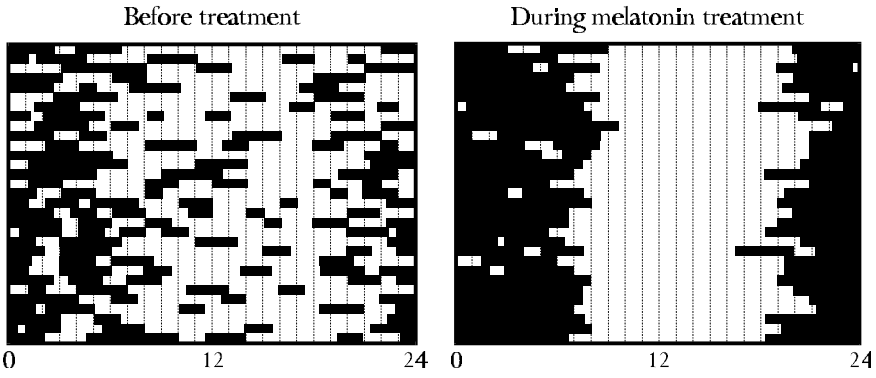

Figure 1.17 Improved sleeping pattern of a child suffering from authistic behavior and developmental delay following treatment with melatonin. Melatonin was administered daily in a dose of 5 mg orally at bedtime. Horizontal axis represents the 24 h cycle from midnight to midnight. Vertical rows represent subsequent days. Black boxes indicate periods of sleep. (from ref.141)

with melatonin at a dose of 2-10 mg resulted in marked improvements of sleep quality and sleep pattern (Fig. 1.17). In all cases there was a substantial improvement in general mood, possibly caused by improved resting. Treatment with melatonin enabled these children to stay with their family.

Special attention deserves insomnia in the elderly. With aging, sleep patterns tend to become disrupted, with night-time wakening and daytime sleepiness. Because it is known that the amplitude of melatonin is reduced with age,[370,371,384] a causal relationship between them is suggested. In fact, peak concentrations of urinary 6-sulphatoxymelatonin appeared to be reduced in elderly people with sleeping problems compared to aged individuals without sleep disorders.[114] There is however only one study in which melatonin has been used in the treatment of age-related insomnia.[113] In a dose of 2 mg, sleep onset was improved, but did not reach the level of significance.

Another demonstration of the causality between reduced melatonin levels and sleep disorders comes from patients that are treated for hypertension with β-adrenergic receptor blockers. A significant decrease in melatonin production was reported in patients treated with metoprolol.[34] The sleep disturbances seen in the same group showed a significant correlation with reduced melatonin amplitude. Although treatment with other β-blockers tended to show similar effects, these were not significant.

Apparently, the absolute amount of plasma melatonin throughout the night influences the quality of sleep. Treatment of insomnia should therefore be directed towards the adjustment of the melatonin profile to a natural situation. Special formulations, or agents that restore the amplitude could be possible objectives.

■ Blindness

The LD cycle is undoubtedly the strongest circadian Zeitgeber. The inability to perceive daylight, as is the case in totally blind people, may therefore result in rhythm abnormalities. Of all blind people, about 10% is considered to be totally blind, that is, without any light perception. Several studies have indicated that about half of these individuals have

free running rhythms of melatonin, cortisol and temperature, generally with a τ-period somewhat larger than 24 h.[235,305] In about one-third of these subjects, the free running rhythms cause sleep disorders. Case studies have reported a synchronizing effect of melatonin in a dose of 5 mg at bedtime on the sleep-wake cycle.[10,375] In a larger study, melatonin has been used in treating five patients by administration of 5 mg at bedtime (22.00 h) for three weeks.[304] Melatonin, cortisol and temperature rhythms were either fully entrained, or phase advanced compared to placebo controls.

Taken together, these results suggest an interesting therapeutic potential of melatonin in the treatment of sleep disorders in totally blind people.

■ Contraceptive

The effects of melatonin on the hypothalamic pituitary axis and therefore the reproductive system have initiated studies aimed at the use of melatonin as an oral contraceptive.[398] When using high doses of melatonin (75 and 300 mg daily) in combination with norethisterone, no peak in LH secretion was observed during the menstrual cycle. Furthermore, plasma FSH levels remained constant. These circumstances prevented ovulation and the increase in progesterone during the luteal phase. The suggested mechanisms of action were antigonadotropic effects on the hypothalamus, such as alterations in the hypothalamic pulsatile GnRH secretion and/or effects on the pituitary release of LH, or even a direct effect on the ovary.

At this point, it must be emphasized that the dosage of melatonin used was extremely high. For most clinical applications, doses of 1-10 mg are used, whereas the antigonadotropic action of melatonin was most pronounced in doses up to 300 mg. There are no reports that using melatonin in low doses 2-3 mg will have a negative effect on the reproductive system.

Because of negative side-effects of estrogens in the present generation of oral contraceptives, replacement by melatonin might be advantageous. Estrogens are suggested to increase the incidence of thromboembolic diseases and the risk of developing pre menopausal breast cancer. Especially increased risk of cancer is impaired by melatonin usage. In fact, because of the proposed oncostatic and immunoenhancing properties of melatonin, the use of melatonin in oral contraceptives might prevent breast cancer, as was recently hypothesized.[61]

However, according to the latest information (personal communications), the development of melatonin as an oral contraceptive has been halted, because of a serious side-effect: pregnancy.

■ Puberty

The influence of melatonin on the hypothalamic pituitary axis and the resulting effects on the reproductive system have suggested a role for melatonin on sexual maturation (for a recent review, see ref. 50). Nearly hundred years ago, an effect of the pineal gland on puberty was suggested, based on the results of a boy suffering from a pineal tumor with precocious puberty. Based on cases like this, involving patients with pineal tumors, a suppressing effect of melatonin on puberty was suggested. This hypothesis was confirmed by an editorial in Science.[161] However, despite that editorial, there is still no general consensus on the role of melatonin in puberty.

In humans, body weight is an important factor in the timing of puberty. The observation that melatonin levels decline with age does not prove a role in development. Increasing body weight with constant melatonin production will result in passively reduced melatonin concentrations. Because many physiological parameters change during development, by itself this does not prove a causal relationship between melatonin and puberty. Stronger evidence comes from subjects with pathologically high or low melatonin levels. A case report described a boy with delayed puberty.[265] It appeared that the pineal gland was enlarged and hyperfunctional, resulting in high plasma melatonin levels. In addition the patient suffered from hypogonadotropic hypogonadism that was consistent with GnRH deficiency. Light treatment during the night was not effective in reducing plasma levels of melatonin, but after several years, the melatonin production started to decline, an effect that was accompanied by maturation of the pituitary and gonadal functions. Similar reports have been described in patients with precocious puberty, which showed reduced melatonin levels, compared to age-matched controls.[173] Many other studies have been published, describing no differences in melatonin levels between subjects with precocious, normal or delayed puberty. Differences in protocol and melatonin assays make it difficult to compare those studies. Nevertheless, precocious puberty appears to be associated with normal or decreased melatonin levels, whereas delayed puberty appears to be associated with normal or increased melatonin levels, strongly suggesting that there is at least a relation between melatonin and reproductive development. The causality as well as the importance under normal physiological conditions of this relationship remains to be cleared.

These data do not directly open a new therapeutic field for melatonin(agonists), but the possible effects on maturation must be considered when using melatonin preparations in young children.

■ Aging

As mentioned previously (page 26), the possible role of melatonin as an anti-aging drug has led to a wide range of speculations, which are readily absorbed by laymen. It must be emphasized at this point that all information regarding the role of melatonin in the aging process has been derived from animal experiments. To my knowledge no clinical studies or reports have been published that indicate a similar effect of melatonin in humans as it has in mice. The practical and ethical problems that would be connected to such studies will probably hold up the availability of clinical data at short notice.

The therapeutical use of melatonin in terms of formulations and dosage schemes will also depend on the mechanism behind its anti-aging effects. Assuming that the chronobiotic activity is responsible for longevity, effective time of administration will probably be limited to the gate of circadian frequency, i.e. in the evening. When the effects are based on immunoenhancing or radical scavenging properties, a rather constant release of melatonin is probably more effective. With the short half-life of melatonin in mind, it seems unlikely that a person can have 24 h radical protection from a drug that is only in the body for 2-3 h.

Taken together, data from animal experiments are premature and clinical data are simply lacking. Nevertheless, the implications for human use are obvious and require full attention.

■ Immune system

The immuno enhancing properties of melatonin may be useful in clinical practice, especially in states of immunodeficiency. The most severe type of immunodeficiency is the acquired immunodeficiency syndrome (AIDS). To date there have been no clinical trials published in which melatonin was used in the treatment of AIDS. Only one review mentioned a study on 11 HIV-infected patients, but despite an increase in the peripheral blood mononuclear cell number, there was no unambiguous enhancement of the immune system.

In cluster headache, a very painful type of primary headache, the natural killer cell activity is decreased. Based on this fact, a study was conducted in which cluster headache patients were treated with daily oral administration of 5 mg melatonin in the evening. Indeed, natural killer cell activity increased following the treatment, but more importantly, the total pain index showed a marked improvement.[101] The mechanism involved may be a combination of enhanced natural killer cell activity and the melatonin induced opioid release. The immunoenhancing role of melatonin may be most interesting in the treatment of cancer which is described below. Especially the increase in natural killer cells and lymphokine activated killer cells as well as the potentiating effect on IL-2 treatment may attribute to a positive impulse for the treatment of cancer.

■ Cancer

Based on the antitumor activity of melatonin in animal studies (see page 22), the role of melatonin in tumor growth and the possible application as an oncostatic agent in clinical use, have been subject of several studies over the past years (for a review, see ref. 24).

The role of melatonin in relation to cancer has mainly been focused on hormone-dependent cancers, such as those of the breast and the prostate. Several studies reported a decline in amplitude of the nocturnal melatonin peak in primary tumors of both breast[23,358] and prostate.[22,25] Interestingly, the reduced production of melatonin appeared to be a transient process, because the development of metastases and treatment with high doses of estrogen were generally accompanied by normalized melatonin profiles. On the other hand, during the initial state of tumor development, melatonin levels can be increased, as was shown in elderly men with so-called incidental prostate cancer, an early stage of cancer with malignant cells that do not grow. Therefore, the conclusion can be drawn that the relationship between pineal gland and cancer is a dynamic one, consisting of various phases during tumor development in which melatonin production is stimulated and inhibited.

As can be seen more often, studies on plasma melatonin levels in relation with tumor growth in patients, do not always point to the same direction. Often different experimental conditions and methodological problems account for the discrepancies. Sometimes, daytime levels are reported, but they are of no use. Especially the nocturnal peak levels bear the most important information. This would imply that blood samples have to be taken during night-time, which is uncomfortable to the patient. The determination of the urinary metabolite, 6-sulphatoxy-melatonin, as a measure for night-time melatonin production, may be an adequate alternative, which involves non-invasive sample collection. Furthermore, the plasma levels of melatonin can be influenced by the patient's drug therapy. The use of agents that increase melatonin production, such as monoamine

oxidase inhibitors and anti-depressants, or agents that decrease melatonin levels, such as β-adrenergic receptor antagonists, is common among elderly people. These factors must be taken into account when interpreting results from different studies.

The actual use of melatonin as an oncostatic agent has been reported several times, with overall positive effects. In *in vitro* studies, melatonin appeared to have antiproliferative characteristics on various breast cancer cell lines,[66] an effect possibly mediated by activation of the estrogen response pathway.[123] One of the first clinical studies using melatonin in cancer patients was described by Lissoni and co-workers.[188] They applied melatonin in doses of 10-20 mg in the afternoon to advanced cancer patients, in which conventional therapies failed. Especially the patients with a performance status of 20 or higher before treatment, responded positively to the melatonin, with marked increases in performance status and increased survival times. Patients with an extremely low performance status before treatment (20), did not respond very well. Subsequent studies including patients suffering from various tumors, indicated a general positive effect of melatonin on performance status and survival rate, but actual regression of the tumor was extremely rare.[189] It must be noticed that these studies involved advanced cancer patients, in which conventional therapy was not effective. More positive results were derived from a combination of melatonin with conventional therapy. In a phase II clinical study, 15 women with metastatic breast cancer, who did not respond to standard tamoxifen therapy, were treated with a combination of melatonin and tamoxifen. In about 30% of the patients, the tumor showed a partial regression. As was seen earlier, quality of life was generally increased.[196]

A number of studies have appeared describing a combination therapy of low doses of interleukin-2 (IL-2) together with melatonin. IL-2 induces an anticancer immune response and is reported to be effective in the treatment of renal cancer and melanoma. However, the IL-2 induced activation of the macrophage system is counteracting the effects of IL-2 by its suppression of the platelet number. Based on the effects of melatonin on the immune system, studies were initiated in which melatonin was co-administered with IL-2.[190,195] It appeared that melatonin could highly potentiate the effects of IL-2. In fact, the treatment with low doses subcutaneously injected IL-2 together with oral administration of 20 mg melatonin, was effective in both the regression of tumors and the stabilization of tumor growth. This beneficial effect was not restricted to renal cancer and melanoma, but also the treatment of colorectal, lung, endocrine and gastric tumors could be enhanced by this combination therapy.[193,192,191] In the case of non-small cell lung cancer, this combination therapy was compared with standard cisplatin and etoposide therapy and appeared to be more effective.[194]

The results of these studies indicate a positive effect of melatonin in the treatment of cancer. The improvement in quality of life may be a result of the sleep-inducing effect as well. The positive action in tumor stabilization and regression, especially when combined with other agents such as IL-2, possibly related to immuno enhancing effects, is a promising development in cancer therapy, but additional studies in other laboratories must be encouraged.

1.9 Pineal microdialysis

The development of the microdialysis technique during the last decade and the increasing scientific interest in the pineal gland and its major product melatonin led to the development of trans pineal microdialysis as described in this thesis. This section describes briefly some characteristics of microdialysis and provides an overview of the application of the microdialysis technique in pineal research. For reviews on details and use of microdialysis, see references 29,146,406 and 410.

■ Microdialysis

In neurochemical research information about neurotransmitter release provides crucial information about neuronal communication. In the early approach, neurotransmitter release was estimated by post-mortem analysis of homogenated tissue. However, whole tissue concentrations often show a poor correlation with extracellular concentrations and in neurotransmitter release, especially this extracellular concentration is of greatest importance.

Early attempts to estimate extracellular concentrations involved the development of the push-pull technique and the *in vivo* voltammetry. Especially the *in vivo* voltammetry has developed to a highly advanced technique by which extremely small amounts of neurotransmitters can be quantified in very small areas. The inability to infuse drugs at the site of measurement and the limitations in terms of identification of compounds have contributed to the demand for additional techniques.

About one decade ago Ungerstedt introduced the use of a dialysis membrane in the monitoring of neurotransmitter release.[377] The membrane, as used in artificial kidneys, provides a barrier between perfusion fluid and the tissue. During perfusion of the membrane, its permeability for compounds with low-molecular weight enables diffusion of neurotransmitters from the extracellular fluid to the perfusion fluid, while large molecules like proteins remain in the tissue. The resulting dialysates are relatively clean and can be assayed directly.

The development of highly sensitive analytical techniques has proven to be stimulatory to microdialysis and often the analytical potential is a limitng factor for its possibilites. The majority of microdialysis experiments are carried out using high-performance liquid chromatography (HPLC) with either electrochemical or fluorescence detection. Such systems combine high sensitivity with broad applicability. The clean dialysates further allow direct injection of the samples into the HPLC-system, without clean-up procedure, the so-called on-line method. The resulting method allows repetitive sampling with high time resolution in unrestricted animals and a direct interaction of the scientist with the experiment.

In addition, the commercially available probes and equipment especially designed for microdialysis have contributed to the routine character that the technique has in many laboratories today. Moreover, the development of new applications in new research areas is a challenging venture.

Basic principles

The basic component of microdialysis is the probe containing the membrane. In early days, the transversal probe was most widely used. It consists of a membrane fixed around a tungsten wire, with both ends capped with a stainless steel tube. During implantation, the tungsten wire is removed and only the membrane is left in the tissue. At a later stage, the concentric probe was introduced, consisting of two fused-silica tubes (inlet and outlet) inserted in the membrane. Because now in- and outlet are located at the same side of the membrane, the probe can be handled like a needle. Varying the length of the probe and using stereotaxic surgery enable the implantation in virtually every brain area.

During the experiment, the probe is continuously perfused. Because the basic principle for sampling is diffusion and because changes in ion concentrations in the extracellular environment can disturb the homeostatic balance, a perfusion fluid that is isotonic to the extracellular fluid, such as Ringer's solution, is used. Relatively small molecules like neurotransmitters can then freely diffuse along their concentration gradient into the perfusion fluid. The actual amount of neurotransmitter that passes the membrane is called the recovery and is of crucial importance in terms of detectability.

Only few parameters that determine recovery can be manipulated. Factors such as pH and tortuosity factor of the tissue,[29] diffusion characteristics of the neurotransmitter and temperature all have fixed values. Some parameters can be changed, but often within specific limits. Characteristics of the membrane can be changed by using different materials. The dialysis area can be increased or decrease by covering parts of the membrane with glue, however the maximal size of this area is limited by the size of the tissue. The parameter that offers the most flexibility is the perfusion rate.

A low perfusion rate enables the perfusion fluid to become more saturated. On the other hand, a higher concentration of neurotransmitter in the perfusion fluid reduces the concentration gradient over the membrane and will slow-down the diffusion process. For a better understanding of this complicated process of diffusion, two types of recovery

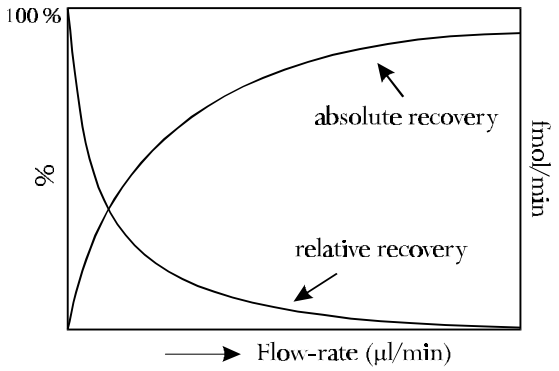

Figure 1.18 The theoretical relationship between recovery and flow-rate. Relative recovery (left axis, %) is calculated as the ratio between dialysate concentration and tissue concentration, while absolute recovery (right axis, fmol/min) is calculated as the amount of compound sampled per minute.

must be considered. One is the relative recovery, defined as the ratio between the final concentration in the perfusion fluid and the concentration in the tissue. A second type is the absolute recovery, defined as the absolute amount of compound that is collected in a certain time interval. The flow-rate determines both kinds of recovery, but in different ways (Fig. 1.18).

When flow-rate would be zero, the concentrations on both sides of the membrane would become equal, resulting in a relative recovery of 100%. When flow-rate is increased, the relative recovery will exponentially decline as indicated in Fig.1.18. When the flow-rate is zero, no volume will be sampled, so the absolute recovery is also zero. When flow-rate increases, increased samples will be gathered with decreased concentrations. The net result is an absolute recovery that increases with flow-rate following a hyperbolic curve. In these situations, a theoretical condition is assumed in which the diffusion process is the only rate-limiting step. This is generally true under *in vitro* conditions, but especially when flow-rates increase to a great extent, other processes, such as migration of the compound towards the membrane in which the tortuosity factor is important, may become rate-limiting. Furthermore, under *in vivo* conditions, tissue characteristics must be taken into account as well. Therefore, the quantification of the exact extracellular concentration is a troublesome task, although not impossible.

Based on the above mentioned considerations, the optimal flow-rate has to be determined. When the flow-rate is too small, the lag-times in the experiment become too long and the system gets extremely sensitive towards dead volumes, leaks etc. When the flow-rate is too large, pressure builds up in the probe and the samples become too large to be analyzed on-line. Generally the optimal flow-rate is the one that results in the largest samples allowed to be on-line analyzed and that does not build up any pressure in the probe. In practice this results in flow-rates of 2-3 µl/min.

Applications

Because microdialysis enables monitoring neurotransmitter release in freely moving animals, it has been applied specifically in brain research. The possibility to dissolve specific agents in the perfusion fluid, resulting in local application of the drug is a convenient way to circumvent the blood-brain barrier. Also this local administration of compounds can be used in pharmacological studies, resolving mainly the involvement of presynaptic receptor systems in the release of neurotransmitters. In this respect, also the use of dual-probe microdialysis must be mentioned. Applying a drug through a probe in one area and measuring the effects on neurotransmitter release in a distant area by a second probe, can reveal important information about neuronal projections in the brain and can be used to study postsynaptic effects.

Recent developments include a wider use in peripheral tissues as well. The implantation of probes in subcutaneous adipose or muscle tissue to monitor glucose, or in organs such as the heart or kidney, and the insertion of a probe in blood vessels provide unique opportunities to monitor the metabolic and neurochemical processes involved in many physiological responses. One peripheral tissue that is of particular interest with respect to the present thesis is the pineal gland.

■ Microdialysis of the pineal gland

The first study describing microdialysis of melatonin in the pineal gland was performed by Azekawa et al.[19] They used a concentric probe and measured melatonin on-line with HPLC coupled to electrochemical detection. The rather complicated surgery, as described in detail later,[20] was necessary because in rats the pineal gland is located just below the confluence of the superior sagittal sinus and the transverse sinus. Damaging these arteries would result in severe bleedings. The electrochemical detection was not always sufficient to measure daytime melatonin levels. Because daytime levels are of crucial importance in circadian studies, alternatives were required.

The use of a transversal probe, as described in this thesis, allowed a much more simplified surgical procedure.[77] In addition, the use of direct fluorescence detection without derivatization resulted in a higher sensitivity, and in the routine measurement of both day- and night-time levels of melatonin.

In addition to the present data, only one other report has been published on pineal microdialysis in the rat, in which the response of rat pineal melatonin production to light was investigated.[150] The probe was similar to the one used by Azekawa et al.[19] but the quantification of melatonin in the dialysates was performed by means of an off-line radioimmuno assay. Two other reports have been published on pineal microdialysis in birds.[118,117] These studies were focussed on circadian rhythmicity of melatonin production, but with relatively low time-resolution (0.5 - 2 h).

In the remainder of this thesis, the development of the trans pineal microdialysis technique and the pharmacological and chronobiological applications are described in full detail.