Central melatonin receptors: Implications for a mode of action

P. J. Morgan and L. M. Williams

Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen (Scotland, U.K.)

Summary. The influence of melatonin on circadian and photoperiodic functions in numerous species is well documented. It is known that the effect of melatonin on circadian rhythmicity is mediated via the suprachiasmatic nucleus (SCN), the biological clock of the brain. It is not known however where the photoperiodic effects of melatonin are mediated. Evidence from brain lesioning and melatonin implant studies point to a site in or near the medial hypothalamus. In contrast to these studies, melatonin receptors have been reported in widespread areas of the brain, the pituitary and in peripheral tissues. The characteristics of the reported melatonin receptors vary widely between studies and consequently no definitive description of a physiologically relevant melatonin receptor has received universal recognition. This review marshals recent evidence for the localization and characterization of the melatonin receptor and discusses these findings in the context of the known effects of the hormone in different species.
Key words. Melatonin; receptors; in vitro; autoradiography; brain; pituitary.

Introduction

Melatonin plays a central role in the timing of reproductive seasonality [1, 33, 41], acting as a neuroendocrine reflection of photoperiod. Despite a great deal of evidence that melatonin exerts its effect in the hypothalamus [23, 24, 27, 42], it is still unknown how the melatonin signal is decoded. Progress in this area has been slow because of inadequate data available on the precise sites of action of melatonin in the brain. Early attempts to identify potential target sites of melatonin action utilized conventional receptor binding assays, using homogenates of potential target tissues and [^{3}H]-melatonin as the radioligand. These studies reported a wide range of tissues which could apparently bind melatonin, including various areas of the brain, the thyroid, and sites in the reproductive organs [10, 12, 13, 49]. Both membrane bound and cytosolic receptors were reported with dissociation equilibrium constants (K_d) in the nanomolar range. Despite these initial studies, there were few reports which either confirmed or extended these findings. More recently, considerable advances in our knowledge on melatonin target sites have been made due to the use of 2-[^{125}I]-melatonin as the radioligand and the technique of in vitro autoradiography. The aim of this review is to consider the recent developments in studies of the melatonin receptor and to relate these findings to a possible mode of action of melatonin.

Localization of melatonin receptors by in vitro autoradiography

Intrinsic to the biological action of any hormone is the existence of highly specific receptor proteins in the target tissue where the hormone exerts its effects. To understand how a hormone exerts its biological actions requires a precise knowledge of the distribution of its receptors. Most evidence indicates that melatonin acts through receptors located in the brain [23, 24, 27], where conventional receptor assays, utilizing crude membrane homogenates, are inappropriate for resolving the fine detail of receptor localization. Autoradiography is the only approach which allows the neuroanatomical resolution necessary to discriminate between different nuclei and their sub-divisions at the cellular level combined with a greatly enhanced sensitivity [36].

Melatonin uptake using in vivo autoradiography

In vivo autoradiography was first used to identify sites of [^{3}H]-melatonin uptake in several brain areas, including the suprachiasmatic nucleus (SCN), in the lamprey and the lizard. In the lamprey in addition to the several regions of the hypothalamus which accumulated [^{3}H]-melatonin, uptake was also observed in the habenular region and in the pineal, whereas localization in the lizard was restricted to the suprachiasmatic nucleus [32]. Uptake has also been observed in the frog retina using the tritiated ligand, where saturable and displaceable uptake was localized in the melanosomes of the retinal pigment epithelium-choroid and the outer plexiform layer of the neural retina [75]. Despite the findings in vivo autoradiography has certain limitations, including rapid metabolism of the radioligand, non-specific uptake and subsequent diffusion of the ligand from the tissue during processing. These problems may explain why this technique has not been applied more widely to the localization of melatonin receptors.

Melatonin binding by in vitro autoradiography

The advent of the technique of in vitro autoradiography combined with the availability of the high specific activity, high affinity radioligand, 2-[^{125}I]-melatonin (see below), has almost certainly led to the greatest progress in the study of the melatonin receptor. Melatonin binding sites were first localized in the SCN and the median eminence (ME) region of the rat hypothalamus [66], and in the SCN, ME/ACN (arcuate nucleus) region, medial preoptic area, pineal and anterior pituitary of the foetal

hamster[72,73]. These sites were visualized by gross morphological techniques, where images were produced on X-ray film from whole brain sections. In a subsequent study of the rat brain, where in vitro autoradiography at the light microscope level was employed, it was revealed that labelling attributed to the ME region was restricted to the pars tuberalis (PT) region of the adenohypophysis; the ME and the pars distalis (PD) of the pituitary did not bind 2-[^{125}I]-melatonin[77]. On dissecting the pituitary from the base of the hypothalamus the cells of the PT may remain attached to, and separate with, either the ME region of the hypothalamus or the PD (anterior pituitary)[76]. Consequently, reports of melatonin receptors in both the ME region and the anterior pituitary, obtained using tissue homogenate assays[63–66] are likely to be the result of the differential partitioning of the cells of the PT between these two regions.

Distribution of central high affinity [^{125}I]-melatonin binding sites in different species

The central [^{125}I]-melatonin binding sites revealed by in vitro autoradiography in the laboratory rat, Syrian and Djungarian hamster and the sheep, show striking differences in distribution (table 1). The functional significance of these interspecific differences is discussed below. At present these brain binding sites can only be described as putative melatonin receptors, as pharmacological characterization of only the non-neural PT/ME site has been made, where high affinities (K_d in the picomolar range) have been identified for the rat, hamster and sheep[49,63–66,79]. Unilateral infusions of excitatory amino acid neurotoxins have been used to destroy neurones in one of the brain areas, the anterior paraventricular nucleus of the thalamus (APVT), of the Syrian hamster, and was shown to destroy the ability of that region to bind [^{125}I]-melatonin. This demonstrates that the binding sites in this area are neuronal[77,79] (fig. 1). Lower affinity (K_d in the nanomolar range) melatonin receptors have been reported in numerous regions of the brain and in the anterior pituitary using tissue homogenate receptor assays (see below). However, attempts to visualize these lower affinity sites have been unsuccessful. Using 800 pM 2-[^{125}I]-melatonin, those regions specifically labelled with only 80 pM radioligand could still be identified, but no new binding sites were discernable. Instead background labelling was found to be very high and homogenously distributed throughout the brain showing negligible displacement in the presence of 1 μM melatonin[76] (fig. 2). Thus as the discrete binding sites identified by in vitro autoradiography can be labelled specifically at

Table 1. Central 2-[^{125}I]-melatonin binding sites identified by in vitro autoradiography

Laboratory rat	Reference	Hamster	Reference	Sheep	Reference	Human	Reference
		(Syrian)					
Suprachiasmatic nucleus (SCN)	79, 78	SCN	71, 79	SCN*		SCN	52
Area postrema (AP)		Preoptic area (POA)		Hippocampus*	45		
Pars tuberalis (PT)		Anterior paraventricular nucleus of the thalamus (APVT)		PT			
		Laternal habenular nucleus (LHN)					
		Habenular commissure					
		Deep pineal					
		Ventromedial nucleus (VMN)					
		Ventral tegmental area (VTA)					
		PT					
		(Syrian)					
SCN	63, 66	Median eminence					
Median eminence		Anterior pituitary					
Choroid plexus							
Anterior pituitary			65				
		(Syrian)					
SCN	73	SCN					
Median eminence		Median eminence	73				
		(Djungarian)					
		SCN					
		Median eminence					
		Medial habenular nucleus					
		Septum					
		Pituitary	73				
		(Djungarian – foetal)					
		SCN					
		Median eminence/					
		Arcuate nucleus region					
		Pineal					
		Anterior pituitary					
		Preoptic area	72				

*(Williams unpublished)

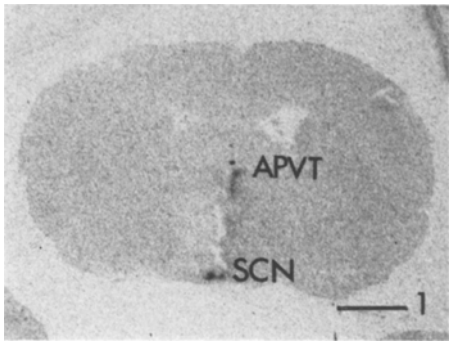

Figure 1. Specific 2[^{125}I]-melatonin binding to a coronal section of Syrian hamster brain bearing a unilateral neurone specific lesion of the anterior hypothalamus and thalamus. Binding to the SCN at the base of the brain is bilateral but binding to the anterior paraventricular nucleus of the thalamus (APVT) is absent on the same side as the lesion. Bar = 2 mm. (By permission of the Journal of Neuroendocrinology.)

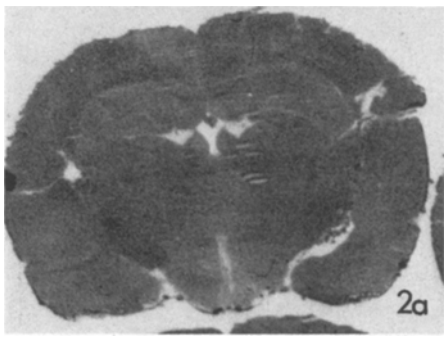

Figure 2 a. 2[^{125}I]-melatonin binding to a coronal section of rat brain after incubation with 800 pM ligand.

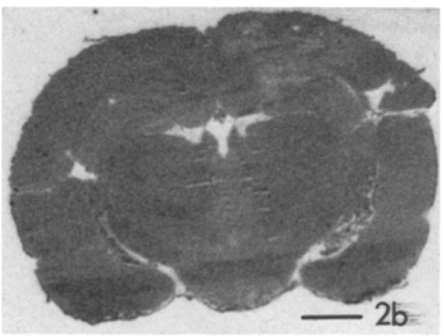

Figure 2 b. Binding to a coronal serial section of rat brain after incubation with 800 pM 2[^{125}I]-melatonin plus 1 μM melatonin. Bar = 2 mm. (By permission of the Journal of Molecular Endocrinology.)

a concentration (80 pM) at least 10 times lower than the K_d of the lower affinity receptors, it would seem that these brain sites are high affinity receptors similar to those found the PT. Further evidence comes from the recent studies on 2-[^{125}I]-melatonin binding sites in the human brain, where an IC_{50} value for melatonin in the SCN of 100 pM was reported[52].

Characterization of melatonin receptors

The pharmacology of melatonin receptors has been studied by numerous groups, and one cannot fail to be struck by the lack of similarity of the binding characteristics for melatonin receptors found by each of the studies. The reasons for the disparate pharmacology are unclear but may be related to the different species and/or tissues used in each of the studies, which in turn may explain the many biological effects attributed to melatonin.

The radioligand

Initial characterization studies utilized [^{3}H]-melatonin as the radioligand, but the low specific activity (ca 30 Ci/mmol) and apparently inconsistent binding of the tritiated ligand[48] made it an unsuitable ligand for pharmacological studies. More recent studies have utilized the radioligand 2-[^{125}I]-melatonin, which was first synthesized and characterized using N.M.R. by Vakkuri et al.[61,62]. As a pharmacological tool 2-[^{125}I]-melatonin has enormous advantages over the tritiated ligand, as it has much higher specific activity (ca 2000 Ci/mmol) allowing femtomole amounts of binding to be measured, it can be counted directly by gamma counting, and for use in in vitro autoradiography much shorter exposure times are required. However, attaching a large atom like iodine to such a small molecule as melatonin could alter its biological activity, devaluing its benefits as a labelling ligand. Fortunately, it has been shown that 2-[^{125}I]-melatonin retains the biological activity of melatonin in both the frog skin melanophore bioassay[28,60] and in influencing foetal testicular development in hamsters, making it an appropriate ligand for binding studies[72]. Furthermore, the molecule seems stable and resistant to degradation by tissue homogenates, as intact 2-[^{125}I]-melatonin can be recovered and isolated by either TLC or HPLC after incubation with membranes prepared from the brain or PT[20,46].

Characterization of binding site identified by in vitro autoradiography

Few studies have examined melatonin receptors utilizing the combined approach of in vitro autoradiography together with homogenate receptor assays. The first study to use this approach revealed the discrete localization of melatonin binding sites in the rat brain[66] and this knowledge allowed homogenate receptor assays to be performed on an identified binding site (ME/PT), revealing a binding site with the lowest equilibrium dissociation constant (see table 2) yet determined[63,66]. More recently in vitro autoradiography has been used to localize melatonin binding sites on the pars tuberalis of the sheep[46] and the hamster[65] and the pharmacological characteristics of this receptor have been described[46,65]. Interestingly, the binding characteristics determined for the rat ME/PT[62,65], the hamster and the ovine PT[46] do show many similarities.

Binding kinetics

The binding of 2-[^{125}I]-melatonin to the ovine PT was found to be profoundly sensitive to temperature. At 37 °C, the association of 2-[^{125}I]-melatonin with the ovine PT binding site was found to occur rapidly over the first 30 min, but equilibrium binding was not reached until 120 min, thereafter remaining stable for up to 6 h. The rate of association at 1 °C was found to be much slower, with negligible binding achieved even after 24 h[46]. The time-course of 2-[^{125}I]-melatonin binding by the PT shows an interesting inter-species difference, as in the hamster equilibrium binding is achieved more rapidly after only 30 min[79] (see fig. 3). A similar time-course has been found for the rat ME/PT, albeit at 28 °C[63]. However, the interspecific difference in binding kinetics for the PT is only slight by comparison to those observed at other sites. Where melatonin binding sites in whole brain homogenates have been studied, those from rat brain have been reported to have a similar time-course to both the rat and hamster PT[37,63,79]. In contrast, extraordinarily rapid kinetics of association have been reported for whole hamster brains, where equilibrium binding is apparently reached after only 5 min at 0 °C[20,48]. Why the rate of association should be so rapid at 0 °C in hamster brain homogenates compared to hamster PT as 37 °C is not clear. 2-[^{125}I]-melatonin has also been used to characterize melatonin receptors in chicken retina, and the binding kinetics for this site are different again. In the chicken retina, equilibrium binding is reached after 3 h at 0 °C and only 9 min at 37 °C[19]. These rather disparate binding kinetics encourage the view that many of the binding sites measured in the different studies are distinct and unrelated.

In most studies the binding of 2-[^{125}I]-melatonin has been shown to be reversible, as addition of excess diplacing ligand (usually melatonin or chloromelatonin) causes the dissociation of 2-[^{125}I]-melatonin from its binding site. From the kinetics of association and dissociation both an association rate constant (k_{+1}) and a dissociation rate constant (k_{-1}) can be measured, from which an estimate of the equilibrium dissociation constant ($K_d = k_{-1}/k_{+1}$) can be calculated. By this method the K_d for the ovine PT (25 pM), the chicken retina (342 pM), the rat brain (45 nM) and the hamster brain (3.08 nM) have been estimated[19,20,37,46].

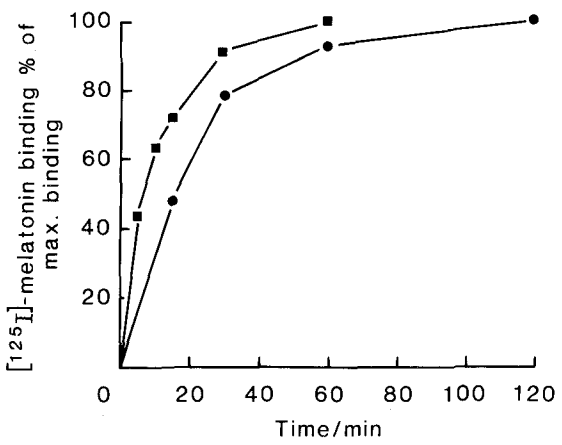

Figure 3. Comparison of the time course of binding for 2[^{125}I]-melatonin to ovine PT (●—●) and hamster PT (■—■) measured at 37 °C; binding is expressed as % of maximal 2[^{125}I]-melatonin bound.

Saturation analysis

The equilibrium dissociation constant can also be derived from saturation studies where fixed concentrations of membrane are incubated with increasing concentrations of radioligand, producing a saturation isotherm of specific binding. Transformation of these data by Scatchard analysis[58] or analysis by more sophisticated computerized approaches[47] provides estimates of both K_d and B_{max} (number of receptor sites/mg protein). As for the kinetics of binding, the estimates of K_d and B_{max} by this method are generally as variable as the tissues used for the binding studies (table 2), although within each study there does seem to be general agreement between the K_d's derived by kinetic and saturation data[19,20,37,46]. However, there are four studies where the estimates of K_d are quite similar, and in each of these studies the PT was used as the source of the receptor. Utilizing a one site binding model[47] a K_d of 32.5 pM was determined for the ovine PT[46], and this is close to the K_d's estimated for the rat ME/PT[63,64,66] and the hamster PT[65,79] (see table 2), indicating that for the PT in at least three species, the melatonin receptor has similar affinity. With regard to the sites of binding in the brain, it is tempting to suggest that the difference in K_d between the rat, the hamster and the chicken could be explained by species differences. However, even within species, there seems to be lack of agreement, as for the hamster one report provides evidence for a single class of binding site[20], whereas another indicates that both high and low affinity sites exist[48].

The variation in B_{max} values (see table 2) is equally intriguing. The ovine PT is a tissue where binding sites were first identified by in vitro autoradiography and one which can readily be dissected out as a discrete entity; it has a B_{max} value of 103 fmol/mg protein[46]. The B_{max} values for the PT of both the rat and hamster are much lower, as it is more difficult to dissect out the PT as a discrete gland in these species. The additional extraneous non-specific tissue increases the total protein, thereby lowering the B_{max} and making its value relative rather than absolute. The B_{max} values for melatonin binding sites in the whole brain range from 5.6 to 123 fmol/mg protein (see table 2); thus considering the discrete localization of melatonin binding sites in the brain as revealed by in vitro autoradiography it is intriguing that B_{max} values are similar to those found for the ovine PT, as one would predict that the high level of extraneous non-specific tissue protein in the brain preparation would lead to extremely low estimates of receptor density.

Table 2. K_d and B_{max} values for 2-[^{125}I]-melatonin and [^{3}H]-melatonin binding sites from various tissues

Tissue	K_d		B_{max} (fmol/mg protein)	Reference
a) Determined using 2-[^{125}I]-melatonin as the radioligand (sp. act. 20–2175 Ci/mmol)				
Ovine pars tuberalis (PT)	32.5	pM	103	46
Rat median eminence (PT)	20.9–60.3	pM	8.5–25.7*	63, 66
Hamster median eminence (PT)	29–59.1	pM	2.5–10.4**	65, 79
Rat anterior pituitary	63.2	pM	3*	63
Hamster anterior pituitary	97.2	pM	6**	65
Whole hamster brain	3.3	nM	110	20
Whole hamster brain	0.32	nM	5.6	48
(synaptosomal membranes)	10.5	nM	123	
Whole rat brain (synaptosomal preparation)	38	nM	81	37
Chicken retina	0.434	nM	74	19
b) Determined using [^{3}H]-melatonin as the radioligand (sp. act. ca 30 Ci/mmol)				
Bovine medial basal hypothalamus	12	nM	8–14	10
Rat hypothalamus	8.65	nM	78	49
hippocampus	11.3	nM	166	
striatum	28	nM	61	
midbrain	302	nM	1370	
Hamster ovary (cytosolic)	6.3	nM	52	13
	0.55	μM	419	

* B_{max} changes with development; ** B_{max} altered by photoperiod.

Melatonin receptor pharmacology

The pharmacology of the melatonin receptor has been explored relatively little, but the K_i/ED_{50} values of different compounds for melatonin receptors taken from different sources are compared in table 3. The order of potency of melatonin and related indoles able to compete with the iodomelatonin binding site on the ovine PT shows some similarities to that of the rat ME/PT and the chicken brain and retina. However, a detailed comparison is precluded as a different range of compounds was tested in each study. Against the ovine PT binding site three levels of competitive potency were identified. Compounds with the highest level of potency were found to have K_i values close to the K_d of iodomelatonin for the binding site (i.e., in the picomolar range). These compounds include 2-iodomelatonin, 2-chloromelatonin and melatonin, and they are structurally characterized by having both a 5-methoxy group, and a N-acetyl group on the side-chain nitrogen of the molecule. Replacement of either of these two functional groups leads to a dramatic drop in potency. The substitution of the 5-methoxy group has a less dramatic effect on the competitive potency of the molecule, as seen in N-acetylserotonin, which has a K_i in the nanomolar range. Those compounds which lack the N-acetyl group have very weak competitive potencies in the micromolar range[46]. The correlation of these two functional groups with the potency of binding has also been made for the chicken retina[19] and the hamster brain[20]. However, despite these apparently similar structural requirements, the order of potency and K_i values for both the chicken retina and the hamster brain are quite different[18]. This has led to the suggestion that two different types of melatonin receptors might exist[18]. ML-1 has been proposed to be a high affinity receptor, encompassing the binding sites of the chicken brain and retina[19] and the rat ME/PT[66]. ML-2 has been proposed as a low affinity binding site to encompass sites with lower binding affinities and different pharmacologies to those common to ML-1, such as those of the hamster brain[20]. However, although the possibility of

Table 3. Comparative competitive potencies of selected compounds against either 2-[^{125}I]-melatonin (K_i) or [^{3}H]-melatonin (ED_{50}) binding in various tissues (nm)

Compound	Ovine PT[46] (K_i)	Chicken retina[19] (K_i)	Hamster brain[20] (K_i)	Hamster brain[48] (synaptosomes) (K_i)	Bovine MBH (ED_{50})[10]
2-iodomelatonin	0.0093	2.5	5.3	0.29	
6-chloromelatonin		4	3.9	6	
2-chloromelatonin	0.0699				
Melatonin	0.1174	6.3	10.8	44	20
N-acetylserotonin	194		7.7		250
ML-23 (N-(2,4-dinitrophenyl)-5-methoxytryptamine)	971				
5-methoxytryptophan	1080	> 100,000	> 100,000		80
5-methoxytryptophol	1230	46,400	303	130	80
5-hydroxytryptamine	3000	> 100,000			
5-methoxytryptamine	33,400	4600	1023	175	250
6-hydroxymelatonin		74	8.6	265	> 10,000

more than one type of binding site always exists, it seems premature to invoke the existence of two receptor types on the basis of present evidence. The kinetics, affinities and pharmacologies of the iodomelatonin binding sites from the various tissues examined are so different that it seems one would need to invoke a different receptor sub-type for each tissue. This is unlikely and it seems prudent therefore not to speculate on the existence of more than one type of melatonin receptor until binding sites from different sources are compared within the same receptor-assay system using the same incubation conditions and separation protocols. However, despite this caveat, it does appear that there is sufficient pharmacological similarity between the studies of the PT binding site from different species to suggest that they do have the same receptor.

Receptor agonists and antagonists

The pharmacological characteristics of the melatonin binding sites in each of the aforementioned studies are consistent with a membrane bound receptor. However, implicit in the term 'receptor' is a functional response, which is manifested as a consequence of receptor site occupation by an agonist. As the information on the location of melatonin receptor sites is still relatively new, few studies have yet addressed the functional responses of the melatonin receptor, and as a consequence our knowledge of those compounds which act as agonists and antagonists is still rudimentary.

Some of the first studies to reveal agonists/antagonists of the melatonin receptor were made on frog skin and isolated melanophore preparations[28,44]. Heward and Hadley[28] demonstrated in frog skin that N-acetyltryptamine can block the activity of melatonin, yet on its own it has no intrinsic biological activity. They concluded therefore that for the frog skin melatonin receptor the N-acetyl substitution is the primary structural determinant which affects the affinity of binding to the receptor whereas the 5-methoxy group primarily determines the activity of the receptor. A similar conclusion has been reached for the retinal melatonin receptor[16]. As 2-iodomelatonin, a molecule with a bulky iodine atom attached to the 2 position, acts as a potent agonist in the melanophore and retinal assays[16,60], the binding of melatonin to its receptor must involve the face of the molecule with carbons 3, 4 and 5 (fig. 4). Although the structural requirements for binding of melatonin and related analogues show considerable similarities, these do not translate into comparable pharmacologies, as melatonin is a more potent agonist than 2-iodomelatonin in the melanophore assay, whereas in the retina the converse is found[16,60].

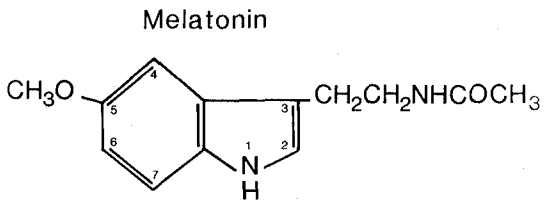

Figure 4. Structure of melatonin (N-acetyl-5-methoxytryptamine) showing the numbering nomenclature.

Two melatonin receptor antagonists have been developed so far. The first is 2-benzyl-N-acetyltryptamine (luzindole, N-0774) which was developed from the observation that N-acetyltryptamine acts as a competitive antagonist against the melatonin receptor in frog skin and the chicken retina[16,18] and as a partial antagonist in the rabbit retina[18]. Substitution of a benzene ring at the 2-position of N-acetyltryptamine produced a competitive antagonist, having no intrinsic biological activity up to 10 μM when tested in the rabbit retina assay[18]. The second melatonin antagonist which has been reported is N-(2,4-dinitrophenyl)-5-methoxytryptamine (ML-23)[39,91]. In the rat, this compound was found to antagonize the inhibitory effect of melatonin on the release of dopamine from the hypothalamus in vitro, and to block both delayed sexual maturation and inhibition of ovulation induced by melatonin[39,85,86]. On the basis of the structural requirements for binding discussed above, one would not predict ML-23 to be a very effective antagonist, because it lacks an N-acetyl group thought to be important for binding. Consistent with this prediction is the inability of ML-23 to antagonize the inhibition of dopamine release from rabbit retina induced by melatonin[18]. Similarly, ML-23 has recently been shown not to inhibit the reproductive effects of melatonin in the Soay ram[40]. Moreover, when allowed to compete with iodomelatonin for binding sites in the ovine PT binding assay, ML-23 was shown to have very weak competitive binding potency[45]. Thus at present the role of ML-23 as a melatonin receptor antagonist would seem restricted to inhibition of the effects of melatonin in the rat.

Factors affecting binding

Circadian rhythmicity

The potential circadian changes in melatonin receptor affinity and density have so far received limited investigation, but again the literature on this subject is inconsistent. Circadian changes in the ability of 1 μM melatonin to inhibit dopamine release from the hypothalamus of the adult rat (CD strain) have been reported[87]. Peak inhibition is observed at 05.00 h and the inhibition minimum at 15.00 h. These changes are not observed in the new-born rat but are apparent one week after birth[87]. However, the diurnal changes in melatonin binding show no correlation, as the same group have reported that whilst the affinity of the receptors in the brain (hypothalamus) remains unchanged over 24 h, receptor density shows circadian variation with the highest density at 18.00 h[90], at which time the inhibition of dopamine release by melatonin is almost at a minimum. The apparent

discrepancy between these results remains to be explained. The situation is further confused by the fact that another study on rat hypothalamus shows circadian changes in both receptor density and affinity[22], with the changes in density showing no correlation with those shown by Zisapel et al.[87]. A clear diurnal rhythm in density of autoradiographic binding has been demonstrated in the SCN of the rat, with a nadir at lights off[36a]. However, it remains to be determined if the rhythms persist in the pinealectomized animal in the absence of endogenous melatonin.

Development

It is known that the levels of melatonin in the foetal circulation of the sheep show diurnal variation[43], and that this rhythm disappears following maternal pinealectomy, indicating that the foetus does not produce its own melatonin rhythm[83,84]. As the temporal information conveyed by the pattern of melatonin secretion experienced by the foetus of the Djungarian hamster and the spiny mouse has profound effects on their post-natal reproductive development[70,71], the foetus must be capable of responding to the maternal signal (Ebling and Foster, this issue). Recently, 2-[^{125}I]-melatonin binding sites have been identified in the foetal hamster brain[72], indicating the importance of melatonin receptors at a very early stage of development. In the rat the developmental changes in the affinity and density of melatonin receptors in the anterior pituitary and median eminence (ME) have been studied[64]. At both sites the receptor affinities were found not to change significantly through the course of development from day 20 of foetal life to day 29 of post-natal life. Receptor density in the ME also did not change, whereas the density in the pituitary progressively decreased with time[64]. In view of the recent demonstration that the ME binding site is really the PT of the adenohypophysis[78], this decrease in B_{max} in the pituitary raises the possibility of melatonin receptors being present in the anterior pituitary during the early days of foetal life which then disappear during the course of foetal development.

Steroids

Steroids play an important modulatory role in regulating gonadotrophin output both during the breeding period and the non-breeding period of seasonally reproductive animals[41]. However, the photoperiodically driven timing (i.e. melatonin action) of the reproductive cycles seems to be steroid independent[21,32,41], and this would seem an essential property of any timing mechanisms. Thus one would predict that steroids would not affect those melatonin receptors involved in translating the photoperiodic response. Nonetheless, in the non-seasonally breeding rat, steroid sensitive melatonin binding sites have been reported[38,88,89]. Therefore, it will be interesting to determine whether melatonin receptors are differentially sensitive to steroid action in seasonally breeding animals.

Inferences for a mode of action

The central sites of melatonin binding, revealed by in vitro autoradiography, are discrete and some appear to be species-specific. It seems unlikely that all sites are involved in the reproductive responses associated with seasonal breeding, however two sites namely the SCN and the PT are common to all species. How does this information help us to understand the mode of action of melatonin, in particular as a hormone that times seasonal breeding?

A role for the SCN

The identification of melatonin binding sites in the SCN of the rat[63,66,76,78], the Syrian and Dungarian hamsters[72,73,77,79], and the sheep (Williams, unpublished) and the human[52] is consistent with it being an important site of action. The SCN is considered to be the 'biological clock' of the brain and it is known to control numerous cerebral and somatic circadian rhythms, including the synthesis and secretion of melatonin by the pineal gland. Although pinealectomy has little effect on circadian rhythms, exogenous melatonin, albeit at pharmacological doses, can reset the locomotor and drinking activity rhythms in rats, an action which is dependent on an intact SCN (see Armstrong, this issue[3,50]) and melatonin has also been shown directly to inhibit both the metabolic and protein synthetic activity of the SCN[12,54]. However, the situation relating to the seasonal control of reproduction is less clear. Evidence from two species suggests that the SCN has no role in mediating the photoperiodically regulated reproductive response, as bilateral lesions of the SCN failed to inhibit melatonin-induced gonadal collapse in the Syrian hamster[6,42] and melatonin-dependent delayed implantation in the spotted skunk[4]. Thus at least in these species the action of melatonin within the SCN appears to be exclusively concerned with circadian rhythmicity (but see Bartness and Goldman, this issue). In the Syrian hamster other areas which bind 2-[^{125}I]-melatonin include the preoptic area, the APVT and the medial region of the lateral habenular nucleus, which together with the SCN form an interlinked neural network[31,67,68]. The preoptic area contains LHRH perikarya[30], whilst the SCN and APVT have been implicated in the circadian control of LHRH release[69]. The SCN is also known to have reciprocal connections with the ventromedial nucleus, which is a weak circadian oscillator[45] and has been identified as a centre important to appetite control[53]. These areas when considered together provide a neural network which could account for the diverse effects of melatonin. It is also possible that the melatonin acting on the SCN is of retinal rather than pineal origin. Melatonin is produced by the retina[74] and melatonin has been visualized by

immunofluorescence in the optic nerve and chiasma[8], which is known to innervate the SCN, although in the frog, evidence indicates that retinal melatonin is rapidly metabolized in situ[9].

A role for the PT

The relative optical densities (O.D.) of [^{125}I]-melatonin binding sites in the brain and pituitary of the rat, Syrian hamster and sheep, reveal that the PT binds the radioligand at the highest concentration in all three species (fig. 5). The localization of melatonin binding sites in this region of the pituitary lends support to the view that the PT is, both morphologically and functionally, a distinct region of the adenohypophysis, and not simply an undifferentiated region of the PD[59]. Although the function of the PT remains to be defined, it may play a role in mediating the photoperiodic effects of melatonin. An immunocytochemical study of the PT from numerous mammalian species has revealed that the PT is composed of both gonadotrophs and thyrotrophs; yet while these cell types are a general and consistent feature of the PT in all mammals studied, their relative proportions vary between species. In contrast, the cellular composition of the PD remains relatively constant, even between species[26]. Consistent with a role involved in mediating photoperiodic responses, immunoreactive TSH cells of the PT of the Djungarian hamster have been shown to change in number after transfer from LD to SD; these changes were not mirrored by the immunoreactive-TSH cells of the PD, indicating a specific photoperiodic response of the PT[80]. However, in view of the low numbers of gonadotrophs and thyrotrophs in the PT relative to the PD, it seems unlikely that these cells in particular are able to influence the endocrine profile dramatically. However the PT is also composed of cells which have the ultrastructural features of peptide secretory cells, but which cannot be visualized using antibodies directed against any of the trophic hormones produced by the PD. These cells are consequently called specific secretory cells[59]. They do not show any changes in response to major experimental perturbations of the endocrine system, such as hypophysectomy, adrenalectomy, thyroidectomy and castration[14], yet pronounced ultrastructural changes have been observed during hibernation and in response to changes in photoperiod[14,81]. The products of these cells remain to be identified. A special feature of the PT is its position between the ME and PD, allowing it to interact with both these regions[14]. In this way it would be possible for melatonin to modulate the signalling between the hypothalamus and the PD. Thus it seems likely that the action of melatonin at the level of the PT may involve an, as yet, unidentified secretory product.

Interpretation of the melatonin signal and the reproductive response

Most experimental evidence indicates that the important component of the melatonin signal is the duration of the elevated night-time levels[5,11,15,25,34,82], which is reflected by a proportional relationship with the length of the scotophase[2,7,29,35,55,57]. (Ebling and Foster; Bartness and Goldman, this issue). Thus the message that melatonin conveys is time. During the transition from long to short days an animal experiences minor daily increments in the melatonin (time) message. However, the reproductive response to the same changing temporal information, conveyed by the melatonin signal, differs according to species. Short day breeders such as sheep respond to the lengthening melatonin signal by becoming reproductively active[41], whereas the response in long day breeders like hamsters is to become reproductively quiescent[51]. Therefore, the same temporal information conveyed through the melatonin signal results in the opposite reproductive response in these two species. In the sheep recent evidence indicates that seasonal breeding is driven by an intrinsic annual rhythm, and the role of the daylength and hence melatonin is to synchronize the circannual rhythm with the seasons[56]. In contrast Syrian hamsters which are maintained on LD remain in a reproductively active state[51] indicating the absence of an underlying circannual rhythm. This suggests that the melatonin decoding system in the sheep is fundamentally different to that of the hamster.

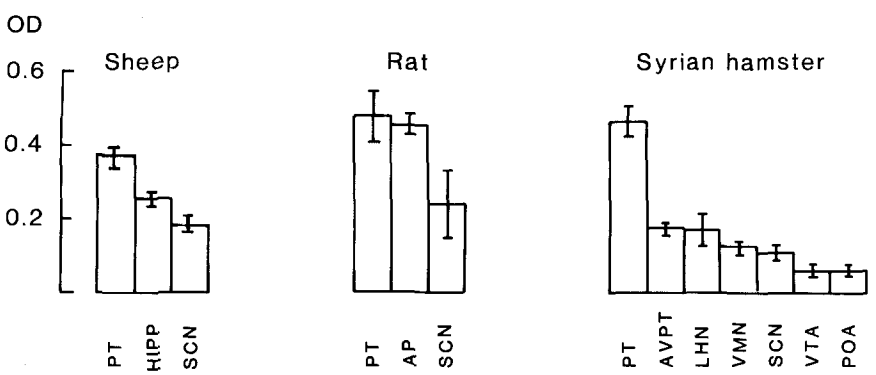

Figure 5. Comparative optical densities (OD) of images produced by 2[^{125}I] melatonin binding on X-ray film. OD are only comparative within species as incubation conditions varied between species (See table 1 for abbreviations).

One explanation of this differential response could be that the target site(s) important to the reproductive response in the sheep is different to that for the hamster. Indeed the marked inter-specific differences in the location of melatonin binding sites might be deemed to support such a hypothesis. In the Syrian hamster and the white-footed mouse, which are both long-day breeders, intra-cerebral implant studies have demonstrated a hypothalamic site of action for the reproductive effects of melatonin[23,24,27]. In the Syrian hamster the SCN is known not to be involved in mediating this response. This leaves the VMN and/or the POA as putative hypothalamic sites of action for the reproductive effects of melatonin. In the sheep, however, the existence of an intrinsic annual rhythm of reproduction pre-supposes its maintenance by an oscillator or clock, which is reset by the melatonin signal, but free runs in its absence. Only three sites of iodomelatonin binding have been identified in the sheep, and of these the SCN is the only likely candidate for such a function. Consequently, although the SCN has been ruled out as the principal site mediating the reproductive response in the hamster it may be premature to do the same in the sheep.

The alternative to the foregoing argument is that melatonin acts through a common target site in both species, yet the difference in the reproductive response occurs through differential output from that site of action. The PT is a common target tissue, which could provide a differential output in response to melatonin due to its species-specific cellular composition[26]. However, the implication of a 'clock' in the control of the annual rhythm of reproduction in sheep, together with the evidence for a hypothalamic target site in rodents, indicates that the PT alone can not mediate the reproductive effects of melatonin and suggest a neural site of action is required. Another common site of action is the SCN, but as this is not involved in the reproductive response of the hamster one would also have to rule out this target site in the sheep. This would suggest that another hypothalamic site is involved in decoding the melatonin message. However, no other hypothalamic binding sites have been yet identified in sheep by in vitro autoradiography (table 1), implying that the neural target sites of primary importance to the reproductive response remain to be localized.

Thus two hypotheses can be marshalled to explain the differential response of LD and SD breeders to melatonin. The first suggests that the target sites important to the reproductive response are different in the two types of breeders, and this is supported by the differential localization of receptors. The second hypothesis proposes that melatonin acts through the same receptor site and the difference in end response is produced through differential output. Implicit in the latter is that the neural target site in the sheep has still to be visualized. On the basis of data presently available it is impossible to distinguish between these two possibilities. However, the precise localization of melatonin binding sites now allows each locus to be assessed for its role in the different responses to melatonin.

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Neural systems underlying photoperiodic time measurement: A blueprint

J. Herbert

Department of Anatomy, University of Cambridge, Downing St., Cambridge CB2 3DY (England)

Summary. This paper briefly reviews the formal properties of the photoperiodic time measurement apparatus of mammals and presents a hypothetical model for the operation of the neural systems responsible for reading and responding to the nocturnal pineal melatonin signal. The primary melatonin readout mechanism is held to be common to all species responsive to melatonin. It seems likely that this mechanism responds to relative changes in the duration and amplitude of the melatonin signal, rather than the absolute levels of melatonin encountered. A series of neural systems which exploit the calendar information provided by the primary readout is envisaged to vary between and within species, depending upon the neuroendocrine response under consideration. Of particular importance is a mechanism for comparing the relative duration of successive melatonin signals. These more complex elements are responsible for phenomena such as the effects of photoperiodic history and photorefractoriness. The brain may be able to encode an accumulated memory of melatonin signals and thereby define longer term intervals within the annual cycle. A series of response elements within the hypothalamus are engaged by the appropriately processed photoperiodic stimuli. For all elements of this model, their anatomical representations are poorly understood or, in certain cases, completely unknown.
Key words. Melatonin; pineal; hypothalamus; photoperiodism; neural timers.

This paper tries to set out a blueprint for the neural systems that may be concerned with the photoperiodic responses of mammals. This blueprint is, inevitably, drawn in outline only. All we can do at the moment is to consider the properties of the photoperiodic signal and the effects it has on dependent functions, and then try to deduce some features of the neural systems that must be responsible for its effects.

Cognitive vs vegetative time measurement

The brain is concerned with measuring time in at least two distinct ways. The first, which is outside the scope of this paper, involves such functions as conscious estimates of the passage of time or the ability to order events, either in the present or in the future, according to their relationships in time and temporal values. These functions are