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Melatonin Receptors: Past, Present and Future

Introduction

The pineal hormone melatonin has a profound impact on seasonal physiology in photoperiodic mammals. These effects are thought to be mediated by melatonin action *via* melatonin receptors in neural sites and in the pituitary *pars tuberalis* (PT). Melatonin also influences circadian rhythms in a wide variety of species, with even more robust effects in birds and reptiles than in mammals (Cassone, 1990; Weaver, 1999). In mammals, the circadian effects of melatonin are thought to be mediated by melatonin receptors in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus. Effects of melatonin on retinal physiology, pancreatic secretory activity, striatal neurochemistry and other responses have also been described.

In this review, we will describe the initial discovery and characterization of melatonin receptors, their molecular identification, and nomenclature. We will also discuss the present state of knowledge regarding sites and molecular mechanisms of melatonin action in a few specific responses. Other chapters in this volume and in the other volumes of this series discuss other aspects of melatonin action, and the reader is referred to these other chapters for more focused discussion on specific physiological systems.

Initial discovery of high-affinity melatonin receptors

Interest in melatonin sites and mechanisms of action existed in the 1970's, but studies conducted in that and even the following decade to identify melatonin receptors were limited in usefulness because the only available radioligands were versions of melatonin labeled with tritium or ^{14}C (Anton-Tay and Wurtman, 1969; Cohen et al., 1978; Cardinali et al., 1979; Vacas and Cardinali, 1980; Niles, 1987). This labeling method produces a radioligand with relatively low specific activity, and thus relatively high concentrations of radioligand were used. We now understand that there is a remarkably low density of melatonin receptors in most tissues, and attempts to detect them using relatively high (nM – μM) concentrations of radioligand leads to a great excess of non-specific over specific binding. As a result, these early studies were essentially doomed by available reagents to be unable to detect the low density of high-affinity melatonin receptors. While published work from the 1970's and early-to-mid 1980's describes melatonin binding sites, these studies are inconsistent with respect to the properties of melatonin binding sites (for review, see Kennaway and Hugel, 1992).

A key breakthrough came in 1984, when Vakkuri and colleagues demonstrated that it is possible to directly iodinate melatonin (Vakkuri et al., 1984a). While their interest was in generating an improved melatonin radioimmunoassay (Vakkuri et al., 1984b), the impact that this discovery has had in identifying melatonin receptors cannot be overstated. 2-^{125}I-iodomelatonin can be labeled to 2200 Ci/mmol, has a low level of non-specific background binding, and has picomolar affinity for melatonin receptors. These qualities have made it the key reagent for identification of melatonin receptors.

Several groups rapidly adopted 2-^{125}I-iodomelatonin for attempts to identify melatonin receptors. Radioreceptor assays using 2-^{125}I-iodomelatonin were developed in several laboratories, including those of Margarita Dubocovich, Peter Morgan, Lennart Niles, Steve Reppert, and Nava Zisapel (Duncan et al., 1986; Laudon and Zisapel, 1986; Dubocovich and Takahashi, 1987; Zisapel, 1988; Pickering and Niles, 1989; Rivkees et al., 1989; Weaver et al., 1989; Pickering and Niles, 1990a). The characteristics of 2-^{125}I-iodomelatonin binding varied between these studies, and a consensus regarding affinity and pharmacological profiles was lacking. It is likely that some part of this difficulty related to the use of relatively high concentrations of radioligand and the use of tissue preparations that contained a relatively low density of melatonin receptors.

The first compelling use of 2-^{125}I-iodomelatonin to identify melatonin receptor binding was accomplished by Dubocovich and Takahashi (1987), who identified high-affinity melatonin binding sites in chicken retina. Dubocovich (1983, 1985) had previously characterized a functional melatonin receptor in rabbit retina by assessing the ability of melatonin and analogues to inhibit calcium-dependent dopamine release. By correlating the affinity of various drugs for inhibition of dopamine release in rabbit retina with the affinity of these same drugs to inhibit 2-^{125}I-iodomelatonin binding in chicken retina, they ascribed function to retinal melatonin binding sites.

Dubocovich and colleagues and Niles and colleagues identified a 2-^{125}I-iodomelatonin binding site in hamster

brain with a fundamentally different pharmacological profile, most notably a relatively higher affinity for N-acetylserotonin and prazosin (Duncan et al., 1986; Niles et al., 1987; Dubocovich, 1988; Duncan et al., 1988; Pickering and Niles, 1989).

To distinguish these two apparent types of melatonin binding site, the first melatonin receptor nomenclature was introduced (Dubocovich, 1988). The sites with the properties identified in chicken retina that were high-affinity and correlated with functional activity were named ML-1 sites, while the lower-affinity, prazosin-sensitive 2-^{125}I-iodomelatonin binding sites characterized in hamster brain were called ML-2 sites (for reviews, see Dubocovich, 1988, 1995).

A further advance in identifying melatonin receptors was the demonstration that 2-^{125}I-iodomelatonin labels anatomically specific sites in brain, as demonstrated by receptor autoradiographic methods. The first such study, published by Vanecek and Illnerova (1987), demonstrated putative melatonin binding sites in the rat suprachiasmatic nucleus. This study was rapidly followed by similar work in other labs (Reppert et al., 1988; Weaver et al., 1988, 1989, 1991b; Duncan et al., 1989; Rivkees et al., 1989b; Laitinen and Saavedra, 1990a,c; Morgan et al., 1994) showing anatomically specific, saturable, high-affinity binding sites ($K_d < 200$ pM) that possessed an ML-1 like pharmacological profile. The rank order of drug potency in inhibiting 2-^{125}I-iodomelatonin binding was 2-iodomelatonin > 6-chloromelatonin > melatonin > 6-hydroxymelatonin > N-acetylserotonin >> 5-hydroxytryptamine. High-affinity melatonin receptors are coupled to G proteins, as sodium ions and GTP analogs inhibit high-affinity 2-^{125}I-iodomelatonin binding (Morgan et al., 1989; Rivkees et al., 1989a; Weaver et al., 1993; Laitinen and Saavedra, 1990a,b,c; Laitinen et al., 1990). Of note, Duncan and colleagues (1989) showed the binding sites with the characteristics of ML-1 and ML-2 sites were both present in hamster brain, further demonstrating that these sites represent different entities (and not just species variation in receptor profile). In some cases, species differences in the binding properties of a receptor can be significant (Oksenberg et al., 1992).

Several studies identified the SCN as a site of melatonin receptor binding in a variety of species (Vanecek and Illnerova, 1987; Reppert et al., 1988; Duncan et al., 1989, for an early review, see Weaver et al., 1991b). This is consistent with the SCN being the site likely to mediate melatonin effects on circadian rhythms. The identification of melatonin receptors in the human SCN (Reppert et al., 1988; Weaver et al., 1993) provides the likely substrate for entraining effects of melatonin in blind people (Lockley et al., 2000).

Another site found to be commonly labeled in autoradiographic studies of melatonin receptor binding was the *pars tuberalis*, a small piece of pituitary tissue that covers the median eminence and pituitary stalk (Williams and Morgan, 1988; Weaver et al., 1991; Morgan et al., 1994). Notably, this area contained the highest density of binding sites in most species. Its location at the interface between the hypothalamus and pituitary led to speculation that this site was important for neuroendocrine responses to melatonin, and indeed this is partially true. Melatonin, acting in the PT, plays an important role in the regulation of pituitary prolactin secretion, and thus contributes to seasonal regulation of moulting and neuroendocrine function (Morgan, 2000; Graham et al., 2002; Lincoln et al., 2006). Additional, species-specific neural sites of melatonin action appear responsible for regulation of gonadotrophic responses (Maywood and Hastings, 1995; Maywood et al., 1996; Lincoln, 1999).

Importantly, early studies employing these autoradiographic methods detected a site-specific distribution of putative receptor binding that correlated well with the distribution of binding sites as assessed by radioreceptor assays conducted on membrane preparations (Vanecek, 1988a; Weaver et al., 1989). Furthermore, tissues identified to have a high density of binding were subsequently found to be sites in which melatonin affected second messenger pathways, particularly G_i-mediated inhibition of forskolin-stimulated cAMP accumulation (Carlson et al., 1989; Morgan et al., 1989, 1995; Vanecek and Vollrath, 1990a; Vanecek, 1995b; see below). Numerous studies thus established that the ML-1 receptor class represents high-affinity, G protein-coupled, functional cell-surface receptors for melatonin.

In contrast, ML-2 type binding sites were not localized in a specific anatomical distribution, but rather appeared to be widespread. In one lab, the assay used to identify the ML-2 site were conducted at coldroom temperatures where it would be unlikely G protein coupling would occur. The ML-2 site has a distinct pharmacological profile (2-iodomelatonin > 6-chloromelatonin > prazosin > N-acetylserotonin > melatonin > 6-hydroxymelatonin >> 5-hydroxytryptamine), and lower affinity (K_d values 1–2 nM) compared to the high-affinity melatonin receptors (Dubocovich, 1988; Pickering and Niles, 1989, 1990).

The unique pharmacological profile of the ML-2 site (namely its high affinity for N-acetyltryptamine) allowed development of a radioligand, 2-[^{125}I]iodo-5-methoxycarbonylamino-N-acetyltryptamine (^{125}I-labeled 5-MCA-NAT, Molinari et al., 1996), and eventual purification of the ML-2 site, leading to its identification (Nosjean et al., 2000). It is not a G protein-coupled receptor. The ML-2 binding site is actually the enzyme, quinone reductase 2 (QR2; Nosjean et al., 2000, 2001). This was proposed based on the isolation of QR2 as a protein binding ^{125}I-labeled 5-MCA-NAT, but was most conclusively demonstrated in subsequent work showing that ^{125}I-labeled 5-MCA-NAT binding is absent in mice with targeted disruption of the quinine reductase 2 gene (Mailliet et al., 2004). Thus, the ML-2 type binding site can be attributed to QR2. The ML-2 binding site was renamed the *MT3*

melatonin binding site by the IUPHAR committee (Dubocovich et al., 1998a; see below). While QR2 is not a cell surface receptor for melatonin, melatonin binding to this enzyme nevertheless may have important effects on cellular physiology (Mailliet et al., 2005).

Nuclear binding sites and putative nuclear receptors have also been described for melatonin. Accumulation of melatonin in the nucleus suggests binding proteins (Acuna-Castroviejo et al., 1994), but the existence and molecular identity of putative nuclear melatonin receptors has not been determined with certainty. Notably, the orphan nuclear receptor, RZRβ, was identified by Carsten Carlberg and colleagues as a nuclear melatonin receptor (Becker-Andre et al., 1994; Steinhilber et al., 1995; for review, see Carlberg and Wiesenberg, 1995). Subsequently, another member of the family of orphan nuclear receptors, RZRα, was shown to have the same properties, and since RZRα is more widely expressed, this receptor was suggested to be responsible for melatonin modulated transcription in the periphery (Wiesenberg et al., 1995). However, other groups have failed to replicate the key finding of melatonin binding to nuclear orphan receptors (Greiner et al., 1996; Hazlerigg et al., 1996a), and indeed even some of the original authors of the publication by Becker-Andre and colleagues (1994) reported in a "Correction" that they were unable to replicate their own findings regarding the proposed activity of melatonin as a ligand for RZRβ (Becker-Andre et al., 1997). This "Correction" is in the literature, but is apparently not published as a retraction because other authors of the paper stood by the original finding (Wiesenberg et al., 1997). There have been no recent reports addressing the proposal that either RZRα or RZRβ is a nuclear melatonin receptor.

Molecular identification of high-affinity melatonin receptors

Initial attempts at molecular identification of melatonin receptors focused on molecular and biochemical methods to isolate receptor protein or cDNA from tissues containing a high density of melatonin receptor binding. Biochemical methods included affinity chromatography and photoaffinity labeling of receptors from lizard and chicken brain and sheep pars tuberalis, all enriched sources of receptor binding (Rivkees et al., 1990; Anis and Zisapel, 1991; Laudon and Zisapel, 1991; Ying and Niles, 1991; Chong et al., 1993). These attempts, in several labs, were unsuccessful in isolating a protein sufficiently pure for amino acid sequence determination.

Molecular approaches included library screening and polymerase chain reaction (PCR) based amplification of potential receptor cDNA's from these tissues as well as from rodent brain. In this current era of fully sequenced genomes, it is important to be reminded of the near-total absence of bioinformatic resources at that time, and the fact that only a handful of G protein-coupled receptor (GPCR) sequences were known. While the PCR-based approach was useful in identifying a number of then-orphan receptors, it did not lead to identification of the first melatonin receptor cDNA, PCR-based approaches only became useful once the structure of the first receptor was identified by other means.

The first melatonin receptor cDNA was isolated by Takashi Ebisawa, working in Steve Reppert's lab at Massachusetts General Hospital (MGH), using an expression cloning strategy (Ebisawa et al., 1994). This approach had successfully been used by colleagues at MGH to isolate receptors for parathyroid hormone (Juppner et al., 1991; Abou-Samra et al., 1992). The tissue source for Ebisawa's effort was a *Xenopus* dermal melanophore cell line. These cells express functional melatonin receptors that regulate melanin aggregation. Indeed, the *Xenopus* dermal melanophore assay was used by Aaron Lerner and colleagues (1958) to isolate melatonin and was used as a bioassay to measure melatonin in fluids for many years (Ralph and Lynch, 1970; Lynch et al., 1975; Sugden et al., 2004). The dermal melanophore cell line used as the source of RNA for the expression cloning study (Ebisawa et al., 1994) was provided by Michael Lerner, one of Aaron Lerner's sons.

The melatonin receptor cDNA isolated from *Xenopus* dermal melanophores encodes a protein of 420 amino acids with seven putative transmembrane regions, consistent with classification of this receptor type within the superfamily of GPCRs (Ebisawa et al., 1994). This receptor was called a "high-affinity melatonin receptor." The characteristics of binding are consistent with a receptor falling in the "ML-1" class, and subsequent work has led to nomenclature systems in which this receptor is the *Xenopus* Mel_{1c} receptor (see below).

More recently, two other isoforms of melatonin receptors have been identified from *Xenopus laevis* skin using a reverse transcription/PCR approach. These receptor cDNAs appear to be alleles of a single locus, and are called the $Mel_{1c(\alpha)}$ and $Mel_{1c(\beta)}$ isoforms (Jockers et al., 1997). Notably, each receptor cDNA encodes a receptor that is 355 amino acid residues in length; this length is much more consistent with the length of other members of the melatonin receptor family than the 420-residue sequence reported by Ebisawa et al. (1994). Jockers and colleagues (1997) propose that the sequence isolated by Ebisawa et al. (1994) could represent either an artifactual sequence present only in the cell line, or a minority sequence expressed in the melanophore cell line that is poorly expressed in frog skin. Interestingly, despite the differences in the carboxyl terminal end of the proteins, the $Mel_{1c(\alpha)}$ and $Mel_{1c(\beta)}$ isoforms do not differ in their gross pharmacological profile from the previously described form (Jockers et al., 1997).

A family of three receptor subtypes

Identification of the first *Xenopus* receptor sequence allowed homology-based screening methods. In other words, having sequence information from one receptor allowed better PCR primers to be designed. Using a PCR-based approach combined with library screening, Reppert and colleagues (1994) succeeded in isolating a high-affinity melatonin receptor from mammals. This high-affinity melatonin receptor also has a pharmacological profile consistent with the "ML-1" class. The distribution of the transcript overlaps beautifully with the distribution of melatonin receptor binding as determined by autoradiography, including expression in the SCN and *pars tuberalis*. This led Reppert et al. (1994) to conclude that this high-affinity receptor subtype is the "receptor that mediates circadian and reproductive responses to melatonin." However, no physiological data were provided to support this contention, and the title was written at a time when the authors were unaware of the existence of other high-affinity melatonin receptors.

Additional efforts at isolating melatonin receptors in mammalian and non-mammalian species led to the realization that a family of three high-affinity melatonin receptors exists in vertebrates (Reppert et al., 1995a, 1995b; for review see Reppert and Weaver, 1995). Each of these receptor subtypes has high affinity for melatonin and a pharmacological profile grossly consistent with the "ML-1" class (Reppert et al., 1994, 1995a, 1995b; Jin et al., 2003). When the need for a nomenclature for multiple subtypes became apparent (Reppert et al., 1995b), these receptors were named the Mel_{1a}, Mel_{1b}, and Mel_{1c} receptor subtypes (Table 1). The first mammalian receptor isolated (Reppert et al., 1994) became the Mel_{1a} receptor, the second one isolated (Reppert et al., 1995a) became the Mel_{1b} receptor. The *Xenopus* melatonin receptor subtype isolated by the original expression cloning strategy (Ebisawa et al., 1994) and found in several other non-mammalian vertebrates (Reppert et al., 1995b) is a subtype distinct from both the Mel_{1a} and Mel_{1b} receptors, and is called the Mel_{1c} receptor.

An IUPHAR receptor nomenclature committee addressed melatonin receptors (Dubocovich et al., 1998a), and as a result the Mel_{1a} receptor is now called the MT1 receptor, while the Mel_{1b} receptor is now designated the MT2 receptor.

Of the family of three molecularly defined, high-affinity melatonin receptor subtypes present in vertebrates, only the MT1 and MT2 receptors are present in mammals. No mammalian homologues of the Mel_{1c} receptor have been isolated yet. Unfortunately, the IUPHAR nomenclature system does not include receptors found only in non-mammalian species, so the Mel_{1c} receptor has no place in this system. The failure of the MT1/MT2 nomenclature system to address the non-mammalian Mel_{1c} receptor subtype, or the structurally related melatonin receptor-related receptor (GPR50, see below), ensures perpetual diversity in nomenclature usage within the melatonin receptor field. Studies in non-mammalian species require the usage of the older subscript-based nomenclature.

Table 1: Melatonin receptor nomenclature, past and present

Original ML System	Cloned Receptor Names	Current nomenclature
ML-1 binding	Mel_{1a}	MT1
	Mel_{1b}	MT2
	Mel_{1c}	not included in nomenclature
ML-2 binding	Quinone reductase type 2	MT3; Quinone reductase type 2

A melatonin receptor-related receptor

A mammalian melatonin receptor-related receptor (MelRR-1, also called GPR50) has been identified (Reppert et al., 1996a; Drew et al., 1998). This receptor is encoded by a gene with the same intron/exon structure as the Mel_{1a}- Mel_{1b}- Mel_{1c}-family of melatonin receptors, and it shares similar amino acid sequences in key regions, making it clear that this receptor is a member of the family of melatonin receptor genes. Human, mouse and sheep homologues of this receptor have been identified, but none of these receptor cDNAs is capable of binding ^{3}H-melatonin or 2-^{125}I-iodomelatonin (Reppert et al., 1996a; Drew et al., 1998; Gubitz and Reppert, 2000). The natural ligand for this receptor has not been identified. The transcript is expressed in a very interesting distribution in neuroendocrine tissues that does not match well with the distribution of melatonin receptor binding (Reppert et al., 1996a; Drew et al., 2001). The relative distribution of GPR50 and melatonin receptor binding is of importance in view of the recent finding that GPR50 heterodimerizes with MT1 and MT2 receptors when co-expressed, and GPR50 heterodimerization inhibits MT1 (but not MT2) receptor binding and function (Levoye et al., 2006). Such dimerization-dependent regulation of receptor function requires cellular co-expression, which may occur in some cell types, but appears not to be the case, generally, in the brain.

Melatonin receptor actions and signal transduction in specific tissues

Melatonin receptor signaling has been studied in native tissues and in cell lines expressing the recombinant receptors. Below we review important findings from a few of the more prominent models regarding receptor expression, signaling and receptor subtype identification. The

models we will discuss are amphibian dermal melanophores, neonatal rat pituitaries, pars tuberalis, suprachiasmatic nucleus, and vascular melatonin receptors.

Amphibian dermal melanophores

Studies of melatonin signal transduction in amphibian dermal melanophores revealed that melatonin acts through a pertussis toxin-sensitive mechanism in promoting melanin aggregation (White et al., 1987), consistent with other work indicating that functional receptors for melatonin antagonize the action of melanocyte-stimulating hormone (MSH) and other agents that increase cAMP accumulation. Pertussis toxin sensitivity is important with respect to showing the involvement of G proteins in melatonin receptor signal transduction. More recent studies in melanophores indicate that melatonin does indeed act through activation of GPCRs (Jockers et al., 1997; for a review of studies on melatonin action in amphibian dermal melanophores, see Sugden et al., 2004).

Neonatal rat pituitary glands

Jeanne Martin and David Klein published a series of studies in the 1970's demonstrating that melatonin inhibits the release of luteinizing hormone (LH) stimulated by luteinizing hormone releasing hormone (LHRH) from neonatal rat pituitary glands (Martin and Klein, 1976; Martin et al., 1977, 1980a,b). The expression of melatonin receptors in the neonatal rat pituitary is high, and there is a rapid, postnatal decline in receptor density (Vanecek, 1988b) caused by endogenous LHRH stimulation *in vivo* (Johnston et al., 2003). Similarly, the effects of melatonin on LHRH-stimulated LH release in rat pituitary are transient (Martin et al., 1977). This effect of melatonin is absent in neonatal hamster pituitary (Bacon et al., 1981) despite the presence of a high density of melatonin receptors (Vanecek and Kosar, 1994).

The discovery that the neonatal rat pituitary has a relatively high concentration of high-affinity melatonin receptor (Vanecek, 1988b) led to revitalized interest in this preparation for characterization of melatonin receptor signal transduction. In an extensive series of studies summarized by Vanecek, (1998a) J. Vanecek and colleagues conducted detailed investigations into melatonin signal transduction in the neonatal rat pituitary. Among the key findings was confirmation that melatonin inhibits cAMP accumulation to inhibit GnRH-induced LH release, and that other signaling pathways were simultaneously activated. Specifically, Vanecek and colleagues provided strong evidence that melatonin could influence cGMP levels, membrane potential, phosphoinositide signaling, and intracellular calcium mobilization in this melatonin receptor-expressing tissue (Vanecek and Vollrath, 1989, 1990a,b; Vanecek and Klein, 1992a,b, 1995a,b; Vanecek, 1995a, 1998b, 1999; Slanar et al., 1997; Zemkova and Vanecek, 1997).

Remarkably, there has been no effort to dissect the receptor subtype mediating response of the neonatal pituitary to melatonin. This would be amenable to dissection using RNA interference (RNAi) approaches for rat pituicytes, or through the use of melatonin receptor-deficient mice.

Pars tuberalis (PT)

The PT was first identified as a site of melatonin receptor binding in the rat by Williams and Morgan (1988). The MT1 melatonin receptor subtype and melatonin receptors are highly expressed in PT tissue of numerous species (Williams and Morgan, 1988; Morgan and Williams, 1989; Weaver et al., 1989, 1991b; Weaver and Reppert, 1990; Reppert et al., 1994). Melatonin receptor binding is abolished in the PT of MT1 melatonin receptor knockout mice (Liu et al., 1997) and, in fact, MT1 receptor knockout mice completely lack detectable melatonin receptor binding by autoradiographic methods (Fig. 1).

The high concentration of melatonin receptors in PT tissue from hamsters and sheep made it a useful tissue for early studies of melatonin receptor signal transduction. These first studies revealed that melatonin inhibited forskolin-stimulated cAMP accumulation through a pertussis toxin-sensitive G protein (Carlson et al., 1989; Morgan et al., 1995). Several studies designed to assess mechanisms for physiological regulation of sensitivity to melatonin (Weaver et al., 1990, 1991a; Carlson et al., 1991) suggested that deficits in sensitivity to melatonin occurred downstream of receptor binding and the initial steps of signaling through inhibition of cAMP accumulation. Extensive studies, primarily by P.J. Morgan, D.G. Hazlerigg and colleagues, identified a variety of melatonin receptor signaling mechanisms in the ovine PT, including signaling through cholera toxin-sensitive G proteins in addition to the previously demonstrated signaling through pertussis toxin-sensitive G proteins (Barrett et al., 1994, 1996, 1998; McNulty et al., 1994; Morgan et al., 1995; Ross et al., 1998).

With respect to physiological functions, recent work has identified the ovine PT as a primary site of melatonin action with respect to regulation of prolactin secretion and circannual rhythms (Morgan, 2000; Hazlerigg et al., 2001; Graham et al., 2002; Lincoln et al., 2003). Allelic variation in the ovine MT1 receptor has been correlated with reproductive seasonality in one study (Pelletier et al., 2000), but not in another (Notter et al., 2003).

In the PT of several species, melatonin rhythmicity is necessary for maintaining rhythmic clock gene expression (von Gall et al., 2002a, 2005; Lincoln et al., 2002, 2006; Jilg et al., 2005; Johnston et al., 2006). Melatonin also participates in heterologous sensitization of adeno-

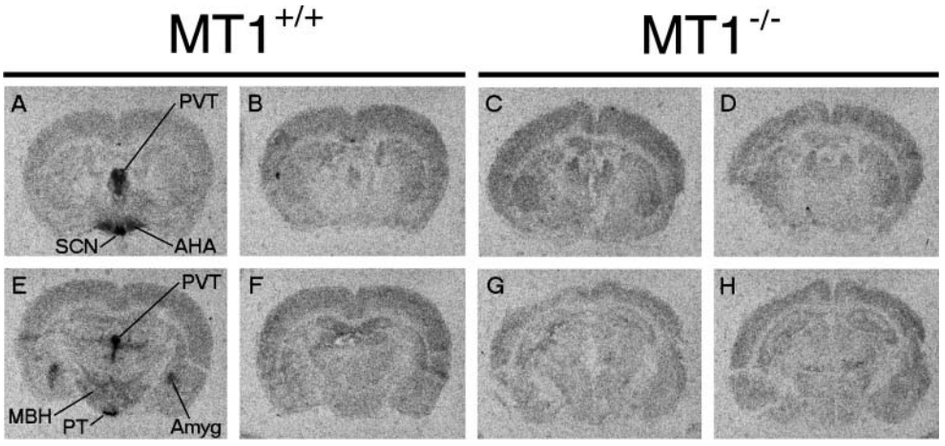

Fig. 1: Melatonin receptor binding in wild-type ($MT1^{+/+}$) and MT1 receptor-deficient ($MT1^{-/-}$) mouse brain. Panels A,C,E,G: Sections at the level of the SCN (top row) and PT (bottom row) were incubated with 2-^{125}I-iodomelatonin (100 pM). Sections were then washed and apposed to film to detect radioactivity. Panels B,D,F,H: Adjacent sections at each level were incubated with 2-^{125}I-iodomelatonin plus an excess (1 μM) of non-radioactive melatonin and processed in parallel to determine non-specific binding. Dark areas in Panels A and E that are not observed in B and F represent sites of specific melatonin receptor binding. Note the absence of readily detectable melatonin receptor binding in the total binding panels from the $MT1^{-/-}$ mouse (Panels C, G). Abbreviations: AHA, anterior hypothalamic area; Amyg, Amygdala; MBH, mediobasal hypothalamus; PVT, paraventricular nucleus of the thalamus; PT, *pars tuberalis*; SCN, suprachiasmatic nucleus.

sine receptors in the rodent PT, with possible implications for regulation of neuroendocrine function (von Gall et al., 2002a).

An interesting feature of melatonin receptor signaling is that photoperiodic responses require melatonin receptor occupancy for long intervals, which requires persistent receptor sensitivity in the face of agonist treatment. Indeed, several studies reveal that long-term melatonin exposure alters post-receptor signaling (Hazlerigg et al., 1993, 1994; Witt-Enderby et al., 1998; Pelisek and Vanecek, 2000; Barrett et al., 2003; Masana et al., 2003).

As noted above, the MT1 receptor is highly expressed in the PT of many species, and it is likely that the MT1 receptor mediates action of melatonin in these species. In isolating a fragment of the receptor cDNA to perform in situ hybridization in Siberian hamster brain, it was discovered that the MT2 receptor of this species contains stop codons within the coding sequence; the hamster MT2 gene does not encode a functional receptor protein (Weaver et al., 1996). It is worth noting that dramatic seasonal reproductive changes occur in the same species, thus the MT2 receptor cannot be necessary for these responses. Furthermore, the most profound circadian responses to melatonin have been demonstrated in developing Syrian hamsters (Davis and Mannion, 1988; Viswanathan et al., 1994), again, a species that lacks a functional MT2 receptor (D. R. Weaver, GenBank accession AY145849). The effects of melatonin in hamster species indicate that the MT2 receptor is not necessary for these responses, and strongly suggest that the MT1 receptor is important for these responses. In sheep, the MT2 receptor transcript seems to be very poorly expressed (Migaud et al., 2005; Xiao et al., 2007. Partial genomic sequences for exon 2 of ovine, porcine and bovine MT2 receptors have been published recently (Xiao et al., 2007).

Suprachiasmatic nucleus (SCN)

The MT1 receptor is expressed in rodent SCN (Reppert et al., 1994; Liu et al., 1997) and in the human SCN (Weaver and Reppert, 1996). Furthermore, as noted above, mice with targeted disruption of the MT1 receptor lack detectable melatonin receptor binding by autoradiographic methods (Liu et al., 1997; Fig. 1).

The MT2 receptor has also been identified in humans and mice (Reppert et al., 1995; Jin et al., 2003a), and a receptor cDNA fragment has been isolated from several other species including rats. The MT2 receptor appears to be expressed at lower density, and may mediate responses to higher concentrations of the hormone. Several compounds with good selectivity for the MT2 receptor (relative to the MT1 receptor) have been developed (Dubocovich et al., 1997; Beresford et al., 1998; Sugden et al., 1999; Davies et al., 2004). Among the most commonly used compounds for experimental purposes is the antagonist, 4P-PDOT, which offers 300- to 22,000-fold selectivity, depending on the assay system used (Dubocovich et al., 1997). Melatonin receptor binding in rodent SCN is resistant to inhibition by the selective MT2 receptor antagonist 4P-PDOT (Dubocovich et al., 1998b), consistent with the interpretation from the MT1 knockout mice noted above that the majority of melatonin receptor binding in brain is due to MT1 receptor expression rather

than MT2 receptor expression (Liu et al., 1997). Nevertheless, responses mediated by the MT2 receptor have been described in the SCN (Dubocovich et al., 1998b, 2005; von Gall et al., 2000; Hunt et al., 2001; for review, see von Gall et al., 2002b; Jin et al., 2003).

Studies by Martha Gillette and colleagues indicate that the electrical activity rhythm within the rat SCN is phase-shifted by melatonin (McArthur et al., 1991, 1997). Studies to address the post-receptor mechanism for this phenomenon reveal an extremely transient activation of protein kinase C (PKC) in SCN slices treated with melatonin, and modulation of PKC activity can mimic or block the effects of melatonin (McArthur et al., 1997). Studies of MT1 melatonin receptor knockout mice support the conclusion that the MT2 receptor plays a role in this response: the *in vitro* phase-shifting response to melatonin persists in SCN slices from $MT1^{-/-}$ mice, but is pertussis-toxin-sensitive (Liu et al., 1997a; Dubocovich et al., 2005). The concentrations of melatonin capable of causing a phase shift in the single unit activity rhythm are remarkable low, 3–10 pM (McArthur et al., 1997; Dubocovich et al., 2005), whereas other *in vitro* responses of SCN slices to melatonin require concentrations of 1–100 nM (reviewed in von Gall et al., 2002b).

Two other responses of SCN slices to melatonin are mediated primarily by the MT1 receptor. The acute inhibition of SCN electrical activity by melatonin (assessed by multiunit recording) is completely absent in MT1 receptor knockout mice, while this response is unaffected in MT2 receptor knockout mice (Liu et al., 1997; Jin et al., 2003). Melatonin also inhibits PACAP-stimulated CREB phosphorylation in SCN slices; this effect is mediated by the MT1 receptor at lower concentrations of the hormone (1–10 nM). A response occurring at higher concentrations (100 nM) is mediated by the MT2 receptor, as in MT1-deficient slices treated with an MT2 antagonist, or in MT1/MT2 double-knockout mice, melatonin does not inhibit PACAP-stimulated CREB phosphorylation (von Gall et al., 2000; for review, see von Gall et al., 2002b; Jin et al., 2003).

With respect to the receptor subtype mediating circadian responses to melatonin *in vivo*, the data are quite mixed and indeed are contradictory. While Dubocovich et al. (1998), reported that the MT2 receptor mediates phase-shifting responses of rats to melatonin, a more recent publication (Dubocovich et al., 2005) indicates that behavioral phase shifts and the acceleration of re-entrainment by melatonin are both absent in MT1 receptor deficient mice. While a proposal of "strain differences" is a reasonable way to reconcile these findings, there are differences even within a single species (mice) with respect to the receptor mediating specific SCN responses (see above) that illustrate that the mechanisms of melatonin action in the SCN are not straightforward.

Vascular melatonin receptors

High-affinity melatonin receptors have been characterized in several vascular tissues. The initial demonstration of melatonin receptors in arteries in the circle of Willis (Viswanathan et al., 1990) was followed by studies characterizing the receptors and demonstrating that they were coupled to G protein-mediated signaling cascades including inhibition of cAMP accumulation through a pertussis toxin-sensitive mechanism (Viswanathan et al., 1993; Capsoni et al., 1994).

The characterization of receptors in vascular beds that could be involved in vascular and thermoregulatory responses led to interest in determining whether melatonin affected vascular tone. Using *in vitro* preparations, it was confirmed that melatonin could indeed influence vascular tone in arteries from several different anatomical locations, including cerebral arteries (Viswanathan et al., 1997), tail artery (Ting et al., 1997, 1999; Doolen et al., 1998; Masana et al., 2002) and coronary vessels (Mahle et al., 1997). While one study reports that a receptor with MT1-like pharmacological profile mediates responses in the rat caudal artery (Ting et al., 1999), another study indicates that an MT2-like receptor mediates responses in this same tissue (Masana et al., 2002). There are multiple responses to melatonin in this preparation (Doolen et al., 1998), and it is possible that the experimental details are critical in producing what appear to be opposite results.

Other tissues

Melatonin receptor-mediated responses have been identified in many other tissues, and complete summarization of this literature is beyond the scope of this review. Of particular interest with respect to dissection of receptor subtypes is work studying melatonin receptor function in hippocampus (Savaskan et al., 2005; Wang et al., 2005; Larson et al., 2006), melatonin effects on mood/depression (Sumaya et al., 2005; Weil et al., 2006), and immune responses (Drazen and Nelson, 2001).

Signal transduction of recombinant receptors expressed in cell lines

The initial cloning papers revealed that each of the G protein-coupled melatonin receptor subtypes could inhibit accumulation of cAMP. This was shown for all *Xenopus* Mel_{1c} receptor isoforms (Ebisawa et al., 1994; Jockers et al., 1997), the human, sheep and mouse MT1 receptors (Reppert et al., 1994; Roca et al., 1996), the human MT2 receptor (Reppert et al., 1995), and the chicken Mel_{1a} and Mel_{1c} receptors (Reppert et al., 1995) and in numerous other papers since these early studies.

Subsequent work has identified additional signaling pathways activated by melatonin receptor occupation in cells expressing recombinant receptors. For example, Godson and Reppert (1997) showed that MT1 receptors signal through parallel pathways, inhibition of adenylyl cyclase and potentiation of phospholipase activation. Activation of both responses was abolished by pertussis toxin (PTX). This suggests that the human MT1 receptor is coupled to the signal transduction pathways through G_i, as this is the only PTX substrate expressed in these cells. Studies by Brydon et al. (1999b), show that MT1 receptor signaling is not only through PTX-sensitive G_i, but is also through PTX-insensitive $G_{q/11}$ proteins that is responsible for mobilization of Ca^{2+} (but see Jarzynka et al., 2006). Additional studies of signaling *via* recombinant melatonin receptors have been performed, but are beyond the scope of this review.

Molecular identification of melatonin receptors provides important reagents

The molecular identification of melatonin receptors has led to a remarkable array of reagents available for study of melatonin receptors. A survey of these uses and a list of representative publications showing these uses is provided.

With the availability of the receptor cDNA nucleotide sequences, it has become possible to study the distribution and regulation of melatonin receptors at the transcriptional level using methods that include quantitative/real-time PCR, solution hybridization, RNAse protection assays, and *in situ* hybridization. The density of melatonin receptor transcripts is so low that in many tissues, Northern blot analysis is not sufficiently sensitive to detect melatonin receptor transcripts.

Availability of sequence information will also allow RNAi based methods to disrupt melatonin receptor function in tissues from many species. The potential of this approach is just beginning to be realized in the melatonin field, where it would be most useful in assessing the receptors responsible for melatonin actions in cultured cells.

The availability of full-length human melatonin receptor cDNAs has allowed screens to identify melatonin receptor ligands. This characterization of recombinant receptors can assess binding selectivity and affinity among receptor subtypes. Expression of recombinant receptors also allows characterization of signaling pathways downstream of receptor activation, and assessment of whether a pharmaceutical is an agonist vs. antagonist using functional assays. Receptor structure-function studies have been conducted on chimeric receptors and receptors with specific residues substituted, to determine residues critical for ligand binding and test hypotheses regarding the properties of the binding site (Conway et al., 1997, 2000, 2001; Kokkola et al., 1998, 2003, 2005; Gubitz and Reppert, 2000).

Biochemical studies of melatonin receptors have been promoted by the availability of epitope-tagged receptors, generated by adding exogenous sequence to the amino- or C-terminus of the receptor. For these studies, it is important to initially show that the tagged receptor retains ligand binding and signaling activity, prior to assessing biochemical interactions. Epitope-tagged receptor proteins have been useful for assessing whether modified proteins are properly trafficked to the membrane (Gubitz and Reppert, 2000), and for identifying physical interactions of the receptor proteins with other proteins by biochemical and imaging methods (Levoye et al., 2006).

The identification of the deduced amino acid sequence of melatonin receptors, obtained from the cDNA sequence has allowed generation of antibodies to the receptor proteins. The low density of melatonin receptors makes this a challenging endeavor. Nevertheless, several investigators have been successful in generating melatonin MT1 receptor antibodies (Brydon et al., 1999a,b; Fujieda et al., 1999, 2000; Dillon et al., 2002; Meyer et al., 2002; Scher et al., 2002, 2003; Rada and Wiechmann, 2006; Wu et al., 2006a,b) and more recently, MT2 receptor antibodies (Savaskan et al., 2005). There are also several commercial outfits marketing antibodies to melatonin receptors. Characterization of melatonin receptor antibodies should include showing the utility of the antisera in western blot identification of over-expressed proteins and the absence of immunoreactivity in MT1/MT2 receptor knockout mice.

Identification of the melatonin receptor gene sequences from several species has allowed definitive understanding of the intron/exon structure of the high-affinity melatonin receptor. An added advantage of this information is that melatonin receptors can be preliminarily identified in other organisms by bioinformatic approaches.

The development of nucleotide probes to melatonin receptor cDNAs allowed isolation of genomic clones and the eventual generation of mice with targeted disruption of the MT1 and MT2 melatonin receptor subtypes (Liu et al., 1997; Jin et al., 2003). These mice have been studied by several investigators to accomplish a molecular dissection of receptors responsible for specific actions of melatonin. Results using these mice to dissect the molecular mechanisms of melatonin actions in the SCN (Liu et al., 1997; Jin et al., 2003; Dubocovich et al., 2005; von Gall et al., 2005) and PT (von Gall et al., 2002a, 2005; Jilg et al., 2005) were discussed briefly above. Other investigators have used the melatonin receptor deficient mice to assess the melatonin receptor subtypes involved in modulating anterior pituitary function (Sheynzon and Korf, 2006), hippocampal long-term potentiation (Wang et al., 2005; Larson et al., 2006), and immune responses (Drazen and Nelson, 2001). It is important to note that some responses to high doses of mel-

atonin may be mediated by QR2 or antioxidant activity of the hormone, rather than through GPCRs. For example, cardioprotective and neuroprotective actions of high-dose melatonin treatment persist in MT1 receptor-deficient mice (Liu et al., 2002; Chen et al., 2003), and further study is needed to determine if these responses are independent of the MT2 receptor as well.

These molecular-based reagents – from gene-specific primers, to antibodies, to knockout mice – provide important new approaches for studying the sites and mechanisms of melatonin action. The future of investigation into "the hormone of darkness" is, ironically, quite bright.

Acknowledgements

During preparation of this article, the authors were supported in part by grants from the DFG (RD, DA525/2-1) and the NIH (DRW, NS56125).

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