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Xiaowei Jin
Harvard Medical School

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Targeted Disruption of the Mouse Mel1b Melatonin Receptor

Xiaowei Jin,1† Charlotte von Gall,2,3‡ Rick L. Pieschl,4 Valentin K. Gribkoff,4 Jorg H. Stehle,2 Steven M. Reppert,1,3‡ and David R. Weaver1,3*  

Laboratory of Developmental Chronobiology, MassGeneral Hospital for Children, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114; Anatomisches Institut II, Johann Wolfgang Goethe-Universität Frankfurt, D-60590 Frankfurt, Germany;1 Department of Neurobiology, University of Massachusetts Medical School, Worcester, Massachusetts 01605; and Neuroscience Drug Discovery, Bristol-Myers Squibb Pharmaceutical Research Institute, Wallingford, Connecticut 06492*  

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Two high-affinity, G protein-coupled melatonin receptor subtypes have been identified in mammals. Targeted disruption of the Mel1a melatonin receptor prevents some, but not all, responses to the hormone, suggesting functional redundancy among receptor subtypes (Liu et al., Neuron 19:91-102, 1997). In the present work, the mouse Mel1b melatonin receptor cDNA was isolated and characterized, and the gene has been disrupted. The cDNA encodes a receptor with high affinity for melatonin and a pharmacological profile consistent with its assignment as encoding a melatonin receptor. Mice with targeted disruption of the Mel1b receptor have no obvious circadian phenotype. Melatonin suppressed multunit electrical activity in the suprachiasmatic nucleus (SCN) of Mel1a receptor-deficient mice as effectively as in wild-type controls. The Mel1b receptor subtype mediates this inhibitory response at moderate ligand concentrations (1 nM). A residual response apparent in Mel1a-Mel1b double-mutant mice, indicating that the Mel1b receptor mediates this effect of melatonin. These data indicate that there is a limited functional redundancy between the receptor subtypes in the SCN. Mice with targeted disruption of melatonin receptor subtypes will allow molecular dissection of other melatonin receptor-mediated responses.

The hormone, melatonin, is produced rhythmically in the vertebrate pineal gland (13). Rhythmic melatonin production plays a critical role in the regulation of reproduction in seasonally breeding mammals (1, 24). In nonmammalian vertebrates, melatonin also plays a major role in the regulation of circadian rhythms (4, 46). While endogenous melatonin appears to play only a subtle role in the regulation of circadian rhythms in mammals, exogenous melatonin influences circadian rhythms in several rodent species and in humans (2, 3, 9, 21, 22, 39, 46, 47). While the magnitude of this phase shifting effect is small for adult mammals, it is nevertheless critical in some situations; e.g., daily melatonin administration appears to be useful for entrainment of non-24-h circadian cycles to the 24-hour day for blind individuals (22).

Two high-affinity receptors for melatonin have been identified in mammals (for reviews, see references 33 and 43). These receptors, the Mel1a and Mel1b receptor subtypes, are members of the G protein-coupled receptor superfamily (29, 30). A melatonin receptor-related receptor (also called H9 and GPR50) has sequence homology to the Mel1a and Mel1b rece-

* Corresponding author. Present address: Department of Neurobiology, Lazarre Research Building, Room 723, University of Massachusetts Medical School, 364 Plantation St., Worcester, MA 01605-2324. Phone: (508) 856-2495. Fax: (508) 856-6266. E-mail: david.weaver@umassmed.edu.  
† Present address: CombinatoRx Inc., Boston, MA 02118.  
‡ Present address: Department of Neurobiology, University of Massachusetts Medical School, Worcester, MA 01605-2324.

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to responses to the hormone, the mouse Mel₁b receptor cDNA was isolated. Finding that the mouse Mel₁b receptor cDNA encodes a functionally relevant melatonin receptor, we then disrupted the Mel₁b receptor gene. Analysis of the mutant mice indicates that the Mel₁b receptor encodes a high-affinity melatonin receptor. The Mel₁b receptor appears to mediate a response to the hormone occurring at higher ligand concentrations in mice lacking the Mel₁a melatonin receptor.

MATERIALS AND METHODS

Isolation and characterization of the mouse Mel₁b receptor cDNA. A fragment of the mouse Mel₁b receptor cDNA isolated by PCR has been previously reported (47). A probe based on this published sequence was used to screen mouse genomic DNA libraries, resulting in isolation of two overlapping genomic clones containing exon 2 (A341 and A351). Genomic DNA encoding exon 1 was isolated from a bacterial artificial chromosome library (Research Genetics, Inc.) screened with the most 5′ end of the intronic sequence from clone A341. In addition, PCR amplification of mouse brain cDNA was performed using primers spanning the intron, to confirm the splicing pattern, as well as along the cDNA to confirm the sequence. A full-length cDNA was isolated by PCR using an overlap-PCR method to “stitch” together exons 1 and 2, producing a silent mutation at the splice site. The integrity of the cDNA construct was confirmed by sequence analysis and compared to the sequence of genomic clones.

Genomic DNA sequence corresponding to the mouse Mel₁b receptor locus has recently been deposited in GenBank. GenBank accession no. NW_000351 represents a genomic contig including the mouse Mel₁b receptor locus. The deduced amino acid sequence of the receptor cDNA (GenBank accession no. XM_146818), based on inferred splicing patterns using GenoScene, differs from that determined above due to the detection of two apparent exons within intron 1, disruption of exon 2 by an apparent intron, and splicing to an additional exon just 5′ of the actual stop codon. Both homology with other members of the melatonin receptor gene family and the RT-PCR data support the conclusion that the Mel₁b receptor locus consists of two exons.

When the nucleotide sequences are translated using a two-exon model, the deduced amino acid sequence of the Mel₁b receptor cDNA reported in GenBank (AAI85489 and NW_000351) still differs from our sequence, due to the apparent insertion of two nucleotides in exon 2 in the portion of sequence encoding the carboxyl terminus of the protein in the GenBank entry. The accuracy of the sequence reported here is supported by homology of our deduced amino acid sequence with the human and Northern pike Mel₁b receptor sequences (12) and is confirmed by the identity of our sequence with the sequence entry in the Celera database (entries mCT7757 and mCG8325). (The only amino acid difference between the sequence reported here and in the Celera database is T237 in our sequence, which is E237 in the Celera and other sequences.) We cannot exclude the possibility that strain differences or other polymorphisms in the genetic sequence exist.

Ligand binding characteristics. To compare the affinity and specificity of the mouse Mel₁a and Mel₁b receptors, the characteristics of these receptor cDNAs were examined in parallel in transiently transfected COS7 cells. Ligand binding studies were conducted using the radioligand 2-[125I]iodomelatonin ([125I]-MEL) as previously described (29, 34). Saturation binding experiments were conducted using [125I]-MEL concentrations of 110 to 150 pM. Data were analyzed as previously described (29, 34). Competitive inhibition studies were conducted using [125I]-MEL concentrations of 110 to 150 pM. Kᵢ values were calculated from 50% inhibitory concentration (IC₅₀) values using the Cheng-Prusoff equation (5): $Kᵢ = IC₅₀/(1 + ([ligand]/Kᵢ))$.

Targeting disruption of the Mel₁b receptor gene. A targeting construct was generated in which the first exon of the Mel₁b receptor gene was disrupted by insertion of a neomycin resistance cassette at a Smal site in exon 1 (Fig. 1). The cassette (PGK-Neo) consists of the phosphoglycerate kinase (PGK) promoter and a neomycin resistance gene and was placed in reverse orientation. The 5′ arm was 2.2 kb; the 3′ arm was 7 kb. The targeting construct was introduced into...
J1 embryonic stem cells by electroporation at the Massachusetts General Hospital Knockout Core facility (En Li, director). For screening, DNA from neomycin-resistant colonies was digested with BamHI and assessed by Southern blotting, using a nonoverlapping 0.8-kb StuI fragment spanning the 5′ end of the construct to screen for targeting events. The targeting event was confirmed by digestion of embryonic stem cell DNA with EcoRI, followed by hybridization with a nonoverlapping probe (based on exon 2) from the 3′ flanking region as a probe (data not shown). A clone with the appropriate banding pattern on Southern blot analysis (#83) was microinjected into blastocysts by the Knockout Core facility. Chimeric mice were bred to females of strain 129/sv, provided by En Li. Heterozygous offspring were bred together. The genotype of the offspring was determined by PCR using primers specific for wild-type and homozygous Mel1a-mutant mice on the 129/sv background.

Southern blot analysis of tail DNA confirmed the targeting event (Fig. 1B). For colony maintenance, mice were genotyped using PCR (Fig. 1C and D). Reactions were performed using a mixture of three primers, allowing detection of both wild-type and targeted alleles in a single reaction (Fig. 1C). A common forward primer in exon 1 (mus1b5′-4, CAGGCCCTCGTGACTGGCCCGG) and a gene-specific reverse primer from intron 1 (mus1b3′-3, 5′ CCTGACCATTAGGACAGAAGCACGG 3′) amplified a 272-bp fragment from the wild-type allele. The common forward primer and a reverse primer based on the sequence of the 3′ end of the PGK-neo cassette (neoc2-2, TGGCCCAAGGCGTACCCGCTTFC) amplified a ca. 350-bp fragment from the targeted allele. Amplification of genomic DNA extracted from tail biopsies was performed using Tag DNA polymerase and a program of 3 min at 94°C for initial denaturation, followed by 35 cycles of denaturation at 94°C (30 s), annealing at 60°C (30 s), and extension at 72°C (1 min). Products were separated on agarose gels and visualized with ethidium bromide using UV trans-illumination (Fig. 1D).

Generation of melatonin receptor-deficient lines on a C3H/He genetic background. Mice with targeted disruption of the Mel1a receptor have been previously described (23). In these mice, exon 1 of the Mel1a receptor gene has been replaced with the PGK-neomycin cassette. To generate animals with the receptor targeting events on a consistent genetic background, a Mel1b receptor-deficient mouse (on the isogenic, 129/sv genetic background) was bred to a C3H/He strain female (C3H, Charles River Laboratories). The C3H strain was used because this strain has been used extensively by others in examining circadian behavioral responses to melatonin (2, 3, 9), and this strain has a rhythmic melatonin production (unlike most other inbred strains of mice; see reference 11). In each generation, heterozygous males were selected and crossed to C3H females. After backcrossing was done for 10 generations, heterozygous male and female mice were interbred to produce wild-type and Mel1b receptor-deficient lines which produce rhythmic melatonin as previously reported (42).

Similarly, a male Mel1b receptor-deficient mouse (on the isogenic, 129/sv genetic background) was bred to a C3H female. Heterozygous male offspring from each generation were backcrossed to C3H females for six generations of backcrossing, at which time male and female heterozygotes were interbred to produce wild-type and Mel1b receptor-deficient lines on the C3H background. Finally, Mel1b receptor-deficient mice on the C3H genetic background were crossed with the Mel1a receptor-deficient mice on the C3H background to generate Mel1a/Mel1b double-mutant mice on a C3H genetic background.

Multinit recordings. Multinit recordings of SCN electrical activity were performed as previously described (14, 23). Briefly, hypothalamic slices were prepared from adult male mice maintained in a 12-h light:12-h dark lighting cycle. Animals were euthanized 2 to 5 h after lights-on. Brains were rapidly dissected and placed into artificial cerebrospinal fluid medium (ACSF) containing 116.3 mM NaCl, 5.4 mM KCl, 1.0 mM NaH2PO4, 26.2 mM NaHCO3, 1.8 mM CaCl2, 0.8 mM MgSO4, 24.6 mM dextrose, and 5-mg/liter gentamicin sulfate (pH 7.35). Slices were prepared using a tissue chopper. Electrical recordings were performed as previously described (14, 23). Experiments were performed on the second day in vitro, 24 h after preparation of the slices. Slices were exposed to vehicle (0.1% ethanol in ACSF) and then to escalating concentrations of melatonin (0.1 to 100 nM) in ACSF containing a maximum of 0.1% ethanol. Data are expressed as percent change in activity compared with control activity recorded in ACSF containing 0.1% ethanol.

Cyclic AMP response element binding protein (CREB) phosphorylation. Pituitary adenyl cyclase activating peptide (PACAP) is a transmitter of the retinohypothalamic tract, the pathway that conveys light-dark information from the retina to the SCN (17). PACAP appears to modulate the effects of glutamate on CREB activity in vitro (17, 40). In vitro application of PACAP to SCN slices induces phosphorylation of CREB on serine residue 133 (20, 40, 41). Phosphorylated CREB (pCREB) is thought to play an important role in the photic regulation of gene expression in the SCN, including the light-induced expression of c-fos and the mPeriod genes (14, 38, 40). Melatonin reduces PACAP-induced pCREB immunoreactivity in SCN slices in a dose-dependent manner (20, 40, 41, 42). Melatonin may thus subtly modulate photic sensitivity of the SCN circadian clock.

Melatonin inhibition of the PACAP-induced CREB phosphorylation state was studied as previously described (41). Briefly, 400-μM coronal brain slices containing the SCN were collected 2 to 6 h after lights-on and maintained in oxygenated medium (145 mM NaCl, 5 mM KCl, 1.8 mM CaCl2, 0.8 mM MgCl2, 10 mM HEPES, and 10 mM glucose [pH 7.35]). At the time corresponding to Zeitgeber Time 10 (ZT10, ca. 10 h after lights-on in the colony room), slices were treated with PACAP (100 nM) or vehicle. Slices were pretreated with melatonin (1 or 100 nM) or vehicle for 15 min prior to PACAP application. Fifteen minutes after PACAP application, slices were fixed with 4% paraformaldehyde. Slices were fixed for 12 to 16 h and then cryo-protected (20% sucrose in phosphate-buffered saline) and sectioned at 14-μm thickness on a cryostat. Sections were mounted on gelatin-coated slides and stored at –20°C until processed for immunohistochemical detection of CREB phosphorylated at serine 133 (pCREB). pCREB was detected by use of a commercially available, polyclonal antibody (Upstate Biotechnology, Lake Placid, N.Y.) at a dilution of 1:1,000. Immunoreaction was visualized with a standard avidin-biotin labeling method (Vector Labs, Burlingame, Calif.) with diaminobenzidine as a chromogen.

Semiquantitative analysis was performed as previously described (41). Briefly, images were digitized (VIDAS, Kontron, Germany), and background staining was defined as the lower threshold. Within the area of the SCN, all cell nuclei showing a pCREB immunoreaction exceeding the threshold were marked. Immunoreactive nuclei were then counted in three sections per animal in a blind manner. The average of six values (number of immunoreactive nuclei per unilateral SCN per 14-μm section) was calculated for each animal. It is not known whether melatonin alters the number of nuclei immunoreactive for pCREB by decreasing the rate of phosphorylation of CREB or by activation of a phosphatase. In this report, “CREB phosphorylation” is used to indicate the static state of CREB phosphorylation (e.g., density of pCREB immunoreactive nuclei), not the rate at which CREB is phosphorylated.

Nucleotide sequence accession number. The cDNA sequence for the mouse Mel1a receptor that was determined in this study has been deposited in GenBank (accession number AY145850).

RESULTS

Isolation and characterization of the mouse Mel1b receptor cDNA. The mouse Mel1b receptor cDNA sequence was determined from genomic DNA and confirmed by amplification of the receptor cDNA from brain RNA (see Materials and Methods for discussion of discrepancies between our sequence and other database entries).

The deduced amino acid sequence of the Mel1b receptor has features shared by other members of the melatonin receptor subfamily of G protein-coupled receptors (Fig. 2). Specifically, the amino acid sequence of the mouse Mel1b receptor is highly related to the human Mel1b receptor, with which it shares 79% identity. The other closely related sequences identified by BLAST searches of the GenBank database are fragments of the Mel1b receptor cDNA from other species, including the Northern pike Mel1b receptor cDNA (12). Amino acid identity of the mouse Mel1a and Mel1b receptors is 50.8%. The mouse Mel1a receptor sequence also has several features conserved in the melatonin receptor family within the superfamily of G protein-coupled receptors. A highly conserved sequence just downstream from the third transmembrane domain (NRY[C/Y]ICHSH) is distinctive; most other receptor families have DRY or ERY in place of NRY (33, 45). In transmembrane domain 7, the melatonin receptor family shares a characteristic motif (NA[I/V][I/V]Y) rather than NPXXY. The presence of potential N-linked glycosylation site in the amino-terminal, putative extracellular domain of the
protein is also a common feature of the melatonin receptor family. Finally, with respect to gene structure, the location of the splice site between exons 1 and 2 is also conserved among all mammalian melatonin receptor genes that have been examined (32, 34, 47). To determine the relative affinity and specificity of mouse melatonin receptors, the characteristics of the mouse Mel1a and Mel1b receptor cDNAs were examined in parallel. 125I-

![Image](http://mcb.asm.org/)

**TABLE 1. Inhibition constants for mouse Mel1a, and Mel1b receptor cDNAs**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>2-Iodomelatonin</th>
<th>Melatonin</th>
<th>N-Acetylsertotonin</th>
</tr>
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<tbody>
<tr>
<td>Mel1a (no. of experiments)</td>
<td>0.045 ± 0.005</td>
<td>0.059 ± 0.017</td>
<td>0.169 (3)</td>
</tr>
<tr>
<td>Mel1b</td>
<td>0.169 (3)</td>
<td>0.159 ± 0.017</td>
<td>0.665 (3)</td>
</tr>
</tbody>
</table>

*Inhibition constants (Ki values) are expressed as means ± SEM, in nanomolar concentrations.
Mel1b receptor did not alter this response to melatonin. With the Mel1a receptor necessary for the suppressive effect of melatonin on SCN neuronal firing (23). Targeted disruption of the Mel1b receptor did not alter this response to melatonin. With respect to inhibition of the PACAP-induced CREB phosphorylation state, melatonin acts through the Mel1a receptor at low concentrations of the hormone (41) (present results). An effect of higher concentrations of melatonin is apparent in Mel1a receptor-deficient mice, and this response is blocked by treatment with the Mel1b receptor antagonist, 4P-PDOT (41). Consistent with this dual regulation, we report here that the SCN of Mel1a-Mel1b double-mutant mice is completely unresponsive to melatonin, even at the higher (100 nM) melatonin concentrations.

DISCUSSION

Melatonin has subtle actions on circadian rhythmicity in mice and in other mammals (46). Mice with targeted disruption of either Mel1a or Mel1b receptor subtype, or the double mutants, do not have a striking overt phenotype. Examination of melatonin receptor-mediated responses does reveal a functional effect of each of the receptor subtypes, however. The Mel1a receptor is necessary for the suppressive effect of melatonin on SCN neuronal firing (23). Targeted disruption of the Mel1b receptor did not alter this response to melatonin. With respect to inhibition of the PACAP-induced CREB phosphorylation state, melatonin acts through the Mel1a receptor at low concentrations of the hormone. Semiquantitative densitometric analysis shows that melatonin reduces the number of pCREB-immunoreactive nuclei in PACAP-treated SCN slices. Values represent labeled nuclei per unilateral SCN and are the mean ± SEM for four to five animals per group. In wild-type mice, melatonin (1 nM) reduces PACAP-induced CREB phosphorylation by >70% (41). In contrast, a 1 nM concentration had no significant effect on mice with targeted disruption of the Mel1a melatonin receptor (Mel1a −/−) (A). At higher melatonin concentrations (100 nM), a more complete suppression occurs even in mice with disruption of the Mel1a receptor subtype (A) (far right). This residual, higher-threshold response is absent in mice with targeted disruption of both the Mel1a and Mel1b receptors (B). ++++, significant difference between vehicle control and PACAP treatment (P < 0.002, t test); **, significant difference versus PACAP group (P < 0.01, Dunnett’s test); NS, not significantly different from PACAP group (P > 0.05; Dunnett’s test).

FIG. 3. Inhibition of SCN multiunit activity by melatonin. Inhibition of SCN electrical activity by melatonin (0.1 to 100 nM) was assessed during midday. Data are plotted as the percent inhibition of electrical activity observed at the 100 nM dose relative to the level of electrical activity from the same slice exposed to vehicle. The left set of bars shows that the inhibition of SCN electrical activity is absent in Mel1a receptor-deficient (−/−) mice (data replotted from reference 23; sample sizes 7 to 11 per group). Right bars show inhibition of SCN activity in experiments conducted for this study, using Mel1b receptor-deficient mice (−/−) (n = 6) and wild-type control (+/+)(n = 11) mice. The inhibition of electrical activity in each group was significant (P < 0.0001, one-sample t tests, versus 0% inhibition), and there was no significant difference between the Mel1b −/− and Mel1b +/+ groups (t test, P = 0.2).

FIG. 4. Inhibition of CREB phosphorylation levels by melatonin. One of the in vitro assays used previously to assess the effects of melatonin on the circadian clock involves monitoring the effect of melatonin on the phase of single-unit firing rate rhythms in SCN slices (19, 23, 26, 47). Liu et al. (23) found that melatonin and 2-iodomelatonin phase-shifted the circadian
rhythm of SCN single-unit activity even in mice with targeted disruption of the Mel1a receptor, although the magnitude of response was lower in the Mel1a receptor-deficient mice at low ligand concentrations. The response persisting in Mel1a receptor-deficient mice was mediated by a pertussis-toxin-sensitive mechanism, indirectly implicating Mel1b receptors as mediating the higher-threshold response. More recently, Hunt et al. (19) reported that the Mel1b receptor subtype mediates the phase-shifting effect of melatonin in the rat SCN, based on studies using subtype-selective antagonists. In contrast, SCN slices from hamsters lacking a functional Mel1b receptor (due to a naturally occurring nonsense mutation) respond to melatonin with phase shifts in single-unit activity (47). These diverse results from the single-unit assay indicate either that there are pronounced species differences in the melatonin receptor subtype mediating the phase shifting response or, more likely, that there is functional redundancy between the receptor subtypes.

While this assay system has been used successfully by our associates and others in the past, in preliminary studies we were unable to obtain reliable single-unit activity rhythms, even with commercially reared wild-type mice, at the time we attempted to examine the responses of Mel1b receptor-deficient mice (C. Liu and S. M. Reppert, unpublished data). In the absence of a robust rhythm with clearly defined phase, assessment of the impact of melatonin on circadian phase was not possible.

We were also unable, in preliminary experiments, to demonstrate a consistent effect of melatonin on locomotor activity rhythms in vivo, even in wild-type mice with the C3H genetic background. This strain has previously been reported to exhibit phase shifts in response to melatonin treatment (2, 3, 9). The mean phase shift occurring in the paradigm described by Dubocovich and colleagues is a less than 1.0-h total shift with daily injections of melatonin for 3 days. Behavioral arousal (such as would occur coincident with handling the animal for injection) has an effect on the rodent circadian system that appears to be as robust as the effect of melatonin (18, 28). In our hands, melatonin injection paradigms did not elicit consistently detectable phase shifts in mice. We were thus unable to use the mutant mice to directly test the hypothesis (9) that the Mel1b receptor mediates behavioral phase shifts.

Data from other systems suggest that both Mel1a and Mel1b receptors contribute to responses to the hormone. Vascular responses to melatonin occurring at low concentrations are mediated primarily by the Mel1a receptor, with a Mel1b-mediated response occurring at higher concentrations (6, 37). This directly parallels our findings regarding the dose-related inhibition of PACAP-induced CREB phosphorylation in the SCN. The acute suppressive effect of melatonin on SCN neuronal firing is mediated by the Mel1a receptor. The Mel1b receptor appears to mediate inhibition of firing in the rat hippocampus, which, unlike the mouse hippocampus, has a significant level of melatonin receptor binding (44). A melatonin-regulated rhythm in mPer1 gene expression in the pars tuberalis of the pituitary is abolished by removal of the pineal gland in hamsters (27); the absence of a functional Mel1b melatonin receptor gene in this species implicates the Mel1a receptor. Consistent with this proposal, pinealectomy or targeted disruption of the Mel1a receptor gene in mice that have rhythmic pineal melatonin synthesis disrupts rhythmic mPer1 expression in the pars tuberalis (42). These findings suggest that the expression of melatonin receptor subtypes varies by tissue and indicate the possibility of functional redundancy when the receptor subtypes are coexpressed.

In summary, targeted disruption of the Mel1b receptor does not have a profound impact on the circadian system of mice. Our data from examining melatonin inhibition of the level of CREB phosphorylation in PACAP-treated SCN slices indicate that the Mel1a receptor mediates the majority of this response to the hormone. At higher melatonin concentrations, a residual impact of melatonin, acting via the Mel1b receptor, is apparent. This difference in the relative contribution of the receptor subtypes is likely not due to differences in the affinity of the receptors per se, but instead may derive from differences in the levels of receptor expression directed by the two genes or by differences in coupling to second messenger pathways. The Mel1a receptor accounts for all the melatonin receptor binding detected in mouse brain in vitro autoradiography (23). The Mel1b receptor, being much more highly expressed, may mediate most responses to the hormone in mice. The Mel1b receptor can also contribute to responses in those tissues in which it is expressed. The availability of mice with targeted disruption of Mel1a, Mel1b, and both receptor subtypes will allow a genetic approach to dissection of additional melatonin receptor-mediated responses.

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