Neuron, Vol. 15, 1003–1015, November, 1995.

Melatonin Receptors Are for the Birds: Molecular Analysis of Two Receptor Subtypes Differentially Expressed in Chick Brain

Steven M. Reppert,* David R. Weaver,* Vincent M. Cassone,† Catherine Godson,* and Lee F. Kolakowski, Jr.‡
*Laboratory of Developmental Chronobiology
Massachusetts General Hospital
Harvard Medical School
Boston, Massachusetts 02114
†Department of Biology
Texas A & M University
College Station, Texas 77843-3258
‡Ina Sue Perlmutter Laboratory
Children's Hospital
Harvard Medical School
Boston, Massachusetts 02115

Summary

Two receptors (CKA and CKB) of the G protein–coupled melatonin receptor family were cloned from chick brain. CKA encodes a protein that is 80% identical at the amino acid level to the human Mel_{1a} melatonin receptor and is thus designated the chick Mel_{1a} melatonin receptor. CKB encodes a protein that is 80% identical to the Xenopus melatonin receptor and defines a new receptor subtype, the Mel_{1c} melatonin receptor, which is distinct from the Mel_{1a} and Mel_{1b} melatonin receptor subtypes. A melatonin receptor family consisting of three subtypes is supported by PCR cloning of distinct melatonin receptor fragments from Xenopus and zebrafish. Expression of CKA and CKB results in similar ligand binding and functional characteristics. The widespread distribution of CKA and CKB mRNA in brain provides a molecular substrate for the profound actions of melatonin in birds.

Introduction

In birds, melatonin is a hormone of critical importance for normal circadian function (for review, see Cassone, 1990). Photoreceptive circadian clocks in the avian pineal gland and retinae rhythmically produce melatonin. The hormone rhythm, in turn, imposes periodicity on structures that ultimately control overt circadian rhythms. The relative contributions of the pineal gland and retinae to circulating melatonin levels varies by species, as does the effect of removal of these organs on circadian function. This potent effect of melatonin in birds is in marked contrast to the situation in mammals, where melatonin has at most a modest, modulatory effect on circadian rhythms (Cassone, 1990; Weaver et al., 1991).

Because of the potent actions of melatonin on the circadian timing system of birds, several groups have used the melatonin agonist 2-[^{125}I]iodomelatonin (^{125}I-Mel) to characterize and map the distribution of melatonin receptors in avian brain (Rivkees et al., 1989; Stehle, 1990; Cassone and Brooks, 1991; Siuciak et al., 1991; Cassone et al., 1995). As in other vertebrates, ^{125}I-Mel binding in birds is of high affinity and sensitive to inhibition by guanine nucleotides. In vitro autoradiography using ^{125}I-Mel shows specific binding in visual systems at several levels of neural organization from the retinae to integrative areas controlling visual function, including structures in the circadian, tectofugal, thalamofugal, and accessory optic systems. This widespread receptor distribution suggests that melatonin exerts temporal control over a broad range of physiological and behavioral events in birds.

Recently, high affinity melatonin receptors of the guanine nucleotide–binding protein (G protein)–coupled receptor superfamily have been cloned from Xenopus laevis and mammals (Ebisawa et al., 1994; Reppert et al., 1994, 1995). These recombinant receptors exhibit pharmacological characteristics similar to each other and to those of endogenous receptors, as defined by ^{125}I-Mel. Two mammalian melatonin receptor subtypes have been identified by molecular cloning studies. The mammalian Mel_{1a} melatonin receptor is expressed in the hypophyseal pars tuberalis and hypothalamic suprachiasmatic nuclei (SCN), presumed sites of the reproductive and circadian actions of melatonin, respectively (Reppert et al., 1994). The mammalian Mel_{1b} melatonin receptor is expressed in retina and may mediate the reported effects of melatonin on retinal physiology in mammals (Reppert et al., 1995). The Mel_{1a} and Mel_{1b} receptors are 60% identical at the amino acid level to each other, and each mammalian subtype is 60% identical to the Xenopus receptor. Thus, it is possible that the frog receptor represents a third melatonin receptor subtype in vertebrates.

We report here the isolation and molecular characterization of two melatonin receptor cDNAs from chick (Gallus domesticus) that are differentially expressed in brain. One receptor, CKA, is the structural homolog of the mammalian Mel_{1a} receptor. The other chick receptor, CKB, is the structural homolog of the Xenopus receptor, showing that a third receptor subtype, now designated the Mel_{1c} melatonin receptor, indeed exists in vertebrates. A melatonin receptor family consisting of three subtypes is further supported by polymerase chain reaction (PCR) cloning of distinct melatonin receptor fragments representing each of the three subtypes from Xenopus and zebrafish (Brachydanio rerio).

Results

Structure of Avian Melatonin Receptors

Fragments of 2 putative melatonin receptors were cloned from chick brain by reverse transcription PCR (RT–PCR). PCR amplification was accomplished using degenerate oligonucleotide primers based on amino acid residues in the third and sixth transmembrane domains conserved among the Xenopus melatonin receptor and the mammalian Mel_{1a} and Mel_{1b} melatonin receptors (see Reppert et

Figure 1. Comparision of the Deduced Amino Acid Sequences of CKA and CKB with the Human Mel_{1a} Melatonin Receptor and the Xenopus Melatonin Receptor

To maximize homologies, gaps (dots) have been introduced into two sequences. The seven presumed transmembrane domains (I–VII) are overlined. Consensus sites for N-linked glycosylation are underlined. Genbank accession numbers for CKA and CKB are U31820 and U31821, respectively. Sequence data for the human and Xenopus receptors are from Reppert et al., 1994, and Ebisawa et al., 1994, respectively.

al., 1994, 1995). Sequence analysis of clones from chicken brain cDNA and genomic libraries that hybridized to the receptor cDNA fragments delineated the 5′ and 3′ ends of the coding region of each receptor. RT–PCR of mRNA from chick brain using specific primers directed at the 5′ and 3′ ends of the coding regions amplified the expected cDNAs; the two cDNA clones were designated CKA and CKB. The PCR-generated cDNAs were subcloned into pcDNA3 for expression studies and sequence analysis. Analysis of genomic clones of CKA and CKB showed that each gene is composed of two exons that encode the coding region. The intron splice site for each chick receptor occurs in the first cytoplasmic loop at the same location as that previously found for both the mammalian Mel_{1a} and Mel_{1b} receptors (Reppert et al., 1994, 1995).

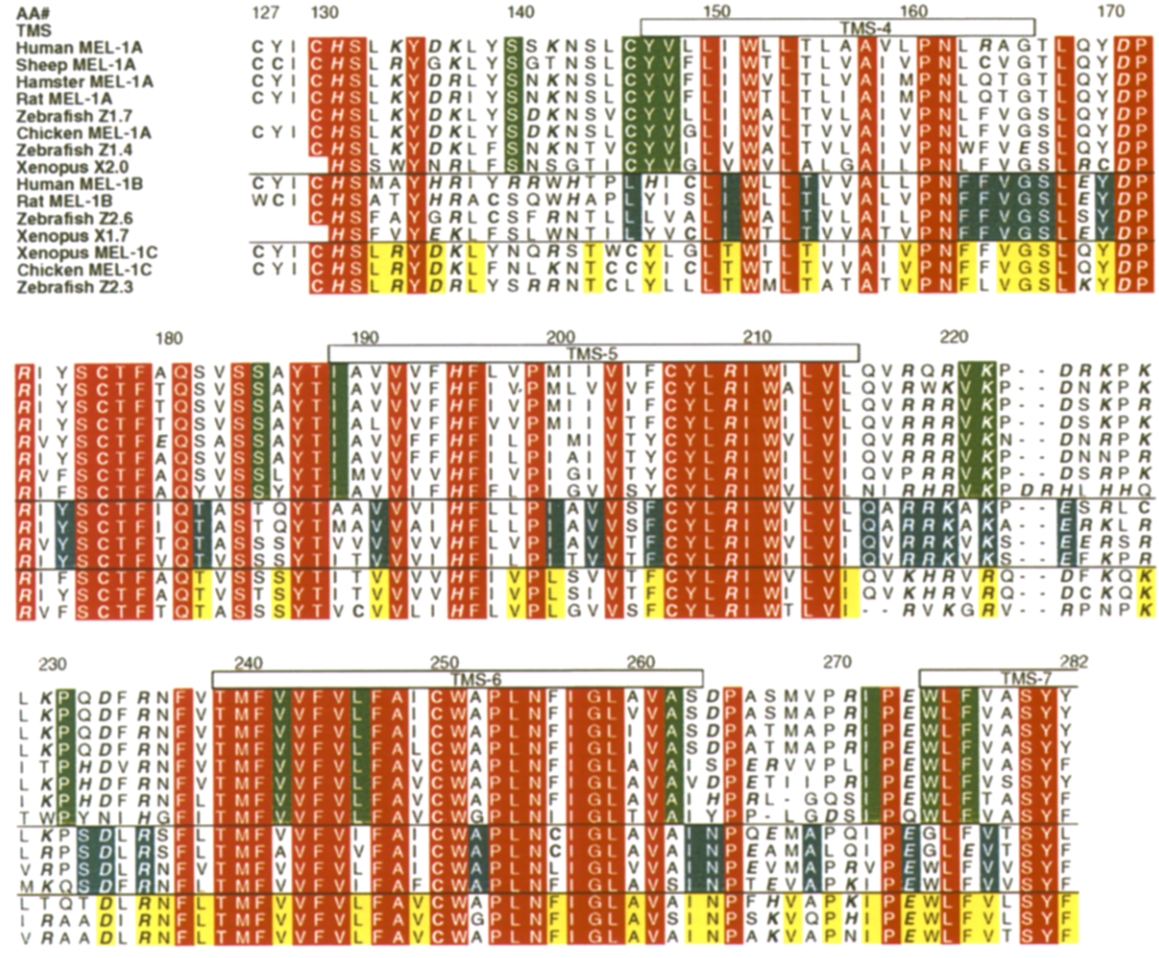
CKA and CKB encode proteins of 353 and 346 amino acids, respectively (Figure 1). Both receptors have structural features that place them in the melatonin receptor group of the prototypic G protein–coupled receptor family. Distinguishing features of this melatonin receptor group include a NRY (rather than DRY) motif just downstream from the third transmembrane domain, a C(C/Y)ICHS motif immediately downstream from NRY, and a NAXXY (rather than NPXXY) motif in transmembrane domain 7 (Figure 1).

CKA and CKB are 68% identical to each other overall at the amino acid level. Within the transmembrane domains, amino acid identity is 77% (Figure 1). The most dissimilar regions of the two avian receptors are in the amino and carboxyl termini and the third cytoplasmic loop. Within the amino terminus, there are two consensus sites for N-linked glycosylation for CKA, and one site in the amino terminus of CKB.

Among all the cloned melatonin receptors, CKA is most similar to the human Mel_{1a} melatonin receptor; there is 80% amino acid identity overall between the two (Figure 1). Furthermore, each has two consensus sites for N-linked glycosylation in the amino terminus. Thus, CKA appears to be the structural homolog of the mammalian Mel_{1a} receptor. CKB is most similar to the Xenopus melatonin receptor; there is 80% amino acid identity overall between the two (Figure 1). Each also has one consensus site for N-linked glycosylation in the amino terminus. One difference between CKB and the Xenopus receptor is that the carboxyl tail of the frog receptor is 66 residues longer. CKB and the Xenopus receptor appear to belong to a third melatonin receptor subtype, the Mel_{1c} melatonin receptor (see below).

Evolutionary Relationships of Melatonin Receptors across Vertebrate Phylogeny Support a Family of Three Subtypes

To provide further evidence of three distinct melatonin receptor subtypes in vertebrates, we used PCR amplification of genomic DNA from Xenopus and zebrafish to amplify additional melatonin receptor fragments from these species. PCR amplification used two sets of degenerate primer pairs. Primers were based on regions near the third transmembrane segment and in the sixth transmembrane segment, with one primer pair based on the peptide sequences AIAINRY and MAYFNSC and another on the peptide sequences INRYCYIC and MAYFNSC. These primer



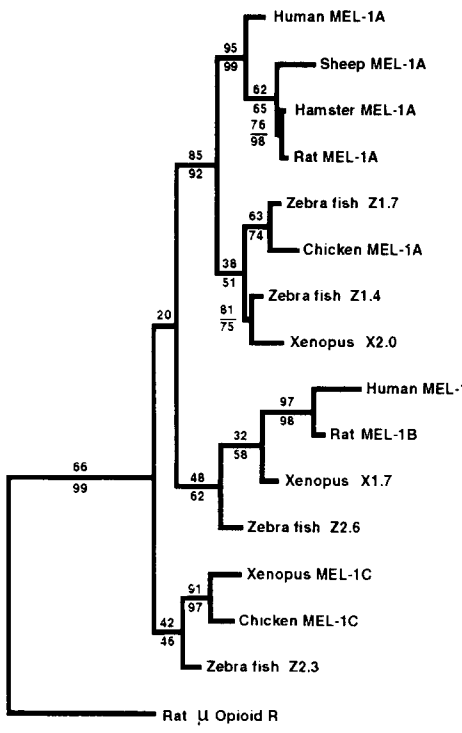


Figure 2. Phylogenetic Analysis of Melatonin Receptor Sequences from Four Vertebrate Classes

(Top) All known melatonin receptors were aligned. Red, consensus among all subtypes; green, consensus among Mel_{1a} receptor members; blue, consensus among Mel_{1b} receptor members; yellow, consensus among Mel_{1c} receptor members.

(Bottom) Phylogenetic tree. The regions comprising transmembrane segments 4–7 were used to calculate a tree using the maximum parsimony method. The numbers above and below the branches are the results of a bootstrap analysis and are related to a confidence limit for the positions of the branches. The rat μ opioid receptor sequence provided an outgroup for the tree. GenBank accession numbers are U31822 (Z1.7), U31823 (Z1.4), U31826 (X2.0), U31824 (Z2.6), U31827 (X1.7), and U31825 (Z2.3).

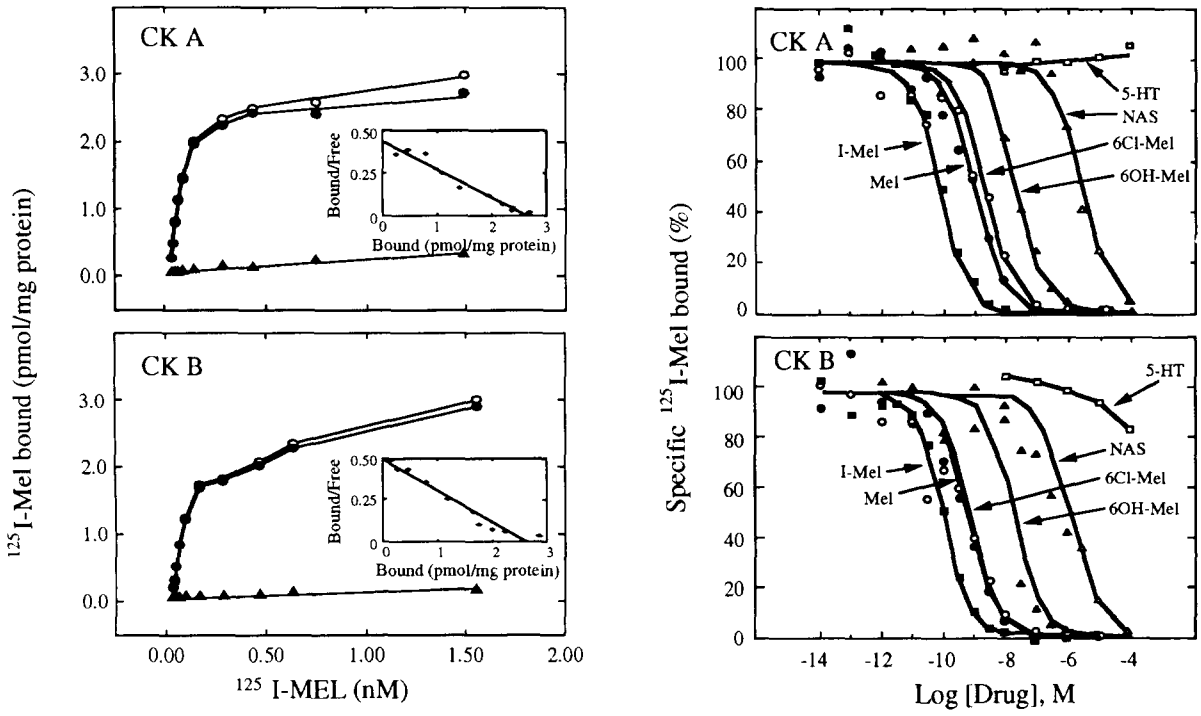


Figure 3. Binding and Pharmacological Properties of Avian Melatonin Receptors

(Left) Expression of CKA and CKB in COS-7 cells assayed by ^{125}I-Mel binding (open circles, total binding; closed circles, specific binding; closed triangles, nonspecific binding [determined in the presence of 10 μM melatonin]). The insets show Scatchard plots of saturation data. The K_D values depicted are 2.2 × 10^{-11} for CKA and 3.1 × 10^{-11} for CKB. B_{max} values are 2.6 pmol/mg of protein for both CKA and CKB. Data shown are representative of three experiments for each receptor cDNA.

(Right) Competition by various ligands for ^{125}I-Mel binding to COS-7 cells transfected with either CKA or CKB. Cells were incubated with 100 pM ^{125}I-Mel and various concentrations of 2-iodomelatonin (I-Mel), melatonin (Mel), 6-chloromelatonin (6Cl-Mel), 6-hydroxymelatonin (6OH-Mel), N-acetyl-5-hydroxytryptamine (NAS), or 5-hydroxytryptamine (5-HT). Nonspecific binding was determined in the presence of 10 μM melatonin. The data shown are representative of three experiments for each receptor. Mean K_i values for CKA are 2.9 × 10^{-11} M (I-Mel) 3.6 × 10^{-10} M (Mel), 7.9 × 10^{-10} M (6Cl-Mel), 7.9 × 10^{-9} M (6OH-Mel), 8.7 × 10^{-7} M (NAS), and >1.0 × 10^{-4} M (5-HT). Mean K_i values for CKB are 4.1 × 10^{-11} M (I-Mel), 3.1 × 10^{-10} M (Mel), 3.8 × 10^{-9} M (6Cl-Mel), 7.1 × 10^{-9} M (6OH-Mel), 4.6 × 10^{-7} M (NAS), and >1.0 × 10^{-4} M (NAS). Inhibition curves were generated by LIGAND using a one site model (Munson and Rodbard, 1980).

sets were designed to avoid the intron known to occur in the first cytoplasmic loop in mammalian and avian receptor genes. Amplified DNA fragments of appropriate length were subcloned and sequenced. Of 30 clones analyzed from Xenopus, 2 novel putative melatonin receptor fragments were identified, along with the Xenopus Mel_{1c} receptor (Ebisawa et al., 1994). Of 30 clones analyzed from zebrafish, 4 different putative melatonin receptor fragments were identified. The alignment of these fragments along with the other cloned melatonin receptor subtypes is depicted in Figure 2.

With sequence information from 15 melatonin receptor fragments spanning four vertebrate classes, a phylogenetic tree was next constructed. The regions of G protein–coupled receptors that are best conserved over wide evolutionary time periods are the transmembrane segments. Thus, we generated an alignment of the regions of melatonin receptors from transmembrane segments 4–7, with the rat μ opioid receptor serving as an outgroup. This alignment was used to calculate the most parsimonious phylogenetic tree using the program PAUP (Swofford, 1991; Kolakowski and Rice, submitted). This tree showed that melatonin receptors are a unique cluster of receptors distinct from their nearest database match (rat μ opioid receptor at 25% amino acid identity; Figure 2). A very similar tree was obtained using the neighbor-join method (Saitou and Nei, 1987). Bootstrap analyses of the tree and the data set showed that the assignment of three melatonin receptor subtypes is strongly supported, with bootstrap values of 85/92 for the Mel_{1a} receptor group, 48/62 for the Mel_{1b} receptor group, and 42/46 for the Mel_{1c} receptor group.

Avian Melatonin Receptor Subtypes Exhibit Similar Binding and Pharmacological Properties

To examine the binding and pharmacological properties of CKA and CKB, the cDNA for each was transiently expressed in COS-7 cells. Scatchard transformation of the saturation data showed that COS-7 cells transfected with either receptor bound ^{125}I-Mel with high affinity (Figure 3, left). The K_D of CKA was 2.3 × 10^{-11} ± 0.2 × 10^{-11} M (mean ± SEM of 3 experiments) with a B_{max} value of 2.4 ± 0.4 pmol/mg protein. The K_D of CKB was 3.1 × 10^{-11} ± 0.1 × 10^{-11} M (mean ± SEM of 3 experiments) with a B_{max} value of 2.1 ± 0.3 pmol/mg protein.

The pharmacological characteristics for inhibition of

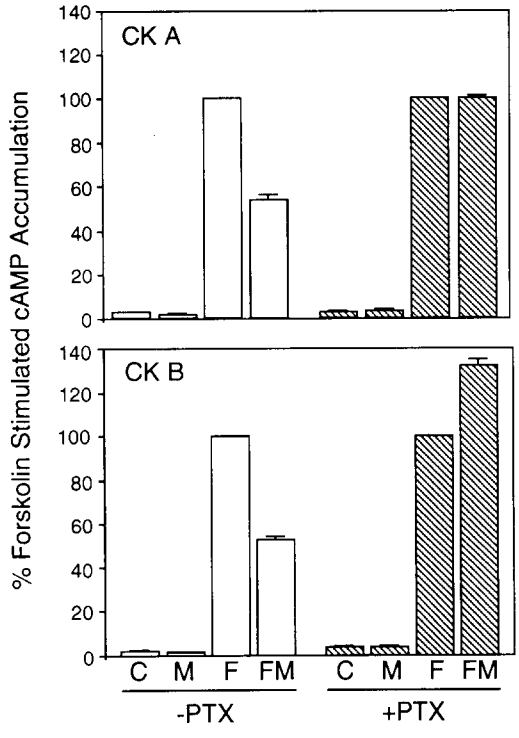


Figure 4. CKA and CKB Couple to G_i

PTX blocks the ability of melatonin to inhibit forskolin-simulated cAMP accumulation. NIH 3T3 cells stably transfected with CKA (top) or CKB (bottom) were preincubated with either vehicle (−PTX) or PTX (+PTX; 100 ng/ml) for 18 hr. Data are expressed as mean ± SEM for three experiments for each clone. For each experiment, values are expressed as a percentage of the mean forskolin-stimulated value (100%). C, basal levels; M, 1 μM melatonin alone; F, 10 μM forskolin alone; FM, 10 μM forskolin plus 1 μM melatonin.

specific ^{125}I-Mel binding in acutely transfected COS-7 cells were next examined. For each of the two avian receptors, the rank order of inhibition of specific ^{125}I-Mel binding by six ligands was 2-iodomelatonin > melatonin ≥ 6-chloromelatonin > 6-hydroxymelatonin > N-acetyl-5-hydroxytryptamine >> 5-hydroxytryptamine (Figure 3, right). Thus, CKA and CKB encode structurally distinct proteins that have indistinguishable affinity constants and binding properties with the ligands examined.

Avian Melatonin Receptors Are Coupled to G_i

Since CKA and CKB appear to be structural homologs of the mammalian Mel_{1a} melatonin receptor and the Xenopus Mel_{1c} melatonin receptor, respectively, we next examined whether the two avian melatonin receptors are coupled to G_i, as are the recombinant mammalian and frog receptors (Ebisawa et al., 1994; Reppert et al., 1994). For these studies, we used clonal lines of NIH 3T3 cells stably transfected with either CKA or CKB (Figure 4). Melatonin (1 μM) did not increase cAMP levels above basal values in cell lines expressing either receptor. Melatonin (1 μM) clearly inhibited the increase in cAMP accumulation induced by 10 μM forskolin in cell lines expressing either CKA or CKB. Importantly, melatonin (1 μM) did not inhibit forskolin-stimulated cAMP accumulation in nontransfected

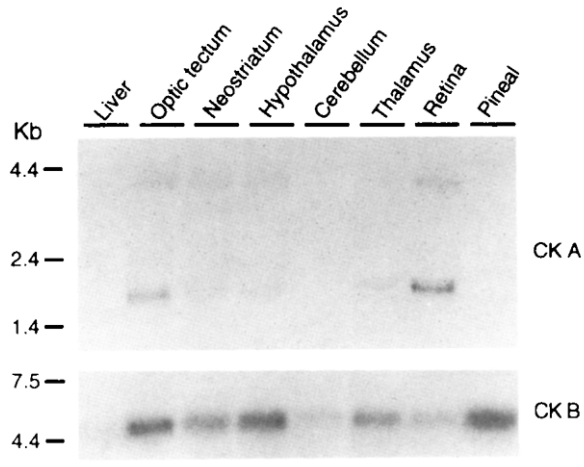


Figure 5. Differential Expression of CKA and CKB mRNA in Chick Brain Assessed by Northern Blot Analysis

Each lane contained 3 μg of poly(A)$^+$ RNA. Location of RNA size markers are indicated on the left. A similar hybridization pattern was found in another blot for each probe. The blots were probed with histone H3.3, which showed equivalent RNA loading among the lanes (data not shown).

NIH 3T3 cells or in cells transfected with vector lacking cDNA. Pertussis toxin (PTX) pretreatment (100 ng/ml for 18 hr) of NIH 3T3 cells stably expressing either receptor blocked the ability of 1 μM melatonin to inhibit the forskolin-stimulated increase in cAMP (Figure 4). Thus, like their frog and mammalian counterparts, both avian receptors can couple to G_i.

Melatonin Receptor Subtypes Are Differentially Expressed in Chick Brain

Northern blot analysis of RNA from liver, pineal gland, and several brain areas probed with CKA or CKB revealed a distinct tissue distribution for each mRNA (Figure 5). Hybridization with CKA revealed two transcripts of 1.8 and 4.0 kb that were prominently expressed in optic tectum and retina, with lesser amounts in neostriatum, hypothalamus, and thalamus. No hybridization signal was apparent in liver, pineal gland, or cerebellum. Hybridization with CKB revealed a single transcript of 4.8 kb that was moderately expressed in optic tectum, neostriatum, hypothalamus, thalamus, and pineal gland, with lower amounts detected in cerebellum and retina. No hybridization signal was apparent in liver. The brain and pineal distribution of mRNA for CKA and CKB by Northern analysis was confirmed by in situ hybridization, as discussed below.

The pattern of ^{125}I-Mel binding by in vitro autoradiography was compared with the patterns of CKA and CKB mRNA expression as assessed by in situ hybridization (Table 1; see Figures 6–8). As previously described, specific ^{125}I-Mel binding is widespread in chick brain, with the highest levels of binding predominating in structures involved in vision (Rivkees et al., 1989; Stehle, 1990; Cassone and Brooks, 1991; Siuciak et al., 1991). These structures include the retina, and retinorecipient relay and integrative structures of the circadian, tectofugal, thalamo-

Table 1. Distribution of Specific ^{125}I-Mel Binding and of mRNA for CKA and CKB

Structure	^{125}I-Mel Binding	CKA	CKB
Retina/optic nerve			
Inner plexiform layer (IPL)	+++	–	–
Inner nuclear layer (INL)	–	+++	++
Ganglion cell layer (GCL)	–	+	–
Optic chiasm (CO)	++	–	+++
Optic tract	++	–	++
Stratum opticum	++	–	+
Retinohypothalamic			
Visual suprachiasmatic nucleus (vSCN)	++	+	+
Ventral supraoptic decussation	+++	–	+
Tectofugal			
Optic tectum (TeO)	+++	+++	++
Stratum griseum et fibrosum (SGF)	+++	++	++
Stratum griseum centrale (SGC)	++	+++	–
Nucleum rotundus (Rt)	+++	+++	–
Perirotundal area (pRt)	++	–	+++
Nucleus triangularis	+++	++	–
Nucleus isthmus parvocellularis	++	–	–
Nucleus isthmus magnocellularis	++	–	–
Ectostriatum (E)	+++	–	–
Periectostriatum	–	–	++
Thalamofugal			
Lateral anterior nucleus	++	+	–
Dorsal lateral nucleus pars lateralis	+	+	–
Dorsal lateral nucleus pars medialis	+	+	++
Ventral lateral geniculate nucleus (GLv)	++	++	++
Hyperstriatum accessorium	++	+	+
Hyperstriatum intercalatus superior	+	+	+
Lamina hyperstriatalis	+	–	+++
Accessory optic			
Ectomammilary nucleus	++	–	–
Nucleus of Edinger-Westphal (EW)	+++	+++	–
Nucleus of the abducens nerve (nVI)	+	++	+++
Dorsal oculomotor nucleus (nIII)	+	+	–
Other neuronal structures			
Olfactory bulb	+	+	++
Neostriatium anterior	++	++	–
Neostriatum intermedium (NI)	+++	+++	–
Neostriatum caudale	++	++	–
Piriform cortex	+	+	–
lobus parolfactorius	+	–	–
Cerebellum (Cb)	–	–	+
Nonneural tissues			
Choroid	++	–	++
Ependyma	–	–	++
Pineal (P)	–	–	+++

Binding and mRNA levels are as follows: +++, high; ++, medium; +, low; –, no binding or hybridization.

fugal, and accessory optic visual pathways (Figures 6 and 7).

Retina/Optic Nerve

Specific ^{125}I-Mel binding was present in the inner plexiform layer of the retina, as previously described (Laitinen and Saavedra, 1990), while nonspecific binding for the radioligand was observed in pigmented epithelium. Specific ^{125}I-Mel binding was also present in the optic chiasm and, to a lesser extent, in the optic tract and stratum opticum.

Emulsion autoradiographs showed that mRNAs for both CKA and CKB were expressed in retina (Figure 8). For CKA, hybridization was very strong in vitread aspects of the inner nuclear layer and, to a lesser extent, in the ganglion cell layer. CKB mRNA was also expressed in the inner nuclear layer, but not in the ganglion cell layer. No hybridization signal was found for CKA in the optic chiasm, optic tract, or stratum opticum, whereas mRNA for CKB was clearly expressed within these three structures.

Circadian/Retinohypothalamic Pathways

The avian retinohypothalamic tract projects contralaterally to the nucleus suprachiasmaticus visualis (vSCN). The vSCN is a discrete nucleus in the lateral anterior hypothalamus between the dorsal supraoptic decussation and the ventral lateral geniculate nucleus (Ehrlich and Mark, 1984; Cassone and Moore, 1987). The vSCN has been shown to be anatomically and immunohistochemically similar to the mammalian SCN (Cassone and Moore, 1987), which is the site of a circadian clock (for review, see Klein et al., 1991). The avian vSCN appears to be the functional homolog of the mammalian SCN, as the vSCN expresses

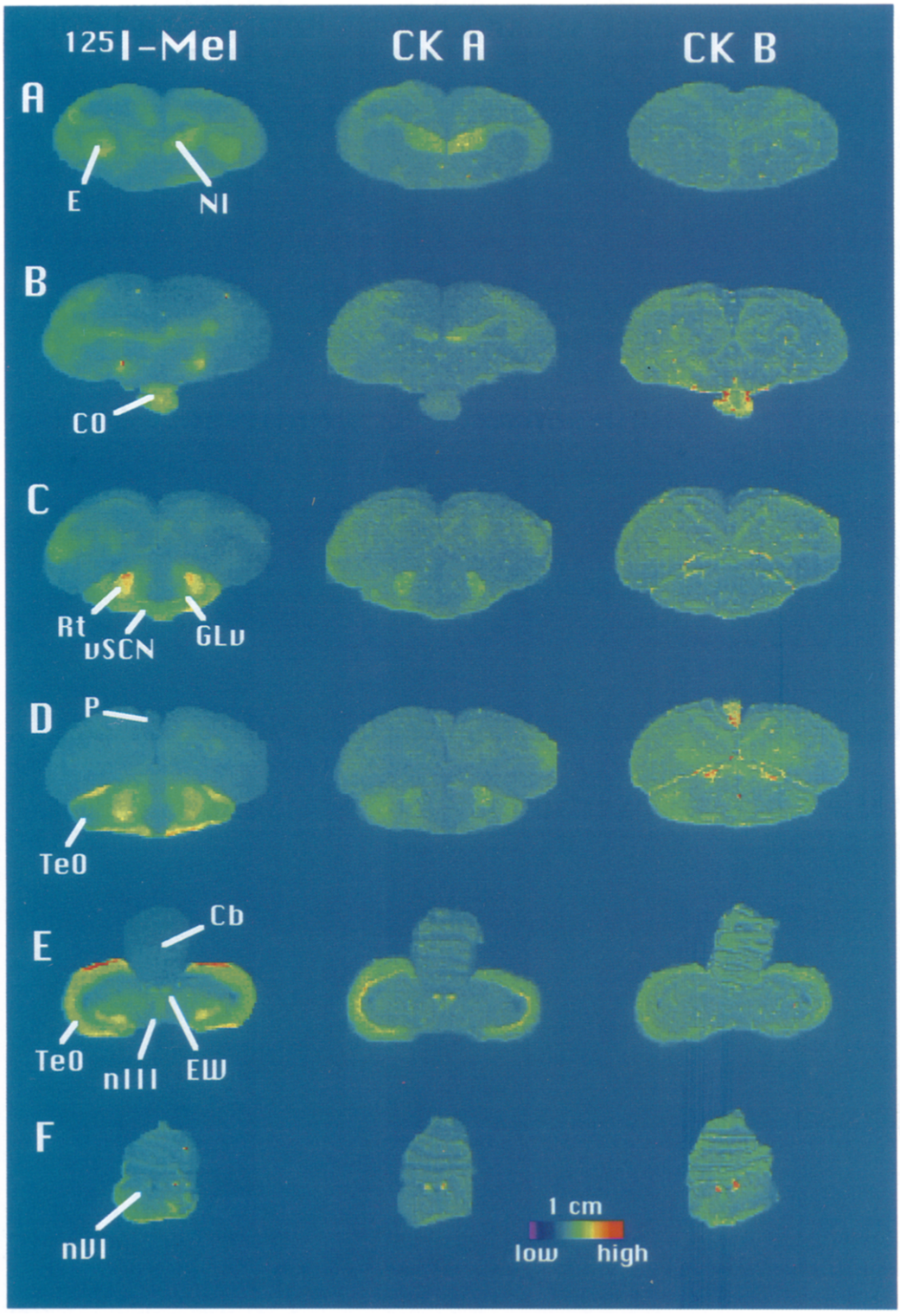


Figure 6. Differential Expression of CKA and CKB mRNA in Chick Brain Assessed by In Situ Hybridization

Distribution of ^{125}I-Mel binding, CKA mRNA, and CKB mRNA in chick brain is depicted. False color images of autoradiographs are shown. Relative intensity of hybridization is indicated by the scale at the bottom of the figure. The panels depict sets of adjacent coronal sections from rostral (top) to caudal (bottom). Abbreviations for the labeled structures are given in Table 1.

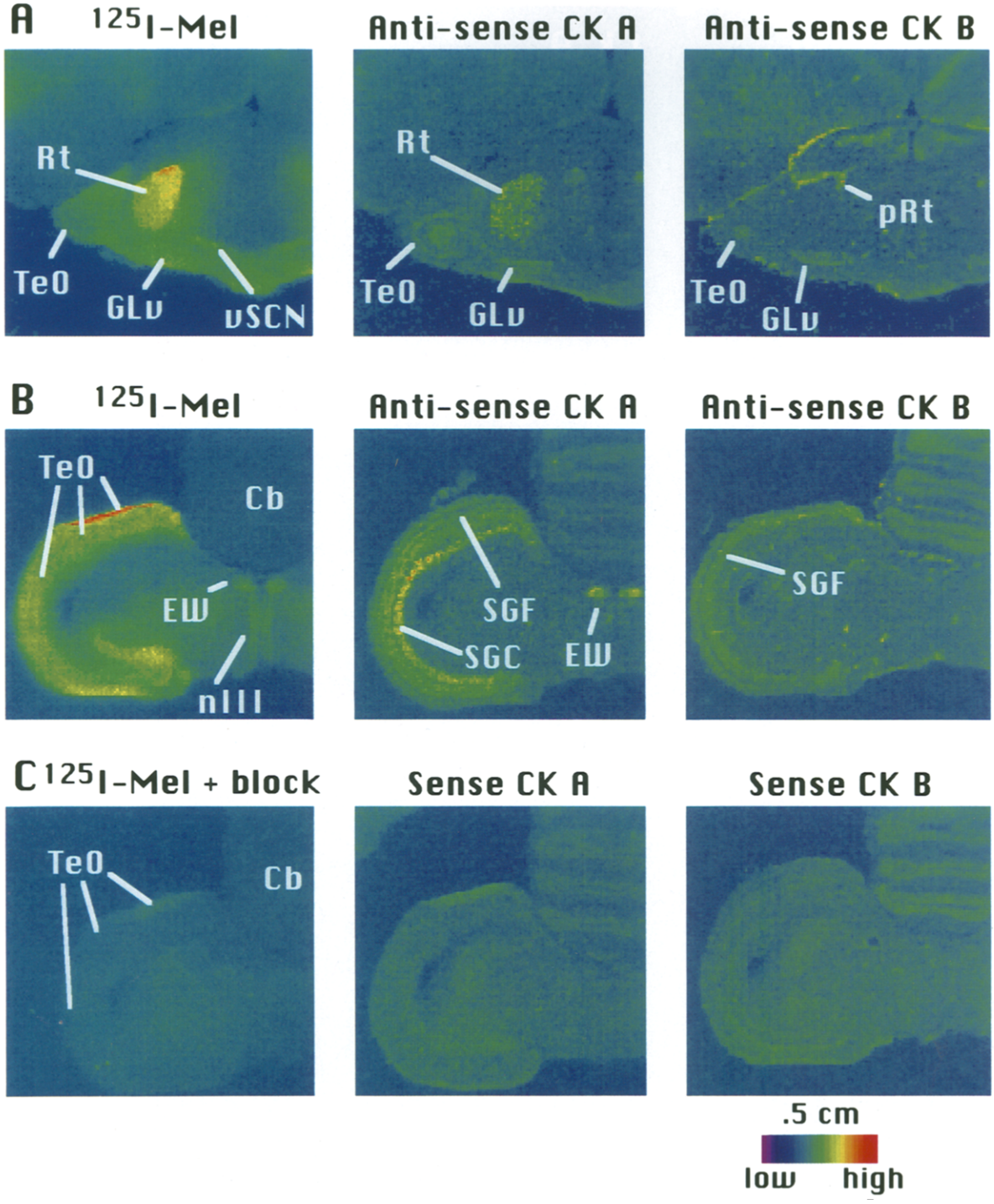


Figure 7. Distribution of ^{125}I-Mel Binding and CKA and CKB mRNA in Selected Brain Areas

False color images of autoradiographs are shown. Abbreviations for the labeled structures are given in Table 1.

circadian rhythms of metabolism in house sparrows in vivo (Cassone, 1988; Lu and Cassone, 1993a) and of electrical activity in Japanese quail in vitro (Juss et al., 1994).

In the present study, as in previous studies (Rivkees et al., 1989; Cassone et al., 1995), the vSCN bound ^{125}I-Mel. The ventral supraoptic decussation, which contains astroglia and neuronal fibers connecting the two vSCNs (V. M. C., unpublished data) also contained specific binding. Emulsion autoradiographs revealed a hybridization signal for both CKA and CKB in the vSCN; the hybridization sig-

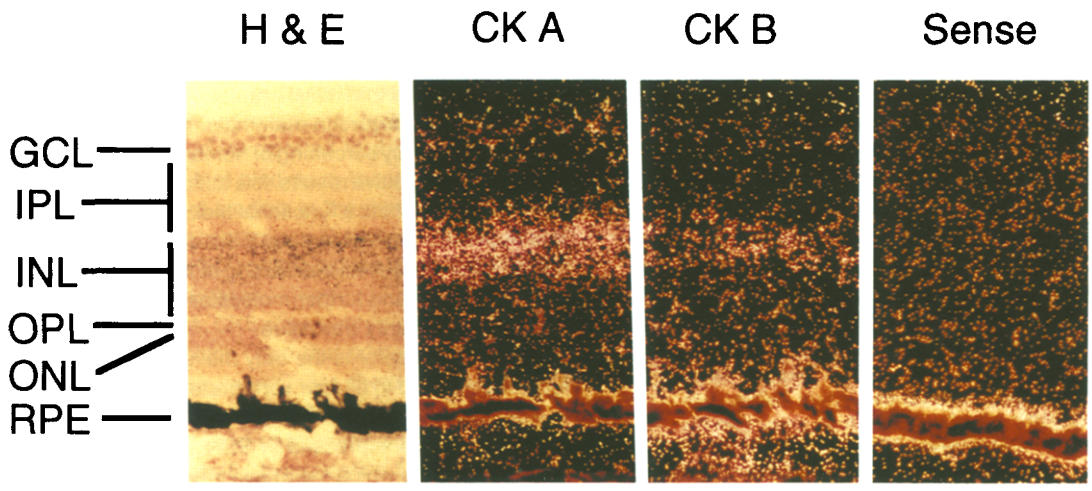


Figure 8. Expression of CKA and CKB mRNA in Chick Retina

Dark-field photomicrographs of emulsion autoradiographs show the distribution of CKA and CKB mRNA in retina. H & E, brightfield photomicrograph of hematoxylin- and eosin-stained section; GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer; RPE, pigment epithelium. Each panel width is 120 μm.

nal for CKB was more intense than that for CKA. For CKB, a clear hybridization signal was also present in the ventral supraoptic decussation.

Tectofugal Visual Pathway

The tectofugal visual pathway is homologous to the mammalian extrageniculocortical pathway (Ehrlich and Mark, 1984; Engelage and Bischof, 1993). Specific ^{125}I-Mel binding was present in all retinorecipient, tectorecipient, and integrative structures of this visual pathway. Structures that exhibited both ^{125}I-Mel binding and hybridization for CKA include the stratum griseum centrale of the optic tectum, nucleus rotundus, and the tectorecipient ventral lateral geniculate nucleus. Inspection of emulsion autoradiographs indicated intense CKA hybridization in magnocellular neurons in the stratum griseum centrale and nucleus rotundus. Furthermore, dense hybridization was present in neuronal cell bodies of the lamina interna of the ventral lateral geniculate nucleus and, to a lesser extent, in the neuropil of that structure. Although ^{125}I-Mel binding was dense within ectostriatum, there was no evidence of hybridization for CKA (see Figure 6A). CKB hybridization was also found in the stratum griseum et fibrosum of the optic tectum and in the neuropil of the ventral lateral geniculate nucleus (see Figure 7B). While there was no detectable CKB hybridization in nucleus rotundus and ectostriatum, there was a hybridization signal in the perirotundal area (see Figure 7A) and in the periectostriatum.

Thalamofugal Visual Pathway

The thalamofugal visual pathway is homologous to the mammalian geniculostriate pathway (Gunturkun et al., 1993). Low levels of ^{125}I-Mel binding were present in the principal optic nucleus and the Wulst. Little to no hybridization for CKA was found in the principal optic nucleus, but a scattered collection of positively hybridizing neurons was found within the hyperstriata of the Wulst. High levels of mRNA for CKB were present in the dorsolateral nucleus of the thalamus, a portion of the principal optic nucleus. There was also increased CKB hybridization in the lamina separating the hyperstriata of the Wulst and other aspects of the forebrain.

Accessory Optic Pathway

Specific ^{125}I-Mel binding was present in the nucleus of the basal optic root (also called ectomammillary nucleus), in the nucleus of Edinger-Westphal, in the nucleus of the abducens nerve, in the dorsal oculomotor nucleus, and within the oculomotor nerve itself (see Figure 6). No binding was present in the cerebellum. Hybridization for CKA was present in the nucleus of Edinger-Westphal, the dorsal oculomotor nucleus, and the nucleus of the abducens nerve, but not in the nucleus of the basal optic root or in the cerebellum (see Figures 6 and 7). Inspection of both films and emulsion autoradiographs suggested hydridization in the cerebellum, but hybridization for the sense probe was equally strong in this structure, indicating this signal is likely nonspecific; no signal for CKA was found by Northern analysis (see Figure 5). There was no detectable hybridization signal for CKB in the nucleus of the basal optic root or the nucleus of Edinger-Westphal. However, there was modest hybridization in cerebellum and a very strong signal within the nucleus of the abducens nerve (see Figure 6F).

Other Structures

Moderate ^{125}I-Mel binding was present in the neostriata of the chick, including neostriatum intermedium, nucleus anterior, and nucleus caudale. Furthermore, ^{125}I-Mel binding was present in the lobus parolfactorius, and low levels of binding were present in the piriform cortex, septomesencephalic tract, and olfactory bulbs. Hybridization for CKA was very strong in the neostriatum intermedium (see Figure 6A). CKA was also present in other neostriatal areas and in the piriform cortex. Although no clear evidence of hybridization for CKA was present in the lobus parolfactorius, hybridization was present in the diagonal band of Broca, which is adjacent to the lobus parolfactorius. Fur-

thermore, hybridization for CKA was present in the olfactory bulbs. For CKB, moderately labeled cells were scattered throughout the brain, giving the appearance of diffuse labeling on film autoradiographs (see Figure 6). Hybridization for CKB was present in the ependyma of all ventricular surfaces, in the walls of arteries, especially within the forebrain, and in the choroid plexuses (see Figure 7A). There was very intense hybridization for CKB in the pineal gland (see Figure 6D; confirmed by Northern analysis). The pineal gland exhibits no detectable ^{125}I-Mel binding.

Discussion

The results show that at least two high affinity melatonin receptor genes mediate the important actions of melatonin in chick brain. Analysis of endogenous melatonin receptor properties by ^{125}I-Mel binding alone does not provide any suggestion that ^{125}I-Mel binding results from the expression of more than one receptor subtype (Rivkees et al., 1989; Stehle, 1990; Cassone and Brooks, 1991; Siuciak et al., 1991). This is now explained by the indistinguishable affinities and pharmacological profiles exhibited by CKA and CKB; the characteristics of each of the recombinant receptors are the same as those exhibited by endogenous melatonin receptors defined by ^{125}I-Mel in avian brain. Thus, the existence of two receptor proteins could be determined only by molecular cloning of the cDNAs for each receptor.

The results also show that a third melatonin receptor subtype, the Mel_{1c} melatonin receptor, exists in vertebrates. Evidence supporting this is the close structural identity CKB shares with the Xenopus melatonin receptor (80% amino acid identity) and bootstrap analysis of the phylogenetic tree. Further evidence of three receptor subtypes in vertebrates is our PCR analysis of additional melatonin receptor fragments from Xenopus and zebrafish. We cloned 2 additional, distinct receptor fragments from Xenopus, 1 of which aligns with the Mel_{1a} receptor group and the other with the Mel_{1b} receptor group. In zebrafish, we cloned 4 different melatonin receptor fragments; bootstrap analysis shows that 2 align with the Mel_{1a} receptor group and 1 each with the Mel_{1b} and Mel_{1c} receptor groups. Thus, a melatonin receptor family consisting of three distinct subtypes is supported by analysis of 15 melatonin receptor fragments spanning four vertebrate classes. It is remarkable that the expressed receptors for each subtype all exhibit similar binding properties, even though they are structurally divergent. Furthermore, G_i appears to be a common signaling pathway for all three subtypes (Ebisawa et al., 1994; Reppert et al., 1994, 1995). The possibility that other intracellular signal transduction cascades are used by these receptors has not yet been fully explored.

We have no evidence that a homolog of the Mel_{1b} melatonin receptor subtype, recently cloned in mammals (Reppert et al., 1995), occurs in birds. We have examined this issue by evaluating numerous PCR-generated clones from chick genomic DNA using degenerate primers that have amplified this subtype from three other vertebrate classes. Furthermore, we have used the human Mel_{1b} melatonin receptor cDNA to probe a chick genomic library at low stringency, again without positive results. We also have no evidence that a Mel_{1c} melatonin receptor subtype (CKB homolog) occurs in mammals. We have used PCR and low stringency screening of human genomic material, again without positive results. It is quite possible that birds have evolved without a functional Mel_{1b} receptor gene and that mammals have evolved without a functional Mel_{1c} receptor gene. However, the cloning of melatonin receptors is in its infancy, and further molecular cloning studies will be needed to clarify this issue.

The Mel_{1a} receptor is the only melatonin receptor subtype so far found in all four vertebrate classes examined. In mammals, where this subtype was first identified, the Mel_{1a} receptor is expressed in SCN and is felt to be the receptor through which melatonin modulates mammalian circadian rhythms (Reppert et al., 1994). It is therefore noteworthy that the avian Mel_{1a} receptor (CKA) is expressed in the vSCN, the avian homolog of the mammalian SCN. The vSCN appears to be a damped circadian oscillator in birds, which is driven by the melatonin rhythm to control rhythmicity in structures that the vSCN innervates (Cassone, 1990). Thus, it appears that the Mel_{1a} receptor subtype may be important for circadian timing across vertebrate phylogeny. CKB mRNA is also present in the vSCN and may contribute to melatonin's effects on this structure.

In chick retina, melatonin inhibits forskolin-stimulated cAMP through a PTX-sensitive mechanism (Iuvone and Gan, 1994). The reported melatonin-mediated inhibition of cAMP in chick retina occurs through receptors that have the same pharmacological characteristics as those we found for recombinant CKA and CKB. However, there are no published reports of melatonin signaling through G_i in avian brain proper. Thus, our finding that recombinant CKA and CKB can clearly couple to G_i is important and identifies a signal transduction pathway through which melatonin may mediate its effects in avian brain. In fact, this may be the transduction cascade through which melatonin inhibits metabolic activity, as assessed by 2-deoxyglucose uptake, in avian brain (Lu and Cassone, 1993a, 1993b). This effect of melatonin appears to be receptor mediated because it is found only in brain sites that specifically bind ^{125}I-Mel (Lu and Cassone, 1993a).

The distribution of mRNAs encoding the two melatonin receptor subtypes is distinct in chick brain and retina. CKA appears to be expressed primarily by neuronal cell bodies in avian brain. Hybridization is clearly visible in neuronal cell bodies in the lamina interna of the ventral lateral geniculate nucleus, in the nucleus rotundus, and in the stratum griseum et fibrosum of the optic tectum. Several areas that express ^{125}I-Mel binding are conspicuously free of hybridization, including the ectostriatum and the isthmic nuclei of the optic tectum (see Figure 6A). It is therefore interesting that ectostriatum receives a major neuronal projection from nucleus rotundus, which does contain CKA mRNA, and both parvocellular isthmic nucleus and magnocellular isthmic nucleus receive afferents from the optic tectum, which has both CKA and CKB mRNA. Binding of ^{125}I-Mel in these areas probably results from receptor protein that is transported to presynaptic terminals. A simi-

lar argument could be made for retinorecipient structures that exhibit high levels of ^{125}I-Mel binding but show no detectable hybridization, such as the ectomammillary nucleus. It is possible that receptor protein produced in retinal ganglion cells is transported to this site, as well as contributing to ^{125}I-Mel binding in the vSCN. This would also explain ^{125}I-Mel binding in the inner plexiform layer of the retina; the receptors are likely presynaptic, arising from CKA and/or CKB receptor protein made in cell bodies in the inner nuclear layer.

In contrast to CKA, CKB shows very strong hybridization in some areas of chick brain that contain very few or no neuronal cell bodies. In particular, there is both hybridization for CKB mRNA and ^{125}I-Mel binding within the optic chiasm, optic tract, and stratum opticum. Moreover, labeled cells with specific hybridization for CKB are scattered throughout the brain. This widespread hybridization and the presence of a hybridization signal in tissues that contain few, if any, neuronal cell bodies suggest that CKB resides, at least in part, on glial cells.

The expression of CKB mRNA in the avian pineal gland, ependyma of the brain ventricles, and cerebellum is enigmatic. We find no evidence of ^{125}I-Mel binding in these structures. The CKB receptor gene may be expressed but not translated in these structures. It is also possible that the receptor gene is translated into a nonfunctional truncated protein due to intronic splice variants.

An important aspect of this work is the finding that the combined expression of two high affinity melatonin receptor genes accounts for the vast majority of ^{125}I-Mel binding found in chick brain. Differential expression in brain suggests that each receptor subtype may have distinct functions and distinct regulatory mechanisms.

Experimental Procedures

PCR

For PCR with degenerate primers, first-strand cDNA or genomic DNA was subjected to 30 cycles of amplification with two oligonucleotide primers (each at 200 nM final concentration). Each reaction cycle consisted of incubations at 94°C for 45 s, 45°C for 2 min, and 72°C for 2 min, with AmpiTaq DNA polymerase (Perkin-Elmer Cetus). The amplified DNA was separated on an agarose gel. DNA bands were subcloned into pCRTMII using a TA Cloning Kit (Invitrogen), and recombinant clones were sequenced. For RT–PCR with specific primers, first-strand cDNA was subjected to 35 cycles of amplification using incubations at 94°C for 45 s, 60°C for 45 s, and 72°C for 2 min. The amplified DNA was separated by agarose gel electrophoresis. DNA bands were subcloned into pcDNA3 (Invitrogen) for expression studies and sequence analysis.

Library Screening

A λgt10 chicken brain cDNA library and a chicken genomic library in EMBL-3 SP6/T7 (Clontech) were plated and transferred to Colony Plaque Screen filters (New England Nuclear). The filters were screened as previously described (Reppert et al., 1994). λ phage that hybridized to the probe was plaque purified.

Expression Studies

COS-7 and NIH 3T3 cells were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, penicillin (50 U/ml), and streptomycin (50 μg/ml) in 5% CO_2 at 37°C.

For ligand binding studies, melatonin receptor cDNAs in pcDNA3 were introduced into COS-7 cells using the diethylaminoethyl dextran method (Cullen, 1987). At 2 or 3 days after transfection, the medium was removed, the dishes were washed with PBS, and the cells were harvested. The cells were then pelleted (2500 rpm for 10 min at 4°C) and stored at −80°C. Whole-cell binding studies were performed by thawing the cells and resuspending them in binding buffer (50 mM Tris–HCl [pH 7.4] with 5 mM $MgCl_2$) at a concentration of 200–500 μg of protein per milliliter. The cell suspension was incubated with ^{125}I-Mel with or without drugs in a total reaction volume of 0.2 ml; the suspension was incubated in a shaker bath for 1.5 hr at 25°C. Reactions were terminated by rapid filtration through Whatman GF/B glass fiber filters. All determinations were done in either duplicate or triplicate. Protein measurements were performed using the Bio-Rad protein assay. Binding data were analyzed by computer using the LIGAND Program of Munson and Rodbard (1980).

Cyclic AMP Studies

For cAMP studies, the receptor cDNA in pcDNA3 was introduced into NIH 3T3 cells using Lipofectamine (GIBCO–BRL). Transformed NIH 3T3 cells resistant to Geneticin, G418 (at 1.0 mg/ml; GIBCO–BRL) were isolated, and clonal lines expressing melatonin receptor binding (>200 fmol/mg total cellular protein) were isolated.

Transformed NIH 3T3 cells were plated in triplicate on 35 mm dishes. After 48 hr, the cells were washed (2 ×) with DMEM and preincubated with 250 μM 3-isobutyl-1-methylxanthine (IBMX) in DMEM for 10 min at 37°C. Cells were then incubated with or without drugs in DMEM with 250 μM IBMX for 10 min at 37°C. At the end of treatment, the medium was aspirated, and 0.5 ml of 50 mM acetic acid was added. The cells were collected, transferred to an Eppendorf tube, boiled for 5 min, and centrifuged (13,750 rpm for 15 min). The supernatant was collected and assayed for cAMP. All treatments were performed on three replicate dishes. cAMP levels were determined in duplicate by radioimmunoassay (New England Nuclear).

Northern Analysis

Poly(A)$^+$ RNA was subjected to electrophoresis through a 1% agarose–formaldehyde gel, blotted onto GeneScreen (New England Nuclear), and hydridized with ^{32}P-labeled CKA or CKB (SA of each, >10^9 cpm/μg). Hydridizing conditions were 50% formamide, 1 M NaCl, 1% SDS, 10% dextran sulfate, and 100 μg/ml denatured salmon sperm at 42°C overnight. The final washing of blots was in 2 × SSC, 1% SDS at 65°C for 1 hr. Blots were exposed at −80°C to X-ray film with an intensifying screen.

In Situ Hybridization

Antisense and sense cRNA probes were generated by in vitro transcription in the presence of [α-^{35}S]thio-UTP (New England Nuclear; 1100–1300 Ci/mmol) as previously described (Reppert et al., 1991). The templates for transcription were cDNAs of the full-length coding regions of CKA and CKB. In situ hybridization was performed as previously described (Reppert et al., 1991). After prehybridization, sections were covered with hybridization buffer containing 1.0 × 10^7 to 1.2 × 10^7 cpm probe/ml, coverslipped, and incubated overnight at 53°C. The next morning, the coverslips were removed, and the slides were washed in 2 × SSC followed by digestion in RNase (10 μg/ml). Slides were washed to a final stringency of 0.1 × SSC at 53°C (two 30 min washes). Autoradiograms were generated by apposing the slides to Kodak Biomax MR film for 2–4 weeks. Slides were then dipped in Kodak NTB-2 emulsion. Emulsion autoradiograms were exposed for 4–6 weeks, developed, and then counterstained with hematoxylin and eosin.

^{125}I-Mel In Vitro Autoradiography

Tissue was processed for autoradiographic localization of ^{125}I-Mel binding sites as previously described (Cassone et al., 1995). Sections were labeled by incubation in buffer (50 mM Tris, 4 mM $CaCl_2$ with 0.1% BSA) containing high specific activity (2200 Ci/mmol) ^{125}I-Mel (30–40 pM). Nonspecific binding was determined in adjacent sections by inclusion of melatonin (1 μM) in the incubation solution. After washing, slides were dried under a stream of cool air. Autoradiographs were generated by apposition of the slides to B-Max Hyperfilm (Amersham) at −80°C for 10 days. Video images of autoradiographs were digitized for analysis of binding.

Tissue Specimens

White leghorn cockerels (14 days old; n = 10) were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine. Five birds were decapitated and their brains, eyes, and livers were rapidly removed and fozen in −40°C isopentane. These tissues were processed for poly(A)$^+$ RNA. The other five birds were transcardially perfused with 100 ml of 0.75% saline and then 100 ml of 0.1 M sodium phosphate buffer with 10% sucrose. Brains and eyes were removed and frozen as above. Brains were transversely sectioned at 20 μm through their entire rostrocaudal extent on a cryostat and thaw mounted onto acetone-cleaned slides coated with Vectabond in ten bins of adjacent sections. Eyes were sectioned transversely and mounted similarly. Bins of adjacent sections were subjected to either ^{125}I-Mel in vitro autoradiography or in situ hybridization to produce a section frequency of 1 per 200 μm for each condition.

DNA Sequencing

Nucleotide sequences were analyzed by the dideoxynucleotide chain termination method of Sanger et al. (1977) using Sequenase (US Biochemical). Sequencing template was double-stranded plasmid. Primers were synthetic oligonucleotides that were either vector specific or derived from sequence information.

Phylogenetic Analysis

Melatonin receptors and the rat μ opioid receptor were aligned using CLUSTAL W (Thompson et al., 1994). The alignments were adjusted manually, and the regions corresponding to transmembrane segments 4–7, as defined by Baldwin (1993), were utilized to generate a PAUP data matrix (Swofford, 1991). A branch and bound search was performed. The most parsimonious tree was found with a tree length of 528. Using PAUP, 1000 replicates of bootstrap analysis were performed. A neighbor-joining tree was calculated using CLUSTAL W and analyzed using the bootstrap procedure (Thompson et al., 1994). The neighbor-joining tree and the maximum parsimony trees do not differ, although the bootstrap values do differ.

Image Analysis and Graphic Representation

Specific ^{125}I-Mel binding was determined by subtracting absorbances from images containing nonspecific binding (100 pM ^{125}I-Mel plus 1 μM melatonin) from those from images containing total binding (100 pM ^{125}I-Mel only) within each structure. Structures that exhibited consistently positive values in 5–10 measurements were deemed to contain specific binding (Cassone et al., 1995). In situ hybridization data were analyzed similarly, but in this case images from hybridizations of sense cRNA probes were subtracted from antisense probes. Structures that consistently exhibited positive values were deemed to contain specific signals.

Images from representative levels of the brain in adjacent histological sections were acquired through a video camera, saved, and converted to Macintosh format TIFF files. These images were then imported into the NIH Image 1.52 program as described (Cassone et al., 1995). Composite figures and pseudocolor representations were produced using this program. Alphanumerics on the figures were produced using Canvas 3.5, and figures were printed on a Tektronix Phaser II dye sublimation printer.

Drugs

^{125}I-Mel was purchased from New England Nuclear. All drugs used in competition studies were purchased from Sigma or Research Biochemicals. PTX was purchased from List. All other chemicals were purchased from Sigma.

Acknowledgments

We thank Matthew Clausen, Jian Huang, and John Lee for expert assistance, Alfred Roca for help with some experiments, Fiona Watson for help with the PCR-generated melatonin receptor fragments, and Wolfgang Driever for providing zebrafish genomic DNA. This work was supported by National Institutes of Health grant DK42125 and a Sponsored Research Agreement from Bristol-Myers Squibb to S. M. R.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *"advertisement"* in accordance with 18 USC Section 1734 solely to indicate this fact.

Received June 14, 1995; revised July 19, 1995.

References

Baldwin, J.M. (1993). The probable arrangement of the helices in G protein-coupled receptors. EMBO J. *12*, 1693–1703.

Cassone, V.M. (1988). Circadian variation in 2-deoxyglucose uptake within the hypothalamic suprachiasmatic region of the house sparrow, *Passer domesticus*. Brain Res. *459*, 178–182.

Cassone, V.M. (1990). Effects of melatonin on vertebrate circadian systems. Trends Neurosci. *13*, 457–464.

Cassone, V.M., and Brooks, D.S. (1991). The sites of melatonin action in the brain of the house sparrow, *Passer domesticus*. J. Exp. Zool. *260*, 302–309.

Cassone, V.M., and Moore, R.Y. (1987). Retinohypothalamic projection and suprachiasmatic nucleus of the house sparrow, *Passer domesticus*. J. Comp. Neurol. *266*, 171–182.

Cassone, V.M., Brooks, D.S., and Kelm, T.A. (1995). Comparative distribution of 2[^{125}I]iodomelatonin binding in the brains of diurnal birds: outgroup analysis with turtles. Brain Behav. Evol. *45*, 241–256.

Cullen, B.R. (1987). Use of eukaryotic expression technology in the functional analysis of cloned genes. Meth. Enzymol. *152*, 684–704.

Ebisawa, T., Karne, S., Lerner, M.R., and Reppert, S.M. (1994). Expression cloning of a high-affinity melatonin receptor from *Xenopus* dermal melanophores. Proc. Natl. Acad. Sci. USA *91*, 6133–6137.

Ehrlich, D., and Mark, R. (1984). An atlas of the primary visual projections in the brain of the chick, *Gallus gallus*. J. Comp. Neurol. *223*, 592–610.

Engelage, J., and Bischof, H.J. (1993). The organization of the tectofugal pathway of birds: a comparative review. In Vision, Brain and Behavior in Birds, H. P. Zeigler and H. J. Bischof, eds. (Cambridge, Massachusetts: MIT Press), pp. 137–158.

Gunturkun, O., Miceli, D., and Wasanabe, M. (1993). Anatomy of the avian thalamofugal pathway. In Vision, Brain and Behavior in Birds, H. P. Zeigler and H. J. Bischof, eds. (Cambridge, Massachusetts: MIT Press), pp. 137–158.

Iuvone, P.M., and Gan, J. (1994). Melatonin receptor-mediated inhibition of cyclic AMP accumulation in chick retinal cell cultures. J. Neurochem. *63*, 118–124.

Juss, R.S., Davies, I.R., Follet, B.K., and Mason, R. (1994). Circadian rhythm in neuronal discharge activity in the quail lateral hypothalamic retinorecipient nucleus (LHRN) recorded *in vitro*. J. Physiol. *475*, 132.

Klein, D.C., Moore, R.Y., and Reppert, S.M., eds. (1991). Suprachiasmatic Nucleus: The Mind's Clock (New York: Oxford Press).

Laitinen, J.T., and Saavedra, J.M. (1990). The chick retinal melatonin receptor revisited: localization and modulation of agonist binding with guanine nucleotides. Brain Res. *528*, 349–352.

Lu, J., and Cassone, V.M. (1993a). Pineal regulation of circadian rhythms of 2-deoxy[^{14}C]glucose and 2-[^{125}I]iodomelatonin binding in the visual system of the house sparrow, *Passer domesticus*. J. Comp. Physiol. (A) *173*, 765–774.

Lu, J., and Cassone, V.M. (1993b). Daily melatonin administration synchronizes circadian patterns of brain metabolism and behavior in pinealectomized house sparrows, *Passer domesticus*. J. Comp. Physiol. (A) *173*, 775–782.

Munson, P.L., and Rodbard, D. (1980). LIGAND: a versatile computerized approach for characterization of ligand-binding systems. Anal. Biochem. *107*, 220–239.

Reppert, S.M., Weaver, D.R., Stehle, J.H., and Rivkees, S.A. (1991). Molecular cloning and characterization of a rat A_1-adenosine receptor that is widely expressed in brain and spinal cord. Mol. Endocrinol. *5*, 1037–1048.

Reppert, S.M., Weaver, D.R., and Ebisawa, T. (1994). Cloning and characterization of a mammalian melatonin receptor that mediates

reproductive and circadian responses. Neuron *13*, 1177–1185.

Reppert, S.M., Godson, C., Mahle, C.D., Weaver, D.R., Slaugenhaupt, S.A., and Gusella, J.F. (1995). Molecular characterization of a second melatonin receptor expressed in human retina and brain: the Mel_{1b} melatonin receptor. Proc. Natl. Acad. Sci. USA *92*, 8734–8738.

Rivkees, S.A., Cassone, V.M., Weaver, D.R., and Reppert, S.M. (1989). Melatonin receptors in avian brain: characterization and localization. Endocrinology *125*, 363–368.

Saitou, N., and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. *4*, 406–425.

Sanger, F., Nicklen, S., and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA *74*, 5463–5467.

Siuciak, J.A., Krause, D.N., and Dubocovich, M.L. (1991). Quantitative pharmacological analysis of 2-^{125}I-iodomelatonin binding sites in discrete areas of the chicken brain. J. Neurosci. *11*, 2855–2864.

Stehle, J. (1990). Melatonin binding sites in brain of the two-day-old chicken: an autoradiographic localisation. J. Neural Transmiss. *81*, 83–89.

Swofford, D.L. (1991). PAUP: Phylogenetic Analaysis Using Parsimony, Version 3.0s+3 (Champaign, Illinois: Illinois Natural History Survey).

Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. *22*, 4673–4680.

Weaver, D.R., Rivkees, S.A., Carlson, L.L., and Reppert, S.M. (1991). Localization of melatonin receptors in mammalian brain. In Suprachiasmatic Nucleus: The Mind's Clock, D. C. Klein, R. Y. Moore, and S. M. Reppert, eds. (New York: Oxford Press), pp. 289–308.

GenBank Accession Number

The GenBank accession numbers for the CKA and CKB sequences reported here are U31820 and U31821, respectively.