Molecular and functional characterization of a partial cDNA encoding a novel chicken brain melatonin receptor**

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Abstract **An approach based on homology probing was used to clone a partial cDNA encoding a novel melatonin (ML) receptor (MLR) from chicken (*Gallus domesticus*) brain. Based on available deduced amino-acid sequence, the chicken MLR (cMLR) displayed greater sequence homology to the frog (*Xenopus*) MLR than cloned human/mammalian receptors, with overall identities of 73% and 66%, respectively. In order to gain functional expression, a chimeric frog/chicken (*flc*)MLR was constructed in which the 5′ end of the *c*MLR, including the N-terminus, TM1 and part of the first intracellular loop was substituted by *f*MLR sequence. [^{125}I]Iodo-ML bound with high affinity (K_d of ~35 pM) to COS-7 cells transiently expressing the *flc*MLR in a saturable and guanine nucleotide-sensitive manner with the following rank order of potency: 2-iodo-ML > ML > 6-Cl-ML > S20750 > 6-OH-ML > S20642 > S20753 > *N*-acetyl-5HT >> 5-HT. Estimated K_i values for these compounds at the *flc*MLR correlated well to those obtained in native chicken brain membranes. In line with the observed structural similarity to the *f*MLR, the *flc*MLR exhibited affinities for ML, 6-Cl-ML and 6-OH-ML ~10-fold lower than mammalian receptors. Functionally, opposing interactions between ML and dopamine receptor signal transduction pathways were observed with ML potently inhibiting dopamine D1A-receptor-mediated cAMP accumulation in cells (HEK-293) transiently co-expressing these receptors. *c*MLR mRNAs were found expressed in chicken brain and kidney with trace levels observed in the lung. The availability of cloned vertebrate MLRs distinct at both the amino acid and pharmacological level from their mammalian counterparts may now allow for the identification of those amino-acid residues and structural motifs that regulate ML-binding specificity and affinity.**

Key words: G-protein coupled; cAMP inhibition; Dopamine D1 receptor

1. Introduction

Melatonin (ML), the primary hormone of the pineal gland, is thought to mediate many of its physiological and behavioral process via neural specific mechanisms. Thus, ML influences the regulation of circadian rhythms, functioning of the neuroendocrine-reproductive axis, visual function, activity levels and sleep in both birds and mammals (for review, see [1–6]). The primary sites of action for these ML-mediated events are receptors located in the suprachiasmatic nuclei and pars tuberalis, the retina and paraventricular nucleus of the thalamus [7–12]. Based on pharmacological and biochemical criteria two ML receptors (MLRs), termed ML_1 and ML_2, have been identified [2,13]. ML_1 receptors are defined by their ability to bind 2-[^{125}I]iodo-ML and various analogs with high affinity (pM) and to inhibit adenylyl cyclase activity [9,14–19]. In contrast, ML_2 receptors display nanomolar affinity for iodo-ML, exhibit a pharmacological profile for ML distinct from the ML_1 receptor and appear to activate second messenger systems other than adenylate cyclase [20].

MLRs have been extensively characterized in *G. domesticus* due to the relatively high density and widespread distribution in both the brain and periphery [8,21–23]. In the retina, ML inhibits dopamine synthesis and release [24] with a pharmacological profile and rank order of potency corresponding to the high-affinity ML_1 receptor [25] found in either the rabbit retina or other neuronal and peripheral tissues from the same or different species ([26,27] and see [2,13]). Moreover, in the retina, many opposing physiological and regulatory actions between dopamine and ML have been documented (see [28] and references therein). These interactions do not appear restricted to the retina, however, but may be evidenced as well in the hypothalamus and the pituitary gland where dopamine and ML regulate hormone release [29,30]. Although the molecular mechanism(s) by which these putative opposing interactions occur are still unknown, possible cross-talk between ML and dopamine D1 receptor-mediated signal transduction events has been suggested [28].

Recently, high-affinity ML_1 receptor cDNAs were isolated and characterized from *Xenopus laevis* dermal melanophores [31] the pars tuberalis of sheep and human hypothalamus [32]. As with multiple dopamine receptors [33,34], MLRs are members of a superfamily of genes encoding G-linked receptors and based on their deduced amino-acid sequence and conservation of structural motifs appear to form a new subclass within this family.

As a step towards examining the molecular mechanisms by which these receptors transduce ML's numerous physiological

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effects and its regulation of dopaminergic signal cascades in both the brain and periphery, we report here on the molecular characterization of a chicken brain partial cDNA appearing to encode a novel MLR.

2. Materials and methods

2.1. Cloning of partial cMLR

A chicken brain cDNA library (Clontech) was screened with a ^{32}P-labeled cDNA fragment (~444 bases) encoding putative transmembrane (TM) domains TM4–7 of the *Xenopus* brain MLR obtained by RT-PCR. ~8×10^5 independent clones were screened under reduced stringency conditions as previously described [35]. Briefly, duplicate nylon filters (Dupont/NEN) were hybridized overnight at 42°C in a solution containing 40% formamide, 50 mM Tris-HCl, pH 7.5, 0.1% sodium pyrophosphate, 0.2% BSA, 0.2% polyvinylpyrolidine, 0.2% Ficoll 400,000, 1% SDS, 0.1% NaCl, 0.1 mg/ml sheared salmon-sperm DNA and a nick-translated (Amersham) ^{32}P-labeled *Xenopus* probe (1.5×10^6 cpm/ml). Filters were washed 2× in 2×SSC/1% SDS for 20 min at 55°C and exposed to autoradiography. One positive clone (~3 kb) was isolated and following tertiary screening was analysed by restriction mapping and Southern blot analysis. A strongly hybridizing 1-kb *Pst*I fragment was subcloned into pSP73 (Promega), sequenced in both directions using the Sanger dideoxy-chain termination method with 7-deaza-dGTP and Sequenase V 2.0 (USB) and with either specific internal (Biotechnology Service Center HSC, Toronto, Canada) or SP6/T7 primers.

2.2. Construction of chimeric frog/chicken MLR

In order to obtain functional receptor activity, we adopted a three primer PCR fusion strategy [36] to construct a cMLR chimeric receptor containing the 5′ region of the cloned *Xenopus* MLR [31]. Briefly, a region within TM2 (a.a...ADLVVA...) of both the frog and chicken cDNAs that displays 83% nucleotide sequence identity was chosen to design two complementary primers (chicken primer A: 5′-GCC GAT CTG GTG GTG GCC TTG TAT-3′ and primer B: 3′-AGA TAA CGG CTA GAC CAC CAC CGG-5′ for *Xenopus*). To amplify the 5′ region of the *Xenopus* MLR, cDNA in pcDNA1 (obtained from Dr. S.M. Reppert) was subjected to PCR using synthetic primer B and the vector primer T7. The entire coding sequence of the cMLR cDNA (encompassing TM2 to 3′-untranslated region) was subjected to PCR using primer A and a primer encoding 3′-untranslated sequence (primer C: 5′-AAC ATT CGA ACT CTA TCA-3′). ~1 μg DNA and 1 μg of each appropriate primer were submitted to 30 cycles of the PCR (1 min at 94°C, 1.5 min at 58°C and 1.5 min at 72°C; Perkin-Elmer/Cetus) with 2.5 U *Taq* polymerase. Amplified DNA (frog ~200 bp and ~900 bp for cMLR) from both reactions were recovered from a 0.8% low-melt agarose gel, purified through GlassMAX (Gibco/BRC) spin columns, mixed and subjected to a second round of PCR using only T7 and C oligonucleotides as primers under conditions described above. An amplified ~1.2-kb product was subcloned into pBluescript SK$^-$. To confirm appropriate splice fusion and the absence of spurious PCR-generated nucleotide errors, the entire chimeric *Xenopus*/*G. domesticus* amplified construct (termed *flc*MLR) was resequenced as described above.

2.3. Cell transfection and ligand-binding analysis

For transient expression studies, the chimeric *flc*MLR was subcloned into the expression vector pCDNA3 (Promega). COS-7 cells were transfected with cesium chloride-purified DNA constructs by electroporation (80 μg DNA/2.5×10^7 cells; 48 Ω, 135 mA, 500 μF), placed into 150-mm plates and cultured for 4–5 days as previously described [37]. COS-7 cells were maintained in Dulbecco's alpha-modified Eagle's medium supplemented with 10% fetal calf serum at 37°C and 5% CO_2. The cells were collected and pelleted at 1000 rpm for 10 min at 4°C and stored at −80°C until use.

[^{125}I]Iodo-ML (2000 Ci/mmol) was prepared as previously described [22]. Cells were thawed, washed and resuspended in 50 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.4, at a concentration of ~300μg protein/ml and incubated (50 μl) with increasing concentrations of [^{125}I]iodo-ML (2–200 pM), in a total reaction vol. of 200 ml for 90 min at room temperature under constant shaking. For competition experiments, cells were

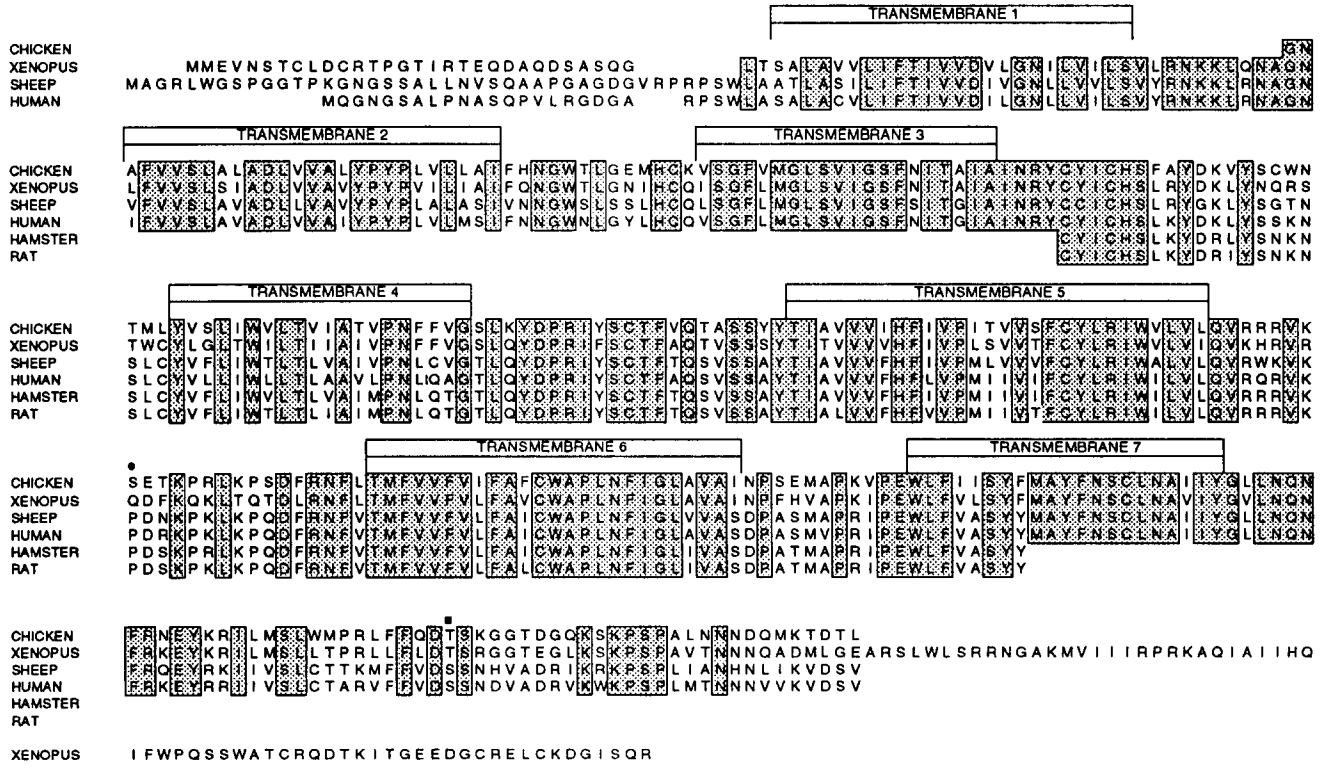

Fig. 1. Deduced amino-acid sequence alignment of the avian MLR and other cloned members of the mammalian and vertebrate MLR family. Boxed and shaded areas denote amino-acid residues conserved between the avian MLR and its vertebrate/mammalian counterparts [31,32]. Putative TM domains are demarcated by boxed regions. Potential phosphorylation sites for protein kinase A (cAMP-dependent) and protein kinase C are indicated by closed circles (●) and closed (■) squares, respectively. Single-letter amino-acid code used.

incubated with 30–40 pM [^{125}I]iodo-ML in the presence of varying concentrations (10^{-12}–10^{-5}M) of competing agents or guanosine-5′-*O*-(3′-thiotriphosphate) (GTPγS) and assayed for ligand-binding activity. Non-specific binding was defined in the presence of 1 μM ML. Assays were terminated by the addition of 3 ml Tris-HCl buffer (4°C) and immediate vacuum filtration through Whatman GF/B filters using a M48R cell harvester. Filters were washed 2 × with 3 ml cold buffer and bound radioactivity measured by a γ-counter at 74% efficiency. In order to directly compare [^{125}I]iodo-ML-binding characteristics of the cloned *flc*MLR expressed in COS-7 cells with native chicken brain MLRs, we assayed both receptor activities in parallel. Brains from *G. domesticus* were homogenized in ice-cold buffer [22] and assayed for MLR-binding activity as described above. Protein concentrations were determined as previously described [22]. Ligand-binding data were analysed by LIGAND or Kaleidagraph (Abelbeck Software) as previously described [35,37].

2.4. cAMP accumulation

HEK-293 cells were transiently co-transfected with *flc*MLR cDNA and the recently cloned chicken dopamine D1A receptor [38] using calcium phosphate precipitation as described [39], placed in 24-well plates and grown for 4 days in Dulbecco's modified Eagle's medium supplemented with 10% FBS [Gibco]. Cells were washed with 0.5 ml prewarmed α-MEM containing 0.5 mM 3-isobutyl-1-methylxanthine and 1 μM propranolol and co-incubated with varying concentrations of ML (10 pM–100 nM) and forskolin (1 μM) or the dopamine D1 selective agonist, SKF-82526 (1 μM) for 15 min at 37°C. Reactions were terminated by the addition of 500 μl 0.2 N HCl, cellular debris pelleted by centrifugation at 500 × *g* and supernatants (12.5 μl) assayed for cAMP formation by RIA (Amersham) as previously described [35]. Mock-transfected (plasmid alone) or untransfected cells did not display endogenous binding or adenylate cyclase activity (data not shown).

2.5. RT-PCR analysis of cMLR mRNA

Total RNA was isolated from chicken brain optic tectum, cerebellum, kidney and lung tissue using TRISOLV (Biotecx Laboratories, Houston, TX) as previously described [35]. ~1 μg total RNA from each tissue was subjected to first-strand cDNA synthesis with 25 pmol of oligo(dT) (Perkin-Elmer/Cetus) and 200 U Superscript reverse transcriptase (Life Technologies) for 30 min at 42°C. The RNA template was removed by the addition of 5 U RNase H for 10 min at 55°C. Single-stranded cDNA samples were purified through GlassMAX spin columns (Life Technologies) and subjected to 30 cycles of PCR amplification (94°C, 1 min; 58°C, 1.5 min; 72°C, 1.5 min) using 0.5 μg of a cMR-specific internal oligonucleotide primer (5′-GAA ATG GCA CCA AAA GTT-3′) encoding sequences within the fourth extracellular loop (MAPKV) and one primer in the 3′-untranslated region (5′-TGA TAG AGT TCG AAT GTT-3′) and 2 U *Taq* DNA polymerase (Perkin-Elmer/Cetus). A DNase control group in which reverse transcriptase was not added was processed in parallel. Amplified products were electrophoresed in a 1% agarose gel, transferred to nylon membranes and probed with γ[^{32}P] end-labeled oligonucleotide internal to the flanking primers (5′-CCT TAA TTC AAT AAT ATA-3′). Blots were washed with 2 × SSC, 1% SDS for 15 min at room temperature followed by a 15-min wash at 42°C and exposed for autoradiography overnight at −70°C.

3. Results and discussion

One hybridizing clone (~3 kb) was isolated from a chicken brain cDNA library and found, after nucleotide sequence analysis, to contain a long open reading frame of 869 nucleotides, displaying marked deduced amino-acid sequence homology to cloned members of the G-protein-linked MLR family [31,32]. The clone, however, did not contain the entire 5′ end of the

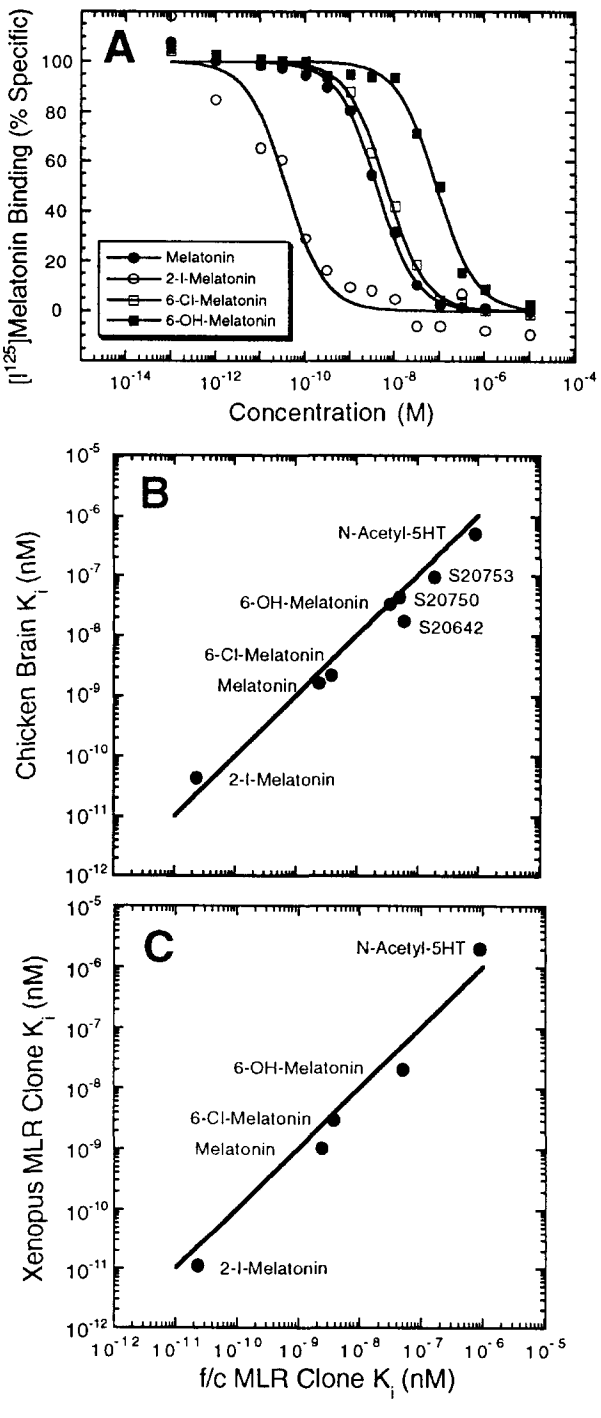

Fig. 2. Pharmacological specificity of [^{125}I]iodo-ML-binding to COS-7 cells expressing the chimeric avian *flc*MLR. COS-7 cells were transfected with a cDNAs encoding the chimeric *flc*MLR and assayed for receptor activity as described in section 2. (A) Representative curves are illustrated for the concentration-dependent inhibition of [^{125}I]iodo-ML-binding (20–40 pM) to expressed *flc*MLRs (~25–40 fmol/mg protein) by ML agonists and other agents as listed. Estimated inhibitory constants (K_i) for these compounds, included in Table 1, were determined by LIGAND or KALEIDAGRAPH and are representative of at least two independent experiments each conducted in duplicate and which varied by <10%. Pharmacological homology between the *flc*MLR and (B) the native chicken brain and (C) cloned *Xenopus* MLR. Correlational plots of estimated inhibitory constants (K_i) of various ML agonists and agents to inhibit [^{125}I]iodo-ML-binding to the cloned *flc*MLR expressed in COS-7 cells with that of chicken brain membranes and of the *Xenopus* MLR expressed in COS-7 cells. The line of identity or equimolarity is indicated. K_i values for *Xenopus* receptors were taken from [31].

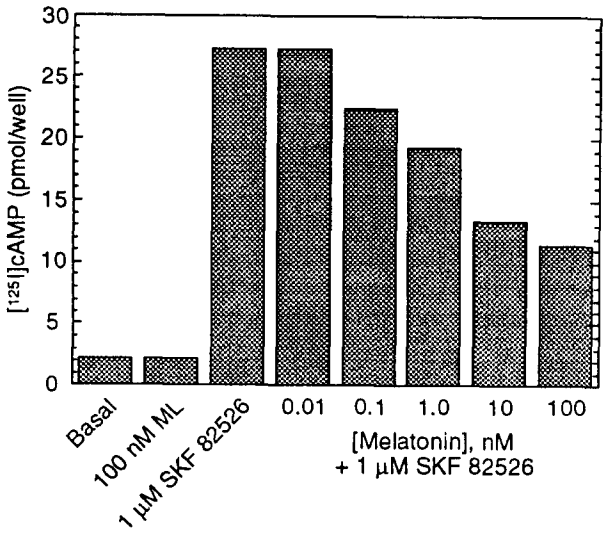

Fig. 3. cAMP accumulation following avian *flc*MLR stimulation. HEK-293 cells transiently co-expressing the *G. domesticus* dopamine D1A and the *flc*MLR at a ratio of ~3:1 were assayed for cAMP accumulation as described in section 2. Following treatment with 1 μM SKF-82526, avian D1A receptor stimulates cAMP production ~20-fold above basal levels. Dopamine D1 receptor stimulation of cAMP accumulation was inhibted, in a concentration-dependent manner by ML with maximal inhibtion (~60%) observed at 100 nM. Results shown are representative of at least three independent experiments each conducted in duplicate. HEK-293 cells transfected with either dopamine D1A or cMLRs alone did not show this interaction. In contrast to the effect of ML, dopamine D1A-mediated stimulation of adenylate cyclase is inhibited by the selective D1 receptor antagonist, SCH-23390 (1 μM) to basal levels (~4 pmol/well).

receptor but contained sequence information encoding the first cytoplasmic loop (beginning at residues GN) to the 3′ end of the coding region (Fig. 1). Repeated attempts, using a variety of techniques, to obtain the 5′ coding region of the cMLR cDNA were unsuccessful (data not shown). The 5′ end of the cMLR cDNA, including the N-terminus and TM1, appears to have been interrupted by intronic sequences (>2 kb) at a position identical with that reported for mammalian MLRs [32].

As depicted in Fig. 1, overall amino-acid sequence identities of the cMLR were 73% identical with that of the *Xenopus* MLR and 67% identical with sheep and human MLRs. Within putative TM domains, amino-acid sequence identities were considerable higher with the cMLR displaying 81 and 80% identity to *Xenopus* and mammalian receptors, respectively. Consistent with the contention that MLRs may represent a distinct subclass of the G-protein-linked receptor family is the conservation of the NRY sequence motif after TM3 and the NAXIY motif found in TM7. Also conserved are consensus sequences for phosphorylation by protein kinase A and protein kinase C within the third intracellular loop and C-terminal tail, respectively, regions thought to be involved in receptor desensitization and regulation [40]. Areas of significant sequence divergence are restricted to putative intra- and extracellular loops and the C-terminal tail. Analysis of deduced amino-acid sequence homologies of these regions of the cMLR reveals identities of 65 and 56% to *Xenopus* and mammalian receptors, respectively. Despite the strong observed sequence homology, the cMLR C-terminus ends at a position identical with those of mammalian receptors and is 65 amino-acid residues shorter than the *Xenopus* receptor.

In order to gain functional expression, we adopted a three primer PCR fusion strategy to construct a chimeric cMLR. Thus, by fusing the N-terminal, TM1 and part of the first intracellular loop of the *Xenopus* MLR (amino-acid residues 1–79) to the cMLR, a full-length MLR cDNA, termed *flc*MLR, was made. We chose to fuse the 5′ end of the *Xenopus*, rather than the cloned human MLR, based on the observed greater nucleotide and deduced amino-acid sequence homology displayed by the cMLR with the *Xenopus* receptor relative to its mammalian counterparts (see above). The entire *flc*MLR was then resequenced and found to contain no PCR-generated nucleotide substitutions/additions/deletions.

We compared the ability of the *flc*MLR to mimic native neuronal avian cMLRs, in terms of its exhibited pharmacological profile and ligand-binding specificity. COS-7 cells transiently expressing the chimeric *flc*MLR bound [^{125}I]iodo-ML (2–200 pM) in a concentration-dependent and saturable manner to a single class of binding site with an estimated K_d of 35 ± 6 pM and B_{max} value, ranging from 20 to 90 fmol/mg protein, respectively. Binding of [^{125}I]iodo-ML (35 pM) to COS-7 cells expressing the *flc*MLR was reduced by ~70% following the addition of 200 μM GTPγS (control: 47 ± 8; GTPgS treated: 16 ± 2 fmol/mg protein), suggesting that expressed receptors are functionally coupled to endogenous subtype-specific G-proteins. Virtually identical results were obtained in native membranes prepared from avian brain (data not shown). Moreover, as depicted in Fig. 2A, [^{125}I]iodo-ML-binding to COS-7 cells expressing the *flc*MLR was inhibited in a concentration-dependent and uniphasic manner (as indexed by Hill coefficients close to unity) by a variety of melatonergic agonists and agents with a rank order of potency and pharmacological profile clearly consistent with a neuronal ML-like receptor. Thus, of all the compounds tested iodo-ML was the most potent inhibitor followed by 6-Cl-ML > S20750 (*N*-[2-(7-methoxy-1-naphthyl)ethyl]crotonamide) > 6-OH-ML > S20642 (*N*-[2-(7-methoxy-1-naphthyl)ethyl)cyclobutane) > S20753 (*N*-(propyl-[2-(7-methoxy-1-naphthyl)ethyl]urea) > *N*-acetyl-serotonin >> 5-HT. Estimated K_i values for these agents are listed in Table 1. Moreover, as illustrated in Fig. 2B, the estimated

Table 1
Inhibitory constant (K_i) values of various compounds for [^{125}I]iodo-ML-binding to the cloned chicken *flc* MLR expressed in COS-7 cells or to native brain membranes

Compound	Cloned	Native	Cloned/Native
	K_i (nM)		
2-iodo-ML	0.023	0.033	0.7
ML	2.4	1.6	1.5
6-chloromelatonin	3.8	2.2	1.7
S-20750	35	17	2.0
6-hydroxymelatonin	49	42	1.2
S-20642	58	33	1.7
S-20753	184	93	1.9
N-Acetyl serotonin	872	500	1.7
Serotonin	>10,000	>10,000	1.0

Inhibitory constants (K_i) of various melatonin agonists and agents for ([^{125}I]iodo-binding to either COS-7 cells tranfected with the avian *flc* MLR cDNA or to chicken brain membranes are listed in order of their potency for the cloned receptor. Values represent the means of at least two independent experiments each conducted in duplicate with estimated K_i values determined by LIGAND and which varied < 10%.

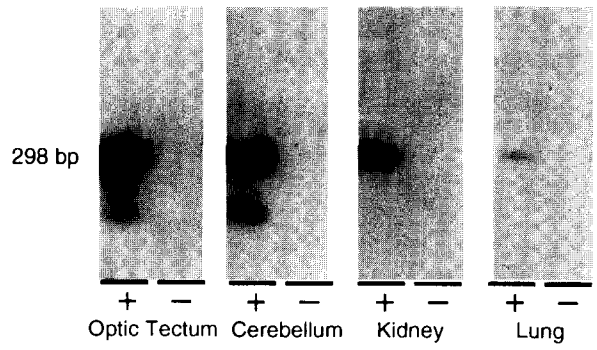

Fig. 4. Tissue-specific distribution of avian MLR mRNA. Samples (1 μg) of *G. domesticus* brain, kidney and lung total mRNA were subjected to reverse transcriptase using oligo(dT) followed by PCR using two additional oligonucleotides as described in section 2. Amplified cDNA products were Southern-blotted and probed with a ^{32}P-labeled oligonucleoide internal to the PCR fragment. MLR transcripts of the appropriate size were evident in chicken forebrain, cerebellum and kidney with trace amounts evident in lung. DNase controls with no reverse transcriptase (−) are located to the right of each hybridizing PCR product. Size of amplified and radiolabeled PCR products (bp) are shown. Blots were subjected to autoradiography for ~12 h.

inhibitory constants (K_i) obtained for the inhibition of [^{125}I]iodo-ML-binding by a series of compounds at the native avian MLR correlate strongly ($r = 0.989$) with K_i values obtained for these agents at the cloned chimeric *flc*MLR with a virtual one to one correspondence in estimated receptor affinity. These data clearly suggest that the addition of the 5′ coding sequence of the *Xenopus* MLR does not appreciable alter the ligand-binding characteristics of the cloned chicken brain MLR.

There are some unique pharmacological characteristics that appear to differentiate vertebrate (frog/chicken) MLRs from their mammalian homologs (sheep/human). Thus, while all cloned MLRs, when expressed in COS-7 cells, display identical affinities for iodo-ML, both the human and sheep MLRs [32] exhibit the inherent ability to bind ML, 6-hydroxy-ML and *N*-acetyl serotonin with higher affinity than either cloned frog [31] or chimeric *flc*MLRs. As depicted in Fig. 2C, and consistent with our proposed classification of these receptors based on amino-acid sequence homologies listed in Fig. 1, of all the agents tested each exhibited affinities for the *Xenopus* MLR that were virtually identical with those obtained from the expressed chimeric *G. domesticus* MLR. In marked contrast, direct comparison of the estimated K_i values of numerous agents at the *flc*MLR with sheep or human receptors [32], although correlative, clearly reveals numerous compounds displaying from 5–10-fold higher affinity for mammalian receptors. Given the lack of strong observed sequence and pharmaclogical homology between the cloned vertebrate and mammalian MLRs, we suggest that the cloned chicken and frog MLRs are not merely species orthologues of mammalian receptors. Indeed, we have isolated a human genomic clone [Liu, F., Brown, G.M and Niznik, H.B. unpubl. data; also see Genbank Accession U25341 (Reppert, S.M. et al.)] which upon sequence analysis displays higher deduced amino-acid sequence homology to the cloned chicken and frog MLRs than those of mammalian origin [32].

In order to assess the ability of the *flc*MLR to functionally inhibit adenylate cyclase activity, we co-transfected HEK-293 cells with both the *flc*MLR and the recently cloned *G. domesticus* dopamine D1A receptor which stimulates the activity of adenylate cyclase in response to selective D1 agonists [38]. As summarized in Fig. 3, in cells co-expressing these receptors the potent D1 receptor agonist SKF-82526 (1 μM) stimulated cAMP accumulation ~20-fold over basal levels. Co-incubation of cells with increasing concentrations of ML inhibited dopamine D1-stimulated cAMP accumulation in these cells. At a concentration of 10–100 nM ML an ~60% decrease in dopamine D1 receptor-mediated adenylate cyclase activity was observed. No endogenous MLR activity was observed in cells only expressing D1 receptors and while 1 μM forskolin stimulated the production of cAMP ~100-fold over basal levels the addition of 100 nM ML resulted in an ~30% decrease of forskolin stimulated cAMP accumulation in HEK-293 cells transiently expressing the cloned *flc*MLR (data not shown). Although it is unclear whether ML- and dopamine D1-like receptors co-exist in the same neurons, evidence for functional ML and dopamine D1 receptor cross-talk regulating the activity of adenylate cyclase in cultured retinal cells has recently been documented [19,28]. The data on cloned receptors provide direct molecular evidence for the existence of functional ML-dopamine receptor interactions at the level of the single cell. The exact subtype-specific G-protein α (Gi/Go) or βγ subunits by which MLR activation inhibits D1 receptor-mediated cAMP accumulation or whether other cloned members of the D1-like receptor family [35,38,41], particularly the dopamine D5 receptor [39,42], are differentially and functionally regulated by specific MLRs is currently unknown. The use of knock out mice for either subtype-specific dopamine receptors [43] or MLRs, when they become available, may help to identity the functional physiological and developmental interactions between these two systems.

As depicted in Fig. 4, RT-PCR analysis of MLR mRNA indicates expression in both chicken brain and peripheral tissues. Southern blots of RT-PCR-amplified products reveals strongly hybridizing bands in chicken forebrain and cerebellum. Similar levels of expression were seen in chicken kidney. In contrast, the chicken lung revealed trace amounts of MLR mRNA. The mRNA distribution patterns of the cloned cMLR are in line with the known distribution patterns of native receptors in avian brain and peripheral tissues, as determined by both ligand-binding and receptor autoradiography [23,26,27,44]. In situ hybridization analysis will be necessary to clearly define the tissue-specific and cellular distribution profile of MLR mRNAs in this species.

We provide molecular evidence for the existence of a chicken brain MLR variant exhibiting amino-acid sequence and pharmacological profiles distinct from mammalian receptor counterparts. Moreover, we show that the chimeric chicken MLR functionally inhibits second messenger dopamine D1 receptor-mediated responses. The availability of species- and receptor-specific MLR cDNAs may provide a more practical approach by which to identify sequence-specific motifs that underlie the expression of unique high-affinity MLR characteristics. Given the degree of exhibited sequence homology among members of the MLR family, observed species- and receptor-specific pharmacological profiles would suggest that changes in key amino-acid residues may translate into major shifts in potency and substrate specificity. As such, the construction of interspecies receptor chimeras may aid in the identification of those regions

involved in the unique pharmacological specificity of MLRs and may ultimately provide the molecular basis for the rational design of therapeutic compounds acting at these receptors for the treatment of various ML-induced endocrine and neuropsychiatric diseased states [5,6].

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