Quantitative Pharmacological Analysis of 2-¹²⁵I-Iodomelatonin Binding Sites in Discrete Areas of the Chicken Brain

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We have localized and characterized 2-¹²⁵I-iodomelatonin binding sites in the chicken brain using *in vitro* quantitative autoradiography. Binding sites were widely distributed throughout the chicken brain, predominantly in regions associated with the visual system. The specific binding of 2-¹²⁵I-iodomelatonin to discrete chicken brain areas was found to be saturable, reversible, and of high affinity. The specific binding of 2-¹²⁵I-iodomelatonin (75 pM) was quantitated for 40 identifiable brain regions. Eight brain regions were chosen for binding characterization and pharmacological analysis: optic tectum, Edinger–Westphal nucleus, oculomotor nucleus, nucleus rotundus, ventral supraoptic decussation, ventrolateral geniculate nucleus, neostriatum, and ectostriatum. These regions showed no rostral-caudal gradient in 2-¹²⁵I-iodomelatonin specific binding, and saturation analysis revealed a single class of high-affinity sites with K_D values in the range of 33–48 pM and receptor site density (B_{max}) ranging from 31 to 58 fmol/mg protein. Competition experiments carried out with various indoles revealed a similar order of pharmacological affinities in these areas: melatonin > 6-chloromelatonin > methoxyluzindole > *N*-acetylserotonin > luzindole >> 5-HT > 5-methoxytryptamine. The affinity constants determined by quantitative autoradiography for these compounds to compete for 2-¹²⁵I-iodomelatonin binding in the optic tectum correlated well with the affinities in chicken brain membranes at 25°C ($r = 0.966$; slope = 0.845; $n = 7$) and 0°C ($r = 0.946$; slope = 0.379; $n = 7$), chicken retinal membranes ($r = 0.973$; slope = 0.759; $n = 7$), and the potency or affinity of these compounds to affect the calcium-dependent release of ³H-dopamine from the rabbit retina ($r = 0.902$; slope = 0.506; $n = 6$). We conclude that the high-affinity sites labeled by 2-¹²⁵I-iodomelatonin in various chicken brain areas have identical binding and pharmacological characteristics to the ML-1 melatonin binding site previously described in chicken brain and retinal membranes and to

the ML-1 melatonin receptor modulating dopamine release from the retina. In the chicken brain, the ML-1 receptor site may mediate functional responses regulated by melatonin.

The avian pineal gland, through the secretion of its primary neurohormone melatonin (MEL; *N*-acetyl-5-methoxytryptamine), appears to be involved in the regulation of circadian locomotor rhythms (Gaston and Menaker, 1968; Menaker and Zimmermann, 1976; Turek et al., 1976; Ebihara and Kawamura, 1981; Simpson and Follet, 1981; Ebihara et al., 1984; Oshima et al., 1989), body temperature (Oshima et al., 1989), feeding behavior (Yamada et al., 1988), sleep-like behavior (Barchas et al., 1967), photoperiodic regulation of reproduction (Johnson and van Tichoven, 1984; Liou et al., 1987; Ohta et al., 1989), and 5-HT levels in different brain regions (Cassone et al., 1986). MEL levels in pineal, serum, brain, and retina fluctuate diurnally, with higher levels during the dark phase (Pang et al., 1974, 1977, 1983; Cassone and Menaker, 1984; Vakkuri et al., 1985). Furthermore, MEL is taken up from the circulation and concentrated in the brain, particularly within the hypothalamus, thalamus, and pons/midbrain, which may be major sites of action of this hormone (Pang et al., 1974; Ralph, 1981; Cassone and Menaker, 1984; Cassone et al., 1986).

Recently, 2-¹²⁵I-iodomelatonin has been used to study MEL receptor sites (Vakkuri et al., 1984; Dubocovich and Takahashi, 1987). Specific 2-¹²⁵I-iodomelatonin binding sites have been demonstrated in the chicken brain using both membrane preparations (Dubocovich et al., 1989, 1990; Rivkees et al., 1989) and autoradiographic localization (Rivkees et al., 1989; Siuciak and Dubocovich, 1989; Dubocovich et al., 1990; Stehle, 1990). In contrast to previous autoradiographic studies using rodents, where MEL binding sites were found to be localized in only a few discrete areas such as the suprachiasmatic nucleus (SCN), the thalamic paraventricular nucleus (PVN), and the median eminence/pars tuberalis (ME/PT) (Duncan et al., 1989; Weaver et al., 1989; Siuciak et al., 1990), binding was widely distributed throughout the entire chicken brain.

2-¹²⁵I-iodomelatonin has been shown to label two pharmacologically distinct sites, ML-1 and ML-2 (Dubocovich, 1988a). The ML-1 site was originally characterized in the chicken and rabbit retina (Dubocovich, 1983, 1985, 1988a; Dubocovich and Takahashi, 1987), where it functions to inhibit ³H-dopamine release (Dubocovich, 1983, 1985). Sites with similar pharmacological characteristics have since been found in the chicken brain (Dubocovich et al., 1989, 1990), rat SCN and median eminence (Vanecek et al., 1987; Laitinen and Saavedra, 1990; Laitinen et al., 1990), and mouse SCN and PVN (Fang et al.,

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Figure 1. Time course of 2-¹²⁵I-iodomelatonin binding in the OT from chicken brain slices using quantitative autoradiography. Coronal chicken brain sections, 20 μ m thick, through the optic lobes were incubated with 75 pM 2-¹²⁵I-iodomelatonin. 2-¹²⁵I-iodomelatonin binding (open circles) was brought to steady state (60 min) before the addition of 3 μ M MEL to all samples (solid circles). At the times indicated, slide-mounted sections were removed and washed as described in Materials and Methods. Values shown are from a representative experiment, with points representing the mean of triplicate determinations (three sections per slide).

1990). Lower-affinity melatonin binding sites (ML-2) have been found in hamster brain membranes (Niles et al., 1987; Duncan et al., 1988, 1989). The most striking pharmacological difference between these two binding sites is the equal potency of MEL and *N*-acetylserotonin (NAS) in competing for 2-¹²⁵I-iodomelatonin binding in hamster brain membranes (Duncan et al., 1988, 1989), in contrast to the ML-1 receptor, where MEL exhibits a much greater potency than NAS (Dubocovich 1983, 1985, 1988a; Dubocovich and Takahashi, 1987).

The aim of this study was to examine the distribution and the binding and pharmacological characteristics of 2-¹²⁵I-iodomelatonin binding sites in discrete areas of the adult chicken brain using quantitative autoradiography. In the chicken brain, 2-¹²⁵I-iodomelatonin labels a high-affinity site with the binding and pharmacological characteristics of the ML-1 MEL receptor previously described in chicken brain and retinal membranes. Knowledge of the distribution and pharmacological characteristics of 2-¹²⁵I-iodomelatonin binding sites in the chicken brain is important for understanding the effects of MEL on behavioral, reproductive, and neurochemical functions.

Materials and Methods

Chemicals and radioligand. 2-¹²⁵I-iodomelatonin was prepared using a modification of the method of Vakkuri et al. (1984). This radioligand had a high specific activity (1800–2000 Ci/mmol) and was stable for at least 60 d. Drugs were obtained from Sigma Chemical Company unless otherwise stated. 6-Chloromelatonin (CLM) was donated by Dr. J. A. Clemens (Eli Lilly Laboratories, Indianapolis, IN). Luzindole (LUZ; 2-benzyl-*N*-acetyltryptamine, N-0774) and 5-methoxyluzindole (MLUZ; N-0745) were donated by Dr. J. Peck [Whitby Research (formerly Nelson Research), Irvine, CA].

Autoradiography. Whole brains from 5–6 week-old male and female chickens (*Gallus domesticus*, white leghorn) were dissected and frozen in 2-methylbutane and stored at -70°C . Chickens were obtained from the slaughterhouse and killed by decapitation between 1100 and 1300 hr. Serial coronal brain sections (20 μ m) were cut on a cryostat (-15°C) and thaw mounted onto gelatin-coated slides. Three alternate sets of sections were generated for the determination of total binding, nonspecific binding, and histological staining. After air drying for 10 min, slides

were stored at -70°C until processing for no more than 1 week. Slide-mounted sections (three per slide) were allowed to air dry for 15 min, preincubated in 150 mM Tris-HCl buffer (pH 7.4, 22°C) for 1 hr to remove endogenous ligand, and then incubated with appropriate concentrations of 2-¹²⁵I-iodomelatonin (0.1 pM to 1 nM) in Tris-HCl buffer with (nonspecific binding) or without (total binding) 3 μ M melatonin for 1 hr, with the exception of kinetic studies, for which the incubation time varied. For competition studies, slides containing three adjacent serial sections were incubated with 75 pM 2-¹²⁵I-iodomelatonin in the absence or presence of various concentrations of drugs (1 pM to 1 mM). Slides were rinsed in ice-cold Tris-HCl buffer (3×5 min each) followed by a rapid rinse in ice-cold distilled water to remove buffer salts. Labeled sections were apposed to Kodak SB5 x-ray film for 3–7 d. The film was developed using Kodak D19 developer (4°C). Tissue sections were stained with thionin, and these were used in conjunction with the atlas of Kuenzel and Masson (1989).

Using a computer-based image analysis system (Imaging Resources Inc., St. Catharines, Ontario, Canada), the optical densities of autoradiograms were measured. ¹⁴C standards (ARC Inc.) were calibrated for use with ¹²⁵I using the method of Miller and Zahniser (1987). For each brain region, the mean density readings from three sections mounted on the same slide were converted into the amount of radioligand bound using the standard reference curve. The protein content in tissue sections was determined from tissue equivalents supplied by the manufacturer of the ¹⁴C standards. The value for nonspecific binding, defined as the binding of 2-¹²⁵I-iodomelatonin not displaced by 3 μ M melatonin, was subtracted from that for total binding to produce the value for specific binding in each region. K_i values from competition experiments were calculated using the method of Cheng and Prusoff (1973), and curve fitting was done using the GRAPHPAD program (The Institute for Scientific Information, Philadelphia, PA).

Results

Incubation conditions

The time course of binding of 75 pM 2-¹²⁵I-iodomelatonin to tissue sections is shown in Figure 1. The binding of 2-¹²⁵I-iodomelatonin was rapid and reversible. At 22°C , 45 min was required to reach equilibrium, and binding remained stable for up to 2 hr. The association rate constant (k_1) from the optic tectum determined from the pseudo-first-order equation was $33.9 \times 10^7 \text{ M}^{-1}$. Dissociation of the bound complex was initiated after 60 min of incubation with the addition of excess competing ligand (3 μ M MEL). The rate constant for dissociation (k_{-1}) from the optic tectum was 0.01439 min^{-1} . The kinetic dissociation constant (K_d) for 2-¹²⁵I-iodomelatonin in the optic tectum calculated from the ratio k_{-1}/k_1 was $41.06 \pm 3.92 \text{ pM}$ ($n = 4$).

Localization

The distribution of 2-¹²⁵I-iodomelatonin binding sites in the chicken brain is shown in Figure 2. 2-¹²⁵I-iodomelatonin binding sites were widely distributed throughout the chicken brain predominantly in regions associated with the visual system. A list of these areas and their abbreviations are shown in Table 1. The specific binding of 2-¹²⁵I-iodomelatonin (75 pM) in different brain areas showed an uneven distribution (Table 1). The highest density of 2-¹²⁵I-iodomelatonin binding sites was found in the OT, T, and R ($>30 \text{ fmol/mg protein}$) as well as in the DSV and the GLV ($>20 \text{ fmol/mg protein}$).

In order to assess whether 2-¹²⁵I-iodomelatonin binding in specific brain areas displayed any rostral-caudal gradient, two entire brains were sectioned and incubated with 75 pM 2-¹²⁵I-iodomelatonin. Figure 3 shows the rostral-caudal distribution of specific binding of 2-¹²⁵I-iodomelatonin throughout selected areas of the chicken brain, demonstrating that binding was uniform from one section to the next in the OT, R, DSV, EW, GLV, OM, NI, and E, areas that were used in further saturation and pharmacological experiments.

Figure 2. Autoradiograms of 2-¹²⁵I-iodomelatonin binding at five selected coronal levels of the chicken brain. A list of the areas and their abbreviations are given in Table 1. Total binding is shown in A–E and corresponds roughly to plates A 10.0 (A), A 8.8 (B), A 6.4 (C), A 5.0 (D), and A 3.4 (E) from the atlas of Kuenzel and Masson (1989). F shows a representative autoradiogram of nonspecific binding defined with 3 μ M MEL.

Saturation studies

Scatchard analysis of saturation binding data was carried out in the following areas: OT, EW, OM, R, DSV, GLV, NI, and E using concentrations of 2-¹²⁵I-iodomelatonin ranging from 0.1 to 1000 pM. Initial saturation experiments using radioligand concentrations extending from 12.5 to 1000 pM revealed only

one high-affinity site; therefore, future experiments were done using smaller concentration ranges. The K_D and B_{max} values for the various areas examined are shown in Table 2. A representative densitometric analysis of saturation binding from one experiment in the OT is shown in Figure 4. Specific binding of 2-¹²⁵I-iodomelatonin (0.1–200 pM) to chicken brain OT increased linearly with concentration of the radioligand from 0.1

Table 1. Distribution and densities of 2-¹²⁵I-iodomelatonin binding in the chicken brain

Brain area	Abbreviation	fmol/mg protein	(n)
Mesencephalic areas			
Optic tectum ^a	OT	37.56 ± 1.84	(22)
Edinger–Westphal nuclei ^b	EW	19.01 ± 1.87	(16)
Oculomotor nuclei ^b	OM	13.03 ± 0.81	(16)
Brachium superior colliculus ^b	BCS	12.58 ± 0.58	(10)
Basal optic root nuclei ^a	BOR	12.26 ± 1.01	(14)
Lentiform mesencephalic nucleus	LM	11.53 ± 1.01	(11)
Magnocellular isthmi nuclei ^b	IMC	10.53 ± 0.65	(14)
Parvocellular isthmi nuclei ^b	IPC	6.13 ± 0.41	(14)
Oculomotor nerve	OMN	6.04 ± 0.42	(12)
Trochlear nerve nucleus	nIV	4.94 ± 0.28	(6)
Diencephalic areas			
Nucleus triangularis ^b	T	56.49 ± 5.63	(10)
Tectothalamic tract	TT	39.80 ± 3.04	(14)
Nucleus rotundus ^b	R	37.52 ± 1.85	(12)
Ventral supraoptic decussation ^a	DSV	25.24 ± 2.02	(14)
Ventrolateral geniculate nucleus ^a	GLV	18.94 ± 1.01	(20)
Dorsolateral geniculate nucleus ^a	GLDP	13.21 ± 0.35	(8)
Pretectal nucleus ^b	PT	15.75 ± 0.82	(8)
Subpretectal nucleus ^b	SP	15.31 ± 0.63	(16)
Pretectal-subpretectal tract	PST	14.91 ± 0.81	(12)
Anterior thalamic area ^a	AT	12.53 ± 0.33	(6)
Lateral habenular nucleus	HL	11.32 ± 0.87	(6)
Optic tract	TRO	6.46 ± 0.26	(16)
Optic chiasm	OC	6.46 ± 0.28	(6)
Lateral hypothalamic area ^a	HY	5.48 ± 0.37	(20)
Dorsolateral thalamic area ^a	DT	4.57 ± 0.15	(20)
Telencephalic sites			
Neostriatum intermedium	NI	11.51 ± 0.52	(20)
Ectostriatum	E	8.56 ± 0.36	(20)
Field L	FL	7.90 ± 0.33	(10)
Area parahippocampus	APH	7.33 ± 0.26	(14)
Neostriatum	N	6.95 ± 0.48	(20)
Hyperstriatum ventrale	HV	5.47 ± 0.22	(20)
Neostriatum caudale	NC	5.23 ± 0.20	(10)
Dorsolateral corticoid area	CDL	4.53 ± 0.31	(12)
Lateral forebrain bundle	LFB	4.06 ± 0.18	(10)
Paraolfactory lobe	LPO	3.88 ± 0.12	(20)
Archistriatum	A	3.78 ± 0.24	(12)
Hyperstriatum accessorium	HA	3.69 ± 0.11	(20)
Olfactory tubercle	TO	3.57 ± 0.07	(4)
Paleostriatum	P	3.35 ± 0.07	(12)
Preoptic area	PO	3.23 ± 0.09	(10)

Values for density of 2-¹²⁵I-iodomelatonin binding (75 pM) are shown as mean ± SEM of independent determinations performed in triplicate (three sections per slide). The number of independent determinations (n) is shown in parentheses.

^a Retinorecipient areas (Ehrlich and Mark, 1984).

^b Thalamic and mesencephalic relay nuclei.

to 75 pM and reached saturation at 100 pM. Similar results were found in all areas examined over the entire 2-¹²⁵I-iodomelatonin concentration range used. Scatchard plots were linear, indicating a single class of high-affinity binding sites (33–48 pM). In the OT, the apparent dissociation constants for 2-¹²⁵I-iodomelatonin derived from kinetic analysis ($K_d = 41.06 \pm 3.92$ pM; $n = 4$) and saturation experiments ($K_d = 43.49 \pm 9.18$ pM; $n = 8$) are in agreement.

Pharmacological properties

Inhibition of 2-¹²⁵I-iodomelatonin binding (75 pM) to chicken brain sections was determined for eight concentrations of one of the following drugs: MEL, CLM, NAS, 5-methoxytryptamine (5-MT), LUZ, 5-methoxyluzindole (MLUZ), and 5-HT. A list of K_i values (mean ± SEM) for all drugs and areas examined is shown in Table 3. A representative analysis of the inhibition of

Figure 3. Rostrocaudal analysis of $2\text{-}^{125}\text{I}$ -iodomelatonin binding throughout the OT, R, DSV, GLV, EW, OM, NI, and E. Sequential coronal $20\text{ }\mu\text{m}$ sections throughout the brain were incubated with 75 pM $2\text{-}^{125}\text{I}$ -iodomelatonin as described in Materials and Methods. The x-axis indicates the distance in microns through all sections in which the indicated brain region appears. Values shown are from a representative experiment.

binding of $2\text{-}^{125}\text{I}$ -iodomelatonin in the OT is shown in Figure 5. The apparent dissociation constants for $2\text{-}^{125}\text{I}$ -iodomelatonin in the OT derived from competition experiments (MEL , $K_i = 44.92\text{ pM} \pm 4.21$; $n = 8$) are in agreement with those derived from kinetic analysis and saturation experiments. In the tectum, MEL and CLM were potent competitors of $2\text{-}^{125}\text{I}$ -iodomelatonin binding ($K_i = 44.92 \pm 4.21\text{ pM}$ and $215.52 \pm 7.24\text{ pM}$, respectively), while MLUZ and NAS were less effective ($K_i = 1.04 \pm 0.04\text{ nM}$ and $294.69 \pm 33.48\text{ nM}$, respectively). The K_i values for LUZ , the competitive MEL receptor antagonist (Dubocovich, 1988b), were also in the nanomolar range ($745.15 \pm 129.45\text{ nM}$). 5-HT and 5-MT were far less potent in displacing $2\text{-}^{125}\text{I}$ -

iodomelatonin in the chicken brain ($K_i = 19.97 \pm 3.38\text{ }\mu\text{M}$ and $459.69 \pm 3.48\text{ }\mu\text{M}$, respectively). All areas displayed an identical order of pharmacological affinity: $\text{MEL} > \text{CLM} > \text{MLUZ} > \text{NAS} > \text{LUZ} \gg 5\text{-HT} > 5\text{-MT}$ (see Table 3).

Correlation between the affinities of various indoles to inhibit $2\text{-}^{125}\text{I}$ -iodomelatonin binding and functional responses in the brain and retina

The pharmacological characteristics of the $2\text{-}^{125}\text{I}$ -iodomelatonin binding sites in discrete areas of the chicken brain using quantitative autoradiographic analysis and those found in chicken brain and retinal membranes (Dubocovich and Takahashi, 1987; Dubocovich et al., 1989; K. Chung and M. L. Dubocovich, unpublished observations) are very similar. Previous studies have shown that the potency of MEL and related agonists to inhibit the calcium-dependent release of ^3H -dopamine from rabbit and chicken retina correlates with the affinities of the same compounds to compete for $2\text{-}^{125}\text{I}$ -iodomelatonin binding sites in chicken retinal membranes (Dubocovich, 1985; Dubocovich and Takahashi, 1987). A high correlation between the affinity of MEL and related compounds to compete for $2\text{-}^{125}\text{I}$ -iodomelatonin binding sites in the chicken brain and chicken retinal membranes has also been shown (Dubocovich et al., 1989). Figure 6A shows the correlation between the affinities of MEL and related compounds to inhibit the binding of $2\text{-}^{125}\text{I}$ -iodomelatonin in the OT (present results; Table 3) and in chicken brain membranes (25°C ; Chung and Dubocovich, unpublished observations). The pharmacological profile of the $2\text{-}^{125}\text{I}$ -iodomelatonin binding site in the OT corresponds closely to that in chicken brain membranes (25°C ; $r = 0.966$; slope = 0.845 ; $n = 7$). These results suggest that $2\text{-}^{125}\text{I}$ -iodomelatonin labels sites of identical pharmacological characteristics in discrete areas of the chicken brain as those found in chicken brain membranes. Similarly, the affinity of the various MEL agonists and putative antagonists in the chicken brain OT (Table 3) correlates with the affinity of these compounds to inhibit $2\text{-}^{125}\text{I}$ -iodomelatonin binding in chicken retinal membranes (25°C ; $r = 0.973$; slope

Figure 4. A, Densitometric analysis of saturation binding in the OT of the chicken brain using quantitative autoradiography. Sequential coronal $20\text{ }\mu\text{m}$ sections through the optic lobe were incubated with eight concentrations of $2\text{-}^{125}\text{I}$ -iodomelatonin ($0.1\text{--}200\text{ pM}$). The specific binding (circles), defined as the difference between total (squares) and nonspecific (triangles), was saturable. B, Scatchard transformation of the saturation data. Values shown are from a representative experiment, with points representing the mean of triplicate determinations (three sections per slide).

Figure 5. Pharmacological characterization of 2-¹²⁵I-iodomelatonin binding in the OT using quantitative autoradiography. Coronal brain sections were incubated with 75 pM 2-¹²⁵I-iodomelatonin in the presence of eight concentrations of one of the indicated drugs. Values shown are from a representative experiment, with points representing the mean of triplicate determinations (three sections per slide). A list of *K_i* values (mean ± SEM) for all drugs and areas examined are shown in Table 3.

= 0.759; *n* = 7; Fig. 6*B*) and in chicken brain membranes (0°C; *r* = 0.946; slope = 0.379; *n* = 7; Fig. 6*C*). Finally, the pharmacological profile of the 2-¹²⁵I-iodomelatonin binding sites in the OT correlates with either the potency of these compounds (i.e., MEL, CLM, NAS, 5-MT, MLUZ) to inhibit the calcium-dependent release of ³H-dopamine or the affinity (i.e., LUZ) for the presynaptic MEL heteroreceptor of rabbit retina (Dubocovich, 1985, 1988b, 1990b) (*r* = 0.902; slope = 0.506, *n* = 6).

Discussion

The localization as well as the binding and pharmacological characteristics of 2-¹²⁵I-iodomelatonin binding sites in discrete areas of the adult chicken brain were determined using *in vitro* autoradiography. Binding sites were widely distributed throughout the entire brain, predominantly in areas associated with the visual system. This study confirms and extends previous qualitative autoradiographic studies of 2-¹²⁵I-iodomelatonin binding site localization in the brain of the 2-d-old chick (Stehle, 1990)

and 12-d-old chick (Rivkees et al., 1989). Although most of the areas displaying specific 2-¹²⁵I-iodomelatonin binding sites are the same in these two studies, there are several significant differences with the present results. First, the studies performed in chicks report binding in several auditory areas, including FL, nucleus ovoidalis, and the dorsolateral mesencephalic nucleus. We found specific 2-¹²⁵I-iodomelatonin binding only in FL in the adult chicken. Second, we did not find binding in the area ventralis of Tsai, an area reported to contain binding sites in the 12-d-old chick (Rivkees et al., 1989). Finally, we report a more extensive distribution of 2-¹²⁵I-iodomelatonin binding sites in the adult chicken brain. Areas of chicken brain showing specific binding of 2-¹²⁵I-iodomelatonin in this study that have not been reported in previous studies include the following: mesencephalic sites—OMN, nIV, BCS; diencephalic sites—PT, SP, PST, TT, TRO, OC, HL, HY, GLDP; telencephalic structures—NC, NI, P, PO, LFB, HV, and CDL. These differences in the distribution of 2-¹²⁵I-iodomelatonin binding between chicken

Table 2. Affinity (*K_D*) and density (*B_{max}*) of 2-¹²⁵I-iodomelatonin binding sites in discrete areas of the chicken brain

Brain area	<i>n</i>	<i>K_D</i> (pM)	<i>B_{max}</i> (fmol/mg protein)
Mesencephalic areas			
Optic tectum (OT)	8	43.49 ± 9.18	57.64 ± 2.10
Edinger–Westphal nucleus (EW)	6	44.62 ± 9.19	41.08 ± 3.25
Oculomotor nucleus (OM)	6	47.72 ± 12.78	33.54 ± 5.74
Diencephalic areas			
Nucleus rotundus (R)	6	44.69 ± 10.14	42.02 ± 6.61
Ventral supraoptic decussation (DSV)	6	33.29 ± 2.11	39.82 ± 4.28
Ventrolateral geniculate nuclei (GLV)	6	35.51 ± 3.87	35.58 ± 3.39
Telencephalic areas			
Neostriatum intermedium (NI)	8	37.94 ± 2.72	36.69 ± 2.85
Ectostriatum (E)	8	45.37 ± 6.94	30.83 ± 3.42

K_D and *B_{max}* values shown represent the mean ± SEM of independent determinations performed in triplicate (three sections per slide). Concentrations of 2-¹²⁵I-iodomelatonin ranged from 0.1 pM to 1 nM.

Figure 6. Correlation between the affinities (K_i values) of compounds for 2- 125 I-iodomelatonin binding sites in the chicken OT using autoradiography and the affinities of compounds to inhibit 2- 125 I-iodomelatonin binding in chicken brain membranes (25°C; A), chicken retinal membranes (25°C; B), and chicken brain membranes (0°C; C) and to affect 3 H-dopamine release from rabbit retina (D). K_i values for chicken brain (0°C) were obtained from Dubocovich et al. (1989); for chicken brain (25°C), from Chung and Dubocovich (unpublished observations); for chicken retinal membranes, from Dubocovich and Takahashi (1987), Dubocovich (1990b), and M. L. Dubocovich (unpublished observations). IC_{50} values for the inhibition of 3 H-dopamine release for various indoles (MEL, CLM, NAS, 5-MT, 5-HT) and the K_b (dissociation constant) value for LUZ were obtained from Dubocovich (1985, 1988b, 1990b). Linear regression of a logarithmic transformation of the data yielded the indicated slopes and correlation coefficients.

and chick brain may represent developmental changes or may be attributed to subtle differences in species and/or experimental conditions.

2- 125 I-iodomelatonin binding in the area referred to as the DSV (Fig. 2) appears to encompass other areas in addition to the DSV as shown in the atlas of Kuenzel and Masson (1989) (W. Kuenzel, personal communication). In this study, specific 2- 125 I-iodomelatonin binding in the DSV includes the nucleus of the supraoptic decussation, which is analogous to the visual SCN (vSCN) described in several studies (Meier, 1973; Gamlin et al., 1982; Cassone and Moore, 1987; Rivkees et al., 1989) and may also include the medial SCN (Hartwig, 1974; Kuenzel

and Masson, 1989). Thus, this entire area displaying 2- 125 I-iodomelatonin binding in this study may actually represent a suprachiasmatic region. Although the functional role of the area referred to as the DSV has not been established, one of its components (i.e., the vSCN) is thought to be analogous to the mammalian SCN, which also contains MEL receptor sites (Laitinen and Saavedra, 1990) and may be involved in mediating the effect of MEL on circadian rhythms.

More extensive analysis of selected brain regions showed the binding of 2- 125 I-iodomelatonin to be saturable, reversible, and to a single high-affinity site. The K_b values for the MEL binding site in the chicken brain (33–48 pM) are in the range of previously

Table 3. Affinities (K_i) for various indoles to inhibit 2-¹²⁵I-iodomelatonin binding in the chicken brain

Brain area	MEL (pM)	CLM (pM)	MLUZ (nM)	NAS (nM)	LUZ (nM)	5-HT (μM)	5-MT (μM)
Mesencephalic areas							
Optic tectum (OT)	44.92 ± 4.21 (8)	215.52 ± 7.24 (4)	1.04 ± 0.04 (4)	294.69 ± 33.48 (4)	745.15 ± 129.45 (4)	19.97 ± 3.38 (6)	459.69 ± 39.18 (4)
Edinger–Westphal nucleus (EW)	71.27 ± 6.51 (6)	205.94 ± 5.72 (4)	2.40 ± 1.26 (4)	256.94 ± 63.61 (4)	616.26 ± 70.16 (4)	16.35 ± 3.73 (4)	748.02 ± 10.79 (4)
Diencephalic areas							
Nucleus rotundus (R)	51.76 ± 7.71 (6)	312.90 ± 19.83 (4)	2.59 ± 0.61 (4)	244.30 ± 53.91 (4)	902.33 ± 139.48 (4)	16.65 ± 3.98 (4)	462.82 ± 66.33 (6)
Ventral supraoptic decussation (DSV)	55.36 ± 7.51 (6)	212.54 ± 15.42 (4)	1.29 ± 0.42 (4)	241.03 ± 32.11 (4)	639.01 ± 124.58 (4)	18.60 ± 1.61 (4)	640.64 ± 186.88 (4)
Ventrolateral geniculate nucleus (GLV)	58.61 ± 9.14 (6)	239.38 ± 14.68 (4)	2.47 ± 0.13 (4)	237.76 ± 11.45 (4)	774.65 ± 85.10 (4)	19.98 ± 0.53 (4)	608.08 ± 174.88 (4)
Telencephalic areas							
Neostriatum intermedium (NI)	37.14 ± 1.67 (8)	210.22 ± 7.16 (4)	2.25 ± 0.23 (6)	266.77 ± 15.76 (4)	843.06 ± 33.93 (4)	17.56 ± 3.41 (4)	655.72 ± 66.04 (4)
Ectostriatum (E)	76.06 ± 18.40 (4)	304.74 ± 11.88 (4)	3.33 ± 0.27 (4)	341.39 ± 13.85 (4)	815.40 ± 76.51 (4)	23.65 ± 8.57 (4)	685.96 ± 38.48 (4)

K_i values shown were calculated by the method of Cheng and Prusoff (1973) from IC_{50} values obtained from analysis of competition curves using the computer program GRAPHPAD. Inhibition of 2-¹²⁵I-iodomelatonin (75 pM) binding to chicken brain slices (20 μm) was determined for eight concentrations of competing drugs. Values shown are the means ± SEM of independent determinations performed in triplicate (three sections per slide). The number of independent determinations (n) is shown in parentheses.

reported affinity constants obtained for the MEL binding site using autoradiography in the rat area postrema and SCN (45.9 and 52.8 pM, respectively; Laitinen and Saavedra, 1990; Laitinen et al., 1990), rat ME (43 pM; Weaver et al., 1989), and mouse thalamic PVN (77.44 pM; Fang et al., 1990). In homogenates of the chicken OT and whole brain, K_D values were 87.2 and 60 pM, respectively (Rivkees et al., 1989; Stehle, 1990). Dubocovich et al. (1989, 1990) reported K_D values of 344, 99, and 56 pM for chicken brain membranes incubated at 0°C, 25°C, and 37°C, respectively. Our data indicate binding to only one site, which is in agreement with previous studies in chicken brain membranes (Dubocovich et al., 1989; Rivkees et al., 1989; Stehle, 1990); however, it is not possible to exclude totally the presence of a lower-affinity site. Laitinen and Saavedra (1990) reported the presence of a low-affinity binding site in the rat SCN (K_D = 761 pM) when wash durations were decreased to 2 min. Therefore, the assay conditions used in the present study (25°C and 15 min wash) may have resulted in preferential examination of high-affinity sites.

The B_{max} values (31–58 fmol/mg protein) obtained in the regions examined are in the range previously reported using membrane preparations in chicken whole brain: 56 fmol/mg protein (Dubocovich et al., 1990) and 37.8 fmol/mg protein (Rivkees et al., 1989).

The pharmacological order of affinities of the indole compounds for 2-¹²⁵I-iodomelatonin binding in this study agrees with previous reports in which MEL binding sites were characterized in chicken brain and in chicken and rabbit retinal homogenates (Dubocovich and Takahashi, 1987; Dubocovich et al., 1989; Dubocovich, 1990a). MEL and MEL analogs (i.e., CLM and MLUZ) possessing a methoxy group on carbon 5 and an acetamido group on carbon 3 of the indole nucleus were the most potent inhibitors of 2-¹²⁵I-iodomelatonin binding. NAS is significantly less potent than MEL at inhibiting the binding of 2-¹²⁵I-iodomelatonin, unlike in the hamster brain, where the

two compounds are equipotent (Dubocovich and Takahashi, 1987; Dubocovich, 1988a; Duncan et al., 1988). Removal of the *N*-acetyl group from the MEL molecule (e.g., 5-MT) caused a dramatic reduction in the potency to inhibit 2-¹²⁵I-iodomelatonin (459–748 μM) in these chicken brain areas. This pharmacological order of affinities suggests that the 5-methoxy group is important for binding, as shown by the low affinity of LUZ for 2-¹²⁵I-iodomelatonin when compared with MLUZ.

The pharmacological affinities of the various indoles on 2-¹²⁵I-iodomelatonin binding determined in discrete areas of chicken brain are very similar to that of the ML-1 MEL receptor found in brain and retina (Dubocovich, 1985, 1988a,b, 1990a,b; Dubocovich and Takahashi, 1987; Dubocovich et al., 1989, 1990). In support of these results, we have shown a correlation between the K_i values obtained in this study and the K_i values to inhibit 2-¹²⁵I-iodomelatonin binding in chicken brain and retinal membranes (Fig. 6A,B). Correlations performed between the affinity constants in the OT versus affinities in chicken brain and retinal membranes at 25°C yielded similar slopes (slopes = 0.845 and 0.759, respectively). In contrast, correlations based on binding studies performed at 0°C in chicken brain membranes resulted in a flattening of the slope (slope = 0.379; Fig. 6C). This may reflect a lower affinity of the radioligand for the MEL binding site at lower temperatures (Dubocovich et al., 1990), possibly as a result of a decoupling of the receptor from a G-protein. Finally, the highly significant correlation found between the affinity of indoles to inhibit 2-¹²⁵I-iodomelatonin binding in tectum, and the potency of the MEL agonists or the affinity of LUZ for the MEL presynaptic receptor to inhibit ³H-dopamine release from retina, suggest that the MEL binding sites found in discrete areas of chicken brain are very likely functional MEL receptors.

The density values obtained for the various areas in the chicken brain demonstrate the heterogeneous distribution of 2-¹²⁵I-iodomelatonin binding sites throughout the chicken brain. A

majority of the areas where binding was found are involved in the receiving or mediation of visual information. Ehrlich and Mark (1984) reported dense visual projections in the lateral and dorsolateral anterior thalamic nuclei, ventrolateral and dorsolateral geniculate nuclei (GLV and GLDP), BOR, and most extensively within the OT. Other areas showing MEL binding sites (see Table 1), although not direct recipients of retinal projections, serve as relay and terminal areas of the visual projection systems. For example, the two major sources of visual input from the thalamus to the telencephalon in bird are the thalamofugal (retino-thalamo-hyperstriatal projection) and tectofugal (retino-tecto-rotundo-ectostriatal projections) systems (Cohen and Karten, 1974). Other areas that exhibit 2-¹²⁵I-iodomelatonin binding receive significant input from retinorecipient areas; for example, the R is a major site of projections from the OT, and the isthmi, pretectal, and subpretectal nuclei also receive tectal afferents. Furthermore, 2-¹²⁵I-iodomelatonin binding sites are also found in areas such as the EW, which are involved in eyeball movements, pupillary contraction, and accommodation (Pearson, 1972). Recently, a retina-SCN-medial EW pathway in the pigeon has been described, which mediates increases in choroidal blood flow in the eye in response to retinal illumination (Fitzgerald and Reiner, 1990). This distribution of MEL receptors suggests that MEL may play a role in influencing various aspects of the avian visual system.

In conclusion, quantitative autoradiographic analysis of 2-¹²⁵I-iodomelatonin binding sites in discrete areas of the chicken brain strongly suggests that these sites are identical to the ML-1 sites found in chicken brain and retinal membranes. The binding and pharmacological characteristics of these MEL binding sites show distinct differences from the sites described previously in hamster brain membranes (Duncan et al., 1988). Furthermore, the high correlations between the affinities of MEL and related indoles in these studies with the potency of these compounds to mediate a functional response in the rabbit retina suggest that in the chicken brain these receptor sites may mediate functional responses regulated by MEL.

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