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<td>Citation</td>
<td>Canadian Journal Of Physiology And Pharmacology, 1995, v. 73 n. 6, p. 685-692</td>
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<tr>
<td>Issued Date</td>
<td>1995</td>
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<tr>
<td>URL</td>
<td><a href="http://hdl.handle.net/10722/42506">http://hdl.handle.net/10722/42506</a></td>
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Ontogeny of 2-[125I]iodomelatonin binding sites in the chicken (Gallus domesticus) kidney and spleen

Y. Song, A.M.S. Poon, G.M. Brown, and S.F. Pang

Abstract: To understand the possible role of melatonin receptors in the development of renal and immune functions, age-related variations of 2-[125I]iodomelatonin binding sites in the chicken kidney and spleen were investigated by radioreceptor assay. Chickens at embryonic day 20, as well as 2 days, 9 days, 2 weeks, 6 weeks, 12 weeks, and 16 weeks after hatching, were kept under a 12 h light : 12 h dark photoperiod and killed at the middle of the light period. Binding sites for 2-[125I]iodomelatonin in membrane preparations of the chicken kidney and spleen were present on embryonic day 20. The maximum binding densities ($B_{\text{max}}$) in the kidney increased to a peak between 9 days and 2 weeks of age, then progressively decreased. $B_{\text{max}}$ values of 2-[125I]iodomelatonin binding sites in the chicken spleen were lower than in the kidney. The peak density in the chicken spleen was recorded at day 2 after hatching and decreased significantly after 6 weeks of age. There were no significant differences in binding affinities ($K_d$) in kidney and spleen of chicken in the different age groups studied. The unity of Hill coefficients of 2-[125I]iodomelatonin binding sites of the chicken kidney and spleen in all age groups tested suggested that only a single class of binding sites was present in these tissues during development. It is proposed that the developmental changes in 2-[125I]iodomelatonin binding sites in the chicken kidney and spleen may be pertinent to the development of diurnal rhythms of kidney functions and the post-pubertal decline in immune functions of the chicken.

Key words: melatonin receptor, renal system, immune system, pineal gland, development, age-related change.

Résumé : On a examiné les variations selon l'âge des sites de fixations de la 2-[125I]iodomélatonine dans la rate et le rein de poulets, en utilisant une méthode faisant appel à des radiorécepteurs. On a maintenu des embryons de poulets de 20 jours, ainsi que des poulets âgés de 2 et 9 jours, et de 2, 6, 12 et 16 semaines dans un cycle photopériodique de 12 h clair : 12 h obscurité, et on les a sacrifiés au milieu de la période de clarté. On a relevé des sites de fixation pour la 2-[125I]iodomélatonine dans les préparations de membranes de la rate et du foie de l'embryon de 20 jours. Dans le rein, les densités de fixation maximales ($B_{\text{max}}$) ont atteint un sommet entre l'âge de 9 jours et 2 semaines, puis ont diminué progressivement. Les $B_{\text{max}}$ des sites de fixation de la 2-[125I]iodomélatonine ont été plus faibles dans la rate qu'au rein. Dans la rate, la densité de crête a été enregistrée à l'âge de 2 jours et a diminué significativement après 6 semaines. Chez tous les groupes examinés, aucune différence significative entre les affinités ($K_d$) de fixation du rein et de la rate n'a été observée ; de plus, l'unité des coefficients de Hill de tous les sites de fixation de la 2-[125I]iodomélatonine du rein et de la rate a indiqué qu'il n'y avait qu'une seule classe de sites de fixation dans ces tissus durant le développement. On suggère que les variations développementales des sites de fixation de la 2-[125I]iodomélatonine dans le rein et la rate de poulets pourraient favoriser le développement des rythmes diurnes des fonctions rénales et le déclin post-pubertaire des fonctions immunes du poulet.

Mots clés : récepteur de mélatonine, système rénal, système immunitaire, glande pinéale, variation liée à l'âge du développement.

[Traduit par la Rédaction]

Received July 21, 1994.

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Introduction

In the past few decades, physiological functions of melatonin have been studied extensively. There is considerable evidence that melatonin may be involved in the regulation of kidney functions (Reiter 1991). In patients with essential hypertension, melatonin administration (2 mg/day) was reported to decrease blood pressure (Birau et al. 1981). This finding is supported by reports in rats that pinealectomy elevates blood pressure (Zanoboni and Zanoboni-Muciaccia 1967) and increases renin secretion (Karppanen and Vapaatalo 1971). Furthermore, in rats, melatonin had an antidiuretic action, which was much less potent than that of serotonin (Arutyunyan et al. 1963), and pinealectomy produced an increase in urine excretion (Karppanen and Vapaatalo 1971). In contrast to these studies in the rat, in hamsters melatonin injections (25 μg/day) increased urinary output (Richardson et al. 1992), indicating that there may be significant species differences in this relationship (Richardson et al. 1992).

A relationship between melatonin and cation metabolism in the rat was proposed on the basis of a change in sodium levels and elevation of circulating calcium, magnesium, and zinc levels produced by suppression of melatonin secretion by constant light (Morton 1990). Csaba and Bokay (1977) found a decrease in circulating levels of magnesium and calcium following the administration of melatonin (10 μg · 100 g⁻¹ body weight · day⁻¹). Furthermore, a reduction in sodium and potassium excretion was reported after pinealectomy (Karppanen and Vapaatalo 1971). The demonstration of 2-[¹²⁵I]iodomelatonin binding sites in kidneys of several mammalian (Song et al. 1993a, 1993b) and avian species (Song et al. 1993d) is consistent with the hypothesis of a direct action of melatonin on the renal system regulating renin secretion and urinary and cation excretion.

Melatonin has been shown to stimulate the mammalian immune system (Maestroni 1993). In birds, melatonin regulates the diurnal rhythms of immune parameters (Skwarlo-Sonta et al. 1991; Rosolowska-Huszca et al. 1991). Pinealectomy modified the circadian rhythms of granulocyte number and serum lysozyme concentration in the chicken, which were restored to normal by daily melatonin injections (10–20 ng/day) (Rosolowska-Huszca et al. 1991). In addition, exogenous melatonin (10–20 ng/day) affects diurnal rhythms of white cell counts and serum agglutinin levels in chickens (Skwarlo-Sonta et al. 1991). However, Skwarlo-Sonta et al. (1992) could not demonstrate any immunostimulatory effects of melatonin (1–100 μg · kg⁻¹ body weight · day⁻¹) in chickens and concluded that the role (and/or) mechanism of action of melatonin may differ between avian and mammalian immune systems. The identification of high affinity 2-[¹²⁵I]iodomelatonin binding sites in the bursa of Fabricius (Liu and Pang 1993), thymus (Liu and Pang 1992), and spleen (Yu et al. 1991; Pang and Pang 1992; Poon et al. 1993) of various avian species led to the suggestion of a direct action of melatonin on the avian immune system (Poon et al. 1994).

Pineal and plasma melatonin levels are elevated during the nocturnal period (Arendt 1985) and seasonal changes are associated with the natural light-dark cycle (Pang et al. 1993b). Age-related changes in pineal and serum melatonin levels have been documented in human (Brown et al. 1979; Pang et al. 1985; Waldhauser et al. 1988), rat (Reiter et al. 1981; Tang and Pang 1988), hamster (Reiter et al. 1980), and chicken (Pang et al. 1993a). It is also well known that the risk of developing hypertension increases with age. Renal circadian rhythms are immature in infants and develop fully after months or even years (Koopman et al. 1989). The circadian rhythm of renal functions, which may be related to the diurnal secretory pattern of melatonin, diminishes with age (Koopman et al. 1989). Therefore, a developmental study of 2-[¹²⁵I]iodomelatonin binding sites in the kidney may provide information relevant to understanding renal melatonin receptor functions.

A decline in immunity with age is also well documented. It has been suggested that the decrease in circulating melatonin levels may contribute to the impaired immunity in aged animals (Pierpaoli and Maestroni 1987; Armstrong and Redman 1991; Grad and Rosenevaiag 1993). If melatonin acts directly on the lymphoid tissue (Poon et al. 1994) and is related to the age-related decline in immunity, developmental changes in lymphoid melatonin receptors may also contribute to the decline in immunity with age. In fact, an age-associated decline in the density of the 2-[¹²⁵I]iodomelatonin binding sites has been reported in rat pituitary (Vanecek 1988; Laitinen et al. 1992), hypothalamus and hippocampus (Zisapel et al. 1989), and caudal and anterior cerebral arteries (Laitinen et al. 1992), and has been related to changes in various neuroendocrine functions with age. In lymphoid tissues, a decrease in 2-[¹²⁵I]iodomelatonin binding in the chicken bursa of Fabricius at about 12 weeks of age has been reported (Liu and Pang 1993). Whether the age-related decline is due to a change in the binding site density or affinity is unknown. Thus, a survey of the developmental changes in lymphoid 2-[¹²⁵I]iodomelatonin binding sites is warranted. The chicken was chosen for these studies because of the higher density of 2-[¹²⁵I]iodomelatonin binding sites in the kidney (Song et al. 1993a) and spleen (Poon et al. 1994) in this species. Moreover, in chicken lymphoid tissues, the spleen may contain a subtype of melatonin receptors similar to that of the kidney (Poon and Pang 1994), whereas the bursa of Fabricius may contain another subtype. Therefore, it is of interest to determine whether the chicken kidney and spleen have similar age-related changes in their expression of 2-[¹²⁵I]iodomelatonin binding sites.

Materials and methods

Chemicals

2-[¹²⁵I]iodomelatonin (specific activity, 2200 Ci/mmol (1 Ci = 37 GBq)) was obtained from NEN Research Products, Dupont Co., Boston, Mass. Other chemicals were purchased from Sigma Chemical Co., St. Louis, Mo.

Animals, housing and sample collection

Male and female chickens (Gallus domesticus) were obtained from the Laboratory Animal Unit, University of Hong Kong, and used in these experiments following the guidelines of the Canadian Council on Animal Care. The chickens were housed under a 12 h light : 12 h dark cycle (lights on 03:00–15:00). Light was provided by ceiling-mounted fluorescent tubes at an intensity of approximately 200 lx at the top of cages. Room temperature and humidity
Fig. 1. Saturation study of 2-[\textsuperscript{125}I]iodomelatonin binding sites in the kidney of Em, 9d, and 16w chickens. (a) Representative saturation studies of 2-[\textsuperscript{125}I]iodomelatonin in the chicken kidney. SB is defined as TB (data not shown) minus NSB. (b) Scatchard transformations of data from Fig. 1a, with K\textsubscript{d} of 28.6, 28.7, and 27.4 pmol/L and B\textsubscript{max} of 2.59, 7.16, and 1.59 fmol/mg protein in kidneys of Em, 9d, and 16w chickens, respectively. (c) Hill plots with coefficients of 0.981, 1.075, and 1.045 in kidneys of Em, 9d, and 16w chickens, respectively.

were maintained at 20–22°C and 60–70%, respectively. Food and water were available ad libitum. Chickens were sacrificed at embryonic day 20 (Em), and at 2 days (2d), 9 days (9d), 2 weeks (2w), 6 weeks (6w), 12 weeks (12w), and 16 weeks (16w) after hatching. For the kidney studies, samples from Em were pooled from kidneys of 3 chickens, and for the spleen assays, Em, 2d, 9d, and 2w samples were pooled from 12–16, 10–12, 7 or 8, and 3 or 4 chicken spleens, respectively. All chickens were decapitated at the midpoint of the light period in the Minor Operation Room of the Laboratory Animal Unit, University of Hong Kong. Kidney and spleen tissues were removed immediately, frozen in liquid nitrogen, and stored at -70°C. Preliminary data showed that there was no significant change in the binding of 2-[\textsuperscript{125}I]iodomelatonin in the chicken kidney and spleen between those stored at -70°C for 1 week and 3 months (Y. Song, A.M.S. Poon, and S.F. Pang, unpublished data).

Membrane preparations
Frozen tissues were thawed and homogenized in 10 volumes (w/v) of ice-cold 0.05 mol/L Tris-HCl buffer (pH 7.4 at 4°C). Homogenates were centrifuged at 40,000 × g for 25 min. Pellets were washed and resuspended in Tris-HCl buffer to a concentration of about 6 g protein/L. The protein concentration of the samples was determined by the method of Lowry et al. (1951), using bovine serum albumen as standard.

Binding assays
The receptor binding assay was carried out by the method previously described (Song and Pang 1992; Pang and Pang 1992). The assay was performed in duplicate. All samples were incubated with 0.005–0.12 nmol/L 2-[\textsuperscript{125}I]iodomelatonin at 37°C for 1 h. Nonspecific binding (NSB) was determined in the presence of 1 μmol/L unlabelled melatonin.
Fig. 2. $B_{\text{max}}$ of 2-[$^{125}$I]iodomelatonin binding sites in the kidneys and spleens of Em, 2d, 9d, 2w, 6w, 12w, and 16w chickens. Data are means ± SEM of 5–10 tests. Statistical analysis showed significant differences in both kidney ($p < 0.01$, ANOVA, $F_{(6,37)} = 10.6$) and spleen ($p < 0.01$, ANOVA, $F_{(6,37)} = 12.3$). *$p < 0.05$, **$p < 0.01$, vs. corresponding highest $B_{\text{max}}$ group.

Specific binding (SB) was calculated by subtracting NSB from total binding (TB). Radioactivity was determined by a gamma counter (Beckman Instruments Inc., Fullerton, Calif.) with 70% efficiency. The maximal binding density ($B_{\text{max}}$) and equilibrium dissociation constant ($K_d$) were obtained from Scatchard analysis (Scatchard 1949).

Statistical analysis
Data are expressed as means ± SEM. Group differences were analyzed by one-way analysis of variance (ANOVA) with Systat 5.2.1, followed by Fisher's least significant difference tests of pairwise comparison (Systat, Inc., Evanston, Ill.), taking $p < 0.05$ as the criterion of significance. Preliminary studies in our laboratory showed that binding of 2-[$^{125}$I]iodomelatonin in the chicken kidney and spleen has no sex difference (Y. Song, A.M.S. Poon, and S.F. Pang, unpublished data). Hence, results of male and female chickens were grouped together for analysis.

Results

Chicken kidney
Binding of 2-[$^{125}$I]iodomelatonin was present in chicken kidneys of all age groups tested. TB and NSB increased over the concentration range (0.005–0.12 nmol/L) of 2-[$^{125}$I]iodomelatonin in all cases tested. SB reached saturation at about 0.07–0.08 nmol/L 2-[$^{125}$I]iodomelatonin (Fig. 1a), which is consistent with data previously reported (Song and Pang 1992; Song et al. 1993a). Scatchard plots showed linear regression, as indicated in Fig. 1b, which shows representative saturation studies of Em, 2w, and 16w old chickens. Hill coefficients approached unity in all the cases tested (Fig. 1c). $B_{\text{max}}$ of 2-[$^{125}$I]iodomelatonin binding sites in the chicken kidneys differed significantly between various age groups ($p < 0.01$, ANOVA, $F_{(6,37)} = 10.6$) (Fig. 2). $B_{\text{max}}$ gradually increased from Em (3.74 ± 0.35 fmol/mg protein) to 9d (8.28 ± 1.20 fmol/mg protein) and reached its peak level at about 9d–2w. After 2w, $B_{\text{max}}$ of 2-[$^{125}$I]iodomelatonin binding sites in the chicken kidney progressively decreased, with $B_{\text{max}} = 2.88 ± 0.40$ fmol/mg protein at 16w. The concentration of 2-[$^{125}$I]iodomelatonin binding sites in the kidney of 16w chicken was about 35% of the 9d group. The $K_d$ ranged from 23.2 to 57.4 pmol/L and showed no significant difference among the various age groups tested ($p > 0.05$, ANOVA, $F_{(6,37)} = 1.04$) (Fig. 3).

Chicken spleen
Binding of 2-[$^{125}$I]iodomelatonin in the chicken spleen was present in all age groups tested. Similar to the data reported by Pang and Pang (1992), TB and NSB of 2-[$^{125}$I]iodomelatonin increased over the range of 0.005–0.12 nmol/L 2-[$^{125}$I]iodomelatonin tested. SB approached saturation at 0.08–0.10 nmol/L 2-[$^{125}$I]iodomelatonin (Fig. 4a). Scatchard plots of different age groups were linear (Fig. 4b), and Hill coefficients approached 1.0 in all the cases examined (Fig. 4c). There were significant differences in the $B_{\text{max}}$ of 2-[$^{125}$I]iodomelatonin binding sites in the chicken spleen at different stages.
Fig. 3. Binding affinity of 2-[^125]Iiodomelatonin binding sites in the kidneys and spleens of Em, 2d, 9d, 2w, 6w, 12w, and 16w chickens. Data are expressed as mean ± SEM of 5–10 examinations. ANOVA shows no significant differences in either kidney (p > 0.05, ANOVA, \( F_{(6,37)} = 1.04 \)) or spleen (p > 0.05, ANOVA, \( F_{(6,47)} = 1.0 \)).

Discussion

Previous reports of 2-[^125]Iiodomelatonin binding sites in the chicken kidney (Song and Pang 1992; Song et al. 1993a) and spleen (Pang and Pang 1992; Poon et al. 1994) indicated that these binding sites were reversible, saturable, specific, and of high affinity. Reported values of \( K_d \) and \( B_{max} \) of 2-[^125]Iiodomelatonin binding sites in the kidney of 800- to 1100-g chickens (about 2–3 months old, \( K_d = 30.3 \pm 5.1 \) pmol/L, \( B_{max} = 2.89 \pm 0.24 \) fmol/mg protein; Song and Pang 1992) are comparable with animals with similar ages in the present study. Comparing our data with those reported earlier in the chicken spleen (Pang and Pang 1992), the binding affinities are in the same range. However, we found a higher \( B_{max} \) in 2-week-old chicks (1.99 ± 0.20 fmol/mg protein) than 12-week-old chickens (1.05 ± 0.12 fmol/mg protein), whereas in that report (Pang and Pang 1992), a lower \( B_{max} \) was recorded in 3-week-old chicks (1.09 ± 0.11 fmol/mg protein) compared with 11-week-old chickens (1.5 ± 0.16 fmol/mg protein). This discrepancy may be due to inter-assay variations in the previous study. In the present experiment, spleens from all age groups were assayed together within a single assay so that differences among age groups could not be due to interassay variations.

In an earlier report by Yu et al. (1991), receptor binding assays were conducted in duck spleen membranes at 4°C for 5 h, whereas the later, detailed characterization of 2-[^125]Iiodomelatonin binding in the chicken spleen (Pang and Pang 1992) was performed at 37°C for 1 h. We have found that, in the chicken spleen, the \( K_d \) and \( B_{max} \) obtained under these two conditions are not significantly different (A.M.S. Poon and S.F. Pang, unpublished data).

The presence of high affinity 2-[^125]Iiodomelatonin binding sites in the chicken kidney and spleen suggests that melatonin may play a role in regulating the renal and immune systems via melatonin receptors during growth and development. Over the growth period (embryonic age 20 days to 165 weeks after hatching), there was an age-related difference in \( B_{max} \) of 2-[^125]Iiodomelatonin binding sites in both the chicken kidney and spleen. However, there was no significant change in \( K_d \). The unity of the Hill coefficient and linearity of Scatchard plots for all age groups tested suggest that a single class of binding sites exists in the chicken kidney and spleen during development.

2-[^125]Iiodomelatonin binding sites in the kidney and spleen of chickens have similar binding affinities. These binding sites may belong to the ML-1 type of melatonin receptors (Song and Pang 1992; Pang and Pang 1992). It has been demonstrated that these binding sites in the kidney (Pang et al. 1993a) and spleen (Poon and Pang 1994) are...
linked to a G-protein; GTPγS decreased both $B_{\text{max}}$ and $K_d$ in the kidney (Pang et al. 1993a), as well as spleen (Poon and Pang 1994). These binding sites in both kidney and spleen may be classified as the same subtype of ML-1-γ according to Pang et al. (1993a). This may explain the similar changes of 2-[$^{125}$I]iodomelatonin binding sites in the kidney and spleen during growth.

It has been reported that 2-[$^{125}$I]iodomelatonin binding sites were detected in the brain from embryonic day 8 chickens (Chong and Sugden 1992). In the rat, 2-[$^{125}$I]iodomelatonin binding sites in the pituitary were found as early as gestation day 15 (Williams et al. 1991) or 20 (Vaneeck 1988). 2-[$^{125}$I]Iodomelatonin binding sites in the 10-day-old hamster fetus could be characterized in the primitive oral pharynx (Rivkees and Reppert 1991). Carlson et al. (1991) reported that 2-[$^{125}$I]iodomelatonin binding sites appeared in the pituitary, pineal gland, olfactory epithelium, and brain of hamster fetus. These findings are consistent with our identification of 2-[$^{125}$I]iodomelatonin binding sites in the kidney and spleen of embryonic chickens. N-Acetyltransferase, a key enzyme of melatonin biosynthesis in the chicken pineal, could be detected at embryonic days 16–19 (Binkley and Geller 1975). Hydroxyindole-O-methyltransferase was also present in primate pineals of near-term fetuses (Reppert et al. 1979, cited in Binkley 1988). The presence of 2-[$^{125}$I]iodomelatonin binding sites in the kidney and spleen of chicken embryo suggests that melatonin plays a role in regulating the renal and immune systems as early as the late embryonic stage.

Previous reports showed that the expression of melatonin binding sites during development varied with both tissues and species. In the rat pituitary, $B_{\text{max}}$ of 2-[$^{125}$I]iodomelatonin binding sites was highest on embryonic day 20 and then decreased progressively during development, with no signifi-
change of $K_d$. However, both $B_{\text{max}}$ and $K_d$ of 2-[125]I-
iodomelatonin binding sites in the median eminence showed
no significant differences with age (Vanecek 1988). In the
area postrema and suprachiasmatic nuclei of rats, melatonin
receptors also did not change during development. The den-
sities of melatonin receptors in the caudal artery, area pos-
trema, and suprachiasmatic nuclei were highest in young rats
(9 days old). There were minor changes in $K_d$, with a lower
$K_d$ being recorded in 9-day-old rats than in 96- and 306-day-
reported that 2-[125]Iiodomelatonin binding sites in the
chicken brain increased during embryonic development and
remained stable during the first 30 days after hatching. The
highest $B_{\text{max}}$ was detected in the 3-month-old chicken. No
significant differences of $K_d$ during development were
detected. In the chicken bursa, binding for 2-[125]Iiodoma-
elatonin was highest in 2-day-old chickens, with an age-
The above findings of minimal changes in $K_d$ of 2-[125]I-
iodomelatonin binding sites with age is consistent with our
present results of similar $K_d$ during development in the
chicken kidney and spleen. Our finding that the $B_{\text{max}}$ of
2-[125]Iiodomelatonin binding sites in the kidney and spleen
was higher in the younger chickens was similar to that
reported in the rat pituitary (Vanecek 1988), arteries and
anterior pituitary gland (Laitinen et al. 1992), and chicken
bursa (Liu and Pang 1992), which differ from the rat median
eminence (Vanecek 1988) and area postrema and suprachas-
matic nuclei (Laitinen et al. 1992) and the chicken brain
(Chong and Sugden 1992). Tissue and species differences
may be responsible for this discrepancy.

Our present studies describe patterns of 2-[125]Iiodo-
elatonin binding sites in the chicken kidney and spleen during
development. Diurnal rhythms of blood pressure (Lemmer
1989), renin secretion (Gordon et al. 1966), glomerular
filtration rate, urine flow, and sodium secretion (Koopman
et al. 1989) have been documented, with higher levels during
the day time. These rhythms may be related to postural
changes, dietary factors, and daily activities (Lemmer 1989;
Koopman et al. 1989). However, regulation by melatonin
cannot be ruled out, as melatonin is a diurnally secreted hor-
mon with peak levels at night (Reiter 1991). In addition, as
noted earlier in the Introduction, melatonin showed effects
on blood pressure (Zanoboni and Zanoboni-Muciaccia 1967;
Karpman and Vapaatalo 1971; Biraar et al. 1981), urine out-
put (Arutyunyan et al. 1963; Karpman and Vapaatalo 1971;
Richardson et al. 1992), and cation metabolism (Karpman
and Vapaatalo 1971; Csaba and Bokay 1977; Morton 1990),
and developmental changes of diurnal renal functions such as
renin secretion, glomerular filtration rate, and urine flow
were reported (Koopman et al. 1989). The age-related
change in the density of 2-[125]Iiodomelatonin binding sites
in the chicken kidney may play a role in the development of
diurnal rhythms of kidney function in the chicken, as well as
in the increasing risk of hypertension in elders. Similarly, the
developmental change in the density of 2-[125]Iiodomelatonin
binding sites in the chicken spleen may be physiologically
relevant. The immune system matures as the animal devel-
ops, reaching maximum function near the time of sexual
maturity and declining after puberty (Kay 1978). The decrease
in binding site density after 6 weeks coincides with
the decline of immunity after puberty. Since melatonin
modulates the immune function, it is possible that the puer-
tal decrease in melatonin receptor density may contribute to
the decline of immune function after puberty. However, the
full relevance of developmental changes of 2-[125]Iiodoma-
elatonin binding sites in the chicken kidney and spleen for the
physiological functions of the immune and renal systems
needs further investigation.

Acknowledgments
This study was supported by the Research Grant Council
grant and Neuroendocrinology Research Fund and Clarke
Foundation grant to S.F. Pang and the Committee on
Research and Conference Grants, University of Hong Kong,
grant to A.M.S. Poon. G.M. Brown is an Ontario Mental
Health Foundation Research Associate. The authors appreci-
te the technical assistance of K.F.L. Tsang, E.K.W. Koo,
and T.K. Yung.

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