2-[125I]-Melatonin binding sites in the central nervous system and neural retina of the frog *Rana perezi*: regulation by light and temperature

Esther Isorna, María J. Delgado, Ana I. Guijarro, Marcos A. López-Patiño, Mercedes Alonso-Bedate, Angel L. Alonso-Gómez*

Departamento de Fisiología (Fisiología Animal II), Facultad de Biología, Universidad Complutense de Madrid, Ciudad Universitaria, 28040 Madrid, Spain

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Abstract

The objective of the present study is to test daily and seasonal changes in 2-[125I]-Melatonin ([125I]-Mel) binding in different brain areas and the retina of the frog *Rana perezi* as well as the possible effect of light and temperature on melatonin receptors. During the day–night cycle, binding of [125I]-Mel showed a clear rhythm in the optic tectum, diencephalon, telencephalon, and neural retina, the binding being higher in the light phase than in the dark phase. By contrast, melatonin receptors did not show any significant summer–winter differences in any of the four tissues studied. In the neural retina, but not in the brain, exposure of frogs to 24 h darkness for one week leads to significantly less [125I]-Mel binding than 24 h light exposure. This darkness-induced reduction of [125I]-Mel binding is not due to a desensitisation of binding sites by high melatonin levels. Thermal acclimation to either 5 or 22°C for one month did not change the affinity (K_d) and density (B_max) of [125I]-Mel binding sites either in the brain or the retina. All these results indicate that there is a daily rhythm in melatonin receptors in the frog brain and retina, and that the light/dark cycle can drive this rhythm in [125I]-Mel binding in the retina. Temperature apparently did not modify [125I]-Mel binding in frogs.

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Keywords: Melatonin receptors; Daily rhythm; *Rana perezi*; Brain; Retina; Temperature

1. Introduction

Melatonin is a hormone synthesised in vertebrate retina and pineal gland during the night, showing low levels during the day in almost all the species studied so far (Pévet, 2003; Reiter, 1993). The origin of circulating melatonin in mammals, birds, and fishes, seems to be mainly the pineal gland (Cassone, 1990; Falcón et al., 2003). However, the lateral eyes could be the main source for this hormone in amphibians. Melatonin levels and the activity of the enzymes involved in its synthesis are higher in the retina than in the pineal gland (Baker et al., 1965; Serino et al., 1993), and daily and seasonal melatonin profiles in plasma are in better correlation with ocular than with pineal melatonin profiles (Delgado and Vivien-Roels, 1989).

Melatonin is a key component of the endocrine system and allows vertebrates to synchronise many physiological functions with the environment (Pévet, 2003; Reiter, 1993). Daily melatonin synthesis shows a clear seasonal pattern closely correlated to environmental factors, the photoperiod being the most important regulator in mammals (Pévet, 2003; Reiter, 1993). However, temperature is the main regulator of daily and seasonal melatonin rhythms in some ectothermic vertebrate species (Alonso-Gómez et al., 1992; Delgado and Vivien-Roels, 1989).
Knowledge concerning melatonin receptors has undergone rapid growth with the development of commercially available 2-[125I]-Melatonin ([125I]-Mel), which allows the characterisation of melatonin binding sites in the brain, retina, and in peripheral tissues of several vertebrate species (Vanecek, 1998). Classical pharmacology suggested the existence of two families of melatonin binding sites formerly called Mel-1 (also ML1) with high affinity and low density, and Mel-2 (or ML2) with low affinity and high density (Dubocovich, 1988). Mel-1 receptors have been cloned (Reppert et al., 1996), and three subtypes have been described: Mel-1a (or MT1), Mel-1b (or MT2), and Mel-1c. All of them belong to the super-family of G protein-coupled receptors (Reppert et al., 1996). Later, Mel-2 sites (or currently MT3) have been identified as an enzyme belonging to the family of the quinone reductases (Nosjean et al., 2000). The physiological role of these binding sites is currently being debated.

Many studies have described daily and seasonal rhythms in melatonin production, but very few have been focused to study the variation in melatonin receptors as a possible step in the regulation of melatonin functions by environmental factors, at least in ectotherms. Thus, day–night changes have been described in the brains of fish (Gaildrat et al., 1998; Iigo et al., 1994), chicken (Yuan et al., 1990), and some mammals (Barrett et al., 1996; Gauer et al., 1993a; Messager et al., 1997; Pelletier et al., 1990; Piketty and Pelletier, 1993; Recio et al., 1996). In amphibians, a diurnal rhythm in melatonin receptors mRNA expression has been described in Xenopus retina (Wiechmann and Smith, 2001). However, no data exist on seasonal changes in melatonin receptors in ectotherms. In addition to photoperiod, temperature is another environmental factor with a marked seasonal rhythm in temperate regions (Rome et al., 1992), and it may directly affect the melatonin receptor function of ectotherms like Rana perezi. To our knowledge, nothing is known about the effect of the temperature on melatonin binding in any ectothermic species.

A previous work described Mel 1-like receptors in the optic tectum, diencephalon, telencephalon, and retina of the frog R. perezi (Isorna et al., 2004), suggesting a key role of melatonin in amphibian physiology. In the present work, we first studied the daily variations of [125I]-Mel binding in different regions of the central nervous system and retina of the frog R. perezi. Second, we studied the effect of constant light and constant darkness (LL, DD) on the affinity and density of melatonin receptors in the optic tectum, diencephalon, telencephalon, and neural retina. Third, we studied the possible differences of [125I]-Mel binding in the frog brain and retina during two seasons of the year, and the effect of a thermal acclimation on the affinity and density of melatonin receptors in the same four central areas.

2. Experimental procedures

2.1. Animals

Adult frogs (R. perezi) from Orense (Spain) were maintained in aquaria with dechlorinated water and were fed with Calliphora sp. larvae twice a week. Unless otherwise noted, the frogs were maintained under a 12L:12D photoperiod and 22 ± 1°C temperature conditions for at least 2 weeks before the experiments.

2.2. Membrane preparation

The animals were decapitated and neural retinas, optic tectum, diencephalon, and telencephalon were dissected, frozen on dry ice, and stored at −80°C until used. Sacrifice was performed during the daytime under natural lighting conditions, and under dim red light during the dark phase of photocyte. Samples were sonicated (six pulses of 3 s at 30 W potency) in 100 μl of assay buffer (50 mM Tris–HCl, 5 mM MgCl2, pH 7.4) and centrifuged for 5 min at 800g to eliminate melatonin granules (Isorna et al., 2004). The supernatant was centrifuged for 10 min at 16,000g to precipitate the cell membranes. The pellet was resuspended in 500 μl of assay buffer and centrifuged again. This washing procedure eliminates tissue melatonin and avoids any interference in the radioligand assay (authors’ unpublished results). Finally, the membranes were resuspended in 100 μl of assay buffer and were stored at −80°C until the binding assays were performed. All membrane manipulations were done at 4°C. Protein concentration in the membranes was determined by Lowry et al.’s (1951) method.

2.3. Binding assays

Binding assays were performed as described by Isorna et al. (2004). Unless otherwise noted, assays were carried out at 25°C in a total volume of 100 μl for 60 min. We used [125I]-Mel as radioligand (2000 Ci/mmol; Amersham International Buckinghamshire, UK), and unlabeled melatonin (1 μM; Sigma Chemical, St. Louis, MO) to quantify the non-specific binding. Radioligand concentrations used were in the range of 50–60 pM except for saturation studies when concentrations from 5 to 100 pM were used. The reaction was stopped by the addition of ice-cold assay buffer (750 μl). Immediate vacuum filtration through 25 mm glass fibre filters (Millipore, APFC) was carried out using a Millipore 1225 cell harvester, and filters were washed with ice-cold assay buffer (4 ml). Next, filter
disks were placed in vials, and radioactivity was quantified in a γ counter (LKB, 1275 minigamma) with 75% efficiency. Specific binding of \([^{125}I]\)-Mel of each sample was calculated by subtracting non-specific from total binding.

2.4. Eyecup culture

Frogs were sacrificed by decapitation, and the eyes were excised. The cornea and lens were removed, and the remnant of the eye (including the retina, pigment epithelium, and sclera) was cultured individually as previously described (Valenciano et al., 1997). Cultures were performed in a saturated atmosphere (95% O\(_2\) and 5% CO\(_2\)) at 25 °C using “Dulbecco’s modified Eagle’s medium” (6.6 g l\(^{-1}\), pH 7.4, GIBCO, Grand Island, NY) as culture medium supplemented with ascorbic acid (0.1 mM), Hepes (20 mM), bovine serum albumin (0.75 g l\(^{-1}\)), NaHCO\(_3\) (35 mM), and gentamicin (50 mg l\(^{-1}\)); all obtained from Sigma Chemical, St. Louis, MO.

2.5. Data analysis

Statistical differences between experimental groups were analysed by a one-way ANOVA followed by the Student–Newman–Keuls post hoc test. Daily rhythms were also analysed by fitting the data to a sinusoidal function by the least squares method described by Duggleby (1981). To test the consistency of the rhythms, we used the principle of a noise/signal ratio less than 0.3 (Delgado et al., 1993). All parameters from sinusoidal function were expressed as the value ± standard error (SE). SE of parameters was based on the residual sum of squares in the least-squares fit. Equilibrium dissociation constants (\(K_a\)) and binding densities (\(B_{\text{max}}\)) from saturation assays were calculated by a non-linear regression of a four-parametrical logistic model using the ALLFIT program (De Lean et al., 1978). Statistical differences in \(K_a\) and \(B_{\text{max}}\) values were evaluated by the extra sum of squares principle (Draper and Smith, 1966).

2.6. Experimental procedure

2.6.1. Daily rhythms of \([^{125}I]\)-Mel binding sites

Frogs maintained at a temperature of 22 ± 1 °C and 12:12 light–dark conditions were killed every 4 h \((n = 6)\) throughout a 24 h daily cycle in March. Density of \([^{125}I]\)-Mel binding in the retina, optic tectum, diencephalon, and telencephalon was determined for six animals per time point.

2.6.2. Effect of constant light and constant darkness on melatonin binding sites

Two groups of seven frogs each were acclimated at two different extreme lighting regimes. One group was exposed to constant light (LL) and the other to constant darkness (DD). Both groups were maintained at 22 ± 1 °C. One week later, animals were sacrificed at the subjective midday (circadian time, CT = 6). The optic tectum, diencephalon, telencephalon, and one neural retina were used to measure melatonin binding density. The other retina was individually pooled for each group, and a saturation study was performed to determine both the affinity constant (\(K_a\)) and \(B_{\text{max}}\) of the melatonin binding sites in the two experimental groups, LL and DD.

2.6.3. Regulation of \([^{125}I]\)-Mel binding sites in neural retina by melatonin

To investigate whether the decrease in \([^{125}I]\)-Mel binding in neural retina of dark-adapted frogs was due to a down-regulation produced by melatonin in constant darkness, frog eyecups were cultured in light, without \((n = 6)\) or with 10 nM melatonin \((n = 6)\) for 7 h. After incubation, neural retinas were extracted from eyecups and washed twice in fresh culture media in order to eliminate the remnant of added melatonin. Next, \([^{125}I]\)-Mel binding was measured in the neural retina after membrane preparation.

2.6.4. Variation of \([^{125}I]\)-Mel binding sites in winter and summer

Frogs were captured in midwinter \((n = 9)\) and midsummer \((n = 9)\) and were maintained in the laboratory at natural environmental conditions for two weeks before the experiment. Animals were sacrificed at midday and \([^{125}I]\)-Mel binding densities of the retina, optic tectum, diencephalon, and telencephalon were individually determined.

2.6.5. Effect of thermal acclimation on \([^{125}I]\)-Mel binding sites

Two groups of frogs were acclimated at 5 °C \((n = 6)\) and 22 °C \((n = 6)\), respectively, under a 12L:12D photoperiod for one month. Animals were sacrificed at ZT = 3 (3 h after light on), and \(K_a\) and \(B_{\text{max}}\) values were measured in the optic tectum, diencephalon, telencephalon, and neural retina.

3. Results

The \([^{125}I]\)-Mel binding showed a significant day–night variation with the highest values at the beginning of photophase \((ZT = 2)\) in the optic tectum, telencephalon, and neural retina (Fig. 1). In the diencephalon, although receptor density was higher during the light phase than during the dark phase, statistical significance was not reached (ANOVA, \(p = 0.09\)). Nevertheless, rhythmic profiles were not identical. The acrophase occurred at the beginning of the light phase in the neural retina, op-
tic tectum, and telencephalon, but it took place during late light phase in the diencephalon.

The daily profile of experimental data allows us to adjust to a sinusoidal model. The parameters estimated from this model are summarised in Table 1. The consistency of the rhythm is confirmed in the neural retina, telencephalon, and diencephalon by the noise/signal ratio of sinusoidal amplitude (<0.3). In the case of the optic tectum, the daily profile was not close to a sinusoidal rhythm. The subtle amplitude of the rhythm with respect to the mesor value (15%) justifies the absence of a significant rhythm (noise/signal = 0.54). The sinusoidal rhythm was more robust in the neural retina, telencephalon, and diencephalon, where the ratio maximum/minimum were 1.5, 1.8, and 2.2, respectively.

In order to test whether this day–night variation of $[^{125}\text{I}]-\text{Mel}$ binding is due to a direct effect of lighting conditions, we maintained the frogs in constant light or darkness for a week. Only the neural retina showed a significant darkness-induced reduction on $[^{125}\text{I}]-\text{Mel}$ binding (Fig. 2). The rest of the studied tissues did not show any response to lighting conditions. Next, we compared the saturation curves of neural retina from the light- (LL) and dark-adapted (DD) frogs. Receptor affinity, estimated as $K_d$, was not modified by the treatment (LL, 15.3 ± 2.4 pM versus DD, 12.7 ± 2.6 pM).

Table 1

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<thead>
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<th>Parameters of cyclic sinusoidal function fitting daily rhythm of $[^{125}\text{I}]-\text{Mel}$ binding in the frog $R.\text{perezi}$</th>
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<tr>
<td><strong>Optic tectum</strong></td>
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<td>MESOR (fmol/mg prot)</td>
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<td>AMPLITUDE (fmol/mg prot)</td>
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<td>ACROPHASE (ZT hours)</td>
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<td>Noise/signal</td>
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Data are shown as the estimation ± SE.

* Rhythm is consistent if noise/signal ≤0.3. (ZT, zeitgeber time).
However, a small reduction in $B_{\text{max}}$ is observed in dark-adapted frogs (LL, 4.7 ± 0.3 fmol/mg prot versus DD, 4.0 ± 0.3 fmol/mg prot).

In order to test if the daily rhythm and dark-induced reduction are due to desensitisation of the retina by exposure to high melatonin levels, eyecups were cultured in the absence ($n = 6$) or presence of 10 nM melatonin ($n = 6$) for 7 h. No significant differences in $[^{125}\text{I}]-\text{Mel}$ binding were observed by comparing control and melatonin supplemented retinas (Fig. 3).

The $[^{125}\text{I}]-\text{Mel}$ binding in neural tissues from frogs maintained under natural conditions in winter (11L:13D photoperiod and 7°C temperature) and summer (13L:11D photoperiod and 18°C of temperature), did not show any significant differences, indicating that, apparently, melatonin receptors did not change in these two times of the year (Fig. 4).

Finally, we have characterised the effect of thermal acclimation on melatonin binding sites. As shown in Table 2, neither the affinity constant ($K_d$) nor the density ($B_{\text{max}}$) changed significantly as a consequence of thermal acclimation in any of the four tissues analysed.

4. Discussion

The present study describes for the first time a daily rhythm of melatonin receptors in the central nervous system and retina of the amphibian $R. \text{perezi}$. The existence of significant daily rhythms in melatonin receptors is apparently highly conserved throughout phylogeny: fish (Gaidrart et al., 1998; Iigo et al., 1994); amphibians (Wiechmann and Smith, 2001); birds (Brooks and Cassone, 1992; Yuan et al., 1990); and mammals (Duncan et al., 1993; Gauer et al., 1993a, 1994; Recio et al., 1996). Nevertheless, such rhythmicity is not a general feature in some brain regions of certain species, such as the suprachiasmatic nuclei of Siberian hamster (Recio et al., 1996) and the chicken optic tectum (Siuciak and Dubocovich, 1993).

Due to the circadian nature of the melatonin message in vertebrates, we could expect that day–night variation of melatonin receptors in target organs were coupled with daily melatonin rhythms. Usually, when a significant rhythm of melatonin receptors exists, the binding is lower during the dark phase of the photocycle. This pattern matches the present results from $R. \text{perezi}$, where a clear nocturnal peak of ocular and plasma melatonin has been described (Delgado and Vivien-Roels, 1989; Delgado et al., 1993). The receptor acrophase in the frog coincides with low melatonin production, at the end of the photophase in the diencephalon, while in the other neural tissues it occurs at the beginning of the light period. These slight tissue-dependent differences in the acrophase have been described previously in some mammalian species (Gauer et al., 1993a; Laitinen et al., 1989; Piketty and Pelletier, 1993; Recio et al., 1996), and their physiological significance is unknown.

The negative correlation between daily rhythms of the hormone and its receptor allows several possible interpretations. Melatonin may determine directly the daily rhythm of receptor density due to a down-regula-
tion mechanism by the nocturnal melatonin levels on their own receptor, as has been suggested in rodents (Gauer et al., 1992, 1994). This possibility seems unlikely for *R. perezi* because the continuous exposure to light or darkness for a week, which inhibits or enhances melatonin synthesis respectively, altered [125I]-Mel binding in the retina, but not in the brain (Fig. 2). In addition, melatonin rhythm in frogs has a much higher amplitude (Delgado and Vivien-Roels, 1989; Delgado et al., 1993) than receptor rhythm (Table 1), suggesting another type of association between melatonin and receptor rhythms for *R. perezi*.

In some mammals, treatments which reduce melatonin production, like light exposure or pinealectomy, increase receptor density without modifying affinity (Gauer et al., 1992; Pelletier et al., 1990), while the administration of exogenous melatonin reversed this effect (Gauer et al., 1993b). This receptor response to the changes in melatonin circulating levels is observed only in species with a pronounced receptor rhythm. In contrast, the arrhythmic melatonin receptors in chicken optic tectum (Siuciak and Dubocovich, 1993), Siberian hamster pars tuberalis and suprachiasmatic nuclei (Duncan et al., 1993), and rat suprachiasmatic nuclei (Laitinen et al., 1992) are not modified by pinealectomy or exogenous melatonin.

The discrepancy in studies performed in the same tissue and species: chicken optic tectum (Brooks and Cassone, 1992; Siuciak and Dubocovich, 1993); or rat pars tuberalis, (Duncan et al., 1993; Gauer et al., 1992) could be justified by a methodological explanation. High nocturnal endogenous melatonin or exogenous administered melatonin could interfere in the radioreceptor assay, apparently reducing the receptor density if membranes are not extensively washed before assay (Duncan et al., 1993). This fact is not applicable in our assays, because the extensive washing in the protocol for preparing membranes eliminates any residual melatonin (Isorna et al., 2004).

In the frog retina, the darkness-induced reduction of [125I]-Mel binding is produced by a decrease in binding sites rather than a decrease in its affinity. This effect could be dependent on the direct photosensitivity of the retina, and in consequence it may not be related to the presence of high melatonin levels. The fact that the addition of exogenous melatonin to *R. perezi* eyecups in culture for 7 h did not reduce [125I]-Mel binding (Fig. 3) suggests that the lower [125I]-Mel binding in the retina of DD-adapted frogs compared to LL-adapted frogs is due to a direct effect of continuous exposure to darkness. Thus, the light/dark cycle could drive not only the rhythm of circulating melatonin but also the receptor rhythm. Target organs receiving photic information directly, like the retina (the present study in *R. perezi*), or indirectly, like the mammalian suprachiasmatic nuclei (Gauer et al., 1994), are included in this group.

Other hypotheses are based on the presence of a clock-driven circadian rhythm of melatonin receptors. This implies a regulation of receptor mRNA expression, as has been described in ovine pars tuberalis (Barrett et al., 1996) and Siberian hamster suprachiasmatic nuclei (Gauer et al., 1997). In the case of anurans, a diurnal rhythm of receptor mRNA abundance in *Xenopus laevis* retina (Wiechmann and Smith, 2001) with peak levels occurring in the light period, and the lowest in the dark phase has been described. Results from *X. laevis* agrees with the [125I]-Mel binding rhythm in the brain and retina of *R. perezi*. Nonetheless, a possible phase-shift of mRNA rhythm with respect to receptor protein must be taken into account. In contrast to the well-documented seasonal rhythms of circulating melatonin in different species of vertebrates (for review see Reiter, 1993), few data exist on seasonal changes in mammalian melatonin receptors, and no studies have been carried out in non-mammalian vertebrates. Target organs involved directly in circadian function, like the suprachiasmatic nuclei, did not show a large variation in melatonin receptor density throughout the year, whereas other brain areas, related with seasonal reproduction, like pars tuberalis showed a marked rhythm (Gauer et al., 1993c; Messager et al., 1997; Recio et al., 1996). In the present study there is no evidence of season-dependent changes in the melatonin receptor (midwinter versus midsummer) in the four brain areas analysed. Nevertheless, it is possible that seasonal rhythms in melatonin receptors in discrete selective brain nuclei cannot be discerned by binding assays.

<table>
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<tr>
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<th>5 °C</th>
<th>22 °C</th>
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<tr>
<td></td>
<td>(B_{\text{max}}) (fmol/mg prot)</td>
<td>(K_d) (pM)</td>
</tr>
<tr>
<td>Optic tectum</td>
<td>13.72 ± 0.70</td>
<td>9.78 ± 1.06</td>
</tr>
<tr>
<td>Diencephalon</td>
<td>8.52 ± 0.36</td>
<td>11.57 ± 1.88</td>
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<tr>
<td>Telencephalon</td>
<td>1.81 ± 0.12</td>
<td>16.66 ± 5.02</td>
</tr>
<tr>
<td>Neural retina</td>
<td>5.22 ± 1.13</td>
<td>18.22 ± 2.93</td>
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</table>

Results are showed as the estimation of the parameter ± SE. \(K_d\) and \(B_{\text{max}}\) were calculated by non-linear regression of a 4-parameter logistic model using the ALLFIT program.
determine such hypothetical seasonal rhythms in melatonin receptors.

In addition to lighting conditions, temperature is a key environmental factor in regulating the synthesis (Alonso-Gómez et al., 1992; Falcón et al., 1994; Masuda et al., 2003; Valenciano et al., 1997) and effects (Alonso-Gómez et al., 1990; Delgado et al., 1992) of melatonin in ectotherms. As a result, hypothetically, an adaptive thermal modulation of melatonin receptor properties could be expected. Neither the affinity constant ($K_d$) nor the density ($B_{max}$) changed significantly in frogs as a consequence of thermal acclimation in any of the tissues analysed. Nevertheless, one month of acclimation is enough to induce thermal adaptive changes in physiological functions in frogs (Rome et al., 1992). In fact, melatonin synthesis is significantly modified by only three weeks of acclimation (Valenciano et al., 1997). The lack of effect of acclimation temperature on melatonin receptors in frogs is in agreement with the similar $[^{125}\text{I}]$-Mel binding found in summer and winter (two seasons with very similar photoperiod but different temperatures). Thus, it can be suggested that, in spite of the relevance of temperature as regulator of the production of the melatonin message, this environmental factor does not affect $[^{125}\text{I}]$-Mel binding. Nevertheless, it cannot be ruled out that temperature affects the transduction of the melatonin signal.

In summary, the present study describes, for the first time in an amphibian species, the rhythm of melatonin receptors in different brain areas and retina, showing a differential regulation by lighting conditions. Brain melatonin receptors are independent of the light exposure, except in the retina. There is no agonist-induced desensitisation of melatonin binding sites in the frog retina, suggesting that the light/dark cycle can drive the daily melatonin receptor rhythm. No seasonal variation has been detected, and temperature has no effect on the density of melatonin binding sites in either the retina nor the brain. This work provides useful information for the understanding of environmental dependent melatonin effects in amphibians. The signal transduction, after the binding of the hormone to its receptor, must be investigated in order to broaden knowledge concerning the mechanisms by which environmental factors modulate tissue response to melatonin.

Acknowledgments

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