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Immunoreactivity of Mel1a-like melatonin receptor and NRH: Quinone reductase enzyme (QR2) in testudine whole embryo and in developing whole retinas

Renata Nunes Silva and Lucia de Fatima Sobral Sampaio*

Laboratório de Bioquímica do Desenvolvimento do Sistema Nervoso, Instituto de Ciências Biológicas, Universidade Federal do Pará, Brazil.

ABSTRACT

Melatonin, the molecule signaling darkness, is possibly related with the development of the vertebrates. In developing chick retinas, Mel1a-like melatonin receptor (Mel1a) has been observed at embryonic day (E) 8 and after. NRH: Quinone reductase enzyme (QR2) is a detoxifying enzyme that binds melatonin analogs in developing chick retinas. In this study, whole mounted E21 embryos and whole retinas of the E21 embryos and juvenile *Kinosternon scorpioides* (JKs) were submitted to immunoassays to investigate the presence of Mel1a and QR2. Texas Red immunofluorescence for QR2 and Mel1a was diffusely localized in whole retinas of the E21 embryos and of the JKs. In E21 whole embryos, Texas Red immunofluorescence for Mel1a was localized in forelimb, hindlimb, peripheral carapace and tail. Mel1a immune signals were also present in choroid fissure of the optic cup, and in a region that corresponds to frontonasal process, mandibular process and maxillary process. QR2 was present in maxillary process region, in lens/iris, forelimb, hindlimb and tail. The results suggest that melatonin must have functions in testudine development by binding Mel1a and QR2.

*Corresponding author: Lucia de Fatima Sobral Sampaio, Instituto de Ciências Biológicas, Universidade Federal do Pará, Rua Augusto Corrêa No. 1, Belém, PA, CEP: 66075-110, Brazil.
lsampaio@ufpa.br

KEYWORDS: *Kinosternon scorpioides*, melatonin, Mel1a-like melatonin receptor, NRH: quinone reductase, development, retina, whole mounted embryo immunoassay

ABBREVIATIONS

SCN, suprachiasmatic nucleus; JKs, 60-day-old juvenile *Kinosternon scorpioides*; E21, 21-day-old embryos; QR2 and NQ02, Ribosyldihydronicotinamide dehydrogenase (quinone), EC 1.10.99.2 (NRH: Quinone Reductase); Mel1a, Mel1a-like melatonin receptor.

INTRODUCTION

Testudines have been used as a model to study the development of vertebrates [1]. The biochemistry, genetics and morphology of the little turtles attest that new subspecies of the Kinosternidae family evolved from *Kinosternon scorpioides* [2]. This subspecies has been cataloged on the basis of its morphological and geographical distribution [3, 4]. *Kinosternon scorpioides* is a testudine whose natural habitat is the region from Panama to Argentina. The reproduction of this species is seasonal, but no circadian rhythmicity is observed for the mating behavior [5, 6]. In the north and northeast of Brazil, studies have been conducted to develop the reproduction of the *Kinosternon scorpioides* in captivity. In the natural habitat as well as in captivity, the duration of the development *in ovo* post-oviposition is 90 days

[7, 8, 9]. Developmental studies using *Kinosternon scorpioides* as a model are difficult because a developmental staging table is still not devised for this species.

Biological rhythms in animals are produced by clock genes or by photoperiod entrainment through the neurohormone melatonin [10]. In vertebrates, melatonin is synthesized mainly in the pineal gland and in the retina [11]. In mammals, the pathway for environmental luminance information is retina → SCN (suprachiasmatic nucleus) → pineal gland. In the pineal gland, the neurochemical signal is translated into endocrine signal by the release of the neurohormone melatonin. This neurohormone also signalizes the absence of light to peripheral clocks [12] localized in cells that present melatonin membrane receptors. A detailed review on the functions and nomenclature of melatonin receptors can be found in Dubocovich *et al*., 2010 [13].

Circadian rhythms are generated in reptiles by biological clocks located in the retina, parietal eye, pineal gland and the SCN [14]. Interestingly in turtle, a widespread dense distribution of the melatonin membrane receptors in the forebrain visual areas, in contrast to the total absence of these receptors in SCN, was shown through 2-[^{125}I]-Iodomelatonin autoradiography binding [15]. In comparative studies on melatonin receptors, using 2-[^{125}I]-Iodomelatonin binding assays, difference was observed in maximal binding site values in brains and retinas from mammalian, avian, reptilian and amphibian species [16]. Other studies showed that the 2-[^{125}I]-Iodomelatonin binding in the brain of mammalian species is concentrated mainly in the pars tuberalis of the adenohypophysis and in the SCN. Thus, 2-[^{125}I]-Iodomelatonin binding is more widespread and densely distributed in brains from reptilian and avian species than in mammalian species [17, 18]. Therefore, melatonin has more circadian functions via receptors in brain structures of the avian, reptilian and amphibian species than in mammalian species.

Melatonin sets biological clocks via the cloned Gi-protein coupled membrane receptor MT1 in mammals and the Mel1a receptor, which is the MT1 homolog receptor cloned from chicken and Xenopus [13]. Immunofluorescence studies showed that MT1 is the first melatonin membrane receptor to appear in bovine embryos [19]. The Mel1a-like melatonin receptor was also characterized by both functional and immunofluorescence studies in embryos of avian [20, 21] and amphibian [22] species. In the mature retina, melatonin functions in retinal circadian rhythmicity [23] and in dark-adapting visual events [24] through this type of membrane receptor [23, 24].

Melatonin also binds to ribosyldihydronicotinamide dehydrogenase (quinone), EC 1.10.99.2 (NRH: Quinone Reductase, NQO2, QR2) [25]. This cytosolic enzyme detoxifies para-quinones by double reduction. However, its physiology is still not entirely understood [26]. Researches indicate that QR2 has a preference for catechol-quinones and estrogen-quinone as substrates [27]. Interestingly, this enzyme is present in retinas of the developing and post-hatched chicks [20]. The incubation of these retinas in a medium containing the melatonin analog 5-MCA-NAT or the natural QR2 cosubstrate NMH (N-methyl-dihydronicotinamide) increases the endogenous dopamine levels. Therefore, QR2 has a catechol-quinone reductase activity in developing retinas [28].

Melatonin modulates reproduction, temperature and adaptive behaviors in mammalian [29], avian [30], amphibian and reptilian [31] species. MT1 is the target in the melatonin signaling of the seasonal reproduction in mammals [32]. A Mel1a receptor is probably related to melatonin functions in seasonal reproductive behavior in testudine species, such as *Kinosternon scorpioides*, but it is still not investigated.

Our previous results showed that chick retinal cells do not differentiate in the absence of the endogenous melatonin or in the presence of the melatonin receptor/binding site antagonist luzindole [33, 34]. Therefore, we suggest that melatonin has functions in vertebrate development through membrane receptors or binding sites. Morphological studies, aiming to investigate the presence of the Mel1a-like melatonin receptor and melatonin binding site QR2 in whole embryos and in developing retinas are valuable for investigating melatonin functions in embryonic developmental stages.

In this study, the presence of the Mel1a-like melatonin membrane receptor (Mel1a) and of the

melatonin binding site QR2 was investigated using immunofluorescence, in the external morphology of a whole mounted 21-day-old (E21) embryo of *Kinosternon scorpioides*. Whole retinas of the E21 embryo and of the 60-day-old juvenile *Kinosternon scorpioides* (JKs) were also investigated for the presence of Mel1a and QR2.

MATERIALS AND METHODS

Materials

Tween 20 and chicken serum were purchased from Sigma Chemical Co., Saint Louis, MO, USA. Texas red (chicken anti-goat IgG-TR: SC-3923), MEL-1-A-R (SC-13186) and NQO2 (N-15. SC-18574) were purchased from Santa Cruz Biotechnology Inc., CA, USA.

Animals

All experiments were done in accordance with Brazilian and international laws for experimental animals. This project was approved by the internal committee (CEPAE-UFPA BIO088-12), obeying Brazilian and international laws regarding the use of the animals in research. Banco de Germoplasma Animal da Embrapa Amazônia Oriental-BAGAM (Salvaterra, PA, Brazil) provided 21-day-old (E21) embryos and 60-day-old juvenile (JKs) *Kinosternon scorpioides* (Linnaeus, 1766) maintained under the equatorial 12-h dark/light cycle and temperature. The same luminance and temperature conditions were maintained in the laboratory. The traffic of the eggs and animals from BAGAM to the laboratory was under approval of the specific committee SISBIO (40839-1). The quantity of the animals used was 12, E21 embryos, and 6, JKs. The E21 *Kinosternon scorpioides* used herein present the same external morphology, indicating that they were at the same developmental stage [35].

Immunoassays

In these assays, non-specific epitopes were blocked by tissue incubation in a Tween 20 (0.5%)/chicken serum (1:400)/PBS solution, all solutions were at pH = 7.4, incubations in antibody solutions were at 4 °C, and each step of the assay was stopped by washing the tissues thrice with PBS for 10 min. The negative controls were prepared by incubating whole embryos and retinas for 72 h, at 4 °C in a PBS solution without the primary antibody. No immunoreactivity was observed in negative controls. Each assay was repeated 3 times.

Immunoassay of whole mounted embryos: Embryos of the *Kinosternon scorpioides*, at E21, were excised from non-embryonic content of eggs and washed in PBS. Subsequently, these embryos underwent the following sequential steps: fixation in a 4% paraformaldehyde/PBS solution for 15 minutes; emulsification by incubation in a Tween 20 (0.5%)/PBS solution for 30 minutes; incubation in a PBS solution containing primary Mel1a (1:100) or QR2 antibody (1: 250) for 48 h; incubation in a PBS solution containing the secondary antibody Texas Red (1:400) for 2 h. The secondary antibody Texas Red binding was stopped by washing thrice while shaking (45 rpm) for 10 minutes. Later, the embryos were analyzed by a Nikon Fluorescence microscope.

Immunofluorescence of whole mounted retinas: E21 *Kinosternon scorpioides* embryos and JKs were sacrificed by decapitation. The optic cups/ eyes were enucleated, put in a calcium and magnesium free phosphate buffered saline; at that point the retinas were dissected. Subsequently, the retinas were washed thrice with PBS and incubated for 15 minutes in 4% paraformaldehyde solution. The emulsification step was done by incubation in a PBS/Tween 20 (0.5%) solution for 30 minutes. Thereafter, the retinas were incubated in a PBS solution containing primary Mel1a (1:100) and QR2 (1:250) for 72 h, washed in PBS, and incubated in a PBS solution containing the secondary antibody Texas Red (1:400) for 2 h. The Texas Red binding was stopped by washing thrice, shaking at 45 rpm for 10 minutes. Once the retinas were dry, they were mounted on slides and visualized by Nikon Fluorescence microscope.

RESULTS

Immunofluorescence for Mel1a-like melatonin receptor and QR2 in *Kinosternon scorpioides* whole embryos

The standard system to study vertebrate embryo development proposed by Ingmar Werneburg [35]

was applied to typify the E21 embryo of the *Kinosternon scorpioides*. This system attributes a code for one or a group of morphological external characters that marks the conclusion of a developmental event that should be included in a stage table. The E21 embryo presents an optic cup with choroid fissure and contour lens/iris (V08e). The maxillary process and frontonasal process are fused (G0lf). The tip of the mandibular process is situated at the level of the frontonasal process (G02g). Forelimb and hindlimb buds are elongated (V12d). The anterior border of the peripheral carapace is not yet defined at this embryonic age (S02c). These codes in parenthesis can be used to compare these E21 embryos with the embryos of other developing species of the Kinosternidae family. Different views of the E21 embryos are showed in the Fig. 1.

Fig. 1. Photographs of the 21-day-old *Kinosternon scorpioides* embryo in different views. Above: Lateral right view (left), dorsal right view (middle) and lateral elongated right view (right) are presented at different magnifications. **Below:** The 21-day-old *Kinosternon scorpioides* in real size, on a common slide of microscopy. Scale bar: 4 mm.

The Mel1a presented a characteristic Texas red immunoreactivity profile in all E21 embryos observed in this study. In the optic cup, Mel1a immunoreactivity was observed mainly in the choroid fissure. Greater immunoreactivity was also observed in regions corresponding to frontonasal process, mandibular process and maxillary process.

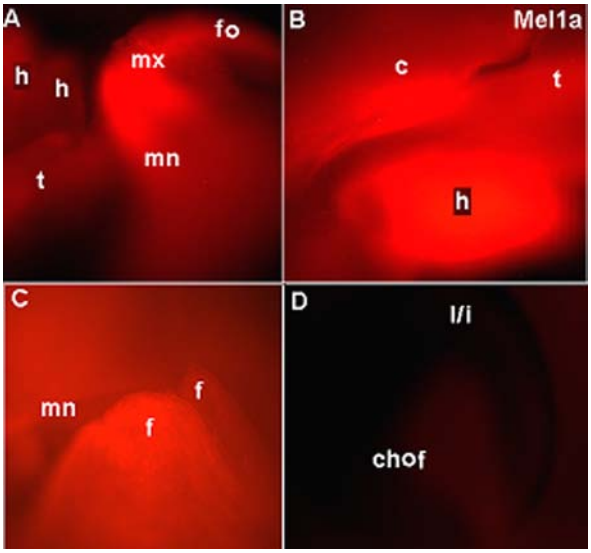

Fig. 2. Localization of the Mel1a-like melatonin receptor in the external morphology of the whole mounted 21-day-old *Kinosternon scorpioides* embryos (E21) shown using Texas red immunofluorescence. A. Anterior lateral view showing Mel1a immunofluorescence in the tail (t), in the hindlimb (h) and in the frontonasal (fo), maxillary (mx) and mandibular processes (mn). Magnifications 25X. **B.** Posterior lateral view showing Mel1a immunofluorescence at the irregular peripheral carapace (c), at tail (t) and at hindlimb (h). Magnifications 40X. **C.** Lateral elongated right view showing Mel1a immunofluorescence in the most external part of the forelimb (f). Magnifications 40X. **D.** Frontal view of the optic cup showing Mel1a immunofluorescence in the choroid fissure (chof) and lens/iris (l/i). Magnifications 40X. This panel is a representative result of the three experiments.

Mel1a immunoreactivity was also observed in the forelimb, hindlimb, peripheral carapace and tail (Fig. 2). No immunoreactivity was observed in the negative controls incubated without the primary antibody.

The Texas red immunofluorescence profile of the QR2 was the same in the E21 embryos tested in this study. This enzyme was localized in the region corresponding to maxillary process. In eye cup, QR2 signal was observed in the lens/iris. Forelimb, hindlimb and tail were also positive for QR2 (Fig. 3). A presumptive epithelial embryonary tissue was also QR2 positive (data not shown).

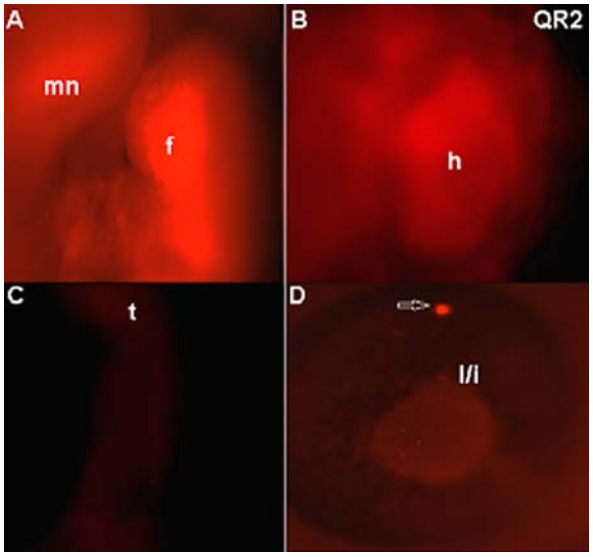

Fig. 3. Localization of the QR2 in the external morphology of the whole mounted 21-day-old *Kinosternon scorpioides* embryos (E21) using Texas red immunofluorescence. A. Right lateral view showing QR2 immunoreactivity at mandibular (mn) region and forelimb (f). Magnifications 25X. **B.** Right lateral view showing QR2 immunoreactivity at hind limb (h). Magnifications 40X. **C.** Left lateral view showing QR2 immunoreactivity at tail (t). Magnifications 40X. **D.** Frontal view of the optic cup showing QR2 immunoreactivity in lens (l) and iris (i). Arrow points to a high signal in the iris. Magnifications 40X. This panel is a representative result of the three experiments.

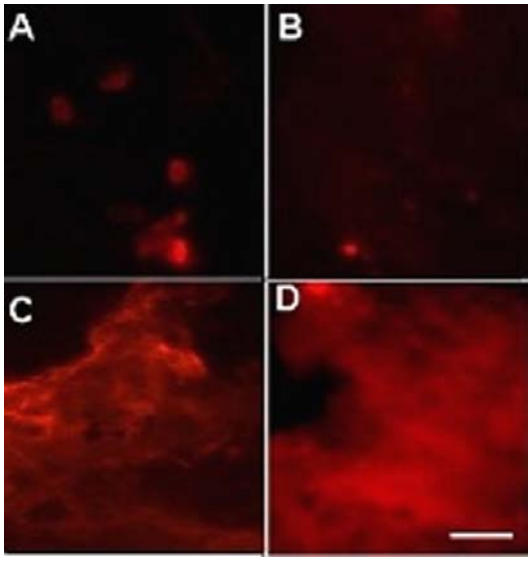

Fig. 4. Mel1a-like melatonin receptor and QR2 in whole retinas from 21-day-old *Kinosternon scorpioides* embryo (E21) and juvenile (JKs) visualized using Texas red immunofluorescence. A, B: Whole neural retinas from E21 embryos; **A.** Mel1a-like melatonin receptor immunoreactivity and **B.** QR2 immunoreactivity. **C**, **D:** Whole neural retinas from JKs; **C.** Mel1a-like melatonin receptor immunoreactivity; **D.** QR2 immunoreactivity. Note that Mel1a-like melatonin receptor and QR2 present different Texas red immunofluorescence pattern. Scale bars 250 μm. This panel is a representative result of the three experiments.

No immunoreactivity was observed in the negative controls incubated without the primary antibody.

Mel1a-like melatonin receptor and QR2 immunofluorescence in E21 embryo and juvenile *Kinosternon scorpioides* whole retinas

An immunofluorescence pattern characteristic of the membrane protein [36] appeared in cells of the E21 embryos and JKs retinas incubated with anti-Mel1a antibody, while an immunofluorescence pattern characteristic of the cytosolic protein [37] was observed in cells of the E21 embryos and JKs retinas incubated with anti-QR2 antibody. An increased immunoreactivity to both Mel1a-like melatonin receptor and QR2 was observed in whole retinas of the E21 embryos and JKs (Fig. 4). No immunoreactivity was observed in negative controls incubated without the primary antibody.

DISCUSSION

There is only one study on *Kinosternon scorpioides* development, in which the initial stages of the development of vitellogenic follicles and developing eggs in the oviduct are shown using ultrasound [7]. Therefore, to the best of our knowledge, an embryo of *Kinosternon scorpioides* is described for the first time in this study. In addition, it is the first time that the Mel1a-like melatonin receptor and the melatonin binding site QR2 are shown in a developing testudine. However, as others of the same class, *Kinosternon scorpioides* may have a delay in its embryonary development in stages prior to oviposition. This inhibition or cessation of the cell division and metabolic activity prevents damage during laying process by the movement of embryos, ensuring that the development is only resumed after oviposition. Thus, all eggs in the oviduct are laid at the same embryonic stage [38]. These observations allowed us to use the number

of days after oviposition to characterize the embryo of the *Kinosternon scorpioides* used in this study.

In this work, the presence of melatonin receptor Mel1a is showed in the areas of ossification such as face, limbs, tail, and carapace of the E21 embryos. These results are in line with the results of mammals showing the participation of the receptor MT1 in the differentiation of osteoblasts [39, 40, 41]. The participation of the Mel1a receptor in the differentiation of osteoblasts in non-mammalian species should indicate that the bone formation is a common evolutionary link. Additional functional studies must be performed to corroborate this hypothesis. Furthermore, Mel1a is present in choroid fissure, which is a blood vessel collection that disappears when the lens is completely formed. The functional significance of this Mel1a localization for eye cup development is unknown. On the other hand, QR2 immunoreactivity was more well distributed in lens/iris than Mel1a. Interestingly, both Mel1a and QR2 are localized in mandibular process, in forelimbs, hindlimb and tail. Additional studies on melatonin functions in development must investigate if this colocalization has functional significance.

QR2 distribution is tissue and species specific in mature vertebrates [27]. Apparently, a unique general aspect about the QR2 distribution in mature tissue/cells of a large number of species is that QR2 is situated in precursors of blood cells in the bone marrow [42], and in skin cells [43]. The present results show that QR2 is also present in precursors of the skin cells in E21 embryos of the *Kinosternon scorpioides* and in retinas from E21 embryos and JKs. QR2 immunofluorescence was found at various stages in developing embryos and post-hatched chicks [20], but in retinas of the mammalian species the presence of this enzyme is unknown. An investigation in mammalian species should attest whether there is a widespread presence of the QR2 in retinas from vertebrates.

Mel1a-like melatonin receptor and QR2 immunofluorescence were found at stages in which retinal cells begin to differentiate in 8-day-old chick embryos [20]. The morphology of the eye cup at E8 chick embryos [44] is similar to the eye cup of the E21 *Kinosternon scorpioides* embryos showed herein. Therefore, the observed immunoreactivity both to Mel1a and QR2 in the retina indicates that they are also present in stages corresponding to retinal differentiation in *Kinosternon scorpioides*. Additionally, melatonin signalizes circadian rhythmicity in the retina of the mammalian species through MT1 receptor [23]; if this type of the receptor is also related to multitasking circadian rhythmicity in avian and reptilian species [45] needs to be investigated.

CONCLUSION

Studies on melatonin functions in animals such as *Kinosternon scorpioides* with reproductive seasonality are useful tools for improving captive management through the control of events related to ambient lighting, which is a signal to the beginning of breeding in these animals. In addition, the results presented in this study suggest that the neurohormone melatonin through the Mel1a membrane receptor and the cytosolic binding site QR2 has a role in the embryonic development.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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