General and Comparative Endocrinology xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Endothelin modulates the circadian expression of non-visual opsins

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ARTICLE INFO

Article history:
Available online xxxx

Keywords:
Xenopus laevis
Melanophores
Melanopsins
Endothelin
Circadian rhythms

ABSTRACT

The non-visual opsin, melanopsin, expressed in the mammalian retina, is considered a circadian photopigment because it is responsible to entrain the endogenous biological clock. This photopigment is also present in the melanophores of *Xenopus laevis*, where it was first described, but its role in these cells is not fully understood. *X. laevis* melanophores respond to light with melanin granule dispersion, the maximal response being achieved at the wavelength of melanopsin maximal excitation. Pigment dispersion can also be triggered by endothelin-3 (ET-3). Here we show that melanin translocation is greater when a blue light pulse was applied in the presence of ET-3. In addition, we demonstrated that mRNA levels of the melanopsins *Opn4x* and *Opn4m* exhibit temporal variation in melanophores under light/dark (LD) cycles or constant darkness, suggesting that this variation is clock-driven. Moreover, under LD cycles the oscillations of both melanopsins show a circadian profile suggesting a role for these opsins in the photoentrainment mechanism. Blue-light pulse decreased *Opn4x* expression, but had no effect on *Opn4m*. ET-3 abolishes the circadian rhythm of expression of both opsins; in addition the hormone increases *Opn4x* expression in a dose-, circadian time- and light-dependent way. ET-3 also increases the expression of its own receptor, in a dose-dependent manner. The variation of melanopsin levels may represent an adaptive mechanism to ensure greater melanophore sensitivity in response to environmental light conditions with ideal magnitude in terms of melanin granule dispersion, and consequently color change.

1. Introduction

Melanin dispersion is an easily observable characteristic of pigment cells in non-mammalian vertebrates, what makes them a useful tool to study key events underlying cell-specific gene expression and function. Melanin synthesis depends on tyrosinase, the step limiting enzyme for melanin production (Hearing, 2011). Transcription factors of the basic helix-loop-helix (bHLH) family such as the microphtalmia (MITF) gene product control tyrosinase transcription by binding to highly conserved E-box elements in the promoter of this gene (Murre et al., 1989; Bentley et al., 1994; Camp et al., 2003; Murisier and Beermann, 2006). Clock proteins are members of the bHLH family able to control up to 10% of cell/tissue transcripts through E-box elements, imposing a rhythmic expression pattern on clock-controlled genes (CCGs) (Panda et al., 2002a; Storch et al., 2002; McIntosh et al., 2010), therefore tyrosinase may be considered a CCG. It is well known that these proteins drive circadian rhythm of several physiological and behavioral processes (Reppert and Weaver, 2001, 2002), hence dispersion and/or production of melanin may also be one of the processes modulated by a temporal mechanism.

Light–dark cycles are responsible to entrain endogenous oscillations, what is fundamental to guarantee the organism temporal organization (Hastings, 1991). In mammals, light-perception by melanopsin-positive retinal ganglion cells leads to clock gene transcription modulation in the central biological clock, thus participating in photo-entrainment (Panda et al., 2002b, 2003). Since the discovery of melanopsin in cultured *Xenopus laevis* dermal melanophores and mouse retina (Provencio et al., 1998, 2002) related sequences have been identified (Bellingham et al., 2002). Non-mammalian vertebrates have two melanopsins, the orthologue of mammalian melanopsin, *Opn4m*, and the orthologue of the melanopsin initially cloned from *X. laevis*, *Opn4x* (Bellingham et al., 2006). *X. laevis* melanophores respond to white light with pigment granule dispersion, with maximal responses at wavelengths between 450 and 470 nm, same range of melanopsin excitation (Isoldi et al., 2005).

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http://dx.doi.org/10.1016/j.ygcen.2014.04.019
0016-6480/

Endothelins (ET) are physiological effectors in pigment cells of mammals and non-mammalian vertebrates (Fujii, 2000). ETs are known to play important roles in cardiovascular regulation, cell proliferation (Kedzierski and Yanagisawa, 2001), and in differentiation and regulation of pigment cells (Filadelfi et al., 2004; Ramanzini et al., 2006; Braasch et al., 2009). There are 3 isoforms of ETs (Bloch et al., 1989; Yanagisawa and Masaki, 1989). Remarkably, ET-1 induces circadian rhythmicity in rat-1 cells (Yagita et al., 2001; Nakahata et al., 2006), mouse fibroblasts (Yagita et al., 2001) and zebrafish ZEM-2S cell line (Farhat et al., 2009). In mammals, two receptor subtypes, ET_A and ET_B, have been found (Arai et al., 1990; Sakurai et al., 1990; Davenport, 2002), and a third subtype (*ETc*) was cloned from amphibian (*X. laevis*) melanophores (Karne et al., 1993).

X. laevis dermal embryonic melanophores possess two melanopsins, one or both of which could be the photoreceptor molecule, responsible for light perception. Upon activation by light, this opsin triggers mechanisms of pigment granule dispersion throughout the cytoplasm (Isoldi et al., 2005).

Since white light and ET-3 induce melanin dispersion in *X. laevis* melanophores (Karne et al., 1993; Isoldi et al., 2005), we decided to use this process to better understand if light and ET-3 can lead to photo-entrainment through melanopsin-mediated mechanisms of gene expression. As a first step, we determined whether blue-light causes melanin granule dispersion as previously seen with white light and how ET-3 affects this response. Then, we determined the temporal pattern of *ETc* receptor and melanopsin gene expression in dermal melanophores of *X. laevis* in culture as well as their modulation by endothelin-3 and light–dark cycles.

2. Material and methods

2.1. Cell culture

X. laevis melanophores (kindly donated by Prof. Mark Rollag, Uniformed Services University of the Health Sciences, USA) were kept in 60% L-15 medium, supplemented with 480 mg/L galactose, 5 mg/L insulin/transferrin/selenium, 4 mg/L uridine, 87.6 mg/L L-glutamine, 25 mg/L L-asparagine, 152 mg/L $CaCl_2$, 49.6 mg/L $MgCl_2$, 51.7 mg/L MgSO4, 0.4 × MEM non-essential amino acids solution, 0.2 × MEM amino acid solution, 0.3 × MEM vitamin solution, 1% HT supplement, 1% antibiotic/antimycotic solution (10,000 U/mL penicillin/10,000 μg/mL streptomycin/25 μg/mL amphotericin), and 10% non-inactivated fetal calf serum (all from Life Technologies, Carlsbad, CA, USA), pH 7.5, at 25 °C.

For the experimental protocols the serum concentration was reduced to 2% and retinaldehyde (Sigma, St. Louis, MO, USA) was added to the cultures to a final concentration of 10-7 M. The addition of retinaldehyde has been shown to be required to maintain the melanophore photosensitivity (Rollag and Lynch, 1993).

2.2. Pigment dispersion assay

X. laevis melanophores were plated (5 × 104 cells/well in a 96-well plate), kept in constant dark (DD) for 24 h and then divided into five groups: (1) cells in DD; (2) cells stimulated with white light (87.85 μwatts/cm^2, Ecolume full-spectrum, white light bulb, model YZ8 W, São Paulo, Brazil) for 10 min; (3) cells stimulated with LED blue-light (87.85–95.17 μwatts/cm^2, 450–475 nm, Golden plus, São Paulo, Brazil) for 10 min; (4) cells treated with 10-8 or 10-9 M ET-3 during 1, 2 or 6 h; (5) cells treated with 10-8 M ET-3 for 1 h and stimulated with blue light for 10 min at the beginning of the hormone treatment.

The melanosome dispersion was quantified by absolute absorbance at 530 nm in a plate reader (SpectraMax 250, Molecular Devices, Germany). One-way ANOVA followed by Tukey's test was used to determine the significance of differences in absorbance between each light stimulus, endothelin group and the control. Differences between hormone doses and control group were determined by two-way ANOVA followed by Bonferroni's test.

2.3. Temporal expression of Opn4x, Opn4m, and endothelin receptor subtype ETC

X. laevis melanophores were plated (2 × 106 cells/25cm^2 flask) and maintained in DD or light–dark cycle (LD 12:12, lights on at 8:00 h, and lights off at 20:00 h) for 7 days. At 8:00 h of the sixth day, the culture flasks were divided into: (1) control cells subject to two medium changes at 8:00 and 14:00 h; (2) hormone-treated cells, subject to a medium change at 8:00 h, to introduce 10^{-9} or 10^{-8} M ET-3 (Calbiochem, USA), and 6 h later (at 14:00 h), to a second medium change for hormone removal. As a negative control for the group subject to medium changes, cells were kept completely undisturbed under DD or LD conditions. On the seventh day (24 h after starting the treatment) total RNA was extracted every 4 h during 24 h.

During the photophase of the light–dark cycle, light intensity was 87.85 μwatts/cm^2 (Ecolume full-spectrum, white light bulb, model YZ8 W). Cells were handled in the dark (medium changes, RNA extraction *etc.*) under red safelight (7 W Konex bulb and Safe-Light filter GBX-2, Kodak, Rochester, NY, USA).

2.4. Blue-light stimulation (450–475 nm)

Cells were kept in DD for 3 days and then stimulated with LED blue-light lamp (87.85–95.17 μwatts/cm^2, 450–475 nm, Golden plus, São Paulo, Brazil) for 10 min on the fourth day (at 8:00 h). Blue light was chosen based on the literature reports that this is the wavelength that maximally stimulates melanopsin (Isoldi et al., 2005; Moraes et al., 2014). Total RNA was extracted 1, 2, 6 and 12 h after light stimulation, simultaneously with control group (maintained in DD, not stimulated, throughout the experiment).

2.5. Total RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)

TRIzol Reagent (Life Technologies, Carlsbad, CA, USA) was used to extract total RNA, the pellet was resuspended in DEPC water and treated with DNase (turbo-DNA-FreeTM, Life Technologies, Carlsbad, CA, USA) to eliminate DNA contamination. The whole procedure followed the manufacturer's instructions. The RNA concentration and quality were assessed using a spectrophotometer (Nanodrop, Willmington, DE, USA), and 1 μg of RNA was reverse transcribed (SuperScript III Reverse Transcriptase, Life Technologies, Carlsbad, CA, USA) with random primers. RT-PCR reactions were performed in a thermocycler (Ependorff, Hauppauge, NY, USA), with the following protocol: 65 °C for 5 min, chill on ice for 1 min, then, after the enzyme addition, 25 °C for 5 min, 50 °C for 50 min, and 70 °C for 15 min.

2.6. Quantitative PCR (qPCR)

The products of RT-PCR were submitted to quantitative PCR reactions in 96-well plates using a pair of primers and a probe specific for the species based on sequences obtained from GenBank (http://www.ncbi.nlm.nih.gov/PubMed), designed using the Primer Express program (Life Technologies, Carlsbad, CA, USA), and synthesized by IDT (Coralville, IA, USA). *18S* ribosomal RNA was used to normalize the values of the genes of interest, as widely used (Goidin et al., 2001; Schmid et al., 2003; Aerts et al., 2004; Zhu and Altmann, 2005; McCurley and Callard, 2008). *18S* RNA does

Please cite this article in press as: Moraes, M.N.d.C.M., et al. Endothelin modulates the circadian expression of non-visual opsins. Gen. Comp. Endocrinol. (2014), http://dx.doi.org/10.1016/j.ygcen.2014.04.019