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RESEARCH ARTICLE

Eye and heart morphogenesis are dependent on melatonin signaling in chick embryos

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ABSTRACT

Calmodulin is vital for chick embryos morphogenesis in the incubation time 48–66 h when the rudimentary C-shaped heart attains an S-shaped pattern and the optic vesicles develop into optic cups. Melatonin is in the extraembryonic yolk sac of the avian egg; melatonin binds calmodulin. The aim of this study was to investigate the function of melatonin in the formation of the chick embryo optic cups and S-shaped heart, by pharmacological methods and immunoassays. Mel1a melatonin receptor immunofluorescence was distributed in the optic cups and rudimentary hearts. We separated embryonated chicken eggs at 48 h of incubation into basal, control and drug-treated groups, with treatment applied in the egg air sac. At 66 h of incubation, embryos were excised from the eggs and analyzed. Embryos from the basal, control (distilled water), melatonin and 6-chloromelatonin (melatonin receptor agonist) groups had regular optic cups and an S-shaped heart, while those from the calmidazolium (calmodulin inhibitor) group did not. Embryos from the luzindole (melatonin receptor antagonist) and prazosin (Mel1c melatonin receptor antagonist) groups did not have regular optic cups. Embryos from the 4-P-PDOT (Mel1b melatonin receptor antagonist) group did not have an S-shaped heart. Previous application of the melatonin, 6-chloromelatonin or forskolin (adenylate cyclase enhancer) prevented the abnormal appearance of chick embryos from the calmidazolium, luzindole, prazosin and 4-P-PDOT groups. However, 6-chloromelatonin and forskolin only partially prevented the development of defective eye cups in embryos from the calmidazolium group. The results suggested that melatonin modulates chick embryo morphogenesis via calmodulin and membrane receptors.

KEY WORDS: Optic cup, S-shaped heart, Calmodulin, Adenylate cyclase, Melatonin receptors, Chicken eggs

INTRODUCTION

The pineal gland produces and secretes melatonin during the hours of darkness. This neurohormone induces entrainment of the circadian and circannual rhythmicity; it also has many other functions in vertebrates (Reppert et al., 1994, 1996; Arendt, 2005). In high concentrations (micromolar), melatonin is a free radical scavenger (Reiter et al., 2002). At low levels (picomolar), melatonin acts by binding to membrane receptors. The cloned Mel1a, Mel1b and Mel1c chicken melatonin membrane receptors are counterparts of the mammalian MT1, MT2 and orphan GPR50 receptors (Brydon et al., 1999; Dufourny et al., 2008). These receptors are coupled to Gi and other G-proteins. Moreover, MT2 (Mel1b) is linked to inhibition of soluble guanylate-cyclase (sGC), leading to inhibition of cyclic guanosine 3′-5′-monophosphate (cGMP) (Tosini et al., 2014). At low concentrations (nanomolar), melatonin also binds to the Ca^{2+}-binding protein calmodulin (Benítez-King et al., 1993).

As a short-term effect, melatonin inhibits calmodulin in the myotube of the newborn rats (de Almeida-Paula et al., 2005). Inhibition of calmodulin also underlies the effect of melatonin on microtubule assembly, as shown in cytoskeletal rearrangements of N1E-115 cells and an *in vitro* system using calmodulin and tubulin purified from sheep brain and cytoskeleton purified from N1E-115 cells (*Mus musculus* neuroblasts) (Huerto-Delgadillo et al., 1994). Additionally, in immortalized rat (Long–Evans fetal) suprachiasmatic nucleus cells, melatonin levels were found to be associated with an increase in protein kinase C (PKC) activity (Rivera-Bermúdez et al., 2003). The same result was observed in an *in vitro* reconstituted enzyme system from MDCK (Madin–Darby canine kidney) epithelial cells, where melatonin stimulated calmodulin phosphorylation by increasing PKC activity, so inhibiting calmodulin (Soto-Vega et al., 2004). However, melatonin was also found to have a long-term effect by inducing an increase in cytosolic calmodulin levels in MDCK cells and N1E-115 cells (Benítez-King et al., 1991). In the hilar zone of the Wistar adult rat hippocampus, melatonin stimulated dendrite formation and complexity by increasing cytosolic calmodulin levels in parallel with the induction of autophosphorylation of PKC, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and extracellular signal-regulated kinase 1/2 (ERK 1/2) (Domínguez-Alonso et al., 2015). Based on these results, the effect of melatonin on calmodulin-related pathways appears to depend on the cellular environment and duration of the hormonal stimulus.

Mel1a, Mel1b and Mel1c mRNA has been reported in Japanese quail oocytes and early embryos (Oblap and Olszańska, 2001). Arylalkylamine *N*-acetyltransferase (AANAT) mRNA, the rate-limiting enzyme in melatonin synthesis, was also found in quail oocytes and embryonated eggs before oviposition (Oblap and Olszańska, 2003). In this avian species, approximately 415.6 pg melatonin was found in the extra-embryonic yolk sac of the eggs during early post-oviposition. It was speculated that melatonin and serotonin form a diffuse neuroendocrine system in the avian egg, which is capable of modulating developmental and metabolic events in the embryo (Olszańska et al., 2007).

In chickens, pineal gland development starts at about 60 h of incubation but distinguishable pinealocytes appear only on the seventh embryonic day (Ohshima and Matsuo, 1988), and a measurable melatonin efflux (5 pg) was obvious on the tenth embryonic day (Möller and Möller, 1990). Rhythmic melatonin synthesis starts *in vitro* after the thirteenth embryonic day (Faluhelyi and Csernus, 2007) and *in vivo* on the seventeenth embryonic day (Csernus et al., 2007). Therefore, the chicken egg melatonin content was the only source of this hormone for the chick embryo during the initial part of late organogenesis, which begins at 48 h of incubation.

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At this stage, the chick embryos have evaginated optic vesicles. Subsequently, the optic vesicles undergo morphogenetic invaginating movements triggered by actomyosin until the optic cup is formed. The driving force was proposed to be provided by calmodulin present in the cytosol of the embryonic cells (Brady and Hilfer, 1982). In the same time frame, 48–66 h of incubation, the embryonic hemodynamic causes rudimentary heart looping, i.e. the C-shaped pattern changes to an S-shape (Martinsen, 2005). Morphogenesis begins earlier in the heart than in the eye (Epstein et al., 1987; Hyer et al., 2003; Martinsen, 2005); this developmental time window allows investigation of the effects of calmodulin on morphogenesis of the rudimentary organs at different developmental time points.

Research on the function of melatonin in vertebrate development is still ongoing. However, preliminary studies have shown that maternal melatonin influences the development of the human fetus, in parallel with the classical maternal melatonin function in the synchronization of fetal biological rhythms with environmental illumination (Voiculescu et al., 2014). In animal models, the Mel1c melatonin receptor has not been investigated in the developing chick retina, but the Mel1a and Mel1b melatonin membrane receptors were observed by immunofluorescence in differentiating chick whole retinas (Sampaio, 2013). A study using chick retinal cells in culture showed that the absence of melatonin or blockage of the melatonin receptors with luzindole, in a straight developmental time window that corresponded to the final part of retinal cell differentiation, hampered the appearance of functional homomeric nicotinic receptors (Sampaio et al., 2005; Sampaio and Markus, 2010). However, in developing chick retinas, the potency of the melatonin receptor antagonists luzindole and 4-P-PDOT was dependent on developmental stage in experiments where they were tested against melatonin inhibitory effects on both cyclic adenosine 3′,5′-monophosphate (cAMP) levels (Sampaio, 2008; Sampaio, 2009) and endogenous dopamine accumulation (Sampaio et al., 2014). Therefore, melatonin appears to have an important role in chick retina differentiation.

The aim of the present study was to investigate a possible function of the hormonal calmodulin inhibitor melatonin on the optic cup and S-shaped heart formation in chick embryos, using pharmacological methods and immunoassays.

MATERIALS AND METHODS

Animals

A local hatchery (Makarú, Ananindeua, Brazil) provided the fertilized chicken eggs [*Gallus gallus domesticus* (Linnaeus 1758)]. All experimental procedures were under the recommendations of the National and International laws on Ethics in Research with Experimental Animal Protocol, code CEUA-UFPA: 229-14.

Drug preparation and doses

The pharmacological tools used were the calmodulin inhibitor calmidazolium (Reid et al., 1990), the positive modulator of adenylate cyclase forskolin (Seamon and Daly, 1981), the melatonin receptor agonist 6-chloromelatonin (Dubocovich, 1988), the non-selective melatonin receptor antagonist luzindole, the Mel1b/MT2 melatonin antagonist 4-P-PDOT (Dubocovich et al., 2010), the Mel1c melatonin receptor antagonist prazosin (Chen et al., 2016), and melatonin.

Melatonin (*N*-acetyl-5-methoxytryptamine), luzindole (*N*-acetyl-2-benzyltryptamine), 6-chloromelatonin (*N*-acetyl-6-chloro-5-methoxytryptamine) and prazosin [1-(4-amino-6,7-dimethoxy-2-quinazolinyl)-4-(2-furanylcarbonyl)piperazine hydrochloride] were purchased from Sigma-Aldrich (St Louis, MO, USA). 4-P-PDOT (cis-4-phenyl-2-propionamidotetralin) was from Tocris Cookson Ltd (Bristol, UK). Calmidazolium {1-[bis(4-chlorophenyl)methyl]-3-[2,4-dichloro-β-(2,4-dichlorobenzyloxy)phenethyl] imidazolium chloride} was purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

Each drug was prepared in ethanol/distilled water v/v (11×10^{-6} mol μl^{-1} ethanol) to give a maximum concentration of 11×10^{-9} mol μl^{-1} ethanol, which was non-toxic for HH12 embryos (Tolosa et al., 2016) in terms of the embryonic external characters studied here. Prazosin (1 mg) was diluted in distilled water (1 ml). Stock solutions were maintained at −20°C.

The maximum concentration of each compound solution was as follows: melatonin 17 µmol l^{-1}, luzindole 1 mmol l^{-1}, 4-P-PDOT 1 µmol l^{-1}, calmidazolium 400 µmol l^{-1}, 6-chloromelatonin 280 µmol l^{-1}, prazosin 1 mmol l^{-1} and forskolin 300 µmol l^{-1}. The total volume of the extra-embryonic contents of chick eggs at 48 h of incubation was approximately 64 ml. The volume per drug applied in the air sac was 10 µl. Therefore, the minimum concentration expected for each substance reaching the embryo was: melatonin 2.65 nmol l^{-1}, calmidazolium 46.87 nmol l^{-1}, luzindole 156.25 nmol l^{-1}, 6-chloromelatonin 43.75 nmol l^{-1}, 4-P-PDOT 0.15 nmol l^{-1}, prazosin 15.62 nmol l^{-1} and forskolin 46.87 nmol l^{-1}.

The dose of each compound per 10 µl volume of egg was as follows: calmidazolium 4.0 nmol; forskolin 0.00143 nmol; melatonin 0.000017, 0.0017 and 0.17 nmol; 6-chloromelatonin 0.0028, 0.028, 0.28, 2.8 and 4 nmol; luzindole 5 and 10 nmol; 4-P-PDOT 0.000001, 0.00001, 0.0001, 0.001 and 0.01 nmol; and prazosin 0.0000042, 0.000042, 0.00042, 0.0042 and 0.042 nmol.

Experimental *in ovo* assay

Embryo development was evaluated using the Hamburger and Hamilton chick embryo staging series (HH) (Hamburger and Hamilton, 1992). The main events during the incubation time scheduled in this study were in accordance with previous studies. Regarding eye development, at the start of experiments (48 h chick embryo), the HH12 stage embryo presented primary optic vesicles. The HH13 stage embryo had formed a lens placode, which started to invaginate in the HH14 stage embryo. The HH15 stage embryo had a completely formed optic cup. The invagination of the lens placode caused a second distinct contour in the region of the iris, which was still unpigmented in the HH18 stage chick embryo (66 h chick embryo) (Hamburger and Hamilton, 1992; Hyer et al., 2003). Regarding heart morphogenesis, at the HH12 stage, the tube heart had acquired a C-shaped form. The C-shaped heart was followed by coiling to attain an S form (S-shaped heart) in the HH18 stage embryos (Hamburger and Hamilton, 1992; Moorman and Christoffels, 2003; Martinsen, 2005).

At incubation day 0, the fertilized chicken eggs were placed vertically (with the air sac at the top) in an automatic incubator (Dove Factories, Vinhedo, São Paulo, Brazil) at 37.78°C and humid atmosphere (55–60% relative humidity). This incubator has a transparent acrylic cover, establishing a natural light–dark cycle 12 h per phase, which is characteristic of the regions located at the equator. All eggs were incubated for 48 h; over this time frame, the embryos develop to HH12 stage. They were then distributed into three groups: basal, control and drug treated. The basal group remained inside the incubator. The control group received only distilled water (10 or 20 µl) because the ethanol quantity (11 nmol μl^{-1}) in the higher concentration solutions used herein is non-toxic (Tolosa et al., 2016). The drug-treated group received a

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volume of 10 µl of each tested drug, or two drugs, with an interval of 10 min between them, achieving a maximum volume of 20 µl. At room temperature (20°C), distilled water and drug(s) were inoculated through a small hole (2 mm) created with the clamp tip in the egg shell, above the air sac. This hole was closed with microporous tape (Cremer SA, Santa Catarina, Brazil). The eggs were returned to the incubator for an additional 18 h of incubation to attain the HH18 stage. The 66 h-incubated eggs were opened at the air sac region, and the embryos were observed visually. In the present experimental conditions, only six embryos from the basal group were not in the HH18 stage and they were not considered for analyses. The embryos were then excised and washed in phosphate-buffered saline (PBS) solution, in which they were immediately analyzed using an optical microscope (Bioval L-1000B, São Paulo, SP, Brazil). The primary results were imaged using a digital color camera (Nikon Coolpix 2300, Tokyo, Japan) coupled to the microscope.

Chick whole-embryo immunoassay

The assays were performed following Silva and Sampaio (2014), except for the use of PBS solution containing Tween 20 (0.5%) and 1:200 normal chicken serum instead of 1:400, and the inclusion of Mel1a blocking peptide solution, which was not in the Silva and Sampaio (2014) immunoassay protocols. Chick embryos at HH12 and HH18 stages were removed from the egg, washed in PBS and incubated in 4% paraformaldehyde/PBS solution for 15 min. The embryos were emulsified, and the non-specific epitopes were blocked in a PBS solution containing Tween 20 (0.5%) and normal chicken serum (1:200) for 30 min. Afterwards, they were incubated in a PBS solution containing primary Mel1a antibody (1:100; Santa Cruz Biotechnology, Paso Robles, CA, USA) or blocking solution or only PBS, for 48 h at 4°C. Each incubation was stopped by washing the embryos with PBS (3 times, 10 min). In the blocking solution, the Mel1a-antibody (4 µg ml^{-1}) was pre-absorbed by binding with Mel1a blocking peptide (Santa Cruz Biotechnology; 20 µg ml^{-1}) in PBS for 2 h at room temperature. After incubation with the primary antibody or blocking solution or PBS, the embryos were washed and incubated in a PBS solution containing anti-goat secondary antibody conjugated to Texas Red (1:300; Santa Cruz Biotechnology) for 2 h at 4°C. The secondary antibody solution was removed, and the embryos were washed thrice in PBS, with shaking (45 rpm), for 10 min. Mel1a immunoreactivity was visualized by Texas Red staining, using the red filter of the fluorescence microscope (Nikon fluorescence microscope, Laboratório de Microscopia Eletronica, Evandro Chagas Institute, Brazil).

Data collection and analysis

In the immunoassays, n=5 embryos per embryonic stage (HH12 and HH18) in three independent experiments were used. Groups with n=20 HH12 embryos were assayed in at least five pharmacological experiments. The number of HH18 embryos with regular morphogenesis (optic cup and S-shaped heart) per group was obtained by the yes/no observation method (Bruns et al., 2015). Data were normalized as a function of the number of embryos presenting regular morphogenesis in each group. Curve adjusting and statistical analysis were done using the program GraphPad Prism 6.00 for Windows (GraphPad Software, Inc., La Jolla, CA, USA). Concentration–response curves were adjusted by non-linear regression. Statistical analyses were performed for comparison of the concentration–response curves and the difference was considered significant at P<0.05 (GraphPad Software, Inc., accessed August 2017, www.graphpad.com; www.graphpad.com/guides/prism/6/statistics/; www.graphpad.com/guides/prism/6/curve-fitting/).

RESULTS

Effects of calmidazolium and forskolin on eye and heart morphogenesis

In the present study, the external morphological characters of the 66 h chick embryos from the basal and the control groups were in accordance with the description of Hamburger and Hamilton (1992) for the HH18 stage chick embryo (Fig. 1A–C). The participation of calmodulin in eye and heart morphogenesis was assessed with the calmodulin inhibitor calmidazolium. All 66 h chick embryos from eggs inoculated with 400 µmol l^{-1} calmidazolium (4 nmol 10 $µl^{-1}$) presented optic cups with an indistinct inner contour in the region of the lens/iris. Additionally, in all 66 h chick embryos from the calmidazolium group, the embryonic heart did not present the S-shape characteristic of chick embryos at HH18 stage (Fig. 1D–F, Table 1).

Forskolin was used to investigate the participation of adenylate cyclase isoforms that are dependent on the Ca^{2+}–calmodulin complex (Wang and Storm, 2003), in eye and heart morphogenesis. In a previous study, forskolin was applied at doses above 1 pmol 10 $µl^{-1}$ into the air sac of embryonated chicken eggs at 48 h of incubation, causing vascular abnormalities in the 66 h chick embryos (Sampaio et al., 2015). Here, the 66 h chick embryos from the forskolin group did not show evidence of embryotoxicity at the dose applied (1.43 pmol 10 $µl^{-1}$). In addition, the application of forskolin (300 µmol l^{-1}) before calmidazolium increased the frequency of S-shaped heart formation in 66 h chick embryos from the calmidazolium group to 100%, while the frequency of normal optic cup formation in embryos from the calmidazolium group was increased to 60% by prior application of forskolin (Table 1).

Effects of melatonin on eye and heart morphogenesis

Melatonin at 0.17, 1.7 and 17 µmol l^{-1} was not embryotoxic at the doses used (0.000017, 0.0017 and 0.17 nmol 10 $µl^{-1}$) (Fig. 1J–L, Table 1). It was applied at the highest dose before calmidazolium to investigate a possible influence of melatonin on eye and heart morphogenesis. Melatonin increased the frequency of regular optic cup and S-shaped heart formation in 66 h chick embryos from the calmidazolium group to 100% (Table 1).

As melatonin and calmidazolium inhibit calmodulin, melatonin could be preventing the dangerous effects of calmidazolium by an antioxidant activity. Therefore, 6-chloromelatonin was tested against calmidazolium effects. This high-affinity, non-selective melatonin receptor agonist binds calmodulin (Benitez-King et al., 1993), but, unlike melatonin, it is not a free radical scavenger (Reiter et al., 2002). No embryotoxicity was observed in 66 h chick embryos developing under the 6-chloromelatonin doses used in this study (0.0028 nmol 10 $µl^{-1}$ to 4 nmol 10 $µl^{-1}$). At the highest concentration, 6-chloromelatonin (280 µmol l^{-1}) increased the frequency of S-shaped heart formation in 66 h chick embryos from the calmidazolium group to 100%, while it increased the frequency of normal optic cup formation to only 50% in the same group (Table 1).

Mel1a immunoreactivity in HH12 and HH18 whole-chick embryos

Previously, a selective ligand for the Mel1a melatonin receptor was not available. Here, a Mel1a immunofluorescence assay was performed in HH12 and HH18 whole embryos from the basal group to investigate the presence of this receptor. In these assays, HH12 and HH18 embryos from the Mel1a antibody negative control (embryos incubated in blocking solution containing the Mel1a antibody adsorbed to Mel1a peptide) and from the secondary

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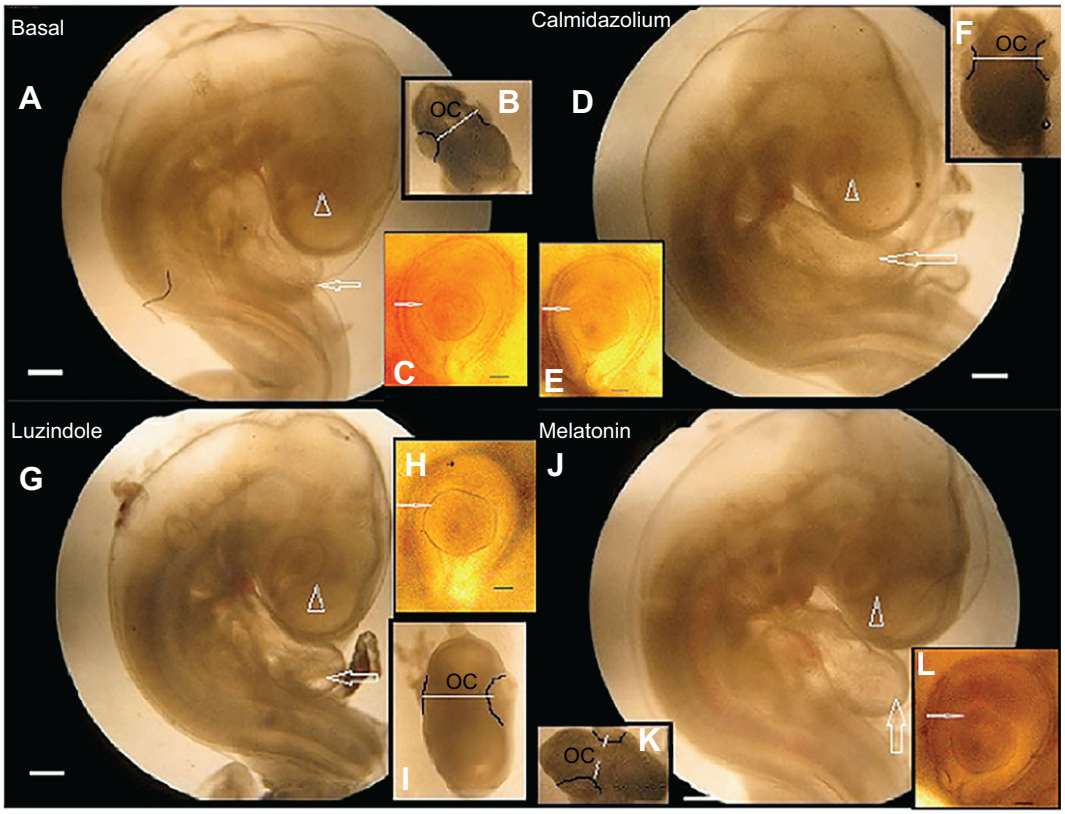

Fig. 1. External morphology of the representative 66 h chick embryos from the basal, calmidazolium, luzindole and melatonin groups. Representative images of the freshly prepared embryos immersed in phosphate-buffered saline (PBS) were obtained by a digital camera coupled to a simple microscope. (A,D,G,J) Images of whole embryos in lateral view. Arrows point to the rudimentary heart, which is S-shaped in the embryos from the basal, luzindole and melatonin groups, but not in embryos from the calmidazolium group. Arrowheads point to the optic cup (OC). (B,F,I,K) Images of whole embryo heads in dorsal view. A white line links the optic cups (OC; base drawn in black). Note that the optic cups appear as a semi-circle in the embryo from the basal (B) and melatonin (K) groups, as a cylinder in the embryo from the luzindole group (I), and as a punctate ledge in the embryo from the calmidazolium group (F). Whole embryos and embryo head scale bar: 50 mm. (C,E,H,L) Images of whole-embryo heads in lateral view, showing a regular optic cup (C,L) and an anomalous optic cup (E,H). Arrows point to the lens/iris region. Scale bar: 0.07 mm. C, E, H and L had identical enhanced contrast using ImageJ software. Representative results were obtained from *n*=20 embryos assayed in at least three independent experiments. Drug concentrations: calmidazolium, 400 μmol l^{-1}; luzindole, 1 mmol l^{-1}; melatonin, 17 μmol l^{-1}.

antibody control (embryos incubated without primary antibody) did not present the Texas Red immunofluorescence signal.

The HH12 chick embryo had a C-shaped heart, which was segmented in the primitive conus, the first ventricular bend and the rudimentary atrium (Männer, 2000; Moorman and Christoffels, 2003). Mel1a-like melatonin receptor immunoreactivity was observed in the outflow tract, including the primitive conus, and in the atrium–ventricular canal (Fig. 2A). The three different morphological sections of the HH12 straight heart tube – the primitive conus, the first ventricular bend and the rudimentary atrium – are shown in Fig. 2B. In eggs containing either HH12 or HH18 chick embryos, Mel1a-like receptor immunoreactivity was observed in the proximal extra-embryonic vessels and in the main vessels from the area vasculosa (Fig. 2C). In the HH18 chick embryos, Mel1a-like receptor immunoreactivity was observed in the outer and inner edges of the optic cup, and in the lens/iris region (Fig. 3A). In the S-shaped heart of the HH18 chick embryo, Mel1a-like receptor immunoreactivity was observed in the end of the primitive conus and in the first ventricle bend (Fig. 3B).

Melatonin receptor pharmacology in chick embryos

The presence of the Mel1a melatonin receptor in HH12 and HH18 whole embryos indicated that melatonin can prevent the defective phenotype of the embryos from the calmidazolium group by binding to membrane receptors. Hence, the non-selective melatonin receptor agonist 6-chloromelatonin, the non-selective melatonin receptor antagonist luzindole, the Mel1b/MT2 melatonin antagonist 4-P-PDOT and the Mel1c melatonin receptor antagonist prazosin were used to investigate the involvement of melatonin membrane receptors in the effects of melatonin on chick eye and heart morphogenesis.

Chick embryos developing in the presence of the melatonin receptor antagonists presented phenotypic abnormalities (Table 1). The embryos from the 500 or 1000 μmol l^{-1} luzindole groups had an S-shaped heart at the doses applied (5 or 10 nmol 10 μl^{-1}). However, in 100% of the embryos from this treatment group we observed a defective optic cup, with an indistinct inner contour in the lens/iris region, which was on a plain above the optic cup (Fig. 1G–I). The highest melatonin dose prevented this phenotype in 100% of the embryos from the luzindole group. The same occurred when 300 μmol l^{-1} forskolin (1.43 pmol 10 μl^{-1}) or 280 μmol l^{-1} 6-chloromelatonin (4 nmol 10 μl^{-1}) was applied, instead of 17 μmol l^{-1} melatonin (0.17 nmol 10 μl^{-1}) (Table 1).

In 66 h chick embryos developing in the presence of the Mel1b/MT2 antagonist 0.1 μmol l^{-1} 4-P-PDOT (0.001 nmol 10 μl^{-1}), a well-formed optic cup was present, while the rudimentary heart did not present the S-shaped phenotype. Prior treatment with 17 μmol l^{-1} melatonin (0.17 nmol 10 μl^{-1}), 280 μmol l^{-1}

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Table 1. Eye and heart morphogenesis are affected by different treatments applied into the air sac of 48 h chicken eggs (*n*=20)

Treatment	No. embryos with normal optic cup*	No. embryos with regular S-shaped heart*	HH12/HH18‡ (*n*/*n*)
None	20	20	20/20
Distilled water§	20	20	20/20
Melatonin (0.17 µmol l^{-1})	20	20	20/20
Melatonin (1.7 µmol l^{-1})	20	20	20/20
Melatonin (17 µmol l^{-1})	20	20	20/20
6-Chloromelatonin (280 µmol l^{-1})	20	20	20/20
Forskolin (300 µmol l^{-1})	20	20	20/20
Calmidazolium (400 µmol l^{-1})	**0**	**0**	20/20
Calmidazolium (400 µmol l^{-1})+melatonin (17 µmol l^{-1})	**20**	**20**	20/20
Calmidazolium (400 µmol l^{-1})+6-Chloromelatonin (280 µmol l^{-1})	**10**	**20**	20/20
Calmidazolium (400 µmol l^{-1})+forskolin (300 µmol l^{-1})	**8**	**20**	20/20
Luzindole (500 µmol l^{-1}; 1000 µmol l^{-1})	**0**	20	20/20
Luzindole (1000 µmol l^{-1})+melatonin (17 µmol l^{-1})	**20**	20	20/20
Luzindole (1000 µmol l^{-1})+6-chloromelatonin (280 µmol l^{-1})	**20**	20	20/20
Luzindole (1000 µmol l^{-1})+forskolin (300 µmol l^{-1})	**20**	20	20/20
4-P-PDOT (0.1 µmol l^{-1})	20	**0**	20/20
4-P-PDOT (0.1 µmol l^{-1})+melatonin (17 µmol l^{-1})	20	**20**	20/20
4-P-PDOT (0.1 µmol l^{-1})+6-chloromelatonin (280 µmol l^{-1})	20	**20**	20/20
4-P-PDOT (0.1 µmol l^{-1})+forskolin (300 µmol l^{-1})	20	**20**	20/20

In each case, 10 µl (§10 or 20 µl) of each drug solution was inoculated into the air sac of the 48 h chicken egg.
*Number of normal embryos per total number of embryos assayed (*n*=20).
‡Number of fertilized eggs inoculated at 48 h incubation (HH12 embryos) per number of viable embryos at 66 h incubation (HH18). HH12 and HH18 relate to the series of regular stages numbered from 1 to 46 as per Hamburger and Hamilton (1992).
Melatonin, 6-chloromelatonin and forskolin were applied 10 min before calmidazolium, luzindole and 4-P-PDOT. Compound effects are in bold.

6-chloromelatonin (4 nmol 10 μl^{-1}) or 300 µmol l^{-1} forskolin (1.43 pmol 10 μl^{-1}) prevented the irregular heart phenotype in 100% of embryos from the 4-P-PDOT group (Table 1). Excision from the egg was enough to cease rudimentary heart beating in the 66 h chick embryos from the basal, control, melatonin, 6-chloromelatonin and luzindole groups. However, this was not the case for 66 h chick embryos from the 4-P-PDOT-group, which presented rhythmical rudimentary heart beats for at least 2 h following excision from the eggs. This sustained heart beat was not present in 66 h chick embryos that developed in eggs inoculated (into the air sac) with 17 µmol l^{-1} melatonin (0.17 nmol 10 μl^{-1}; *n*=20), 300 µmol l^{-1} forskolin (1.43 pmol 10 μl^{-1}; *n*=20) or 280 µmol l^{-1} 6-chloromelatonin (4 nmol 10 μl^{-1}; *n*=20), 10 min before 0.1 µmol l^{-1} 4-P-PDOT (0.001 nmol 10 μl^{-1}) application. The frequency of 66 h chick embryos with regular heart morphogenesis (S-shaped heart) in each 4-P-PDOT group was dependent on the concentration applied ($logIC_{50}$=−6.49±0.16, R^2=0.95) (Fig. 4A). In addition to the abnormal rudimentary heart phenotype characterized by the absence of complete looping (Fig. 4B), the frequency of the chick embryos presenting with ceased heart beating after excision from the egg was also reduced by 4-P-PDOT in a concentration-dependent manner ($logIC_{50}$=−6.71±0.18, R^2=0.96) (Fig. 4A). These 4-P-PDOT effects appeared to be associated because their concentration–response curves were not statistically different (*P*=0.2954, α=0.05, $F_{1,8}$=1.253) (Fig. 4A).

6-Chloromelatonin was applied before luzindole and 4-P-PDOT to confirm that these ligands are binding to melatonin receptors in 48–66 h chick embryos. The frequency of 66 h chick embryos with an S-shaped heart from the 0.1 µmol l^{-1} 4-P-PDOT group was increased by prior application of 6-chloromelatonin in a concentration-dependent manner ($logEC_{50}$=−4.83±0.24, R^2=0.91) (Fig. 5A). The frequency of 66 h chick embryos with normal eye cups from the 1 mmol l^{-1} luzindole group was also increased in a concentration-dependent manner by prior application of 6-chloromelatonin ($logEC_{50}$=−5.55±0.05, R^2=0.99) (Fig. 5B). Statistical analysis of these concentration–response curves showed that they were different (*P*=0.0252; α=0.05; $F_{1,6}$=8.782) (Fig. 5).

Prazosin is an α1-adrenergic receptor antagonist (Studer and Piepho, 1993), an NRH:quinone reductase antagonist (Pegan et al., 2011) and a Mel1c melatonin receptor antagonist (Chen et al., 2016). In this study, prazosin (0.1 µmol l^{-1} to 1 mmol l^{-1}) was used to assess the activity of the Mel1c melatonin receptor in developing chick embryos. All embryos from the prazosin groups had a small S-shaped heart (Fig. 6E) at all doses applied (0.0000042 to 0.042 nmol 10 μl^{-1}; *n*=20 per dose), and embryos from the 1 mmol l^{-1} prazosin group presented a slight edema. These effects remained even in the presence of 17 µmol l^{-1} melatonin (0.17 nmol 10 μl^{-1}; *n*=20). At lower prazosin doses (0.0000042 and 0.000042 nmol 10 μl^{-1}), these rudimentary hearts gave rhythmic bursts that remained even when the 66 h chick embryos were excised from the eggs (*n*=20 per dose), except when 17 µmol l^{-1} melatonin (0.17 nmol 10 μl^{-1}) was applied before prazosin (*n*=20).

Prazosin also triggered an alteration in rudimentary eye morphogenesis. The reduction in the frequency of 66 h chick embryos with normal eye cups from prazosin groups was concentration dependent ($logEC_{50}$=−5.03±0.02, R^2=0.90) (Fig. 6A). Melatonin shifted the prazosin concentration–response curve to the right (Fig. 6A). The defective rudimentary eye observed in the 66 h chick embryos from the prazosin groups presented the lens/iris region on a plain above the inner edge of the defective optic cups (Fig. 6C–E), when compared with the 66 h chick embryos from the basal group (Fig. 6B and Fig. 1A,C).

DISCUSSION

Melatonin modulates chick embryo morphogenesis via calmodulin

Over the developmental time frame investigated in the present study, the inhibition of calmodulin by calmidazolium impeded eye and heart morphogenesis. This result was in agreement with a

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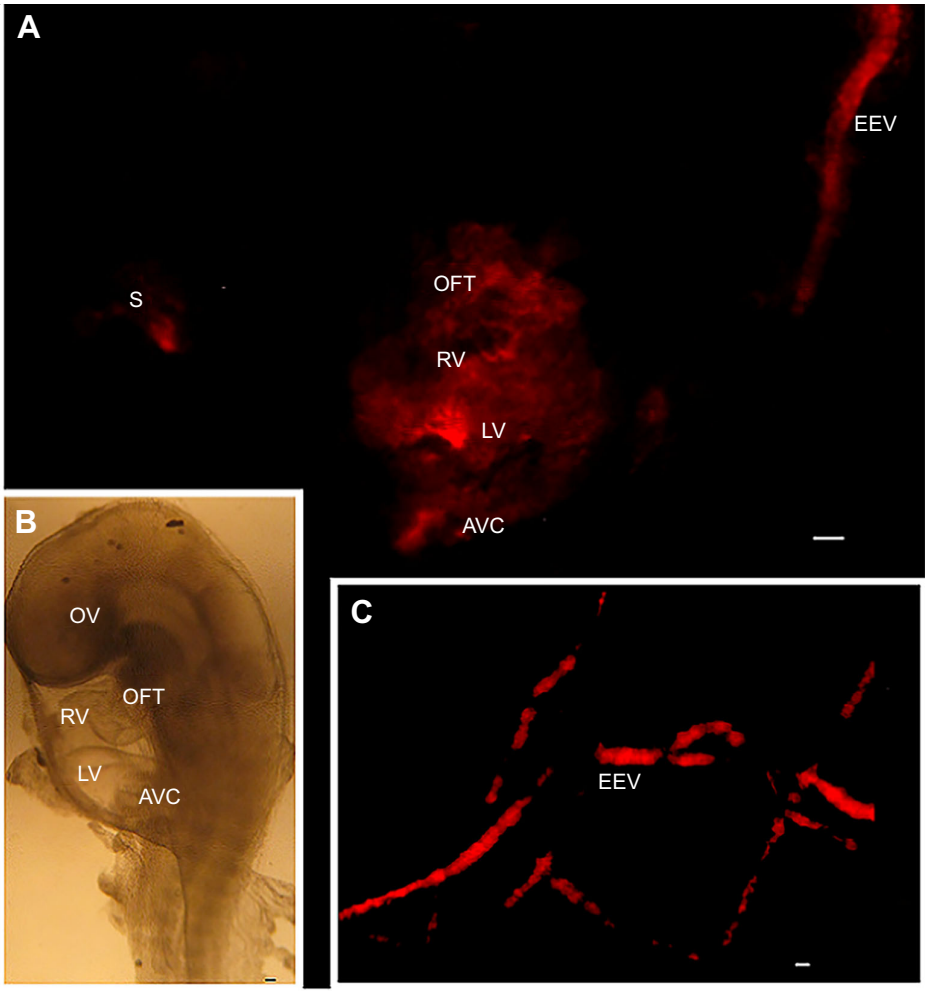

Fig. 2. Mel1a melatonin receptor immunoreactivity in the HH12 chick embryo and extraembryonic vessels visualized with Texas Red fluorescence. (A) Image of the HH12 embryo in the right ventral position. Rudimentary heart presenting Mel1a immunoreactivity in the outflow tract (OFT), right ventricle (RV), left ventricle (LV) and atrium–ventricular canal (AVC), especially in the midline of the straight heart tube, which at HH12 is on the right side. The labeled somite (S) is in line with the heart. (B) Image of a freshly prepared HH12 chick embryo in left dorsal position, showing the optic vesicle (OV) and the rudimentary C-shaped heart with outflow tract (OFT), right ventricle (RV), left ventricle (LV) and atrium-ventricular canal (AVC). (C) Mel1a immunoreactivity in the extraembryonic vessels (EEV) of the HH18 chick embryo in the lateral position. Mel1a immunoreactivity was not observed in the negative control embryos previously incubated in MT1 antibody plus MT1 blocking peptide solution. Representative results were obtained from *n*=5 embryos assayed in three independent experiments. Scale bars: (A) 30 μm; (B) 20 μm; (C) 25 μm.

previous study in chick embryos that linked the calcium-binding protein calmodulin with the formation of the optic cup (Brady and Hilfer, 1982) and the S-shaped heart (Midgett et al., 2014). Additionally, the results from experiments using forskolin point to a function for Ca^{2+}–calmodulin-dependent adenylate cyclase in heart and eye morphogenesis, even in developmental stages characterized by low levels of adenylate cyclase in chick embryos (Hejnova et al., 2014).

It was previously suggested that melatonin and serotonin modulate organogenesis in embryonated avian eggs (Olszańska et al., 2007). This hypothesis was supported by the present study because supplementation with melatonin prevented the defective

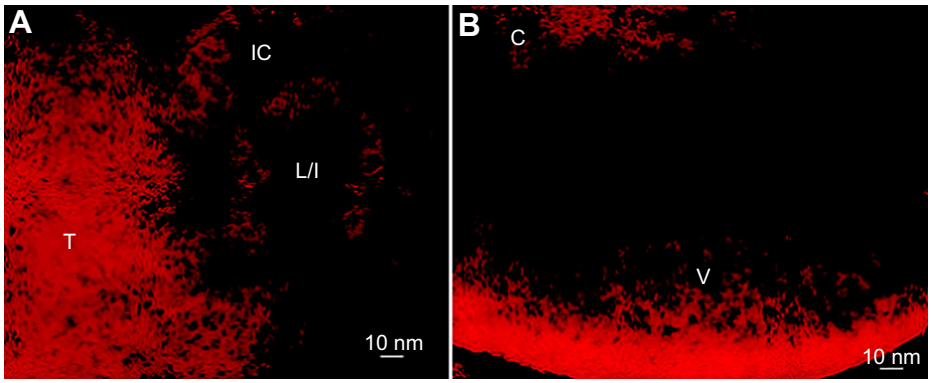

Fig. 3. Mel1a melatonin receptor immunoreactivity in the rudimentary heart and optic cup of the HH18 chick embryo visualized with Texas Red fluorescence. Image of the HH18 embryo in the front-lateral position. (A) Mel1a immunoreactivity is distributed in the optic cup inside edges (IC) and in the contour of the region corresponding to the lens/iris (L/I). Strong immunoreactivity appears in the telencephalon region (T). (B) Mel1a immunoreactivity is in the primitive conus region of the right ventricle (C) and in the edge of the primitive left ventricle (V). Mel1a immunoreactivity was not observed in embryos from the negative control that were previously incubated in solution containing MT1 antibody plus MT1 blocking peptide. Representative results were obtained from *n*=5 embryos assayed in three independent experiments.

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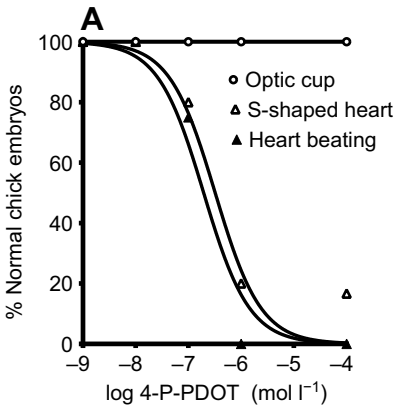

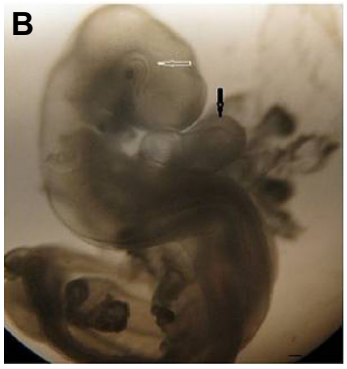

Fig. 4. Effect of 4-P-PDOT on chick embryo morphogenesis. (A) Effect of increasing concentrations (mol l^{-1}) of 4-P-PDOT (applied into the air sac of 48 h embryonated chicken eggs) on the frequency of S-shaped heart patterning, normal cessation of heart beating post-excision from the egg, and optic cup formation in 66 h chick embryos. Each point corresponds to the percentage of normal chick embryos per treatment group. (B) Representative image of a freshly prepared 66 h chick embryo immersed in PBS from the 0.1 µmol l^{-1} 4-P-PDOT group. Black arrow points to the rudimentary heart without S-shaped patterning. White arrow points to the optic cup. Scale bar: 50 mm. *n*=20 embryos per group were assayed in three experiments.

phenotypes caused by calmidazolium or melatonin receptor antagonists in chick embryos. Therefore, the melatonin present in the yolk sac of embryonated chicken eggs can modulate eye and heart morphogenesis. The coupling of melatonin receptors with the Gi protein is the most studied mechanism underlying melatonin effects. Therefore, it was hypothesized that the melatonin antagonists disrupted chick embryo morphogenesis by increasing basal cAMP levels. However, forskolin prevented the luzindole- and 4-P-PDOT-dependent effects on morphogenesis. Thus, effects of melatonin on chick embryo morphogenesis did not appear to occur by inhibition of cAMP levels.

The higher 6-chloromelatonin dose only partially reduced the frequency of embryos from the calmidazolium group presenting with defective eye morphogenesis. Therefore, melatonin appears to be functioning not only via membrane receptors but also possibly by melatonin binding sites. Taking into account that optic cup formation occurs by rearrangement of the cytoskeleton (Fuhrmann, 2010), which was modulated by melatonin through calmodulin (Benítez-King and Antón-Tay, 1993), melatonin may trigger chick embryo eye morphogenesis by positive modulation of calmodulin pathways.

Role of Mel1a and Mel1b in S-shaped heart formation

The mammalian counterpart of the Mel1a melatonin receptor MT1 was found in the bovine blastocysts (Sampaio et al., 2012). In the stages corresponding to the middle of late organogenesis, this receptor was also shown by immunoassay to occur in the embryonic chick whole retina (Sampaio, 2013), and in whole-embryo and juvenile retina of Testudinata species (Silva and Sampaio, 2014). In whole chick embryos, Mel1a-like receptor immunofluorescence had a membrane protein pattern, like that found in the entire chick embryo retina (Sampaio, 2013) and in the testudinatum whole embryo and whole retinas (Silva and Sampaio, 2014). In this study, the Mel1a-like receptor was found in the HH12 chick embryo. The Mel1a melatonin receptor was shown to occur in the outflow tract and in the proximal vessels of the rudimentary heart in chick embryos. This receptor was also found in cardiovascular tissues of the embryonic and post-hatching chick (Pang et al., 2002) and in the heart of the mature mouse (Naji et al., 2004). As melatonin has a protective role in the mature cardiovascular system via receptors (Paulis and Simko, 2007), this neurohormone may act via Mel1a from early stages of embryonic development. Also, Mel1a was found in the main vessels of the area vasculosa and its mammalian counterpart MT1 was found in the human placenta (Soliman et al., 2015), suggesting that this melatonin receptor takes part in melatonin functions in vertebrate development.

The area vasculosa vessels in embryonated eggs were apparently unaffected by all treatments tested, except for prazosin (1 mmol l^{-1}), which caused a slight hemorrhagic point in the sinus terminal region of the area vasculosa. Therefore, the calmidazolium-, luzindole- and 4-P-PDOT-triggered disruption of chick embryos morphogenesis did not appear to be a function of the alteration of embryonic vessel permeability that causes edema syndrome, which was the main cause of embryotoxicity in chick embryos (Chernoff and Rogers, 2010), at least in the doses used in the present study. Indeed, melatonin was not acting as a direct free radical scavenger (Maitra and Hasan, 2016) because 6-chloromelatonin, which is not an antioxidant like melatonin, prevented the appearance of the damaged eye and heart phenotypes observed in embryos from the calmidazolium and melatonin receptor antagonist groups.

In mature tissues, melatonin effects on blood vessel diameter were dependent on concentration, as shown in a study using vascular smooth muscles from rat (Fischer 344) caudal arteries, where low melatonin concentrations potentiated vasoconstriction induced by phenylephrine, possibly by the MT1 melatonin receptor, while higher melatonin concentrations resulted in vasodilatation, which was blocked by the selective MT2 antagonist 4-P-PDOT (Doolen et al., 1998). In heart morphogenesis, the balance between vasoconstriction and vasodilatation is necessary to stabilize the form and function of the heart, ensuring complete cardiac looping, which will create the asymmetry in the heart chambers (Midgett et al., 2014). In the present study, Mel1a was located in the embryonic chick heart and vessels, and Mel1a and Mel1b blockage by the non-selective melatonin receptor antagonist luzindole did not hamper rudimentary heart looping. Also, the heart from embryos that developed in the presence of the Mel1c melatonin receptor antagonist prazosin (a vasodilator) was S-shaped. In contrast, the Mel1b melatonin receptor antagonist 4-P-PDOT affected both rudimentary heart patterning and beating. The same preventative effect that melatonin had against defective heart morphogenesis in embryos from the 4-P-PDOT group was shown to occur when melatonin was replaced by 6-chloromelatonin. Thus, the present results suggest that the Mel1a and Mel1b melatonin receptors were involved in the pathways responsible for maintaining the balance between vasoconstriction and vasodilatation that resulted in the S-shaped heart patterning, and that an imbalance in favor of vasodilatation is less detrimental than vasoconstriction for rudimentary heart looping.

Role of Mel1a and Mel1c in eye cup formation

The Mel1a melatonin receptor was observed in the inner edges of the optic cup and iris/lens region of HH18 stage whole embryos, but it was not observed in the optic vesicles of HH12 stage embryos.

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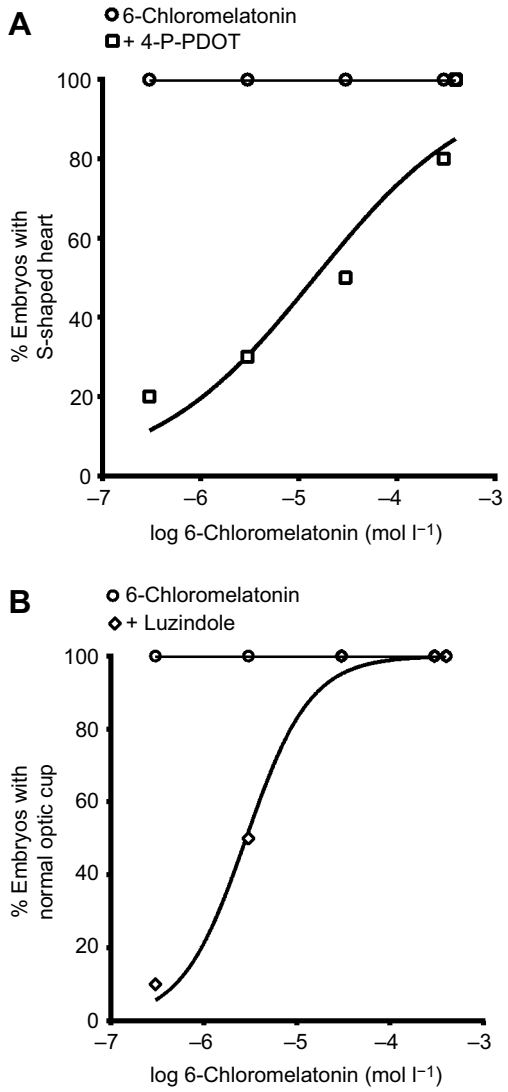

Fig. 5. Effects of 6-chloromelatonin on 4-P-PDOT and luzindole disruption of chick embryo morphogenesis. Effect of increasing concentrations of 6-chloromelatonin (applied into the air sac of 48 h-incubated embryonated chicken eggs) on the frequency of S-shaped heart and optic cup formation in 66 h chick embryos. (A) 6-Chloromelatonin (mol l^{-1}) was applied 10 min before 4-P-PDOT (0.1 µmol l^{-1}). (B) 6-Chloromelatonin (mol l^{-1}) was applied 10 min before luzindole (1 mmol l^{-1}). Each point corresponds to the percentage of normal embryos per treatment group. *n*=20 embryos per group were assayed in three experiments.

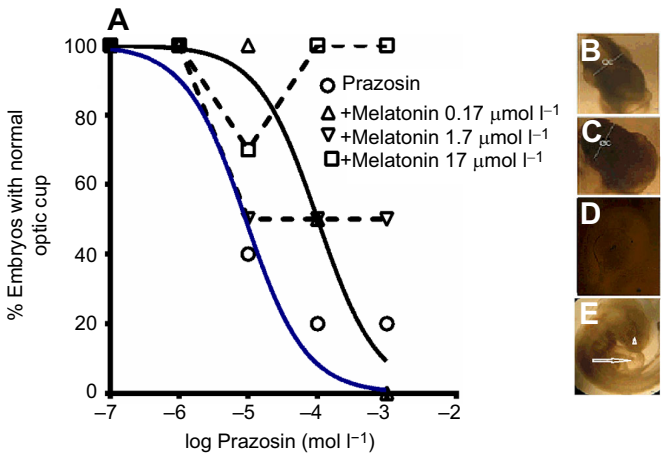

Fig. 6. Effect of melatonin on prazosin-disturbed optic cup formation. (A) Effect of increasing concentrations of prazosin (mol l^{-1}) (applied into the air sac of 48 h-incubated embryonated chicken eggs) on the frequency of normal optic cup formation in 66 h chick embryos. Melatonin was applied 10 min before prazosin. Normal eye cups were present in 100% of the embryos treated only with melatonin (0.17, 1.7 and 17 µmol l^{-1}). Two points (circles) of the prazosin concentration–response curve (blue line) are masked by the squares. Each point corresponds to the percentage of normal embryos per treatment group. (B–E) Images of the freshly prepared 66 h chick embryos immersed in PBS from the basal (B) and 1 mmol l^{-1} prazosin group (C–E). B and C show the dorsal views of the embryo head, highlighting the optic cup pairs with a white line. D shows a lateral view of the rudimentary eye. Scale bar: 0.02 mm. E is a lateral view of the whole 66 h chick embryo. Arrow points to the heart, and arrowhead points to the optic cup. B, C and E scale bars: 50 mm. *n*=20 embryos per group were assayed in three experiments.

Therefore, we infer that luzindole affected developmental events that occurred after the invagination of the optic vesicles. Interestingly, the lens vesicle began to form through invagination of the lens placode after HH14, and it was not dependent on optic cup formation, which occurred from HH11 to HH13+ stages by contact between the optic vesicle and the pre-lens ectoderm (Hyer et al., 2003; Fuhrmann, 2010). The 66 h chick embryos with anomalous rudimentary eyes from the luzindole, prazosin and calmidazolium groups presented, to different degrees, a defective localization of the lens/iris region. Calmidazolium, luzindole and prazosin inoculation at HH12 seemed to induce some issues in the lens placode and optic vesicle coordinate invaginating movements. Melatonin prevented all these issues and so the invagination of the lens placode was probably dependent on melatonin. Embryos from the luzindole group had defects in the eye cup that did not mirror those of the chick embryos from the prazosin group or the calmodulin group. It could be inferred, therefore, that luzindole did not antagonize only the Mel1c melatonin membrane receptor or the calcium-binding protein calmodulin, because it also appeared to antagonize a Mel1a-like membrane receptor. Consequently, the Mel1a and Mel1c membrane receptors were probably taking part in the pathways triggered by melatonin in optic cup formation.

Melatonin receptor pharmacology in developing chick embryos

Melatonin receptors are coupled with G-proteins (Jockers et al., 2016). Hence, melatonin binding affinity would be a function of the drug→receptor→G-protein ternary complex (Kenakin, 2017). It was previously shown that 4-P-PDOT and luzindole did not inhibit the melatonin effect on cAMP accumulation in chick retinas in the early differentiation stages (embryonic day 8). The 4-P-PDOT antagonist effect was shown to occur only after early synaptogenesis (embryonic day 14), and the luzindole antagonist effect was shown to occur only after retinal differentiation (2-days post-hatching) (Sampaio, 2008). The same developmental profile for the luzindole antagonist effect was found in differentiating retinal cells in culture (Sampaio et al., 2005; Sampaio and Markus, 2010). Here, rudimentary heart morphogenesis was not hampered by luzindole but it was by 4-P-PDOT, which was not capable of impeding optic cup formation. Additionally, a small inhibitory effect of 6-chloromelatonin on the action of 4-P-PDOT in the rudimentary heart was observed when compared with its effectiveness in the prevention of luzindole-triggered disruption in the rudimentary eye, which is less differentiated than the heart, in the developmental time frame studied here. In a [^{3}H]melatonin competition binding assay, carried out in mature cells, with a low level of the receptors coupled

to G-proteins, the ranked K_i order of potency was melatonin≥6-chloromelatonin>4-P-PDOT>luzindole. In the same cells, but with a high level of the receptors coupled to G-proteins, the ranked order of potency was reversed (Legros et al., 2014). Another study showed that in the heart, the Gi and Gs protein cellular content was very low before chick embryonic day 4, increasing only from day 4 to the levels observed in post-hatched chicks (Hejnova et al., 2014). The amount of G-protein present in the tissues of the embryos used in this study must be low given the high relative efficacy of 6-chloromelatonin against luzindole-triggered disruption of optic cup formation. The low level of G-protein may be responsible for the low potency of the melatonin receptor antagonist. This is because both the potency of the melatonin receptor antagonists and the G-protein levels were dependent on tissue differentiation. Also, the appearance of the Mel1a melatonin receptor seemed to depend on the progress of organogenesis because it was observed in the rudimentary eye after it appeared in the heart. Accordingly, in the developmental time frame studied here, the melatonin-triggered effects on eye morphogenesis appear to occur more via calmodulin than via melatonin receptors. However, the participation of melatonin receptors seems to be more significant than that of calmodulin in the effects of melatonin on heart morphogenesis.

Conclusions

Our results suggest that the melatonin content of the yolk sacs of embryonated chicken eggs modulates S-shaped heart patterning and optic cup formation in the embryo. Melatonin appears to signal via calmodulin, Mel1a and Mel1c membrane receptors in optic cup formation, but mainly via Mel1a and Mel1b membrane receptors in heart morphogenesis. Consequently, disruption in the melatonin system in these developmental stages can lead to heart and optical congenital diseases.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: L.F.S.S.; Methodology: R.C.N., L.F.S.S.; Validation: R.C.N., L.F.S.S.; Formal analysis: R.C.N., L.F.S.S.; Investigation: R.C.N., L.F.S.S.; Resources: L.F.S.S.; Writing - original draft: R.C.N., L.F.S.S.; Writing - review & editing: L.F.S.S.; Visualization: L.F.S.S.; Supervision: L.F.S.S.; Project administration: L.F.S.S.; Funding acquisition: L.F.S.S.

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