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Cellular localization of melatonin receptor Mel1b in pigeon retina☆

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ABSTRACT

Melatonin, an important neuromodulator involved in circadian rhythms, modulates a series of physiological processes via activating its specific receptors, namely Mel1a (MT_1), Mel1b (MT_2) and Mel1c receptors. In this work, the localization of Mel1b receptor was studied in pigeon retina using double immunohistochemistry staining and confocal scanning microscopy. Our results showed that Mel1b receptor widely existed in the outer segment of photoreceptors and in the somata of dopaminergic amacrine cells, cholinergic amacrine cells, glycinergic AII amacrine cells, conventional ganglion cells and intrinsically photosensitive retinal ganglion cells, while horizontal cells, bipolar cells and Müller glial cells seemed to lack immunoreactivity of Mel1b receptor. That multiple types of retinal cells expressing Mel1b receptor suggests melatonin may directly modulate the activities of retina via activating Mel1b receptor.

1. Introduction

In vertebrates indoleamine hormone melatonin, acting as a neuromodulator, regulates various physiological processes, such as sleep-wake cycle, pubertal development, core body temperature, oncogenesis, immune function and seasonal adaptation (Pandi-Perumal et al., 2008), in the central nervous system (CNS). In nonmammalian vertebrates three subtypes of specific receptor, namely Mel1a (corresponds to mammalian MT_1), Mel1b (corresponds to mammalian MT_2) and Mel1c receptor, have been isolated, cloned, and sequenced (Pandi-Perumal et al., 2008; Reppert et al., 1995; Reppert et al., 1994; Tosini et al., 2012; Huang et al., 2013; Klosen et al., 2019). In the vertebrate retina melatonin, acting as a paracrine hormone, is synthesized and secreted in a circadian manner with high level at night and low level during the daytime, and regulates various types of neurons in retina to modulate various visual activities, including resting potentials (Fischer et al., 2008; Nao-i et al., 1989), membrane conductances (Cosci et al., 1997), and multiple types of membrane channels (van den Top et al., 2001; Yang et al., 2011).

Recent works have described significant high-resolution room-temperature X-ray free electron laser structures of human MT_1 receptor (Stauch et al., 2019) and human MT_2 receptor (Johansson et al., 2019) respectively. Then drug development will be facilitated the design of future tool compounds and therapeutic agents to contribute to maintain natural circadian rhythms against shift work, travel, and ubiquitous artificial lighting (Huang et al., 2013; Wiechmann and Sherry, 2013). Once the compounds or agents are developed, they will be bound to use to target melatonin receptors.

Our previous study showed the expression of MT_1 and MT_2 receptors in the rat, and we found the co-expression of MT_1 and MT_2 receptors in M1-type intrinsically photosensitive retinal ganglion cells (GC) (Sheng et al., 2015). However, current studies suggests that the cellular expression of these receptors is highly species-dependent and neuron subtype-dependent (Huang et al., 2013). That may be indeed related to the divergent biological rhythms between nocturnal animals and diurnal animals. Lots of works about the localization of melatonin receptors have been done in the retina of nocturnal animals, such as rat (Yang et al., 2011; Sheng et al., 2015; Mennenga et al., 1991; Fujieda

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et al., 1999; Zhao et al., 2010), mouse (Sengupta et al., 2011; Baba et al., 2009) and guinea pig (Fujieda et al., 2000). Although these studies are fruitful, most of them focus on MT_1 receptor. In other words, information regarding retinal expression of MT_2 or Mel1b receptor in vertebrates is rather scant, letting alone MT_2 or Mel1b receptor in the retina of diurnal animals. In diurnal animals, such as human, melatonin exerts its neuromodulation widely (Reppert et al., 1995; Wiechmann and Smith, 2001; Scher et al., 2003; Scher et al., 2002; Ikegami et al., 2009). Morphological study on mRNA expression shows that melatonin receptors exist in different layers in the chick retina (Natesan and Cassone, 2002). Wiechmann and his colleagues' work shows that Mel1b receptor is located in rod and cone photoreceptors, horizontal cells (HCs) and GCs, but not in dopaminergic and GABAnergic amacrine cells (AC) in retina of *Xenopus laevis* (Wiechmann et al., 2004), which is quite different from our results in pigeon retina (see below). Above all, detailed research on the systematical cellular localization of MT_2 or Mel1b receptor is urgently needed both in retina of diurnal animal and nocturnal animal.

The flying bird retina with one or two foveae has high acuity regions. The pigeon has two regions of high retinal GCs density and one fovea (Querubin et al., 2009), which makes the visual acuity of the pigeon be comparable to it in human eyes (Rodrigues et al., 2016). These characteristics prompt us to introduce the pigeon as a new model system to study the vision. The purpose of this work is to demonstrate the cellular localization of Mel1b receptor in pigeon retina. We found that immunofluorescence for Mel1b receptor existed in rods and cones, dopaminergic ACs, cholinergic ACs, glycinergic AII ACs, conventional GCs and intrinsically photosensitive retinal GCs, while no obvious labeling for Mel1b receptor was detected in the somata of HCs, bipolar cells (BCs) and Mülller cells.

2. Materials and methods

2.1. Animals

1-year-old pigeons (*Columba livia* domestica) with free access to food and water were used in this research. Pigeon house was kept at a temperature from 22 °C to 28 °C and under a humidity of 40 ± 10%. Pigeons were housed for at least 20 days in a 14-h light (~1000 lx): 10-h dark (LD) cycle before experiments. To avoid any possible diurnal impacts on protein expression, all retinas were harvested and immediately fixed 5–7 h after light onset (Zeitgeber Time 5–7). A total of 12 pigeons were sacrificed in the present study. Use and handling of animals were strictly in accordance with the U.S. National Institutes of Health guidelines for the Care and were approved by Institutional Animal Care and Use Committees of Qilu University of Technology. All efforts were made to minimize the number of animals used and their suffering.

2.2. Tissue preparation

Pigeons were deeply anesthetized with 20% urethane and eyecups were immediately immersed in fresh 4% paraformaldehyde in 0.1 M phosphatic buffered saline (PBS, pH 7.4) for 30 min. After fixation, the eyecups were chilled sequentially in 10% (*w/v*), 20% and 30% sucrose in 0.1 M PBS at 4 °C, and then embedded in optimal cutting temperature compound (OCT, Miles Inc., Elkhart, IN, USA). A freezing microtome (Leica Microsystems, Wetzlar, Germany) was used to cut frozen sections vertically at 14 μm thickness, and sections were then mounted on gelatin chromium-coated slides and stored at −20 °C for further processing.

2.3. Antibodies

The Mel1b receptor antibody (Sheng et al., 2015), being purified from rabbits, targets the epitope of the 3rd intracellular loop (amino acid residues 232–246: (C) RKAKATRKLRLRPSD) of the mouse MT_2 receptor (1:100 dilution, AMR-032, Alomone laboratories, Jerusalem, Israel). Then a series of well-established primary antibodies (see Table 1 for a complete list) and secondary antibodies (see Table 2 for a complete list) were used during immunohistochemistry double-staining and Western blot experiments.

Table 1
Primary antibodies used in this study.

Antigen	Host	Source	Working dilution
Mel1b	Rabbit	AMR-032, Alomone laboratories, Israel	H: 1:100; W:1:1000
Rho4D2	Mouse	ab98887, Abcam plc., UK	H: 1:2000
S-opsin	Goat	sc-14363, Santa Cruz Biotechnology, USA	H: 1:500
L/M-opsin	Goat	sc-22117, Santa Cruz Biotechnology, USA	H: 1:500
CB	Mouse	300, Swant, Switzerland	H: 1:2000
ChX10	Sheep	ab16141, Abcam plc., UK	H: 1:400
TH	Mouse	T1299, Sigma, USA	H: 1:5000
ChAT	Goat	AB144P, Chemicon, USA	H: 1:800
PV	Mouse	235, Swant, Switzerland	H: 1:1000
Brn3a	Goat	sc-31984, Santa Cruz Biotechnology, USA	H: 1:800
Melanopsin	Mouse	sc-515838, Santa Cruz Biotechnology, USA	H: 1:100
CRALBP	Mouse	ab15051, Abcam plc., UK	H: 1:500

Abbreviations: Rho4D2, Rhodopsin; CB, calbindin D-28 k; ChAT, choline acetyltransferase; TH, tyrosine hydroxylase; Gly, glycine; PV, parvalbumin; CRALBP, cellular retinaldehyde-binding protein; H, immunohistochemistry; W, Western blot.

2.4. Immunohistochemistry

Sections were blocked in a buffer containing 6% normal donkey serum, 0.2% Triton X-100 in 0.1 M PBS for 1.5 h at room temperature, and then incubated with the primary antibodies in incubation buffer (3% normal donkey serum, 1% bovine serum albumin and 0.2% Triton X-100 in 0.1 M PBS) at 4 °C for 2 days. After 6 rinses in PBS, binding sites of the primary antibodies were revealed by further incubating with the fluorescent second antibodies for 1.5 h at room temperature in the dark. In control experiments, the entire procedure was identical except that the sections were incubated in a mixture of Mel1b receptor antibody and an excessive dose of corresponding immunizing peptide (AMR-032, Alomone laboratories), which were mixed for 5 h in advance. All double labeling experiments have been performed on at least 3 birds.

2.5. Confocal laser scanning microscopy and data analysis

The sections were viewed and immunofluorescence images were acquired at 40× magnification using a Fluoview FV1000 confocal laser scanning biological microscope (Olympus Corporation, Tokyo, Japan). To avoid any possible reconstruction stacking artifact, double-labeling was precisely evaluated by sequential scanning on single-layer optical sections. The settings (laser intensity, gain and contrast) of the confocal microscope were the same for the photographs shown in Fig. 1B and B'. All analysis were performed offline. Images were resized and adjusted for global brightness and contrast using the software Photoshop CS3 (Adobe Systems, San Jose, CA, USA).

2.6. Western blot

Equivalent amounts of freshly extracted retinal lysate (20 μg/lane) were electrophoresed on 10% SDS-PAGE and then electrophoretically transferred onto PVDF membranes. Non-specific binding was blocked in a blocking buffer (pH 7.6) containing 20 mM Tris-HCl, 137 mM NaCl,

Table 2
Secondary antibodies used in this study.

Description	Source	Working dilution
Alexa Fluor 488-conjugated donkey anti-rabbit IgG	A-21206, Invitrogen, USA	H: 1:200
Alexa Fluor 555-conjugated donkey anti-mouse IgG	A-31570, Invitrogen, USA	H: 1:200
Alexa Fluor 555-conjugated donkey anti-goat IgG	A-21432, Invitrogen, USA	H: 1:200
Alexa Fluor 555-conjugated donkey anti-sheep IgG	A-21436, Invitrogen, USA	H: 1:200
Horseradish peroxidase-linked donkey anti-rabbit IgG	31,458, Millipore, USA	W: 1:2000

Abbreviations: H, immunohistochemistry; W, Western blot.

0.1% Tween-20 (TBST) and 3% (*w/v*) skimmed milk for 1.5 h at room temperature. The blots were then incubated with the Mel1b receptor antibody used in immunohistochemistry experiment overnight on a shaker in 4 °C, followed by horseradish peroxidase (HRP)-linked donkey anti-rabbit IgG (see Table 2) for 1.5 h at room temperature. Immunoblots were visualized by enhanced chemiluminescence (ECL, Amersham Biosciences, Piscataway, NJ, USA), and finally captured using ChemiDoc XRS System with Image Lab software (Bio-Rad, Hercules, CA, USA). To estimate the molecular weight (MW), a widely-used pre-stained protein ladder (26,617, Thermo Scientific, Waltham, MA, USA) was used. In control experiments, the blots were incubated in a mixture of Mel1b receptor antibody and an excessive dose of corresponding antigen peptide.

3. Results

3.1. Expression profile of Mel1b receptor in pigeon retina

Western blot analysis of Mel1b receptor in the retinal homogenates of pigeon showed that the antibody against Mel1b receptor clearly recognized a robust band at approximately 45 kDa (Fig. 1A). The band of 45 kDa corresponded to the previous study (Reppert et al., 1995; Zhao et al., 2010). No such immunoreactive band was visible when the antibody was pre-incubated with the corresponding antigen peptide (Fig. 1A'). These data indicated that the protein recognized by the antibody used in this work may indeed be Mel1b receptor.

In vertical sections of pigeon retina (Fig. 1B), Mel1b receptor signal was visible in the outer retina, which may be the OS of rods and/or cones. A few Mel1b receptor signals were also clearly observed in the INL. Based on the positions and shapes of these cells, they might be HCs, BCs, ACs, displaced GCs or Müller cells. Meanwhile, immunostaining for Mel1b receptor was clear in the GCL, which could be displaced ACs and/or GCs. However, no obvious Mel1b receptor immunolabeling was detected in the outer plexiform layer (OPL) and inner plexiform layer (IPL). When the antibody was pre-treated with the antigen peptide, only a low level of background was visible (Fig. 1B'). Fig. 1B″ shows the multiple layers of retina clearly.

3.2. Cellular localization of Mel1b receptor in the rods and cones

To explore the cellular localization of Mel1b receptor in the outer retina of pigeon, we firstly conducted double fluorescent labeling experiments using the antibody against Mel1b receptor along with specific and reliable markers to mark photoreceptors. Fig. 2A-A", graphs of the vertical section from pigeon retina, double-immunolabeled with the antibodies against Mel1b receptor and Rho4D2 (a rod marker (Ghosh et al., 2009; Ritchey et al., 2010)) showed the Mel1b receptor was co-labeled with the Rho4D2. Quantitative analysis showed that Mel1b immunoreactivity signals were detectable in the OS of all rods (n = 138) observed in 10 retinal sections (see Table 3, the same below). Fig. 2B-B" showed graphs of the vertical section from pigeon retina double-immunolabeled with the antibodies against Mel1b receptor and short-wave-opsin (S-opsin, a blue cone marker (Ji et al., 2012; Ortin-Martinez et al., 2014)). Overlapping signals revealed that Mel1b receptor was detectable in the OS of blue cones. Totally, 55 S-opsin-positive blue cones observed in 9 retinal sections were all Mel1b-positive. Fig. 2C-C", graphs of the vertical section double-immunolabeled with the antibodies against Mel1b receptor and long/medium-wave-opsin (L/M-opsin, a widely used marker for red/green cone (Ji et al., 2012)). Double immunolabeling revealed that Mel1b receptor-immunoreactive was observed obviously in the OS of red/green cones (33 out of 33 cells from 3 sections).

3.3. Cellular localization of Mel1b receptor in the HCs and BCs

We further studied whether HCs and BCs expressed Mel1b receptor or not. Fig. 3A-A", graphs of the vertical section double-immunolabeled with the antibodies against Mel1b receptor and CB (a marker for HCs (Rabie et al., 1985; Liu et al., 2011)). CB-immunostained HCs were located at the distal margin of the INL. As shown in the merged image

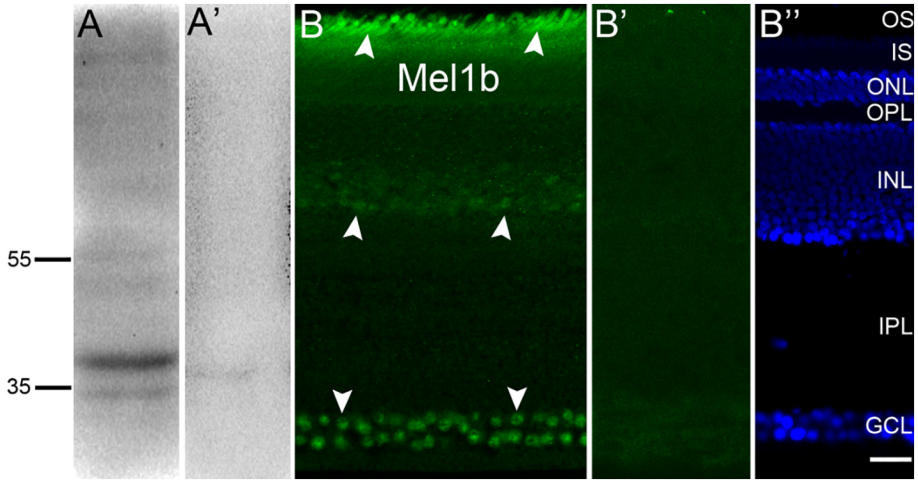

Fig. 1. Expression of Mel1b receptor in pigeon retina. (A) Western blot analysis of whole pigeon retina homogenates for the Mel1b antibody marks a main band of ~45 kDa. MW scale (kDa) is shown on the left. (A') No band was detected when primary antibody was pre-absorbed with the antigen peptide. (B) Micrograph of the immunostaining profile of the Mel1b antibody in pigeon retina. Bright fluorescent signal for Mel1b receptor exists in the OS of photoreceptors, INL and GCL. (B') Micrograph of vertical section reveals that no obvious signal is visible when the Mel1b antibody is pre-absorbed by the immunizing peptide. (B″) Micrograph of DAPI stained retina shows the multiple layers of a retinal section. Representative cells are indicated by arrowheads. OS, outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; MW, molecular weight. Scale bar = 20 μm.

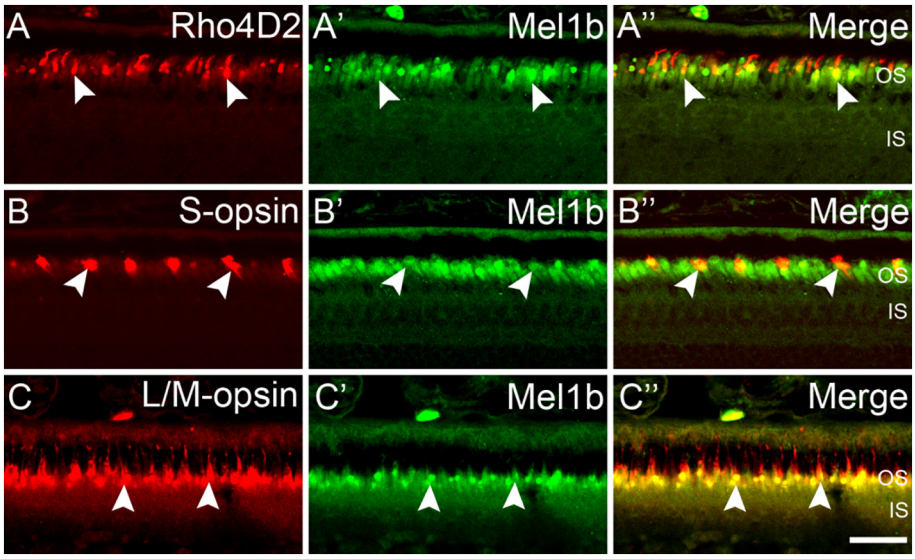

Fig. 2. Confocal laser scanning fluorescence micrographs, showing the distribution of Mel1b receptor in rods and cones. (A–A") Co-labeling of Rho4D2 (A, red) and Mel1b (A', green) in a rod-dominant section. Note that Mel1b immunostaining is visible in Rho4D2-labeled OS of rods. (B–B″) Co-labeling of S-opsin (B, red) and Mel1b (B', green) in a cone-rich vertical section. Merged image (B″) reveals the Mel1b receptor is more widely present in OS of blue cone. (C–C″) Co-labeling of L/M-opsin (C, red) and Mel1b (C′, green) in a cone-rich section. Yellowish in merged image (C″) indicates Mel1b receptor is expressed in red/green cones. Representative cells are indicated by arrowheads. Scale bar = 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Positive rates for Mel1b receptor in retinal cells.

Cell type	Positives	%
Rod	138/138	100
Blue cone	55/55	100
Red/green cone	33/33	100
HC	0/26	0
BC	0/583	0
Dopaminergic AC	12/12	100
Cholinergic AC	103/105	98
AII AC	71/71	100
Brn3a-labeled GC	443/443	100
IpRGC	10/10	100
Müller cell	0/94	0

(Fig. 3A"), the CB-positive HCs were not Mel1b receptor-labeled. Totally, 26 CB-positive cells from 10 vertical sections were all found to be Mel1b-negative. Double labeling experiments with the antibodies against Mel1b receptor (Fig. 3B) and ChX10 (a specific pan-BC marker (Whitaker and Cooper, 2009; Zhang et al., 2012), Fig. 3B') showed that the somata of the ChX10-positive BCs appeared to be lack of Mel1b receptor. A total of 583 ChX10-positive cells in 23 vertical sections were identified in the INL, and all of them were not co-labeled by Mel1b receptor.

3.4. Cellular localization of Mel1b receptor in the ACs

Our data indicated that Mel1b receptor might not exist in the HCs and BCs, we then conducted double labeling experiments of Mel1b receptor in combination with special markers for different subtypes of ACs to investigate whether the Mel1b receptor-related fluorescence in the INL was signaled from the ACs.

The dopaminergic AC, labeled by TH (Scher et al., 2002; NguyenLegros et al., 1997), was one category of GABAergic AC. Dopaminergic AC (Kosaka et al., 1987; Wulle and Wagner, 1990; Contini and Raviola, 2003), which was located almost exclusively in the innermost row of the INL with its processes mainly arborizing in the outermost stratum of the IPL. Double immunolabeling using anti-Mel1b receptor and anti-TH antibodies revealed that dopaminergic ACs exhibited strong Mel1b signal (Fig. 4A-A"). All of the dopaminergic ACs observed in this study were co-labeled by Mel1b receptor (12 out of 12 cells from 9 sections). The cholinergic AC was another subpopulation of GABAergic AC (Kosaka et al., 1988). ChAT-positive cholinergic ACs were situated either in the INL or in the GCL, forming two mirror-symmetric populations, and their processes formed two distinct bands in S2 and S4 of the IPL (Voigt, 1986). Fig. 4B-B" showed ChAT-positive cell were strongly Mel1b receptor-positive, while no obvious Mel1b receptor signal existed in the two bands formed by the processes of the cholinergic ACs. 105 cells with strong immunofluorescence signal for ChAT were observed in 7 retinal sections, and they were mostly Mel1b-positive (103 out of 105 cells). PV-immunolabeled AII AC (Wassle et al., 1993), characterized by their bushy dendritic trees terminating at different levels within the IPL, was the most prominent subtype of glycinergic AC. Double immunostaining experiment unequivocally demonstrated Mel1b receptor was clearly expressed in AII ACs, while their processes were not clearly labeled by Mel1b receptor (Fig. 4C-C"). In a quantitative analysis involving 71 AII ACs collected from 18 vertical sections, none of these AII ACs were not Mel1b-immunoreactive.

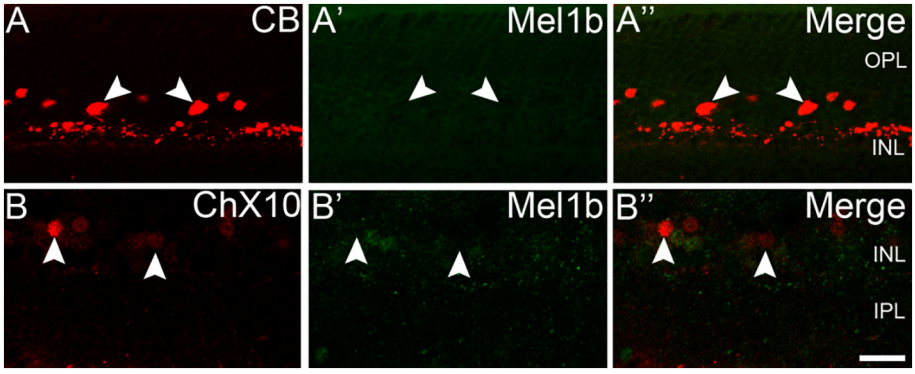

Fig. 3. Confocal laser scanning fluorescence micrographs, showing the distribution of Mel1b receptor in HCs and BCs. (A–A") Co-labeling of CB (A, red) and Mel1b (A', green) in vertical section. Note that Mel1b immunoreactivity is not detectable in CB-labeled HCs. (B–B″) Co-labeling of ChX10 (B, red) and Mel1b (B′, green) in vertical section. No overlap in merged image (B″) indicates ChX10-positive BCs are not immunostained by Mel1b receptor. Representative cells are indicated by arrowheads. Scale bar = 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

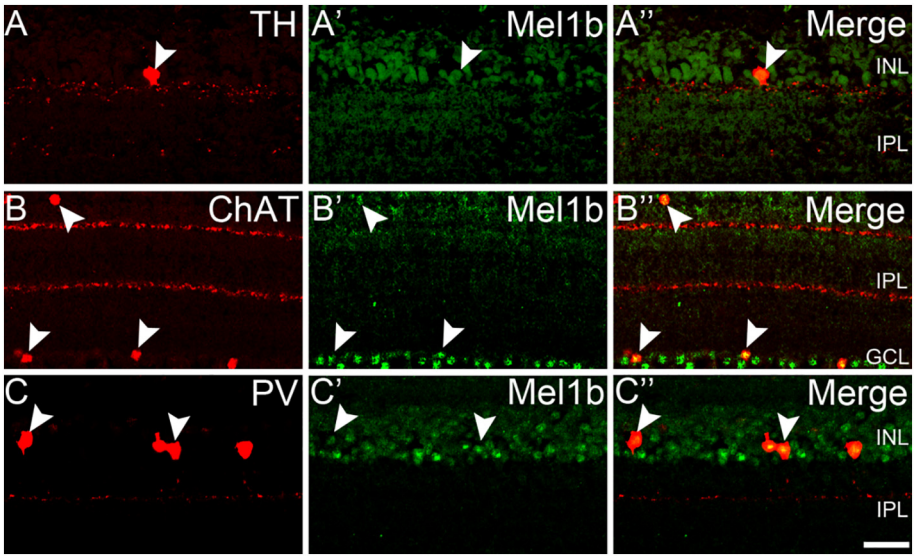

Fig. 4. Confocal laser scanning fluorescence micrographs, showing the distribution of Mel1b receptor in different AC subtypes. (A–A") Co-labeling of TH (A, red) and Mel1b (A', green) in vertical section. Note that Mel1b immunoreactivity is visible in TH-labeled dopaminergic ACs in the innermost part of the INL. One representative neuron (A") double labeled with TH and Mel1b is indicated by arrowhead. (B–B″) Co-labeling of ChAT (B, red) and Mel1b (B′, green) in vertical section. Yellowish in overlapped image (B″) indicates ChAT-labeled mirror-symmetric cholinergic ACs are Mel1b receptor immunoreactive (arrowheads). (C–C″) Co-labeling of PV (C, red) and Mel1b (C′, green) in vertical section. Superimposed image (C″) with yellowish reveals PV-labeled glycinergic ACs are labeled by Mel1b receptor (arrowheads). Note that processes of ACs above seem to be Mel1b-negative, Scale bar = 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. Cellular localization of Mel1b receptor in the GCs

The Mel1b receptor-positive cells in the GCL could be either GCs or displaced ACs (Fig. 1A). For further confirmation that GCs indeed expressed Mel1b receptor, we then performed double staining of Mel1b receptor and Brn3a (Fig. 5A-A"). The overlapping staining patterns indicated that Brn3a-labeled GCs (Nadal-Nicolas et al., 2009) were co-labeled by Mel1b receptor (443 out of 443 cells, 13 vertical sections). It was noteworthy that in the GCL some neurons, which were Mel1b-positive but Brn3a-negative, might be displaced ACs (such as cholinergic AC in Fig. 4B-B") or Brn3a-negative GCs. The ipRGC, which project to SCN to set circadian clock, was a special melanopsin-containing GCs (Berson et al., 2002; Hattar et al., 2002). This subtype GC could not be labeled by Brn3a, but it could be immunoreactive with melanopsin (Jain et al., 2012). Double labeling using the antibodies against Mel1b receptor and melanopsin revealed that all melanopsin-positive ipRGCs we found existed in the innermost row of the INL, but not in the GCL, and Mel1b receptor was expressed in the somata of ipRGCs (10 out of 10 cells, 7 vertical sections, Fig. 5B-B").

3.6. Cellular localization of Mel1b receptor in the Müller cells

The Müller cell with soma in the INL was the principal glial cell of the vertebrate retina. Two main trunks of the Müller cells moved toward both limiting surfaces of the retina. In the nerve fiber layer (NFL)/GCL, the trunks were enlarged to endfeet, while the trunks in the ONL ensheathed photoreceptor cell bodies (Fig. 6A). They played a crucial role in supporting the neurons (Subirada et al., 2018). Double-labeling of Mel1b receptor and CRALBP (a specific marker for labeling both somata and processes of Müller cells (Wurm et al., 2009)) was conducted (Fig. 6A-A"). Our data showed no overlap of the staining of Mel1b receptor and CRALBP (0 out of 94 cells, 9 vertical sections), which indicated that Müller cells might not express Mel1b receptor.

4. Discussion

Melatonin, coordinating with its specific receptors in the retina, is a major modulator of rhythmic activities, such as sleep (Pandi-Perumal et al., 2008; Huang et al., 2013; Dubocovich and Markowska, 2005). Abundant studies focused on Mel1a or MT_1 receptor have been done, but very few data about Mel1b or MT_2 receptor are now available. Like the Mel1a or MT_1 receptor, Mel1b or MT_2 receptor is also involved in retinal information processing (Yang et al., 2011; Zhao et al., 2010; Savaskan et al., 2007; Wiechmann and Sherry, 2012). Moreover, circadian rhythm-related Mel1b or MT_2 receptor is unstudied well on retinal cellular localization in nocturnal animals and diurnal animals. Our lab's previous studies show the melatonin receptor MT_2 is immunocytochemically located in the Rod-ON-BCs, RGCs and ipRGCs in the adult rat (Yang et al., 2011; Sheng et al., 2015; Zhao et al., 2010). While Wiechmann and his collogues show us Mel1b receptor is found in OFF-BCs, ACs and GCs in the retina of *Xenopus laevis* (Wiechmann and Sherry, 2012). However, their findings above are different from their

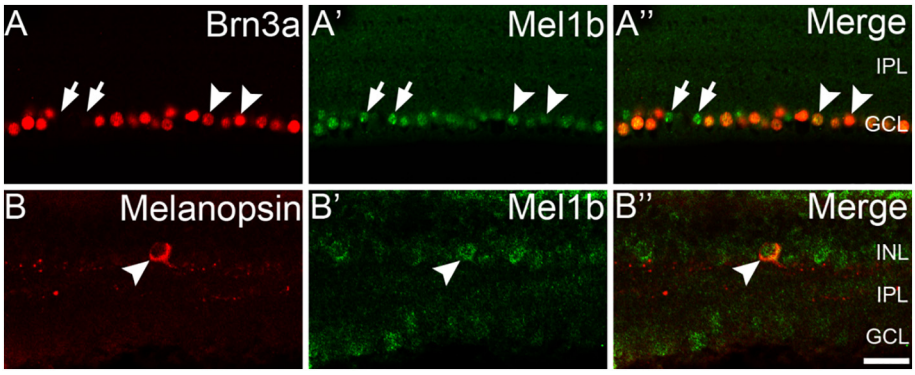

Fig. 5. Confocal laser scanning fluorescence micrographs, showing the distribution of Mel1b receptor in retinal GCs. (A–A") Co-labeling of Brn3a (A, red) and Mel1b (A', green) in vertical section. Note that Mel1b immunoreactivity is visible in Brn3a-labeled GCs. Two representative neurons double labeled with Brn3a and Mel1b (A", yellow) are indicated by arrowheads. Many neurons, lacking Brn3a immunoreactive but Mel1b receptor-positive in the GCL (indicated by arrows), may be Brn3a-negative GCs or displaced ACs. (B–B″) Co-labeling of melanopsin (B, red) and Mel1b (B′, green) in vertical section. Merged image (B″) with yellowish indicates co-localization of melanopsin and Mel1b in a displaced melanopsin-positive ipRGC in the INL (arrowhead). Scale bar = 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

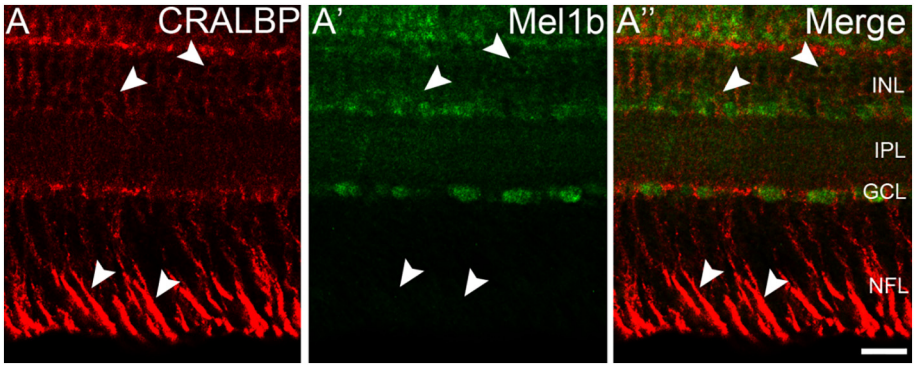

Fig. 6. Confocal laser scanning fluorescence micrographs, showing the distribution of Mel1b receptor in Müller cells. (A-A") Co-labeling of CRALBP (A, red) and Mel1b (A', green) in vertical section. Note that Mel1b is not detectable in CRALBP-labeled Müller cells, no matter in somata or in processes of glial Müller cells. Representative cells are indicated by arrowheads. NFL, nerve fiber layer. Scale bar = 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

previous study that Mel1b melatonin receptor is expressed in the inner segments (IS) of rod and cone photoreceptors, HCs and GCs, but not dopaminergic ACs and GABAergic ACs in *Xenopus laevis* retina (Wiechmann et al., 2004). In regard to the specificity of the Mel1b receptor antibodies, Western blot analysis demonstrate that the major immunoreactive bands approximately correspond to 35, 42, 45, and 80 KDa in the neural retina, which might lead to non-specific staining in Wiechmann's studies. Savaskan and his colleagues' studies show that MT_2 receptor is localized to IS of photoreceptors, BCs, GCs, OPL and IPL in human retina (Savaskan et al., 2007). However, better understanding on biological rhythm requires us to investigate the detailed cellular location of Mel1b or MT_2 receptor systematically.

In our study, we demonstrated more evidence about detailed cellular localization of Mel1b receptor in pigeon retina by double immunofluorescence labeling. Mel1b receptor-positive signals were found in OS of rods and cones, and somata of dopaminergic ACs, cholinergic ACs, glycinergic AII ACs, conventional GCs and ipRGCs. However, we failed to detect obvious Mel1b receptor-immunoreactive in either HCs or BCs who seem to be the major source of the processes in the OPL. And no clear Mel1b receptor-immunoreactive was detected in the IPL. Currently, there is few, if any, evidence available regarding whether Müller cells express Mel1b receptor or not. Our results have proved Mel1b receptor might be negative in this glia cells in pigeon retina. The prominent neuron subtype-dependence of Mel1b receptor expression consistently shows the complicated and crucial regulation of melatonin system. However it seems that both in the diurnal animals and nocturnal animals Mel1b receptor or MT_2 receptor always exists in GCs, the only type of output neurons in the retina. That indicates us melatonin system may regulate visual center indirectly in a circadian manner no matter in diurnal animals or nocturnal animals. These difference of Mel1b receptor or MT_2 receptor expression and its abundant physiological function in the retina between diurnal animals and nocturnal animals might be used to explain the diverse circadian rhythms. It is noteworthy that the similarities and differences of cellular localization of Mel1b or MT_2 receptor seem unclear in diurnal animals or nocturnal animals, and much more research works need to be done.

Melatonin system, functionally coupled with circadian rhythm-related dopamine system or melanopsin system, is involved in modulating retinal rhythmicity (Sengupta et al., 2011; Ribelayga et al., 2004; Sakamoto et al., 2005; Pack et al., 2015). In this study, we provided new potential evidence, at least in part, to indicate the interactions between melatonin system and dopamine system or melanopsin system because of Mel1b receptor being indeed expressed in both dopaminergic ACs and melanopsin-positive GCs. However, that whether the other two melatonin targets Mel1a and Mel1c receptors owing the similar cellular localization on dopaminergic ACs and melanopsin-positive GCs remains unclear. And more works focusing on the positive interactions between melatonin system and dopamine system or melanopsin system, even the interaction of these three systems, should be done for further. Rods and cones response to light directly and modulate visual functions (Pandi-Perumal et al., 2008; Kolb and Famiglietti, 1974; Hack et al., 1999; Hattar et al., 2003; Hannibal et al., 2007). ACs modulate GCs a lot and GCs are the only type of output neuron in the retina. Interestingly, Mel1b receptor prefers to be expressed in both outer neurons (rods and cones) and inner neurons (ACs and GCs), which may imply melatonin system modulates the retinal rhythmicity from the beginning and at the end of signal pathway in the retina, and then maintains the circadian rhythm in CNS.

References

Baba, K., Pozdeyev, N., Mazzoni, F., Contreras-Alcantara, S., Liu, C., Kasamatsu, M., Martinez-Merlos, T., Strettoi, E., Iuvone, P.M., Tosini, G., 2009. Melatonin modulates visual function and cell viability in the mouse retina via the MT1 melatonin receptor. Proc. Natl. Acad. Sci. U. S. A. 106, 15043–15048.

Berson, D.M., Dunn, F.A., Takao, M., 2002. Phototransduction by retinal ganglion cells that set the circadian clock. Science 295, 1070–1073.

Contini, M., Raviola, E., 2003. GABAergic synapses made by a retinal dopaminergic neuron. Proc. Natl. Acad. Sci. U. S. A. 100, 1358–1363.

Cosci, B., Longoni, B., Marchiafava, P.L., 1997. Melatonin induces membrane conductance changes in isolated retinal rod receptor cells. Life Sci. 60, 1885–1889.

Dubocovich, M.L., Markowska, M., 2005. Functional MT1 and MT2 melatonin receptors in mammals. Endocrine 27, 101–110.

Fischer, T.W., Zmijewski, M.A., Wortsman, J., Slominski, A., 2008. Melatonin maintains mitochondrial membrane potential and attenuates activation of initiator (casp-9) and effector caspases (casp-3/casp-7) and PARP in UVR-exposed HaCaT keratinocytes. J. Pineal Res. 44, 397–407.

Fujieda, H., Hamadanizadeh, S.A., Wankiewicz, E., Pang, S.F., Brown, G.M., 1999. Expression of mt1 melatonin receptor in rat retina: evidence for multiple cell targets for melatonin. Neuroscience 93, 793–799.

Fujieda, H., Scher, J., Hamadanizadeh, S.A., Wankiewicz, E., Pang, S.F., Brown, G.M., 2000. Dopaminergic and GABAergic amacrine cells are direct targets of melatonin: immunocytochemical study of mt1 melatonin receptor in guinea pig retina. Vis. Neurosci. 17, 63–70.

Ghosh, F., Arne´r, K., Engelsberg, K., 2009. Isolation of photoreceptors in the cultured full-thickness fetal rat retina. Invest. Opthalmol. Visual Sci. 50, 826.

Hack, I., Peichl, L., Brandstatter, J.H., 1999. An alternative pathway for rod signals in the rodent retina: rod photoreceptors, cone bipolar cells, and the localization of glutamate receptors. Proc. Natl. Acad. Sci. U. S. A. 96, 14130–14135.

Hannibal, J., Georg, B., Fahrenkrug, J., 2007. Melanopsin changes in neonatal albino rat independent of rods and cones. Neuroreport 18, 81–85.

Hattar, S., Liao, H.W., Takao, M., Berson, D.M., Yau, K.W., 2002. Melanopsin-containing retinal ganglion cells: architecture, projections, and intrinsic photosensitivity. Science 295, 1065–1070.

Hattar, S., Lucas, R.J., Mrosovsky, N., Thompson, S., Douglas, R.H., Hankins, M.W., Lem, J., Biel, M., Hofmann, F., Foster, R.G., Yau, K.W., 2003. Melanopsin and rod-cone photoreceptive systems account for all major accessory visual functions in mice. Nature 424, 76–81.

Huang, H., Wang, Z., Weng, S.J., Sun, X.H., Yang, X.L., 2013. Neuromodulatory role of melatonin in retinal information processing. Prog. Retin. Eye Res. 32, 64–87.

Ikegami, T., Motohashi, E., Doi, H., Hattori, A., Ando, H., 2009. Synchronized diurnal and circadian expressions of four subtypes of melatonin receptor genes in the diencephalon of a puffer fish with lunar-related spawning cycles. Neurosci. Lett. 462, 58–63.

Jain, V., Ravindran, E., Dhingra, N.K., 2012. Differential expression of Brn3 transcription factors in intrinsically photosensitive retinal ganglion cells in mouse. J. Comp. Neurol. 520, 742–755.

Ji, Y., Zhu, C.L., Grzywacz, N.M., Lee, E.J., 2012. Rearrangement of the cone mosaic in the retina of the rat model of retinitis pigmentosa. J. Comp. Neurol. 520, 874–888.

Johansson, L.C., Stauch, B., McCorvy, J.D., Han, G.W., Patel, N., Huang, X.-P., Batyuk, A., Gati, C., Slocum, S.T., Li, C., Grandner, J.M., Hao, S., Olsen, R.H.J., Tribo, A.R., Zaare, S., Zhu, L., Zatsepin, N.A., Weierstall, U., Yous, S., Stevens, R.C., Liu, W., Roth, B.L., Katritch, V., Cherezov, V., 2019. XFEL structures of the human MT2 melatonin receptor reveal the basis of subtype selectivity. Nature. 569 (7755), 289–292.

Klosen, P., Lapmanee, S., Schuster, C., Guardiola, B., Hicks, D., Pevet, P., Felder-Schmittbuhl, M.P., 2019. MT1 and MT2 melatonin receptors are expressed in non-overlapping neuronal populations. J. Pineal Res. 67.
Kolb, H., Famiglietti, E.V., 1974. Rod and cone pathways in the inner plexiform layer of cat retina. Science 186, 47–49.
Kosaka, T., Kosaka, K., Hataguchi, Y., Nagatsu, I., Wu, J.Y., Ottersen, O.P., Stormmathisen, J., Hama, K., 1987. Catecholaminergic neurons containing Gaba-like and or glutamic-acid decarboxylase-like immunoreactivities in various brain-regions of the rat. Exp. Brain Res. 66, 191–210.
Kosaka, T., Tauchi, M., Dahl, J.L., 1988. Cholinergic neurons containing Gaba-like and or glutamic-acid decarboxylase-like immunoreactivities in various brain-regions of the rat. Exp. Brain Res. 70, 605–617.
Liu, F., Xu, G.Z., Wang, L., Jiang, S.X., Yang, X.L., Zhong, Y.M., 2011. Gene expression and protein distribution of orexins and orexin receptors in rat retina. Neuroscience 189, 146–155.
Mennenga, K., Ueck, M., Reiter, R.J., 1991. Immunohistological localization of melatonin in the pineal gland and retina of the rat. J. Pineal Res. 10, 159–164.
Nadal-Nicolas, F.M., Jimenez-Lopez, M., Sobrado-Calvo, P., Nieto-Lopez, L., Canovas-Martinez, I., Salinas-Navarro, M., Vidal-Sanz, M., Agudo, M., 2009. Brn3a as a marker of retinal ganglion cells: qualitative and quantitative time course studies in naive and optic nerve-injured retinas. Invest. Ophthalmol. Vis. Sci. 50, 3860–3868.
Nao-i, N., Nilsson, S.E., Gallemore, R.P., Steinberg, R.H., 1989. Effects of melatonin on the chick retinal pigment epithelium: membrane potentials and light-evoked responses. Exp. Eye Res. 49, 573–589.
Natesan, A.K., Cassone, V.M., 2002. Melatonin receptor mRNA localization and rhythmicity in the retina of the domestic chick, Gallus domesticus. Vis. Neurosci. 19, 265–274.
NguyenLegros, J., VersauxBotteri, C., Savy, C., 1997. Dopaminergic and GABAergic retinal cell populations in mammals. Microsc. Res. Tech. 36, 26–42.
Ortin-Martinez, A., Nadal-Nicolas, F.M., Jimenez-Lopez, M., Alburquerque-Bejar, J.J., Nieto-Lopez, L., Garcia-Ayuso, D., Villegas-Perez, M.P., Vidal-Sanz, M., Agudo-Barriuso, M., 2014. Number and distribution of mouse retinal cone photoreceptors: differences between an albino (Swiss) and a pigmented (C57/BL6) strain. PLoS One 9.
Pack, W., Hill, D.D., Wong, K.Y., 2015. Melatonin modulates M4-type ganglion-cell photoreceptors. Neuroscience 303, 178–188.
Pandi-Perumal, S.R., Trakht, I., Srinivasan, V., Spence, D.W., Maestroni, G.J., Zisapel, N., Cardinali, D.P., 2008. Physiological effects of melatonin: role of melatonin receptors and signal transduction pathways. Prog. Neurobiol. 85, 335–353.
Querubin, A., Lee, H.R., Provis, J.M., O'Brien, K.M.B., 2009. Photoreceptor and ganglion cell topographies correlate with information convergence and high acuity regions in the adult pigeon (*Columba livia*) retina. J. Comp. Neurol. 517, 711–722.
Rabie, A., Thomasset, M., Parkes, C.O., Clavel, M.C., 1985. Immunocytochemical detection of 28000-mw calcium-binding protein in horizontal cells of the rat retina. Cell Tissue Res. 240, 493–496.
Reppert, S.M., Weaver, D.R., Ebisawa, T., 1994. Cloning and characterization of a mammalian melatonin receptor that mediates reproductive and circadian responses. Neuron 13, 1177–1185.
Reppert, S.M., Godson, C., Mahle, C.D., Weaver, D.R., Slaugenhaupt, S.A., Gusella, J.F., 1995. Molecular characterization of a second melatonin receptor expressed in human retina and brain: the Mel1b melatonin receptor. Proc. Natl. Acad. Sci. U. S. A. 92, 8734–8738.
Ribelayga, C., Wang, Y., Mangel, S.C., 2004. A circadian clock in the fish retina regulates dopamine release via activation of melatonin receptors. J. Physiol. 554, 467–482.
Ritchey, E.R., Bongini, R.E., Code, K.A., Zelinka, C., Petersen-Jones, S., Fischer, A.J., 2010. The pattern of expression of guanine nucleotide-binding protein β3 in the retina is conserved across vertebrate species. Neuroscience 169, 1376–1391.
Rodrigues, T., Krawczyk, M., Skowronska-Krawczyk, D., Matter-Sadzinski, L., Matter, J.M., 2016. Delayed neurogenesis with respect to eye growth shapes the pigeon retina for high visual acuity. Development 143, 4701–4712.
Sakamoto, K., Liu, C., Kasamatsu, M., Pozdeyev, N.V., Iuvone, P.M., Tosini, G., 2005. Dopamine regulates melanopsin mRNA expression in intrinsically photosensitive retinal ganglion cells. Eur. J. Neurosci. 22, 3129–3136.
Savaskan, E., Jockers, R., Ayoub, M., Angeloni, D., Fraschini, F., Flammer, J., Eckert, A., Muller-Spahn, F., Meyer, P., 2007. The MT2 melatonin receptor subtype is present in human retina and decreases in Alzheimer's disease. Curr. Alzheimer Res. 4, 47–51.
Scher, J., Wankiewicz, E., Brown, G.M., Fujieda, H., 2002. MT(1) melatonin receptor in the human retina: expression and localization. Invest. Ophthalmol. Vis. Sci. 43, 889–897.
Scher, J., Wankiewicz, E., Brown, G.M., Fujieda, H., 2003. AII amacrine cells express the MT1 melatonin receptor in human and macaque retina. Exp. Eye Res. 77, 375–382.
Sengupta, A., Baba, K., Mazzoni, F., Pozdeyev, N.V., Strettoi, E., Iuvone, P.M., Tosini, G., 2011. Localization of melatonin receptor 1 in mouse retina and its role in the circadian regulation of the electroretinogram and dopamine levels. PLoS One 6, e24483.
Sheng, W.L., Chen, W.Y., Yang, X.L., Zhong, Y.M., Weng, S.J., 2015. Co-expression of two subtypes of melatonin receptor on rat M1-type intrinsically photosensitive retinal ganglion cells. PLoS One 10, e0117967.
Stauch, B., Johansson, L.C., McCorvy, J.D., Patel, N., Han, G.W., Huang, X.-P., Gati, C., Batyuk, A., Slocum, S.T., Ishchenko, A., Brehm, W., White, T.A., Michaelian, N., Madsen, C., Zhu, L., Grant, T.D., Grandner, J.M., Shiriaeva, A., Olsen, R.H.J., Tribo, A.R., Yous, S., Stevens, R.C., Weierstall, U., Katritch, V., Roth, B.L., Liu, W., Cherezov, V., 2019. Structural basis of ligand recognition at the human MT1 melatonin receptor. Nature. 569 (7755), 284–288.
Subirada, P.V., Paz, M.C., Ridano, M.E., Lorenc, V.E., Vaglienti, M.V., Barcelona, P.F., Luna, J.D., Sanchez, M.C., 2018. A journey into the retina: Muller glia commanding survival and death. Eur. J. Neurosci. 47, 1429–1443.
Tosini, G., Baba, K., Hwang, C.K., Iuvone, P.M., 2012. Melatonin: an underappreciated player in retinal physiology and pathophysiology. Exp. Eye Res. 103, 82–89.
van den Top, M., Buijs, R.M., Ruijter, J.M., Delagrange, P., Spanswick, D., Hermes, M.L., 2001. Melatonin generates an outward potassium current in rat suprachiasmatic nucleus neurones in vitro independent of their circadian rhythm. Neuroscience 107, 99–108.
Voigt, T., 1986. Cholinergic Amacrine cells in the rat retina. J. Comp. Neurol. 248, 19–35.
Wassle, H., Grunert, U., Rohrenbeck, J., 1993. Immunocytochemical staining of AII-amacrine cells in the rat retina with antibodies against parvalbumin. J. Comp. Neurol. 332, 407–420.
Whitaker, C.M., Cooper, N.G.F., 2009. The novel distribution of phosphodiesterase-4 subtypes within the rat retina. Neuroscience 163, 1277–1291.
Wiechmann, A.F., Sherry, D.M., 2012. Melatonin receptors are anatomically organized to modulate transmission specifically to cone pathways in the retina of Xenopus laevis. J. Comp. Neurol. 520, 1115–1127.
Wiechmann, A.F., Sherry, D.M., 2013. Role of melatonin and its receptors in the vertebrate retina. Int. Rev. Cell Mol. Biol. 300, 211–242.
Wiechmann, A.F., Smith, A.R., 2001. Melatonin receptor RNA is expressed in photoreceptors and displays a diurnal rhythm in Xenopus retina. Mol. Brain Res. 91, 104–111.
Wiechmann, A.F., Udin, S.B., Summers Rada, J.A., 2004. Localization of Mel1b melatonin receptor-like immunoreactivity in ocular tissues of *Xenopus laevis*. Exp. Eye Res. 79, 585–594.
Wulle, I., Wagner, H.J., 1990. Gaba and tyrosine-hydroxylase immunocytochemistry reveal different patterns of colocalization in retinal neurons of various vertebrates. J. Comp. Neurol. 296, 173–178.
Wurm, A., Erdmann, I., Bringmann, A., Reichenbach, A., Pannicke, T., 2009. Expression and function of P2Y receptors on Muller cells of the postnatal rat retina. Glia 57, 1680–1690.
Yang, X.F., Miao, Y., Ping, Y., Wu, H.J., Yang, X.L., Wang, Z., 2011. Melatonin inhibits tetraethylammonium-sensitive potassium channels of rod ON type bipolar cells via MT2 receptors in rat retina. Neuroscience 173, 19–29.
Zhang, P.P., Yang, X.L., Zhong, Y.M., 2012. Cellular localization of P2Y(6) receptor in rat retina. Neuroscience 220, 62–69.
Zhao, W.J., Zhang, M., Miao, Y., Yang, X.L., Wang, Z., 2010. Melatonin potentiates glycine currents through a PLC/PKC signalling pathway in rat retinal ganglion cells. J. Physiol. 588, 2605–2619.