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Présentée par

Virginie Laurent-Gydé, MCF

Institut des Neurosciences Cellulaires et Intégratives, UPR CNRS 3212

Equipe « Rythme, vie et mort de la rétine »

Melatonin synthesis regulation and role in the mammalian retina

Soutenue le 18 Mars 2016 devant le jury:

Rapporteur interne : Dr. Valérie Simonneaux, Strasbourg

Garant d'habilitation : Dr. David Hicks, Strasbourg

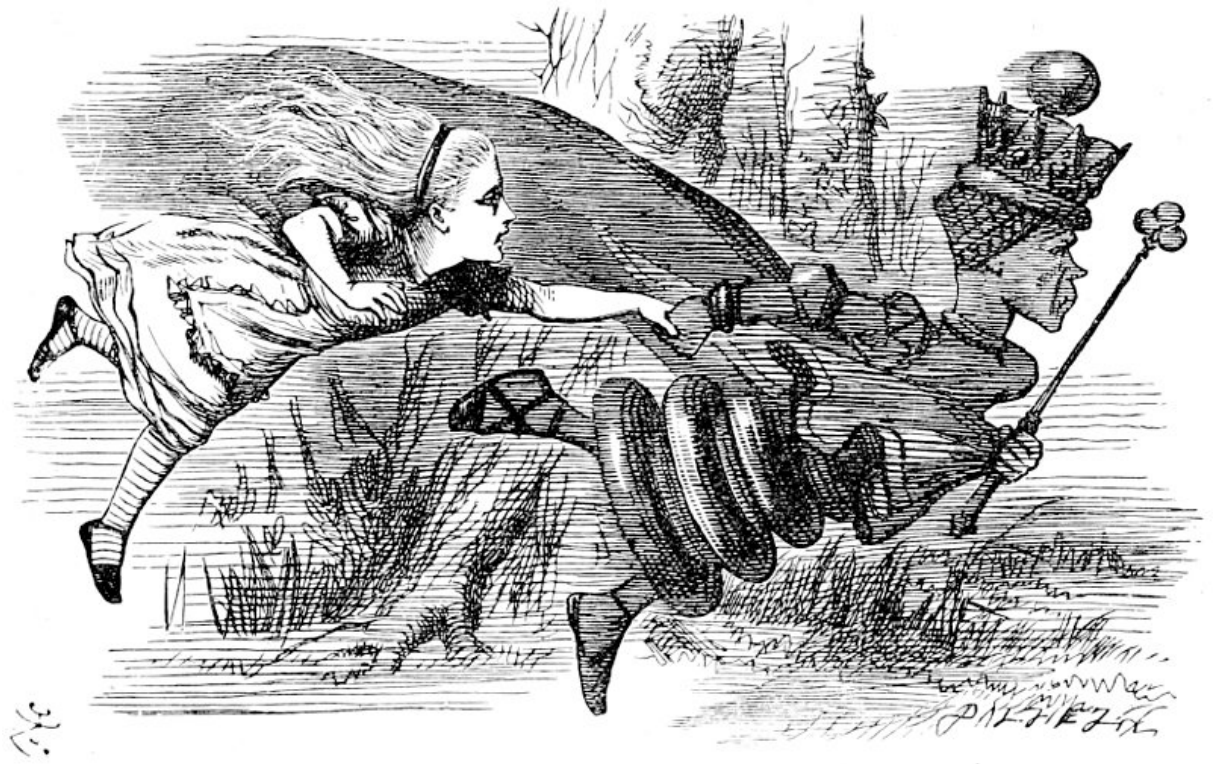
Examinateur: Dr. Ouria Dkhissi-Benyahya, Lyon

Examinateur : Dr. Thomas Bourgeron, Paris

Rapporteur externe : Pr. Jack Falcon, Banyuls/Mer

Rapporteur externe : Pr. Christian Grimm, Zurich

Foreword:



“The Red Queen hypothesis was proposed over 40 years ago by the late evolutionary biologist **Leigh Van Valen.** It advanced evolutionary thinking beyond **the idea that organisms were merely matched to their physical environment by suggesting that interactions between species (such as between hosts and parasites, predators and prey) would also be important in driving evolutionary change.** In essence, the Red Queen hypothesis states that in the battle for resources, species must continuously evolve just to keep up with their enemies, who themselves also evolve in response. The result is that species constantly change but, relative to their enemies, don’t actually get any fitter like running on an evolutionary treadmill. The name for the theory came from Lewis Carroll’s ‘Through the Looking Glass’ (aka Alice in Wonderland). Alice finds herself in a race with the Red Queen, and despite running as fast as she can, Alice stays in the same place.” **Quote from the Royal Society blog** http://blogs.royalsociety.org/publishing/darwin-review-2014/

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Brief Scientific biography:

Thanks to a high school teacher who used with dexterity the typical -"Données Initiales, Problème, Hypothèse, Tests, Résultats, Interprétations, Conclusions"-(DIPHTeRIC) scientific approach to teach biology, I was hooked by life sciences. Then, I chose animal biology as a favorite field and allied biochemistry to neurosciences to define my own path (DEUG, License, Maitrise, DEA). Following a PhD on the renin-angiotensin system of the leech *Theromyzon tessulatum* which was mostly biochemistry (Laurent V. et al, 1995, 1996, 1997, 1998, 2000), I undertook a first post-doctoral training in Dr Elwyn Isaac and Dr David Coates' laboratory at the University of Leeds, UK. These researchers are expert in the biochemistry and genetics of *C. elegans* enzymes. I acquired knowledge of molecular tools and we isolated a new aminopeptidase from the worm (Laurent et al., 2001). I then sought to come back to neurosciences and patho-physiology issues. So, I moved to Iris Lindberg's laboratory in LSUHSC, New Orleans, LA for a 3-years post-doc study on a mouse model of Cushing's disease, an endocrine disorder linked to ACTH hypersecretion. We were able to demonstrate the importance of dopamine in the control of ACTH secretion and that adrenalectomy rescues 7B2 null mice mortality (Laurent V. et al, 2002). I came back to France in Dr. de Keyser's team at the Institut Cochin, Paris to work on the vasopressin receptor V3 (V1b), a useful marker for ACTH producing cells to study tumors. In the meantime I applied for several Assistant Professor jobs and got a position in Strasbourg as a "Maitre de Conférences, section CNU 68 (Biologie des organismes)". From September 2003 to 2007, I worked in the team of Mireille Masson-Pévet on the link between Melatonin, Rorβ, reverbα transcription factors and the circadian system (Agez L. et al, 2007; 2009). At the end of 2007, coincident with the five years renewal of the Institut des Neurosciences Cellulaires et Intégratives, I joined the team of Dr. David Hicks, named « Horloge circadienne et physiopathologie des photorécepteurs" (currently termed "Rhythm, life and death in the retina"). Since this change, we have been developing an original research topic on melatonin within the mammalian retina. I would like to point out that there have been two motors to drive my research path: i) deciphering enzyme structure, action and regulation; and ii) exploring their link with neurohormone production in physiological and pathological contexts. I have used this dynamic to pursue the research that will be developed here in order to obtain the HDR grade.

First, our choice of the animal model, named *Arvicanthis ansorgei* (Rodentia, Thomas 1910) and the synthesis and regulation of melatonin production in the retina of this diurnal animal will be thoroughly discussed. In a second section, we will focus on melatonin and its role on photoreceptor phagocytosis in both *Arvicanthis* and mouse retinas. Thirdly, new scientific questions and the associated future studies will be developed. Finally, a conclusion chapter includes the hypothetical model regarding melatonin and mammalian retina.

Part I: Melatonin synthesis and regulation in the retina of the diurnal rodent: *Arvicanthis ansorgei*

I. *Arvicanthis ansorgei* as a model

Arvicanthis ansorgei Thomas 1910 (referred as *Arvicanthis ansorgei* hereafter) is a diurnal Muridae exclusively African which shares common characteristics with other *Arvicanthis* species such as *Arvicanthis niloticus* partly related to their diurnality (Challet et al. 2002). In Africa, *Arvicanthis ansorgei* refers to the unstriped or Nile grass rat, widely distributed in savannas and grasslands of sub-Saharan countries such as the southern part of Senegal and Mali. *Arvicanthis ansorgei* breeding colony was initiated in Strasbourg in 1998 by Bruno Sicard and Paul Pévet from animals trapped in the southern part of Mali. The colony is nowadays hosted in the Chronobiotron in the Institut des Neurosciences Cellulaires et Intégratives (INCI) under the care of Dominique Ciocca (Figure 1). Our team has been working for years on the retina of this diurnal rodent for scientific purposes that will be quoted or discussed in details hereafter. Briefly, *Arvicanthis* possesses a retina whose morphology has two great advantages i) compared to nocturnal rats and mice with only 1-3% cones (Chang Jin et al 1998; Szél and Röhlich 1992) this population makes up 33% of total photoreceptors (PR) in *Arvicanthis* (Bobu and Hicks 2006). As available evidence suggests cones are an important site of melatonin production (Niki et al. 1998), and cones are essential to human eyesight, enabling high acuity chromatic vision (Harrison 1953; Otake, Gowdy, and Cicerone 2000), this species constitutes a potentially valuable experimental model; ii) these cones are neatly arranged in two rows of cell bodies overlying the rods, a feature which facilitates discrimination between photoreceptor types (Bobu and Hicks 2006). Additionally, I will discuss at length a potential third advantage iii) the large cone contingent combined to higher number of **melanopsin type 1 intrinsically photosensitive retinal ganglion cell (M1-type ipRGC)** and higher sensitivity to light of type I ipRGC might reflect specific adaptations of the diurnal animal regarding light irradiance over time (visual and non visual circuits e.g. circadian behavior) (Karnas et al. 2013)

At this stage, I should mention that I used female *Arvicanthis* retinas for all the studies discussed here. We initially chose to work on females because these were readily available in our animal facility and there were no published studies on possible sexual dimorphism related to rodent retinal melatonin that could influence our choice. It is acknowledged that studies should be repeated with *Arvicanthis* males to clarify this question, as studies in rat and human suggest retinal gender differences linked to age and hormonal environment (Salyer et al. 2001; Wexler et al 2010). However, this point will not be discussed further as the focus will be on melatonin production and roles in female *Arvicanthis* retinas.

Arvicanthis ansorgei from the breeding colony
Credit : Coralie Gianesini

Figure 1: *Arvicanthis ansorgei* adult from our breeding colony in Strasbourg.

I.A. Retina of *Arvicanthis ansorgei* versus nocturnal rodent's retina

The retina of *Arvicanthis ansorgei* has been thoroughly studied by our team (Bobu and Hicks 2006) and displays a multilayer structure similar to the one of *Arvicanthis niloticus* (Gaillard et al. 2008) but distinct from nocturnal rodent. The retinal pigmentary epithelium (RPE), a monolayer of cells containing nuclei, mitochondria, melanin granules and phagosomes lies on top of the outer retina (Figure 2).

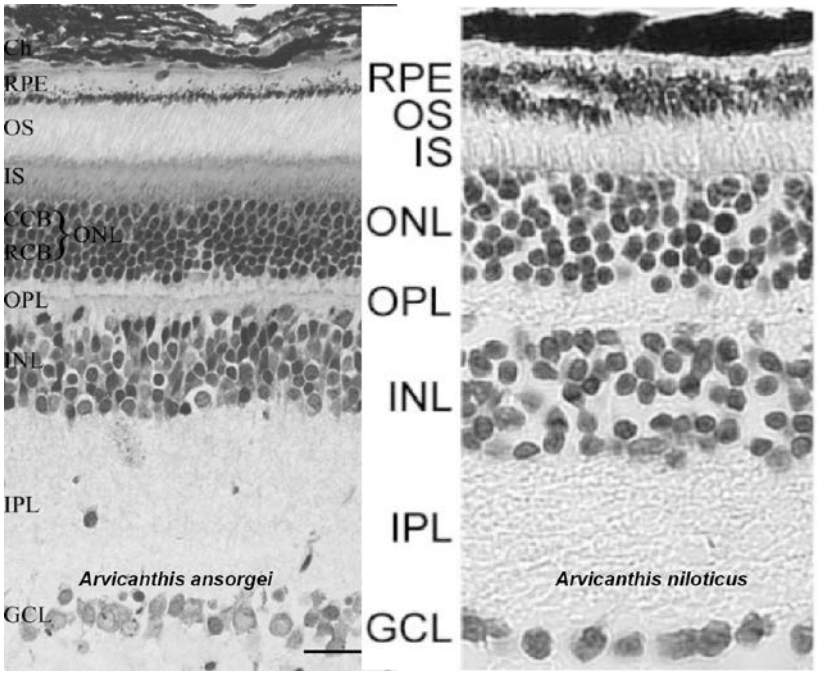


Figure 2: *Arvicanthis* sp. retina structure:

Left Panel: toluidine-blue stained semithin section of retina from adult *Arvicanthis ansorgei*. The typical layered structure of mature retina is also apparent for *Arvicanthis niloticus* retina: right panel: ONL; constituted by the cone [CCB] and rod [RCB] cell bodies. Ch, choroid; GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; IS: photoreceptor inner segments; OPL, outer plexiform layer, RPE: retinal pigment epithelium. Scale bar, 20 µm. Scale for right panel is 25 µm. From (Bobu and Hicks 2006; Gaillard et al. 2008).

I.A.1. The outer retina

In contact with the RPE, the outer segments of cone and rod photoreceptors (PRs) are orderly arranged (Figure 3) and attached to the inner segments (IS) by their connecting cilium. The outer nuclear layer (ONL) contains the rod and cone nuclei and the axons extending to synaptic terminals where neurotransmission to bipolar and horizontal cells occurs. *Arvicanthis ansorgei* ONL is organized in 6 superimposed rows of photoreceptor cell bodies, very similar to other diurnal retinas such as the primate one (Linberg et al. 2001) but with cones neatly arranged in two rows of cell bodies overlying the rods, a feature which facilitates discrimination between photoreceptor types. This anatomy appears uniform across the retina, from center to mid periphery, but toward the far periphery, the ONL thins to four to five cell bodies, with only a distal row of cones, and three to four rows of rods (Bobu and Hicks 2006). As mentioned above *Arvicanthis ansorgei* possesses 33% cone photoreceptors, 35-40% in *Arvicanthis niloticus* (Gaillard et al. 2008) versus 2.8% in mice (Chang Jin et al 1998) and less than 1% in rat (Szel et al. 1992). This structure contrasts with the ONL of nocturnal animals such as mouse and rat whose retinas contain around 98% rods (6.4 million and stacked in 10-12 rows in mice (Figure 2). Synapses of the photoreceptors are forming the most external plexiform layer termed outer plexiform layer (OPL) which conveys the photic information to bipolar cells, modulated by horizontal cells (la Cour and Ehinger 2005).

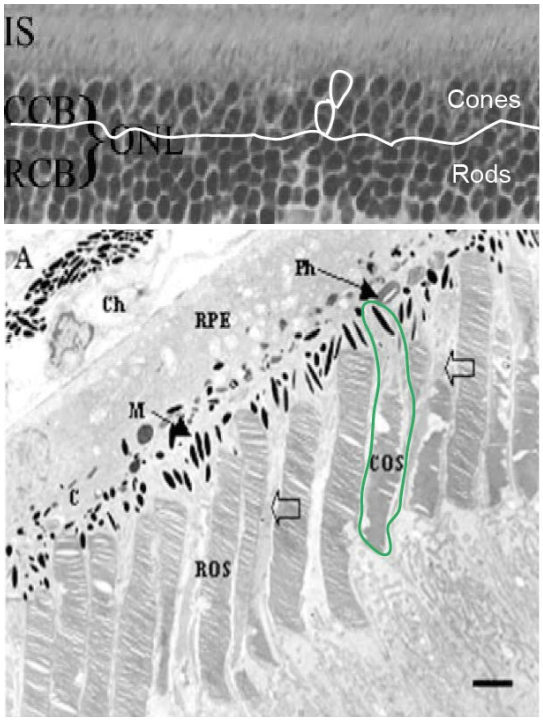


Figure 3: Outer retina of *Arvicanthis ansorgei*

Top panel: Focus on *Arvicanthis ansorgei* ONL with two rows of cone-type cell bodies (white drawing) neatly arranged on top of rod cell bodies. **Lower panel**: Electron microscopic image of the PR–RPE interface in adult *A. ansorgei*. Shown are representative views of rod (ROS) and cone (COS) outer segments apposed to the RPE. COS outlined in green expose a tapered shape and lower position, slightly greater electron density, and greater artifactual damage during tissue processing. In addition, traces of cone matrix sheath are visible surrounding them (open arrows). Several phagosomes (Ph) are present within the RPE cytoplasm (tissue obtained at 0900 hours). Ch, choroid; M, melanin granules. Scale bar, 1 µm.

I.A.2. The inner retina

Below the OPL, from top to bottom, horizontal, bipolar, Müller and amacrine body cells constitute the inner nuclear layer. These cells are stacked in 5 rows as observed for the human and *Arvicanthis niloticus* retinas, but also quite similar to other rodents (Figure 4). However, our team has not thoroughly characterized the INL of *A. ansorgei* retina. Based on general information from other mammalian retinas, bipolar cells relay information between PR and retinal ganglion cells (RGC).

Horizontal and amacrine cells are respectively localized in the external and internal part of the INL. These cells combine and modify the PR synaptic signal prior to final transmission to RGC. These changes allow adjustment of sensitivity for bright and dim light vision. They also permit shaping of visual responses by processes such as lateral inhibition which increases contrast and sharpness of the image. INL also contains the nuclei of Müller cells, the main glial cells of the retina, the cell bodies of which span the entire retina (Kolb 2005).

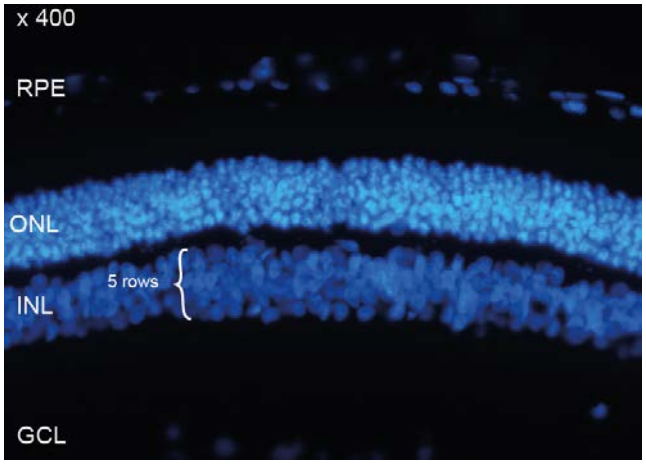


Figure 4: DAPI staining of a 10 µm section of *Arvicanthis ansorgei* retina:

Photograph is centered on the INL composed of 5 rows of cell bodies.

The inner plexiform layer (IPL) separates the INL from the GCL, i.e. the most inner nuclear layer of the tissue and contains the synapses of the bipolar, amacrine and ganglion cells. Information from bipolar cells are modulated by amacrine cells and transferred to the ganglion cells (la Cour and Ehinger 2005). Bipolar cells and Horizontal cells are not the subject of this work and thus will not be discussed. For more information see reviews by (Thoreson and Mangel 2012) and online Webvision: Bipolar Cell Pathways in the Vertebrate Retina by Ralph Nelson and Victoria Connaughton ; S-Potentials and Horizontal Cells by Ido Perlman, Helga Kolb and Ralph Nelson.

Finally, the innermost part of the retina is the ganglion cell layer (GCL) (Figure 5) containing ganglion cell bodies and those of displaced amacrine cells (40% in *Arvicanthis ansorgei* (Bobu and Hicks 2006) versus 60% in mice retina (Chang Jin et al 1998). The RGC transfer light information from the retina to the brain. The axons of the RGC form bundles within the nerve fiber layer (NFL) and converge towards the optic papilla e to enter the optic nerve(Kolb 2005). 1 to 2% of the RGC are the ipRGC which contain the recently discovered photopigment melanopsin (Karnas et al. 2013). These cells have photoadapatative functions distinct from the image visual function that will be further detailed in the paragraph on ganglion cells below. Finally, astrocytes are restricted to the NFL where they ensheathe axons as well as blood vessels.

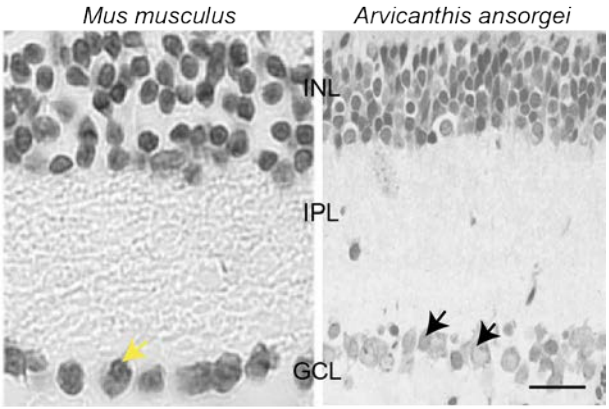


Figure 5: Retina GCL layer comparison in mouse and *Arvivanthis ansorgei*.

GCL in *Arvicanthis ansorgei* appears as a heterogeneous layer (black arrows) compared with mouse retina GCL (yellow arrow pointing to one cell body aligned with the others). *A. ansorgei* photograph adapted from (Bobu and Hicks 2006)

I.A.3. Blood supply of the retina

There are two sources of blood supply to the retina in most mammals: the central retinal artery and the choroidal blood vessels. The choroid receives the greatest blood flow (65-85%) (GlattHJ & Henkind 1979) and is vital for the maintenance of the outer retina (particularly the photoreceptors) and the remaining 20-30% flows to the retina through the central retinal artery from the optic nerve head to nourish the inner retinal layers. The arterial intraretinal branches then supply three layers of capillary networks i.e. 1) the radial peripapillary capillaries and 2) an inner and 3) an outer layer of capillaries. The precapillary small veins drain into venules and through the corresponding venous system to the central retinal vein. Recently, Ramos et al have described a mouse retinal whole-mount observation by confocal laser technology that allows the microscopal analysis of the entire retina combined with the use of fluorescent markers (Ramos et al. 2013). Our team has examined *Arvicanthis ansorgei* retinas by scanning laser ophthalmoscopy and vascularization appears similar to the mouse (Boudard et al., 2010). This is contrary to what was observed in the guinea pig or beaver retinas (other diurnal rodents) where the tissue is devoid of vessels which are restricted to the optic nerve head (Ramos et al. 2013).

I.B. The different cell types :

I.B.1. The photoreceptors: distribution, structure, connections and roles:

As in almost all mammals, photoreceptors are of three types in *Arvicanthis ansorgei* retina: two basic categories: rods and cones in the ONL and the ipRGCs in the GCL. The “classic” photosensors (rods and cones) lie on the outermost retina against the RPE. Light must therefore travel through the thickness of the retina before striking and activating the rods and cones. Subsequently, the phototransduction cascade creates an electrical message that stimulates neurons of the retina and is transmitted to the brain (visual image formation) in the form of a spiking discharge pattern of the ganglion cells. Besides, melanopsin (photopigment) producing ipRGC are responsible for non-image forming physiological functions, such as circadian photoentrainment, pupillary light reflex and masking responses to light (Karnas et al. 2013).

I.B.1.a Cone and rod distribution:

Arvicanthis ansorgei retina is composed of 33% cones and 67 % rods, approximately 10-15 times more cones than mice and rats (Figure 6). Therefore, *A. ansorgei* retina belongs to the diurnal mammals group whose retina display from 8% to 95% cones (Leo 2000). Among them, ground squirrels (approximately 90% cones), an pigs (15%-20% cones, (Hendrickson and Hicks 2002) all share a particularly cone-rich retina. However, only a few diurnal species are known to have more cones than rods (the ground squirrel quoted above, the tree shrew Tupaia has 95% cones (Müller and Peichl 1989). The old world primates, and humans, possess a central macula where cone density is maximum, reaching 100% in the foveola. Cone densities generally are higher in diurnal than in nocturnal species but all Mammals have a duplex retina containing both types.

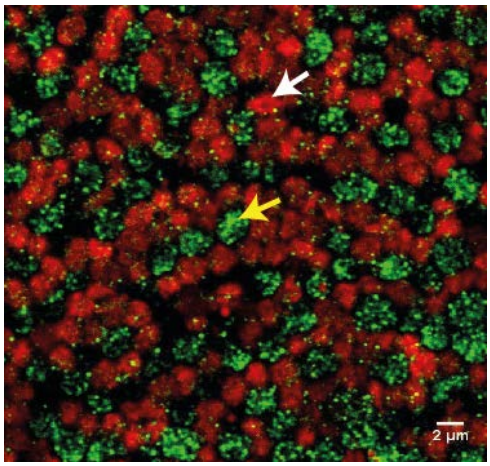


Figure 6: *Arvicanthis ansorgei* whole-mounted retina.

The optical section was fixed at the level of the IS. Rod IS are specifically stained with anti-phosducin antibody (White arrow), while cone IS are visible with anti-AA-NAT (green staining and yellow arrow) in numerous cells. This photograph allows the estimation of 33% cones present in the diurnal rodent retina. Credit: Coralie Gianesini.

I.B.1.b Cone and rod structure:

In *Arvicanthis ansorgei* retina and at the ultrastructural level, rod outer segments (OS) appear as cylindrical structures with stacked membrane discs clearly visible. Cone OS are narrower, more conical shaped, in lower position than the rod ones and slightly more electron dense. Rod OS is filled with closed discs of double membranes enveloped by separate plasma membranes. On the contrary, Cone OS contains invaginations of a single cell membrane that form a stack of membranous disks where photopigments exist as transmembrane proteins (Figure 7). Traces of cone matrix sheath are visible surrounding them. Apical processes from the RPE envelope the OS of both rods and cones (Bobu and Hicks 2006). The OS are attached to the photoreceptor IS by a connecting cilium. As for other mammals the IS contain mitochondria, ribosomes and Golgi bodies where opsin molecules are assembled and then integrated in OS discs. Photoreceptor cell bodies contain nuclei, below which are short axons terminating in synaptic contactswhere neurotransmission to second order neurons occurs.

Interestingly, recent studies have demonstrated that nuclear architecture of rod (not cones) photoreceptor cells differs fundamentally in nocturnal and diurnal mammals (Solovei et al 2009; Solovei et al. 2013). The rod nuclei of diurnal retinas possess most heterochromatin situated at the periphery and euchromatin residing toward the interior whereas rod nuclei in nocturnal retinas display an opposite organization remodeled during the terminal differentiation of these cells. The same authors identified lamin B receptor and Lamin A/C as main factors involved in the chromatin remodeling. Investigation of these features in *Arvicanthis ansorgei* retina indicates that these animals possess nocturnal-type rod nuclear architecture (Frohns, Hicks in preparation). As outlined in Solovei et al studies, the inverted pattern of rod nuclear architecture appeared very early in the evolution of

mammals, seemingly as an adaptation to nocturnal vision since this chromation organization facilitates light transmission to the OS; whereas the conventional nuclear pattern was reacquired independently in mammals that readopted a diurnal lifestyle. These data make *Arvicanthis ansorgei* a species at a "transitional" state in evolution, since they possess nocturnal rod features but abundant cones, thin ONL and behavioral properties consistent with a diurnal lifestyle.

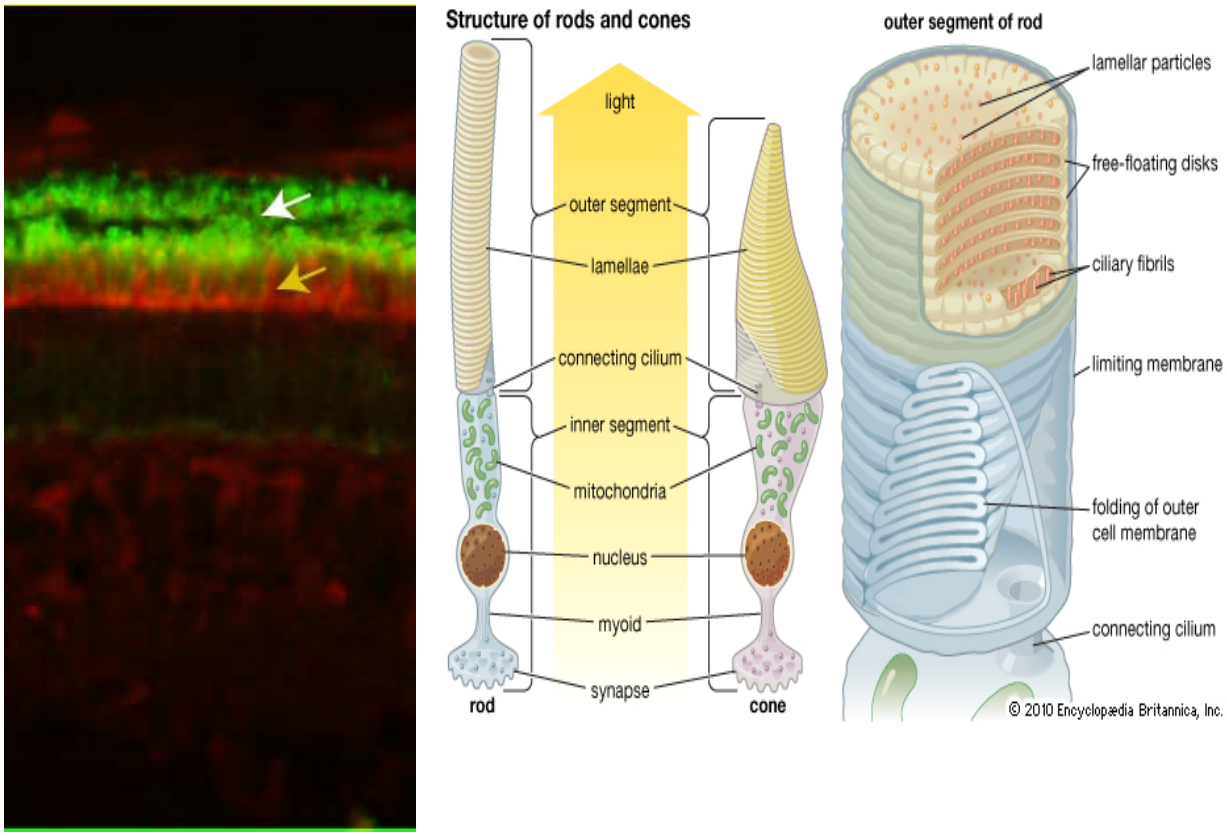


Figure 7: Cone and rod photoreceptors:

Left panel: *Arvicanthis ansorgei* retina. White arrows points to rhodopsin staining (green) in the rod outer segments. Yellow arrow indicates cone transducin presence in the cone inner segments. **Right panel**: Schema of rod and cone structures; from Encyclopedia Britannica.

I.B.1.c Cone and rod pigments:

Arvicanthis ansorgei retina contains two types of cone photoreceptor but a single type of rod photoreceptor. In addition to differences in their architecture, photoreceptor types reflect the visual pigment composition of OS and their biological relevance (Figure 8). Despite a 430 million years old common ancestor of jawed vertebrates with an eye fundamentally like ours, (that has evolved differently to optimize color vision) the lower mammals such as rodents have retained two spectral classes (Peichl 2005). Namely, M and S cones are distinguished by the portion of the visible spectrum to which each is maximally sensitive.

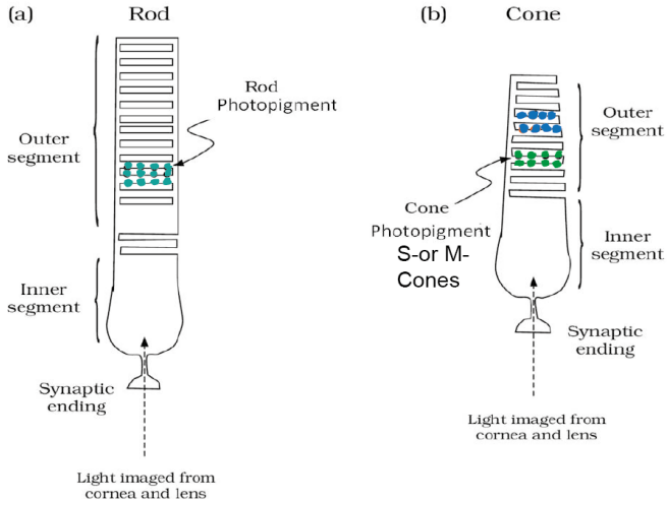
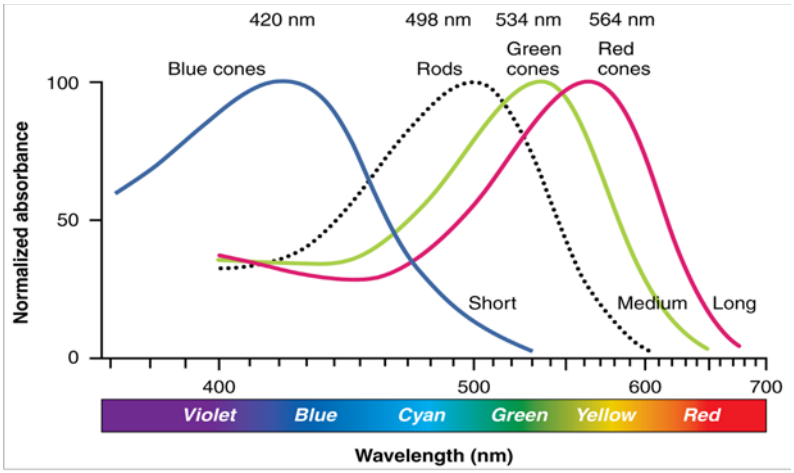


Figure 8: Photopigments in Mammalian rod and cone photoreceptors

Rods and cones contain light absorbing pigments that initiate vision. Light enters the photoreceptors through the inner segment and is funneled to the outer segment that contains the photopigment. Rodents possess M- and S-cones illustrated here by blue and green pigments on the cone drawing. Modified from https://foundationsofvision.stanford.edu/chapter-3-the-photoreceptor-mosaic/

M cones correspond to middle wavelength cones (λmax ~ 530-537 nm) and S cones for short wavelength sensitive (λmax ~ 415-430 nm). Only a few old world primates have evolved trichromatic vision and express "L" (long wavelength) cones most sensitive to low-frequency photons (λmax ~ 555-565 nm, Jacobs et al, 1998) Figure 9.



"1416 Color Sensitivity" by OpenStax College - Anatomy & Physiology, Connexions Web site.

Figure 9: Normalized human photoreceptor absorbances for different wavelengths of light

Mapping studies of SW cones across *Arvicanthis ansorgei* entire retina show a significantly higher number in the central field than in the far periphery. There is no detectable variation in their number as a function of retinal quadrant (Bobu and Hicks 2006). This result differs from data in mouse retina where both cone spectral classes are distributed in a heterogeneous pattern with SW-cones predominantly located in the ventral field (Szél and Röhlich 1992; Szel et al. 1992). Besides, numerous sections taken across the retina did not reveal a specific arrangement of M cones. Comparison of M-cone opsin levels in rat and *Arvicanthis ansorgei* displays a correlation between cone abundance and level of proteins, e.g. more M-cone opsins in the latter. However, S-cone opsin level is identical to the rat one. We may explain this similarity by a low S-cone to M-cone ratio, or to a lower amount of S-cone opsin per cell, or both.

Co-localization of *Arvicanthis ansorgei* S- and M-cone opsins has not been studied because both anti-mouse opsins antibodies were obtained in rabbits. However, co-localization of opsins has been demonstrated in a few rodent species including the house mouse, the pouched mouse (*Saccostomus campestris*), the Siberian hamster, and even human adult retinas (Cornish et al. 2004; Lukáts et al. 2005). Rods express uniquely rhodopsinand our team has observed a lower amount of rhodopsin in *Arvicanthis ansorgei* retina compared to the rat, a result consistent with the reduced rod population. Besides, we have examined the daily transcription profiles of visual pigment genes (*Opn1sws, Opn1mws, Opn2, Opn4,*) and selected PR genes such as *Arr3* or *Aa-nat* (Bobu et al 2013). These genes are under circadian control (see master clock section below), tightly synchronized among one another (morning maxima, except *Aanat* which will be discussed in part 3). A single 24 h cycle of constant light is sufficient to greatly modify these profiles. The results thus demonstrate strong circadian regulation of both clock genes with several putative outputs and underscore the dramatic consequences of altered lighting regimes.

I.B.1.d Cone and rod connections:

Retina converts light information into electric messages- phototransduction- conveyed to the image-forming structures of the brain. In addition to their distinct shape, photopigments and distribution, rods and cones have specific synaptic connections. Arrangement of the circuits that transmit rod and cone information to RGC contributes to the different characteristics of scotopic (rod) and photopic (cone) vision (Figure 10). Rod and cone pathways are strictly separated: rods connect to rod bipolar cells and cones to cones bipolar cells. Cone bipolar cells form synapses with RGC whereas rod bipolar cells do not directly contact RGC but feed into cone bipolar cells via a population of amacrine cells. Visual sensitivity and spatial resolution (acuity) are conflicting fundamental capacities (Peichl 2005). Sensitivity requires the summation of the signals of many photoreceptors in order to optimize the signal-to noise ratio, thus precluding spatial resolution. Such convergence is a hallmark of the rod pathway. Good spatial resolution requires low convergence and stimuli of high brightness or color contrast and reasonably high light levels. This is a task for the cones, which have low convergence onto cone bipolar cells and ganglion cells that preserves the fine-grain information to higher processing stages.

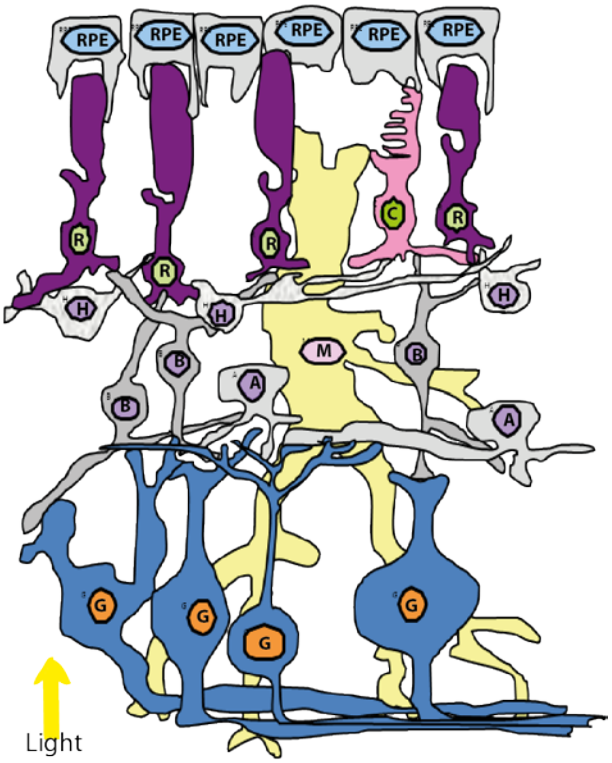


Figure 10: Schematic representation of mammalian retina cell types and connections.

Rod (R) and cone (C) photoreceptors are displayed in a cross-sectional depiction of the retina also showing connections of these photoreceptors to retinal pigment epithelium (RPE) distally and relaying cells (bipolar (B), horizontal (H), amacrine (A), ganglion (G)) proximally. Modified from an original drawing by Marie-Paule Felder-Schmittbuhl.

However, the spatial resolution capacity of a retina is determined by its ganglion cells, because in most mammals even the smallest ganglion cells in the *Area centralis*, the retinal region of highest neuron density and hence visual acuity, sum the inputs of several cones. The only known exception is the primate fovea, where the 1:1 connection between cones and ganglion cells conserves the "pixilation" of the cone mosaic down to the ganglion cells (by the so-called midget RGC system). In other species such as *Arvicanthis ansorgei*, a higher cone density also indicates better visual acuity, but the acuity cannot be calculated directly from that density unless the convergence ratio of cones to ganglion cells is known. At present, we have not determined the ratio of cones to ganglion cells in our animal model.

In human, the wiring of rods and cones with RGC are different in the peripheral and central retina. Indeed, in the periphery there is high convergence of the rods onto their postsynaptic neurons: one RGC carries information of a large number of photoreceptors (Figure 10). More convergence makes the rod system a better detector of light, because small signals from many rods are pooled to generate a large response in the bipolar cell. However, such convergence also reduces the spatial resolution of the rod system. At the opposite, in the cone rich central retina, a low convergence of cone is found: one cone cell is connected to one bipolar cell itself contacting one RGC (Figure 10). As stated above, the one-to-one relationship of cones to bipolar and ganglion cells maximizes visual acuity (Mustafi et al 2009).

I.B.1.e Cone and rod roles:

Phototransduction:

Phototransduction is the process through which light energy is converted into electrical signals. Visual phototransduction occurs in the retina through the rods, cones and ipRGC (cell type discussed in a section below). Indeed, the rod system is extremely sensitive to light (responding to one photon, (Cicerone 1976; Rodieck and Rushton 1976) and used for vision at low light levels (scotopic or night vision). Rods allow a grey nuance vision and are sensitive to contrast and movement perception but they have a low spatial resolution. Therefore, the cone system is the one responsible for high visual acuity. Cones are less sensitive to light (100 photons are required to compare with a one photon-rod response) and account for color discrimination in daylight (photopic) condition (Sugita 1988).

General mechanism (not *Arvicanthis ansorgei* specific)

Photoreceptors can respond to light by virtue of their containing a visual pigment embedded in the bi-lipid membranous discs that make up the OS. The visual pigments (**G protein coupled receptors: GPCR**) are either cone opsins (specific to cones) or rhodopsin (specific to rods) bound to a chromophore derived from vitamin A known as retinal. The Figure 11 below illustrates phototransduction in rods. First, absorption of a photon by retinal changes its conformation from the 11-cis form to all-trans retinal. This event triggers conformational changes in rhodopsin resulting in binding and activation of the transducin G protein located in the cytosol. Then, bound guanosine diphosphate (GDP) is exchanged for guanosine triphosphate (GTP) and dissociation of the transducin α subunit occurs. In turn the α-subunit-GTP complex activates cyclic guanosine monophosphate-dependent phosphodiesterase (cGMP-PDE) that hydrolyses cGMP to 5'-GMP. The resulting decrease in the cytoplasmic free cGMP concentration leads to the closure of the sodium channels on the plasma membrane. Channel closure blocks entry of cations and causes hyperpolarization of the cell due to the ongoing sodium pump activity and potassium current.

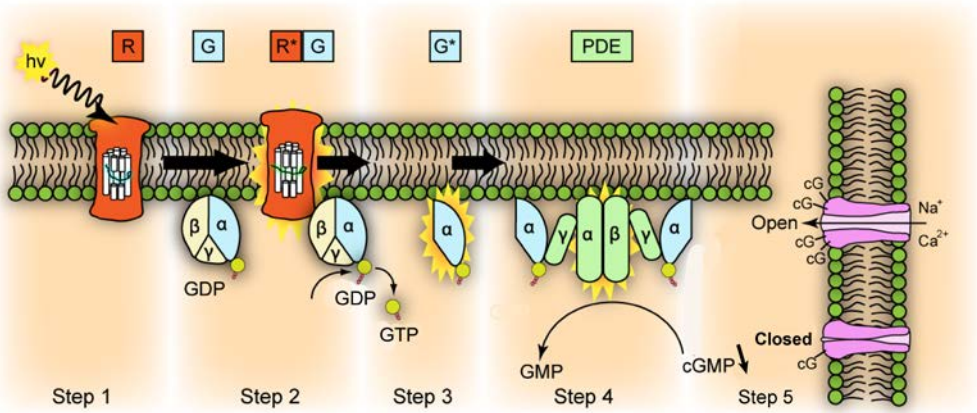


Figure 11: Schematic representation of the activation of vertebrate rod phototransduction.

Step 1: a photon (hv) is absorbed by the retinal bound to the rhodopsin (R). Step 2: the activated rhodopsin (R*) binds the G protein heterotrimere (G). Step 3: activated G protein (G*) catalyzes the exchange of GDP for GTP, which produces the active Gα*-GTP. Step 4: Two Gα*-GTP bind to PDE γ subunits, thereby releasing the inhibition on the catalytic α and β subunits. This leads to activated PDE, which in turn catalyzes the hydrolysis of cGMP. Step 5: The subsequent decrease of the free cytoplasmic cGMP concentration leads to the closure of

the cGMP-gated channels on the plasma membrane which blocks the influx of cations into the OS, and results in hyperpolarization of the cell. Adapted from (Fu 2010).

The activation phase of phototransduction is terminated by phosphorylation of the visual pigment by rhodopsin kinase which leads to binding of arrestin and de-activation of the receptor. In parallel, free trans-retinal undergoes a series of biochemical steps (known as the visual cycle) within the adjacent RPE to be reconverted into 11-cis-retinal.

An essential and seemingly paradoxical feature of rod and cone signaling is that they are more active in the dark than in the light, since glutamate release is constitutive under depolarizing conditions (when the cation channels are open). Light stimulation and hyperpolarization of rod and cone photoreceptors results in decreased glutamate neurotransmitter release in the OPL. There are two types of BC, called "on" and "off", which express distinct sub-types of glutamate receptor (GluR): in the case of "off" BC these are ionotropic GluR which directly signal glutamate binding, ie. reduced glutamate release following illumination leads to reduced iGluR stimulation and hyperpolarisation, thus switching off the cells (hence they are "turned off" by light); on the contrary, "on" BC express a unique metabotropic GluR6, inversely coupled to cation channels that hold the cells in a hyperpolarized state when glutamate release is high. Hence decreased glutamate concentration during light stimulation leads to decreased activation of mGluR6, the cation channel blockage is stopped and the cell depolarizes (they are "turned on" by light). These two parallel pathways, "on" and "off", are responsible for generating a primary visual image composed of darker areas (shadows, dark hues) and brighter ones (illuminated surfaces). Additionally to the light signal transduction pathway (PR-bipolar cell-RGC), photoreceptor signal is modulated by horizontal and amacrine cells prior to final transmission to RGC.

Cone response to photons:

The probability that a photon will be absorbed by a cone is based on three factors in temporal order: I) The direction of arrival of the photon (most efficient when along the long axis of cone); ii) The frequency of the photon (only 67% are of the correct frequency to photoisomerize visual pigment molecules due to the spectral content of the signal); iii) The type of cone (L, M, or S-type) (Mustafi et al 2009). Cone photoreceptors are less sensitive and have a faster response to light than rods (for review of the differences see (Miller, James L. M 1994). Although phototransduction in cones is broadly similar to that in rods, there should also be some important differences that would be responsible for the differences in sensitivity and response kinetics. These differences are thought to reside mainly in the biochemical components of the phototransduction process (Ebrey and Koutalos 2001). In the cone, membrane shape and composition are critical determinants of its extended activity in light, in contrast to rods. In cones, the protein molecules of the biochemical cascade are located on a continuously folded plasma membrane rather than on separated disc membranes (Figure 12). Thus, cone membranes offer a much larger surface area for rapid exchange of substances between the cell exterior and interior, such as chromophore transfer for pigment regeneration and fast calcium dynamics during light adaptation (Fu and Yau 2007). Moreover, organization of opsins in mammalian cone cells has yet to be determined (Mustafi et al 2009) but could influence phototranduction. Another, perhaps relevant, difference between rods and cones is the surface to

volume ratio, which would affect how long it would take equal numbers of transporters (on the surface) to effect changes in the concentration of the Ca^{2+} (of the outer segment volume).

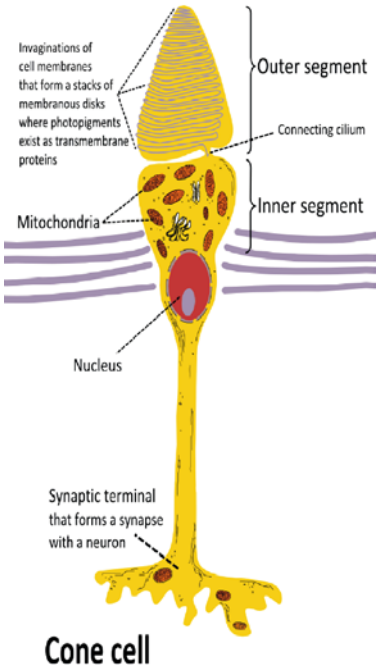


Figure 12: Cone structure

Cone cells have a cone-like shape at one end where pigment filters incoming light. They are typically 40–50 µm long, and their diameter varies from 0.5 to 4.0 µm, being smallest and most tightly packed at the center of the primate eye at the fovea. The S cones are a little larger than the M-and L- cones. Credit: Ivo Kruusamägi

Besides its visual function, retina is also involved in non visual function including different neuronal pathways. The first **non visual function is the pupillary reflex (PLR)**, a task performed by rod, cone and ipRGC. Second, the **major retinal non visual function is the photo-entrainment of biological events** as described below.

Pupillary reflex: not *Arvicanthis ansorgei* specific

The PLR serves image vision by reducing the saturation of rod and cone photoreceptors by light, increasing depth of field, and improving resolution. PLR also protects the retina from the potentially damaging effects of exposure to bright light. The PLR is mediated by ipRGC that projects directly to the olivary pretectal nucleus in the midbrain (Hattar et al 2002) (Figure 13).

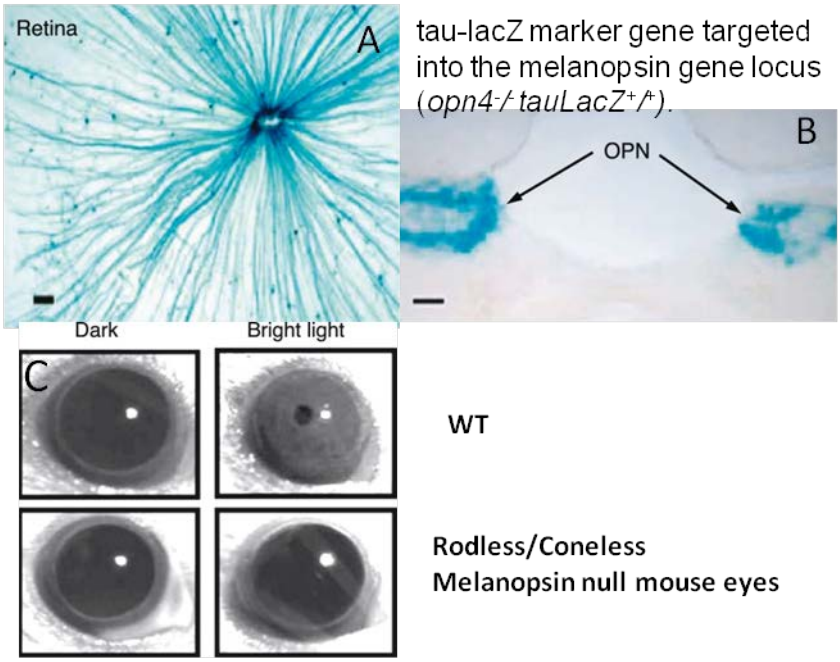


Figure 13: Pupillary light reflex pathway.

Panel A: flat-mount retina of a mouse with the tau-lacZ marker gene targeted into the melanopsin gene locus (*opn4-/-tau-LacZ+/+*). Blue color shows X-gal staining of the β-galactosidase activity coded by *tau-lacZ* in the ipRGCs. IpRGC axons converge to the optic disk. Scale bar is 100 µm. Panel B: olivary pretectal nucleus (OPN) is a major target of ipRGCs. Coronal section, scale bar is 100 µm. Panel C: PLR is controlled by rod, cone and ipRGCs. Adapted from (Tri Hoang Do 2010)

Rod and cone photoreceptors provide inputs to ipRGCs but melanopsin cells are not required for pattern-forming vision (Güler et al. 2008). In contrast, PLR and other non visual light responses are abolished if rod-cone and melanopsin signaling pathways are all disrupted in mice (Hattar et al 2003). In humans, rods and cones are capable of driving the initial rapid constriction of the pupils in response to light (Alpern and Campbell 1962), whereas the PLR is most sensitive to short wavelength blue light during exposure to continuous light (Zaidi et al. 2007), suggesting a primary role for melanopsin.

For light intensities below the threshold of activation for ipRGCs, spectral responses of the PLR during exposure to continuous light are consistent with **a role for rods**, with little or no contribution from cones (McDougal and Gamlin 2010). To assess both rod and cone roles in PLR, Gooley et al have studied the PLR in the retina of a blind patient with intact non visual responses to light but without a functional outer retina. These authors show that rod-cone photoreceptors play an important role in driving pupillary constriction during exposure to continuous low-irradiance light. Rod-cone photoreceptors mediate rapid constriction of the pupils following light stimulus onset and allow the pupils to track high-frequency intermittent light stimuli. With intermittent light in the 0.1-4Hz range, they prevent pupillary escape and enhance their constriction for at least 30 minutes. Their conclusion states that **cones** are involved in the phenomenon. They hypothesize that short intermittent dark pulses give cone photoreceptors the opportunity to dark adapt before each light pulse, thus preventing pupillary escape (Gooley et al 2012). Therefore, both rod and cone PR subtly participate in the PLR in the mammalian retina.

I.C. Non image forming visual function: Photo-entrainment of biological clocks

I.C.1. Introduction

Numerous biological functions display daily and seasonal variations in a way to anticipate and adapt to the upcoming cyclic changes in environmental conditions including light level, temperature, food availability, the presence of predators or hosts (Figure 14 below).

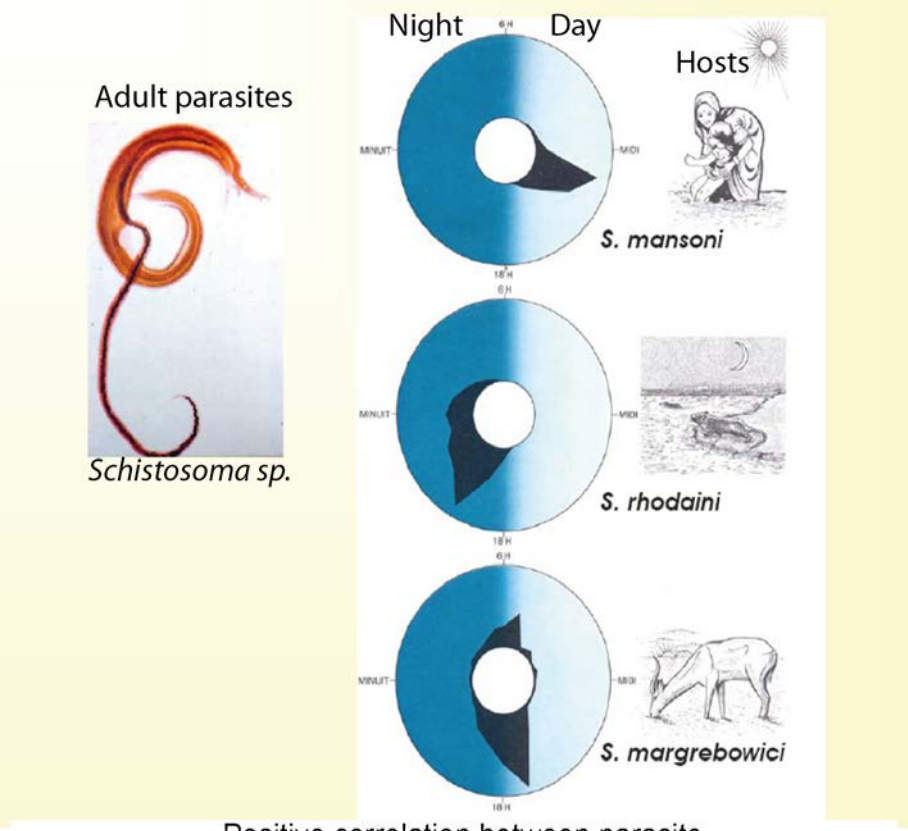


Figure 14: Illustration of the biological rhythms adaptation between the Plathyhelminth parasite *Schistosoma* and its Mammalian hosts. Claudes Combes 1988.

Indeed, we have known since Antiquity that living organisms including animals go through daily variations of physiological functions and behaviors adjusted to 24 hours (24h) light-dark cycle; about equal to the rotation of Earth around its axis. However, it is only during the 20th century that physiologists have overcome the apparent contradiction between the physical environment influence on the daily rhythms of living organisms and their endogenous homeostasis, to demonstrate the existence of accurate endogenously organized timing systems termed **biological clocks**. What are biological rhythms? Biological rhythms are repeated fluctuations of biological events such as hormone secretion, body temperature, sleep/wake cycle mainly characterized by the period (duration of one fluctuation) and amplitude (Figure 15). That said, several criteria must be met in order to confirm that a particular variable is under endogenous circadian control (detailed in (Silver and Kriegsfeld 2014). Chronobiology examines periodic (cyclic) phenomena in living organisms and their adaptation to solar, lunar and seasons- related rhythms. I will focus on rhythms with periods close to those of the astronomical day named **circadian** rhythms. Therefore, properties of a circadian clock will be briefly explained before going closer to our topic: the photo-entrainment of biological clocks.

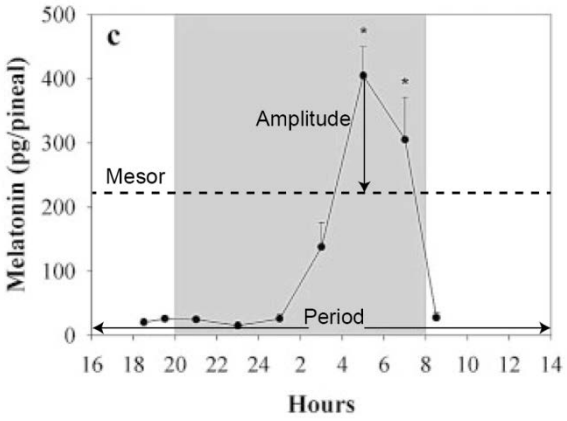


Figure 15: Daily profile of melatonin in the pineal of *Arvicanthis ansorgei.*

Animals were raised under a 12-h light: 12-h dark cycle (lights on from 08.00 h), from (Garidou et al 2002). Among the parameters used to describe a biological rhythm, Period is the time elapsed for one complete oscillation or cycle. Mesor (midline estimating statistic of rhythm. Amplitude is the difference between the peak (or trough) and the mesor of rhythm.

I.C.2. Properties of a circadian clock

First, a clock distributes an output, assessed as a biological rhythm and in constant conditions the period of circadian rhythms is approximately, but not precisely, 24 h. Nonetheless, rhythms driven by daily cues such as the LD cycle or geophysical signals would be precisely 24 h. To adjust its endogenous period to the light/dark cycle, a clock must possess a setting mechanism to link the internal oscillations to the astronomical position (time) of the sun. The most potent synchronizers or Zeitgebers (literally time givers) capable of resetting the clock are the light/dark cycle (LD=12 hours light/12 hours dark), food consumption, activity/rest cycle (Figure 16). Second, unlike most biological processes, where increased temperatures hasten biochemical processes and decreased temperatures have the opposite effect, circadian rhythms are temperature compensated, such that the period of the rhythm is unaltered by temperature changes (Pittendrigh and Caldarola 1973). This result rules out the possibility that daily changes in temperature are responsible for circadian rhythmicity, although some rhythms can be entrained to cycles in temperature. Together, these findings provide strong suggestive evidence for the endogenous regulation of circadian rhythms.

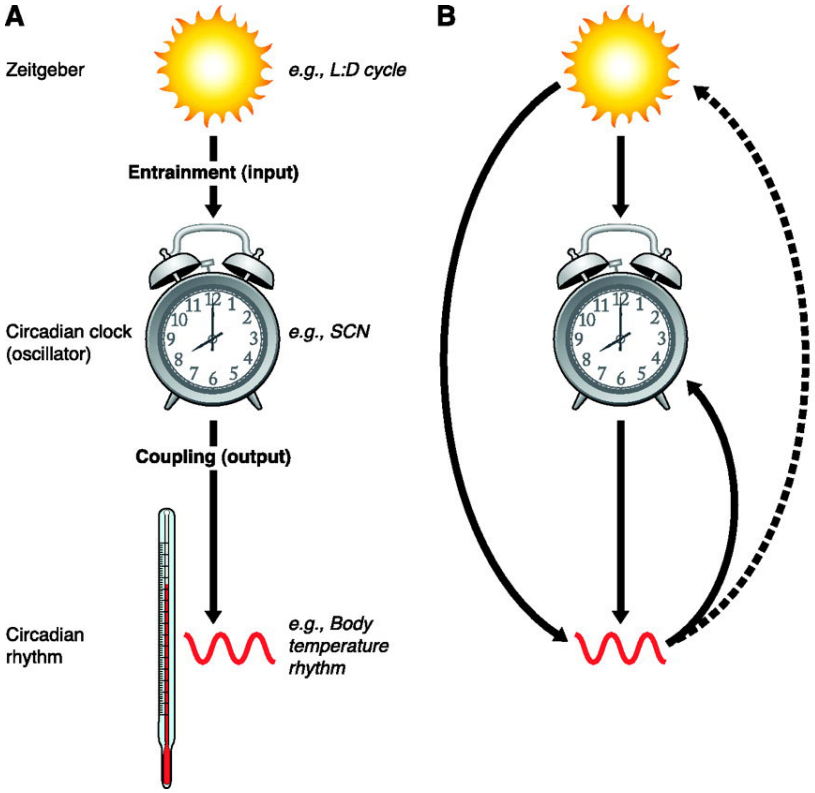


Figure 16: Simplified scheme of the circadian system.

A: a linear model which includes an endogenous oscillator (e.g., the hypothalamic suprachiasmatic nuclei), the environmental entrainment agent (zeitgeber), and the output that governs overt biological rhythms. B: a more complex view based on solid evidence emphasizes a nonlinear relationship between the components of the model. Clock-controlled rhythms often include variables that feedback into the endogenous oscillator, in a similar fashion to physiological homeostatic mechanisms. Moreover, these variables might affect the input pathway (e.g., a clock-controlled circadian rhythm of photoreceptor structures). On the other hand, input signals might directly affect physiological and behavioral variables without the participation of the clock (masking). Adapted from (Colombek 2010).

I.C.3. The brain master clock:

In 1972, two laboratories showed that the destruction of a very discrete hypothalamic area, the suprachiasmatic nuclei (SCN) led to permanent loss of circadian rhythms (Moore and Eichler 1972; Stephan and Zucker 1972). The finding of a neural locus for circadian timekeeping provides definitive evidence for the endogenous control of circadian rhythmicity. The circadian clock is characterized by a hierarchical principle. The SCN central clock is entrained by environmental light conditions. In turn, the central clock entrains the peripheral clocks through various hormonal and neuronal signals, which dictate the oscillating gene expression in most other organ systems (Hogenesch and Ueda 2011). **Melatonin, as a prominent output of the central circadian clock, modulates a wide array of physiological functions** such as the sleep/wake cycle (Burke et al 2013), reproduction (Barrett and Bolborea 2012; Simonneaux 2011) and immune modulation (Sande et al. 2008). **Melatonin is also synthesized *de novo* in the vertebrate retina** where it can exert local effects on photoreceptor phagocytosis, neuronal electric activity, dark adaptation and light sensitivity, by way of specific MT_1 and MT_2 receptors expressed in the eye (references in our publication (Gianesini et al 2015)).

At the cellular level, circadian rhythms are generated by interlocked transcriptional/translational feedback loops consisting of 'clock' genes and their protein products (reviewed in (Hastings et al 2014; Partch et al 2014; Zhang and Kay 2010) (Figure 17). In mammals, the core feedback loop consists of two transcriptional activators, CLOCK and BMAL1, and two transcriptional repressors, the PERIOD (PER) and CRYPTOCHROME (CRY) proteins. In the morning, CLOCK and BMAL1 activate transcription of the *Per* (*Per1*, *Per2* and *Per3*) and *Cry* (*Cry1* and *Cry2*) genes by binding to the E-box (CACGTG) domain on their gene promoters. Over the course of the day, *Per* and *Cry* genes are translated into their respective proteins. PER and CRY proteins form heterodimers late in the day that translocate from the cytoplasm to the cell nucleus to inhibit CLOCK: BMAL1-mediated transcription. The timing of nuclear entry is balanced by regulatory kinases that phosphorylate the PER and CRY proteins, leading to their degradation (Lowrey PL 2000); (Shanware et al. 2011). REV-ERBα/ROR-binding elements (Preitner et al. 2002) act to regulate *Bmal1* transcription via a secondary feedback loop. The transcriptional retinoid-related orphan receptor (ROR) is a transcriptional activator of *Bmal1*, whereas REV-ERBα, an orphan nuclear receptor, negatively regulates *Bmal1*. The same CLOCK: BMAL1 mechanism controlling *Per* and *Cry* gene transcription also controls transcription of REV-ERBα. This secondary feedback loop produces rhythmic expression of BMAL1, further stabilizing the clockwork. Then, a third loop has been added to the clockwork with D-site of albumin promoter-binding promoter (DBP) and E4 promoter binding rotein 4 (E4BP4) which synergistically regulate the expression of D-box-containing genes including *Per*.

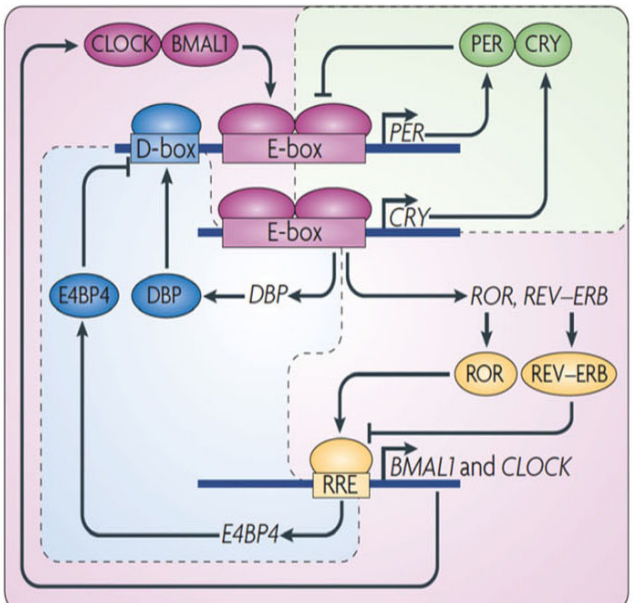


Figure 17: Molecular clockwork machinery

A triple interlocking loops model was developed in the past decade, including the PER–CRY loop, which is the primary loop, and the ROR–REV-ERB-associated loop and D-site of albumin promoter-binding protein (DBP)-E4 promoter-binding protein 4 (E4BP4)-associated loop. In addition to the primary loop, which was previously recognized as the core loop; transcriptional regulation of CLOCK and BMAL1 is controlled by ROR transcriptional activators and the dimeric REV-ERB repressors [by binding to REV response element (RRE)], the expression of which is governed by CLOCK–BMAL1 activity (through binding to E-box-containing DNA elements). Furthermore, the transcriptional activator DBP (the expression of which is controlled by an E-box) and E4BP4 (the expression of which is controlled by RRE) synergistically regulate the expression of D-box-containing genes, including PER. (Silver and Kriegsfeld 2014)

Moreover, hundreds of clock modifiers have now been identified through a genome-wide screen, some of which directly or indirectly associate with known clock components (Silver and Kriegsfeld 2014).

Finally, there have been hints of the existence of oscillation in the absence of transcriptional and translational feedback loops. One of the most striking examples is the discovery of circadian oscillations in the red blood cells which lack a nucleus and therefore lack the genetic clock

mechanism (O'Neill and Reddy 2011). We must thus consider the cross-talk between the transcriptional and the "cytoplasmic-metabolic" clock components to study cell type-specific program of expression.

I.C.4. *Arvicanthis ansorgei* Master clock:

Arvicanthis ansorgei master clock is located in the SCN (Figure 18) (Caldelas et al. 2003) and has been well studied by teams of our Institute (INCI, Strasbourg). Circadian profile and photic regulation of clock genes in the SCN share clear similarities with nocturnal mammals (Caldelas et al. 2003). However, as a clock output the temporal organization of their locomotor activity shows a diurnal frequently crepuscular, pattern of activity. Clock parameters have also been determined such as the phase-response curve (Pittendrigh and Daan 1976) to photic and non photic synchronizing cues and the master clock period **tau** which ranges from 23.6h to 24,4h (Challet et al. 2002). Finally, *per1* and *per2* expressing neurons have been characterized as well as the circadian expression profile of SCN essential neuropeptides (Dardente et al 2004; Dardente et al. 2002).

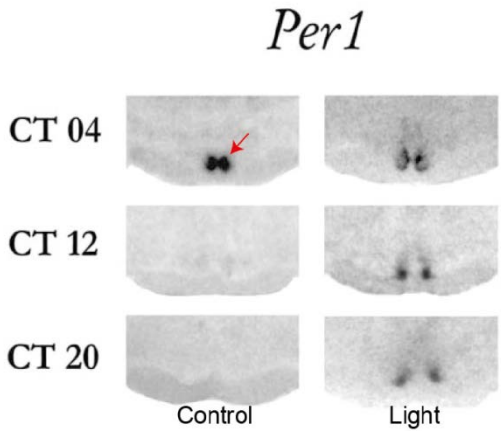


Figure 18: Circadian expression and photic modulation of *Per1* mRNA in *Arvicanthis ansorgei* SCN

Per1 mRNA expression is analyzed in coronal sections of the suprachiasmatic nucleus (red arrow) in *Arvicanthis ansorgei* exposed to light (Light: 100 lux during 1 h) or not (Control) during the subjective day (CT 04 or CT 08), the early (CT 12) and late (CT 20) subjective night. Adapted from (Caldelas et al. 2003)

After this long but necessary description of the circadian clocks, we may recall that the light entry in the body goes through the entire retina to stimulate rods and cones and ipRGCs (see below) to convey non visual information to the central clock.

I.C.5. The photoentrainment of the master clock:

I.C.5.a Cone and rod path:

The major work of Rollag MD, Hattar S., Saper C.B., Foster R. teams to name few (review) on mouse retina has allowed the discovery of a subpopulation of ganglion cells expressing a new photopigment termed melanopsin (ipRGC) (Provencio et al. 1998). Subsequently, their work has highlighted the necessity of bearing the 3 photoreceptor types (rod, cone, ipRGC) to achieve non visual communication with central brain and in particular with SCN (Hattar et al 2003). Indeed, the retinohypothalamic tract relays non-visual information from the outside world via ipRGC to

synchronize the master clock in the SCN (Figure 19). Rod and cone signals contribute to the light response in the SCN and to circadian entrainment through stimulation of the ipRGCs (Aggelopoulos and Meissl 2000). In rats, the majority of visually responsive SCN neurons studied under dark adaptation receive rod input (92% of examined cells). When studied under light adaptation, most visually responsive SCN neurons (77%) respond to input from cones. The presence of these neuronal responses suggests a role for rod and cone photoreceptors in SCN function (Aggelopoulos and Meissl 2000). In addition, Van Diepen et al (Van Diepen et al. 2013) have demonstrated, with *opn4*-/- and *rd/rd* mice, that SCN exhibit sustained firing levels in the absence of melanopsin, and across a broad range of wavelengths. The relative contribution of melanopsin, rods, and cones remains to be determined, and is most likely dependent on the intensity and wavelength composition of the light source. They suggest that at lower to intermediate irradiances, classical photoreceptors play a role in transmitting light information to the SCN. At low light intensities, only rods are stimulated and clear tonic responses are observed in the SCN. At high light intensity (rod saturation) the authors could see an increment in SCN light-response levels, presumably mediated by cones.

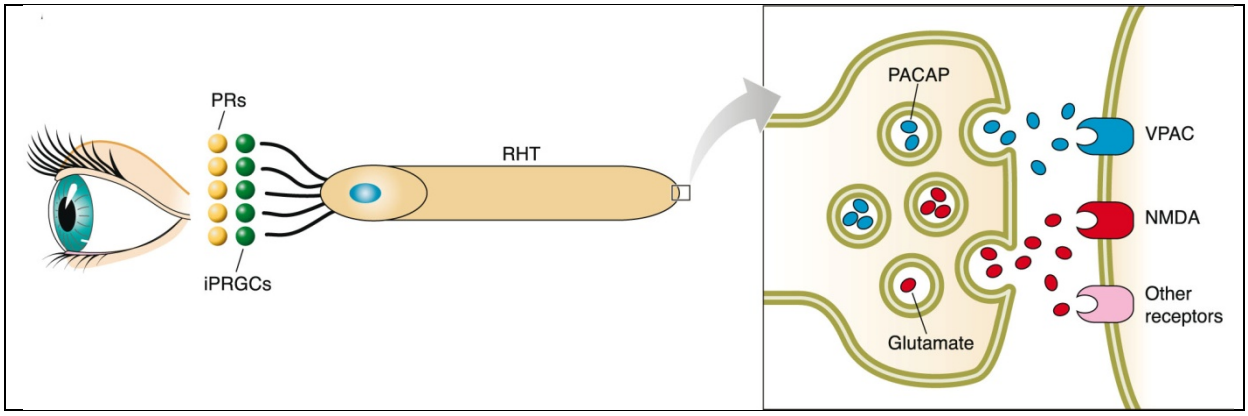


Figure 19: Schematic representation of the retino-hypothalamic tract

The retino-hypothalamic tract originates in the retinal ganglion cells (including photoreceptive GCs) and innervating (through glutamate and PACAP neurotransmission) the ventral part of the SCN. VPAC: vasoactive intestinal peptide receptor, NMDA: N-methylD-aspartate receoptor. Adapted from (Colombek 2010).

Also, a switch from nocturnal to diurnal activity (output of the clock) was observed in wild type mice under light/dark cycles with scotopic levels of light during the light phase, suggesting that the state of photoreceptors can play an important role in determining phasing of activity in diurnal and nocturnal species (Doyle et al. 2008). Finally, the ablation of the melanopsin gene leads to **partial** impairment of the pupil reflex, light-induced phase delays of the circadian clock and period lengthening of the circadian rhythm in constant light. These different works demonstrate direct and indirect rod and cone links with SCN and raise more questions about the contribution of each photoreceptor type in the temporal niche switch. **Our work has focused on melatonin in the retina of the diurnal rodent *Arvicanthis ansorgei* (as will be reported below) and our results highlight cones and GCL as essential cells for melatonin synthesis and action. *Arvicanthis ansorgei* possesses 33% of cones and display a bimodal diurnal activity. Therefore, we can ask these questions: Do more cones imply more irradiance information sent to the SCN? Does cone melatonin play a role in the transmission of UV light information to SCN? Or is this task shared with ipRGC? I will discuss these questions in the "Melatonin and light" paragraph.**

The third acknowledged role of cone and rod cells in the non-visual flow of information going to the central brain is the modulation at least via the SCN, of the sleep-wake cycle in Mammals. Hubbard et al report that the mirrored effects of light and darkness on sleep/wake cycle are mediated through melanopsin but also rod-cone based phototransduction. These effects depend on time of day, are mainly melanopsin-phototransduction based during the dark phase and rods/cones pathways during the light phase (daytime) (Hubbard et al. 2013). To sum up, rod and cone cells are essential for the photoentrainement of the clocks **but ipRGCs expressing melanopsin are essential for all major non-visual functions in Mammals.** Our team, in collaboration with H. Meissl, has characterized these specific subpopulations of ganglion cells in the retina of *Arvicanthis ansorgei* (Karnas et al. 2013).

I.D. The Ganglion Cells: in Mammals (including *Arvicanthis ansorgei)*

The RGC receive visual information from photoreceptors via two intermediate neuron types: bipolar cells and retina amacrine cells (see sections below). RGC collectively transmit image-forming and non-image forming visual information from the retina in the form of action potential trains to several regions in the thalamus, hypothalamus, and midbrain. RGC vary significantly in terms of their size, connections, and responses to visual stimulation but they all share the defining property of having a long axon that extends into the brain. In the retina of *Arvicanthis ansorgei*, ganglion cells axons are visualized with specific anti-neurofilament antibody (Figure 20 below). These axons form the optic nerve, optic chiasm, and optic tract. The ipRGC, forming only a small percentage of RGC contribute little to form vision, but their axons form the retinohypothalamic tract. RGC spontaneously fire action potentials at a base rate while at rest. 'ON' type ganglion cell fibers respond with a transient burst to light onset, and a sustained elevated discharge rate throughout the photic stimulation.'ON-OFF' fibers respond with discharge bursts at the onset and cessation of light stimuli, but are otherwise quiet. 'OFF' type ganglion cell fibers are quiet until the stimulus light is turned off, whereupon they respond with a sustain burst of impulses (see Ralph Nelson, Webvision). Recent data suggest that mouse retina has approximately 20 RGC types (Masland 2012). This number reflects the fact that besides an input from the 12 types of bipolar cells described in (Ghosh et al. 2004), RGC are shaped also by the contribution of amacrine cells, so that additional functional channels are created at the retina exit (Taylor and Smith 2011). I will focus on the ipRGC-melanopsin cells as it is relevant to circadian rhythms and our work on melatonin.

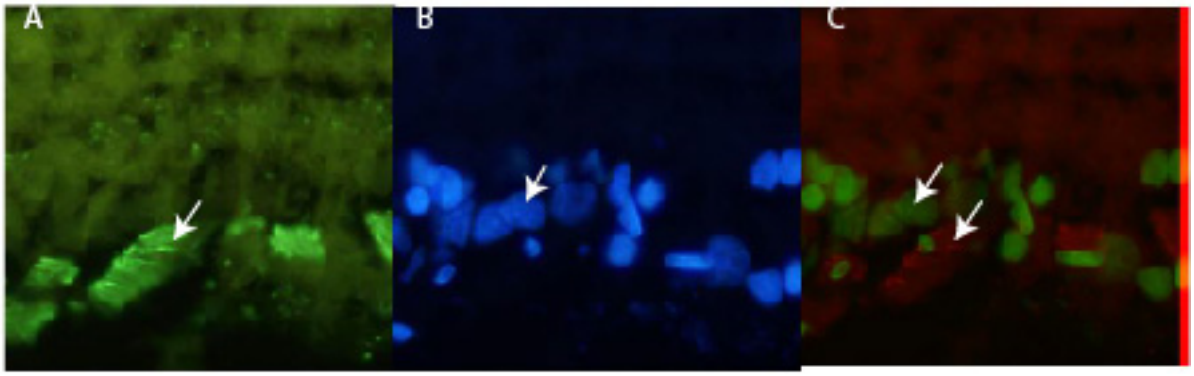


Figure 20: Ganglion cells layer in the retina of *Arvicanthis ansorgei*.

Panel A: Ganglion cell axons are labeled with anti-neurofilament light chain (anti-NFL 68/70 kDa) as pointed by the white arrow; **Panel B** depicts the DAPI staining of the ganglion cell nuclei (white arrow). **Panel C** represents the merged images (A and B). Magnification is X400.

I.D.1. Melanopsin photopigment and phototransduction:

Melanopsin is a seven transmembrane G-protein coupled receptor (GPCR) with all the key structural features of an opsin whose peak spectral sensitivity is 480 nm (Provencio et al. 1998). In mammals, one melanopsin gene has been identified (in contrast to other vertebrate classes which contain at least two (Bellingham et al. 2006), but splice variants have been reported (Pires et al. 2009) whose functional relevance is yet unclear. Phylogenetic analysis suggests that mammalian melanopsin is more closely related to the invertebrate opsins than the classical vertebrate visual opsins (Koyanagi and Terakita 2008; Provencio et al. 1998). Thus, ipRGCs phototransduction pathway leads to the depolarization of photoreceptors through Gq-mediated signaling cascade and opening of a transient receptor potential (TRP) channel (Peirson and Foster 2006). This scheme differs from the rod/ cone phototransduction which leads to their hyperpolarization (see section above). To date, the precise cellular mechanisms of phototransduction in ipRGCs have not been fully characterized. *In vivo* melanopsin binds 11-cis retinal as its chromophore and converts this to all-trans retinal following absorption of a photon (Walker et al. 2008). Interestingly, there is strong evidence that melanopsin is capable of acting as a bistable photopigment (Melyan et al. 2005; Mure et al. 2007; Panda et al. 2005) and is capable of spontaneously regenerating 11-cis chromophore from all-trans retinal. However, data by (Wang 2010) show that *Drosophila* photoreceptors require bistability and also the use of pigment cells to regenerate 11-cis retinal (11-CRAL). Moreover, lack of all trans retinal (ATRAL) detection in mouse dark-adapted ipRGCs support an additional light-independent mechanism for chromophore regeneration proposed by (Schmidt et al 2011) and also work by (Doyle et al. 2008)on the RPE65 KO mouse. These researchers suggest that Müller cells may assist ipRGC in the chromophore regeneration, the same way as it is described for cones (Figure 21). To sum up, melanopsin phototranduction is not completely elucidated and raises intriguing questions notably about its relation with the cone/rod systems within the retina.

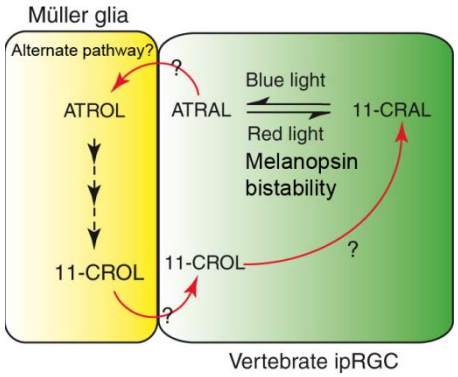


Figure 21: A proposed pathway for chromophore regeneration in ipRGCs.

Schmidt et al propose that ipRGCs also use a chromophore regeneration pathway requiring the Müller glia, similar to cones. Red lines indicate proposed steps for chromophore regeneration in ipRGCs and question marks indicate that these pathways have not been demonstrated, although the dependence of ipRGCs on RPE65 is well documented (Schmidt et al 2011)

I.D.2. Intrisically photosensitive retinal ganglion cells (ipRGC):

I.D.2.a Diversity of ipRGCs:

Recent studies indicate that ipRGCs are composed of different subtypes, each with unique morphological and physiological properties and potentially distinct functional roles. The ipRGCs subtypes contribute differently to non image and image forming behaviors. The three well-characterized subtypes are the M1 ipRGCs which stratify in the outermost sublamina of the IPL, the M2 ipRGCs which stratify in the innermost sublamina of the IPL, and the bistratified M3 ipRGCs, with dendrites in both inner and outer sublamina. In addition to differences in dendritic stratification, M2 cells have larger soma sizes and larger, more highly branched dendritic arbors, and lower expression of melanopsin than M1 cells (Ecker et al. 2010; Schmidt and Kofuji 2009). Detailed study of the M3 ipRGCs has revealed that these bistratified cells, in contrast to other bistratified RGCs, show variability in the proportion of dendritic stratification in the inner and outer sublaminae (Schmidt and Kofuji 2011). In *Arvicanthis ansorgei*, M1 ipRGCs make 74% of ipRGC cells, a much higher figure than what is known for the mouse 30-44%. Few M1-type cells possess somata displaced to the inner nuclear layer. Consequently, M2 ipRGCs are in low number (about 25%) and possibly include non-M1 unidentified cells (Karnas et al. 2013) (Figure 22). Recently, the Cre/Lox system (Ecker et al. 2010) used within the melanopsin gene has allowed the discovery of two additional subtypes of ipRGCs, M4 and M5 (Schmidt and Kofuji 2011). Both of these cell types stratify in the inner sublamina of the IPL. M4 cells have the largest soma of any described ipRGC subtype, as well as larger and even more complex dendritic arbors than M2 cells (Ecker et al. 2010). By contrast, M5 ipRGCs have small, highly branched arbors arrayed uniformly around the soma. These two new subtypes cannot be stained with even the most sensitive melanopsin antibody, although they do display a consistent yet weak intrinsic light response. For the same reason, these subtypes could not be reliably identified in *Arvicanthis ansorgei* retina.

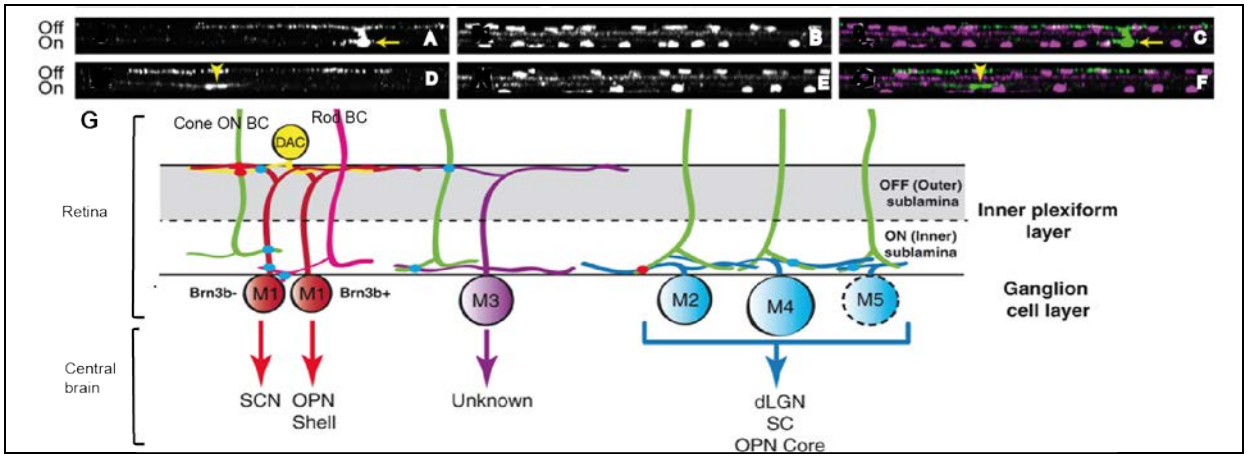


Figure 22: Morphology of M1 and M2 ipRGCs in the retina of *Arvicanthis ansorgei*.

A-C: Side view and stratification level of M1 cell (soma indicated by an arrow). **D-F**: Side view and stratification level of the M2 cell (soma indicated by an arrowhead); note that in (M) and (O) also the intense OFF plexus of processes from melanopsin cells is visible. **G) Schematic illustration of the connectivity and location of the five distinct morphological subtypes (M1-M5) of ipRGCs and projections to their predominant targets in the brain:** The ganglion cell layer contains conventional ganglion cells (not shown) and the ipRGCs. M1 ipRGCs stratify in the OFF sublamina (red); M2, M4, M5, stratify in the ON sublamina (blue); and M3, stratify in the ON and OFF sublamina (purple) of the IPL of the retina. M4 ipRGCs have the largest cell body size, and M1 cells have smaller body size than M2-M4 cells. The cell body size of M5 is not known (dotted line). The proportion of ON and OFF stratification in M3 ipRGCs varies considerably between cells. Recent findings suggest that the M1 subtype consists of two distinct subpopulations that are molecularly defined by the expression of the Brn3b transcription factor. Red dots indicate synaptic connections for which both functional and anatomical evidence

exists. Blue dots indicate synaptic connections for which either functional or anatomical evidence exists.. IpRGC subtypes project to distinct nonimage and image-forming nuclei in the brain. M1 cells predominantly project to non-image forming centers such as the suprachiasmatic nucleus (SCN) to control circadian photoentrainment and the shell of the OPN to control the pupillary light reflex. M1 innervation of brain targets could be further divided by Brn3b expression with Brn3b-negative (Brn3b-) M1 ipRGCs predominantly projecting to the SCN. M3 brain targets are completely unknown at this time. M2, M4 and M5 are included together because no specific genetic marker exists for a single subtype. Collectively, they project to image forming areas in the brain such as the lateral geniculate nucleus (LGN) and the superior colliculus (SC), but also to the core of the OPN of which no specific function is assigned to this brain region. Retrograde analysis confirms that M2 cells project minimally to the SCN and strongly to the OPN. BC: bipolar cell, DAC: dopamine amacrine cell. Adapted from (Karnas et al. 2013; Schmidt and Kofuji 2009).

I.D.2.b Different light-induced electrical properties:

Furthermore, the morphological diversity of mouse ipRGCs has been correlated to distinct physiological properties. M1 cells have larger, more sensitive intrinsic light-evoked responses, whereas M2 cells have significantly smaller light responses that are at least one log unit less sensitive to light (Schmidt and Kofuji 2009). Moreover, M1 cells have higher input resistance, a more depolarized resting membrane potential, and spike at lower frequencies than M2 cells (Schmidt and Kofuji 2009). Electrophysiological analyses of the rare M3 cells demonstrated that these cells are remarkably invariable, albeit the dendritic variability within this subtype. M4 and M5 subtypes have smaller and even less sensitive intrinsic light responses than M2 cells (Ecker et al. 2010). Atypical ipRGC photoreceptors can also act as conventional RGCs by receiving extrinsic (synaptic) rod/cone input via bipolar cells (Belenky et al. 2003; Viney et al. 2007; Wong et al. 2007).

Regarding *Arvicanthis ansorgei*, multi-electrode arrays (MEA) of ipRGCs from retinas of newborn (P0-P3) animals show 3 different response types distinguished by their sensitivity to light, response strength, latency and duration. On the whole, this study indicates that the basic morphological and physiological features of ipRGCs in *Arvicanthis* are comparable to mice. However, differences were observed with respect to the preponderance of M1 subtype, expression of certain phenotypic markers, firing rate levels and elevated sensitivity of type I *Arvicanthis* ipRGCs to short light flashes (Karnas et al. 2013).

I.D.2.c Intra-retinal connections:

The usual paradigm is that RGC with dendrites stratifying in the outer sublamina of the IPL receive excitatory inputs from bipolar cells that responds to light decrements (OFF), whereas RGCs with dendrites stratifying in the inner sublamina of the IPL receive synaptic inputs from bipolar cells that respond to light increment (ON). Contrary to what is expected given the position of each ipRGC subtype, both ON (M2) OFF (M1) and ON-OFF stratifying (M3) ipRGC subtypes receive predominantly ON-input. In fact, ON bipolar cells make synapses with M1 ipRGC dendrites present within the OFF sublamina of the IPL (Schmidt and Kofuji 2011). M1 ipRGC dendrites also colocalize with dopaminergic amacrine cells.

In *Arvicanthis ansorgei*, as described above displaced cells are stratified in the outer IPL (receiving OFF signals?), similar to M1 ipRGCs but about 40% of amacrine cells are also displaced towards the GCL (David Hicks, personal communication). It is intriguing that observations by (Zhang et al 2008)

have also suggested that mouse ipRGCs signal back to dopaminergic amacrine cells in the opposite direction of classical neuronal circuits although their function is unknown. M2 and M3 cells activity depends principally on input from the outer retina triggered by rods and cones. Besides, anatomical studies demonstrate a direct input from rod bipolar cells onto M1 ipRGCs (Ostergaard et al 2007).

I.D.2.d Projections:

Using the *Opn4tau–lacZ* line, M1 ipRGCs were shown to project to the SCN and the shell of the olivary pretectal nucleus (OPN) for mediation of circadian photoentrainment and the pupil constriction, respectively (Schmidt et al 2011). Most importantly for our purpose is that multisynaptic projections via SCN are responsible for the ipRGC regulation of pineal melatonin, placing the neurohormone as a main output of the circadian clock (Larsen 1998). In addition, M1 ipRGCs project to other brain regions involved in circadian behaviors such as the intergeniculate leaflet (IGL) and the ventral lateral geniculate nucleus (vLGN) (Gooley et al. 2003; Hattar et al. 2006).They also project to the supraoptic nucleus, ventral subparaventricular zone, medial amygdala, lateral habenula and could therefore mediate the effects of light on the hypothalamic regulation of sleep, behavior and physiology (Hatori and Panda 2010). In *Arvicanthis niloticus*, the heterogeneity of ipRGC central projections might suggest a function associated to non visual photoreception which could influence temporal niche switching. Anterograde tracing studies in *Arvicanthis niloticus* showed reduced ipRGC innervations of the vLGN compared to nocturnal rats (Todd et al 2012). However recent studies from Langel et al report the relative input to different brain regions for both ipRGCs and RGCs. We can note that there are as many PACAP/melanopsin RGC cells projecting to the SCN than non PACAP/non melanopsin RGC cells (Langel et al. 2015). Non-M1 ipRGC subtypes innervate additional targets in the brain such as the core of the OPN (unknown function, (Schmidt et al 2011). They also contribute substantial synaptic input to the dorsal lateral geniculate nucleus (dLGN), a structure involved in object localization and discrimination, and the SC. Complete study with genetic mouse models is described and illustrated in (Ecker et al. 2010). In that case, ipRGCS projections to the LGN and SC might provide irradiance information for image forming vision (Figure 23) (Hatori and Panda 2010; Karnas et al. 2013). Finally, recent description of sparse ipRGCs projections to the posterior thalamus juxtaposed to dura-sensitive neurons are proposed to mediate the light exacerbation of migraine pain (Noseda et al 2010).

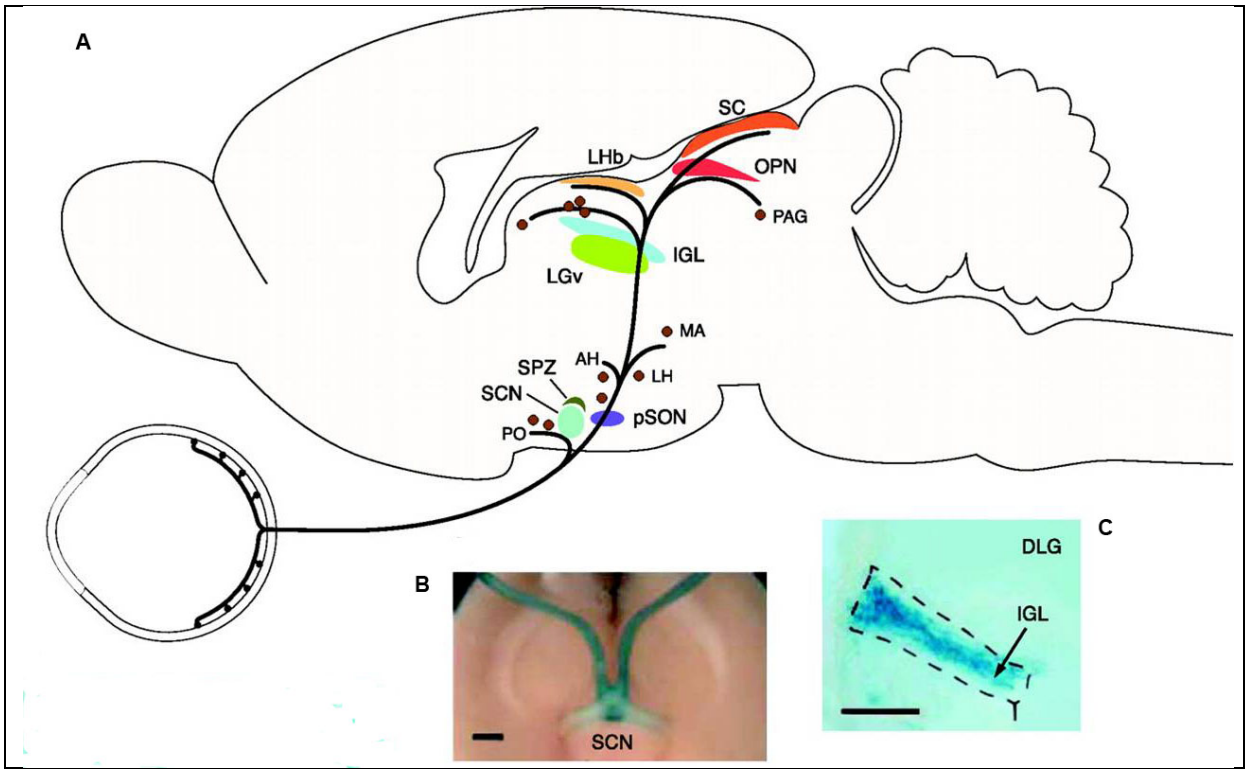


Figure 23: Brain targets of ipRGCs.

A) Schematic sagittal view of the mouse brain showing a sampling of regions innervated by ipRGCs. PO, preoptic area; SCN, suprachiasmatic nucleus; SPZ, subparaventricular zone; pSON, peri-supraoptic nucleus; AH, anterior hypothalamic nucleus; LH, lateral hypothalamus; MA, medial amygdaloid nucleus; LGv, ventral lateral geniculate nucleus; IGL, intergeniculate leaflet; BST, bed nucleus of the stria terminalis; LGd, dorsal lateral geniculate nucleus; LHb, lateral habenula; SC, superior colliculus; OPN, olivary pretectal nucleus; PAG, periaqueductal gray. B: ventral view of the *opn4*$^{-/-}$ tauLacZ$^{-/-}$ brain showing ipRGC axons running in the optic nerve and innervating the suprachiasmatic nuclei (SCN). Scale bar is 1 mm. C: prominent innervation of the intergeniculate leaflet (IGL) by ipRGC axons. Coronal section, scale bar is 100 µm. DLG, dorsal lateral geniculate nucleus. From (Tri Hoang Do 2010).

The data gathered in these studies give solid information of the connections between the retina, central brain and behavior but raise more questions: What are the ipRGCs subtypes involved in the modulation of vision tasks in the vLGN? Do ipRGCs have direct access to ventrolateral preoptic nucleus (VLPO) for sleep modulation or to other thalamic nuclei involved in alertness? What is the role of non M1 ipRGC on the OPN core? What is the ipRGCs projection site involved in the temporal niche integration? Our model *Arvicanthis ansorgei* is among the only diurnal characterized species, and appears relevant to address some of these questions which will be discussed in the "Melatonin and light" part below.

I.D.2.e Behavioral outputs of ipRGCs in nocturnal and diurnal Mammals:

As already stated above, ipRGCs are the primary actors of the light modulated **non image forming visual functions**. M1 ipRGCs were shown to project to the SCN for mediation of circadian photoentrainment (Schmidt et al 2011). The circadian clock, in turn, affects a number of functions, such as melatonin release, body temperature regulation and feeding behavior. Other aspects of

mammalian biology that are influenced by great changes of environment light include: blood pressure, alertness, mood, sleep/wake (Lockley and Gooley 2006; Vandewalle et al. 2006; Zaidi et al. 2007). Recently, Gompf et al have reported that glutamate transmission from the ipRGCs is necessary for normal light entrainment of the SCN at moderate light levels, but residual transmission-possibly by PACAP in ipRGCs or by other RGC-can weakly entrain animals, particularly at very high light levels (Gompf et al. 2015). Same results were obtained independently with a similar mouse genotype (*Opn4Cre*/+ vesicular glutamate transporter 2 (*VGLUT2fl/fl*)) by (Purrier et al 2014). These data are the first to identify the glutamate transmission as a major path to SCN-controlled activity. However, the glutamate transmission is not unique and more work need to be done on PACAP and RGC cells to elucidate all the components of circadian locomotion regulation.

I.D.2.f Masking effect of light:

In addition to these effects, light can also have direct effect (SCN-independent) stimulatory or suppressive effects on locomotor behavior. These mechanisms have been termed **"masking"** effects because, although they do not entrain the clock, they can "mask" its true phase. Therefore, in addition to aligning sleep and waking with time of day, light also acutely promotes alertness in humans, and sleep in nocturnal species. Several groups recently demonstrated that alteration of the light input to the brain by genetic ablation or inhibition of phototransduction components (rods and cones, *Opn4*, or ipRGCs) severely affects both sleep and waking (Schmidt et al 2011). Thus, Hubbard et al have proposed a model wherein light directly influence the flip-flop switch, e.g. the VLPO sleep-active neurons in nocturnal animals, shifting the reciprocal inhibitory interaction toward sleep promotion. Conversely, a lack of VLPO activation possibly associated with light-induced activation of the locus coeruleus (LC) and hypothetically other wake-promoting systems (for instance raphe nuclei-5-HT) shift toward arousal in diurnal species (Hubbard et al. 2013). However, it remains to be determined whether the SCN, beyond its clock function, may also influence sleep and waking through relaying these direct effects of light.

In *Arvicanthis ansorgei,* the same team has found that sleep and waking are mainly regulated by a crepuscular component with a bimodal waking distribution with light/dark/light transitions (Hubbard et al. 2013). Nevertheless the main sleep regulatory mechanisms classically described in mammals are conserved in *Arvicanthis Ansorgei*. For instance, **melatonin and its rate-limiting enzyme, arylalkylamine N-acetyltransferase (AA-NAT) are expressed in *Arvicanthis ansorgei* pineal gland during the night or dark phase, similarly to the distribution observed in human** (Garidou et al 2002) whereas most laboratory mice strains used in research do not express melatonin (i.e. C57BL6/J, 129/Sv). Indeed, pineal melatonin production is restricted to dark hours by appropriately phased circadian rhythm of pineal stimulation and extreme sensitivity of melatonin synthesis to light inhibition (Klein et al 1972). Finally, Melatonin is being studied for its beneficial impact on sleep-related neuronal dysfunction, which occurs in neurological disorders such as Alzheimer's disease and autism (Kwon et al. 2015). **This suggests that *Arvicanthis ansorgei* may represent a useful model for future direct light effect and sleep research, especially from the perspective of deciphering the temporal niche switch anatomical substrate as already mentioned above. The data presented in this section together with the ones reported in the "cone/rod pathways" part above, converge towards elements that will be discussed in the "Melatonin and light" part: The question**

will be addressed under the light of retinal melatonin synthesis and regulation as it is our main research project. How direct and indirect effect of light act on a diurnal Mammal behavior (including the retinal melatonin protagonist).

I.E. Amacrine cells

Ganglion cells receive inputs from bipolar cells but a second direct input comes from **amacrine cells (AmC)**. These interneurons occupy the IPL where bipolar cells and retinal ganglion cells form synapses. They feed back to the bipolar cells that drive them, synapse upon ganglion cells and on each other (Masland 2012). There are at least 33 different subtypes of AmC based just on their dendrite morphology and stratification (major work in the rabbit retina as well as more recent studies on mouse retina (Pozdeyev et al 2008). Also, amacrine diversity is evidently related to ganglion cell diversity. So far, about 15-20 different ganglion cell types have been identified (Masland 2012). Rare wide-field GC and AmC types exist and can cover the entire retina creating functional networks still unknown. So researchers are far from providing a complete morpho-functional cartography of the mammalian ACs.

Nevertheless, AmC are known as mostly inhibitory neurons using gamma-butyric acid (GABA) and glycine but they also achieve paracrine modulating actions through acetylcholine, dopamine or factors undiscovered yet. Dopamine AmC are widely spreading (1) carry out diffuse release of dopamine, (2) simultaneously carry out traditional synaptic release, (3) are dual neurotransmitter neurons, releasing both dopamine and GABA, and (4) modulate the activity of many retinal neurons during light and dark adaptation. Dopamine release is associated with light adaptation, circadian rhythms and classically opposed to melatonin action in the retina (McMahon et al 2014). Retinal Dopamine and melatonin cross-talk is one important aspect of melatonin regulation we have considered in our team work (see section below). In *Arvicanthis ansorgei*, dopamine amacrine cells are classically located in the inner nuclear layer.

Other AmC known functions are to synapse and modulate retinal ganglion cells and/ or bipolar cells in the IPL, create functional subunits within the receptive fields of many ganglion cells, contribute to vertical communication within the retinal layers. Through their connections with other retinal cells at synapses and release of neurotransmitters, AmCs contribute to the detection of directional motion and control high sensitivity in scotopic vision through connections with rod and cone bipolar cells. Besides, it is interesting to note that almost all AmCs communicate among several strata of the IPL. For instance, the multistratification of narrow-field AmC is that gap junctions (a linkage of two adjacent cells consisting of a system of channels extending across a gap from one cell to the other, allowing the passage of ions and small molecules) allow them to be excitatory at one place and inhibitory in another.

Finally, highly specialized amacrine cells can create a specific receptive field property in a single type of GC (ground squirrel retina, (Chen and Wei 2012; Sher and DeVries 2012). Indeed, when excited by the blue-ON bipolar cell, this amacrine cell performs a sign inversion: it inhibits the GC cell upon which it synapses, thus creating a GC responding to a blue stimulus by slowing its firing (Masland 2012).

I.F. Muller cells

The **Müller cell (MC)** is the principal glial cell in the vertebrate retina. These radial glial cells span the entire thickness of the retina and ensheath all retinal neuronal somata and processes. MC population forms a dense, regular pattern and constitutes an anatomical link between the neurons and the compartments with which these exchange molecules, i.e., the retinal blood vessels, the vitreous body, and the subretinal space. This link is also largely functional and MCs are equipped with different ion channels, ligand receptors, transmembraneous, transporter molecules, and enzymes (Bringmann et al. 2006). They bear a high density of specialized K^+ channels. The high K^+ conductance of the MC membrane, accompanied by a very negative resting membrane potential of about -80 mV, is characteristic for normal mature MC and is required for all supportive functions of the cells. Indeed, from early stages of retinal development, they are essential in creating and maintaining the neuroretinal architecture and support neuronal survival and regular information processing.

Among their numerous functions, we can note that they convert all-trans retinal into 11-cis-retinol (Das et al. 1992), release it into the extracellular space for uptake by cone photoreceptors, and possibly for ganglion cells as discussed above in the GC section (Schmidt et al 2011). They are involved in the glucose metabolism, regulate the blood flow or maintain the ion and water homeostasis of the retinal tissue including the pH (Bringmann et al. 2006). By contrast to photoreceptors MCs are strikingly resistant to ischemia, anoxia, or hypoglycemia (Silver et al 1997; Stone et al. 1999) and become “reactive” in response to every pathological alteration of the retina termed MC gliosis (for review: (Bringmann et al. 2006). Of relevance for our purpose, is that MCs play a major part in the recycling of neurotransmitters (glutamate, GABA, glycine) in glio-neuronal interaction thereby participating in ammonia detoxification or free radicals elimination (Figure 5 (Bringmann et al. 2006)).

Besides, one study reports the presence of MT_1 and MT_2 melatonin receptors on cultured MCs and could be also involved in the neuron-MC cross-talk either in the neuroprotection context or for the circadian events regulation (Jaeger et al. 2015; Jiang et al. 2012). However, we need to remain cautious about MT_2 immunodetection as no reliable MT_2 antibody is known so far. In *Arvicanthis ansorgei*, Müller cells can be observed with anti-mouse glutamine synthase staining (Figure 24). MCs nuclei occupy the middle of INL as described for rat MCs (Lindqvist et al. 2010) while processes span the entire retina.

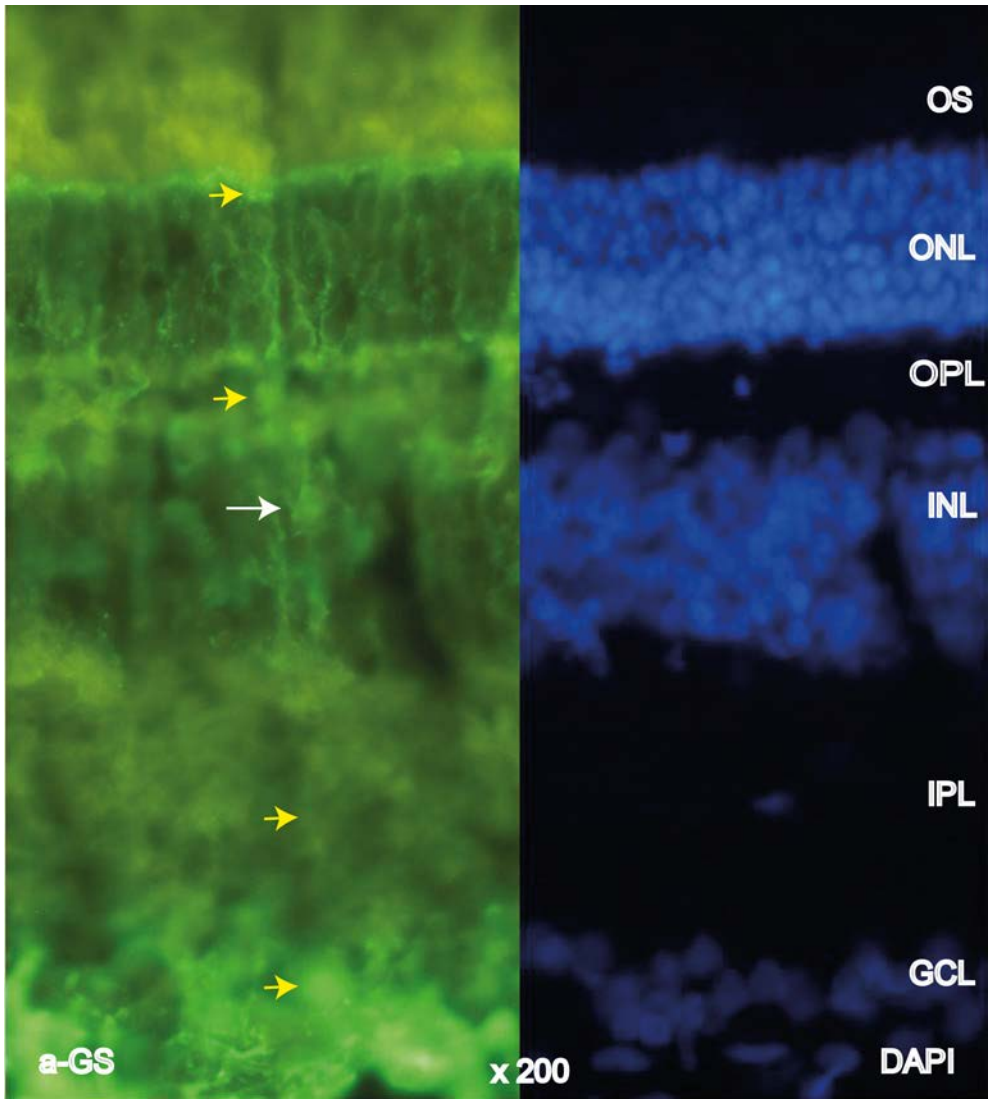


Figure 24: Photographs of glutamine synthetase (GS) immunodetection and DAPI staining of *Arvicanthis ansorgei* retina.

White arrow outlines Müller cell body in the INL. Yellow arrows point to Müller cell processes spanning the whole retina. GS immunostaing was achieved on 10µm transversal retina sections. Photograph reflects x 200 magnification. OS: outer segment, ONL: outer nuclear layer, OPL: outer plexiformlayer, INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer.

I.G. Summary:

Overall, the *Arvicanthis* structural data presented above and confronted to other Mammals make our diurnal muridae the most relevant model to study retinal melatonin synthesis and role. Take home messages so far are the following:

- Photoreceptors include 33% of cones and ipRGCs which are the candidate cell types for melatonin synthesis.
- Photoreceptors are equipped with all the usual known pigments: rhodopsin, cone opsins, melanopsin but also with clock genes.

- Inner retina presents bipolar, horizontal, amacrine and Müller cell bodies organized as in the human retina and blood vessels span all over the retina in a classical mammalian way.
- GCL contains clock genes and at least 3 differently functional ipRGCs. Two resemble mouse M1 and M2 ipRGC types.
- Data in *Arvicanthis ansorgei* and *niloticus* suggest classical (not all characterized) wiring between GCL and central brain regarding visual image forming and non visual forming functions (circadian clock, sleep/wake cycle, alertness…).
- *Arvicanthis ansorgei* is a diurnal, bimodal-type animal whose retina is particularly well suited to study the effects of highly variable light irradiance.

In addition to its critical functions in the brain and well-characterized synthesis in the pineal gland, melatonin is also produced in the retina, where it is thought to be involved in processes such as phototransduction, electrical activity and phagocytosis of shed photoreceptor membranes; and also play a role in pathological changes, e.g. increased vulnerability to light damage at night, or protection against oxidative stress (Baba et al, 2013; Alarma-Estrany et al., 2007 ; Peters & Cassone, 2005). The expression of this indoleamine is rhythmic, under the dual control **of a circadian oscillator** and changes in ambient lighting. These processes synchronize retinal physiology with daily fluctuations in light levels and darkness (Herzog and Block, 1999). **Notwithstanding, the cellular and molecular control of melatonin synthesis in the mammalian retina, as well as its principal function(s) are still unknown.**

II. Mammalian retinal clock:

The retinal clock was first discovered in *Xenopus Laevis* with the observation of a circadian regulation of **arylalkylamine N-acetyltransferase (AA-NAT)** enzyme activity in LD and DD (Iuvone and Besharse 1983). As a reminder, AA-NAT is the penultimate enzyme of melatonin synthesis pathway (Coon et al 1995). Here, I will focus on the mammalian circadian clock and its main link with the neurohormone melatonin. From studies on cultured hamster retina, it has been established that the retinal clock presents all the parameters of an independent circadian clock (self-sustained oscillations, adjust its period to the astronomical time, temperature compensated) (Tosini and Menaker 1998). The clock(s) have now been characterized in other species including rat (Buonfiglio et al. 2014; Sakamoto et al 2004) and mice (Ruan et al. 2008; Tosini and Menaker 1998).

II.A. Molecular organization:

Clock genes are widely expressed in the neural retina (Peirson et al. 2006; Sandu et al 2011; Schneider et al. 2010) including core genes with a profile not different from those reported from other tissues. *Bmal1, Per1, Cry1, Clock* genes are each required individually in order for the retina to express circadian rhythms; whereas *Per2, Per3 and Cry2* are dispensable (Ruan et al. 2012). In rat retina, clock genes are present in photoreceptors, but the presence of a functional circadian clock is questioned (McMahon et al 2014; Sandu et al 2011). In the mouse retina, the situation looks more complicated. Clock genes (but not all depending on the method) are expressed in classic photoreceptors (Ruan et al 2006). However, INL neurons such as dopamine neurons co-express *Per1, Per2, Cry1, Cry2, Clock, Bmal1* (Dorenbos 2007). These results are challenged by one study which proposes the reverse (Dkhissi-Benyahya et al. 2013), i.e., photoreceptors express all the clock gene transcripts and the INL does not. Unexpectedly, melanopsin and its signaling appear to play a role in

the functioning of the photoreceptor clockwork. Finally Liu et al have localized the clock proteins (CLOCK, BMAL1, NPAS2, PERIOD1, PERIOD2 and CRYPTOCHROME2) in the outer and inner retina, but these proteins only display a diurnal and circadian variation in the cones (Liu et al 2012).

We have analyzed the clock genes (*Bmal1, Per1, Per2, Cry1, Cry2*) transcripts under LD, DD, LL conditions in the retina of *Arvicanthis ansorgei* (Figure 25). Results are the following: i) under LD conditions, expression profiles of the different clock genes examined appear synchronized to maximal values at or shortly after dawn; and ii) roughly similar profiles are maintained under DD for many of these genes, indicating that they are driven by circadian clock mechanisms; and iii) they are in the majority of cases greatly perturbed by LL (see (Bobu et al 2013). These expression profiles are quite different from those published for retinas of nocturnal rodents: Previous studies using whole or fractionated rat retinas have shown clock gene acrophases to occur predominantly at the day/night transition (Van Hook et al 2012; Sengupta et al. 2011). Taken together with the global early morning maxima in *Arvicanthis* retinal output genes (*Aanat, Opn4*), the findings suggest differences in retinas from nocturnal versus diurnal species, possibly related to visual physiology and retinal cellular composition (Bobu et al 2013). In brief, in the mammalian retina, we do not know yet which cells generate the clock(s).

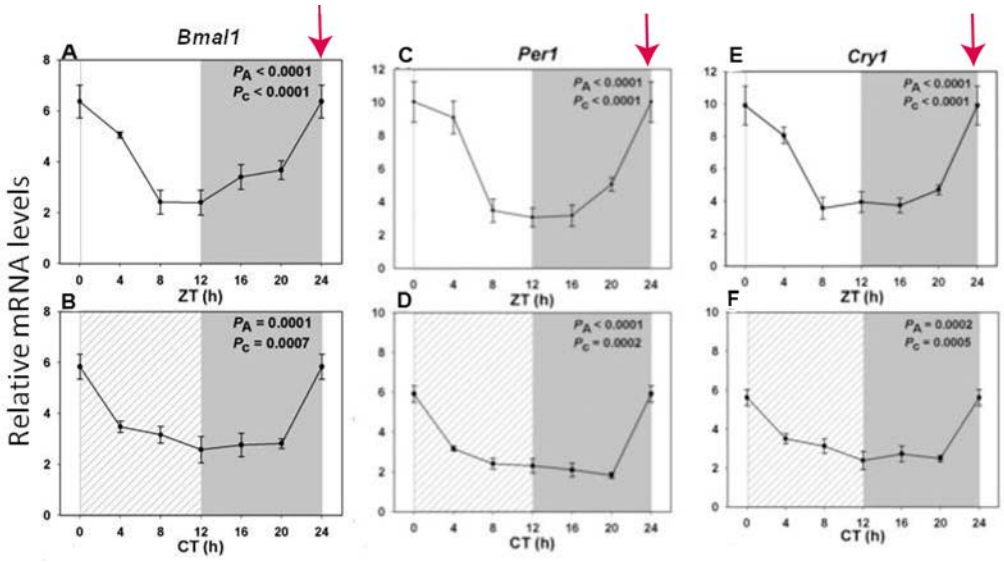


Figure 25: Expression profile of clock genes in the whole retina of *Arvicanthis ansorgei*.

A, C, E: In a 12 h light: 12 h dark cycle (LD) Bmal1, Per1 and Cry1 exhibit a rhythmic expression pattern with the peak value shortly after light onset (n=3-6 per time point). **B, D, F:** Rhythmicity was maintained although dampened in constant dark (DD) (n=6 per time point). Illumination conditions are depicted as solid white (day) and solid grey (night) areas in LD, right hatched (subjective day) and solid grey (subjective night) areas in constant dark (DD) and solid white (subjective day). Animals were killed every 3 or 4 h across the 24 h period, and RNA extracted from retinal tissue. RNA expression levels were quantified by real time PCR. One-way analysis of variance (ANOVA) and cosinor levels of significance (Pa and Pc respectively) are given in the upper right corner of each panel. Adapted from (Bobu et al 2013).

II.B. What is the cellular organization of the retinal circadian clock(s)?

Depending on the species and the techniques used, this is the up to date consensus (Jaeger et al. 2015; McMahon et al 2014). There are likely multiple sites of molecular circadian rhythm generation

within the mammalian retina. Clock genes are lowly expressed in the photoreceptors maybe primarily in cones (mouse) and may express circadian rhythms (rat). In addition, clock gene expression is concentrated within the inner nuclear layer where GABA/DA amacrine and Müller cells can express circadian variations of clock genes transcripts (Rat and mouse). The inner circadian clock may influence photoreceptors and other retinal neurons through the rhythmic secretion of dopamine but is independent from photoreceptors and melatonin. Finally, the GCL including ipRGCs and displaced amacrine cells is involved in the generation and entrainment of retinal circadian clocks (Figure 26).

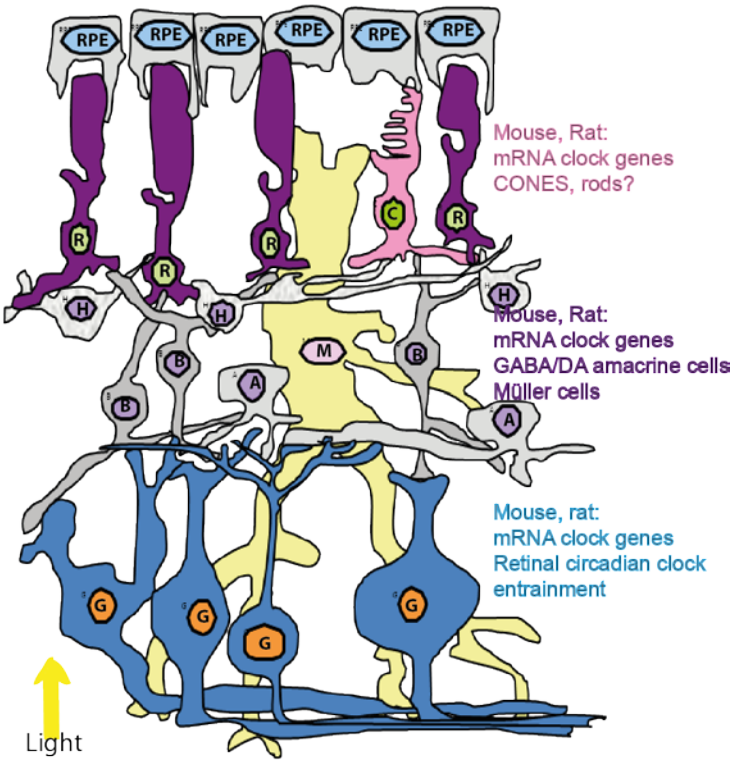


Figure 26: Consensus on the cellular organization of the retinal clock.

Clock genes are lowly expressed in the outer nuclear layer. They are well expressed in the INL and the GCL. GCL is involved in the generation and entrainment of the retinal circadian clock. Retinal pigment epithelium (RPE) distally, cone (C), rod (R) and relaying cells (bipolar (B), horizontal (H), amacrine (A), ganglion (G) proximally.

II.C. Is intercellular coupling necessary for rhythm generation?

Cells may have 3 ways to ensure their circadian rhythms coordination in order to get to the tissue-level organization: i) neurotransmission: melatonin, dopamine, GABA are secreted with a circadian rhythm (Doyle et al (1) 2002; Doyle et al. 2002; Jaliffa et al. 2001; Tosini and Menaker 1996) and could synchronize cells throughout the retina ii) Gap junctions could serve to couple clock cell populations as maybe expected with GABA/DA amacrine cells. Iii) Spike-mediated transmission as seen in the SCN to maintain cellular synchronization and tissue-level rhythms (Maywood et al. 2006). Until now, results are not conclusive. Taken separately neurotransmitters have not proven really necessary for cellular coupling (Jaeger et al. 2015; Ruan et al. 2008). Gap junctions appear to play a role even though a limited one in the coupling of oscillators in the rat retina (Jaeger et al. 2015). Overall, the results indicate the importance of direct, cell to cell connection in the retina clock but more studies are necessary to understand the mechanisms at the cell level resolution.

II.D. Functions of the retinal oscillators:

The retinal clock (or coordinated multioscillators) is essential to drive many processes thereby anticipating the normal cycle of photopic and scotopic visual conditions. Indeed, retina adapts to the LD cycle. Another important function of the retinal circadian clock resides in its ability to gate sensory information. Indeed Mammalian retinal clock receives light and sends the photic message to different structures of the central brain. A wide array of established functions driven by the retinal clock is depicted in the Figure 27. For this work, we'll keep in mind that melatonin release (Iuvone and Besharse 1983; Tosini and Menaker 1998, 1996) dopamine synthesis (Doyle et al (1) 2002; Doyle et al. 2002), GABA turnover rate and release (Jaliffa et al. 2001), rod disk shedding (Teirstein et al 1980) and pigment expressions (Bobu et al 2013) are all regulated in a circadian manner. Moreover, the mammalian retinal clock and its output influence cell survival and growth processes in the eye including photoreceptors degeneration from light damage (Grewal et al 2006). Finally, some genes are under retinal clock and SCN clock control (*Per2*, *Dbp* but not *Aa-nat*) because SCN lesion leads to the loss of their mRNA rhythm in the retina (Sakamoto et al 2000). Of interest is also the photoreceptor disk shedding or phagocytosis; indeed this robust circadian event is controlled by both photoreceptors and retinal pigmentary epithelium (Baba et al 2010).

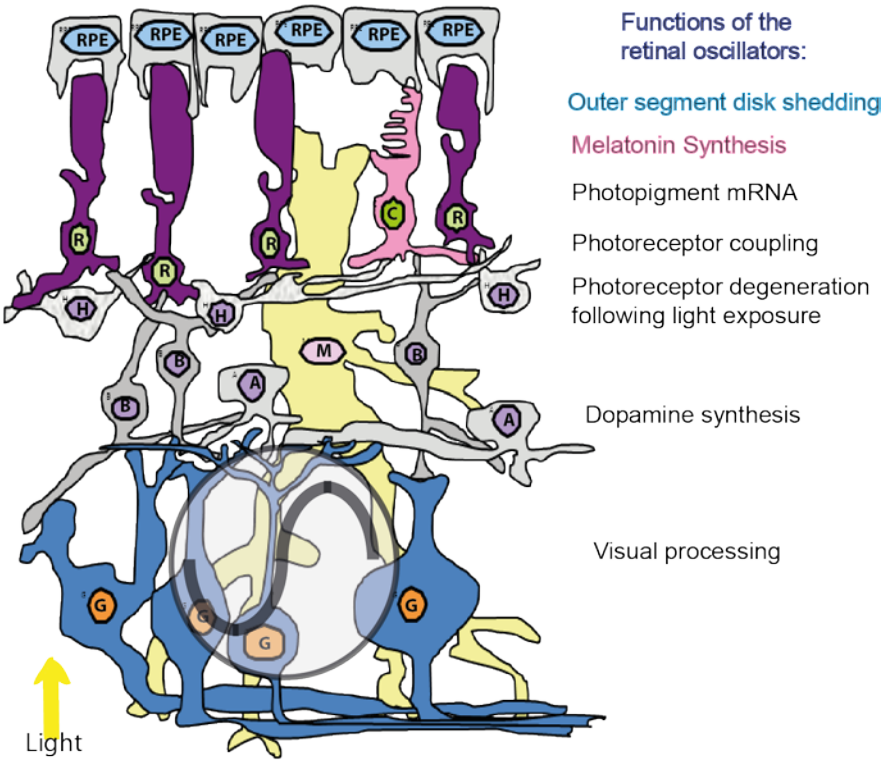


Figure 27: The circadian clock system in the mammalian retina controls several functions.

Many studies have shown that rhythms are under direct control of the retinal circadian clock system. Recent studies have also indicated that the many different cell types within the eye contain circadian clocks that interact to modulate many ocular functions. Shown here are several known circadian processes in the retina and the eye, with their approximate location identified by retinal layer. Adapted from (McMahon et al 2014)

III. Pineal gland and Melatonin: Time giver

III.A. The pineal gland, first source of melatonin in the body:

The mammalian pineal gland or *epiphysis cerebri* is a neuroendocrine gland secreting the hormone melatonin (Ganguly et al 2002; Lerner et al. 1958; Stehle et al 2002) as the primary source in the body. The gland is derived from the neural tube and located at the border between the mesencephalon and the diencephalon of the brain. Neuroanatomically, the pineal is described as a part of the epithalamus and thereby as a part of the diencephalon. The pineal gland is an impaired structure localized in the rostral part of the superior colliculi near the third ventricle in all mammals except rodent (Møller and Baeres 2002). Indeed in rodents the pineal gland is localized at the intersection between both cerebral hemispheres and the cerebellum (Figure 28).

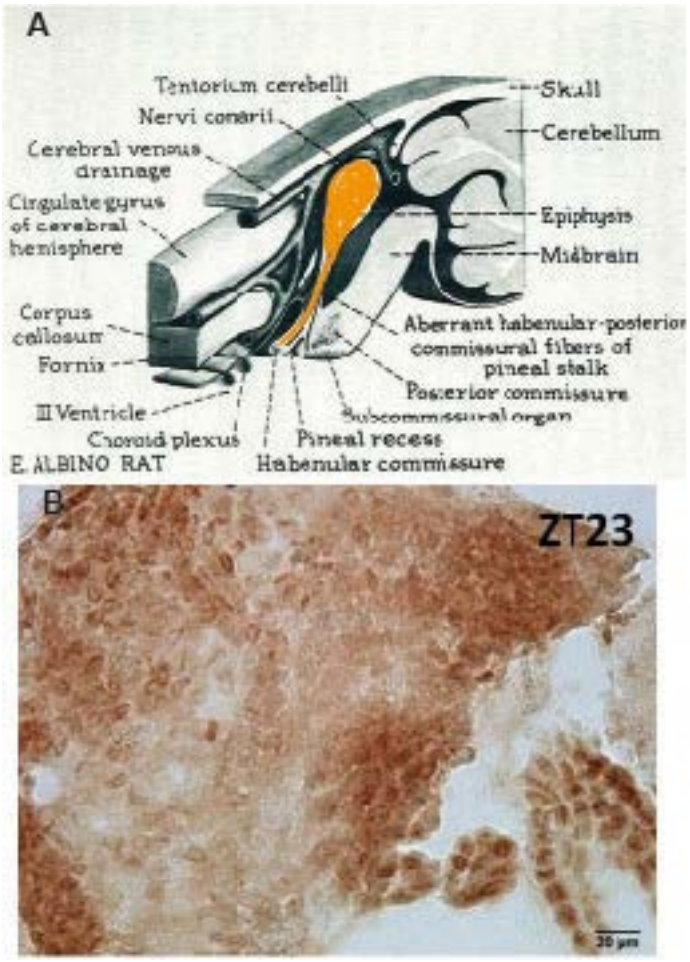


Figure 28: Pineal gland in rodents.

Panel A Diagrammatic representation depicting longitudinal slice from the diencephalic roof region of the rat brain. Orange coloring outlines the epiphysis. Adapted from The pineal book by Wurtman J., Axelrod J. and Kelly D.E. **Panel B** is a photograph of an *Arvicanthis ansorgei* pineal gland slice immunostained with anti-AA-NAT at ZT23 when protein expression is the highest.

I

Arvicanthis ansorgei possesses a smaller pineal gland than the rat one but similarly located at the intersection of the cerebellum and both hemispheres. This structure is tightly covered by the *dura* matter and plated to the skull. Consequently, *Arvicanthis ansorgei* pineal is difficult to collect but we have calculated based on 5 pineal glands a mean of 7.5 mg total proteins versus 10 mg proteins in rat pineal gland (Figure 28). In all mammals investigated five cell types have been described: the hormone-producing pinealocyte, the interstitial cell, the perivascular phagocyte, neurons, and peptidergic neuron-like cells. The main component (90% of cells) considered here is the pinealocyte-type which secretes melatonin.

III.A.1. Pineal melatonin synthesis:

Carey Pratt McCord and Floyd P. Allen (January 1917) were the first to report the presence in cow pineal, of a biological active compound able to lighten tadpole skin, by contracting the dark **epidermal melanophores** (The Journal of Experimental Zoology, volume 23, 1917). In 1958, Aaron B. Lerner and colleagues at Yale University isolated the hormone from bovine **pineal gland** extracts and named it **melatonin**. The chemical nature of melatonin is N-acetyl-5-methoxytryptamine.

Melatonin is synthesized from the essential amino acid tryptophane, which is first converted into 5-hydroxytryptophan before its decarboxylation into serotonin. The conversion of serotonin to melatonin involves two major enzymatic steps. The first is N-acetylation by arylalkylamine N-acetyltransferase (AA-NAT) to yield N-acetylserotonin (NAS). The second step is the transfer of a methyl group to the N-acetylserotonin to yield melatonin through the action of hydroxyindole-O-methyltransferase (HIOMT) Figure 29.

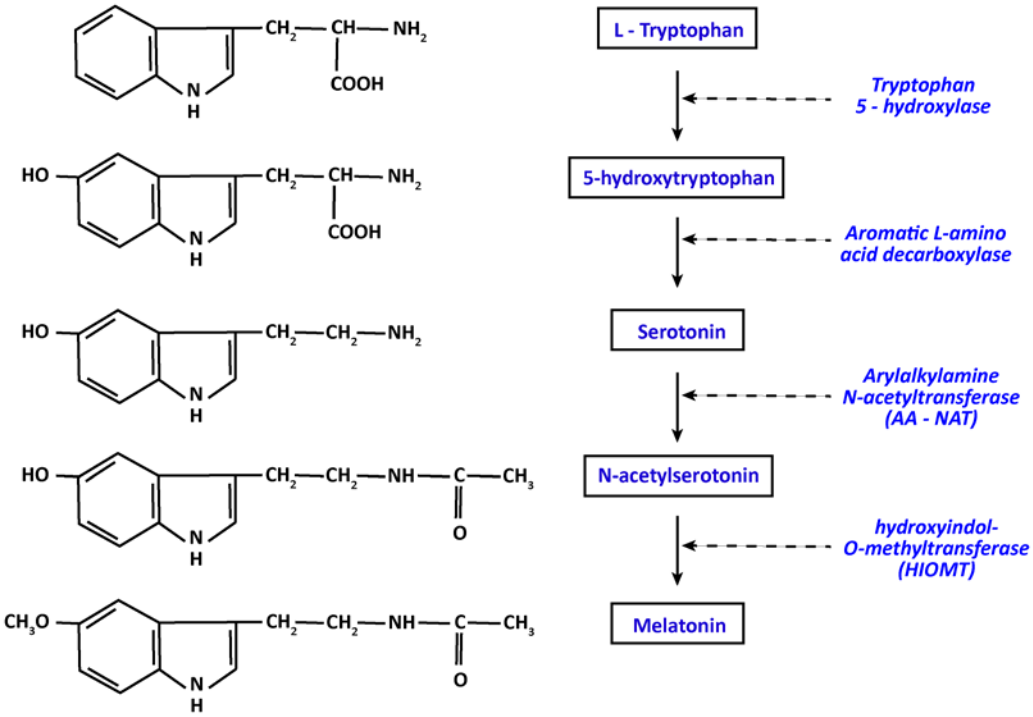


Figure 29: Melatonin synthetic pathway.

Melatonin synthesis begins with bloodstream amino acid tryptophan up-take and its subsequent 5-hydroxylation by tryptophane hydroxylase. 5-hydroxytryptophan is then converted to serotonin by the action of an aromatic L-amino acid decarboxylase. The neurotransmitter is acetylated by arylalkylamine N-acetyltransferase (AA-NAT) to N--acetylserotonin, which is subsequently O-methylated and converted to melatonin by hydroxyindole-O-methyltransferase (HIOMT). Modified from (Konturek, Konturek, and Brzozowski 2006).

Melatonin is amphiphile [composed of a hydrophobic element (indole nucleus) and a hydrophilic moiety (long carbon chain)] and diffuses across cell membranes as well as cell layers. Early studies with melatonin-acetyl-^{3}H report that unlike serotonin, its access to the central nervous system is not hindered by a blood brain barrier. One hour after intravenous injections of the radioactive compound to cats, the indole concentrates in the pineal gland, parts of the eye and the ovary (Wurtman, Richard J. 1968).

III.A.2. Pineal melatonin catabolism:

The hormone rapidly disappears from the blood, with a half-life of approximately 35 min (Cardinali et al 1972; Waldhauser et al. 1984). In human bloodstream, melatonin is converted to **6-hydroxymelatonin** by the liver, which clears 92 to 97% of circulating melatonin (Tetsuo et al 1980; Young et al 1985). Then 6-hydroxymelatonin is metabolized and excreted in urine as the sulfate metabolite (50 to 80%) and glucuronide (5 to 30%) (Ma et al. 2008). This catabolism is not necessarily true for tissue melatonin. Especially in the central nervous system, oxidative pyrrole-ring cleavage prevails and no 6-hydroxymelatonin was detected after melatonin injection into the *cisterna magna*. This may be particularly important because much more melatonin is released via the pineal recess into the cerebrospinal fluid than into the circulation (Tricoire et al. 2002). In mouse, the most abundant metabolite found in urine is the glucuronide metabolite (75-88%) (Kennaway et al. 2002; Ma et al. 2008). In the brain, the primary cleavage product is N-acetyl-N-formyl-5-methoxykynuramine (**AFMK**), which is deformylated either by arylamine formamidase or hemoperoxidases to N-acetyl-5-methoxykynuramine (**AMK**). AFMK and AMK also form metabolites by interactions with reactive oxygen and nitrogen species (Hardeland R 2005).

III.A.3. Melatonin synthesis is driven through multisynaptic neuronal pathways:

As already stated above, pineal gland is the primary source of melatonin in Vertebrates and is under the control of a multisynaptic neuronal pathway Larsen et al., 1998). The light information first received by the retina is conveyed to the main circadian clock in the SCN via the **retinal-hypothalamic tract** (Gillette and Mitchell 2002; Hannibal 2002) (Figure 30). The SCN **controls** melatonin secretion by the pineal gland via **the hypothalamo-pineal tract**. This pathway includes the paraventricular nucleus (**PVN**) which innerves the intermediolateral cells of the spinal cord (**IML**). The IML projects onto the superior cervical ganglia (**SCG**) and the SCG sends noradrenergic (**NA**) fibers (Kappers 1960) to the pineal gland. This pathway is specifically activated at night and releases NA into the pineal perivascular space leading to melatonin synthesis. The implantation of this pathway starts after birth to be effective after 3 weeks (Kappers 1960). Note that melatonin profile is similar in diurnal and nocturnal animal (*Arvicanthis ansorgei* and *Rattus norvegicus* respectively) pineal gland.

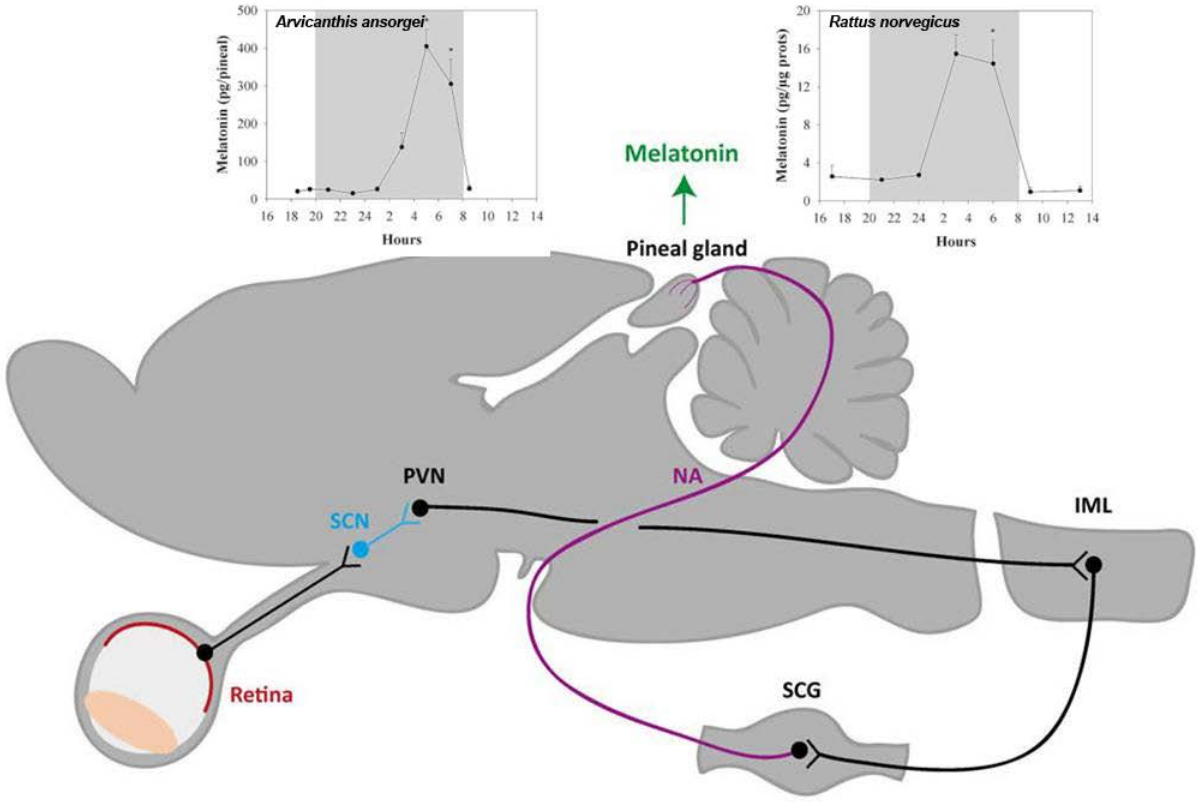


Figure 30: Diagram of the retinal-hypothalamic and hypothalamo-pineal tracts.

Light information is transmitted by retinal ganglion cells to the SCN via the retinal-hypothalamic tract. SCN uses the hypothalamo-pineal tract to control melatonin synthesis by the pineal gland. *Arvicanthis ansorgei* and *Rattus norvegicus* melatonin profiles are presented on the top left and right panels respectively (Garidou et al. 2002). SCN: suprachiasmatic nucleus; PVN: paraventricular nucleus; IML: intermediolateral cells of the spinal cord; SCG: superior cervical ganglia; NA: noradrenergic. Modified from (Simonneaux 2011).

III.A.4. Regulation of melatonin synthesis:

In the pineal gland, adrenergic receptors control the circadian synthesis of melatonin (Deguchi and Axelrod 1972). Nocturnal NA activation of these receptors leads to activation of different signaling pathways. First, the **β1-adrenergic receptor** is positively coupled to the adenylyl cyclase (**AC**) via a G stimulatory protein (Gs) and leads to an increase in the cytoplasmic cyclic adenosine 3',5'-monophosphate (**cAMP**) level (Strada 1972). Within the cell, cAMP activates protein kinase A (**PKA)** (Fontana and Lovenberg 1971)). Secondly, the **α1-adrenergic receptor** is coupled to the phospholipase C (PLC) and to a calcium channel. Activated α1-adrenergic receptors increase the level of intracellular Ca^{2+} and protein kinase C (**PKC**) (Ho et al 1988). During the night, the activation of AC is potentiated in presence of the Ca^{2+}/calmoduline (Ca^{2+}/CAM) complex (Tzavara et al. 1996). This "cross-talk" causes a large and rapid increase in cAMP (Vanecek 1985). The activation of cAMP/PKA pathway induces the phosphorylation of cyclic AMP-responsive element-binding protein (**CREB**) which binds the cyclic AMP-responsive element (CRE) (Foulkes et al. 1996) and induces the transcription of cAMP dependent genes such as ***Aa-nat***. Phosphorylated CREB (P-CREB) can also bind the promoter of the CRE modulator (CREM) gene, and can induce the transcription of inducible cAMP early repressor (*Icer*). **ICER** is a powerful repressor of cAMP induced transcription (Stehle et al 1993) and modulates cAMP response.

III.B. The limiting enzymes of melatonin synthetic pathway: AA-NAT and HIOMT

III.B.1. -Arylalkylamine N-acetyltransferase (AA-NAT):

S. Coon and D. Klein major work on AA-NAT has been fruitful. Indeed their work span the *Aa-nat*/AA-NAT basic structures and the enzyme activity, the regulatory features; its crucial role in melatonin synthesis and the enzyme potential active role in photoreception evolution (Baler et al 1997; Falcon et al 2014; Klein 2007). **In the IV part of this work, I will point out several chosen aspects which will then serve the discussion about retinal AA-NAT in our diurnal rodent *Arvicanthis ansorgei*.** AA-NAT is mainly known as the penultimate enzyme in the melatonin biosynthesis. This active molecule catalyzes the N-acetylation of arylalkylamines using acetyl CoA (AcCoA) as the acetyl group donor. AA-NAT is a fascinating compound which is thought to have played a role in the 500 My evolution of the putative photodetector ancestor of the pineal gland and the retina (Falcon et al 2014). *Aanat* and *Aanat* homologs form the AA-NAT family, which is part of the large (histone acetyltransferase) ***Gcn5*-related acetyltransferase (GNAT) superfamily** (Falcon et al 2014) All GNAT family members share common structural features associated with AcCoA binding; in addition, each member family has unique properties reflecting substrate specificity for each of a wide range of substrates (*e.g.* aminoglycosides, diamines, puromycin, histones, and arylalkylamines). AA-NAT family members have only been found in Gram positive bacteria, fungi, algae, cephalochordates, and vertebrates (see (Klein 2007) for discussion). During evolution, changes have occurred between the emergence of cephalochordates and then vertebrates. Indeed, AA-NAT has a specific catalytic core, and flanking regulatory regions which make it 1000 more efficient than the yeast homolog. *Aa-nat* genes are expressed at high levels in the pinealocyte and retinal photoreceptor, reflecting their evolutionary origin from a common ancestral photodetector.

III.B.1.a *Aa-nat* gene:

Aa-nat gene has been isolated in most studied mammals such as rat (Borjigin et al 1995), sheep (Coon et al 1995), human (Coon et al 1996) monkey (Klein et al 1997) mice (Roseboom 1998), chicken (Bernard et al 1997) and fish (Bégay et al. 1998). The human gene is at the 17q25 locus on chromosome 17 (Coon et al 1996) in the rat 10q32.3 chromosome and in the mouse 11th chromosome in position E2.3-2.3 (Roseboom 1998). The portion of *Aa-nat* gene encoding the mRNA transcript is approximately 2.5 kilobases (kb) in human, 1.9 kb in sheep and 3.8 kb in chicken (Klein et al 1997). The gene is composed of 4 exons and 3 introns in human (Coon et al 1996; Klein et al 1996). Studied mammalian *Aa-nat* genes carry conserved motifs in the transcripts such as the same C- and N-terminal cyclic AMP-dependent protein kinase regulatory sites that could participate in the stability and controlled AA-NAT activity (Coon et al 1995). In *Arvicanthis ansorgei*, we have cloned *Aa-nat* from retinas and its sequence is 92 % to *Arvicanthis niloticus Aa-nat* and present 85% homology with the mouse *Aa-nat* (Figure 31). These data fit with all studied mammalian *Aa-nat* which are relatively close homologs (Coon et al 1996).

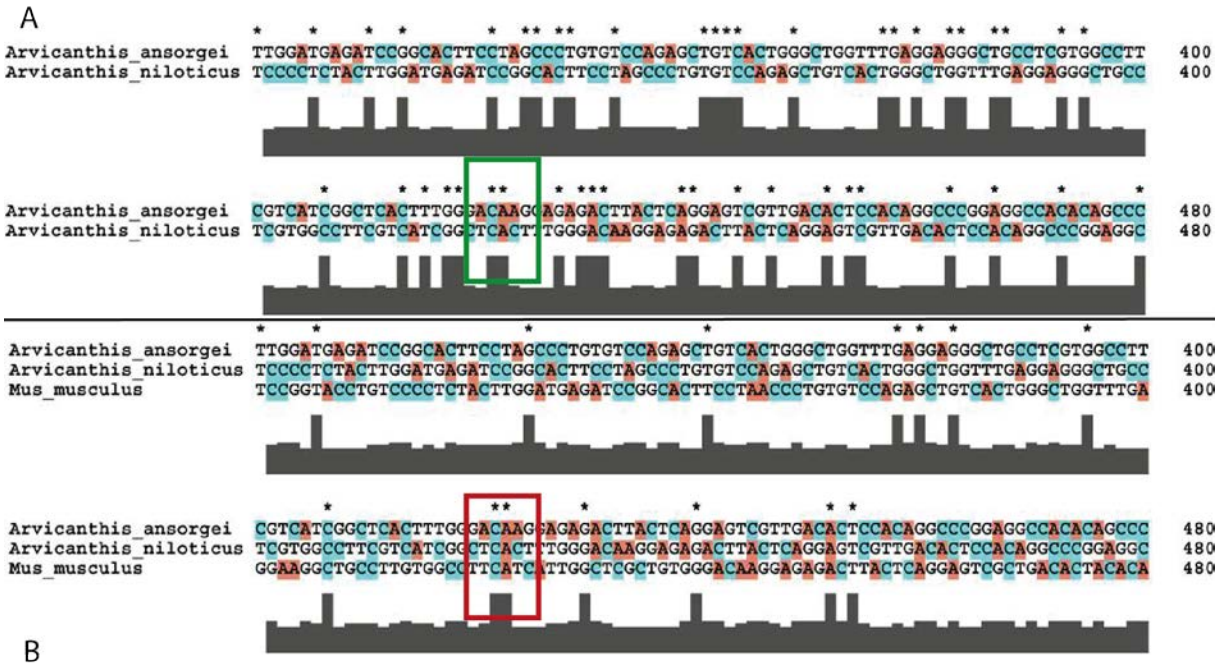


Figure 31: Alignment of *Arvicanthis ansorgei Aa-nat* partial coding sequence with *Arvicanthis niloticus* and *Mus musculus Aa-nat* transcripts.

A) Top panel illustrates close homology of *Arvicanthis* species *Aa-nat* coding sequences (92%). **B) Bottom panel** shows *Arvicanthis* sp. *Aa-nat* sequence comparison with the mouse one and reveals 85% homology with 100 % identity islets (green and red boxes) among the analyzed rodent *Aa-nats*. Identical residues are marked by stars. A: orange, C: blue

III.B.1.b *Aa-nat* gene promoter:

The *Aa-nat* gene promoter (2160 base pairs (bp)) contains different sites for transcription factor binding (Figure 32): i) P-CREB induced by cAMP binds CRE (Baler et al 1997; Burke et al. 1999). Ii) **Immediate early gene (IEG)** type transcription factors such as **activating protein 1 (AP1)** bind AP1 motif (Karin and Smeal 1992). Iii) The complex CLOCK/BMAL1 binds the E -box, implying that clock genes can act on *Aa-nat* transcription (Chen and Baler 2000). This is in general the clock activation pathway. To turn off AA-NAT, the suppressive effect of light decreases cAMP levels leading to dissociation of the AANAT/14-3-3 complex, followed by inactivation and proteosomal destruction. In addition, E-box elements contribute to the blockage of ectopic expression (Klein, 2007). Moreover, the *Aa-nat* gene first intron also contains E-box elements, which contribute to the gene's pineal specific expression (Humphries et al. 2007). iv) **CRX/OTX** transcription factors bind photoreceptor conserved elements such as **Pineal Regulatory Element (PIRE)**, only expressed in the pineal gland and the retina (Li et al. 1998).

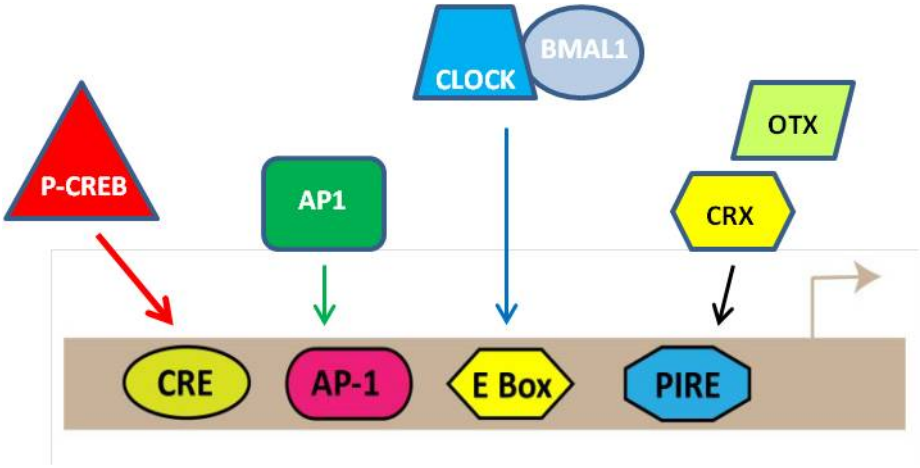


Figure 32: *Aa-nat* gene promoter.

P-CREB transcription factor binds to CRE. AP1 binds to AP-1 while the CLOCK-BMAL1 complex binds to the E-box. CRX/OTX transcription factors binds photoreceptors conserved elements such as PIRE only in the pineal gland and the retina.

III.B.1.c *Aa-nat* mRNA:

Aa-nat gene transcription produces only one mRNA (as assessed by Northern blot analysis) in rat, sheep, human, monkey, chicken (Klein et al 1997). On the contrary, in some fishes, data suggest alternate splicing of the mRNA leading to different possible AA-NAT isoforms (3 in trout, 2 in pike, 1 in Zebra-striped fish) (Falcón 1999). *Aa-nat* mRNA presents a significant variation between day and night, with a 150 fold increase at night in rat, chicken, monkey and pike pineal gland (Klein et al 1997). At the opposite, in trout and ungulates, *Aa-nat* mRNA is weakly increased at night (Falcón 1999; Ganguly et al 2002; Klein et al 1997; Stehle et al. 2001). In *Arvicanthis ansorgei*, pineal *Aa-nat* profile is vastly similar to the rat one with a circadian regulation and a peak in the middle of the dark period (Figure 33).

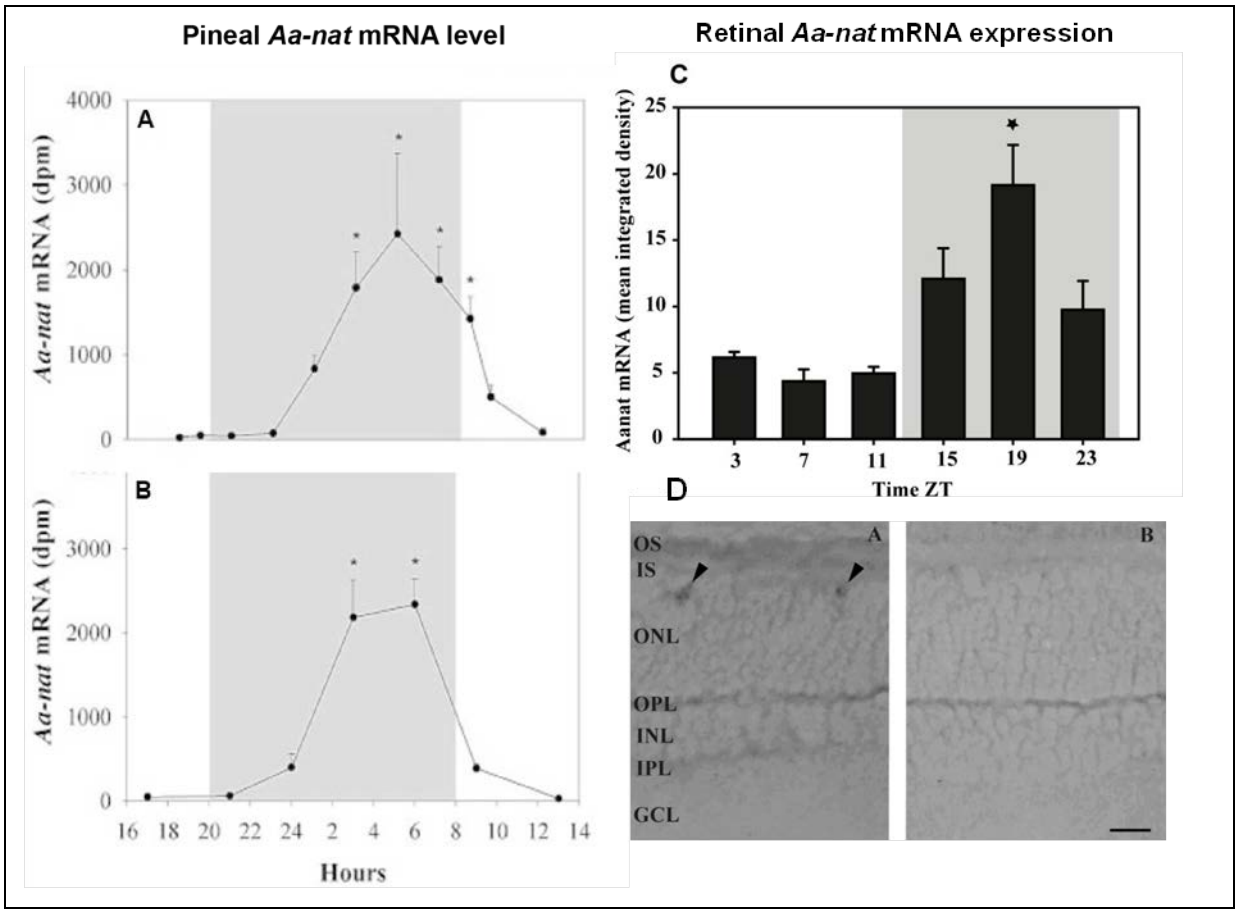


Figure 33: Pineal and retinal *Aa-nat* circadian profile in *Arvicanthis ansorgei* and *Rattus norvegicus*.

Panels A-B *Aa-nat* mRNA level rises up to 100 times the basal level at ZT20 in *Arvicanthis ansorgei* and *Rattus norvegicus* respectively. Animals were raised in 12:12 LD conditions. Adapted from (Garidou et al 2002). **Panel C**: Retinal *Aa-nat* profile is similar to the pineal gland one but the peak occurs slightly in advance at ZT19. **Panel D**: photographs of *Aa-nat* mRNA *in situ* hybridization on 10 µm rat retina cryosections at ZT18. Left panel is with the antisense probe and arrows point to *Aa-nat* expression in the ONL and especially in cones. Right panel shows the sense probe control.

III.B.1.d AA-NAT protein/Enzyme E.C. 2.3.1.87:

AA-NAT is a globular 23 kDa cytosolic protein that forms a reversible complex with 14-3-3 zeta proteins (14-3-3 proteins are a family of conserved regulatory molecules expressed in all eukaryotic cells). The catalytic core of AA-NAT forms a cavity encompassing the arylalkylamine and AcCoA binding pockets (Figure 34 A). Arylalkylamines bind in a funnel-shaped pocket formed by 3 protein loops which contact the aromatic ring of substrates via aromatic residues. Loops 2 and 3 are relatively rigid compared to loop 1 which is floppy. Loop 1 is longer (CPL amino acids) in AA-NAT compared with homologs. The additional three amino acids may be important for binding or catalysis or both. AA-NAT preferentially acetylates arylethylamines, including the naturally occurring amines serotonin, tyramine, dopamine, tryptamine, phenylethylamine, and synthetic naphthyl-, benzofuryl- and benzothienylethylamines.

AA-NAT harbors N- and C- regulatory regions which are crucial because it enables cAMP to control rapid activation/inactivation and protection/degradation switching. This is achieved by phosphorylating the Ser/Thr residues in the PKA/14-3-3 motifs (Figure 34 B). The N-terminal regulatory region is about 30 residues in length with the primary structure of the PKA/14-3-3 motif (28RRHpTLP33). The sequence external to this site is disordered, except for a lysine (Lys10) which is thought to be critical for proteosomal proteolysis. The C-terminal regulatory region contains the PKA/14-3-3 motif 200RRNpSG(C/R) 205. AA-NAT can bind to 14-3-3 proteins via either PKA/14-3-3 motif; however, binding via both is required for activation. When AA-NAT and 14-3-3 zeta bind to each other, this favors the optimal configuration of loop 1 and increases affinity for arylalkylamines. Interestingly, it also shields AA-NAT from phosphatases and degradation as well as thiol-dependent inactivation (Klein 2007). Rat AA-NAT displays an optimal activity at 37°C and pH 6.8 in presence of AcCOA to yield NAS in very low amount during the day but with a strong increase at night (Parfitt et al 1975). The correlation AA-NAT protein/activity is robust. Both exhibit circadian variations with the maximum around ZT20 (middle of the night) but diverse amplitudes (species-dependent) suggesting distinct regulation of the molecule (Klein et al 1997). To sum up, vertebrate AA-NAT protein and activity are regulated via binding to 14-3-3 proteins.

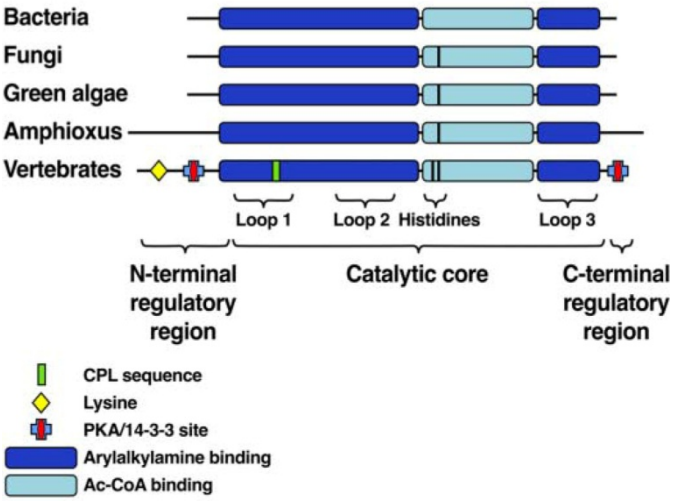


Figure 34 A: 0rganization of AA-NAT and AA-NAT homologs.

The central catalytic core is flanked by regulatory regions, each of which possesses a PKA site. The N-terminal PKA site is located within a sequence converted to a binding motif upon phosphorylation (Ganguly et al 2002)

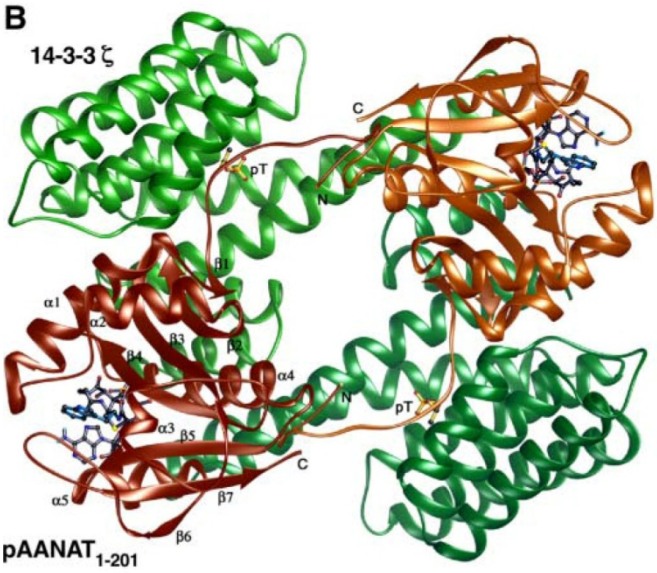


Figure 34B: The AA-NAT/14-3-3 ξ complex

Complex is viewed from above the 14-3-3 ξ dimer half-pipe (Obsil et al. 2001).

III.B.2. Hydroxyindole-O-methyltransferase/N-acetylserotonin-methyltransferase (HIOMT/ASMT) E.C. 2.1.1.4:

HIOMT/ASMT is the ultimate enzyme of pineal and retinal melatonin synthesis (Axelrod 1961). HIOMT uses N-acetylserotonin (NAS) as a substrate to generate melatonin. As stated before though, AA-NAT has always been considered the limiting enzyme of rhythmic melatonin synthesis (see all literature cited in the AA-NAT section) and has been widely studied. By contrast, HIOMT data are scarce and sometimes puzzling: virtually no HIOMT activity detected in Macaque retina (Coon et al. 2002). However, HIOMT is in theory the limiting enzyme of the neurohormone synthetic pathway. This biochemical requirement led us to consider its presence, activity, in the retina of our diurnal model *Arvicanthis ansorgei*.

III.B.2.a *Hiomt* gene:

Hiomt cDNA was cloned from bovine (Ishidaz et al 1987) then chicken (Voisin et al 1992), human (Donohue et al 1992) and rat (Gauer and Craft 1996) pineal glands. The main *hiomt* gene trait is the weak homology or high divergence in its sequence between all the studied species. The rat *hiomt* presents only 65%, 63%, 59%, homology with respectively bovine, human and chicken *hiomt* genes (Gauer and Craft 1996) (Figure 35). The human *Hiomt* gene has been assigned to the **pseudo-autosomal region (PAR1)** of the X and Y chromosome (Rodriguez et al. 1994). Note that in pseudo-autosomal regions, genes are inherited just like any other autosomal gene.

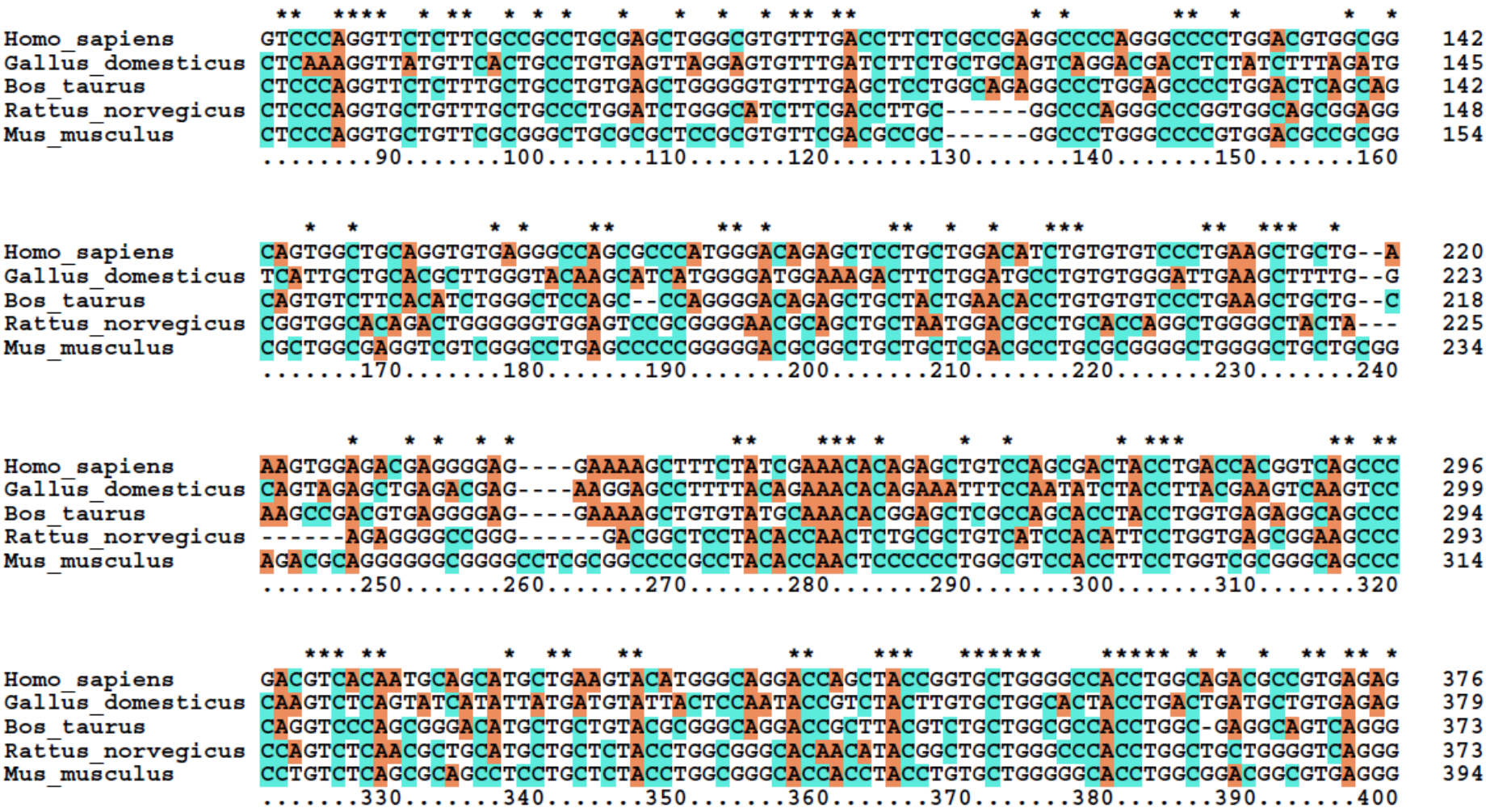


Figure 35: Alignment of coding sequences comparing HIOMT from human, chicken, bovine, rat, mouse.

Identical residues are marked by stars. (81-400 residues). A: orange; C: blue.

The human *Hiomt* gene is complex in that it contains **two distinct putative promoters** (A and B) driving differential splicing. Three distinct HIOMT messages with different coding regions can be amplified from retina, brain, and pineal tissues. The promoter also bears CRE, AP1 and PIRE motifs already identified in the *Aa-nat* promoter (See *Aa-nat* section above). Rodriguez et al suggest that promoter B makes all three *Hiomt* messages and that it is expressed **in all the retinas tested albeit in different amounts** (the expression in retina seems to be transient, with basically only two of the eight retinas tested showing expression). Promoter B is thus probably responsible for the low level HIOMT expression observed in all the samples examined. **Promoter A** is more difficult to assess. The expression of this promoter **seems to be exclusive to retina** with the finding of the element (CAATTAAG) supportive of retina-specificity. Indeed, Kikuchi *et al.* have found this element in several photoreceptor-specific genes, *e.g.* rhodopsins, interphotoreceptor retinoid-binding protein, and arrestin, from widely varied species such as *Drosophila,* chicken, cow, rat, mouse, and humans. This sequence has been named **photoreceptor-conserved element** or **PCE-1**. Promoter A could be responsive to a retina-specific circadian rhythm while promoter B could be more generic, with low level expression in all tissues except for pineal.

By contrast with the human enzyme, rat *Hiomt* codes for a single transcript expressed in the pineal gland and in the retina under a circadian control (Gauer and Craft 1996). More recently, a study of mouse melatonin synthesis has focused on *Hiomt* gene and demonstrated that murine *Hiomt* gene is also located in the PAR of sex chromosome but is extremely polymorphic due to a highly recombination rate (Soriano et al. 1987). Consequently, the most popular mouse strain C57/BL6 harbors two mutations in *Hiomt* gene which reduce drastically HIOMT protein expression whereas C3H/HE and CBA/J exhibit active HIOMT proteins and produce melatonin (Kasahara et al. 2010).

Based on these highly variable data, no progress has been made toward a consensus on *hiomt* and melatonin expression in the Mammalian retina. Our work in *Arvicanthis ansorgei* retina has focused on the HIOMT expression with rat tools (anti-rat HIOMT) as we were not able to obtain a reliable *Hiomt* RNA probe in order to perform *in situ* hybridization (see results in the article below).

III.B.2.b *Hiomt* mRNA

Rat pineal *Hiomt* mRNA exhibits a day/night variation with low amplitude which is positively correlated to HIOMT activity (Ribelayga et al 1999). The other available data regarding *Hiomt* mRNA comes from the chicken pineal (Grève et al. 1996) Chicken pineal gland *Hiomt* transcription is under circadian control with a peak at midday contrary to the usual melatonin synthesis rule (High at night). No analysis of Mammalian retinal spatio-temporal *Hiomt* mRNA expression exists so far.

III.B.2.c HIOMT protein/activity:

Recently, the crystal structure of human HIOMT has been unveiled. This HIOMT monomer has an apparent molecular weight of 39 kDa and is composed of two domains. The N-terminal domain intertwines several helixes with another monomer to form the physiologically active dimer. The C-terminal domain is typical of other **S-adenosyl-L-methionine (SAM) -dependent O-**

methyltransferases (Botros et al. 2013). As expected, the overall fold of the SAM binding site is strictly conserved, and the major differences with other SAM methyltransferases occur at the second substrate binding site, which defines the specificity of these enzymes for NAS.

HIOMT sequence is highly variable among all studied species. Indeed, rat HIOMT presents 66%, 69%, 60% sequence homology with respectively bovine, chicken and human HIOMTs (Nakane et al 1983). These data agree with the two isoforms possibly generated by alternate splicing (3 distinct transcripts), at least known for the human enzyme (NCBI, http://www.ncbi.nlm.nih.gov/gene/438). Interestingly, HIOMT protein (contrary to AA-NAT) is very stable with a half-life superior to 24 hours (Bernard et al. 1993).

Pioneer studies on HIOMT activity (in vivo affinity studies) have demonstrated that the enzyme preferentially methoxyls NAS (50-80 %), then 5-hydroxytryptophol (15-30 %), serotonin (around 10 %) and finally 5-hydroxy-tryptophane and 5-hydroxyindole acetic acid (less than 5 %) (Axelrod 1961; Cardinali et al 1972; Morton 1987; MortonDJ 1986). Rat HIOMT displays an optimal activity at 37°C, pH 7.9 with cofactor SAM to convert NAS into melatonin (Ribelayga et al 1997, 1998, 1999). The weak increase of HIOMT activity (0.7 nmole/pineal gland/hour during the day to 2 nmol/pineal gland/hour during the night) at night correlating with the feeble variation of mRNA expression and its long half life demonstrate a different dynamic of HIOMT metabolism compared with the one of AA-NAT (Ribelayga et al 1999). This suggests that HIOMT may not be involved in the short term regulation (day/night) synthesis, a role largely attributed to AA-NAT.

In the rat, rabbit and hen **retinas**, HIOMT activity profiles are all similar and do not depend on a circadian control (Nowak et al 1989). Moreover HIOMT activities are low in retina compared to the pineal HIOMT enzyme profiles and are probably tied to low amount of the enzyme itself in the retina (Gauer and Craft 1996). Finally, non human primate retinal HIOMT has always been considered as nearly undetectable (Coon et al. 2002) and no other studies have since reported data on mammalian retinal HIOMT. We have addressed the question of HIOMT presence, activity and localization in *Arvicanthis ansorgei* retina using notably anti-rat HIOMT 1478. I wish to thank warmly Dr. Thomas Bourgeron (Institut Pasteur, Paris) for providing several HIOMT antibodies he had made for his own studies on human HIOMT and the associated peptides. Anti-rat HIOMT 1478 was the only antibody we could use reliably (based on cross-reaction among Muridae) in our immunohistochemistry experiments. We then bought a second antibody to perform additional controls (BIOS bs-6961R anti-human HIOMT/ASMT and the blocking peptide bs-6961P).

III.C. Melatonin in the eye:

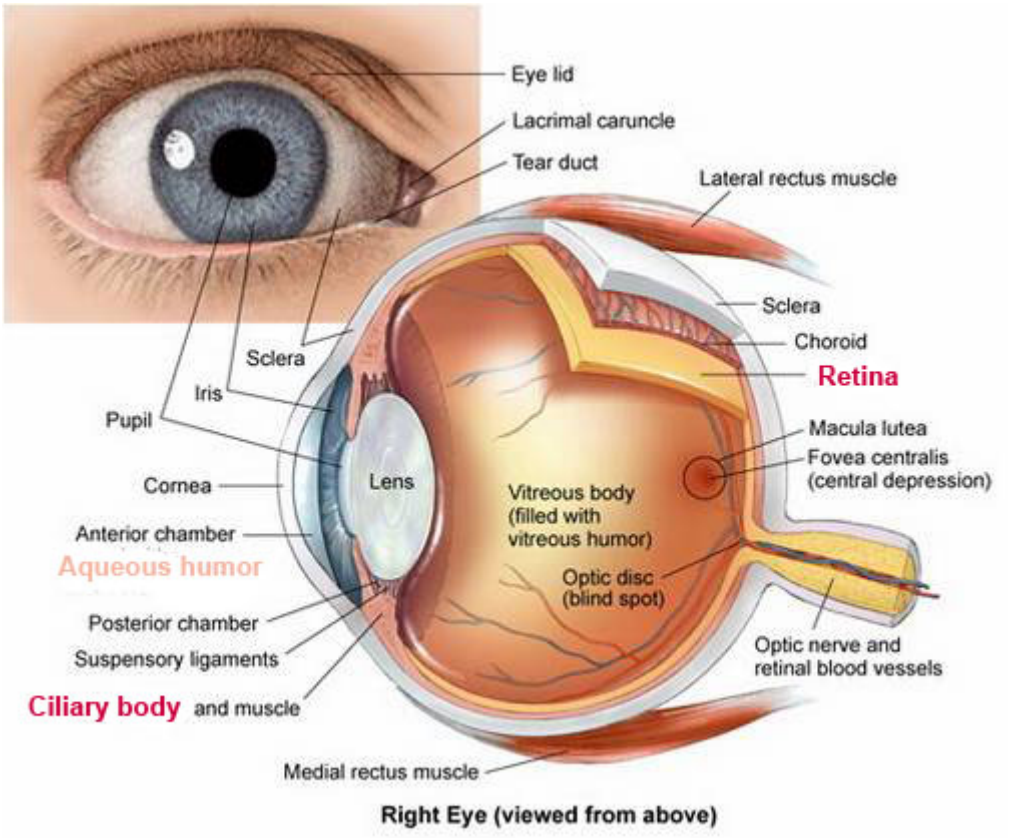


Figure 36: Anatomy of the human eye.

Red legends point out the retina and the ciliary body which are melatonin sources in the eye. In pink, aqueous humor fills the anterior chamber and contains melatonin. Modified from http://diagramreview.com/eye-diagram-and-functions/eye-diagram/

Few data support melatonin synthesis in other parts of the eye than the retina. One is the ciliary epithelium of the ciliary body (Figure 36). Two layers compose the ciliary epithelium: an outer pigmented epithelium, and an inner non-pigmented epithelium (Figure 37), which is the layer that secretes the aqueous humor in a circadian manner (Wiechmann and Summers 2008). The circadian rhythm of aqueous humor secretion could influence **intraocular pressure (IOP)** and melatonin is acting on IOP (Alcantara et al 2011). In fact, AA-NAT and HIOMT activity as well as melatonin have been detected in the human ciliary body (Martin XD et al 1992). AA-NAT activity and melatonin are classically rising at night in the ciliary body of chicken and rabbits (Aimoto T. et al 1985). Finally, pinealectomy does not change the daily rhythm of melatonin in the ciliary body, suggesting that melatonin rhythm in this organ is independent of the bloodstream source (Wiechmann and Summers 2008). Besides, AA-NAT, HIOMT activity and melatonin have been measured in rat and rabbit lenses. Interestingly, AA-NAT activity profile displays a daily variation whereas HIOMT activity does not appear to fluctuate (Abe et al. 1999). Finally, Mhatre et al demonstrated the presence and activity of both AA-NAT and HIOMT in the lachrymal glands of Syrian hamsters (Mhatre 1988). However, only speculation can be made about the use of melatonin synthesis in the lens and the lachrymal gland because no specific role has been defined in these structures. Local melatonin production may be necessary to stimulate correctly nearby melatonin receptors (discussed in a section below).

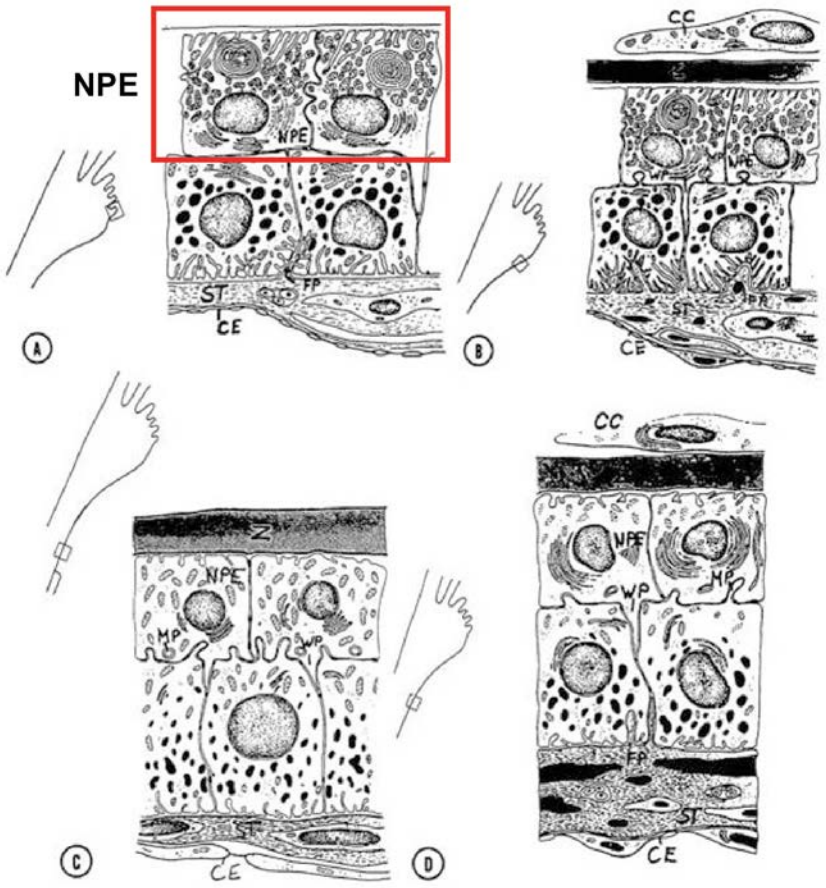


Figure 37: Schematic of variation in primate ciliary epithelial bilayer fine structure from anterior process to posterior base.

CC: covering cells, CE: capillary endothelium, FP: fingerlike projections, MP: mushroomlike process, **NPE: non pigment epithelium**, ST: stromal layer, WP: wedgelike process, Z: zonular fibers. (Association for Research in Vision and Ophthalmology). **Panel A:** Red rectangle highloghts NPE which secretes aqueous humor.

This wide first part was meant to give all the keys to come to the heart of the main scientific question we have addressed since I joined David Hicks team to work on *Arvicanthis ansorgei* retina, a diurnal rodent with a rich-cone retina. In addition, my second goal was to point out several questions for future studies. We started the project in the retina with the pineal melatonin background and with an impression of fragmented datas on retinal melatonin synthesis and function. The article presented below answer the following questions: **Which retinal cell types are involved in melatonin synthesis? How Aa-nat/AA-NAT and Hiomt/HIOMT regulated? Is diurnality versus nocturnality an important factor here? What are melatonin possible roles in *Arvicanthis ansorgei* retina (melatonin receptor-type 1 related)?**

Melatonin in the retina

IV. Melatonin in the retina of *Arvicanthis ansorgei*

I. Melatonin in the retina of *Arvicanthis ansorgei*

Unique regulation of the melatonin synthetic pathway in the retina of diurnal female *Arvicanthis ansorgei* (Rodentia)

Coralie Gianesini[1], Daniel Clesse[2], Gianluca Tosini[3], David Hicks[1] and Virginie Laurent[1]

[1]CNRS UPR 3212, Institute for Cellular and Integrative Neurosciences, 5 rue Blaise Pascal, F-67084, Strasbourg, France

[2]CNRS UMR 7364, Laboratoire de Neurosciences Cognitives et Adaptatives, Université de Strasbourg, Faculté de Psychologie, 12 rue Goethe, F-67000 Strasbourg

[3]Department of Pharmacology and Toxicology, Morehouse School of Medicine, 720 Westview Drive, Atlanta, GA 30110, United States

Corresponding author: Virginie Laurent, CNRS UPR 3212, Institute for Cellular and Integrative Neurosciences, 5 rue Blaise Pascal, F-67084, Strasbourg, France. gydelaurent@inci-cnrs.unistra.fr

Abstract

Knowledge about melatonin synthesis and its potential roles within the retina remains fragmented, especially in mammals where studies have focused on the penultimate enzyme of melatonin synthesis Arylalkylamine N-acetyltransferase (AA-NAT), whereas the final enzyme necessary for melatonin production is Hydroxyindole-O-methytransferase (HIOMT). We explored multiple parameters of the melatonin synthetic pathway in the cone-rich retina of a diurnal rodent, *Arvicanthis ansorgei*, cones being previously implicated as probable reservoirs of melatonin production. We analyzed the temporal and spatial expression of *Aa-nat* mRNA and AA-NAT protein, and enzymatic activity of AA-NAT, HIOMT, as well as the high-affinity melatonin receptor MT_1 and melatonin itself. We report that *Aa-nat* mRNA was localized principally to cones and ganglion cells (RGC) with opposing cyclic expression, being maximal in cones during the night, and maximal in RGC in the daytime. AA-NAT protein was also immunolocalized to these same populations, and was globally present and active throughout the 24 hour (h) period. HIOMT immunolocalization largely mirrored that of AA-NAT, but expression levels and activity were extremely low and remained uniform throughout the 24 h period. MT_1 showed a complementary expression pattern to the synthetic enzymes, present in rod photoreceptors, some inner retinal neurons and RGC. Surprisingly, melatonin levels were consistently low throughout the day-night cycle, in accordance with the low expression and activity levels of HIOMT. These data demonstrate that the melatonin synthetic pathway in a diurnal rodent differs from that described for other tissues and species (both nocturnal and diurnal), the contrasting phase expression in photoreceptors and RGC suggesting distinct roles in these populations.

Introduction

Melatonin is a ubiquitous molecule retained throughout evolution from bacteria to humans acting as a substrate for multiple functions ranging from antioxidant properties to circadian clock output *(1,2)*. In mammals, melatonin is a neurohormone synthesized principally at night by the pineal gland *(3)*. Circadian regulation of this indolamine is controlled by a poly-synaptic pathway commencing with retinal photoreception, neural transmission to the hypothalamus and relays within the autonomic nervous system. Then transduction of the neural message into a biochemical output occurs within the pineal gland *(3,4)*. Melatonin, as a prominent output of the central circadian clock, modulates a wide array of physiological functions such as the sleep/wake cycle *(5)*, reproduction *(6,7)* and immune modulation *(8)*. Melatonin is also synthesized *de novo* in the vertebrate retina where it can exert local effects on photoreceptor phagocytosis *(9,10)*, neuronal electric activity *(11,12)*, dark adaptation *(13)* and light sensitivity *(14)*, by way of specific MT_1 and MT_2 receptors expressed in the eye *(15,16)*. In nocturnal rodents MT_1 receptors are localized in photoreceptors, inner nuclear layer (INL) and retinal ganglion cells (RGC) *(12)*. Furthermore, a melatonin-proficient mouse that lacks MT_1 displays reduced viability of photoreceptors and RGC during aging *(11)*. Nevertheless, our knowledge about melatonin synthesis, regulation and precise role(s) remains fragmented in mammalian retina. The available evidence shows that melatonin synthesis in nocturnal rodent retinas shares many classical features with that of the pineal gland, such as the biochemical substrates (tryptophan and serotonin) and enzymatic machinery (notably AA-NAT and HIOMT) *(17,18)*. AA-NAT, under the control of light, a retinal circadian clock and proteasome formation, represents the limiting enzyme of rhythmic melatonin secretion *(19)*. As in the pineal gland, within the retina *Aa-nat* mRNA is maximal in the middle of the night *(20,21)* when melatonin synthesis takes place.

Localization studies in the retina have revealed contrasting results: whereas one study detected *Aa-nat* mRNA exclusively within a subpopulation of photoreceptor cells that resemble cones in number and distribution *(22)*, another group reported a much wider expression pattern, throughout the photoreceptors and in the INL and RGC *(23)*. In chicken retina, *Aa-nat* mRNA is mainly detected in photoreceptors *(24)* and at a lower level in RGC *(25)*. Compared to rodents, primate retina show a very different spatial and temporal expression pattern of both *Aa-nat* and AA-NAT. Primate *Aa-nat* levels are virtually equal between night and day, while AA-NAT shows marked nightly increase *(26)*. In rodents, expression levels of *Aa-nat* vary greatly between night and day. These differences indicate that control of melatonin synthesis occurs at the level of transcription in rodents, while in primates this control is principally post-translational *(26)*. HIOMT is the true rate-limiting step of N-acetyl serotonin (NAS, the product of AA-NAT) conversion into melatonin *(27)*. HIOMT/*hiomt* biology is still poorly understood, partly because the level of *hiomt* gene expression is low and constitutive in rat retina *(28)*, partly because HIOMT has not been detected in non-human primate eyes *(26)*, and also because its sequence is extremely divergent between species *(29)*. Only chicken retinas express HIOMT protein in photoreceptors *(30)*. The aim of this study was therefore to characterize the retinal melatonin synthetic pathway as completely as possible, through analyzing the spatial and temporal expression patterns of *Aa-nat*, AA-NAT, *Hiomt* and HIOMT, as well as their relative activities.

Emphasis is made on the use of a diurnal rodent *Arvicanthis ansorgei (31)*: this species possesses a retina whose morphology has two great advantages: i) compared to nocturnal rats and mice with only 1-3% cones *(32,33)*, this population makes up 33% of total photoreceptors in *Arvicanthis (34)*. As available evidence suggests cones are an important site of melatonin production *(22)*, and cones are essential to human eyesight, enabling high acuity chromatic vision *(35,36)*, this species constitutes a potentially valuable experimental model; ii) these cones are neatly arranged in two rows of cell bodies overlying the rods, a feature which facilitates discrimination between photoreceptor types *(34)*. Finally the photic niche of this species makes it simpler to extrapolate towards other diurnal species including humans. Our data indicate that the melatonin synthetic pathway in *Arvicanthis* differs in several respects from both nocturnal rodent and diurnal primate (and chicken) retinas, contrasting with the accepted models of melatonin regulation and functional effects.

Materials and methods

Animals

This study was conducted with young adult (3-6 month) female Sudanian Unstriped Grass Rats *Arvicanthis ansorgei*, raised in our animal facilities (Chronobiotron) under conditions of cyclic light (12 h white light, ~100 lux) and darkness (LD), as previously described *(34)*. In some experimental series, animals were maintained in constant darkness (DD) for 24 h prior to tissue collection, performed during the following 24 h, still under DD. Animals were anesthetized by isoflurane inhalation and immediately decapitated every 4 h over a period of 24 h. Zeitgeber Time 0 (ZT0) and circadian time (CT) 0 are defined as the beginning of the day (real or subjective), when lights are turned on (or expected to be turned on), 07:00 h. For immunohistochemistry and *in situ* hybridization studies, eyes were treated as detailed previously *(34)*. Regarding all other experiments, retinas were rapidly isolated by slitting the cornea with a scalpel blade, discarding the lens and vitreous and snap frozen individually in sterile Eppendorf® tubes in liquid nitrogen. Animal treatment and experimentation were in agreement with institutional and national guidelines, and adhered to the Association for Research in Vision and Ophthalmology Guidelines for Use of Animals, to the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the Animal Use and Care Committee from Strasbourg. The experimental procedures were covered by European authorization to perform animal experimentation (CREMEAS, AL/24/31/02/13).

In situ hybridization

We used a previously published protocol *(37)* with some modifications. Sense and anti-sense riboprobes were transcribed from linearized plasmide (pBK-CMV phagemid) containing a 1311 base pairs rat *Aa-nat* cDNA (Stratagene®, La Jolla, CA, USA) in the presence of digoxygenin-labeled nucleotide (Roche, Meylan, France). Frozen retinal sections were post-fixed for 20 min with 4% formalin in 100 mM PBS before dehydration and rehydration through a sequence of 70%, 95%, 100%, 95%, 70% ethanol. Sections were digested by 0.5 µg/mL proteinase K (Roche, Meylan France) at 37°C for 30 min. Hybridization was performed at 54°C with 200 ng/mL labeled sense or antisense probes in 50% formamide, 5X Standard Sodium Citrate (SSC), 5X Denhardt's solution, 0.1% Tween20, 0.04% DEPC and 1 mg/mL salmon sperm DNA. Sections were treated with RNAse A (AMRESCO) at 3 µg/mL in NTE buffer (Tris 100 mM, Nacl 400 mM, EDTA 25mM) at 37°C 30 min. Alkaline phosphatase activity

was detected after 2 h. Relative quantification of *Aa-nat* mRNA transcripts is representative of the subjective mean staining within the photoreceptor (outer nuclear layer or ONL), INL and ganglion cell layer (GCL) from four retinal sections of each of three different retinas (n=3) for each time point.

Immunohistochemistry

The protocol was adapted from published procedures *(38)*. For AA-NAT and HIOMT immunostaining, antigen retrieval was performed in Tris-EDTA buffer (Tris 10 mM, EDTA 1 mM, pH 8) for 1 h at 95°C followed by 2 h slow cooling at room temperature. Then retinas were permeabilized with Triton X-100 (0.1% in PBS) for 5 min, and sections were saturated in blocking buffer (3% BSA, 0.05% Tween-20, 0.1% Triton -X100 and 0.1% sodium azide in PBS) for 1 h. Retinas were incubated overnight at 4°C with different primary antibodies diluted in the blocking buffer: rabbit polyclonal cone arrestin (generous gift of Dr. C. Craft, Doheny Eye Institute, USC, Los Angeles, USA) and was used at 1/5000 dilution; rabbit polyclonal anti-rat AA-NAT 3314 was a generous gift from Dr D.C Klein, National Institute of Child Health and Human Development (Bethesda, Maryland, USA), 1/1000; rabbit polyclonal anti-rat HIOMT 1478 was a generous gift of Dr T. Bourgeron, Institut Pasteur (Paris, France), 1/250; rabbit polyclonal anti-mouse MT_1 *(12)*, 1/400. Sections were then incubated with the appropriate secondary antibody (Jackson Labs, Bar Harbor, ME; 711-066-152) and the Avidin/Biotin/horseradish peroxydase complex (Vectastain ABC kit, Vector Laboratories). Detection of AA-NAT, HIOMT and MT_1 signals was conducted using 3,3-diaminobenzidine (0.5 mg/mL, Sigma) with H_2O_2 (0,003%) in Tris Buffer Imidazole (TBI) for 5 min, 10 min and 15 min respectively. The sections were dehydrated in graded ethanol followed by toluene baths before mounting and observation under a microscope (Nikon Optiphot 2). Images were obtained by CCD camera video capture linked to a dedicated PC containing image analysis software (Nikon BIA).

Immunohistochemistry of whole-mounted retina

The protocol is according to published methods from our laboratory *(39)* with some modifications. Briefly, fixed retinas were treated for antigenic reactivation as described above. Samples were blocked with PBS buffer containing 3% BSA, 0.1%Tween-20, 0.1% NaN3 for 48 h. Then retinas were immersed in the primary antibody solution for 48 h at 4°C. Antibodies used were: AA-NAT 3314, 1/500 dilution; HIOMT 1478, 1/250; goat polyclonal anti-human Brn3 (Santa Cruz, CA; #sc-6026), 1/200; mouse monoclonal phosducin, 1/400 *(40)*, and mouse monoclonal RET-P3 *(41)*, 1/50. Brn3 expression is restricted to nuclei of RGC in *Arvicanthis (42)*; phosducin is specific to rod cytoplasm and provides a robust marker of rod IS; RET-P3 (of which the antigen is unknown) is specific to rod cell bodies and provides a reliable marker of this compartment. Samples were incubated overnight in corresponding secondary antibodies: goat anti-rabbit IgG-Alexa488 or -Alexa594 diluted 1/500 (Invitrogen) for AA-NAT and HIOMT, donkey anti-goat IgG-Alexa488 for Brn3, and rabbit anti-mouse IgG-Alexa488 or Alexa594 for anti-phosducin and RET-P3. Retinas were flattened onto microscope slides with mounting medium (Prolong, Invitrogen) and observed using a confocal microscope (Leica).

Western blotting

The protocol was adapted from a previous publication *(34)*: equal amounts of total protein (24 µg/lane) were separated by electrophoresis and transferred to polyvinyl difluoride (PVDF) membranes (Millipore; Immobilon-P). The membranes were blocked and incubated with anti-AA-NAT 3314 diluted 1/15000 in blocking buffer overnight at 4°C. Membranes were rinsed in TBS Tween-20

0.2% and incubated with the secondary antibody (goat anti rabbit-HRP, Jackson 111-035-144) diluted 1/200000 in TBS Tween 0.2% with 0.25% fish gelatin for 2 h. The signal was developed by chemoluminescence (kit Amersham^{TM}, GE Healthcare) and revealed by autoradiography (CL-XPosure films, Thermo Scientific). Analyses of immunoblots were performed on five retinas from different animals at each time point. Normalization was achieved against actin protein detected on the same membrane.

Enzymatic assays

Analyses of HIOMT and AA-NAT enzyme activities were performed in *Arvicanthis ansorgei* retinas as previously described for pineal gland *(43,44)*. Individual left retinas were sonicated in 100 µL of phosphate buffer (0.05 M; pH 8.25) for determination of HIOMT activity. Right retinas were sonicated in phosphate buffer (0.05 M, pH 6.8) containing 0.35 mM acetyl-CoA (Sigma) for AA-NAT activity determination. HIOMT and AA-NAT activities are expressed as pmoles/mg protein/hour.

HPLC measurements

Each frozen retina was homogenized by ultrasonication in 100 µL of mobile phase [1% methanol (V/V), 50 mM citric acid, 40 mM Na_2HPO_4, 0.8 mM EDTA and 0.3 mM Octan Sulfonic Acid. The samples were centrifuged (13000 rpm -16100 g) for 30 min and supernatants were analyzed for dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) with an HPLC system using an amperometric electrochemical detector (HPLC-ED; Decade II Antec, Leyden, Netherlands). For the detection, the working potential was set at 0.6 V. The temperature of the column and cell detection was maintained at 34°C. Each 20 µL sample was injected on a 100 mm length, 3 mm internal diameter, C18 column (Kinetex C18- and XB-C18 -2.6µ 100A, Phenomenex, Torrance, CA, USA). For the isocratic separation, the flow rate was kept at 0.8 mL/min. Standard solutions of dopamine and DOPAC (Sigma Aldrich, Saint-Quentin Fallavier, France) were diluted in the same mobile phase in order to obtain a 3 points standard curve for each standard for the quantification of the samples.

Melatonin quantification by ELISA

Melatonin ELISA kit (RE54021, IBL international GMBH) was used to perform the melatonin immunoassay following the protocol described in the kit instructions. For comparative purposes we analyzed melatonin levels both in diurnal *Arvicanthis* but also nocturnal mouse retinas, the latter involving the melatonin-competent C3H strain *(11)*. Briefly, Retinas, standards and controls were individually extracted in ammonium acetate buffer with protease inhibitor. The protein concentration was determined for each retina by a Bradford protein assay. Samples were incubated during 17 h at 4°C with the melatonin antiserum (rabbit polyclonal). The optical density (OD) was measured at 405 nm. The obtained ODs of the standards are plotted against their concentration. The results are presented in pg melatonin/mg protein.

Statistical analysis

Data are given as the mean ± SEM of n = 3-5 animals. Statistical analysis was performed with one-way analysis of variance (ANOVA) using the Sigma plot software. The significant level was set at $p < 0.05$. When required, ANOVA were followed by post-hoc Holm-Sidak test.

Results

Aa-nat mRNA expression in cone photoreceptors and RGC of *Arvicanthis ansorgei* shows phase-inversed expression under cyclic light conditions

We performed *in situ* hybridization studies with digoxygenin-conjugated RNA probes. Since previous publications for nocturnal rodents report a nocturnal peak in *Aa-nat* RNA levels, we first examined retinal sections prepared at ZT19. Staining was observed uniquely within and lying directly above the two scleral-most rows of cells of the outer nuclear layer (ONL) **(Figures 1A, B)**, with no detectable signal elsewhere in the retina (lower ONL, INL and GCL respectively). At the middle of the day, ZT6, hybridization signal was no longer apparent in the ONL but was now detected strongly in the GCL, and weakly in the INL **(Figure 1C)**. These localizations are shown at higher magnification in **Figures 1D-H**. We used anti-cone arrestin antibody to identify the cell type expressing *Aa-nat* at ZT19 **(Figures 1D, E)**: *Aa-nat* transcripts were localized to the apical cytoplasm and inner segments (IS) of cones. As stated earlier, GCL was not labeled at ZT19 **(Figure 1F)**, but at ZT6 labeling was no longer detectable in cones **(Figure 1G)**, whereas positive signal was clearly seen in the GCL **(Figure 1H)**. Sense probe controls did not demonstrate any signal **(Figure 1I)**. Validation of the *Aa-nat* probe was performed with rat retina, and similar results were obtained (at ZT19 we observed scattered staining of individual cells located near the sclera margin, corresponding to cones: **Supplemental Figure 1**).We examined *Aa-nat* hybridization in sections taken across the entire 24 h period, and used a semi-quantitative scale to record expression levels and cellular localization **(Table 1)**. It was seen that expression within the cones was faint or undetectable throughout the day, but intense during the night; expression within the INL was always weak but could be seen at both night and day; and expression in the GCL was moderate to strong during the day but below detection levels at night.

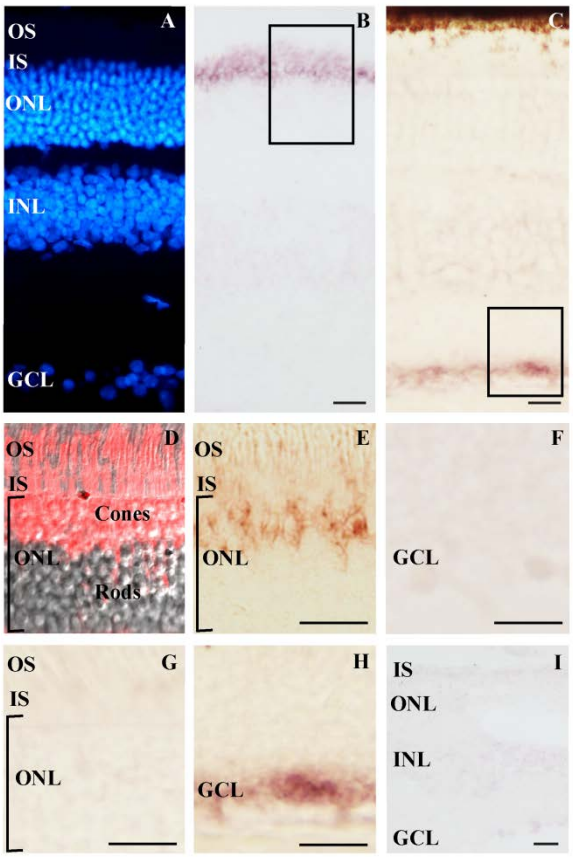
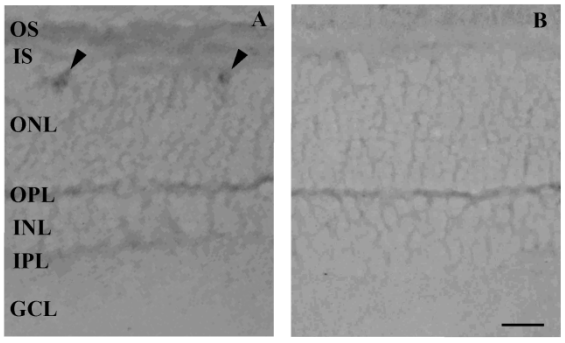


Figure 1: *Aa-nat* gene is expressed in the cones and ganglion cells of *Arvicanthis ansorgei* retina.

A) DAPI staining of a 10 µM section of *Arvicanthis ansorgei* retina indicates the area of interest: from top to bottom: OS: photoreceptor outer segments; IS: photoreceptor inner segments; ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer. **B)** *Aa-nat* antisense probe at ZT19 (night time) stains IS and the upper part of the ONL. **C)** *Aa-nat* antisense probe at ZT6 stains GCL. **D)** Immunostaining of *Arvicanthis ansorgei* retina with anti-cone arrestin (red) clearly showing ONL organization with cone cell bodies lying on top of rods. **E)** At ZT19, *Aa-nat* mRNA staining is restricted to the two uppermost cell body rows and IS. **F)** At ZT19 *Aa-nat* antisense probe does not stain GCL. **G, H)** At ZT6 *Aa-nat* antisense probe stains GCL (H) but not ONL (G). **I)** Use of *Aa-nat* sense probe at ZT19 does not stain any structures. Scale bar = 10 µm. Additional abbreviations: RPE: retinal pigmented epithelium.



Supplemental Figure 1: *Aa-nat* gene is expressed in the cones of rat retina.

A) In ZT19 rat retina, hybridized *Aa-nat* anti sense probe is detected in some cells of the ONL (arrowheads), corresponding to the number and position of cones. **B)** *Aa-nat* sense probe shows no staining in the ONL. Abbreviations as in Figure 1.

	ZT3	ZT6	ZT7	ZT11	ZT15	ZT19	ZT23
Photoreceptor	+	0	0	+/-	++	+++	++
INL	+/-	+	0	+/-	0	0	+
GCL	+	++	+	+/-	0	0	0

Table 1: Semi-quantification of *Aa-nat* expression in the retina of *Arvicanthis ansorgei*.

Aa-nat gene displays an opposing cyclic expression in cone and ganglion cell layers of *Arvicanthis ansorgei* retina. n= 3 animals for each time point. +++: strong; ++: moderate; +: weak; +/-: very weak; 0: absent. ZT: Zeitgeber Time with ZT0 = light on.

AA-NAT is expressed in many cells of the *Arvicanthis ansorgei* retina

Immunohistochemistry was performed on transverse sections prepared from retinas fixed at different times in the LD cycle **(Figure 2)**. At ZT23, AA-NAT immunoreactivity was strong and widespread, seen in IS, cone cell bodies, throughout most of the INL and inner plexiform layer (IPL), and within a subset of cells in the GCL **(Figures 2A-C)**. The lower area of the ONL, corresponding to rod cell bodies, showed no staining above background levels **(Figures 2A, B)**, while the outer segments (OS) were devoid of label. In sections of retinas fixed in the daytime (ZT7), AA-NAT staining was overall weaker, albeit still detectable in the cone cell bodies and the majority of the INL, IPL and GCL. Rods, OS and horizontal cells were unstained **(Figure 2D)**. Positive controls with sections of pineal gland obtained at ZT23 showed generalized strong staining **(Figure 2E)** and negative controls using sections of cortex or omission of primary antibody only showed background label **(Figures 2F,G respectively)**.

The cone-specific localization of AA-NAT was confirmed by performing double label immunofluorescent staining on whole retina. Staining with a rod-specific antibody (anti-phosducin) filled the cytoplasm in rod IS, forming a meshwork pattern across the retinal surface (Figure 2H); the unstained interstices corresponded to the profiles of cone IS **(Figure 2H)**. AA-NAT staining also revealed a meshwork aspect of stained profiles **(Figure 2I)**. Merged images demonstrated that AA-NAT immunoreactivity co-localized with the dark spaces (cones) between phosducin-positive profiles **(Figure 2J)**. However not all cone profiles were immunostained, and weak AA-NAT immunostaining was also observed in rods. We did not assess markers for INL neurons, but as AA-NAT immunoreactivity was previously seen in the GCL, we performed similar double immunofluorescence studies at this level. Brn3 was localized in RGC nuclei **(Figures 2K, M)**, while AA-NAT staining was seen within the cytoplasm of these cells **(Figures 2L, M)**. AA-NAT was also detected in numerous Brn3-immunonegative cells **(Figure 2M)**.

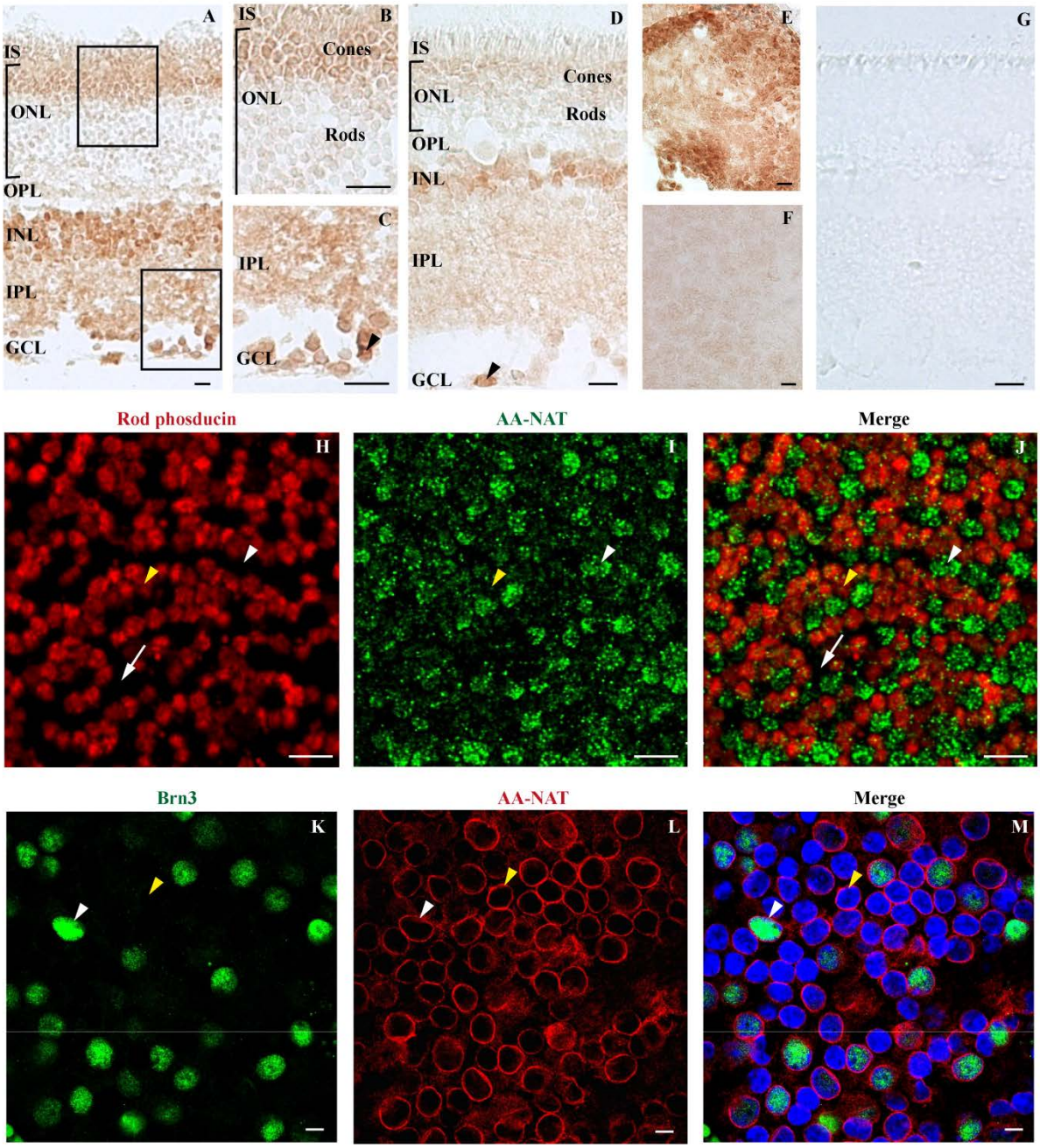


Figure 2: AA-NAT is localized in many cells in the retina of *A. ansorgei*.

Panels A) to G) represent analysis of AA-NAT localization by immunoperoxidase methods. **A)** At ZT23, AA-NAT staining was clearly visible in IS, upper ONL (cones), INL, IPL (inner plexiform layer) and GCL. The lower ONL (rods) is unlabelled. **B)** Enlargement of the ONL confirms that AA-NAT expression is restricted to cone cell bodies and IS. **C)** Enlargement of the IPL and GCL confirms expression in these areas. Black arrowhead indicates immunostained cell in GCL. **D)** Staining is likewise present at ZT7 although notably fainter at the level of the cones. Panels **E)** and **F)** are respectively positive and negative controls of AA-NAT detection at ZT23, in the pineal gland and cortex respectively of *Arvicanthis ansorgei*. **G)** Control without primary antibody. Scale bar for A-G = 20µm. **Panels H) to M)** fluorescence immunohistochemistry of whole mounted retinas at ZT23. **Panels H) to J)**: the optical section was fixed at the level of the IS. **H)** Rod IS are specifically stained with anti-phosducin antibody (yellow arrowhead), while cone IS are unstained and visible as dark profiles (white arrowhead). **I)** AA-NAT (green staining) is seen in numerous profiles. **J)** When both fields are merged, it can be seen that AA-NAT is weakly present in rod IS but strongly expressed in many (but not all) cone IS. An unstained cone IS is shown

by the white arrow in H and J. **K) to M)** optical sections fixed at the level of the GCL. **K)** Retinal ganglion cells (RGC) are stained with Brn3 antibody, visible within numerous nuclei (green staining). **L)** AA-NAT (red staining) is also detected in many cells as annular profiles corresponding to cytoplasm. **M)** Merged images show that AANAT is expressed in all Brn3-positive RGC (white arrowhead in K-M, as well as Brn3-negative cells (yellow arrowhead in K-M). Total cell nuclei are counter-stained with DAPI (blue). Scale bar for H-M = 5 µm. Abbreviations as in Figure 1.

To obtain information about the quantitative expression profile for AA-NAT protein in *Arvicanthis* retina, we performed western blotting analyses at similar time points across the 24 h period **(Figure 3A)**. Identical results were obtained using two different antibodies raised against AA-NAT. In both cases an intense immunoreactive band of the expected molecular weight for AA-NAT could be observed. There was considerable variation in immunoreactive intensity within sample groups (even though sibling animals were raised under identical housing conditions), and the only time point that gave reproducibly low expression was ZT3 **(Figure 3A)**. We observed that AA-NAT expression was high across the remainder of the day and early night, rising further at ZT19 and reaching a maximum at ZT23, around 10 fold higher levels than at ZT3 ($p > 0.05$, one-way ANOVA) **(Figure 3B)**. A similar profile of virtually continuous AA-NAT expression (except for CT3) was also observed in *Arvicanthis* raised under DD conditions **(Figure 3C-D)**. AA-NAT protein levels were elevated throughout the night with a peak at CT23, and showed intense expression in the middle of the day at CT7 ($p < 0.05$, one-way ANOVA) **(Figure 3D)**.To complete the characterization of AA-NAT temporal expression in *Arvivcanthis* retina, the enzymatic activity was assayed over the 24 h period. Values were weakly correlated with the expression levels in that the enzyme was constitutively active throughout the day and night cycle **(Figure 3E)**. There was no significant increase of activity at night ($p > 0.05$, one-way ANOVA).

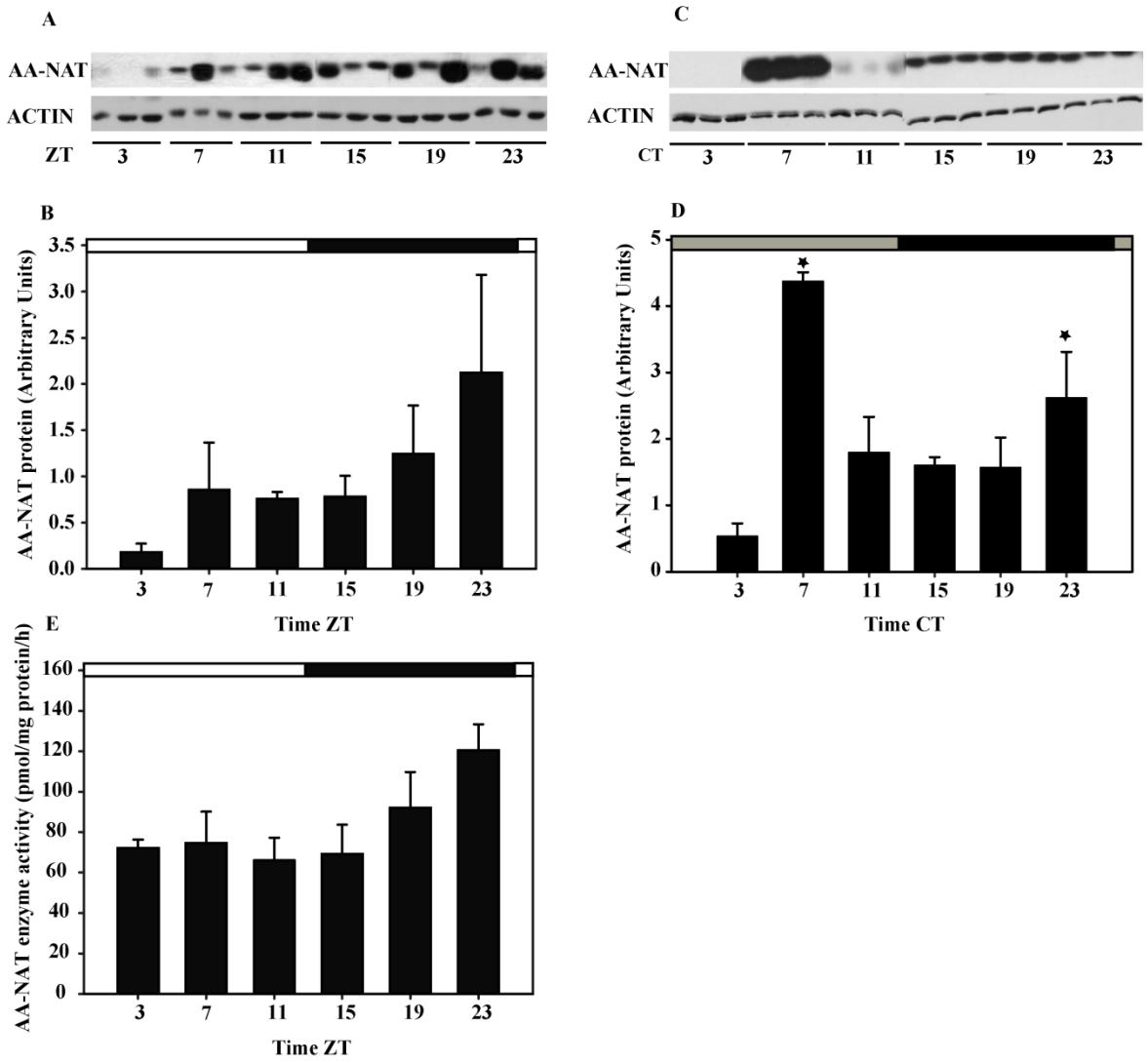


Figure 3: AA-NAT expression and activity are high at night but persist throughout daytime.

A) Western blotting analysis of AA-NAT expression in retinas from 3 different animals per time point reveals the presence of the protein AA-NAT throughout the 24 h. Data indicate that AA-NAT expression across 24 hours is overall higher at night but is also elevated at every point sampled in the daytime except ZT3. It is also evident that there was great variability between individuals within a sample time. **B)** Densitometric quantification of AA-NAT on western blots (n=5) confirms the general increase in the dark period as well as the substantial expression in late day, and the low expression at ZT3 ($p > 0.05$, one-way ANOVA).**C)** Western blotting analysis of AA-NAT expression in retinas (1 animal per time point, the same sample is repeated 3 times) reveals the presence of AA-NAT protein throughout the 24 h. Data indicate that AA-NAT expression is higher at CT7 but remains elevated at night. **D)** Densitometric quantification of AA-NAT protein as seen on western-blots (n = 3, three distinct experiments) demonstrates that the maximum of AA-NAT is at CT7 ($p < 0.001$, one-way ANOVA, Holm-Sidak test). The lowest expression is at CT3. Data are the mean values ± SEM of 3 animals analyzed in 3 independent experiments. **E)** AA-NAT activity is uniform throughout the day and early night, then increases slightly at ZT19 and peaks at ZT23 ($p > 0.05$, one-way ANOVA). Data are the mean values ± SEM of 4 animals. Light bar represents day time, black bar represents night-time, grey bar represents the subjective night.

HIOMT is expressed in photoreceptors, OPL and GCL in *Arvicanthis ansorgei* retina

Although we could not investigate *Hiomt* RNA expression in *Arvicanthis*, due to lack of cross-reactivity with an RNA probe specific for rat HIOMT (data not shown), we were able to perform immunohistochemical studies. Immunolabeled HIOMT was detected in IS, OPL, IPL and GCL at ZT23 **(Figure 4A)** but was not visible within the ONL or INL. HIOMT immunostaining was similar at ZT7 **(Figure 4B)**. Pineal gland and cortex were respectively used as positive and negative controls for HIOMT immunolabeling specificity **(Figures 4C, D)**. No signal was detected when the primary antibody was omitted **(Figure 4E)**. As for AA-NAT, to confirm HIOMT distribution we performed double label immunohistochemistry on whole mounted retina **(Figures 4F-K)**. As before, phosducin-positive profiles correspond to rod IS, while the black spaces represent cone IS **(Figure 4 F)**. HIOMT appeared as patchy staining **(Figure 4G)**. Merged images demonstrated that HIOMT was present throughout the IS layer, but more strongly in cones **(Figure 4H)**. At the level of the GCL, Brn3 staining was seen in RGC nuclei **(Figures 4I, K)**. HIOMT staining was localized in the cytoplasm of Brn3-immunopositive RGC **(Figures 4J, K)**, as well as many Brn3-immunonegative cells. In summary, use of multiple independent approaches indicated that within the *Arvicanthis* retina, despite phase-inversed expression of *Aa-nat* in cones (maximum at night) and GCL (maximum at day), AA-NAT and HIOMT are produced together in cones and RGC throughout the 24h period.

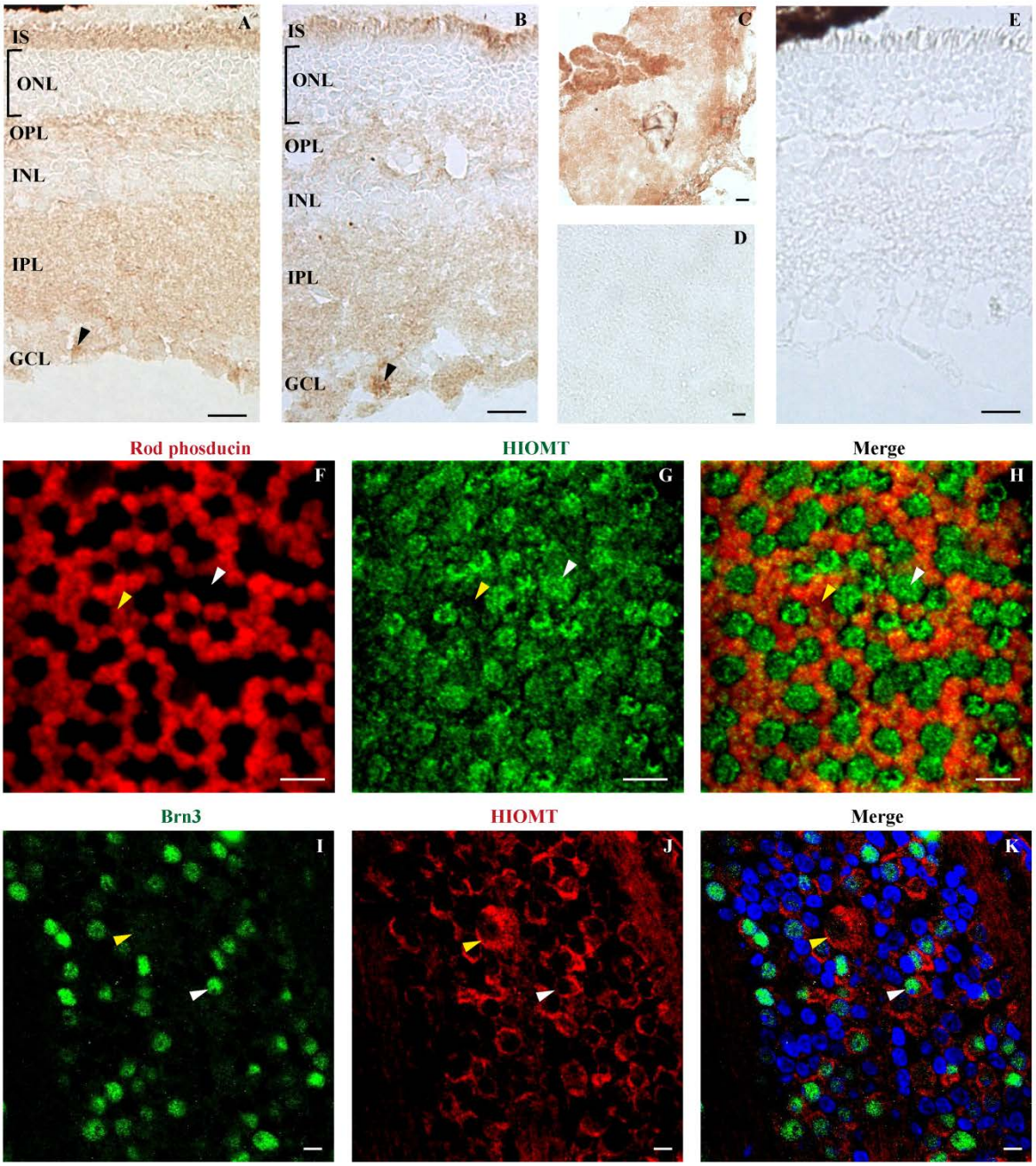


Figure 4: HIOMT is expressed in photoreceptors, synaptic layers and RGC in the retina of *Arvicanthis ansorgei.*

Panels A) to F) represent analysis of HIOMT localization using immunoperoxidase techniques. **A)** At ZT23, HIOMT staining can be seen in IS, OPL, IPL and GCL. **B)** Staining of similar intensity and distribution is also present at ZT7. Black arrowhead indicates immunostained cell in GCL. **C)** and **D)** are respectively positive and negative controls of HIOMT detection at ZT23 in sections of pineal gland and cortex of *Arvicanthis ansorgei*. **E)** Control without primary antibody. Scale bar for A-E = 20 µm. **Panels F) to K):** fluorescence immunohistochemistry of whole mounted retinas at ZT23. **Panels F) to H):** optical section fixed at the level of the IS. **F)** As in figure 3, rod IS are heavily stained by anti-phosducin antibody (yellow arrowhead), leaving unstained cone IS visible as black spaces (white arrowhead). **G)** HIOMT immunostaining is seen as circular profiles. **H)** Merged images reveal that HIOMT is mostly expressed in cone IS, with weaker staining in the surrounding rod IS. Panels **I) to K):** optical sections fixed at the level of the GCL. **I)** As in figure 3, RGC are

stained with Brn3 antibody, visible within numerous nuclei (green staining). **J)** HIOMT is visible as circular profiles corresponding to cell cytoplasm (red). **K)** Merged images indicate a co-localization between HIOMT and Brn3-positive RGC (white arrowheads in I-K), as well as Brn3-negative profiles (yellow arrowheads in I-K). Scale bar for F-K = 5 µm. Abbreviations as in Figure 3.

Melatonin concentrations are constitutively low in the *Arvicanthis ansorgei* retina

HIOMT enzyme activity in *Arvicanthis ansorgei* retina was very low (fiftyfold less) compared to AA-NAT activity **(compare Figures 5A and 3E)**, but was detectable throughout the 24 h cycle at a roughly similar level. ELISA quantification of melatonin concentrations was similarly low and uniform across the day/night cycle **(Figure 5B)**. We also attempted to identify melatonin by HPLC. There was no spike in the elution profile corresponding to purified melatonin standard, even at high signal amplification (data not shown). Furthermore, we also assayed for NAS (the end product of AA-NAT) by HPLC, and as for melatonin there was no detectable peak (data not shown). For comparative purposes, we also quantified melatonin in retinas isolated from C3H melatonin-competent mice. In this case, there was a notable rhythm of melatonin concentration, peaking in the night at fourfold higher values than that of *Arvicanthis* **(Supplemental Figure 2)**. As retinal melatonin and dopamine synthesis are seen in anti-phase in many species, we measured dopamine expression in *Arvicanthis ansorgei*. Using HPLC we measured levels of dopamine and its major metabolite, dihydroxyphenyl acetic acid (DOPAC). Dopamine levels showed a significant increase at the middle of the day (ZT7) **(Figure 5C)**, and likewise DOPAC levels were maximal at ZT7, decreasing towards the end of the day and remaining low through the night **(Figure 5D)**.

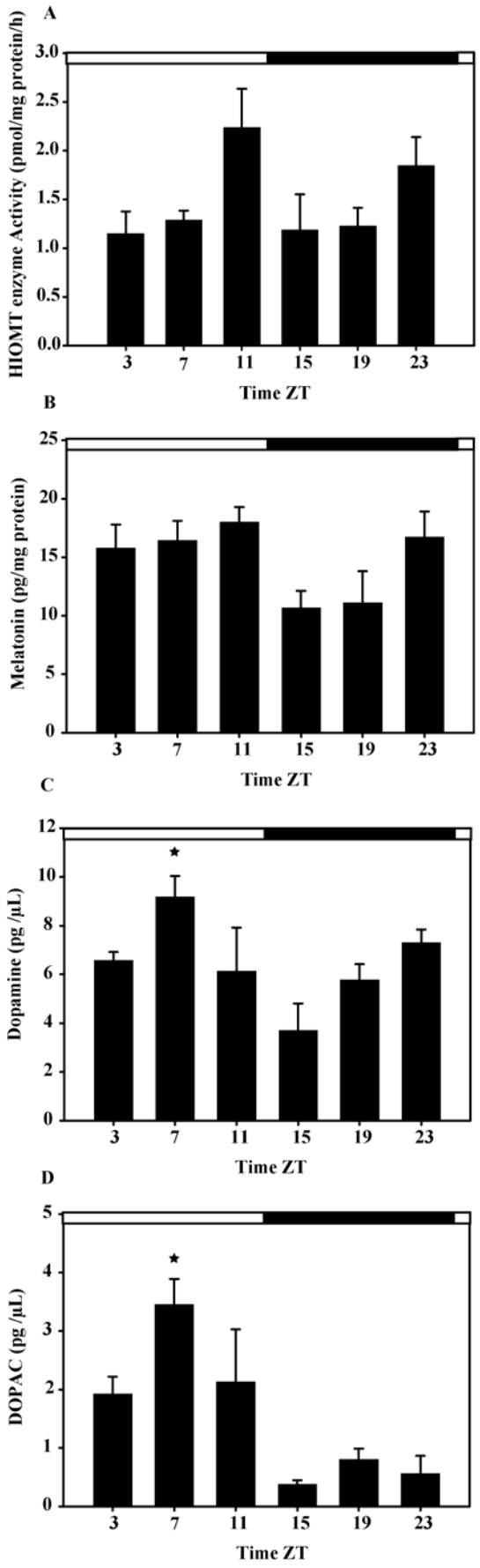
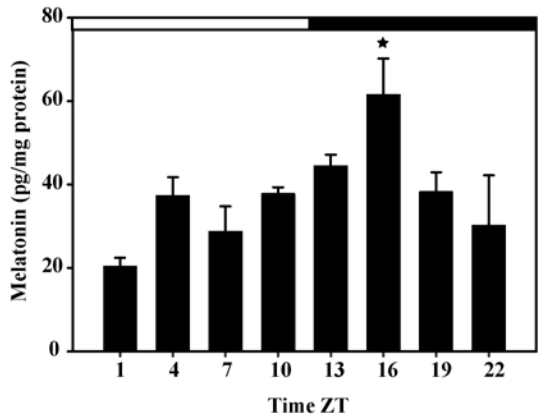


Figure 5: HIOMT activity and melatonin are present throughout 24 hours.

A) Daily profile of HIOMT enzymatic activity (n = 4 retinas per point) was obtained by radioactive assay performed on retinas of *Arvicanthis ansorgei* raised under 12h light/12h dark cycles. HIOMT enzyme activity appears constitutive with small increases at the end of the day (ZT11) and night (ZT23) periods ($p > 0.05$, one-way ANOVA). **B)** Melatonin concentrations were measured by ELISA in 5 *Arvicanthis* retinas for each time point. Melatonin is constitutively present at low levels ($p > 0.05$, one-way ANOVA). **Panels C) and D):** Dopamine and dihydroxyphenyl acetic acid (DOPAC) were analyzed by HPLC (n = 4 retinas per time point). Dopamine concentrations were fairly uniform but exhibited a daytime peak at ZT7 ($p=0.035$, one-way ANOVA followed by Holm Sidak test); DOPAC levels were markedly higher during the day period, also peaking at ZT7 ($p=0.001$, one-way ANOVA followed by Holm Sidak test).



Supplemental Figure 2: Melatonin increases at night in the mouse retina.

Melatonin concentrations were measured by ELISA in 6 melatonin proficient mouse retinas for each time point. Melatonin levels rose gradually during the day and early night to peak at ZT16, threefold higher than the lowest level at ZT1 ($p = 0.05$, one-way ANOVA, Holm-Sidak test).

MT_1 receptors show a complementary distribution to AA-NAT/HIOMT in *Arvicanthis ansorgei* retina

An important component of melatonin action resides in the presence and function of its receptors MT_1 and MT_2. Although we were unable to perform immunolocalization studies of MT_2, using a specific MT_1 antibody we detected immunoreactivity in rod cell bodies, within selected cells of the INL (especially the innermost row corresponding to the position of amacrine cells) and strong labeling in most cells of the GCL, with no difference across the light/dark cycle **(Figures 6A, B)**. In an opposite pattern to AA-NAT, immunostaining was low in cone cells. Positive and negative controls, respectively *pars tuberalis* and cortex, showed strong immunostaining and absence of label respectively **(Figures 6C, D)**. Control retinas without primary antibody showed no labeling **(Figure 6E)**. Confirmation of MT_1 expression on rod cell bodies **(Figure 6G, H)** was provided by co-localization studies using RET-P3, a specific marker of this compartment **(Figures 6F, H)**. We also studied the cell-specific localization of MT_1 in the GCL: Brn3 **(Figure 6I)** and MT_1 immunostaining **(Figure 6J)** overlapped within many cells **(Figure 6K)**. As for AA-NAT and HIOMT, MT1 was also present in many Brn3-negative cells.

The totality of these data concerning the melatonin synthetic pathway in retina, along with those reported in the literature for other mammalian and non-mammalian species are summarized in **Table 2**.

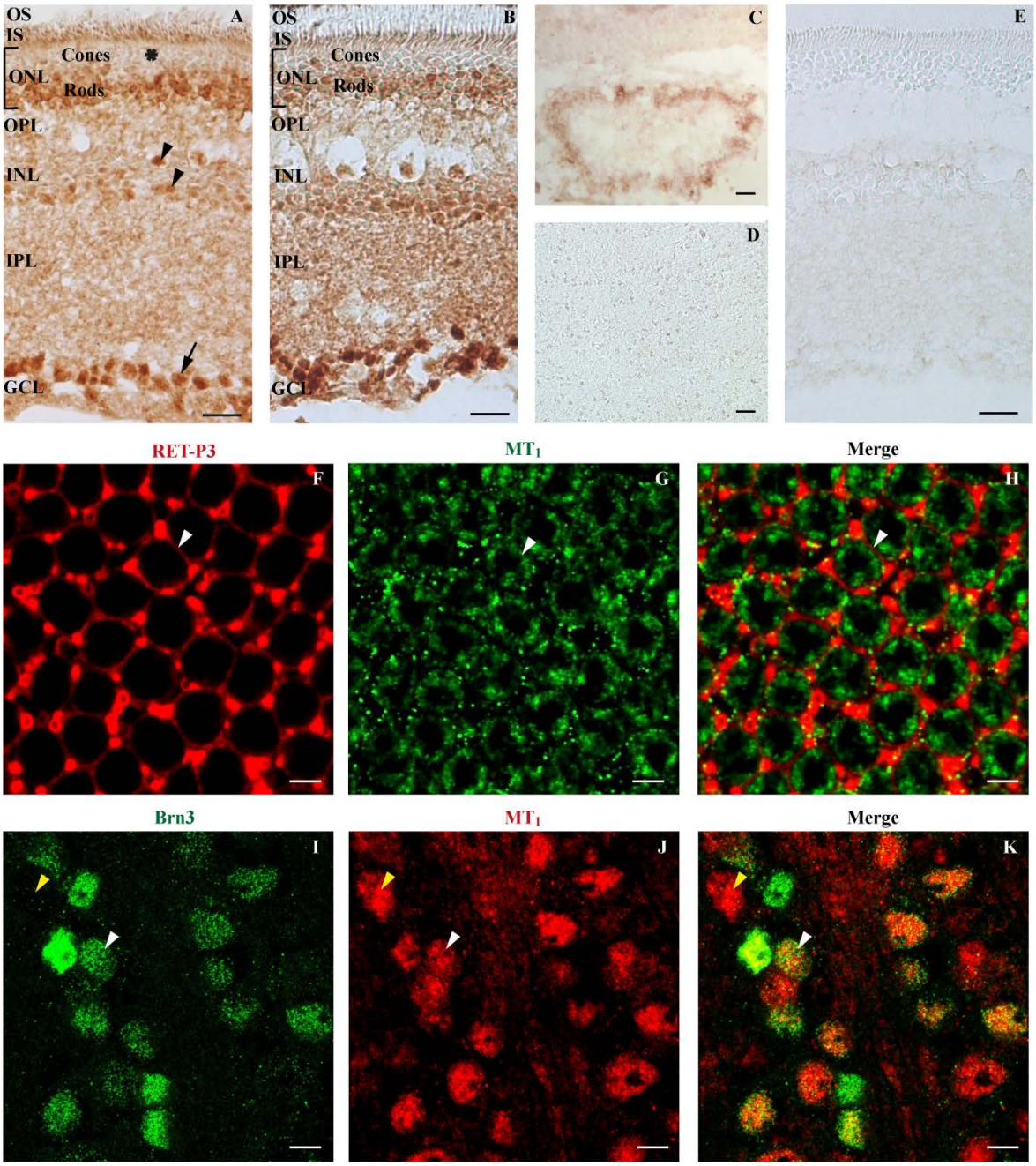


Figure 6: Melatonin receptor MT_1 is expressed in the rods, inner nuclear layer and ganglion cells in the retina of *A. ansorgei*.

Panels A) to E) represent analysis of MT_1 localization using immunoperoxidase techniques. **A)** Arrowheads point to specific MT_1 staining at ZT23 in the INL (upper arrowhead corresponding to horizontal cell; lower arrowhead corresponding to amacrine cell) Arrow points to MT_1 staining in GCL. Strong staining was also seen throughout the lower ONL (rod cell bodies), and more moderately in IS, OPL and IPL. Notably, MT_1 staining was weak in cone cell bodies (asterisk). **B)** MT_1 staining is also present at ZT7 during daytime, with similar distribution and intensity. **Panels C) and D)** are respectively positive and negative controls of MT_1 detection at ZT23 in sections of *pars tuberalis* and cortex of *Arvicanthis ansorgei*. **E)** Control without primary antibody. Scale bar for A-E = 20 µm. **Panels F) to K):** fluorescence immunochemistry of MT_1 expression at ZT23 in whole mounted retinas. **Panels F) to H):** optical section fixed at the level of the lower ONL (rod cell bodies). **F)** Rod cell bodies are outlined by RetP3 binding (staining in red, white arrowhead). **G)** The same field immunostained for MT_1 shows widespread binding (staining in green). **H)** Merged images reveal co-localization of MT_1 and rod cell

body plasma membranes. **Panels I) to K):** optical section fixed at the level of the GCL. **I)** As in Figure 3, RGC are stained with Brn3 antibody, visible within numerous nuclei (green staining). **J)** The same field immunostained for MT_1 shows widespread binding (in red). **K)** Merged images demonstrate co-localization between MT_1 and Brn3-positive RGC (white arrowhead in I-K). There are also Brn3-negative cells stained with MT_1 (yellow arrowhead in I-K) as for AA-NAT and HIOMT analysis. Scale bar for F-K = 5 µm. Abbreviations as in Figure 3.

	Arvicanthis	Rat/Mouse	Monkey	Chicken	Trout	Other
***Aa-nat* mRNA** Qualitative and quantitative expression during 24 h	CCB, CIS +++ at ZT19, GCL ++ at ZT6. In PR, N>D ~4x; in RGC: D>N by ISH. *(60):* N>D ~20x by qPCR.	**Rat:** *(22):* CCB +++ at ZT17, 0 at ZT5. *(23):* ONL +++ at ZT20, + at ZT8; INL, GCL ++ at all ZT. *(21):* N>D ~3x. **Mouse:** *(20):* N>D ~2x.	*(26):* time of day not specified. ONL +++; INL ++; GCL +. N=D.	*(24):* ONL+++ at ZT18, ++ at ZT6; INL and GCL + at all ZT. N>D ~4x. *(25):* PR +++ at ZT22, + at ZT3; GCL +++ at ZT3, 0 at ZT22. Under DD: PR, N>D ~3x; RGC, D>N ~6x.	*(48):* ONL, vitreal-most INL, GCL +++ at night; ONL +, other layers 0 at day. N>D ~5x.	**Human:** *(72):* whole retina +++. **Bovine:** *(73):* whole retina +++. **Ovine:** *(74):* N = D.
AA-NAT protein	CCB, CIS, INL, IPL, GCL +++ at ZT23; CCB, CIS +, INL, IPL, GCL ++ at ZT7. Present at all times except ZT3. N>D ~10x.	nd	*(26):* N>D ~5x.	*(47):* N>D ~4.5x.	*(48):* D>N ~2.5x.	nd
AA-NAT activity	~100 pmol/mg prot/h. Present at all times, small increase at night, ~1.5x.	**Rat:** *(75):* 300 pmol/retina/h. N >D ~6x.	*(26):* ~25000 pmol/mg prot/h. N>D ~5x.	*(50):* ~1000 pmol/mg prot/h in PR; 400 pmol/mg prot/h in RGC. In PR, N>D ~2x; in RGC D>N ~1.5x. *(47):* ~24000 pmol/mg prot/h. N>D ~18x. *(24):* N>D ~8x. *(76):* ~15000 pmol/mg prot/h. N>D ~15x. *(25):* under DD: ~2000 pmol/mg prot/h in PR; ~800 pmol/mg prot/h in RGC. In PR, N>D ~6x; in RGC, D>N ~2x. *(75):* 800 pmol/mg retinal tissue/h. N>D ~8x.	*(48):* ~2800 pmol/mg prot/h. D>N ~4x.	***Xenopus laevis*: *(77):*** ~24000 pmol/mg prot/h. N>D ~6x. **Rabbit:** *(75):* 40 pmol/mg retinal tissue/h. *N>D ~8x.* Mongolian gerbil: *(78):* 7000 pmol/mg protein/h. N>D ~1.8x.
***Hiomt* mRNA**		**Rat:** *(data not show):* CCB, CIS, GCL +, INL+/- at night; CCB 0, GCL +, INL+/- at day. *(28):* +, N=D.	*(26):* +/-, N=D.	*(54):* IS, ONL, OPL.	*(48):* ONL +, vitreal-most INL, GCL +/-. N=D.	**Human:** *(79):* +/- *(80):* +

	Arvicanthis	Rat/Mouse	Monkey	Chicken	Trout	Other
HIOMT protein	CIS, OPL, IPL, GCL +. N=D.	**Rat:** (53): ONL, IS +++; INL, GCL +.	nd	*(30):* ONL +++, GCL ++.	nd	**Bovine:** *(53):* ONL, IPL +/- (51): 0 **Lizard:** *(53):* CCB, CIS. Human: *(53):* ONL, IS. *(79):* 0
HIOMT activity	~2 pmol/mg prot/h. N=D.	**Rat:** *(28):* ~20 pmol/mg prot/h. N=D. *(79):* ~200 pmol/mg prot/h. *(75):* 20 pmol/retina/h. N=D.	nd	*(75):* ~20 pmol/mg retinal tissue/h. N=D.	nd	**Human:** *(79):* 0 **Bovine:** *(79):* 0 **Rabbit:** *(75):* 5 pmol/mg retinal tissue/h. N=D.
Melatonin	~18 pg/mg prot. N=D.	**Mouse:** ~60 pg/mg prot. N>D ~3x. **Rat:** *(81):* ONL, INL. N>D ~4x. *(61):* ~150 pg/retina. N>D ~2x. *(62):* ~1000 pg/retina. N>D ~5x. *(82):* 60 pg/retina. N>D ~2x.	nd	**Chick:** *(76):* ~1 ng/mg prot. N>D ~4x. *(63):* ~1000 pg/retina. N>D ~4x. *(25):* under DD: ~40000 pg/mg prot in PR; ~4000 pg/mg prot in RGC; +/- in INL. N>D in PR ~13x; D>N in RGC ~7x. *(45):* under DD: ~1000 pg/mg prot in RGC. D>N ~5x.	**Sea bass:** (83): D>N or N=D.	**Golden Hamster:** *(84):* D>N. **Bovine:** *(85):* 3000 pg/retina.
Dopamine	~10 pg/µL. D>N ~2x.	**Rat:** *(86):* ~200 pg/mg wet weight. D>N ~2x. *(87):* ~3000 pg/mg prot. D>N ~1.5x. *(88):* ~30 pmol/retina. D>N ~1.5x. **MouseBALB/c:** *(89):* ~700 pg/retina. D=N.	*(90):* ~6 pmol/10 min/retina. D>N ~2x.	*(68):* ~2000 pg/mg prot. D>N ~2x.	**Cichlid fish:** *(91):* ~2000 pg/retina. D>N ~1.5x.	**Rabbit:** *(92):*~300 pg/mg wet tissue. D>N ~1.5x. **Human:** *(93):* ~3000 pg/mg prot. D>N ~2.5x.

Table 2: Species comparison of the melatonin synthetic pathway within the retina.

Compilation of published data and those from the present study underline variations in the melatonin pathway of most studied vertebrates. We have indicated the relative change in gene or protein expression between night and day using italic. Activity values were all expressed in pmol/h/mg prot to allow comparison between species. Abbreviations as in figure 3. Additional abbreviations: CCB: cone cell body; CIS: cone inner segment; DD: dark/dark; D: day values; N: night values; n.d: not determined; PR: photoreceptors. Staining intensity is represented as follows: +++: strong; ++: moderate; +: weak; +/-: very weak; 0: absent.

Discussion

Vertebrate retina has long been known to synthesize melatonin thought to modulate various physiological processes within the eye. However, knowledge of the melatonin synthetic pathway has never been examined in a systematic fashion, is fragmentary in mammalian retina, and limited principally to nocturnal species (mouse and rat). Published studies have focused on the penultimate enzyme of melatonin synthesis, AA-NAT, whereas expression and activity of the final enzyme in the pathway, HIOMT, are still poorly understood. In the work reported here we performed systematic localization and expression of multiple components of the melatonin synthetic pathway in a diurnal rodent *Arvicanthis ansorgei*, arguably a closer model to human central cone-rich retina. In stark contrast to nocturnal rodent retina, *Aa-nat* mRNA expression showed differential phase expression in distinct cell populations, and AA-NAT was abundantly expressed throughout the 24 h light/dark cycle, although actual melatonin levels were low at all times.

Differential expression of AA-NAT, HIOMT and MT_1 suggests distinct roles in Arvicanthis ansorgei retina

Within the photoreceptors, *Aa-nat* expression was abundantly detected in cones exclusively during the night, whereas levels were below the detection limit in rods, consistent with data from rat retina *(22)* **(Supplemental Figure 1)**, as well as the other cell types in the retina. In contrast, *Aa-nat* expression appeared in RGC during the day, in phase opposition to that of cones. To the best of our knowledge, this is the first description of two phase-opposed sites of *Aa-nat* transcription in mammals, similar to the situation in chicken retina *(45)* **(Table 1)**. Immunodetection of AA-NAT has proved very problematic in previous studies, possibly because AA-NAT is strongly associated with the protein chaperone 14-3-3 *(46)* leading to inaccessibility of epitopes to antibodies directed against AA-NAT. We initially compared multiple immunohistochemical procedures, and optimal results for frozen sections were achieved using antigen retrieval techniques followed by immunoperoxidase protocols. To the best of our knowledge this is the first demonstration of AA-NAT protein localization in the retina, where it was clearly found in cones, many cells within the INL and in the majority of neurons within the GCL. Contrary to mRNA expression, protein was also highly expressed during the day, under both normal cyclic and constant darkness conditions (except for ZT3/CT3). To explain this profile, we propose that AA-NAT detected at night derives from cones, and is under strict light/circadian control similar to the scheme hypothesized by Tosini, Iuvone and colleagues. Previous studies in rat retina proposed that light decreases Ca^{2+} and cAMP levels leading to activation of proteasome proteolysis *(19)*. Studies in chicken demonstrated that during the day AA-NAT phosphorylation by PKA is decreased, leading to dissociation from the chaperone 14-3-3 and subsequent degradation by the proteasome *(46,47)*. AA-NAT detected during the day comes from the GCL, and constitutes an independent light-insensitive pool. Similar hypotheses have been advanced for trout retinas *(48)*, where AA-NAT expression is also detected during the day. This model is depicted in **Figure 7**. AA-NAT in the GCL co-localized with both Brn3-positive and -negative neurons, indicating that both RGC and possibly displaced amacrine cells [which constitute ~40% of neurons in the GCL *(33)* and are Brn3-immunonegative] synthesize AA-NAT. One previous study in rats employing a fluorescence-based *in situ* hybridization approach showed widespread distribution of *Aa-nat* mRNA throughout the retina *(23)*. Also, widespread *Aa-nat* distribution was seen in the macaque monkey retina *(26)*, and in cones and GCL of chicken retina *(49,50)* **(Table 2)**.

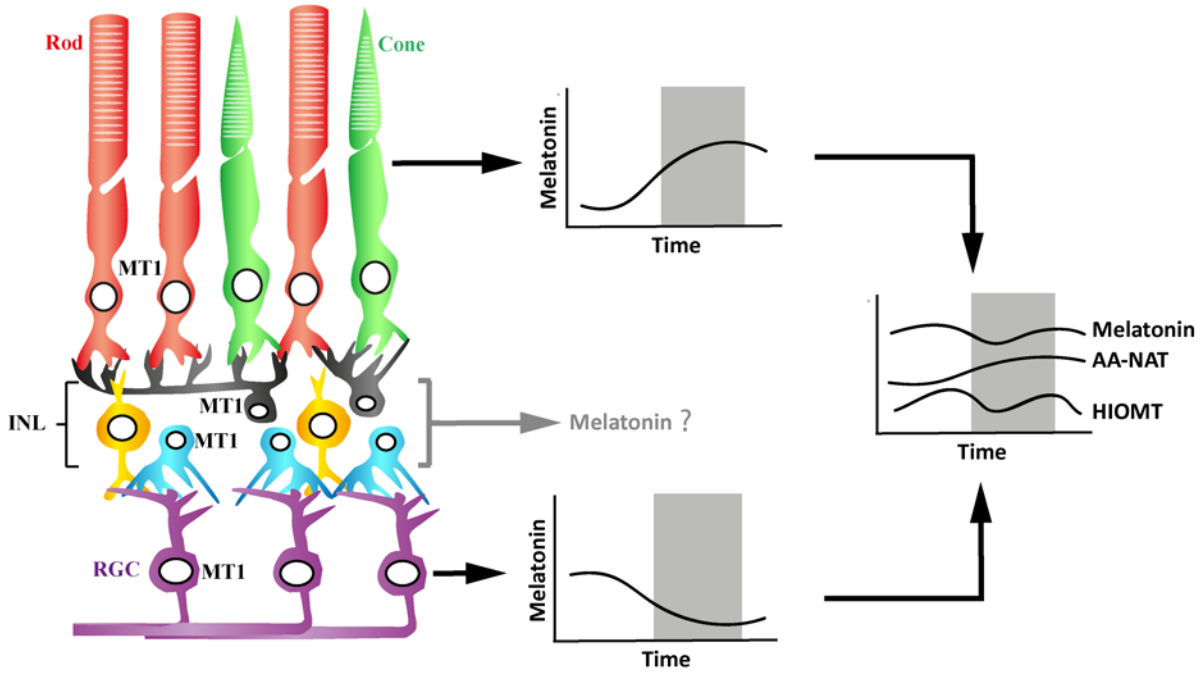


Figure 7: Model of melatonin synthesis in *Arvicanthis ansorgei* retina.

Middle upper scheme: melatonin is produced by cones at night. Lower middle diagram: melatonin is produced by RGCs during the day. Constitutive melatonin level in the retina may reflect the cumulative PRs and RGCs melatonin. Right scheme: similarly, AA-NAT expression throughout the 24 hours could result from integration of PR nocturnal AA-NAT and RGC daytime AA-NAT productions. The same hypothesis can be proposed for HIOMT.

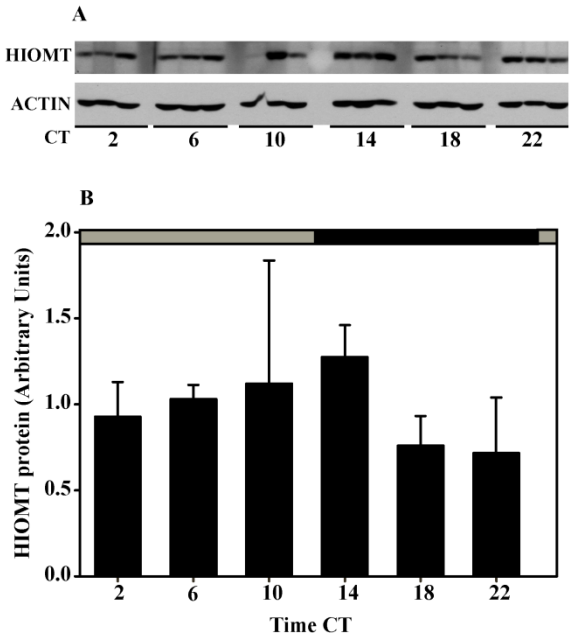
HIOMT represents the ultimate step in melatonin production and is therefore crucial for understanding its synthetic pathway. Previous studies in rat retina have revealed very faint expression of *Hiomt* *(28)*, and it was virtually undetectable in bovine *(51)* and macaque retinas *(26)*. Although we were not successful in obtaining reliable *in situ* hybridization images, HIOMT immunoreactivity was detected in photoreceptor IS, the two synaptic layers and GCL. *In situ* hybridization studies have been hampered by the lack of specific probes due to the highly divergent *Hiomt* sequences as demonstrated between mouse strains *(52)*. Previous studies showed HIOMT immunoreactivity in IS of several mammalian [rat, bovine and human *(53)*] and non mammalian species [chicken *(30,54)* and lizard *(53)*] **(Table 2)**. Hence in *Arvicanthis*, HIOMT was expressed in two of the same compartments as AA-NAT, mainly cone IS and RGC. It has been stated that co-expression of these two enzymes leads to production of melatonin *(55)*, implicating cones and GCL as major foyers of melatonin synthesis in *Arvicanthis* retina.

MT_1 receptor distribution was complementary to that of the supposed sites of melatonin synthesis: within the ONL, MT_1 expression was high in rod cell bodies; within the INL it was limited to scattered cells lying along the vitreal-most border, possibly a sub-population of amacrine cells; and within the GCL it was strongly expressed by many Brn3 positive RGC. Receptor expression was below detection limits in cones, indicating that in this species cones might release melatonin which targets uniquely rods. A recent study proposes that melatonin increases light sensitivity in the retina of mice via MT_1/MT_2 heteromer, suggesting that melatonin could be involved in dark adaptation *(14)*. A

physiological role for melatonin is further supported by rod and RGC loss in MT1-deficient mice *(11)*, although it is not known if such effects occur in *Arvicanthis*.

AA-NAT and HIOMT expression indicates constitutive production of melatonin.

It is generally accepted in pineal gland and retina that AA-NAT is under strict circadian and light control resulting in highly predominant nocturnal activity *(56–58)*. *Aa-nat* transcripts display a marked nocturnal maximum in *Arvicanthis ansorgei* cones, consistent with the known regulation of *Aa-nat* in the pineal gland of rat *(59)* and retina of nocturnal rodents *(20,21)* primates *(26)* **(Table 2)**, as well as in the pineal gland of *Avicanthis ansorgei* itself *(57)*. Furthermore using quantitative PCR we previously demonstrated cyclic expression profiles of *Aa-nat* in the retina of *Arvicanthis ansorgei* maintained under different lighting regimens *(60)*. It is important to note that the day-to-night ratio in the retina is much lower than in the pineal gland, around fourfold as previously shown for rat, mouse *(20,21)*, chicken *(24)* and primates *(26)* **(Table 2)**.We also performed western blotting analyses of AA-NAT expression across the 24 h. Although there was much individual variation, the overall pattern displayed a nocturnal maximum but with sustained expression throughout most of the day. Coon et al. *(26)* showed a nocturnal peak of AA-NAT levels in monkey retina and Iuvone et al. *(47)* reported AA-NAT protein expression across 24 h in the chicken retina with a peak at night **(Table 2)**. Our analysis of HIOMT protein expression during 24 h was performed in DD condition and displays a constitutive pattern **(Supplemental Figure 3)**. This result is coherent with the HIOMT activity **(Figure 5A)** performed in LD condition.



Supplemental Figure 3.

A) Western blotting analysis of HIOMT expression in retinas from 3 different animals per time point reveals the presence of HIOMT protein throughout the 24 h. **B)** Densitometric quantification of HIOMT (n=3 per time point) confirms the presence of HIOMT over the 24 h ($p > 0.05$, one-way ANOVA). White, black and grey bars represent day time, night-time, and the subjective night respectively. **C)** HIOMT peptide-antibody absorption control displays a reduced staining of *Arvicanthis* retina. Scale bar = 20 µm.

Different regulation and functions of the melatonin synthetic pathway in a diurnal rodent retina compared to other species

Taking together our observations and those reported in the literature, *Arvicanthis* shows similarities and differences to all described tissues. Some important data are missing, but within the Rodentia there are clear distinctions between nocturnal and diurnal species **(Table 2)**. Although only very low uniform levels of *Hiomt* have been reported in rat retina *(28)*, levels of melatonin are relatively high at night in both the rat *(61,62)* and mouse **(present study, Supplemental Figure 2)**. If cones are to be

considered a major source of retinal melatonin, as is known for chickens *(25,46)* and suggested by the strong expression of *Aa-nat* mRNA and AA-NAT protein in both nocturnal *(22)* and diurnal **(present study)** rodents, then *Arvicanthis* should exhibit relatively high values compared to the mouse. The contrary is true, and approximation to cone numbers indicates *Arvicanthis* has actually fifty-fold less melatonin than mouse retina **(Table 2)**. Although this study does not address the question directly, one possibility is that melatonin is exported towards the bloodstream. Given the level of melatonin production in *Arvicanthis* retina (10-15 pg/mg protein across the entire day/night cycle) compared to the pineal gland [estimates from published data give peak night values of 530 pg/mg protein, day values of 25 pg/mg protein: *(57)*], any retinal contribution to circulating melatonin would be minor, at least at night. Pinealectomy reduces the nocturnal plasma melatonin value by 80% in chicks, and bilateral enucleation further reduces the value by another 9% *(63)*. These results suggest that 9% of plasma melatonin originates from the eyes, hence retina which is the major melatonin production site in this organ. Combined with the widespread distribution of MT_1 receptors, these findings suggest that melatonin is synthesized in the retina principally for local purposes *(64)*.

As stated above, *Arvicanthis* pineal melatonin is strongly regulated in a cyclic manner, with the nocturnal peak being ~100 fold daytime levels *(57)*. The uniformly low values for retinal melatonin across the 24 h period indicate that circulating melatonin does not enter the retina, contrary to observations in the mouse *(65)*. It has been suggested that in primate retinas, in which HIOMT levels are also extremely low, that rather than synthesizing melatonin, AA-NAT plays a role in detoxification by acetylating arylalkylamines and preventing them depleting the photoreceptors of retinaldehyde *(58)*. Adding our immunolocalization data to this hypothesis suggests such reactions occur specifically in cones and that they are somehow preferentially vulnerable to depletion compared to rods. Primate retinas show large differences in day versus night levels of AA-NAT protein and activity *(26)*; this was not the case for *Arvicanthis*, which although showing AA-NAT predominantly at night also exhibits robust expression in the latter half of the day, whereas activity was unchanged across the 24 h period with a low increase at night **(Table 2)**. There is thus only weak correlation between AA-NAT expression levels and AA-NAT activity in *Arvicanthis*. It is noteworthy that AA-NAT actually generates NAS, which has been shown recently to exhibit pronounced intrinsic biological activity in the mouse. Notably, it binds to trkB receptors with high affinity, and can act as a ligand to trkB and thereby exert neuroprotective effects *(66,67)*. However, NAS was undetectable in *Arvicanthis* retina, at any time of day or night, even when extracts were prepared from pooled retinas (data not shown). So this does not seem to be a viable explanation for the role of AA-NAT in this species. In *Arvicanthis* retina, melatonin and dopamine production is not clearly opposed on a day/night basis **(Figure 5)**. These results differ from the previously described interplay between both compounds where dopamine inhibits AA-NAT activity, and melatonin in turn inhibits dopamine release, forming a mutual inhibition loop in retina of chicken and mammals *(68–70)*.

To summarize, although AA-NAT and HIOMT can be observed in *Arvicanthis* retina across most of the 24 h period, enzyme activity levels are actually low and melatonin itself is synthesized at correspondingly low levels. AA-NAT within photoreceptors (principally cones) may fulfill a detoxifying function, or even exert some neuroprotective effect. This is intriguing given that the retinas in this species are highly resistant to intense light damage *(71)*, and may represent an adaptation to exposure to potentially damaging light levels. Nevertheless the presence of MT_1 receptors indicates the low melatonin amounts are still functional and melatonin action may differ in photoreceptors

compared to inner retinal neurons: *Arvicanthis* exhibits a diurnal or bi-modal activity pattern *(31)* and is hence active in variable light intensities. Thus, melatonin may modulate the responsiveness of rod photoreceptors under dim lighting (dark adaptation).We also demonstrate that RGC contain all the machinery necessary to produce melatonin and respond to it. Published studies support a role for melatonin signaling through high affinity receptors in the survival of this cell-type *(11)*, and parallel studies demonstrate that MT_1 activation is necessary for cone survival during aging *(Gianesini et al., manuscript in preparation)*. In conclusion, our data describe a unique melatonin pathway in the retina of *Arvicanthis,* indicating distinct physiological adaptation of diurnal versus nocturnal rodents, and highlights the fine tuning of melatonin regulation in each layer of the retina.

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V. Melatonin synthetic pathway: Additional discussion

In *Arvicanthis ansorgei* retina, melatonin synthetic pathway reveals differences and common features with the pineal gland melatonin source which can be summarized as follows**. In the outer nuclear layer** and especially cones (see section IV.A.I), *Aa-nat*/AA-NAT and *Hiomt*/HIOMT expressions and activities are light and circadian clock driven (Gianesini et al 2015). All the studied parameters echo the well known pineal pathway. Only levels of enzymes and melatonin expression are much lower than those of the pineal gland (see article above and for review in nocturnal rodent: (Tosini et al 2012). This aspect can be discussed in the IV.A.2.Enzymes paragraph and the melatonin role in IV.B.4.paragraph below.

In the ganglion cell layer, we found intriguing differences in the melatonin synthetic pathway: *Aa-nat*/AA-NAT and *hiomt/*HIOMT are lowly expressed, apparently loosely regulated, e.g. no significant circadian variations. Melatonin synthetic pathway can be discussed in the light of the retinal clockwork (see section IV.B.3). The enzyme activities are positively correlated to the protein expressions. Most importantly, active enzymes are present throughout the 24 hours. The regulation appears light independent (see section IV.B below). Finally, the resulting retinal melatonin profile is a low expression (see ELISA with *Arvicanthis* and mouse samples) present throughout the day. It is the first time that the second source of melatonin in a mammalian organism is dissected at the cell level. We can apprehend the importance of the different retinal cell-type and layers physiology (see IV.A.I) and ask whether it is relevant for its potential roles (see section IV.B below). We must also ask the question of whether the retinal melatonin synthetic pathway involved is specific of a diurnal species and more generally part of the diurnal switch (see sections IV.B6 and IV.B.7). We have also assessed retinal DA and DOPAC which display a circadian variation with a maximum during the day. This data follows the pattern observed in the retina of nocturnal rodents (McMahon et al 2014). The melatonin-dopamine cross-talk appears also present in *Arvicanthis ansorgei* retina see section IV.B.5.

V.A.1. Which retinal cell types are involved in melatonin synthesis?

In *Arvicanthis ansorgei* retina, we have localized enzymes (transcripts and proteins) of the melatonin synthetic pathway in cones (not the rods) and in the ganglion cell layer (data in the article above). Enzymes are also express at low levels in the INL as described in other species such as the rat (Liu et al. 2004) and monkey (Coon et al. 2002) but we did not investigate further this aspect.

V.A.1.a Cones:

These data are the first to localize melatonin synthesis at the cellular level. This result is consistent with the previous study by Niki et al in the rat retina (Niki et al. 1998) but our model was particularly relevant due to the abundance of cones and their precise alignment in the ONL. The regulation of AA-NAT and HIOMT in the cones appears similar to what has been described before in other rodent retinas (Tosini et al 2012). We observe high expression of transcripts, proteins (at least AA-NAT) and enzyme activity at night. However, all the machinery is toned down compared to the pineal gland which is the dedicated melatonin factory (reviewed in table 2 (Gianesini et al 2015)). The Low level of cone melatonin production compared to the pineal level might be related to its specific

autocrine/paracrine role in the retina (see paragraph below). Note that we have detected melatonin receptors type 1 in the photoreceptor population and rather in the rods (see article above). In any case, the general regulation is reminiscent of the pineal melatonin pathway.

One explanation may come from studies by J. Arien Kappers, Richard M. Eakin, Jean-Pierre Collin and David Klein have established the common cellular origin of pineal and retinal photoreceptors (Klein 2004). The genetic similarity has then been described. Notably; the melatonin synthetic pathway was depicted in the retina, albeit questioned in the mammalian retina, and at the opposite, molecules of the phototransduction cascade were isolated in the pineal gland (Klein 2004). Nevertheless the thorough study of AA-NAT evolution points out that vertebrate AA-NAT may be the result of a 500 Myears molecule evolution succeeding the evolution of eye and retina. This event would possibly include a horizontal gene transfer from another phylum to the vertebrate one (Falcon et al 2014; Klein 2004) and a strong function-driven evolution to switch from amine detoxification to melatonin production (Falcon et al 2014). Overall, the literature and our study support the photoreceptors as a source of melatonin, which first represents the signal of darkness. Melatonin role (s) will be further discussed below.

Perspectives:

The evident experiment we should perform on *Arvicanthis ansorgei* retinas is a colocalization between a cone-specific marker (cone phosducine) AA-NAT and HIOMT to give a definite proof of the photoreceptor type involved in melatonin synthesis. The only obstacle to that work is to find cross-reacting (for *Arvicanthis ansorgei*) antibodies (cone marker and AA-NAT) obtained from different species so that the secondary antibody step can be achieved.

V.A.1.b Ganglion cell layer : Daytime player

The second melatonin source within *Arvicanthis ansorgei* retina is in the GCL: Although the contribution to total retinal melatonin appears to be modest, it could be acting locally to regulate the physiology of the inner retinal circuitries during the light period (Valdez et al. 2012).

First, melatonin can control the daily rhythms of scotopic and photopic ERG in C3H/$f_{+/+}$ mice (melatonin proficient mice without genetic-dependent early retinal degeneration) whereas the circadian regulation of ERG is abolished in C3H/$MT_2^{-/-}$ and C3H/$MT_1^{-/-}$ (Baba et al 2009, 2013). Mice lacking melanopsin *(Opn4$^{-/-}$)* exhibit diurnal variation in the cone ERG, ie amplitude and implicit time (time to peak) circadian rhythm. However, the magnitude of these diurnal differences was reduced compared to WT mice. These events inhibit the effect of optimizing photopic vision at a time of day when it is likely to be employed (Barnard et al. 2006). **These results suggest that ipRGCs have important function in optimizing classical visual pathways according to the time of the day** (Barnard et al. 2006). Authors propose that ipRGCs provide photoentrainment of retinal oscillators and that the loss of melanopsin may result in de-synchronization of cellular clocks and abolition of coherent circadian signaling within the retina. **We can speculate that RGCs daytime melatonin, as an output of the clock, could act as a time-giver and help synchronizing retinal signaling.**

To further sustain this hypothesis, *Arvicanthis ansorgei Opn4* transcript expression (analyzed under different light conditions, Figure 38) peaks at dawn (night/day transition, see green arrow) in LD conditions (12:12) while the peak is shifted to early night (CT15) in LL (constant light). In constant

darkness (DD), rhythmic expression of *Opn4* is lost (Bobu et al 2013). Therefore, *Opn4* is controlled by light and a circadian clock. If GCL daytime melatonin acts as an output of the clock it could synchronize *Opn4* gene expression to participate in the retinal signaling. Indeed *Aa-nat* transcript peaks a few hours before *Opn4* in LD conditions, even though it is the total retinal pool of *Aa-nat* (see red arrow, Figure 38).

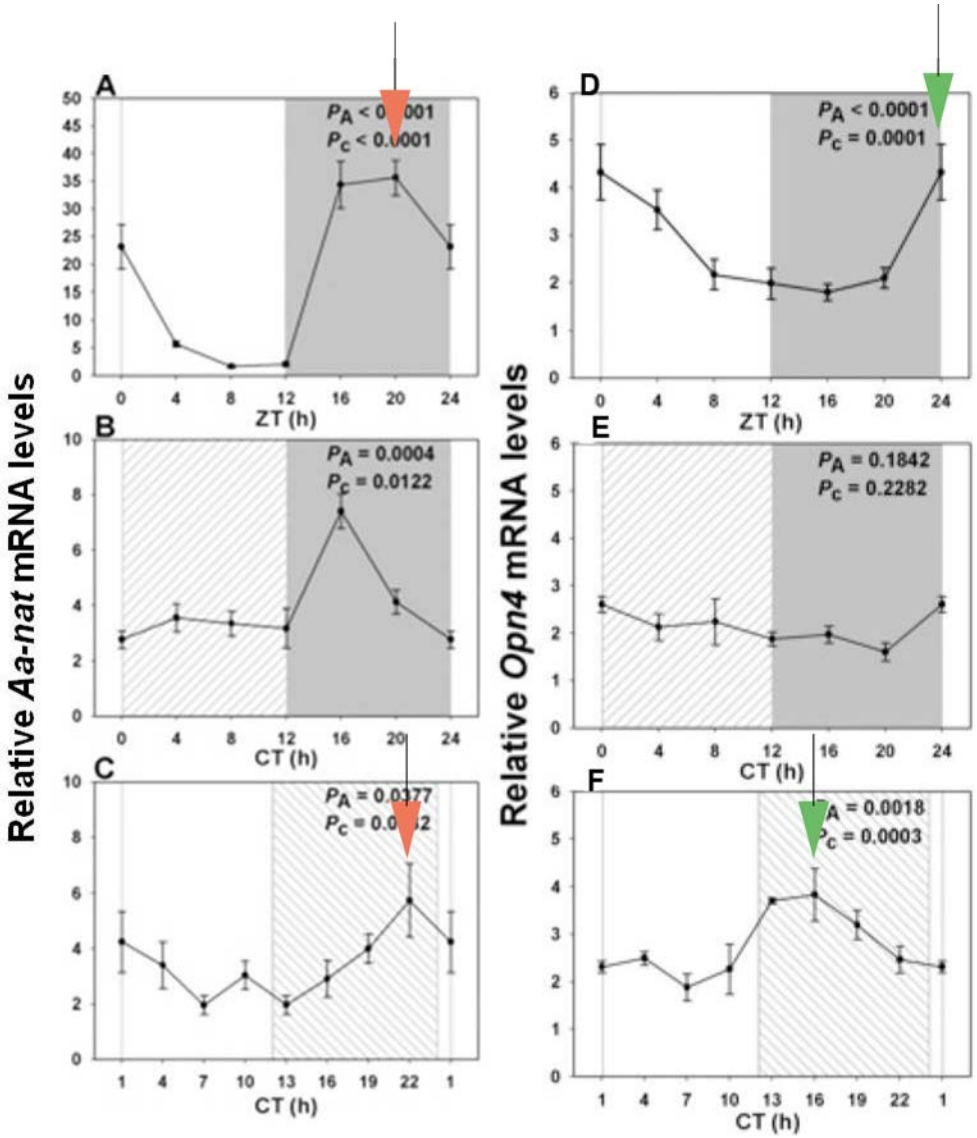


Figure 38: *Aa-nat* and *Opn4* mRNA expression over 24h under different lighting conditions in the retina of *Arvicanthis ansorgei*.

A-C *Aa-nat* expression level: **A.** In a 12 h light: 12h dark cycle (LD) there is a strongly rhythmic pattern with maximal expression at ZT20, and a nadir 12 h later (n=3-6 per time point). **B:** In constant dark (DD) the profile is attenuated, with a peak at CT16 (n=5-6 per time point). **C:** In constant light (LL) there is only a small phase shift, such that peak values are at CT22 (n=4 per time point). **D:** In a 12 h light: 12 h dark cycle (LD) there is a rhythmic pattern with maximal expression close to "dawn" (night/day transition), and a nadir 12 h later (n=3–6 per time point). **E:** Melanopsin expression profile is attenuated in constant dark (DD) and does not attain significance (n=6 per time point). **F:** However, in constant light (LL) there is again a large phase shift, such that peak values now occurs in early night (CT15; n=4 per time point). Illumination conditions are depicted as solid white (day) and solid grey (night) areas in LD, right hatched (subjective day) and solid grey (subjective night)

areas in constant dark (DD) and solid white (subjective day) and left hatched (subjective night) areas in constant light (LL). Adapted from (Bobu et al 2013).

Perspectives:

To address this question of melatonin and melanopsin cooperation, we have two ways. First ex vivo, we can keep retina explants in culture and apply melatonin at different times of day/night and measure neuron electric activities by MEA. The expected result would be a change in the electric activity of the ganglion cell layer following daytime melatonin injection. The difficulty resides in the identification of the ipRGCs involved and the supra-physiological effect of melatonin here. We will base our work on the previously identified ipRGCs and their specific activity signature such as the one of type I ipRGCs in the retina of *Arvicanthis ansorgei* (see figure 6 (Karnas et al. 2013).

Secondly, we can separate the different layers of the retina in order to analyze the molecules of one cell-type or at least from one isolated layer. Such work has been initiated by our co-workers and recently published for rat and mouse retina clock (Jaeger et al. 2015). This method includes the separation of the different layers by vibratome. Then, we analyze each sample for a specific cell-type enrichment with cell markers by PCR (cone photopigment, tyrosine hydroxylase for amacrine cells, rhodopsin, melanopsin) or Western-blotting. We can adapt this setting for *Arvicanthis ansorgei* retina. The second option is to do laser microdissection to try to reach the same result although with a better resolution but less material. Then, we can better define melatonin synthetic pathway parameters in relation with melanopsin expression and synchronization of circadian signaling within the ganglion cell layer or the retina (gap junction regulation for instance see (Völgyi et al. 2013)).

V.A.2. AA-NAT and HIOMT in diurnal and nocturnal models: Two enzymes, two regulations

V.A.2.a AA-NAT and 14-3-3 protein:

As stated in the III.B.1.d paragraph above, vertebrate AA-NAT protein and activity are regulated via binding to 14-3-3 proteins. Indeed, during the night, chicken retinal AA-NAT is phosphorylated and forms a complex with the 14-3-3 protein, leading to a protection of AA-NAT from degradation (Pozdeyev et al. 2006). To test whether this regulation also occurs in *Arvicanthis ansorgei* retina and during the day (which is different to what is known so far), we studied the expression of the phosphorylated (P) form of AA-NAT across the 24 hours.

We performed western blotting analyses of *Arvicanthis* retinas collected every 4 hours across the 24 hours period to analyze P-AA-NAT protein (Figure 39 A). We detected an immunoreactive band at the expected size of 26 kDa corresponding to AA-NAT. There was considerable variation in immunoreactive intensity within sample groups (even though animals were raised under identical conditions) (Figure 39 B). We observed P-AA-NAT expression over the 24 hours rising at ZT11 but reaching a maximum during the night (at ZT23) (Figure 39 B). The unphosphorylated (UnP) form of AA-NAT was also detected by western blotting analyses (Figure 39 C). UnP-AA-NAT was present over the 24 hours with a peak at ZT15, a slight decrease at ZT23 and a minimum at ZT11 (Figure 39 D). Our results display a maximum level of AA-NAT expression (P and UnP) at night but expression persists throughout daytime (Gianesini et al 2015). The 14-3-3 protein was detected over the 24 hours in the retina of *Arvicanthis ansorgei* (Figure 39 E) and exhibited the same profile as the one of P-AA-NAT

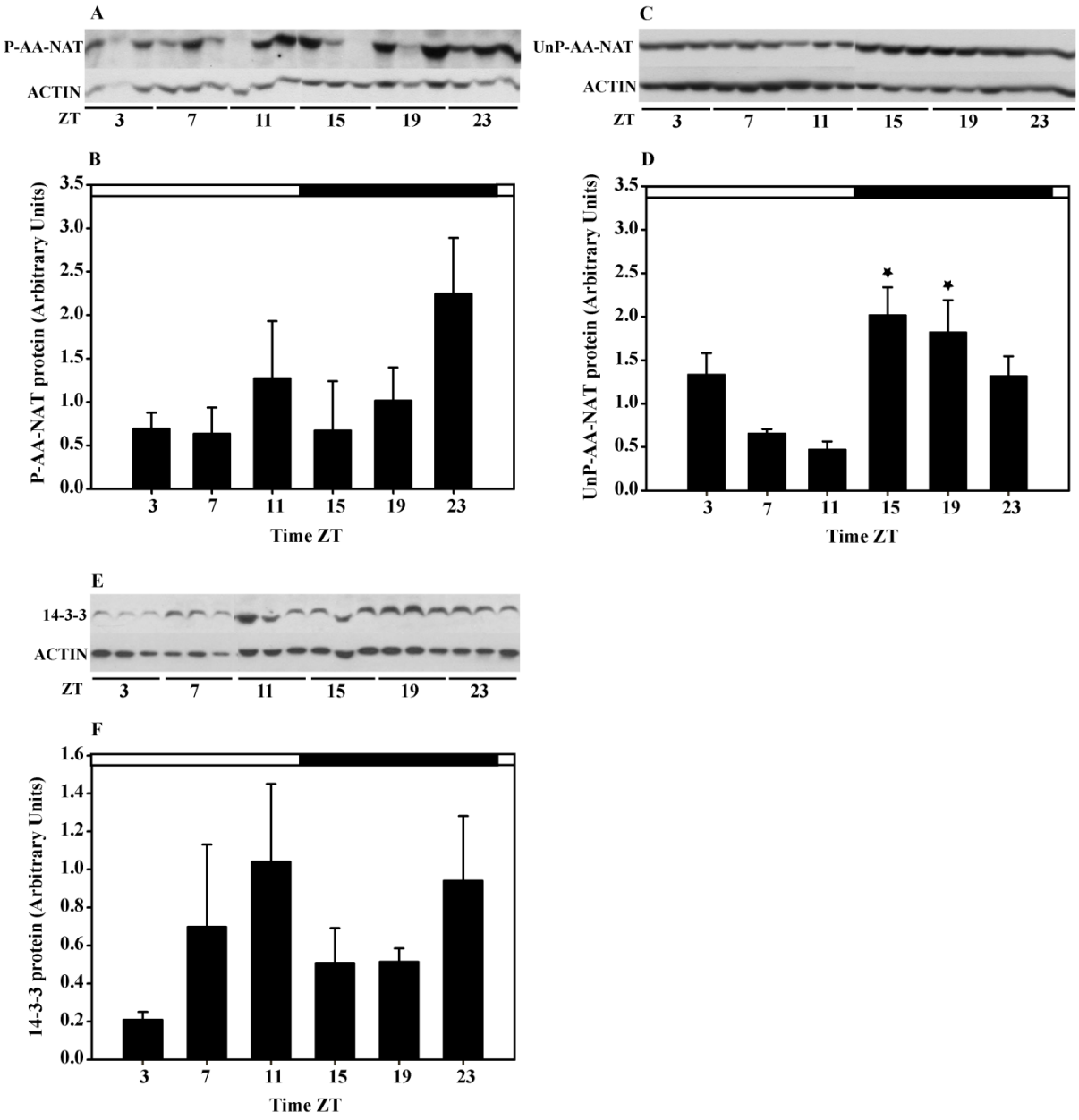
(Figure 39 F). These results suggest that 14-3-3 is present in the retina at the same time as P-AA-NAT allowing a possible interaction between both proteins.

Perspectives:

Interestingly, 14-3-3 profile is the lowest at ZT3, the time point when P-AA-NAT is almost absent from the studied retinas (LD and DD conditions), further supporting the role of 14-3-3 in stabilizing AA-NAT protein at the other time points. The relationship between 14-3-3 and AA-NAT activity is less clear in *Arvicanthis ansorgei* retina because the activity is constant throughout the day Fgure 3C in (Gianesini et al 2015). The necessity of analyzing the AA-NAT/14-3-3 independently in each layer (photoreceptors and GCL) is obvious. Furthermore, a formal proof of the interaction between AA-NAT and 14-3-3 could be obtained by co-immunoprecipitation or by **proximity ligation assay (PLA)** (Söderberg et al. 2006).

Figure 39: Phosphorylated AA-NAT and 14-3-3 expression exhibit the same profile.

A) Western blotting analysis of phosphorylated (P)-AA-NAT expression (antibody anti AA-NAT 3352, gift from David Klein) in retinas from 3 different animals per time point reveals the presence of P-AA-NAT protein throughout the 24 hours. Data indicate that AA-NAT expression across 24 hours is overall higher at the end of the night. It is also evident that there was great variability between individuals within sampling time. **B)** Densitometric quantification of P-AA-NAT in western blots (n = 3) confirms a constitutive expression with modest increases at the end of the night (ZT23) and day (ZT11) ($p > 0.05$, one-way ANOVA). **C)** Western blotting analysis of unphosphorylated (UnP)-AA-NAT (antibody anti AA-NAT 3305) expression in retinas from 3 different animals per time point. UnP-AA-NAT is present over the 24 hours. **D)** Densitometric quantification of UnP-AA-NAT in western blots (n = 3) underlines a peak at the beginning of the dark period ($p = 0.005$, one-way ANOVA, Holm-Sidak test). **E)** Western blotting analysis of 14-3-3 expression in retinas from 1 animal per time point (same sample repeated 3 times for each ZT) reveals the presence of the protein 14-3-3 throughout the 24 hours. **F)** Densitometric quantification of 14-3-3 in western blots (n = 3) confirms a constitutive expression with slight increases at the end of the night (ZT23) and day (ZT11) ($p > 0.05$, one-way ANOVA). Data are the mean values ± SEM of 3 animals analyzed in 3 independent experiments or per time point. Black bars represent night-time; light bars represent day-time.



V.A.2.b AA-NAT and HIOMT in DD conditions:

To discriminate between light and circadian control of AA-NAT and HIOMT (Figure S3 in (Gianesini et al 2015)), we performed AA-NAT and HIOMT western blotting analyses across the 24 hours period with retinas from animals raised 4 days in DD. We observed an intense immunoreactive band at the expected molecular weight of AA-NAT and HIOMT. However the similarity stops here. On one hand, AA-NAT expression reaches a peak at CT7 and slightly decreases over the subjective night. LD AA-NAT expression (Figure 3A in (Gianesini et al 2015)) does not exhibit the peak at CT7 suggesting that in LD conditions, the light inhibits the peak of AA-NAT in the middle of the day. These results suggest that AA-NAT may be degraded during the day only in LD conditions. On the other hand, HIOMT is present throughout the 24 hours apparently not regulated by the light. This is a preliminary result and cannot

be interpreted as long as we do not have the HIOMT LD profile. This is the first experiment to complete now that we have two different anti-HIOMT antibodies and optimal technical conditions. We should also repeat the experiment with a minimum of 5 animals per time point for statistical purposes as inter-individual variations in HIOMT and AA-NAT expression is great.

Overall, these observations are quite logical as mammal AA-NAT half-life is of a few minutes whereas the HIOMT half life is very stable and goes over 24 hours (Gauer and Craft 1996). AA-NAT is then quickly down-regulated by light masking the circadian control while HIOMT protein escapes light inhibition. **To sum up, the regulation of AA-NAT and HIOMT can be (the other regulation is at the transcriptional level) post-translational although their circadian variations are not strong or even arguable and their dual role is not so pronounced in *Arvicanthis ansorgei* retina.** Indeed, AA-NAT is present throughout the 24 hours and is de facto less the rate limiting enzyme of rhythmic melatonin synthesis during the day whereas the HIOMT, the usual rate limiting enzyme of night melatonin appears rather constitutive, so much less the night melatonin limiting enzyme.

Perspectives:

The classical model is not robust in the diurnal rodent. We should investigate this mechanism with special attention to the retina layer and cell-specific melatonin synthetic pathway as well as the low level of the enzymes apparently sufficient for retina physiology (see IV.B.4 paragraph discussion below). We could compare HIOMT expression over 24 hours in *Arvicanthis ansorgei* pineal gland and the different separated layers of the retina (ONL or even cones layer and GCL) to get an insight into HIOMT regulation. Vibratome or laser microdissection techniques could be used to isolate *A. ansorgei* cones. Ganglion cells are largely immunopositive for AA-NAT and HIOMT (Figures 3L and 4J respectively in (Gianesini et al 2015)) and could be first analyzed as a layer. Different photoperiod (LD, DD and LL) could help refine the light control of AA-NAT and verify whether it acts or not on HIOMT expression.

V.A.2.c *Hiomt* in cones and GCL: the nocturnal point of view

Hiomt could not be analyzed in *Arvicanthis ansorgei* retina. So far, nobody has cloned the diurnal rodent gene despite all the attempts over the years using PCR probes. We came to the conclusion that sequencing of *Arvicanthis ansorgei* (project initiated by David Hicks in collaboration with Donald Zack at the John Hopkins Hospital, Baltimore, MD USA) genome would eventually allow studying the retinal gene expression.

Why is it virtually impossible to get a glimpse of *hiomt*?

As reported above in the III.B.2.a paragraph, the only well studied mammalian *Hiomt* genes (usually termed *Asmt*) are the Human and the mouse genes. Otherwise, information comes from the chicken *Hiomt* gene (Bernard et al. 1993; Grève et al. 1996; Voisin et al 1992). First, the Human gene belongs to the 29 identified genes that constitute the PAR1 at the end of the short arm of sex chromosomes. This region normally behaves like an autosomal region for genetic material inheritance although this is the region involved in crossing over and recombination. In addition to this highly recombinant region (20 fold higher than the genomic average), the substitution rate is significantly elevated in the intron of *Asmt* gene and confirm that recombination (crossing over) may be mutagenic (Filatov and Gerrard 2003). Moreover, *Asmt* gene bears 2 promoters (A and B) coding for 3 transcripts which

translate into 2 isoforms (isoform 3 does not apparently display an enzyme activity)(Rodriguez et al. 1994). In brief different promoters and regulations sites imply a variety of mechanisms to regulate *Asmt* expression. The equivalent of Promoter A could be specific of the retina and therefore participate in the low expression, albeit circadian, of *hiomt* gene observed in rat retina by (Gauer and Craft 1996). We must also not forget a second important aspect of gene regulation which is the epigenetic part but no data are currently available regarding *Asmt*. Thirdly, mouse *Asmt* gene is highly polymorphic (elevated recombination rate) and also located in the PAR of X and Y chromosomes (Soriano et al. 1987). Consequently, the most common strain C57Bl6 harbors two mutations in *Hiomt* gene which reduce drastically HIOMT protein expression whereas C3H and CBA strains display functional enzymes (Kasahara et al. 2010).

Arvicanthis ansorgei belongs to the Muridae family and it is probable that *Asmt* gene be located in the same sex chromosomal regions than the other mammalian species. Therefore, *Arvicanthis Asmt* gene sequence could be really different from the mouse one. That would explain the difficulty to clone it with the common molecular tools. Moreover, mutations in the promoter or intron regulatory regions might be responsible for the low expression of HIOMT observed (direct correlation between low gene expression and low translation).

Finally, numerous Human *Asmt* gene mutations either in the promoter (Autism spectrum disorders (Melke et al. 2008)) or rare mutations in other gene regions (bipolar disorder (Etain et al. 2012)) have been identified and linked to psychiatric disorders. These mutations entrain a lower HIOMT/ASMT activity and therefore low melatonin level in the organism. We will discuss melatonin role in the paragraph below but a normal status of the hormone (nocturnal rise, sufficient amplitude) seems required for normal circadian behaviors (sleep/wake cycle notably).

***Hiomt* is specifically expressed in cones and GCL of the rat retina:**

We then used the nocturnal rat to determine the cellular localization of *hiomt*. For that purpose, we used a digoxigenin-labeled riboprobe specific for rat *hiomt*. Note that this riboprobe gave no signal in *Arvicanthis ansorgei* retina. We hence restricted our observations to rat retina, obtained across the 24 hours light/dark cycle. *In situ* hybridization of rat retinas collected at ZT19 display expression in rare loci along the scleral border of the ONL and weak expression in INL and GCL **(**Figure 40 B). This pattern suggests that staining is restricted to cones, since they represent < 2 % of total photoreceptors in the rat. To confirm this, we performed *in situ* hybridization for *hiomt* followed immediately with immunostaining against cone arrestin. Although the harsh treatment of sections reduced antibody efficacy, immunostaining was localized to rare cytoplasmic profiles (arrow) (Figure 40 D). *Hiomt* mRNA label (white label) was overlying immunostained cells (Figure 40 F). Expression of *hiomt* mRNA in cones was not visible during the day (Figure 40 C, H). On the other hand GCL expression is higher during the day (Figure 40 C, I). Weak staining was also observed in the INL during day and night (Figure 40 B, C).

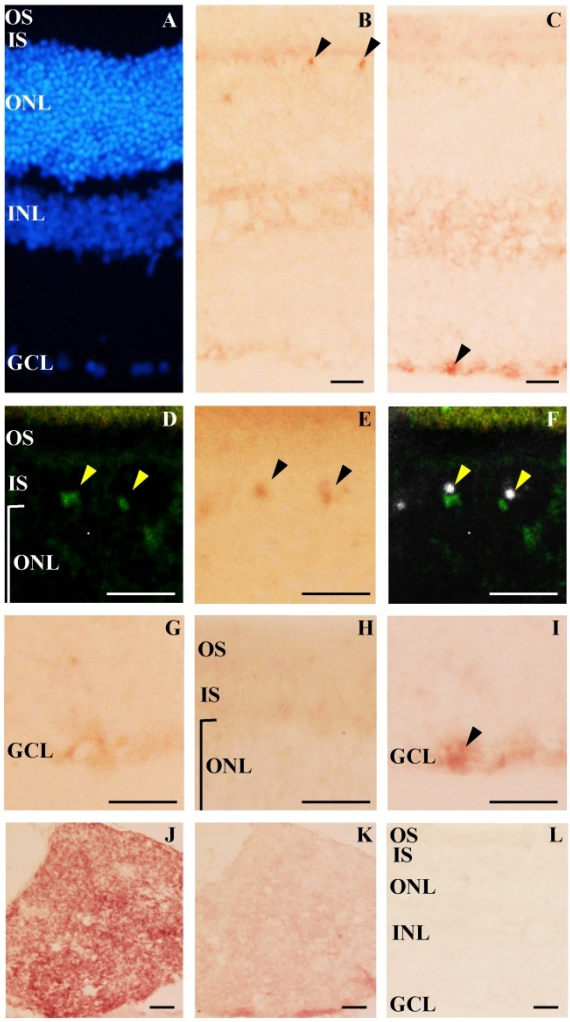


Figure40: *Hiomt* gene is expressed in the cones and GCL of rat retina.

A) DAPI staining of a 10 µm rat retina section indicates the area of interest: from top to bottom: OS, IS, ONL, INL, GCL. **B)** *Hiomt* antisense probe labeling at ZT23 (night time) is detected in cells of the ONL and more weakly the INL and GCL. **C)** *Hiomt* staining with antisens probe at ZT7 (day time) appears mainly in the GCL and weakly in the INL. **D)** Immunostaining of rat retina with anti-cone arrestin (green) shows cone segment (arrow). **E)** Enlargement of ONL at ZT23 shows *hiomt* staining in 2 cells (arrow). **F)** Overlapping of *hiomt* and cone arrestin images reveal that *hiomt* staining (show in white) is restricted to the cone segments (arrow). **G)** Enlargement of GCL at ZT23 reveals a weak *hiomt* staining. **H)** Enlargement of ONL at ZT7 shows no *hiomt* staining. **I)** Enlargement of GCL at ZT7 reveals strong *hiomt* staining. **J)** Positive control with *hiomt* antisense probe in rat pineal gland at ZT23. **K) and L)** Use of *hiomt* sense probe at ZT23 on pineal gland and retina tissues respectively does not reveal labeling in any structures. Scale bar = 10 µm.

Perspectives:

The results obtained in rat retina are consistent with our data regarding *Aa-nat* and melatonin synthesis in *Arvicanthis ansorgei*. **Next step will be to do** in situ hybridization on *Arvicanthis* retina sections with specific *Hiomt* probe (when *Arvicanthis* genome is sequenced) in order to confirm our model. The classical circadian and light regulation of AA-NAT/HIOMT does not seem valid in *Arvicanthis ansorgei*.

Does AA-NAT fulfill other tasks in *Arvicanthis ansorgei* retina?

Cones make melatonin at night. However, AA-NAT is present throughout the nycthemeron in these cells and in GCL. In addition, AA-NAT activity is elevated compared to HIOMT's. This suggests that AA-NAT may fulfill another role besides melatonin synthesis.

First, AA-NAT generates NAS, which has recently been shown to exhibit pronounced intrinsic biological activity. Notably, it binds to **trkB** (BDNF receptor) with high affinity and thereby exerts neuroprotective effects (Jang et al. 2010; Shen et al. 2012). We analyzed retinas of *Arvicanthis ansorgei* by HPLC in order to detect NAS. However we could not detect NAS in the samples probably because its level is low and inferior to the detection threshold of HPLC. AA-NAT higher expression than that of HIOMT does not account for NAS production in *Arvicanthis* retina.

Second, another maybe underestimated role is a function in neurotransmission. Fish AA-NAT1 (two isoforms in the fish retina) is able to acetylate dopamine in vitro. In addition, both **tyrosine hydroxylase (TH** limiting enzyme of dopamine synthesis) and AA-NAT1 are expressed in amacrine cells of the fish retina. N-acetyl-dopamine was also detected in the fish retina (Zilberman-Peled et al 2006). In *Arvicanthis ansorgei* retina, AA-NAT and HIOMT are present and active during the day, as DA and DOPAC, a situation similar to the fish melatonin regulation. Moreover, AA-NAT and HIOMT are present in the GCL and at a low level in the INL. As we know that the *Arvicanthis* GCL contains 40% displaced amacrine cells and that INL contains the rest, we can speculate a possible link between AA-NAT activity and dopamine metabolism (Figure 41). Another reason for such hypothesis is the low amount of retinal melatonin detected in the diurnal rodent. Comparison of *Arvicanthis* and mouse melatonin levels (ELISA, see article Gianesini et al, 2015) reveals a lower hormonal level in the former. **In future studies**, co-localization (confocal microscopy on whole mounted retinas) of AA-NAT and TH could be performed on *Arvicanthis* tissues to underpin this hypothesis.

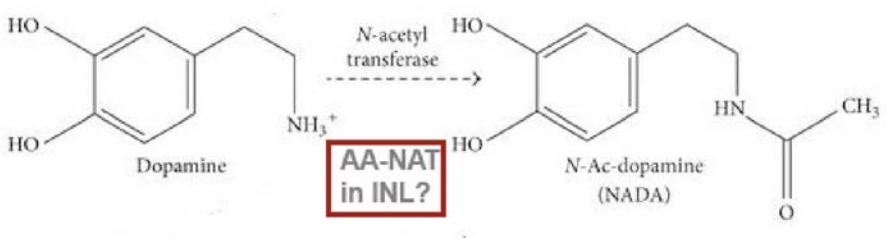


Figure 41: Hypothetical role of AA-NAT in *Arvicanthis ansorgei* INL.

As described for fish retina or insect melanin formation, an N-acetyl transferase can catabolize dopamine into N-Ac dopamine.

Finally, AA-NAT has been described to participate in the detoxification of phototransduction products (Klein 2004, 2007). Cones of *Arvicanthis* contain AA-NAT. In PRs, retinaldehyde (essential for photon

capture) are depleted by reacting with arylalkylamines (tyramine, serotonin, tryptamine). This generates toxic **bis-retinyl arylalkylamine (A2AA)**. AA-NAT prevents A2AA formation by N-acetylating the arylalkylamines and thereby help detoxify the cell (Klein 2004). Previous results from our team showed that *Arvicanthis ansorgei* retinas are highly resistant to intense light damage (Boudard et al. 2010). This phenomenon may represent an adaptation to high ambient light conditions to which *Arvicanthis* is exposed in its natural environment. We can suppose that AA-NAT play a role in cones detoxification in the diurnal rodent retina.

AA-NAT and HIOMT in *Arvicanthis ansorgei* retina:

AA-NAT and HIOMT in *Arvicanthis* retina are well coordinated to produce melatonin at night in cones and during the day in the GCL. Mainly clock driven transcripts are translated in somehow almost constitutive active AA-NAT and HIOMT proteins. However, *Aa-nat*/AA-NAT depends on light and clock whereas hiomt/HIOMT regulation is subtle and still unknown (Figure 42 model)**.**

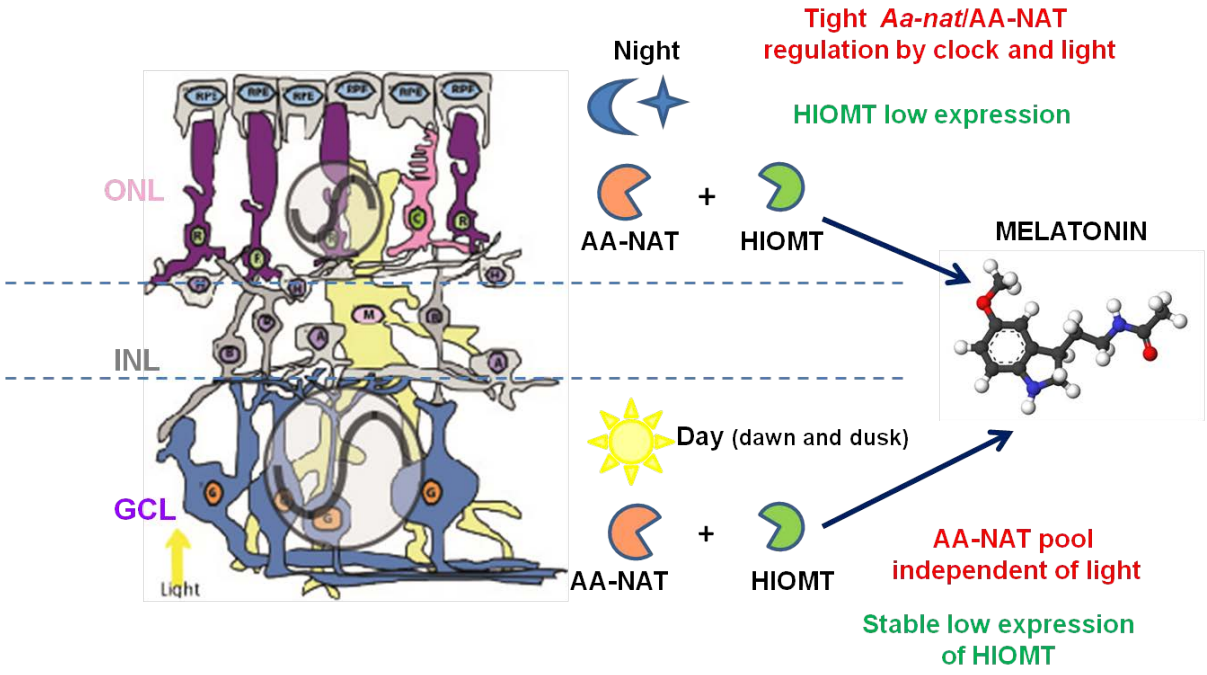


Figure 42: Hypothetical model of AA-NAT and HIOMT regulation and roles in *Arvicanthis ansorgei* retina.

Tightly regulated AA-NAT triggers melatonin synthesis pathway. Low stable HIOMT appears devoted to melatonin synthesis in the diurnal animal retina. Each retina layer might regulate melatonin synthesis according to local needs.

i) AA-NAT appears to have evolved for more than 500 My to be as fit as possible in the Mammal phylum in order **to convert circadian and light message into neurohormonal cue**, e.g. melatonin (Klein 2007). Only, in retina, AA-NAT might enter both main cascades: melatonin synthesis and phototransduction/detoxification. Besides, **HIOMT** is produced from a highly polymorphic unstable evolving gene in low level but present over the 24 hours, regulated by unknown mechanisms (specific promoter, phosphorylation, proteasome; cell-specific epigenetic regulation?) **to synthesize**

melatonin. Indeed, *Hiomt* expression could be driven by the equivalent of human *Hiomt* Promoter B in the retina of *Arvicanthis ansorgei* (Rodriguez et al. 1994). Moreover, epigenetic and/or post-translational regulations such as phosphorylation could also limit HIOMT expression as observed in our samples. Therefore AA-NAT might be tightly regulated to ensure both functions at the right time whereas HIOMT is the limiting enzyme of melatonin synthesis and both HIOMT activity and melatonin expression are positively correlated in our hands. In addition, no physiological import of melatonin has been observed (see discussion in paragraph IV.B.4 below) so HIOMT is required in the retina to synthesize melatonin**. ii) *Arvicanthis ansorgei* is a diurnal bi-modal species whose retina is exposed to strong variations of light irradiance** and melatonin synthetic pathway regulation may be of importance to support retina physiology (see Melatonin and light paragraph below).

It appears that the melatonin synthetic pathway in *Arvicanthis ansorgei* is tuned to perform its task but sufficiently controlled not to impact on the species reproduction unlike other Muridae (see (Kasahara et al. 2010; Roseboom 1998).

Perspectives:

In future studies, we should analyze retinal AA-NAT putative other roles in *Arvicanthis ansorgei* by in vitro tests with retina extracts and substrates such as arylalkylamines (reflecting the in vivo prevention of toxic A2AA formation (Klein 2004, 2007)). Then, we should do the same experiment in mouse retinas which bear a mutated AA-NAT: C57/Bl6 to compare if more toxic compounds accumulate in their cones (Roseboom 1998). Finally, we could create conditional AA-NAT and HIOMT knock-in to target the cones and the GCL in the retinas of melatonin proficient/deficient mice to analyze functions discussed above (melatonin synthesis, detoxification or dopamine acetylation). *Arvicanthis ansorgei* cannot be used as a transgenic animal yet. A current obstacle to this goal is the lack of knowledge of the reproductive cycle in this species. Indeed, we do not know the oestrus cycle parameters, the required super-ovulation conditions and the optimal in vitro maintenance of oocytes.

V.B. Melatonin role (s):

Our work identifies at least two main sites of melatonin production: cones and RGCs of *Arvicanthis ansorgei* retina. AA-NAT and HIOMT expressions were also seen within the INL, but were not further characterized in this layer. However, the key to melatonin physiological action resides in the presence of its receptors. **Two melatonin receptors type 1 and 2** (MT_1 and MT_2) have been identified in the Mammal retina (Dubocovich 1995; Dubocovitch 1983). MT_1 is present in all the layers of *Arvicanthis ansorgei* retina as described in our article part IV. Briefly, MT_1 immunostaining is strong in the rods, ganglion cells, INL cells (horizontal and amacrine cells most probably) and more moderate in the IPL and OPL. This wide distribution is consistent with two hypotheses discussed in the following paragraphs: **one is the autocrine/paracrine role of melatonin**. This implies diffusion of the amphiphilic melatonin from the ONL and GCL towards its receptors in INL, IPL, OPL. The second applies to **the potential multiple tasks of the indolamine** which will be discussed below: cross-talk with dopamine, circadian clock messages, visual sensitivity, melatonin and non visual functions, photoreceptor disk shedding (see also (Tosini et al 2012). MT_2 was not analyzed by lack of reliable biological tools (antibodies). MT_3 homolog to the quinone reductase 2 (with a lower melatonin

affinity) has been described but no data exists for the retina (Dubocovich et al 2010; Nosjean et al. 2000).

V.B.1. Melatonin receptors MT_1 and MT_2 background:

V.B.1.a Melatonin receptors structure

The first pharmacological characterization of a functional mammalian melatonin receptor (Dubocovitch 1983) and the cloning of the first human melatonin receptor (Reppert et al 1994) came 25 and 36 years respectively after melatonin discovery. Demonstration that melatonin receptors are coupled to a G protein took place in 1987 in *Xenopus laevis* dermal melanophores (White et al 1987).

In mammals, two GPCRs have been identified and termed **MT_1 and MT_2** (ortholog of Mel_{1a} and Mel_{1b} respectively for non mammalian) (and also named MTNR1A and MTNR1B respectively referring to human genes (Bouatia-Naji et al. 2009). These receptors present a general structure consisting of seven transmembrane α-helical segments connected by alternating intracellular and extracellular loops. The amino terminus is located on the extracellular side whereas the carboxyl terminus is in the intracellular side (Figure 43).

The human MT_1 and MT_2 melatonin receptors encode proteins of 350 and 362 amino acids, respectively. The amino acid homology for the human MT_1 and MT_2 receptors is approximately 60 % overall and 73 % within the trans-membrane domains. The amino terminus of the MT_1 melatonin receptor contains two consensus sites for N-terminal asparagine-linked glycosylation (Figure 43) whereas that of the MT_2 shows only one site.

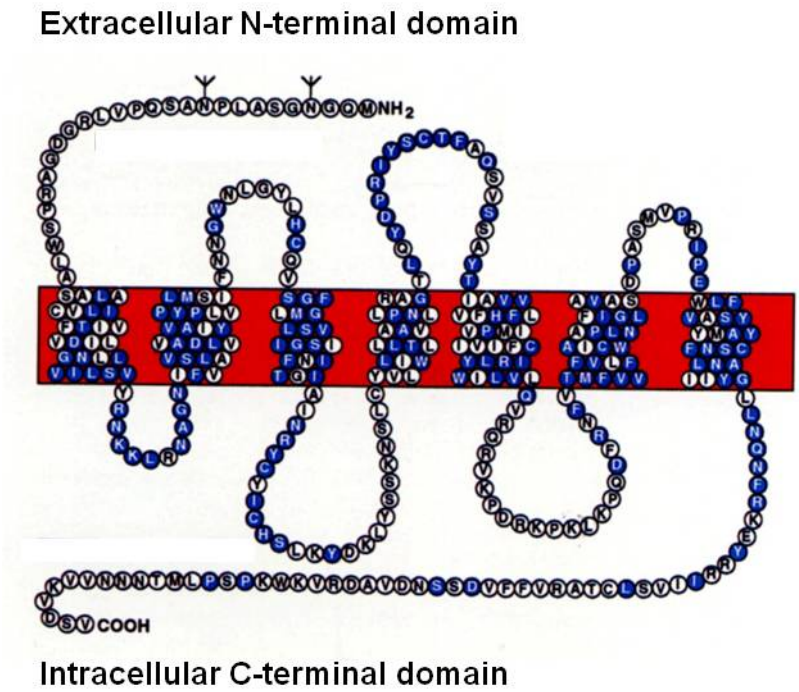


Figure 43: Membrane topology of the human MT1 (hMT1) melatonin receptor.

Gray circles denote identical amino acids in the hMT1 and hMT2 melatonin receptors. Both glycosylation sites on the hMT1 receptor are denoted (Y) in the N terminus. Adapted from (Reppert and Weaver 1995).

Human MT_1 and MT_2 melatonin receptors genes bear two exons separated by a 13 kb intron (Reppert and Weaver 1995; Roca et al 1996; Slaugenhaupt 1995). Human MT_1 receptor gene is localized in the 4q35.1 chromosome locus while it is in the chromosome 8 of the mouse (Roca et al 1996; Slaugenhaupt 1995). The human MT_2 melatonin receptor resides on 11q21-22 chromosome, while mouse MT_2 belongs to chromosome 9 (Reppert and Weaver 1995). MT_1 and MT_2 are considered high affinity melatonin receptors (ligands with picomolar binding affinity (Dubocovich 1995). Besides there is another receptor with lower melatonin affinity (nanomolar range) which has been designated **MT_3** melatonin receptor. However this receptor is the homologue of a human quinone reductase 2 (Nosjean et al. 2000) and is apparently not involved in melatonin function.

Pharmacological properties have always been the principal mean to distinguish and decipher melatonin receptors signaling because until now, melatonin receptors antibodies have proven mostly non reliable. Briefly, the first competitive melatonin antagonist discovered was the **luzindole** (Dubocovich 1988) which is **an antagonist for MT_1/MT_2 heteromers** (Baba et al 2013) **and MT_2 homomers**. Another selective antagonist which has been identified, the cis-4-phenyl-2-propionamidotetralin (**4P-PDOT**) presents higher affinity for MT_2 receptor mouse (22 fold (Baba et al 2013)), human (300 fold (Dubocovich et al 2010)). 4P-PDOT is also an antagonist of **MT_1/MT_2 heteromers** (Baba et al 2013). The N-butanoyl 2-[9-methoxy-6H-isoindolo[2.1-a]indol-11-yl]ethanamine (**IIK7**) has been identified as a selective **agonist for MT_2 receptors** in mouse (1000 fold higher affinity **for MT_2 versus MT_1 receptor** (Baba et al 2013)) and in human (90 fold higher affinity for MT_2 (Teh et al 1999)) Low doses of IIK7 only activate MT_2 receptors but high doses of IIK7 **activate MT_2 and MT_1** (Baba et al 2013). These results indicate that despite a high overall conservation of pharmacological properties between species, there might be some important differences in MT_1 and MT_2 selective compounds between human and mouse (Tosini et al 2014).

V.B.1.b Melatonin receptors signaling pathways

MT_1 and MT_2 are coupled to an **inhibitory G protein (G_i)** which stimulates cAMP pathway inhibition and **ERK1/2** pathway activation (Guillaume et al. 2008) (Figure 44). MT_1 can also bind to the G_q protein which stimulate PLC pathway (Brydon et al. 1999; Tosini et al 2014) (Figure 44). Furthermore, inhibition of the guanylyl cyclase pathway was shown to be MT_2 specific (Jockers et al. 1997; Petit et al. 1999) (Figure 44). Melatonin can also increase the activation of phosphatidylinositol 3 kinase (PI3K) and serine/threonine protein kinase (AKT). Melatonin can thus stimulate the survival pathway (Faria et al. 2013; Ha et al. 2006).

Depending on the cellular background, the species and receptor types, only 15-40 % of the MT_1 and MT_2 receptors are in the uncoupled state (not coupled to a G protein). MT_1 and MT_2 form different complexes between agonist/receptor/G protein. These complexes bind melatonin ligands with different affinities (Tosini et al 2014). Indeed, MT_1 and MT_2 form different receptor signaling complexes. Multi-PDZ domain protein 1 (MUPP1) binds to G_i protein and promotes interaction between G_i and MT_1 receptor (Guillaume et al. 2008). In addition, MT_1 receptor binds to G_i protein and the regulator of G-protein signaling 20 (RGS20). MT_1/G_i/RGS20 complex is asymmetric between the different protomers of receptor dimers. The concept of asymmetry in receptor dimers suggests that each protomer fulfils its specific task (Maurice et al. 2010) (Figure 44).

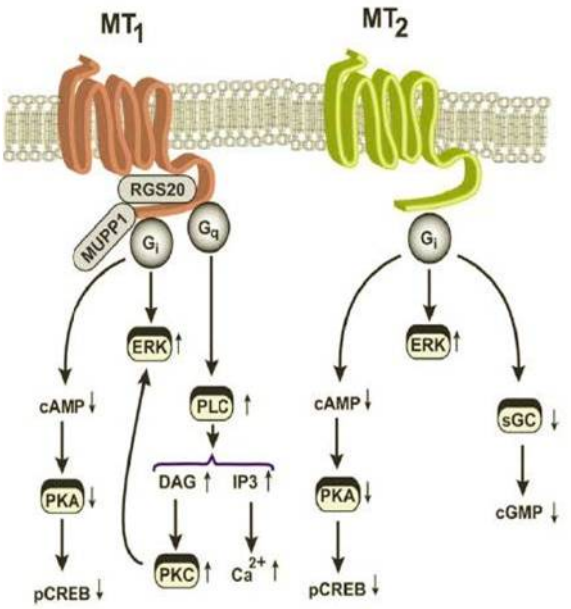


Figure 44: Principal melatonin-receptor signaling pathways.

Depending on the type of melatonin receptor complexes present in cells (MT_1 homomers, MT_2 homomers) the depicted signaling pathways are activated upon melatonin stimulation. DAG: diacylglycerol; ERK: extracellular signal-regulated kinase; IP3: inositol triphosphate; MUPP1: multi-PDZ domain protein 1; pCREB: phosphorylated-cAMP-response element-binding protein; PLC: phospholipase C; PKA: protein kinase A; PKC: protein kinase C; RGS20: regulator of G protein signaling 20; sGC: soluble guanylyl cyclase; cGMP: cyclic guanosine 3', 5'-monophosphate (Tosini et al 2014).

V.B.1.c Melatonin receptors in the retina:

MT_1 and MT_2 mRNA and proteins have been detected in the retina of many vertebrate species with major work from Wiechmann et al on *Xenopus laevis* tissue (Wiechmann and Sherry 2013). The table 1 below, an extract from Coralie Gianesini's PhD thesis (2015) compares the distribution and level of both receptors expression in the retina of Vertebrates. No specific antibody is yet available to localize MT_2 receptor in rodents. Therefore, the lack of information regarding MT_2 is great as illustrated in the table 1.

Table 1: Species comparison of melatonin receptors expression within the retina.

Data show retinal melatonin receptors localization and expression of most studied Vertebrates. Relative change between night and day are indicated by blue color. ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer; CCB: cone cell body; CIS: cone inner segment; COS: cone outer segment; RIS: rod inner segment; ROS: Rod outer segment; PR: photoreceptors; RGC: ganglion cells; RPE: retinal pigmentary epithelium; D: day values; N: night values; nd: not determined. Staining intensity are represented as follows: +++: strong; ++: moderate; +: weak; +/-: very weak; 0: absent.

	Rat/Mouse	Monkey	Chicken	Trout	Other
***MT1 (Mel1a)* mRNA**	**Mouse:** (Baba et al 2009)*:* ONL, INL +++, GCL +. **Young rat:** (Fujieda et al. 1999)*:* GC, amacrine cells, horizontal cells +++.	nd	**Chick:** (Natesan and Cassone 2002)*:* IS, INL, GCL. D>N.	nd	**Xenopus**: (Wiechmann and Smith 2001)*:* retina, RPE
MT1 (Mel1a)	**Rat:** (Fujieda et al. 1999)*:* OPL, IPL +++. **Young rat:** (Fujieda et al. 1999)*:* OPL, horizontal cells +++, IPL +. **Mouse:** (Sengupta et al. 2011)*:* COS, ROS +++, RGC ++, OPL +.	(Scher et al. 2003)*:* amacrine cells.	**Chick:** (Rada and Wiechmann 2006)*:* IS, ONL, OPL, horizontal and amacrine cells, IPL, GCL. Peak at evening.	**Carp**: (Huang and Yang 2005)*:* OPL, horizontal cells.	**Human:** (Meyer et al. 2002)*:* GCL, IS +++. (Savaskan et al. 2002)*:* IS +++, GCL ++, amacrine cells and IPL +. (Scher et al. 2002)*:* RIS, horizontal cells, amacrine cells, IPL, RGC **Guinea pig**: (Fujieda et al. 2000)*:* GCL +++, amacrine cells, IPL, OPL ++. ***Xenopus laevis:*** (Wiechmann, Udin, and Summers Rada 2004)*:* OPL (horizontal cells that ramify OPL).
MT2 (Mel1b) mRNA	**Mouse:** (Baba et al 2013)*:* ONL, INL.	nd	**Chick:** *(Natesan, 2002):* IS, ONL, INL, GCL.	nd	***Xenopus laevis*:** (Wiechmann and Smith 2001)*:* RPE, IS, INL, GCL. D>N.
MT2 (Mel1b)	nd	nd	**Chick:** (Rada and Wiechmann 2006)*:* Peak at evening.	nd	**Human:** (Savaskan et al. 2007)**:** IS, OPL, bipolar cells, IPL, RGC ***Xenopus laevis*:** (Wiechmann, Udin, and Summers Rada 2004)*:* RPE, IS, OPL (horizontal cells that ramify OPL), GCL.
MT1/MT2	**Mouse:** (Baba et al 2013)*:* PR.	nd	nd	nd	nd

V.B.1.d MT_3: homolog to quinine reductase:

As mentioned earlier, a « MT_3 » receptor has been characterized but is a homolog to the quinine reductase 2 (Nosjean et al. 2000). However, melatonin has been shown to down-regulate pro-oxidant enzymes (such as oxyde nitric synthase) and up-regulate antioxidant enzyme (such as superoxyde dismutase) (Hardeland R 2005). Melatonin could also act directly as a free-radical scavenger by interacting with several reactive oxygen and nitrogen species (Hardeland R 2005). Oxido-reductive properties of the quinine reductase could explain the antioxidant role of melatonin when the hormone binds to MT_3 (Vella et al. 2005). Studies demonstrate that melatonin can act as an antioxydant in PR. Indeed melatonin reduces the light-induced oxidative processes in PR at picomolar and low nanomolar concentrations (Marchiafava and Longoni 1999). Melatonin may also have protective effects on RPE cells. Indeed melatonin decreases ischemia induced apoptosis in human RPE cells (Osborne et al 1998) and protects RPE cells against hydrogen peroxide (H_2O_2) induced cell death (Liang et al 2004). On the contrary, melatonin exhibits potent prooxidant effects at micromolar concentration, i.e. above the physiological melatonin concentration (Marchiafava and Longoni 1999).

Perspectives:

We have collected in collaboration with Etienne Challet (Team "Regulation of circadian clocks", Institut des Neurosciences Cellulaires et Intégratives, Strasbourg), retinas from male C3H/MT_3KO mice in order to test the putative role of melatonin in oxidative stress. Our (with David Hicks) project involves administration of exogenous melatonin or melatonin inhibition to assess its effect on visual function of the retina and photoreceptor survival. In vivo, we will record electroretinograms from the diurnal rodent (Single flash ERG, 6 Hz Flicker ERG) or MT_3KO mice (absence of quinine reductase 2, the type 3 melatonin receptor) placed under photopic (cone stimulation) or scotopic conditions (rod recruitment) without or with exogenous melatonin in order to identify target cells and examine their survival. The use of MT_3KO mice is advantageous to study melatonin in pathological conditions. First this mouse lacks the quinine reductase 2 which is thought to counteract oxidative stress (Fu et al 2008); secondly, this model carries the *rd1* mutation in the GMPc phosphodiesterase gene, which leads to rapid rod degeneration and to a hyperoxic environment for cones (Komeima et al. 2006). We will examine kinetics of cone death in the WT mice with *rd1* mutation only, and MT_3KO. Then we shall treat these animals with anti-oxidants (e.g. Vitamin E, DHA) and/or melatonin to record their action on photoreceptor survival.

V.B.2. Melatonin and the retinal clock(s):

There are likely multiple sites of molecular circadian rhythm generation within the mammalian retina (Jaeger et al. 2015; McMahon et al 2014). Overall, **ONL and photoreceptors** (cones) may constitute an oscillator although controversial (mouse, rat). In addition, clock gene expression is concentrated within the **inner nuclear layer** where GABA/DA amacrine and Müller cells can express circadian variations of clock genes transcripts (Rat and mouse). The inner circadian clock may influence photoreceptors and other retinal neurons through the rhythmic secretion of dopamine but is independent from photoreceptors and melatonin (Sengupta et al. 2011). Finally, the **GCL including**

ipRGCs and displaced amacrine cells is involved in the generation and entrainment of retinal circadian clocks.

V.B.2.a Circadian control of melatonin production in *Arvicanthis ansorgei* retina

The melatonin synthetic pathway mirrors the putative retinal circadian oscillator locations. However the different compounds are more or less driven by the clocks. *Aa-nat*, *Hiomt* (not determined in *Arvicanthis* but in the rat retina) gene transcripts are clock regulated whereas AA-NAT/HIOMT are varying slightly over 24 hours but not significantly (Figure 45). Nevertheless, our interpretation is that the clock-driven control of all the parameters may appear more robust if analysis goes down to each retinal layer or even to the cell-types involved (see article Gianesini et al, 2015 above). **Then, the next question is whether melatonin conveys the temporal message to modulate retinal functions and which ones?** This question is developed as a project described in the future studies chapter.

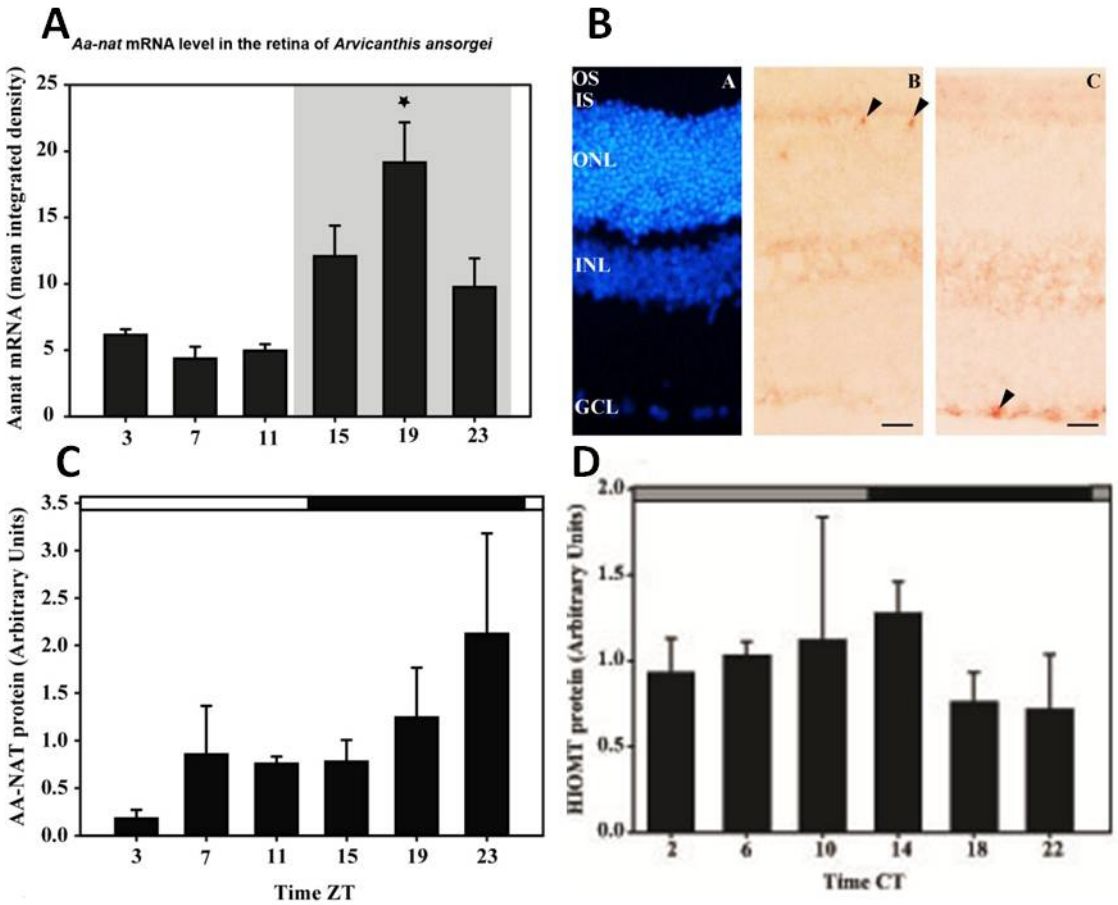


Figure 45: Daily variations of *Aa-nat*/AA-NAT and *Hiomt*/HIOMT in the retina of rodents.

Panel A: Only *Aa-nat* mRNA expression displays a significant peak during the night while **(panel C)** AA-NAT rises at night but is present over the 24 hours in the retina of *Arvicanthis ansorgei*. **Panel B:** Rat *Hiomt* is present in both ONL and INL (as for *Aa-nat*) while HIOMT in DD conditions shows a constitutive profile over the nycthemeron **(panel D).**

V.B.2.b Melatonin temporal message in the ONL:

In *Arvicanthis ansorgei* retina, melatonin is synthesized in the cones and might act on rods as MT_1 receptors are localized on these photoreceptors (paracrine action) and to a lesser extent on cones (autocrine). This last result differs from the mouse retina where MT_1 is expressed by cones (Sengupta et al. 2011). As mentioned above, melatonin could act on other parts of the retina as MT_1 are widespread in this tissue and the hormone can diffuse but probably not further (see paragraph IV.3.a below). Moreover, Sengupta et al also report that dopamine (amacrine cells) circadian rhythm is independent from photoreceptor melatonin (Sengupta et al. 2011). **Therefore, ONL melatonin may transmit the clock circadian message locally.**

The potentially most important role of melatonin in the normal retina is the modulation of **daily renewal of photoreceptor outer segment membrane** (Besharse and Dunis 1983). This melatonin function is one of my ongoing research projects. This constitutes the **chapter II** of this manuscript.

Aa-nat, AA-NAT are controlled by a clock in the ONL of *Arvicanthis* and regulation appears similar to the mouse retinal melatonin synthetic pathway. Therefore, local melatonin is probably more elevated at night. Its main function in that case is **to increase visual sensitivity** and facilitate dark adaptation. Even though *Arvicanthis* is a diurnal species, its locomotor activity is usually bimodal around day/transition and the reverse. Retinal adaptation to obscurity might be equally important than adaptation to high light irradiance. This may be achieved as described in other species by increasing horizontal cell coupling through inhibition of dopamine release (Harsanyi and Mangel 1992; Iuvone and Gan 1995). Other inner retina neurons are potential melatonin targets. Melatonin enhances GABA-ergic inhibition transmission of light evoked dopamine release (Boatright 1994). Melatonin potentiates glutamate induced currents from isolated cone-driven horizontal cells in the carp retina by increasing the binding activity of the glutamate receptor (Huang and Yang 2005). This causes the depolarization of the H1 horizontal cell membrane potential, and a reduction in their light response.

Melatonin may also **act directly on retinal photoreceptors** via its MT_1 receptors mainly located on rod in *Arvicanthis ansorgei* retina. In *Xenopus laevis*, melatonin acting through melatonin receptors directly stimulates the responsiveness of rod photoreceptor to light (Wiechmann et al. 2003). Melatonin proficient mice exhibit daily rhythms in the scotopic and photopic ERG and removals of melatonin receptors inhibit ERG daily rhythm (Baba et al 2009, 2013). Depending on the species, administration of exogenous melatonin modulates the amplitude of ERG (Lu et al 1995; Wiechmann et al. 2003). In Human, oral administration of melatonin during the day decreases the amplitude of the cone ERG. Authors suggest that naturally increasing melatonin in the evening may decrease the impact of the cone system (day vision) in order to better promote the rod system (night vision) and by doing so, ensuring that the most suitable visual function is enhanced according to the time of the day (Gagné et al. 2009).

Melatonin can exert **both beneficial and detrimental effects on photoreceptors**: on one hand it may play an important role in protecting them from oxidative stress (Marchiafava and Longoni 1999), preventing apoptosis (Liang et al. 2001) and also promotes photoreceptor viability during aging (Baba et al 2009), on the other hand it can also sensitize photoreceptors to light-induced damage (Sugawara et al. 1998). Some studies involve melatonin in the pathogenesis of **age-related macular degeneration (AMD)**. AMD is a slow and progressive disease of the macula, i.e. the central part of the retina where cone density is maximal, and constitutes the leading cause of irreversible visual loss

in the western world (Coleman et al. 2010). Yi et al., (2005) reported that daily administration of melatonin (3 mg) may protect the retina and delay the progression of AMD (Yi et al. 2005); while Rosen et al. (2009) described that production of melatonin is decreased in AMD patients with respect to age-matched controls (Rosen et al. 2009). These findings suggest that a deficiency in melatonin may play a role in the progression of AMD.

In conclusion, melatonin can modulate ERG and visual sensitivity circadian rhythms, in a species dependent manner as well as cell viability.

Perspectives:

Coralie Gianesini (PhD grade obtained in June 2015) has spent a year in Dr. Gianluca Tosini's laboratory to test the hypothesis of a role for melatonin in cone survival. Her work demonstrates that cone viability is affected by interruption of melatonin signaling (Gianesini et al 2016).

V.B.2.c Melatonin temporal message in the GCL:

Melatonin synthesis by *Arvicanthis* GCL is perhaps the most intriguing part of our results. Indeed, melatonin synthetic pathway is distinct from what has been reported so far (largely discussed above). *Aa-nat*/AA-NAT/HIOMT proteins and activities are expressed during the day. **What for?** Moreover, MT_1 are strongly expressed at ZT23 and ZT7. We have not determined the daily profile of MT_1 expression but it is present day and night which is consistent with melatonin action also during the day. Cell population is mixed in *Arvicanthis* GC: ganglion cells, ipRGC and displaced amacrine cells (40%). We have detected AA-NAT and HIOMT in ganglion cells (Brn3 positive cells) but also in other cells (Brn3 negative cells) which could be displaced amacrine cells. This means that melatonin production may emanate from ganglion cells (ipRGC and others) and amacrine cells in this layer and integrate different functional circuits.

Perspectives:

Whether the melatonin clock controlled component is as robust as in the rod and cones photoreceptors is not clear in *Arvicanthis ansorgei*. However, the protein kinase B or *Akt* and *Phospho-Akt* (gene belonging to the survival pathway) display a circadian profile which seems impaired in the GCL of MT_1 and MT_2 KO mice (Gianesini Coralie, personal communication). Melatonin would be in that case important for the circadian modulation of target genes in the GCL.

As mentioned earlier, GCL (isolated layer) melatonin synthetic pathway should be examined under LD, DD and LL conditions. In parallel, the same analysis could be done in C3H mice and C3H/$f^{+/+}$. Indeed, C3H (Komeima et al. 2006) are melatonin proficient mice but with the *rd* degeneration so in a few weeks, retina photoreception is reduced to the GCL. Thus, these mice have the sole GCL melatonin source to examine. C3H $f^{+/+}$ are the perfect controls in that *rd* mutation has been removed (Baba et al 2009).

Melatonin main roles in the GCL could be of two sorts: either participate in the light information treatment (see paragraph iV.B.5) to visual ends or modulate non visual functions especially with ipRGCs (see paragraph IV.B.6).

V.B.2.d Does melatonin feedback on the clock?

As an output of the circadian clock, melatonin may feedback on the molecular clockwork. Indeed studies report that melatonin influences the amplitude and phase of clock gene transcripts such as *Per1* and *Cry1* in the mouse retina (Dinet and Korf 2007; Dinet et al. 2007). A recent study in the retina of melatonin proficient mice demonstrated that melatonin signaling (via MT_1 and MT_2) may be important for the regulation of clock genes expression in the INL or GCL but not in PR (Hiragaki et al. 2014). No data are available yet in *Arvicanthis ansorgei* regarding melatonin feedback on the GCL clock. To make a prediction is perilous because GCL melatonin synthetic pathway is not clearly circadian. The hormone is present at all times and at a really low level.

V.B.3. Is retina an endocrine gland?

Our work addressed this question indirectly because we examined a whole hormonal synthetic pathway in neurons. Endocrine gland means " a ductless gland that produces and secretes hormones into the blood or lymph nodes, affecting metabolism and other body processes ». **Does retina fit in with this definition?** Here is our (with Coralie Gianesini) contribution to the still open debate:

V.B.3.a Does retinal melatonin contribute to the bloodstream pool?

One possibility is that melatonin is exported towards the bloodstream. Given the level of melatonin production in *Arvicanthis ansorgei* retina (10-15 pg/mg protein across the entire day/night cycle) compared to the pineal gland (estimates from published data give peak night values of 530 pg/mg protein, day values of 25 pg/mg protein) any retinal contribution to circulating melatonin would be minor, at least at night. Pinealectomy reduces the nocturnal plasma melatonin value by 80% in chicks, and bilateral enucleation further reduces the value by another 9%. These results suggest that 9% of plasma melatonin originates from the eyes, hence retina which is the major melatonin production site in this organ (Reppert and Sagar 1983). The authors conclude that only a minor level of bloodstream melatonin comes from retina. Indeed another study reports that the retinal melatonin production does not seem to contribute significantly to plasma levels (Vaughan and Reiter 1986). Combined with the widespread distribution of MT_1 receptors, these findings suggest that melatonin is synthesized in the retina principally for local purposes (Vanecek 1998).

Pinealectomy and/or bilateral enucleation could be done in *Arvicanthis ansorgei* to measure plasma melatonin level either by radioimmunoassay or ELISA in order to determine precisely the contribution of each source to the organism. With the present data, we cannot really answer the question of whether retina is an endocrine gland per se.

Another experiment to do, would be prepare RPE explants from *Arvicanthis* eyes, put them in a dish culture on a membrane and test whether melatonin can enter the tissue without being degraded. This experiment could help understand whether circulating melatonin does or not enter the retina.

V.B.3.b Does circulating melatonin enter the retina?

Pineal gland melatonin is released in the bloodstream and irrigates all the body. Since retina is highly vascularized, pineal melatonin possibly enters in the retina.

AA-NAT and melatonin day/night expression levels in the pineal gland and the retina of *Arvicanthis ansorgei* are here compared to determine whether pineal melatonin enter in the retina in

physiological condition (and *in vivo*). *Arvicanthis ansorgei* pineal gland exhibits a daily profile of *aa-nat* mRNA, AA-NAT activity and melatonin with nocturnal peaks (Garidou et al 2002) (Table 2). The day-to-night ratio and level of expression observed in the retina is much lower than that in the pineal gland (Table 2). Comparison between pineal gland and retina values suggests that bloodstream melatonin contributes weakly to the retinal level in *Arvicanthis ansorgei*.

	***Arvicanthis* pineal gland**	***Arvicanthis* retina**
***aa-nat* mRNA**	(Garidou et al. 2002)*:* N>D ~250 X	In PR, N>D ~4x; in RGC, D>N by ISH. (Bobu et al 2013)*:* N>D ~20x by qPCR.
AA-NAT protein	nd	Present at all times except ZT3. N>D ~10x.
AA-NAT activity	(Garidou et al. 2002)*:* 1000 pmol/pineal/h. N>D ~10 X	~100 pmol/mg prot/h. Present at all times, small increase at night, ~x1.5.
Melatonin	(Garidou et al. 2002)*:* 400 pg/pineal. N>D ~20 X	~18 pg/mgprot. N=D.

Table 2: Circadian variation of AA-NAT and melatonin in *Arvicanthis ansorgei* pineal gland and retina.

N: night value; D: day value; nd: not determined.

However it is important to note that pharmacological concentration of melatonin administrated in the circulation reaches the retina. Indeed, in human, daytime oral administration of melatonin (when endogenous level is low) modifies cone ERG, suggesting that bloodstream hormone reaches the eye (Gagné et al. 2009). Similarly, daytime intraperitoneal injection of melatonin in C3H mice (melatonin proficient mice) increases rods light sensitivity (Baba et al 2009, 2013). Under DD conditions, daily intraperitoneal melatonin injections at night in C57/Bl6 mice (melatonin deficient mice) restore retinal melatonin and dopamine rhythmicity (Doyle et al. 2002).

V.B.4. Melatonin cross-talk with dopamine:

Melatonin and dopamine have long been considered the dark and the light “knights”, respectively, of the retinal physiology (Tosini et al 2008) because melatonin is produced at night (Tosini and Menaker 1998, 1996) and dopamine peaks during the day. As such, they may merely be output of the circadian clock (McMahon et al 2014). These compounds are rather well studied neurohormones but more work is to be done to dissect **direct and indirect effects of melatonin on dopamine** and how collaboration between both affects retinal physiology (photoreceptor phagocytosis for instance) and more importantly for humans, retinal pathology.

V.B.4.a Background:

Melatonin inhibits the release of dopamine through melatonin receptors (Dubocovitch 1983); (Boatright 1994); (Ribelayga et al 2004) and dopamine inhibits the synthesis of melatonin by acting on D2-like dopamine receptors (Nguyen-Legros et al. 1996; Tosini and Dirden 2000; Zawilska and Iuvone 1992). Dopamine is synthesized from a unique cell population within the INL which are amacrine cells (Witkovsky et al. 2004) The circadian rhythm of dopamine release is dependent on melatonin (Dubocovitch 1983) Indeed dopamine is no longer rhythmic in mice that are genetically incapable of synthesizing melatonin (Doyle et al. 2002; Nir, Haque, and Iuvone 2000; Pozdeyev et al 2008). On the contrary, the circadian rhythm of *aa-nat* mRNA is maintained when the dopamine

rhythm is abolished (Sakamoto et al 2006), probably because *Aa-nat* gene is directly controlled by the circadian clock via CLOCK/BMAL1 and the E box. **These results suggest that the circadian regulation of dopamine depends on melatonin but melatonin rhythm is independent of the dopamine presence.** Dopamine has different roles in the retina. The hormone regulates protein phosphorylation in photoreceptors (Pozdeyev et al 2008) and modulates the response of ipRGCs to light (Van Hook et al 2012). Moreover, dopamine modulates the rhythm of genes coding for melanopsin and PACAP (Sakamoto et al. 2005), controls the expression of *Adcy1* (gene coding for AC) (Jackson 2011) **and influences the phagocytosis of PRs** (Masri 1996) as well as their coupling via gap junctions (Ribelayga et al 2008). Dopamine sensitizes the bipolar cells of rods to light via GABA (Herrmann et al. 2011). Finally, dopamine may play a role on the surviving of PR in a murine model of retinal degeneration (*rd* mouse) (Ogilvie and Speck 2002) and on the ocular refraction in a primate model of myopia (Iuvone et al 1991). In conclusion, melatonin modulates dopamine release to control retinal circadian rhythms and essential retinal functions.

V.B.4.b Melatonin and Dopamine in the retina of *Arvicanthis ansorgei*:

Our data may support retina adaptation to darkness through melatonin/dopamine interplay: a fundamental aspect of mammal retina function (McMahon et al 2014). In our study, dopamine/DOPAC levels rise at ZT7 in the middle of the day and overlap with the daytime melatonin increase. These results differ from the previously described interplay between both compounds. As dopamine and melatonin are measured in the entire retina, we have not determined the rhythmic profile of dopamine and melatonin in PRs. However it is likely that melatonin peaks during the night in PRs (see article and discussion above) and thereby inhibits the production of dopamine at night. One study in chicken shows that intraocular injection of dopamine during the night significantly inhibits AA-NAT activity in PRs implying that the feedback loop between melatonin/dopamine occurs at least in PRs (Valdez et al. 2012).

As stated above, dopamine modulates the rhythm of melanopsin and PACAP genes in mouse retina (Sakamoto et al. 2005). In *Arvicanthis ansorgei*, dopamine displays a daily profile and melatonin possesses MT_1 receptors on the IPL and GCL. As melatonin is known to regulate the rhythm of dopamine secretion, melatonin may indirectly act on ipRGC physiology or directly via its MT_1 (MT_2? no data available) receptors. The second hypothesis (to be tested in *Arvicanthis ansorgei*) would be privileged for two reasons. Melatonin receptors MT_1 are required for circadian regulation of photic ERG (Sengupta et al. 2011) and this is also obtained with the removal of melanopsin producing cells (Barnard et al. 2006). These two studies suggest that melatonin may affect the circadian regulation of the photic ERG by acting on MT_1 receptors present on the ipRGCs.

As mentioned in the paragraph about “Melatonin and the temporal message in the ONL”, we could use *Arvicanthis ansorgei* in vivo to measure the effect of intravitreal melatonin injections on ERG However, we should prepare retinal explants of *Arvicanthis* retinas to analyze the activity of the ipRGC because the only relevant method is the MEA. The second good model to test this would be the C3H mouse with only RGCs left and specially ipRGCs when rod and cone degeneration has occurred. The effect of melatonin application on C3H retina explants would then only be relates to the GCL and ipRGCs.

V.B.4.c Does melatonin influence dopamine signaling for photoreceptor phagocytosis?

This is a question I could have asked in the chapter II because it is an ongoing project related to the role of melatonin on photoreceptor phagocytosis. The reason I am inserting this discussion here is because of the model used to answer, even partially this question. I am currently collaborating with Dr. Gianluca Tosini (Morehouse Medical center, Atlanta, GA, USA) to analyze photoreceptor (rod) phagocytosis in **dopamine receptor type 2 knock-out (D_2RKO)** mice retinas. I thank him for lending me this part of unpublished data. Preliminary results show that phagocytosis rhythm is indeed altered in the D_2RKO mice compared to the WT animals (Figure 46 Lower right panel). D_2RKO mice display phagosomes but no phagocytosis rhythm. Moreover, our collaborator Dr. Gianluca Tosini analyzed the D_2R associated signaling pathway that could play a role on phagocytosis. Interestingly, **FAK (focal adhesion protein)/ FAK-P-Y397 (phosphorylated FAK)** proteins have opposed profiles in WT and D_2RKO mice. FAK phosphorylation correlates well with phagocytosis peak at ZT1 (beginning of the day) in WT mice. The reverse is true for D_2RKO in which no rhythmic phagocytosis is observed and phosphorylation of FAK seems downregulated. Overall, dopamine appears to play a role on photoreceptor phagocytosis.

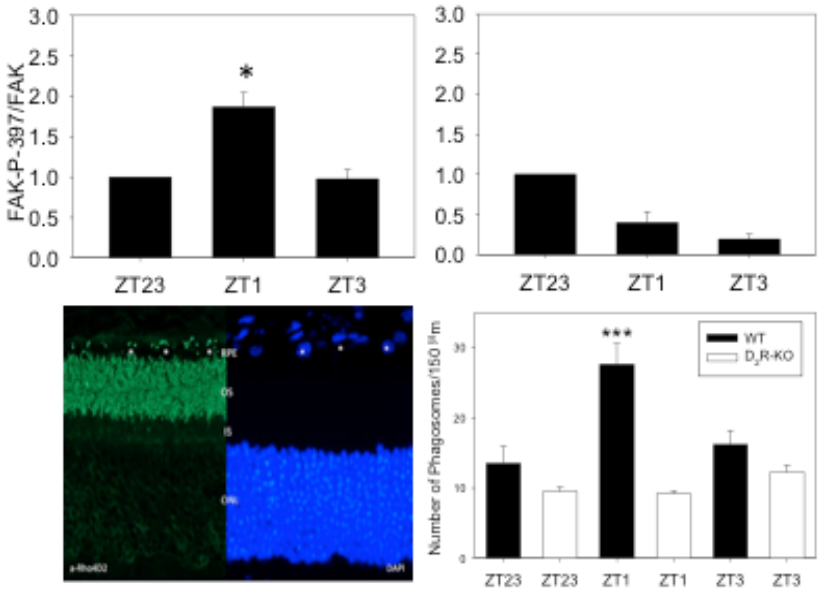


Figure 46: Dopamine regulates photoreceptor phagocytosis:

Upper panels: Control mice (left) show a significant increase in FAK-P-Y397/FAK 1 hr after light onset (One-way Anova, $P < 0.05$) whereas D_2RKOs mice (right) display a significant decrease in FAK-P-Y397/FAK after light onset (One-way Anova, $P < 0.05$). Results are presented as mean +\- SEM. N=3/group. Lower panels (left) Representative photomicrographs of the methodology used to count phagosomes in mouse RPE (Bobu and Hicks 2006). (Right) Phagosomes number measured in WT and D_2RKOs mice at 3 different Zeitgeber times (ZT 23, 1 and 3). Consistent with previous data, the number of phagosomes increase shortly after light onset in WT (one-way ANOVA, $P< 0.01$, followed by post-hoc test, N=4-6 for each time point) but not in D_2RKOs.

V.B.5. Melatonin and light:

V.B.5.a The importance of cones:

Hattar et al have demonstrated that the 3 photoreceptor types (rod, cone, ipRGC) are required to achieve non visual communication with central brain and in particular with SCN (Hattar et al 2003). In rats, the majority of visually responsive SCN neurons studied under dark adaptation receive rod input (92% of examined cells). When studied under light adaptation, most visually responsive SCN neurons (77%) respond to input from cones. The presence of these neuronal responses suggests a role for rod and cone photoreceptors in SCN function (Aggelopoulos and Meissl 2000). In addition, Van Diepen et al (Van Diepen et al. 2013) have demonstrated, with *opn4*$^{-/-}$ and *rd/rd* mice, that SCN exhibit sustained firing levels in the absence of melanopsin, and across a broad range of wavelengths. The relative contribution of melanopsin, rods, and cones remains to be determined, and is most likely dependent on the intensity and wavelength composition of the light source. They suggest that at lower to intermediate irradiances, **classical photoreceptors play a role in transmitting light information to the SCN.** At low light intensities, only rods are stimulated and clear tonic responses are observed in the SCN. At high light intensity (rod saturation) the authors could see an increment in SCN light-response levels, presumably mediated by cones. Also, a switch from nocturnal to diurnal activity (output of the clock) was observed in wild type mice under light/dark cycles with scotopic levels of light, suggesting **that the state of photoreceptors can play an important role in determining phasing of activity** in diurnal and nocturnal species (Doyle et al. 2008).

Shuboni et al have demonstrated that *Arvicanthis niloticus* (the closest known species to our model) displays a positive masking dominant phenotype which translates by a strong behavioral response to light during the dark phase (locomotor activity)(Shuboni et al. 2012). Cones and rods are the cells involved in this phenomenon in mice (Mrosovsky et al 1999; Redlin and Mrosovsky 1999) have argued that the retina and brain anatomical substrates for the difference between positive and negative masking in nocturnal rodents are respectively the photoreceptors and the intergeniculate nucleus. In summary, the diurnal rodent behaves differently from the nocturnal rodent in that is strongly dependent on light. **The mechanisms mediating positive and negative masking pathways, in the retina and central brain, have not been investigated in a diurnal species.**

V.B.5.b Perspectives:

Our work has focused on melatonin in the retina of the diurnal rodent *Arvicanthis ansorgei* and our results highlight cones and GCL (including ipRGCs) as essential cells for melatonin synthesis and action. Thus, two interesting questions already mentioned in the "photoentrainment of the master clock" part are the following:

Do more cones imply "diurnal" information sent to the central brain? What is the ipRGCs projection site involved in the temporal niche integration?

(Bobu and Hicks 2006; Karnas et al. 2013) have demonstrated that *Arvicanthis ansorgei* possesses a large cone contingent and M1-type ipRGCs that display a higher sensitivity to light. The logical question to ask is whether the elevated number of cones combined to a higher number of M1-type ipRGC might reflect specific adaptations of the diurnal animal regarding light irradiance over time (visual and non visual circuits, aka circadian behavior). We could try to answer this question by exposing animals to high irradiance light (masking protocol of 1-3 hours light during the dark phase)

or the reverse expose the animals to darkness (masking protocol of 1-3 hours darkness during the light phase) in order to measure SCN phase (locomotor activity, clock genes) and validate or not the upstream place of the temporal niche switch (retina) compared to the Master clock.

However, to examine the mechanism and the recruitment of the cones and ipRGCs, we should put *Arvicanthis ansorgei* retinas explants in culture to do MEA analysis with light or dark stimulation and measure cell electrical activity. We could then see if the cones are massively connected to the M1-type ipRGCs and what type of activity it elicits. Moreover, Gompf et al have reported that glutamate transmission from the ipRGCs is necessary for normal light entrainment of the SCN at moderate light levels, **but residual transmission-possibly by PACAP in ipRGCs or by other RGCs can weakly entrain animals, particularly at very high light levels** (Gompf et al. 2015). It would be interesting to discriminate the ipRGCs and/or other GCL involved in the high irradiance conditions in the retina of *Arvicanthis ansorgei*. Co-localization between M1-Type cells at least and PACAP/glutamate markers could be performed.

Finally, anterograde tracing studies in *Arvicanthis niloticus* showed reduced ipRGC innervations of the vLGN compared to nocturnal rats (Todd et al 2012). Anterograde tracing studies should be performed in *Arvicanthis ansorgei* to identify precisely if M1-type cells are indeed projecting to structure like vLGNs that could treat the light information in a diurnal fashion.

V.B.6. Blue light, melatonin and ipRGC:

Blue light has become the new cutting edge challenge as it has invaded human life as well as other life beings (animals, plants) and is disturbing basic physiology such as sleep/wake cycle, circadian timing or alertness with consequences on human health (Chang et al. 2014; Schmoll et al. 2011). Indeed, blue light is present in public lightings and most of all electronic device screens and stimulates, via the retina, brain areas in charge of alertness, wakefulness (Silver and Kriegsfeld 2014). Therefore, retina is central in the translation of light information into behavior. Central brain wiring and behavior tests will not be discussed although it is a whole interesting research project but we will rather focus on the interaction between light, melatonin and ipRGCs in the diurnal model *Arvicanthis ansorgei* and other appropriate models.

V.B.6.a Does melatonin modulate light message transmission to the SCN?

In *Arvicanthis ansorgei*, melatonin is produced in the cones and GCL. Cone melatonin synthetic pathway resembles a circadian clock output (as described in other species (McMahon et al 2014) stronger during the dark period and could help dark adaptation of the retina. However global retinal melatonin is present throughout the 24 hours and this raises the question of another function. GCL appears to produce little but constitutive melatonin even though MT_1 are present on rods, OPL, INL, IPL and GCL.

V.B.6.b Working hypothesis:

Our finding echoes the prevalence of clock protein expression in cones and ipRGCs of the mouse retina (Liu et al 2012). A recent study has demonstrated the involvement of ultraviolet sensitive

(UVS) cones in ipRGC-SCN dialogue in response to UV light (Van Oosterhout et al. 2012). In addition, it is known that cone signals can be relayed to the SCN via pathways from ipRGCs in order to drive circadian photoentrainment (Tri Hoang Do 2010). Irradiance at dawn has been shown to provide an important cue for the entrainment of the mammalian circadian clock (Hattar et al 2002; Tri Hoang Do 2010). As melatonin is an output of the clock and is produced both in cones and GCL at night, it could participate in the communication between cones and ipRGCs. Moreover, MT_1 receptors are expressed in RGCs and INL of *Arvicanthis ansorgei* retina.

V.B.6.c Perspectives:

In vivo, intravitreal injections of pharmacological doses of melatonin or MT_1 inhibitors to *Arvicanthis ansorgei* could be performed in order to measure SCN outputs (locomotor activity, clock genes and clock controlled genes). ***Nrl*** mice (housed in the Chronobiotron) could be used as positive controls as they do not have any rod photoreceptors.

Does melatonin regulate melanopsin expression in ipRGC?

Melatonin receptors MT_1 are present in the GCL including ipRGCs. In addition, a recent study by (Sheng et al. 2015) report that MT_1 and MT_2 are only present on M1-type ipRGCs in mice. Moreover, in the light of our data (Bobu et al 2013) and (Gianesini et al 2015); *Aa-nat*/AA-NAT/ HIOMT/Melatonin/*Opn4* all display a rise at the night/day transition so they could be expressed in the same cells (Figure 47). This issue can be addressed by immunohistochemistry on 10 µm retina sections. We could perform co-localization between AA-NAT and melanopsin, HIOMT and melanopsin.

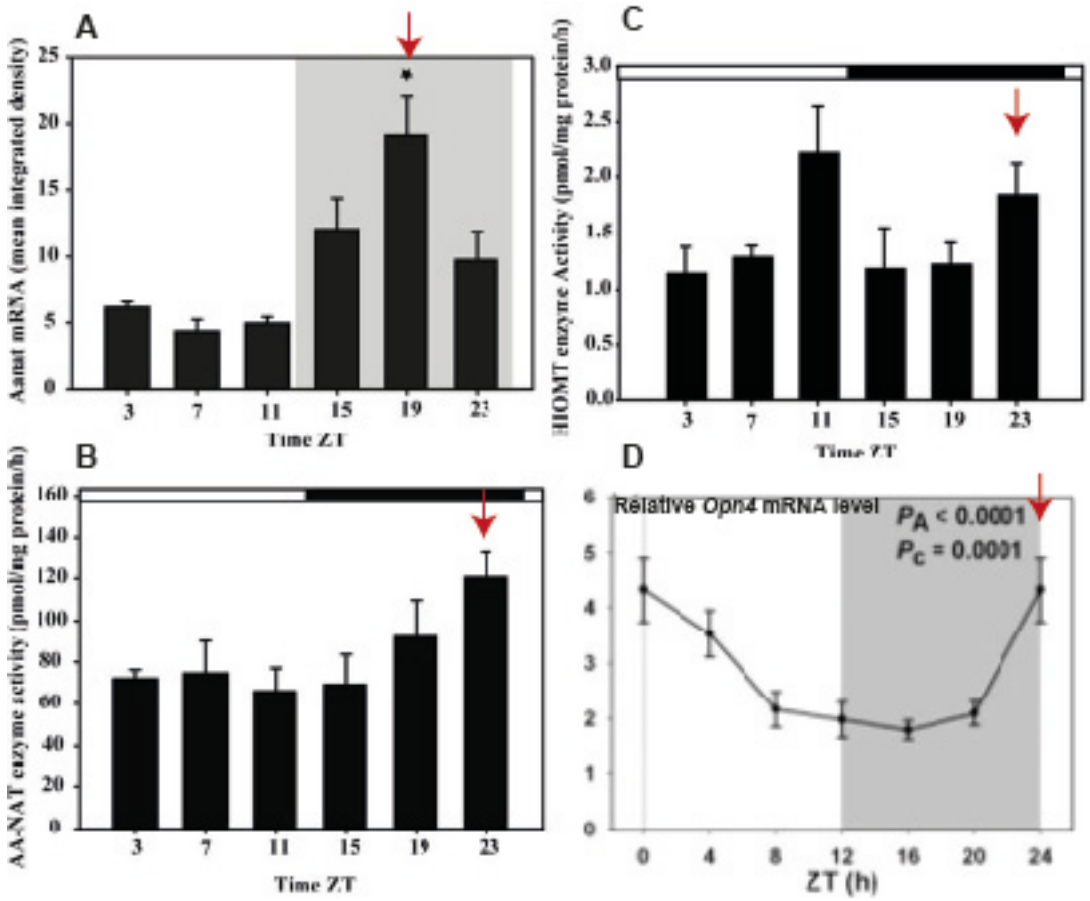


Figure 47: Daily expression of *Aa-nat*/AA-NAT, HIOMT, *Opn4* in the retina of *Arvicanthis ansorgei*.

Panel A: *Aa-nat* expression peaks at ZT19 and is followed by AA-NAT ativity maximum at ZT23 (**panel B**). **Panel C**: HIOMT activity is also elevated at ZT23 and *Opn4* mRNA expression is the highest at dawn. Red arrows indicate the time of maximum expression for each considered parameter.These data are extracted from (Bobu et al 2013; Gianesini et al 2015).

The best model to test the hypothesis would be the C3Hf^{+}/$^{+}$ MT_1 KO mouse because it suppresses the melatonin signaling and we can measure melanopsin expression. As for *Arvicanthis ansorgei* which appears to be the most relevant model compared to the human retina, we should separate the GCL from the other layers and makes cell culture. Melatonin could be applied to the GCL culture and the melanopsin profile assessed. On the contrary, the GCL could be treated with MT_1/MT_2 antagonists Luzindole or 4-PPDOT and then assess melanopsin gene/protein expression over 24 hours.

VI. Summary:

AA-NAT, HIOMT and melatonin are the three protagonists of the indolamine synthetic pathway but we may argue that they each possess a physiological significance of their own. Here are listed the main findings in *Arvicanthis ansorgei* retina discussed in this chapter:

- Phospho-AA-NAT and the chaperone 14-3-3 display a similar profile over the 24 hours in the whole retina. AA-NAT is therefore present and active day and night. AA-NAT is strongly regulated by light and the circadian clock as seen with LD and DD conditions.
- Post-translational events (Phosphorylation?) seem to regulate AA-NAT and HIOMT.

- AA-NAT may have a detoxification role in cones and have dopamine as a target in the INL (hypothesis to test).
- *Hiomt*/HIOMT is also expressed in cones and GCL of rat retina.
- Whole retina AA-NAT, HIOMT and Melatonin are present at low levels over the 24 hours but the immunohistochemistry study in each layer reveals a fine tuned regulation with at least 2 melatonin production sites: cones (night) and GCL (day) with opposed maxima.
- Melatonin synthesized in cones (with MT_1 on rods mainly) may represent the classical darkness signal and serves dark adaptation/increase visual sensitivity. Melatonin may also endorse the clock output role to regulate essential events such as photoreceptor phagocytosis (see chapter II). The neurohormone could modulate photoreceptor phagocytosis directly (MT_1/MT_2 receptors in RPE (Alarma-Estrany and Pintor 2007)) or indirectly via the rhythmic expression of dopamine. Dopamine is indeed involved in the photoreceptor phagocytosis (D_2RKO mice do not display the morning phagocytosis peak). The crosstalk Melatonin/Dopamine seems to exist in *Arvicanthis ansorgei*. Moreover, the work of Coralie Gianesini demonstrates the role of melatonin in cone survival (Gianesini et al 2016).
- Melatonin is also potentially a retinal clock messenger in the GCL. Melatonin synthesized by some ganglion cells including ipRGCs and possibly displaced amacrine cells is regulated differently from the ONL. HIOMT is always present and active. The circadian (DD conditions) analysis of HIOMT expression does not show a significant variation over the 24 hours. HIOMT half life is over 24 hours and it may be cell-specifically regulated (epigenetic regulation?) to perform melatonin synthesis even when light is present. Melatonin produced by ipRGCs may act in an autocrine/paracrine manner in the GCL and/or INL (MT_1 is distributed in INL, IPL and GCL).

II: Melatonin and photoreceptor phagocytosis

Part II: Melatonin and photoreceptor phagocytosis:

I. Does melatonin play a role on photoreceptor phagocytosis?

Retinal biology and especially photoreceptor physiology are clock dependent (Jaeger et al. 2015; McMahon et al 2014) in order to adapt to light variations over the nycthemeron. Melatonin, considered the body "time giver", modulates essential events in the retina: light sensitivity, rod phototransduction, electric activity rhythm, intraocular pressure regulation (Alarma-Estrany and Pintor 2007; Baba et al 2013; Peters and Cassone 2005; Wiechmann et al. 2003). In *Arvicanthis ansorgei*, our team has found that photoreceptor phagocytosis, a vital phenomenon for these cells, presents a rhythmic circadian profile (Bobu and Hicks 2009). Melatonin could regulate photoreceptor phagocytosis as previously suggested for other species (Besharse and Dunis 1983). **A major concern for human health is that phagocytosis disruption, notably its rhythm, may participate in these cells destruction and eventually lead to vision loss as seen with AMD** (Finnemann et al 2002; Rosen et al. 2009). Based on these data and in collaboration with Dr. Gianluca Tosini (Neuroscience Institute, Morehouse School of Medicine, Atlanta, GA 30310, USA), we have analyzed photoreceptor phagocytosis in the retina of $C3Hf^{+/+}$ MT_1KO or MT_2KO or WT mice to assess melatonin role on this vital process for outer nuclear photoreceptors.

First, the work of Ralph Jokers (Institut Cochin, Paris, expert on MT_1 and MT_2 structure and function) and of our collaborator Gianluca Tosini on the characterization of MT_1/MT_2 heteromer and associated signaling in the mouse retina is presented below. Thus, it is important to go back to the photoreceptor phagocytosis (= disk shedding) background with its regulation and to give a complete description of the RPE before discussing our data about the role of melatonin on photoreceptor phagocytosis.

II. MT_1/MT_2 heteromers in the retina:

The widely observed co-expression of MT_1 and MT_2 (in mouse, chicken and human) and the potential of both receptors to form heteromeric complexes in vitro are particularly interesting. A recent study reports for the first time the presence of the heterodimer in the mouse retina where they are responsible for the melatonin dependant increase in light sensitivity at night. MT_1/MT_2 heteromer is coupled to the G_i/cAMP pathway and a G_q/PLC pathway (Figure 48) (Baba et al 2013). Studies on mouse MT_1/MT_2 heteromers indicate a significant amplification of G_q/PLC pathway activation in cells co-expressing both receptors types with improved amplitude and $\mathbf{EC_{50}}$ values as compared to cells expressing MT_1 alone (tenfold difference) (Baba et al 2013). MT_2 receptor alone was not able to activate G_q/PLC pathway and MT_1/MT_2 cannot activated the pathway when MT_2 is inactive. The authors propose that in MT_1/MT_2 heteromer**, MT_2 can be considered as a positive allosteric regulator of MT_1 receptor in respect to the G_q/PLC activation** (Baba et al 2013). The propensity of MT_1/MT_2 heteromer and MT_1 homomer formation is similar, whereas MT_2 homomer formation is 3-4 fold lower, suggesting that the **MT_2 receptor preferentially exists as heteromeric complexes with MT_1 or as monomers** (Ayoub et al. 2004)

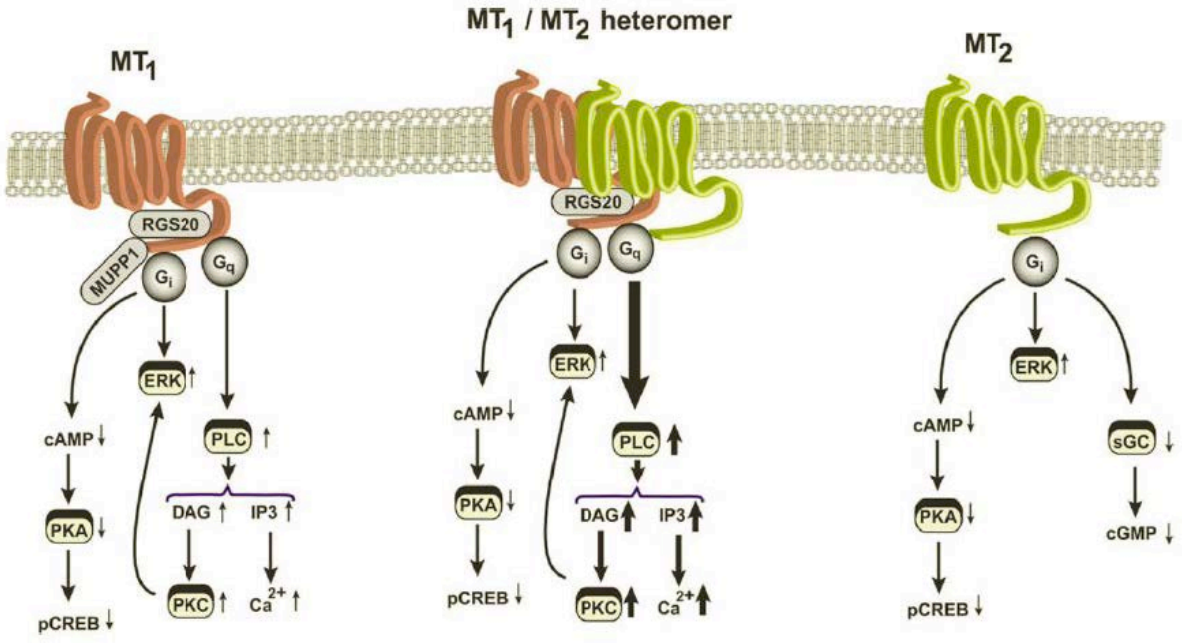


Figure 48: Melatonin receptor signaling in the retina.

Depending on the cells, different types of melatonin receptor complexes are present (MT_1 homomers, MT_2 homomers, MT_1/MT_2 heteromers) and different signaling pathways are activated upon melatonin stimulation. Thickness of the arrows represents the potency and efficiency of the pathway activation. DAG: diacylglycerol; ERK: extracellular signal-regulated kinase; IP3: inositol triphosphate; MUPP1: multi-PDZ domain protein 1; pCREB: phosphorylated-cAMP-response element-binding protein; PLC: phospholipase C; PKA: protein kinase A; PKC: protein kinase C; RGS20: regulator of G protein signaling 20; sGC: soluble guanylyl cyclase (Tosini et al 2014).

III. Photoreceptor phagocytosis /Disk shedding:

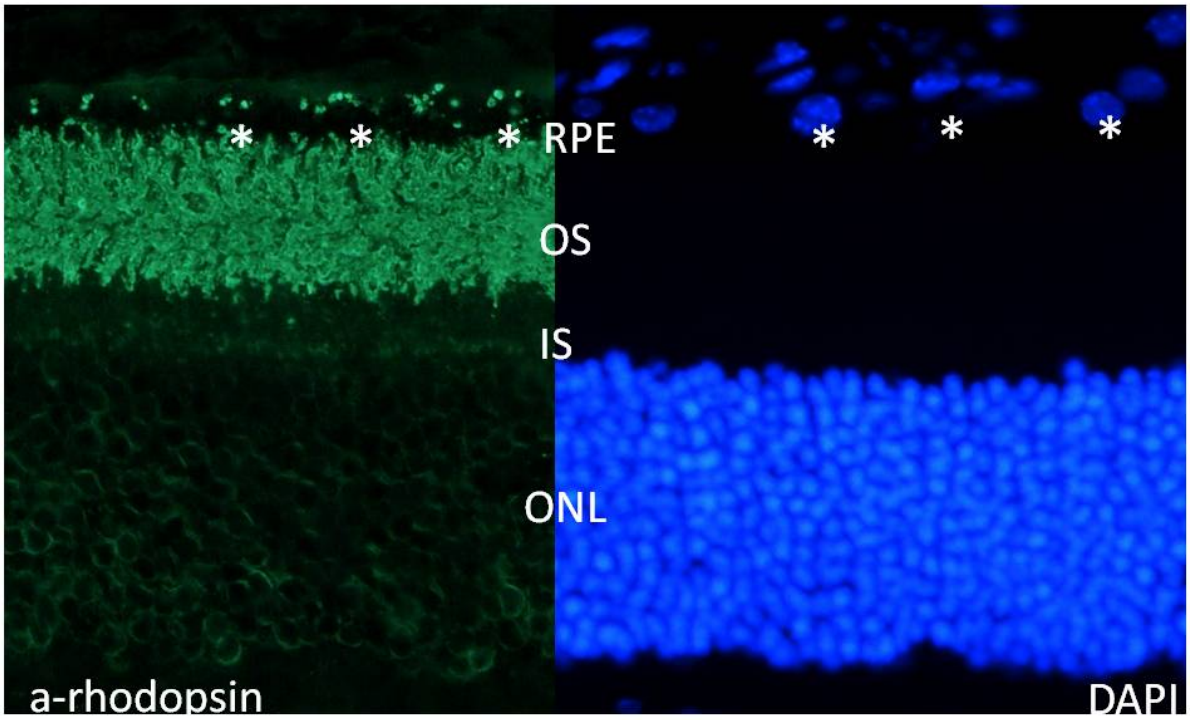


Figure 49: Photoreceptor phagocytosis in the mouse retina.

Left panel: numerous phagosomes, small green spheres above the white stars, are visualized with anti-rhodopsin (anti-Rho4D2) inside the RPE cells. **Right panel:** DAPI staining outlines the different cell layers of the retina. Stars indicate RPE nuclei. Immunostaining was achieved on 10µm transversal cryosections. Magnification: x400.

Each rod-type PR regenerates its OS within 10 days (Strauss 2005). PR turnover is composed of two balanced phases: an anabolic part involving RNA, transcription, protein synthesis, generation of new membranes; and a catabolic part consisting of removal of OS membrane, digestion and recycling of some cellular components. **This catabolic aspect corresponds to PR phagocytosis by the RPE. However it is also termed disk shedding because the process is driven both by the photoreceptor and the RPE.** Why is it happening? First, the complete replacement of the OS every 10-14 days serves to prevent secretion of the bisretinoid compounds that are precursors of RPE lipofuscin (granules that accumulate in the lysosomal compartment over time). Second, phagocytosis might represent a defense against chronic oxidative stress because PRs are submitted to light radiation, high level of oxygen and derivatives and possess a high amount of polyunsaturated fatty acids. Therefore to maintain the excitability of PR, the OS undergo a constant renewal process. OS are newly built from the base of outer segments, at the cilium. The tips of the POS that contain the highest concentration of radicals, photo-damaged proteins, and lipids are shed from the photoreceptors. Through coordinated OS tip shedding and formation of new OS, a constant length of the OS is maintained. Shed OS are phagocytosed by the RPE (Sparrow 2010; Strauss 2005) see Figure 49 above.

III.A. The retinal pigment epithelium:

The RPE is a monolayer of pigmented cells forming a part of the blood/retina barrier. The apical membrane of the RPE faces the photoreceptor outer segments (Figure 50). Long apical microvilli surround the light-sensitive outer segments establishing a complex of close structural interaction. With its basolateral membrane the RPE faces Bruch's membrane, which separates the RPE from fenestrated endothelium of the choriocapillaris (Figure 50).

As a layer of pigmented cells the **I)** RPE absorbs the light energy focused by the lens on the retina. **II)** The RPE transports ions, water, and metabolic end products from the subretinal space to the blood. **III)** The RPE takes up nutrients such as glucose, retinol, and fatty acids from the blood and delivers these nutrients to photoreceptors. **IV)** RPE takes part in the visual cycle. Indeed, retinal is transported to the RPE, reisomerized to 11-cis-retinal and transported back to photoreceptors, to maintain the photoreceptor excitability (Figure 50). **V)** Furthermore, the voltage dependent ion conductance of the apical membrane enables the RPE to stabilize ion composition in the subretinal space, which is essential for the maintenance of photoreceptor. **VI)** Finally, **the third aspect in the maintenance of photoreceptor excitability is the phagocytosis of photoreceptor outer segments (Figure 50). Vii)** In addition, the RPE is able to secrete a variety of growth factors helping to maintain the structural integrity of choriocapillaris endothelium and photoreceptors (Strauss 2005).

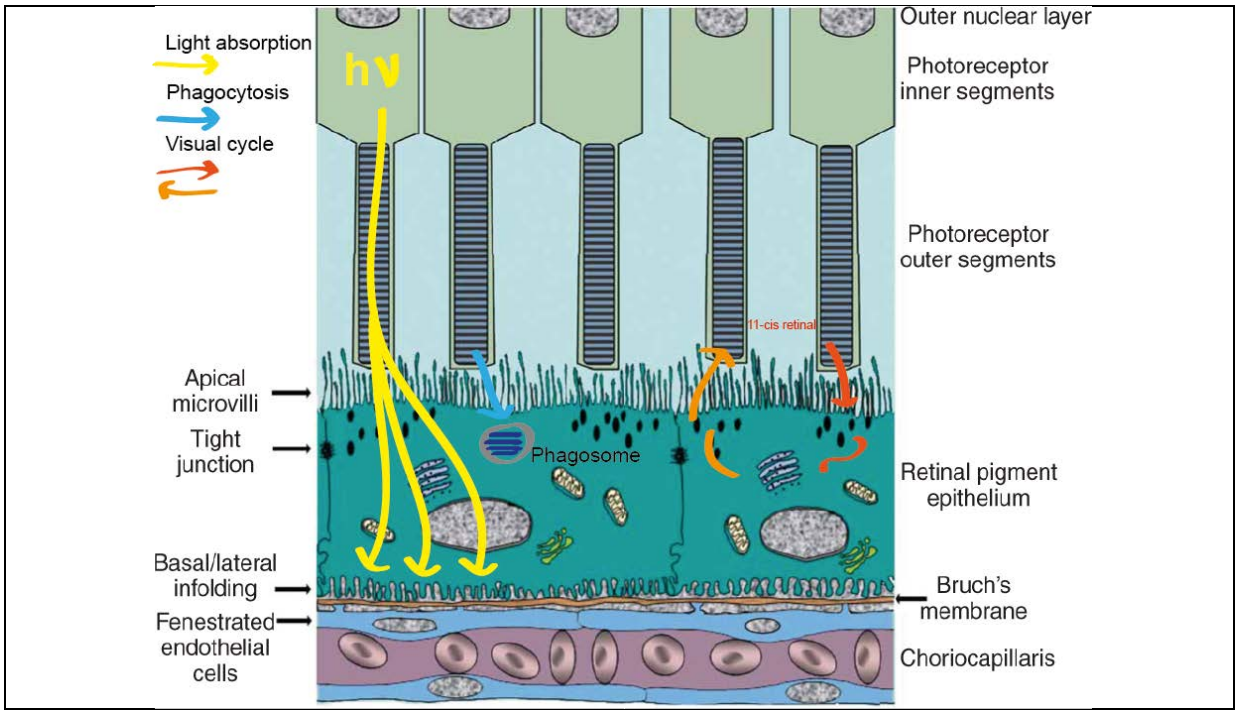


Figure 50: Diagram of the outer retina illustrating the polarized nature of the retinal pigment epithelium.

The RPE monolayer is close to the photoreceptor inner and outer segments, Bruch's membrane and the choriocapillaris. The RPE shows apical microvilli, tight junctions and basal/lateral infolding. The endothelial cells of the choroid are fenestrated. Colored arrows summarize the principal RPE functions. Modified from (Sonoda et al. 2009).

III.B. Photoreceptors and the disk shedding:

The stacks of discs containing visual pigment molecules in the outer segments of the photoreceptors are constantly renewed. New discs are added at the base of the outer segment at the cilium while old discs are displaced up the outer segment and are pinched off at the tips and engulfed by the apical processes of the pigment epithelium. The different steps of photoreceptor phagocytosis are illustrated on the schema below (Figure 51). These discarded discs become phagosomes in the pigment epithelial cells.

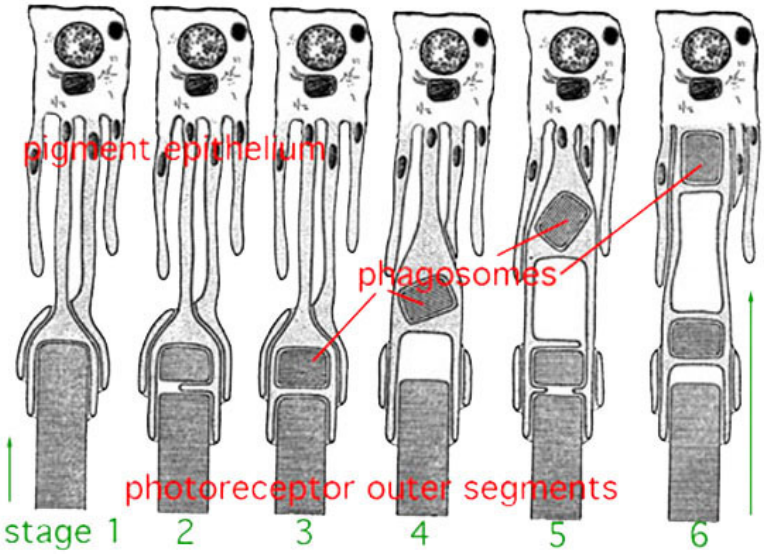


Figure 51: Representation of disk shedding and phagosome retrieval into the RPE by Helga Kolb.

The stacks of discs containing visual pigment molecules in the photoreceptor outer segments are constantly renewed. New discs are added at the cilium, the base of the outer segment. At the same time old discs are displaced up the outer segment and are pinched off at the tips and engulfed by the apical processes of the RPE. These discarded discs are phagosomes in the pigment epithelial cells. The phagocytosis/disk shedding event is circadian There is a burst of disc shedding at light on in the morning.

They are then broken down by lysis. Photoreceptor outer segment discs are phagocytosed by the pigment epithelium in a diurnal cycle. Cone outer segments differ from rod outer segments in several respects. Firstly, they are shorter and more conical with a wider base and tapering shape compared with those of rods. Secondly, as mentioned in the chapter I, their discs are connected to the plasma membrane throughout the extent of the outer segment, and thus they are open to extracellular space. Apical processes of the pigment epithelium phagocytize pieces of cone outer segments, just like they do the rod outer segments, but at a different time in the diurnal cycle compared with rods i.e. at light off-set compared light-onset (Besharse et al 1982).

III.B.1. Molecular Mechanism:

These molecular aspects have been established in the rod photoreceptor, so they may not be completely representative of cone phagocytosis.

The **initial step in phagocytosis is the specific binding of** OS at the apical membrane of the RPE. This triggers the activation of an intracellular second-messenger cascade, which in turn activates the ingestion of bound OS. **A mannose 6 phosphate receptor, CD36, αVβ5 integrin, mertk, CD81, L type calcium channel** (and MT_1/MT_2 which will be discussed hereafter) receptors have all been described as regulators of OS phagocytosis (references in (Sparrow 2010)). The macrophage scavenger receptor CD36 was found to regulate the rate of OS internalization. Binding of OS to the **receptor tyrosine kinase c-Mer (MerTK)** leads to phosphorylation and activation of the second-messenger cascade. Binding of OS requires the activation of αVβ5-integrin receptors which interact with MerTK via the FAK signaling pathway. In brief, OS binding to αVβ5-integrin receptors activates FAK, which in turn phosphorylates MerTK, leading to activation of the second-messenger cascade. Subsequent OS internalization coincides with dissociation of activated FAK from αVβ5. CD81, a tetraspannin appears to form a functional complex with αVβ5 integrin to facilitate OS binding. L-type calcium channels display bidirectional interaction with αVβ5 integrin.

Stitt and colleagues have identified the closely related growth-arrest–specific **protein 6 (Gas6)** and **Protein S (ProS)** as preferred **ligands for MerTK** (Stitt et al. 1995). Gas6 is necessary for OS phagocytosis. A third soluble identified ligand is **milk fat globule epidermal rowth factor 8** (MFG-E8) which exhibits high affinity binding for $\alpha V\beta 5$ (Hanayama et al. 2002).

Activation of different membrane-bound receptors triggers a variety of downstream signaling pathways, which play either direct or indirect roles in the phagocytic process (Figure 52). Briefly, activation of MerTK triggers the second messenger **inositol 1,4,5-triphosphate (InsP3)** cascade which increase phosphorylation of proteins and also causes a subsequent increase in intracellular Ca^{2+}. The **cAMP second messenger** system is an important modulator of the phagocytosis rate. Finally, downstream Annexin A2 could be involved in later stages since this downstream intracellular signaling molecule is necessary for phagosome processing to proceed (Law 2009).

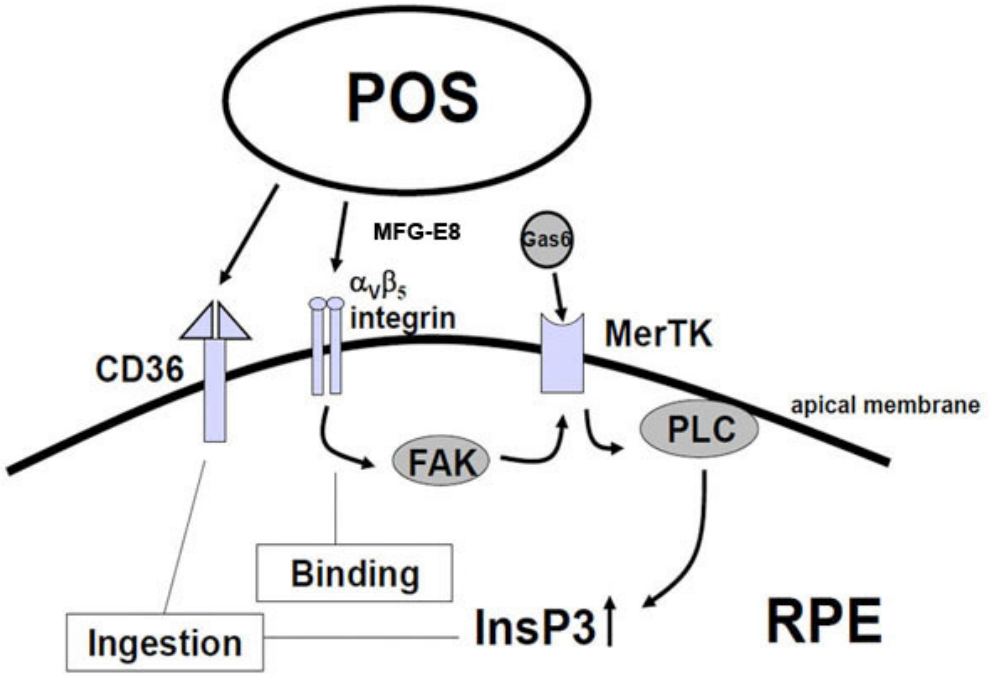


Figure 52: Molecular aspects of photoreceptor phagocytosis.

The initiation starts with binding of photoreceptor outer membranes. The event of binding is transduced into an intracellular signal, a rise in intracellular inositol 1,4,5-trisphosphate (InsP3), which in turn leads to ingestion of the bound photoreceptor outer segment membranes. Binding is likely mediated by integrins, and signal transduction occurs through the receptor tyrosine kinase MerTK. Ingestion involves the macrophage receptor CD36. Coordinate signal transduction occurs through integrin and MerTK interaction involving the focal adhesion kinase (FAK). CD36, macrophage phagocytosis receptor; FAK, focal adhesion kinase; Gas6, growth-arrest-specific protein 6; MFG-E8, milk fat globule EGF 8, another ligand for $\alpha V\beta 5$ integrin receptor;MerTK, receptor tyrosine kinase c-mer;PLC, phospholipase C; POS, photoreceptor outer segment. Modified from (Strauss 2005).

III.B.2. Phagocytosis regulation: light and clock roles

Disk shedding and phagocytosis occur every day in a synchronized fashion shortly after the onset of light for rod (Besharse et al 1977). There **is a burst of disc shedding at light on in the morning,** as

seen with the increased numbers of phagosomes in the pigment epithelium shortly thereafter (Young 1971). Interestingly, rod phagocytosis exhibits a marked morning rise in activity in every species examined: e.g., fish, frogs, birds, and mammals such as rats, cats and monkeys (references in (Sparrow 2010)). There is often a secondary smaller peak occurring during the night, and seems to correspond to clock activity "priming" the system to prepare for light onset and dawn. The rhythms of disk shedding and phagocytosis persist in constant darkness, indicating that they are under control of circadian clocks (Grace et al 1996, 1999; LaVail 1976; Teirstein et al 1980; Terman et al 1993). Experimental evidence indicates that the circadian clock controlling disk shedding is located in the eye, possibly in photoreceptors (Teirstein et al 1980; Terman et al 1993). However, it must be noted that the photoreceptors are not the sole controller of disk shedding since it has been reported that close proximity of photoreceptors and RPE is necessary before the disk shedding can occur (Matsumoto 1987; Williams et al 1987) and lysosomal fusion in the RPE can be triggered by light and circadian signals (Bosch et al 1993). A recently published article has shown that the diurnal rhythm in exposure of phosphatidylserine by rod outer segments, which appears to play role in disk shedding and phagocytosis, is not entirely controlled by the photoreceptors, but RPE cells participate in the synchronization of this process (Ruggiero et al 2012).

III.B.3. Melatonin and phagocytosis:

Although the mechanism is not deciphered yet, melatonin is thought to play a role in the morning phagocytic peak, since subcuteneous inplants containing melatonin increase phagosome numbers (White and Fisher 1989). On the other hand, strains of mice which do not synthesize retinal melatonin exhibit normal cyclic phagocytosis (Grace et al 1999). MT_1 activation decreases cAMP through adenylyl cyclase inhibition, while MT_2 inhibits both adenylyl cyclase and the soluble pathways(von Gall et al 2002). Moreover, MT_1 are present in rods of *Arvicanthis ansorgei* and photoreceptors of mice (Sengupta et al. 2011) and MT_1 and MT_2 are present on RPE cells (Alarma-Estrany and Pintor 2007).

Our team leader David Hicks is an expert on photoreceptor phagocytosis and is interested in the physiology of cones. He has assessed photoreceptor phagocytosis in *Arvicanthis ansorgei* retina under different lighting conditions. The diurnal animal retina goes through a typical morning peak of phagocytosis and a second smaller peak at night both regulated by a retinal clock and light (Bobu and Hicks 2006, 2009).

We then decided to test whether melatonin could influence photoreceptor phagocytosis in vivo. I learned and adapted the immunocytochemical technique to assess phagosome numbers as described in (Bobu and Hicks 2006). In collaboration with Gianluca Tosini who has produced the C3H $f^{+/+}$ MT_1KO and C3H $f^{+/+}$ MT_2KO (Baba et al 2009), we examined these mice and their WT controls for the rod photoreceptor phagocytosis.

III.B.3.a Quantification of phagocytosis during the LD cycle:

The eyes of C3HWT or C3H-$MT_1^{-/-}$ or C3H-$MT_2^{-/-}$ mice were collected every 3 hours over a 24h period and fixed for 8 hours in paraformaldehyde (PAF) 4% before being stored in PBS at 4°C. Three eyes per genotype and per time points were used for the following experiment. Prior to cryoprotection, eye balls were dissected into two parts at the optic nerve level removing cornea, lens and aqueous

humor. Both halves were then incubated, one hour each, successively in 10%, 20%, 30% sucrose PBS solutions. Each half-eye was individually included in Tissue-Tek ®OCT™ compound and frozen in liquid nitrogen before being kept at -80°C. The cryoprotected eyes were cut into 10 µm slices using a cryostat (Leica CM3050 S) prior doing immunohistochemistry in order to bring out phagosomes. For that purpose, slides with retina and RPE slices (4 for each genotype at every time points) were thawed, dried and sections were permeabilized 5 minutes in a 0.1% Triton X-100 solution (Sigma-Aldrich™). Slices were next immersed in blocking buffer (3% Bovine serum albumin, 0.1% Tween-20 (Euromedex®), 0.1% sodium azide in PBS) for one hour. Then, sections were incubated with blocking buffer and anti-rhodopsin antibody Rho4D2 at a 1/40000 dilution overnight at 4°C. The following day, slices were washed with PBS for one hour before the application of the secondary antibody Alexa 488 1/400 and DAPI 1/400 diluted in blocking buffer. Sections were thus incubated 2 hours and washed in PBS for 45 minutes. Slides were mounted in Glycerol/PBS 1:1 prior to observation under the fluorescence microscope.

Phagosomes count: DAPI use allowed precise visualization of each retina-RPE morphology while phagosome counting was rendered possible by **rhodopsin immunodetection**. For each mouse, 4 sections were analysed. Each section was divided into 21 grids of 150 µm on which phagosomes were visually identified and counted. Data presented are the means of the n° of phagosomes per 150 µm retinal section.

Results:

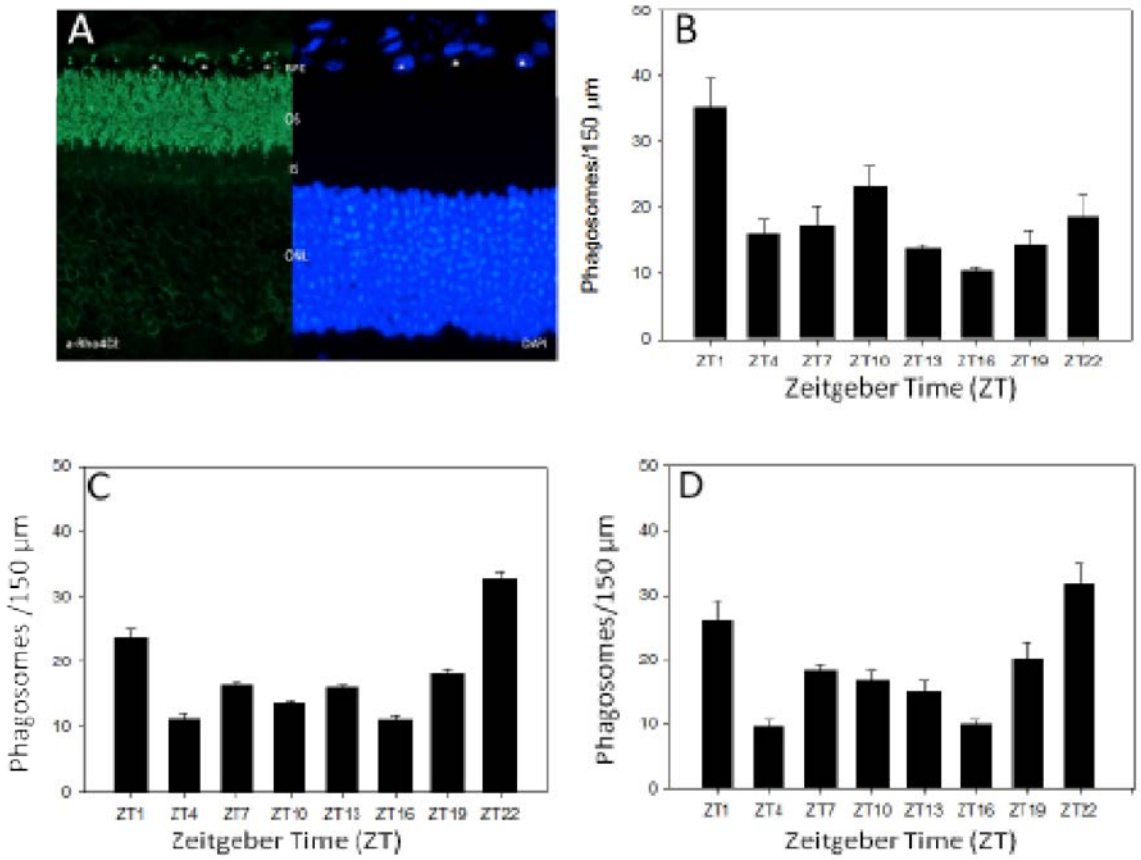


Figure 53: Representative photomicrographs of phagosomes counting in mouse RPE (A)

B) Daily rhythm of phagosomes number measured in WT (C3H-$f^{+/+}$); $MT_1^{-/-}$ (C) and $MT_2^{-/-}$ (D) mice at 8 different Zeitgeber times (ZT). A daily rhythm of phagosomes number is present in all three genotypes (one-way ANOVA, P< 0.01, in all cases. N=4-6 for each time point). In WT mice, the peak of phagosomes number occurred at ZT1 (i.e., 1-hr after light onset) whereas in $MT_1^{-\backslash-}$ and $MT_2^{-\backslash-}$ mice the peak occurred 3 hr earlier (ZT22), when the mice were still in darkness. Results are presented as mean +/- SEM. (Bobu and Hicks 2006).

Quantification of phagosomes as a function of the time of the day showed that in C3H-$f^{+/+}$ phagocytic activity peaks 1 hour after light onset (ZT1; Figure 53 B). Phagosome counts are lower for the other points, reaching a minimum in early night (ZT16; Figure 53 B). **In both $MT_1^{-/-}$ and $MT_2^{-/-}$ mice the peak of phagocytic activity occurs at ZT22 (i.e., 3-hrs before than in C3H-$f^{+/+}$)** while the minimum is observed at ZT16 (Figure 53 C, D). Summation of phagosome counts for each measured time point over the entire 24-hour period demonstrates that the total mean phagocytic activity does not change among the three genotypes (C3H-$f^{+/+}$ = 18.91+/- 1.669 (SEM); $MT_1^{-/-}$ = 18.47 +/- 1.42; $MT_2^{-/-}$ = 18.17 +/- 1.42; ANOVA, P > 0.9). These data suggest that lack of MT_1 or MT_2 receptors affects the timing of the daily rhythm in phagocytosis. As mentioned above, Coralie Gianesini's data with the same mouse

strains support a role for melatonin in cone viability during aging (article in preparation). In addition, D2RKO mice display no rhythm of phagocytic activity. **All together, melatonin could modulate the phase of phagocytosis via the hereromer MT_1/MT_2 while dopamine could control the amplitude of phagocytosis via the D_2R. Finally, lack of melatonin receptors in aging mice is associated with an increase in cell death. Melatonin is thus important for normal photoreceptor maintenance.** These data are part of a complete work on melatonin and photoreceptor phagocytosis that will be published with Gianluca Tosini.

III: Future studies

Part III: Future studies

I. Melatonin Receptor Function in Neural Circuits in the Retina

(MElatonin Receptors and Retinal RhYthms: MERRRY)

I have written this project with another team member Marie-Paule Felder-Schmittbuhl, PhD researcher and Dr. Christophe P. Ribelayga, Ph.D. Assistant Professor of Ophthalmology and Visual Science; University of Texas Medical School at Houston, Texas, United States of America. This project was submitted to the ANR-PRC call on October the 15th of 2015. If the project gets financial support, I will be in charge of the first objective and participate to the second led by Marie-Paule Felder-Schmittbuhl. Christophe P. Ribelayga, an expert on retinal electrophysiology will perform experiments described in the third scientific objective.

I.A. Scientific objectives and methods

The leading cause of blindness and low vision in industrialized countries are primarily age-related eye diseases such as age-related macular degeneration and glaucoma (one million people affected in both cases, in France). Understanding normal retinal function as well as the modifications that occur in the retina as a result of aging is essential if we want to develop therapeutic interventions that may help slow down or even reverse the pathological aging processes. It has long been known that the neurohormone melatonin, which is locally synthetized and released in the retinal tissue, is a key player in the control of retinal function and a potent modulator of photoreceptor death (Iuvone et al. 2005; Tosini et al 2012). Melatonin production is rhythmically controlled by a retinal circadian clock and high at night. Its rhythmic actions on retinal function manifest as daily rhythms of one or more components of the electroretinogram (Baba et al 2009, 2013). The G-protein coupled melatonin receptors MT1 and MT2 have been identified in most retinal nuclear layers and shown to mediate the actions of melatonin on retinal function. In addition, it has recently become clear that melatonin signalling displays trophic actions on retinal neurons. Knocking out MT1 receptors leads to an age-related decrease in photoreceptor cells (Baba et al 2009) and increases intraocular pressure and retinal ganglion cells death (Alcantara et al 2011). Melatonin also modulates phagocytosis of photoreceptor outer segments (White and Fisher 1989). Thus, melatonin signalling is required for normal function and maintenance of the retina. Yet, at present, little is known about the precise cellular distribution of melatonin receptors in the retina or of their contribution to the modulation of specific neural circuits in the retina or of the retinal clock activity.

Specific Objective 1: Obtain a precise cartography of melatonin receptor distribution in the retina. To date our knowledge of the expression pattern of melatonin receptors in the rodent retina is based on *in situ* hybridization and immunohistochemical studies ((Baba et al 2009, 2013; Fujieda et al. 1999; Gianesini et al 2015). We have developed new animal models in which *LacZ* has been knocked in the *mt1* (*Mtnr1a-LacZ*-KI) or *mt2* (*Mtnr1b-LacZ*-KI) gene. We will use *LacZ* as a reporter together with appropriate retina cell markers to precisely map the cell types that express melatonin receptors.

Specific Objective 2: Determine the role of melatonin signalling in retinal clock function. Previous studies have shown that melatonin signalling significantly affects the pattern of clock gene expression in the retina; an effect that is however different depending on the retina layer ((Dinet and Korf 2007; Hiragaki et al. 2014). We have developed a unique tool to assay clock gene expression and clock activity in individual or paired retina cellular layers (Jaeger et al. 2015). We will use this tool to characterize the effects of melatonin on retinal clock function. In particular, we will evaluate the robustness of the clock in the absence of melatonin signalling using our mice with targeted deletion of melatonin receptors.

Specific Objective 3: Characterize the role of melatonin in the circadian control of photoreceptor physiology. Previous studies have shown that the light response kinetics of photoreceptors and the strength of electrically coupling between photoreceptors are modulated by a circadian clock (Jin et al. 2015; Ribelayga et al 2008; Zhang et al. 2015). The involvement of melatonin in this process remains elusive as it is possible that melatonin acts primarily by suppressing dopamine, which in turn modulates the gap junction conductance between photoreceptors (Li et al 2013). In addition, melatonin may act directly on the photoreceptors through its receptors (Baba et al 2013) and modulate the gap junction conductance and/or the photoresponse. We have developed unique electrophysiology tools to record the light responses and gap junction conductance of mouse photoreceptors (Jin et al. 2015). We will use these tools to determine the role of melatonin in the circadian regulation of photoreceptor coupling and/or light responses.

Specific Objective 1: Obtain a precise cartography of melatonin receptor distribution in the retina.

Anatomical distribution of MT1 and MT2 receptors has been extensively investigated using 2-[^{125}I] iodomelatonin binding, *in situ* hybridization and immunohistochemistry (Wiechmann and Summers 2008) for review. In the rodent retina, melatonin receptors are expressed in all three nuclear layers (Baba et al 2009; Fujieda et al. 2000; Gianesini et al 2015; Sengupta et al. 2011). To identify precisely which cell types in the retina express melatonin receptors, we have developed a genetic approach, a strategy that has not been used so far in this field. We have created animals in which the reporter *LacZ* has been knocked in the *Mtnr1a* or the *Mtnr1b* genes (*Mtnr1a-LacZ*-KI and *Mtnr1b-LacZ*-KI mice; ES cells from KOMP-Velocigene - UC Davis). We will precisely map the expression sites of the X-GAL staining or of immunolabelled LacZ using appropriate retina cell markers. We have validated the approach by confirming the major sites of expression of *Mtnr1a* gene in the adult brain (Klosen et al., unpublished data). In addition, we will generate new conditional knock in mice, in which the *Cre* recombinase will be inserted into the *Mtnr1a* or the *Mtnr1b* locus. Upon breeding with a lox-P-flanked reporter line, this strategy is expected to enhance the signal-to-noise ratio of the expression of the reporter and allow refinement/confirmation of the distribution sites of the receptors. To overcome the problem of broad expression of the receptors during prenatal development (Davis 1997), we will use the Cre-ER$_{T2}$ recombinase for knock in into the *Mtnr1a* (presently under generation) or the *Mtnr1b* locus. In this case, CRE activity will be induced by tamoxifen in the adult to drive expression of *Cre* in melatonin receptor expression sites and, upon prior breading with a reporter line, to allow the detection of the reporter in melatonin receptor expression sites. All these studies (including objectives 2 and 3) will be performed on a melatonin-proficient background requiring prior backcrossing of the genetic models. *This specific objective will provide new data on the identity of the cells that express melatonin receptors in the retina.*

Specific Objective 2: Determine the role of melatonin signalling in retinal clock function.

Melatonin signalling plays a central role in the genesis of circadian rhythms in the retina (Iuvone et al. 2005; McMahon et al 2014) for reviews. Yet, in the absence of a melatonin rhythm or when photoreceptors (which are the primary locus of melatonin synthesis) are lost, the retinal clock mechanism remains functional, as evidenced by the robust *in vitro* rhythms in bioluminescence observed in *mPer2*$_{Luc}$ retinal explants from melatonin-deficient (C57Bl6) mice (Jaeger et al. 2015; Ruan et al. 2008) or from mice in which the photoreceptors have degenerated (*rd1* mice (Ruan et al 2006)). However, recent studies have suggested that melatonin signalling modulates the pattern of clock gene expression in the retina (Dinet and Korf 2007; Dinet et al. 2007; Hiragaki et al. 2014). These studies also suggested that the effects of melatonin on clock gene expression are different depending on the retinal layer. To further evaluate the role of melatonin in the retinal clock activity, we will cross the *mPer2*$_{Luc}$ mouse line with either the *Mtnr1a-LacZ*-KI or the *Mtnr1b-LacZ*-KI line. We will compare the phase and amplitude of the *mPer2*$_{Luc}$-generated rhythms of bioluminescence in wild type mice and in mice with targeted deletion of melatonin receptors. This approach will be complemented with precise pharmacological manipulations of melatonin synthesis and/or signalling. We will use whole retinas but will extend our analysis to individual nuclear retina layers we can obtain with a vibratome technique (Jaeger et al. 2015). Comparing results on whole retinas withthose obtained on individual layers will help us determine how melatonin affects clock activity within each layer but also how melatonin controls the overall retinal clock activity (interlayer/intercellular). We will also analyse single-cell oscillatory capacity using transversal retina sections and imaging coupled to bioluminescence (Luminoview, Olympus). *This specific objective will generate new data on the effects of melatonin signalling on clock activity within single layers and single cells in the retina.*

Specific Objective 3: Characterize the role of melatonin in the circadian control of photoreceptor physiology.

Recent studies have suggested that the strength of electrical coupling between photoreceptors changes between day and night (Jin et al. 2015; Li et al 2013; Ribelayga et al 2008; Zhang et al. 2015). In addition, the light responses of photoreceptors also vary according to the time of day, although it is still unclear whether the daily variations are indirectly related to a change in coupling or due to a direct modulation of the photoresponse (Jin et al. 2015; Ribelayga et al 2008). Circadian rhythms in photoreceptor coupling and photoresponse kinetics are present in melatonin-proficient mouse strains (Jin et al. 2015; Katti, Butler, and Sekaran 2013; Ribelayga et al 2008; Zhang et al. 2015) but are absent in melatonin-deficient strains (Katti, Butler, and Sekaran 2013; Li et al 2013), supporting a role for melatonin in these processes. Also in support of a role for melatonin in the control of photoreceptor function, melatonin receptors are expressed in both rods and cones (Baba et al 2013). However, the effects of melatonin on photoreceptors may also be indirect and mediated by dopamine. Indeed, melatonin suppresses dopamine release and thereby generates a dopamine rhythm with low levels of retinal dopamine at night (McMahon et al 2014). Dopamine modulates photoreceptor coupling and light responses through dopamine D4 receptors expressed in photoreceptors (Jin et al. 2015; Li et al 2013; Ribelayga et al 2008). Here we propose to take advantage of our mice with targeted deletion of melatonin receptors to clarify the role of melatonin in the regulation of photoreceptor coupling and/or light response properties. Specifically, we will use a pharmacogenetic approach and test the effects of exogenously applied melatonin on rod-rod, rod-cone and cone-cone coupling and rod and cone light responses in the presence or absence of

pharmacological inhibition of dopamine effect (i.e. dopamine D4-antagonist), in wild type mice and in our mutant animals. We have recently developed the tools to record from single or pairs of mouse photoreceptors (Jin et al. 2015). Based on preliminary data (Ribelayga and Jin, unpublished data), we hypothesize that most of the effects of melatonin on photoreceptor behaviour are mediated by dopamine. We will thus also test the effects of exogenous dopamine on photoreceptor coupling and light response properties in our mutant model to determine whether the presence of melatonin receptors is required for dopamine receptor function. Nothing is known about the potential cross-talk between these 2 types of receptors and this would represent a new aspect of the interactions between the melatonin and the dopamine systems. *Results from this objective will provide new data on the role of melatonin in the control of information processing in the retina.*

I.B. Significance and general impact

The proposed research is innovative and will be of great impact. Specifically, our work will reveal the precise identity of the retinal cells that express the melatonin receptors. Moreover, the highly innovative genetic approach that we have developed here with the creation of the *Mtnr1a-LacZ*-KI and *Mtnr1b-LacZ*-KI lines will be used to answer critical questions of physiology. It is known that melatonin, which is an output of the retinal clock, in turn affects retinal clock function and that the effects of melatonin are heterogeneous throughout the layers. We will take the analysis of the effects of melatonin on clock function from the tissue level down to the layer and to the cellular level. Our experiments will provide unprecedented detail on how melatonin controls clock activity within single retinal cells but also how melatonin contributes to the coupling of clocks within different cell types/retinal layers. Finally, we will characterize the role of melatonin in the control of a specific retinal circuit: the network of coupled photoreceptors. As melatonin signalling is critical for photoreceptor survival, understanding the normal function of melatonin in this particular network is essential. The project is based on the joined, transnational efforts from highly competent, complementary scientific partners and the combination of outstanding ex vivo and in vitro approaches in dedicated/novel mice models. It constitutes an unprecedented opportunity to understand how vision is organized by the dynamic interaction of cell-specific autonomous oscillators and the role that melatonin and its receptors play therein. Given the central role this molecule plays in retina biology, it is expected that yielded results also provide fundamental knowledge about potential visual effects linked to manipulation of the melatonin system in patients.

II. Intrinsically photosensitive ganglion cells, melatonin and photoperiod in the rodents

A Master 2 student in neuroscience: Bastien Leclercq; has been recruited to perform the following study from January to June 2016 which anticipate a thesis project on photoperiod integration by the mammalian retina and its axonal projections.

Besides retina, melatonin is synthesized at night by the pineal gland with a strong daily rhythm. The timing of its production correlates with the duration of the period of darkness. Thus melatonin signaling is believed to be the internal signal by which the organisms may perceive the seasonal changes in the photoperiod and regulate the reproductive cycles. In the seasonal mammals,melatonin acts on the pituitary *pars tuberalis* to influence TSH production which in turn

regulates the seasonal rhythm of reproduction (e.g., hamsters, sheep, and many others), and transduces the seasonal changes (Revel et al 2009; Simonneaux et al. 2013). However, the mechanisms by which seasonal variations of photic information are transmitted to the central brain are unknown. Intrinsically photosensitive retinal ganglion cells (ipRGCs) have been shown to project to the suprachiasmatic nuclei (SCN) for mediation of circadian photoentrainment (Schmidt and Kofuji 2011) and pineal melatonin secretion (Larsen 1998). Thus, the question we aim to address is the following: **Do ipRGC play a role in integration of photoperiodic information in mice?** Although the reproductive system of the mouse is generally thought to be non sensitive to photoperiod, the rodent can perceive these seasonal light variations. Indeed, mouse retino-hypothalamic tract is similar to those of photoperiodic species (Youngstrom and Nunez 1986) and even common laboratory strain C57/Bl6 mice display photoperiodic changes in depression-like behavior and brain serotonin content (Otsuka et al. 2014). The master 2 project will include the following steps: 1) C3H melatonin proficient mice will be used as a model to examine ipRGCs. Indeed, the C3H strain (Komeima et al. 2006) harbors the *rd1* mutation (retinal degeneration 1, cGMP-dependent phosphodiesterase) and within few weeks mice lose >99% rod and ~90% cone photoreceptors, leaving only ipRGC to mediate photoreception. Groups of adult (3 month) animals housed in short photoperiod (8 hours white light, 16 hours dark) or long photoperiod (16 hours white light, 8 hours dark) will be examined for their pineal melatonin profile. Serum samples (30 µl) will be collected every 2 hrs across the L/D cycle by the "tail snip" method. 2) Immunohistochemistry will be performed on the retinas to monitor ganglion cell number as it was reported to vary in CBA/J mice with *rd1* (Ruggiero et al. 2010). 3) In parallel, we will use an immunotoxin-based approach to specifically disrupt ipRGC function (Targeted destruction of photosensitive retinal ganglion cells with a saporin conjugate alters the effects of light on mouse circadian rhythms (Göz et al. 2008)), to analyze photoperiodism integration in similar groups of mice to those stated in step 1. Similar experiments (same age, short and long photoperiods) will also be performed in melatonin-proficient mice strains (CBA/CaJ) with normal retinal structure (ie. presence of all three photoreceptor populations) for comparison. Melatonin measurement will then be achieved by ELISA. 4) We will make retinal explants of these various mouse models to assess ipRGCs functional response to light using the "multi-electrode array" approach. We will record ipRGCs neuronal activity in presence or not of melatonin. Then, in order to determine which retinal cells project to SCN, retrograde labeling by cholera toxin injection into the SCN will be conducted. RGC markers (melanopsin, Brn3a-c, Thy1, NF-200) will be used to identify back-labeled cells. These data will determine whether ipRGCs control photoperiodic changes.

III. Melatonin and Alzheimer's disease

III.A. Background:

The retina is a second major site of melatonin synthesis, but the role(s) of melatonin within the eye remain only partly understood. In the retina, melatonin can exert local effects on neuronal electric activity (Sengupta et al. 2011), photoreceptor phagocytosis (Besharse and Dunis 1983), and light sensitivity (Baba et al 2013), by way of specific MT_1 receptors in photoreceptors, inner nuclear layer and ganglion cells (Sengupta et al. 2011).We have been studying melatonin regulation in the cone-rich diurnal rodent *Arvicanthis ansorgei* and our results indicate that cones possess the synthetic machinery (Chapter I(Gianesini et al 2015)). Cone photoreceptors are essential to human eyesight,

enabling high acuity chromatic vision (Harrison 1953; Otake, Gowdy, and Cicerone 2000). Cone degeneration is a major underlying cause of human blindness and is associated with pathologies developing during aging (such as **Alzheimer's Disease, AD**. (Finnemann et al 2002). Melatonin could be involved in such conditions in several respects: i) studies in non-primate mammal suggest that melatonin could protect the retina, promoting cell viability (Baba et al 2009; Yi et al. 2005); ii) Perturbed circadian regulation of melatonin profiles seem to affect aged people health (Jenwitheesuk et al. 2014). A unique study on human AD retinas claims that MT1 is up-regulated in ganglion cells of AD patients; but down-regulated in photoreceptors (Savaskan et al. 2002). These findings are in opposition to the disappearance of cells observed in the retina of AD and glaucoma patients with an increase in MT_1 expression.

III.B. Aim of the study:

Our aim with the present study is to examine the issue in human retina, using newly developed molecular probes. Namely, we shall endeavour to determine expression of MT_1 and the limiting enzymes of melatonin synthesis in the retina of different AD patients and controls. These data will help establishing the changes in melatonin regulation occurring during the progression of AD. We will use a new antibody raised against MT_1, compared with the ones used in all previous studies (Sengupta et al. 2011) and others against key melatonin synthetic enzymes. We will also use specific cell markers (rhodopsin or cone transducin for the photoreceptors, melanopsin or *Brn3* for ganglion cells) to see whether melatonin regulation is correlated with disappearance of distinct populations. Thus, we will use fixed ocular tissue from a series of AD donors at different stages of the disease (and at least one control non-AD retina). Samples have been obtained upon scientific project application from Dr.Thomas Beach, Head of the Pathology service, Banner Sun Health Research Institute, Sun City, AZ, USA thomas.beach@sunhealth.org. Firuzeh Rajabian, a PhD student in our team, possesses additional training on human vision and will assist me for the experiments.

The role of melatonin in retinal aging and disease: perspectives

In *Arvicanthis ansorgei*, we have deciphered a specifically regulated melatonin pathway occurring in cones and ganglion cells depending on light and clock controls. We have preliminary results showing that melatonin is important for neuron survival with aging (Gianesini et al 2016) adding to the previous results about retina resistance to drug damage (Boudard et al. 2010). We may argue that both *Arvicanthis* cone-rich structure and its specific melatonin synthesis regulation may not only reflect the retina physiological adaptation to light irradiance over time but also a way to study neuroprotection of the macula in a closer model to primate than the commonly used nocturnal rodents.

Partie IIIbis : Etudes futures

I Introduction: La mélatonine dans la rétine des mammifères

Les pathologies de l'œil conduisent à la cécité et touchent des centaines de millions de personnes dans le monde (Rosen et al. 2009), représentant un enjeu colossal de société et de santé (à titre d'exemple : 1 Million de gens sont touchés par la dégénérescence maculaire liée à l'âge en France (DMLA)). La rétine possède deux types de photorécepteurs essentiels pour la vision : les cônes et bâtonnets. Les batônnets sont 1000 fois plus sensibles que les cônes et sont indispensables pour la vision de nuit. De plus leur prédominance (120 millions contre 6 millions de cônes dans la rétine humaine ; 97% des photorécepteurs totaux chez la souris) a fait que l'essentiel des informations expérimentales concerne ce type de photorécepteurs. Or ce sont les cônes qui déterminent la vision photopique, sont responsables de la discrimination des couleurs, et assurent une haute acuité visuelle chez l'homme. En outre, les personnes âgées sont très affectées par la DMLA, due à une inflammation chronique de la choroïde qui se répercute sur la santé de la macula riche en cônes. Les enjeux cliniques des pathologies rétiniennes dépendent donc directement des cônes et la prévention de leur perte constitue un objectif essentiel dans la mise en place de traitement. Pour y parvenir, la compréhension approfondie de leur biologie normale est incontournable. La mélatonine, hormone synthétisée et sécrétée localement dans la rétine est connue pour participer à des fonctions essentielles de ce tissu (Iuvone, 2005 ; Tosini et al, 2012). En effet, elle est impliquée dans différents aspects telles que l'activité électrique neuronale (Sengupta et al. 2011), la phagocytose des photorécepteurs (J. C. Besharse and Dunis 1983), l'adaptation à l'obscurité (J. C. Besharse and Dunis 1983), ou la sensibilité à la lumière (Baba et al. 2009, 2013). En outre, la mélatonine semble protéger la rétine et retarder la progression de la DMLA (Yi et al, 2005). Cependant, les informations concernant la synthèse et la régulation de la mélatonine restent éparses. **Nous avons donc entrepris l'étude exhaustive de la synthèse de la mélatonine afin par la suite de mieux comprendre les rôles potentiels de cette hormone dans la physiologie des photorécepteurs, notamment les cônes, chez les mammifères.**

II- Derniers travaux réalisés:

1) La voie de synthèse de la mélatonine dans la rétine:

Nos études ont été réalisées chez le rongeur diurne *Arvicanthis ansorgei* qui présente l'avantage d'avoir une rétine ressemblante à la rétine centrale humaine, très riche en photorécepteurs de type cône (33 % contre 3 % pour les souris). Ces cellules sont potentiellement impliquées dans la synthèse de la mélatonine (Niki et al. 1998). Dans le but de caractériser le profil d'expression de la mélatonine dans la rétine, nous avons entrepris une analyse qualitative et quantitative de l'expression spatiale et temporelle des deux enzymes de synthèse l'ArylAlkylamine-N-AcetylTransferase (*Aa-nat)*/AA-NAT et l'HydroxyIndol-O-MethylTransferase (*Hiomt)*/HIOMT, ainsi que du récepteur de la mélatonine MT_1. Cependant, les données montrent que la mélatonine est produite en faible quantité dans la rétine d'*Arvicanthis ansorgei* et très difficilement mesurable, ce qui a nécessité deux années de mise au point des outils (détection de la mélatonine par RIA ou HPLC, spécificité des anticorps, sondes ARN). Mes résultats suggèrent que la régulation de la synthèse de la mélatonine dans la rétine du rongeur diurne *Arvicanthis ansorgei* est remarquable à plusieurs égards. Brièvement, les données montrent

l'importance de prendre en compte des voies de synthèse multiples: une voie semblable à celle de la glande pinéale, dans la couche nucléaire externe de la rétine et en particulier dans les cônes ; et l'autre distincte, apparemment constitutive dans la couche des cellules ganglionnaires. Chez cette espèce de rongeur diurne, l'AA-NAT pourrait avoir un autre rôle que celui de production de la mélatonine, notamment un rôle de détoxification des cônes (Klein DC, *JBC* 2007), cellules exposées à de forts stress oxydatifs durant la période de lumière. La présence d'HIOMT et de mélatonine en faible quantité mais de façon constante pourrait être adaptée au besoin physiologique d'*Arvicanthis ansorgei*.

2) Rôle de la mélatonine dans la phagocytose des photorécepteurs:

La mélatonine est impliquée dans le renouvellement et la phagocytose des photorécepteurs par l'épithélium pigmenté adjacent. (J. C. and M. I. Besharse 1983; White and Fisher 1989). Ce recyclage intensif est essentiel pour la function et la survie de ces cellules, parce qu'une perturbation de la phagocytose engendre la destruction des photorécepteurs (Mullen et LaVail, 1976). Une altération de l'efficacité de la phagocytose pourrait également participer à la DMLA. Afin d'approfondir le rôle de la mélatonine dans ce processus, et en collaboration avec le Dr Gianluca Tosini (Neuroscience Institute, Morehouse School of Medicine, Atlanta, GA 30310, USA), j'ai optimisé la technique (Bobu et al, 2006) d'analyse de la phagocytose des photorécepteurs dans la rétine des souris $C3Hf^{+/+}$ (souche compétente pour la production de mélatonine mais ne porte pas la mutation *rd*1 :*retinal degeneration 1, phosphodiesterase cGMP dependante*, qui provoque la dégénérescence), les souris MT_1KO et MT_2KO (souris KO pour les récepteurs de la mélatonine) et leurs contrôles sauvages. J'ai également fait l'analyse sur la rétine des souris KO pour le récepteur de la dopamine D2, étroitement lié à l'action de la mélatonine. Mes résultats montrent que les souris dépourvues de récepteurs de la mélatonine (des deux types) présentent un pic de phagocytose en fin de nuit en avance de phase, c'est-à-dire plus tôt que la normale alors que l'absence de récepteurs de la dopamine abolit complètement le rythme de phagoctose. **Ces données suggèrent donc que la mélatonine et la dopamine sont impliquées dans la régulation de ce phénomène physiologique.** L'ensemble des résultats est en cours de rédaction avec le Dr. Gianluca Tosini.
J'ai initié ces travaux en 2008 puis j'ai dirigé Coralie Gianesini d'abord en Master 2 de Neurosciences 2010/2011 et en thèse ainsi que Delphine Hennequin en Master 2 Neurosciences en 2013/2014. Ce projet a permis la rédaction de trois articles (deux déjà publiés, un autre en révision, plus un quatrième en préparation). Il m'a permis de signer en dernier auteur l'article publié Gianesini et coll. dans *Endocrinology* et d'endosser pleinement le rôle de Directeur de Thèse (soutenue le 26 juin 2015). **Les résultats concernant la synthèse et la régulation de la mélatonine établis, je souhaite orienter ma recherche vers les rôles de la mélatonine dans la rétine en conditions physiologique et pathologique. Je propose donc un projet (n°1) multidisciplinaire pour tenter de comprendre les rôles de la mélatonine sur les circuits neuronaux de la rétine. D'autre part, j'ai écrit un projet (n°2) pour mieux comprendre le rôle de la mélatonine dans la rétine de patients atteints de la maladie d'Alzheimer. Enfin, j'examinerai l'implication des photorécepteurs de la rétine et de la mélatonine exo-rétinienne dans l'intégration de la photopériode: projet n°3.**

III-Projets:

1) Le rôle des récepteurs de la mélatonine sur les circuits neuronaux de la rétine.

(Acronyme: MElatonin Receptors and Retinal RhYthms: MERRRY)

J'ai écrit ce projet avec un autre membre de notre équipe le Dr. Marie-Paule Felder-Schmittbuhl et le Dr. Christophe P. Ribelayga, assistant Professeur d'Ophtalmologie et Science de la Vision à University of Texas Medical School à Houston, Texas, USA. Ce projet a été déposé pour une demande de financement ANR-PRC le 15 Octobre 2015. Le cas échéant, j'aurais en charge la réalisation de l'objectif 1 et participerait à l'objectif 2 porté par Marie-Paule Felder-Schmittbuhl. Christophe P. Ribelayga, expert en électrophysiologie mènera les expériences précisées dans l'objectif 3.

Le rythme de production de la mélatonine est contrôlé par une horloge rétinienne et son maximum est atteint la nuit. Ses actions rythmiques sur la fonction de la rétine se manifestent par les rythmes journaliers d'un ou plusieurs composants de l'électrorétinogramme (Baba et al, 2009 ; 2013). Les récepteurs couplés aux protéines G de la mélatonine: MT1 et MT2 sont à priori présents dans toutes les couches nucléaires de la rétine et sont impliqués dans les fonctions locales de l'hormone (Baba et al, 2009, 2013). En outre, il est clairement apparu que la voie de signalisation de la mélatonine a des actions trophiques sur les neurones de la rétine. En effet, l'absence de MT1 conduit à la diminution du nombre de photorécepteurs au cours du vieillissement (Baba et al, 2009), à une augmentation de la pression intraoculaire et à la mort des cellules ganglionnaires (Alcantara-Contreras et al, 2011). La mélatonine module aussi la phagocytose des photorécepteurs (White and Fisher, 1989). En résumé, la voie de signalisation de la mélatonine est nécessaire pour un fonctionnement normal et la maintenance de la rétine. Cependant, à ce jour, la distribution exacte des récepteurs de la mélatonine dans la rétine ; leur contribution à la modulation de circuits neuronaux ou sur l'activité de l'horloge rétinienne sont peu connus.

Objectif spécifique 1: Obtenir une cartographie précise de la distribution des récepteurs de la mélatonine dans la rétine.

La distribution anatomique des récepteurs MT1 et MT2 a été largement étudiée en analysant les sites de liaison [^{125}I] iodomelatonin, l'hybridation *in situ* et de l'immunohistochimie (revue de Wiechmann and Summers 2008). Dans la rétine de rongeurs, les récepteurs de la mélatonine sont exprimés dans les 3 couches nucléaires (Fujieda et al. 2000; Baba et al, 2009; Sengupta et al, 2011; Gianesini et al, 2015). Pour identifier précisément quels types cellulaires de la rétine expriment MT1 et /ou MT2, nous avons développé une approche génétique inédite dans ce domaine. Nous avons crée des animaux dans lesquels *LacZ* a été introduit dan les gènes *Mtnr1a* or the *Mtnr1b* (souris *Mtnr1a-LacZ*-KI et *Mtnr1b-LacZ*-KI; cellules ES du KOMP-Velocigene -UC Davis). Nous allons précisément déterminer les sites d'expression du marquage X-GAL ou du marquage anti-LacZ en utilisant des marqueurs cellulaires de la rétine appropriés. Nous avons validé cette approche en confirmant les sites majeurs d'expression de *Mtnr1a* dans le cerveau de souris adultes (Klosen et al, non publié). De plus, nous génèrerons de nouvelles souris KO conditionnelles, dans lesquelles la Cre recombinase sera insérée dans les loci *Mtnr1a*-ou *Mtnr1b.* Après croisement avec une lignée reporter Lox-P, cette stratégie devrait améliorer le ratio signal/bruit de l'expression du reporter et permettre une confirmation/rafinement de la distribution des récepteurs de la mélatonine. Pour résoudre le problème d'une expression étendue des récepteurs pendant le développement prénatal (Davis, 1997), nous utiliserons la recombinase Cre-ERT2 pour faire un KI dans les loci *Mtnr1a*-ou

Mtnr1. Ces études (objectifs suivants également) seront conduites dans des souris qui produisent de la mélatonine, ce qui nécessitera un rétrocroisement dans le fond génétique approprié.

Objectif spécifique 2 : Déterminer le role de la signalisation de la mélatonine dans la fonction de l'horloge rétinienne.

La voie de signalisation de la mélatonine joue un rôle central dans la genèse des rythmes circadiens dans la rétine (revues de Iuvone et al, 2005; McMahon et al, 2014). Cependant, en absence de mélatonine ou quand les photorécepteurs ont disparu, le mécanisme de l'horloge rétinienne reste fonctionnel. En effet, les rythmes enregistrés sur explants de rétines de souris *Per2Luc* sur fond génétique C57Bl6 qui ne produisent pas de mélatonine (Jaeger et al, 2015, Ruan et al, 2008) sont robustes. Néanmoins des études parallèles suggèrent que la mélatonine module le patron d'expression des gènes horloges dans la rétine (Dinet et al, 2007 ; Hiragaki et al, 2014). Pour mieux évaluer le rôle de la mélatonine sur l'activité de l'horloge, nous croiserons la lignée de souris *Per2*^{Luc} avec soit les souris *Mtnr1a-LacZ*-KI ou la lignée *Mtnr1b-LacZ*-KI. Nous comparerons la phase et l'amplitude des rythmes de bioluminescence générés par les souris *Per2*^{Luc} sauvages ou avec la délétion ciblée de MT1 ou MT2. Cette approche sera complétée par des manipulations pharmacologiques précises de la synthèse ou voie de signalisation de la mélatonine. Nous utiliserons des rétines entières mais étendrons notre analyse à chaque couche nucléaire obtenue par coupe au vibratome (Jaeger et al, 2015). En comparant les résultats obtenus sur rétines entières avec ceux des différentes couches isolées nous pourrons déterminer comment la mélatonine affecte l'activité de l'horloge dans chaque population cellulaire mais aussi comment elle contrôle l'activité générale de l'horloge de la rétine (inter-couche, inter-cellule). Nous analyserons la capacité oscillatoire d'une cellule unique sur coupe transversale de rétine grâce à une caméra couplée à l'analyse de la bioluminescence (Luminoview, Olympus).

Objectif spécifique 3 : Caractériser le rôle de la mélatonine dans le contrôle circadien de la physiologie du photorécepteur.

Des études précédentes ont montré que la cinétique de réponse à la lumière des photorécepteurs et la force du couplage électrique entre photorécepteurs étaient contrôlés par une horloge (Ribelayga et al, 2008; Jin et al, 2015; Zhang et al, 2015). L'implication de la mélatonine dans cet événement n'est pas clairement établie. La mélatonine pourrait inhiber la dopamine, qui à son tour module la conductance des jonctions communicantes entre photorécepteurs (Li et al, 2013) ou agir directement via ses récepteurs MT1 et/ou MT2. Dr. Christophe P. Ribelayga (University of Texas Medical School de Houston, TX USA) notre collaborateur est un expert de renommée mondiale en électrophysiologie de la rétine (Jin et al, 2015; Jin and Ribelayga, 2015). Il se propose d'enregistrer les réponses à la lumière et la conductance des jonctions communicantes des photorécepteurs (bâtonnet-bâtonnet, bâtonnet-cône, cône-cône). Pour cette étude, le Dr. C.P. Ribelayga utilisera nos modèles murins *Mtnr1a-LacZ*-KI et *Mtnr1b-LacZ*-KI afin de clarifier le rôle de la mélatonine dans la régulation du couplage des photorécepteurs et/ou leurs propriétés de réponse à la lumière.

2) La mélatonine et la maladie d'Alzheimer:

La mélatonine synthétisée dans les cônes serait impliquée dans les pathologies de l'oeil liées à l'âge comme la maladie d'Alzheimer (AD) (WuYF et Swaab DF, *JBR* 2005). En effet, les études chez les mammifères non primates suggèrent que la mélatonine protège la rétine, en promouvant la survie cellulaire (Baba et al. 2009); le dérèglement de la composante circadienne de la voie de synthèse de la mélatonine a des effets néfastes sur la santé des personnes âgées (Jenwitheesuk et al. 2014). De plus, une etude unique sur des rétines humaines AD rapporte que les récepteurs de la mélatonine MT_1 sont surexprimés dans les cellules ganglionnaires de patients AD; mais sous exprimés dans les photorécepteurs (Savaskan et al. 2002). Ces données sont en opposition avec celles des patients atteints de glaucome qui présentent une augmentation de l'expression de MT_1. Notre but est de réexaminer la voie de synthèse de la mélatonine dans la rétine humaine. En effet, nous déterminerons l'expression de MT_1 et des enzymes limitantes de synthèse de la mélatonine dans la rétine de différents patients AD et contrôles. Ces données permettront d'établir les changements de regulation de la mélatonine qui interviennent au cours de la progression de la maladie d'Alzheimer. Nous utiliserons un nouvel anticorps dirigé contre MT_1 validé dans la rétine de mammifères (Sengupta et al, 2011) ainsi que d'autres anticorps dirigés contre les enzymes clés de la synthèse de l'indolamine. Nous utiliserons des marqueurs cellulaires spécifiques (rhodopsine ou cone transducine des cônes pour les photorecepteurs, mélanopsine ou Brn3 pour les cellules ganglionnaires) pour voir si la régulation de la mélatonine est correlée avec la disparition de populations cellulaires particulières. Pour cela, nous allons utiliser des tissus oculaires fixes d'une série de donneurs AD à différents stades de la maladie (et au moins une rétine contrôle saine/non-AD). Les échantillons ont été récemment obtenus du Dr. Thomas Beach, Chef de Service de la Pathologie, Banner Sun Health Research Inst., Sun City, AZ, USA (thomas.beach@sunhealth.org) sur projet scientifique.

3) Cellules ganglionnaires intrinsèquement sensibles (ipRGC), mélatonine et détection de la photopériode chez les rongeurs :

En dehors de la rétine, la mélatonine est synthétisée et libérée par la glande pinéale, avec un rythme journalier très marqué et une durée de synthèse nocturne dépendante de la durée de la nuit. Aussi, la mélatonine est considérée comme le messager interne par lequel les organismes perçoivent le changement saisonnier de la photopériode. Chez les mammifères saisonniers il a été montré que la mélatonine agit sur la *pars tuberalis* de l'hypophyse pour réguler la production de TSH qui, en aval contrôle le rythme saisonnier de la reproduction (Simonneaux et al, 2013 ; Revel et al, 2009). Cependant, le mécanisme par lequel les variations saisonnières des informations photiques sont transmises au cerveau n'est pas connu. Les ipRGCs projettent vers les noyaux suprachiasmatiques (SCN) de l'hypothalamus pour moduler le photoentrainement circadien (Schmidt et al, 2011) et la sécrétion de mélatonine par la glande pinéale (Larsen 1998). Le but de ce projet est de déterminer si les ipRGC jouent un rôle dans l'intégration saisonnière de la photopériode chez la souris. Bien que le système reproducteur de la souris soit en général décrit comme non sensible à la photopériode, ce rongeur perçoit les variations saisonnières de lumière et la voie rétino-hypothalamique de la souris est similaire à celles des espèces photopériodiques (Youngstrom et al, 1986). De plus la souche commune de souris C57/Bl6 présente des changements photopériodiques des taux de sérotonine cérébrale et de son comportement ressemblants à de la dépression et (Otsuka et al, 2014). **Le projet comprendra les étapes suivantes** : 1) Des souris C3H qui produisent de la mélatonine seront utilisées comme modèle pour examiner les ipRGCs. En effet, ces souris (Komeima et al, 2006) portent la

mutation *rd1* et en quelques semaines perdent plus de 99% des bâtonnets et environ 90% des cônes, ne laissant que les ipRGCs pour assurer la photoréception. Des groupes de souris adultes (3 mois) maintenus en photopériode courte (8 heures de lumière blanche, 16 heures d'obscurité) ou longue (16 heures de lumière blanche, 8 heures d'obscurité) seront examinés pour leur profil de mélatonine provenant de la glande pinéale. Des prélèvements de sang (20 µl) seront effectués toutes les deux heures sur une période de 24 heures. 2) Le nombre de cellules ganglionnaires sera évalué par immunocytochimie car il peut varier comme cela a été démontré chez la souche CBA/J porteuses de la mutation *rd1* (Ruggiero et al 2010). 3) En parallèle, nous utiliserons une immunotoxine pour détruire spécifiquement les ipRGCs (destruction ciblée des ipRGCs avec un conjugué saporin qui altère les effets de la lumière sur les rythmes circadiens de la souris, Göz et al, PLoSOne, 2008) afin d'analyser l'intégration de la photopériode à l'aide de groupes de souris identiques à ceux utilisés pour l'étape 1. Des expériences similaires (souris de même âge, photopériodes courte et longue, dosage de la mélatonine par ELISA) seront réalisées avec des souris contrôles qui produisent de la mélatonine et possèdent une rétine intacte (CBA/N sans *rd1*). 4) Nous mesurerons la réponse fonctionnelle à la lumière des ipRGCs par l'approche de « multi-electrode array », utilisant des explants rétiniens in vitro. L'activité neuronale sera enregistrée en présence ou absence de mélatonine perfusée. **Ces travaux détermineront si les ipRGCs contrôlent les changements photopériodiques dans l'organisme.** Par la suite (inclus dans le projet de thèse), afin de cartographier les projections cellulaires issues de la rétine vers les SCN, nous injecterons pour un marquage cellulaire rétrograde, de la toxine diphtérique dans les SCN. Des marqueurs de cellules ganglionnaires (i.e. mélanopsine, brn3, Thy1, NFL-200) permettront l'identification des cellules avec toxines. **Un étudiant de Master 2 de Neurosciences: Bastien Leclercq, a été recruté pour mener cette étude qui anticipe le sujet de thèse sur l'intégration par les mammifères de la photopériode, au niveau de la rétine et de ses projections axonales.**

IV: Conclusions

Part IV: Conclusions

Arvicanthis ansorgei is a valuable model to study retina physiology. We have deciphered the complete melatonin synthetic pathway in a diurnal rodent with a cone-rich retina (Figure 55). The emphasis can be made on the confirmed roles of melatonin in this tissue in which it modulates essential functions such as photoreceptor phagocytosis and cell survival. Moreover, we have demonstrated the importance of considering local synthesis, regulation and action of the neurohormone as well as its dialogue with dopamine. Along the path, we have identified AA-NAT and HIOMT as independent molecules endowed with potential supplementary tasks (detoxification for AA-NAT, O-methylation of other 5 methoxyindoles for HIOMT) and with divergent expression levels or regulation. Indeed, MT_1, AA-NAT and HIOMT are present in *Arvicanthis ansorgei* ganglion cells. In our preliminary results, a subpopulation of human ganglion cells appears to display HIOMT immunoreactivity. (Figure 54)

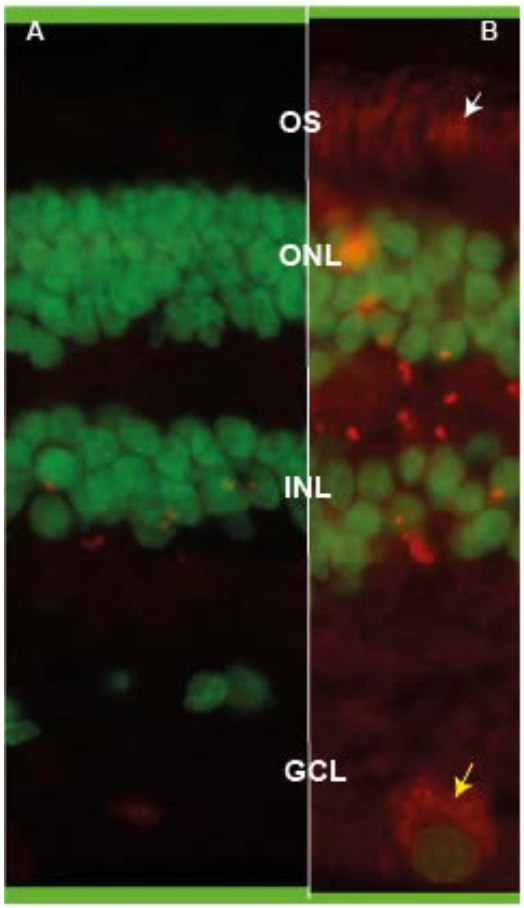


Figure 54: ASMT immunoreactivity in human retina.

Photographs are merged images with DAPI staining (green) and ASMT immunodetection (red). Panel A is the control without antibody. Panel B displays ASMT detection principally in the GCL (yellow arrow) and a faint positive immunoreactivity in the photoreceptors (white arrow). Magnification is X400.

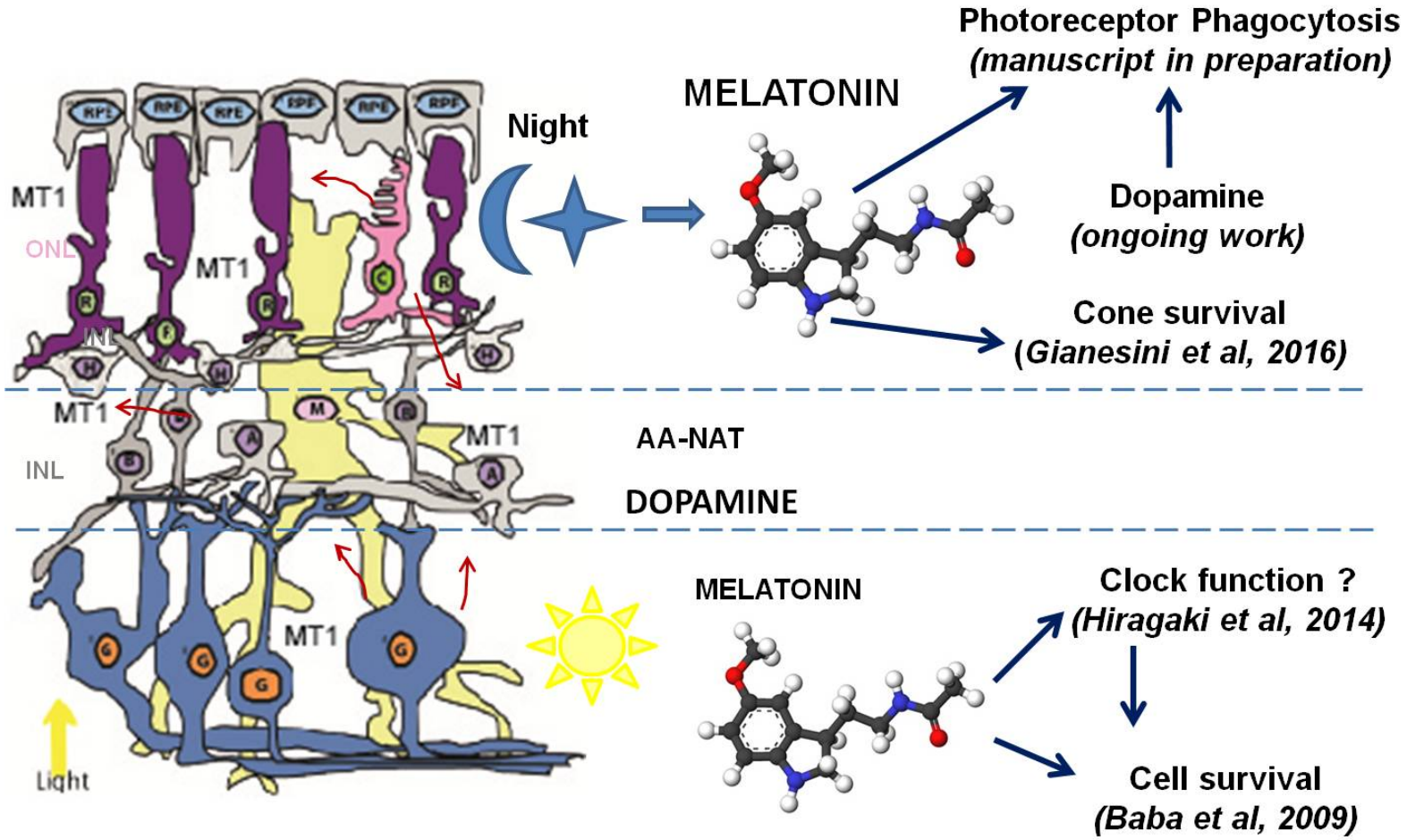


Figure 55: Model of Melatonin synthesis and regulation in the retina of *Arvicanthis ansorgei*.

MT_1 receptors are distributed throughout the retina as illustrated on the left diagram. Dashed lines materialize the separation between ONL, INL and GCL. **Top part:** ONL melatonin is produced at night to modulate retina adaptation to darkness as well as photoreceptor phagocytosis and cone survival**. Bottom part**: GCL melatonin level is low and apparently uniform over the 24 hours to fulfill roles that are not yet determined. Note thet both AA-NAT and dopamine are produced in the INL.

Overall, we can propose, with the projects developed in the precedent chapter, to tackle new scientific questions as follows:

Does melatonin and melatonin signaling play a role in the retinal clock function and photoreceptor physiology? The MERRY project should address this question.

Do ipRGCs convey photoperiod information toward central brain and impact behavior? The second project involves mouse mutant models with targeted disruption of retinal cell populations that will be exposed to short and long photoperiods. These experiments will allow us to determine the specific role of ipRGCs in the photoperiod integration.

How does melatonin synthetic pathway help cones survive in retinas exposed to high irradiance light variations overtime. The analysis of AD patient's retinas should provide new information on the regulation of the melatonin pathway in each cell type during aging.

General conclusion

Until now, all basic research on retina has gathered precise knowledge on each cell type (e.g. cone, rod, ipRGC, amacrine cells…) and their general organization towards vision build-up and non visual functions achievement. Our team contribution resides in the knowledge of the neurohormone melatonin synthetic pathway and its functions (electric activity modulation, photoreceptor phagocytosis) within the retina. However, our task is now to establish how physiological networks: cell coupling, circadian regulation, light photoperiod encoding, are born, regulated and modified so the information goes correctly to the central brain. Moreover, we have to determine which factors are critical to keep notably aging retinas in healthy state. We and collaborators may start addressing these issues with data including melatonin and dopamine molecules, both intertwined in the retina light/dark cycles-dependent functions.

Part V: Bibliography

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Part VI: *Curriculum vitae*

Virginie Laurent-Gydé, Enseignant-Chercheur

43 ans, mariée, 2 enfants (2006, 2011)

Coordonnées professionnelles :

Institut des Neurosciences Cellulaires et Intégratives (INCI), UPR 3212 CNRS

Equipe « Rythme, vie et mort de la rétine »

5, rue Blaise Pascal F-67084 Strasbourg Tel (+33) 03 88 45 66 75 Fax (+33) 03 88 45 66 54

E-mail: gydelaurent@inci-cnrs.unistra.fr ou gydelaurent@unistra.fr

Fonction: Maître de Conférences, poste 0574 NUMEN : 15S0319751FUO **Section du CNU:** 68

Etablissement actuel: Université de Strasbourg. 4, rue Blaise Pascal 67081 Strasbourg

Titre universitaire Français: Docteur de l'Université des Sciences et Technologies de Lille **(USTL)** en Sciences de la Vie et de la Santé. La thèse intitulée « Le système rénine-angiotensine chez la sangsue *Theromyzon tessulatum* » a été soutenue le 9 février 1998 à Villeneuve d'Ascq. Directeur de Thèse: Pr. Michel Salzet.

DIPLOMES:

1998 : **Doctorat en Biologie-Santé** (USTL)**.**

1995 : **DEA en Biologie-Santé** (USTL)**.** Options Neurosciences-Biochimie

1994 : Maitrise de Biologie cellulaire (USTL) Options Immunologie-Endocrinologie-Génétique microbienne.

1993 : **Licence de Biologie cellulaire**(USTL)**.** Options Biochimie- Biologie des organismes

1992 : Diplôme d'Etudes Universitaires Générales -Deug. B- (USTL)**.** Option Chimie-Biologie-Stage de 2 mois en laboratoire de recherche INSERM.

EXPERIENCES PROFESSIONNELLES:

Sept-2003-présent : Maître de Conférences à l'Université de Strasbourg.
Demande de financement ANR-PRC, première phase le 15 Octobre 2015
(cf. fichier résumé du projet)
2006 : Subvention fixe de l'Association pour la Recherche contre le Cancer
2004 : Obtention de 40000 Euros suite à l'appel d'offre du Conseil Scientifique de l'Université Louis Pasteur

Décembre 2002-Août 2003 : Chercheur post-doctorat à l'Institut Cochin-INSERM U 567
2002 : Bourse de la Fondation pour la Recherche Médicale

1999-2002 : Chercheur post-doctorat en Biochimie et Biologie Moléculaire à l`Université d`Etat de Louisiane -LSUHSC- (New Orleans, LA, USA).

2000: Prix NSF présentation orale et poster « Gordon Conference »: Hormonal and Neural Peptide Biosynthesis

1998-1999 : Chercheur post-doctorat en Biologie Moléculaire à l`Université de Leeds (U.K.).
1998 : Lauréat de la bourse LAVOISIER du Ministère des Affaires Etrangères

1997-1998 : Attaché Temporaire d'Enseignement et de Recherche (ATER) à l'Université des Sciences et Technologies de Lille (USTL).

1995-1998 : Doctorant dans l`UPRES A CNRS 8017 "Endocrinologie des Annélides" de l'USTL.

DOMAINES DE COMPETENCES:

Biologie moléculaire: Techniques de clonage d'ADN, PCR, RT-PCR quantitative en temps réel, étude spatio-temporelle de l`expression d`un gène à l`aide des gènes rapporteurs Luciférase et *green fluorescent protein (gfp)*, hybridation *in situ.*

Biochimie: Chromatographies d'affinité, chromatographie liquide à haute pression (phase inverse) à détection électrochimique, électrophorèses analytiques, enzymologie, marquage radiométabolique (^{35}S).

Biologie cellulaire: Culture primaire de cellules hypophysaires de souris et fractionnement cellulaire. Culture de lignées cellulaires humaines et de rat. Coupes au cryostat d'yeux de rongeurs. Evaluation de la phagocytose des photorécepteurs de la rétine en microscopie à épifluorescence (Bobu et Hicks, 2006). Techniques d'analyse de la rétine de rongeurs : immunocytochimie, dissection rétine à plat, vibratome.

Immunologie: Dosages radioimmunologiques (RIA) et immunoenzymatiques (ELISA, DIA), radiomarquage de peptides à l'^{125}I, Western Blot, immunoprécipitation, immunohistochimie.

Animaux: Gestion de la production de mutants génétiques, surrénalectomie bilatérale, introduction sous-cutanée de comprimés de corticostérone, injections intrapéritonéales, génotypage.

LISTE DES TRAVAUX:

Gianesini C., Hiragaki S., Contreras-Alcantara S., Laurent V.**,** Hicks D., Tosini G.**;** Cone viability is affected by interruption of melatonin signaling. **Investigative Ophthalmology and Visual Science** 2016, Jan 1; 57(1):94-104.

Gianesini C., Clesse D., Tosini G., Hicks D., Laurent V. ; Unique regulation of the melatonin synthetic pathway in the retina of diurnal female *Arvicanthis ansorgei* (Rodentia). **Endocrinology**, 2015, Sep; 156 (9):3292-308.

Bobu C., Sandu C., Laurent V., Felder-Schmittbuhl M-P., Hicks D. Prolonged light exposure induces widespread phase shifting in the circadian clock and visual pigment gene expression of the *Arvicanthis ansorgei* retina. **Molecular Vision** 2013; vol. 19.

Agez L., Laurent V., Guerrero HY., Pevet P., Masson-Pevet M., Gauer F. Endogenous melatonin provides an effective circadian message to both the suprachiasmatic nuclei and the pars tuberalis of the rat. **J. Pineal Res.**, 2009 Jan; 46(1): 95-105.

Agez L., Laurent V., Pevet P., Masson-Pevet M., Gauer F. Melatonin affects nuclear orphan receptors mRNA in the rat suprachiasmatic nuclei. **Neuroscience**, 2007 Jan 19 ; 144(2) :522-30.

Pevet P., Agez L., Bothorel B., Saboureau M., Gauer F., Laurent V., Masson-Pevet M. Melatonin in the multi-oscillatory mammalian circadian world. **Chronobiol. Int**., 2006, 23 (1-2) : 39-51.

Peinado, J.R, Laurent, V., Lee, S.N., Peng, B.W., Pintar, J.E., Steiner, D.F. and I. Lindberg. Strain-dependent influences on the hypothalamo-pituitary-adrenal axis profoundly affect the 7B2 null phenotype. **Endocrinology**, 2005 Aug ; 146 (8) : 3438-44.

Laurent, V., Jaubert-Miazza, L., Desjardins, R., Day, R., Lindberg, I. Biosynthesis of Proopiomelanocortin-derived peptides in Prohormone convertase 2 and 7B2 null mice. **Endocrinology**, 2004 Oct. 145 (2): 519-28. Epub 2003 Oct. 23.

Wynsky-Sommerer, R., Grouselle, D., Rougeot C, Laurent V, David, J.P., Delacourte A, Dournaud P., Seidah, N.G., Lindberg I., Trottier, S. & Epelbaum J. The proprotein convertase PC2 is involved in the maturation of prosomatostatin to somatostatin-14 but not in the somatostatin deficit in Alzheimer's disease. **Neuroscience** 2003; 122 (2): 437-47.

Laurent, V. and Lindberg, I. Mini-RIA: adaptation of conventional ^{125}I-labeled radioimmunoassay to a 96-tube format. **Anal. Biochemistry**, Oct. 1; 309(1):143, 2002.

Zhu, X., Zhou, A., Dey, A., Norrbom, C., Carroll, R., Zhang, C., Laurent, V., Lindberg, I, Ugleholdt, R., Holst, J.J., Steiner, D.F. Disruption of PC1/3 expression in mice causes dwarfism and multiple neuroendocrine peptide processing defects. **Proc. Natl. Acad. Sci.** Aug 6; 99 (16): 10293-8, 2002.

Laurent, V., Kimble, A., Peng, B., Zhu, P., Pintar, J.E., Steiner, D.F., and Lindberg, I. Mortality in 7B2 null mice can be rescued by adrenalectomy: involvement of dopamine in ACTH hypersecretion. **Proc. Natl. Acad. Sci.** March 5. Vol. 99, nº5, 3087-3092, 2002.

Laurent, V., Coates, D., et Isaac, R.E. Functional expression and characterization of the aminopeptidase P of *C. elegans*. **Eur. J. Biochem.** 268 (20): 5430-8, 2001.

Laurent, V., Salzet, B., Verger-Bocquet, M., Bernet, F., et Salzet, M. Morphine-like substance in leech ganglia, **Eur. J. Biochem**. 267, 2354-2361, 2000.

Laurent, V., Stefano, G.B., et Salzet, M. Leech angiotensin converting enzyme. **Annals New York Academy of Sciences**, May 15; 839:500-2, 1998.

Salzet, M., Salzet-Raveillon, B., Cocquerelle, C., Verger-Bocquet, M., Pryor, S.C., Rialas, C.M., Laurent, V., et Stefano, G.B. Leech Immunocytes Contain Proopiomelanocortin (Nitric Oxide Mediates Hemolymph Proopiomelanocortin Processing). **The Journal of Immunology**, 159: 5400-5411, 1997c.

Vandenbulcke, F., Laurent, V., Verger-Bocquet, M., Stefano, G.B., et Salzet, M. The leech angiotensin-converting-like enzymes: cellular localization and biochemical identifications of a membranar and a soluble forms. **Mol. Brain. Res**., 49, 229-237, 1997b.

Laurent, V., Stefano, G.B., et Salzet, M. Presence and biochemical properties of a Molluscan angiotensin-converting-like enzyme. **Regul. Peptides**, 69, 53-61, 1997a.

Laurent, V., Salzet, M. Metabolism of enkephalins in head membranes of the leech Theromyzon tessulatum by peptidases: Isolation of an enkephalin-degrading aminopeptidase. **Regul. Peptides**, 65, 123-131, 1996c.

Laurent, V., Salzet, M. Metabolism of angiotensins by head membranes of the leech *Theromyzon tessulatum.* **FEBS Lett**., 384, 123-127, 1996b.

Laurent, V., Salzet, M. Biochemical properties of an angiotensin-converting enzyme in the leech *Theromyzon tessulatum*, **Peptides**, 17 (5), 737-745, 1996a.

Laurent, V., Salzet, M. Isolation of a neuropeptide-degrading endopeptidase from the leech *Theromyzon tessulatum.* **Eur. J. Biochem**., 233, 186-191, 1995d.

Laurent, V., Salzet, M. Isolation of a renin-like enzyme from the leech *Theromyzon tessulatum,* **Peptides**, vol. 16(8), 1351-1358, 1995c.

Laurent, V., Salzet, M. A comparison of the N-terminal sequence of the leech angiotensin converting-like enzyme with forms of Vertebrate angiotensin converting enzyme, **Neurosc. Lett**., 198, 60-64, 1995b.

Laurent, V., Bulet, P., et Salzet, M. A comparison of the leech angiotensin I-like molecule with forms of Vertebrates angiotensinogens : an hormonal system conserved in course of evolution. **Neurosc. Lett**., 190 : 175-178, 1995a.

COMMUNICATIONS- SEMINAIRES :

1-Gianesini C., Hicks D., Tosini G., Laurent V.

Title: Novel regulation of melatonin synthetic pathways in the retina of the diurnal rodent *Arvicanthis ansorgei*, Gordon Conference, Pineal Melatonin: Comparative Approaches to Human Health and Disease –Galveston - Texas – USA, 2014.

2- Tosini G., Laurent V., Baba K., Hiragaki S., Contreras-Alcantara S., D.Hicks, 2013

Titre: Modulation of the daily rhythm in disk shedding by melatonin in the mouse. The Association for Research on Vision and Ophthalmology (ARVO) meeting, May 5-9th 2013. Communication orale.

3-V. Laurent, D. Hicks. 2010

Titre: Role of melatonin in photoreceptor phagocytosis in the rodent retina. ARVO meeting, May 2-6th 2010.

4-Laurent V, Sandu C, Felder-Schmittbuhl M-P, Bobu C, Hicks D. 2009

Titre: Aryl-Alkylamine-N Acetyltransferase (*Aa-nat*) expression in the cones of *Arvicanthis ansorgei* retina.XI Congress of the European Biological Rhythms Society in association with the Japanese Society for Chronobiology. August 22-28.

5- L. Agez, V. Laurent , I. Ryadnova, P. Pevet, M. Masson-Pevet et Gauer F. 2004

Titre : La mélatonine régule le niveau d'expression des facteurs de transcription rorb et rev-erbα dans la *pars tuberalis* mais pas dans les noyaux suprachiasmatiques du rat. 32ème Congrès de la Société de Neuroendocrinologie (SNE) du 16 au 18 septembre 2004. Communication affichée.

6- Peinado, J.R., Lee, S-N., Laurent, V., Peng, B.W., Pintar, J.E., Steiner, D.F. and Iris Lindberg, 2004.

Titre: Strain-dependent differences in pituitary dopaminergic regulation contribute to the different phenotypes of the 7B2 and PC2 nulls. The Endocrine Society's 86th Annual Meeting. June 16-19th. Communication affichée.

7-Laurent, V., Jaubert-Miazza, L., Smith, K., Zhou, A., Desjardins, R., Day, R. and Lindberg, I, 2002

Titre: Biosynthesis of POMC-derived peptides in Prohormone Convertase knock-out mice. Gordon Research Conference: "Hormonal and Neural Peptide Biosynthesis". July 21-26th 2002. Communication affichée.

8- Laurent, V., Kimble, A., Zhu, P., Steiner, D.F., and Lindberg, I. 2001

Titre: Recent progress on the 7B2 knockout mouse. The Endocrine Society's 83rd Annual Meeting. June 20-23rd 2001. Communication affichée.

9- Laurent, V., Kimble, A., and Lindberg, I. 2000

Titre: Recent progress on the 7B2 KO mouse. Gordon Research Conference: "Hormonal and Neural Peptide Biosynthesis". July 16-21th 2000. **Communication orale et affichée**. Double prix NSF pour le séminaire et le poster.

10- Stancombe, P., Laurent, V., Isaac, R.E., and Coates, D. 1999

Titre : Zinc-Metallopeptidases expression in *C. elegans.* 12th International *C. elegans* Meeting. University of Wisconsin-Madison. June 2-June 6. Communication affichée.

11-Laurent, V., Salzet, M. 1999

Titre : ACE, NEP and the Renin Angiotensin System in the leech. Annual meeting of the Society for Experimental Biology. **Edimbourg 22-26 March 1999. Communication orale.**

12-Laurent, V., Vandenbulcke, F., Verger-Bocquet, M., Salzet, M. 1996

Titre : The leech angiotensin-converting enzyme. 18th Conference of European Comparative Endocrinologists. Rouen 10-14 sept. Communication affichée.

13-Salzet, M., Verger-Bocquet, M., Wattez, C., Laurent, V., et Malecha, J. 1995

Titre : Un système Rénine-Angiotensine chez les Hirudinées. Colloque Lille-ULB (le 30.05.95). Communication affichée.

14-Laurent, V., Salzet, M. 1995

Titre : Isolation and biochemical characterization of a renin and an angiotensin-converting-like enzymes in the leech *Theromyzon tessulatum.*12th International Symposium on Neurosecretion. The peptidergic neuron. Kiel, Allemagne. Communication affichée.

15-Laurent, V., Salzet, M., Verger-Bocquet, M., Malecha, J. 1995

Titre : Un système rénine-angiotensine chez les Hirudinées. 2ème colloque des Neurosciences, Lyon. Communication affichée.

ENCADREMENT:

La liste ci-dessous référence tous(tes) les étudiant(e)s que j'ai encadré(e)s en stage de recherche en laboratoire depuis mon post-doctorat aux USA jusqu'à aujourd'hui.

2015-2016 : Bastien Leclercq, Master 2 Neurosciences, Université de Strasbourg

2011-2015: Thèse de Mlle Coralie Gianesini soutenue le 26 Juin 2015, Université de Strasbourg

2013-2014: Delphine Hennequin, Master2 Neurosciences, Université de Strasbourg

2013: Arnaud Bubeck, Master1 Science technologie et Société, Université de Strasbourg

2010-2011: Coralie Gianesini, M2 Neurosciences, Université de Strasbourg

2010: Adeline Normand, BTS 1ère année, Lycée Jean Rostand, Strasbourg

2010: Ekaterina Ivanova, 2ème année, Université de Strasbourg

2010: Aline Autret, 3ème année, Université de Strasbourg

2010: Nicolas Imbert, BTS 2ème année, Université de Strasbourg

2008-2009: Coralie Gianesini, M1 Sciences de la Vie, Université Henri Poincaré de Nancy

2009: Marion Dehlinger, M1 Neurosciences, Université de Strasbourg

2009: Sarah MacDonald, 3ème année de Licence, University of Exeter, Angleterre

2009: Virginie Gabel, M1 Neurosciences, Université de Strasbourg

2008: Amandine Grimm, M1 Neurosciences, Université de Strasbourg

2006-2007: Marilyn Ewert, M2 Ecophysiologie Ethologie, Université de Strasbourg

2005-2006 : Hélène Cordier, M2 Sciences de la Vie et de la Terre, Université de Strasbourg

2004-2006: Céline Roth, BTS stages de 1ère et 2ème années, Lycée Jean Rostand, Strasbourg

1999-2002: Encadrement d'étudiants en thèse et de techniciens dans le laboratoire du Dr. Lindberg au Louisiana State University Health Sciences Center (New Orleans, LA, USA).

ACTIVITÉS D'ENSEIGNEMENT :

Sept 2003-présent :

Depuis mon recrutement à l'Université Louis Pasteur intégrée ensuite dans l'Université de Strasbourg (UDS), je travaille au sein de la Faculté des Sciences de la Vie en tant que Maître de Conférences. J'enseigne la Biologie Animale de la première année de licence Sciences de la Vie jusqu'en Master 2 (SVT et Ecophysiologie/Ethologie). J'ai développé des **cours sur les relations interspécifiques des êtres vivants** (Parasitisme, symbiose, mutualisme… et l'Évolution) enseignés sous formes de cours magistraux (20-200 étudiants), TD, TP (25-30 étudiants) aux étudiants de toutes années. J'effectue également des cours magistraux de Biologie cellulaire en L1.

Je suis responsable de deux Unités d'Enseignements (UE) intitulées **« Biologie des organismes » et « Stage en situation » pour les étudiants de 3ème année de Licence Sciences de la Vie parcours préparation au Professorat des Écoles**. Je coordonne également les stages d'ASTEP (Accompagnement en Science et Technologie à l'Ecole Primaire) pour la Faculté des Sciences de la Vie en relation avec Mme Clarisse Huguenard-Devaux, responsable de ce dispositif pour l'UDS. Ces stages proposés aux étudiants à partir de la troisième année de licence se font en lien avec le rectorat et l'inspection académique. Ils ont pour but de favoriser un échange de compétences et connaissances entre les maîtres de stage habilités par le rectorat et les étudiants, des scientifiques apprentis- enseignants. Les étudiants valident ainsi une UE de 3 ects dans leur cursus et enrichissent leur parcours scientifique.

J'effectue de l'enseignement de **méthodologie scientifique** par différents TD qui s'échelonnent de la première année de licence (actuellement -Méthodologie du Travail Universitaire et Démarche Scientifique- (MTUDS) en L1, TD –Phylogénie et anatomie comparée des Métazoaires -(PAM) en L2) jusqu'au Master. Ces enseignements ont un but commun : accroître et développer des compétences scientifiques : poser les bonnes questions, faire des hypothèses, trouver des méthodes, analyser et discuter les résultats, apporter de nouvelles idées. La version la plus élaborée de cet enseignement est le travail avec deux collègues enseignant-chercheurs, chercheurs et un groupe de 4-5 étudiants sur un concept scientifique (1 structure cérébrale et une pathologie par exemple) pendant 3 mois de manière à partir d'une idée et réussir à construire un projet scientifique. Ceci en passant par les étapes clés d'études bibliographiques, stages techniques, discussions, restitutions orales, évaluations etc. Cet enseignement est réalisé en première année du Master de Neurosciences.

Je prends part aux **entretiens individuels** (initialement dispositif « Plan Réussite ») que nous (équipe pédagogique de la Licence Sciences de la Vie) proposons aux étudiants afin de les aider à améliorer leurs performances académiques et/ ou à construire leur projet professionnel. Je participe également aux sessions intitulées « Prêt pour l'emploi» organisées par l'espace Avenir de l'UDS depuis 4 ans. Ceci me donne la possibilité d'entrer en contact avec des professionnels du recrutement de divers secteurs d'activités. Je peux ainsi mieux aider les étudiants à la construction de leur projet professionnel en 1ère et 3ème années dans les UE d'Accompagnement au Projet de l'Etudiant.

En 2015, j'ai organisé 2 **sessions de formation (1 jour) appelée « De l'œil au cerveau » avec la Maison pour la Science en Alsace** (MSA) http://www.maisons-pour-la-science.org/fr/alsace. Cette formation s'adresse aux Enseignants du secondaire spécialisés en SVT et Chimie/Physique. Ce programme inclut des travaux pratiques pour réaliser de l'immunohistochimie sur des rétines de rongeurs, une démonstration de la technique d'électrorétinogramme, la visite du Chronobiotron (animalerie dédiée à la chronobiologie) et un séminaire intitulé « la rétine, les rythmes biologiques et le sommeil ». Le but de cette formation est de rendre accessible le travail réalisé en laboratoire par les chercheurs, aux enseignants du secondaire. Cette formation est renouvelée le 25 et 26 février 2016.

RESPONSABILITÉS ADMINISTRATIVES :

Depuis l'année Universitaire 2005/2006, j'ai repris la **direction d'études du parcours Préparation au Professorat des Ecoles** niveau L3 de la Licence Mention Sciences de la Vie. Depuis la rentrée universitaire 2010/2011, je suis également directeur des études pour la L2 parcours Professorat des Ecoles. Cette responsabilité administrative comprend **l'établissement de l'offre de formation** avec l'équipe pédagogique de la Faculté des Sciences de la Vie, **l'organisation des enseignements, des examens et des jurys ; la coordination des enseignants, la préparation des emplois du temps etc. afin qu'une trentaine d'étudiants puissent réaliser leurs 2ème et 3ème année de Licence dans les meilleures conditions.** En effet, cette Licence au parcours d'abord scientifique (Biologie, Géologie, Chimie) est complétée par des UE de préprofessionnalisation (cf. l'Ecole Supérieure du Professorat et de l'Education= ESPE), du Français et Culture Générale, des Mathématiques, de la communication scientifique (TICE), des Sciences Physiques adaptées aux écoliers, des options de Psychologie, un stage en situation de 4 semaines en Ecole primaire (dispositif adossé à l'ASTEP cité haut). Ce parcours de licence prépare donc les étudiants à l'entrée dans le Master Métiers de l'Enseignement, de l'Education et de la Formation (composante ESPE de l'Université de Strasbourg) parcours Professorat des Ecoles ou au Master de Communication Scientifique de l'Université de Strasbourg et dans ce cas à une carrière dans l'animation scientifique.

Part VII: Acknowledgement

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…/…

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« Quoi que tu rêves d'entreprendre, commence-le. L'audace a du génie, du pouvoir, de la magie. » Citation de Johann Wolfgang Von Goethe