Aus dem Institut/der Klinik für Schlafmedizin

der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Regulation of Melanopsin and PACAP mRNA by Light, Circadian and Sleep Homeostatic Processes

zur Erlangung des akademischen Grades

Doctor medicinae (Dr. med.)

vorgelegt der Medizinischen Fakultät

Charité – Universitätsmedizin Berlin

von

Claire-Marie Gropp

aus München

Datum der Promotion: 22/06/2014

Table of contents

Figures

Abbreviations

ANOVA: analysis of variance

cAMP: cyclic adenosine monophosphate

cDNA: complementary deoxyribonucleic acid

CO2: carbon dioxide

CT: circadian time

Ct: cycle thresholds

DD: dark-dark exposure

EEG: electroencephalogram

IGL: intergeniculate leaflet

INCI: institut des neurosciences cellulaires et intégratives

IP3: inositol trisphosphate

ipRGC: intrinsically photosensitive retinal ganglion cells

LD: light-dark exposure

LH: lateral hypothalamus

LL: light-light exposure

mRNA: messenger ribonucleic acid

NREM sleep: non-rapid eye movement sleep

OPN: olivary pretectal nucleus

Opn4: melanopsin

PAC1: pituitary adenylate cyclase activating polypeptide receptor-1

PACAP: pituitary adenylate cyclase activating polypeptide

PLC: phospholipase C

PLR: pupillary light reflex

REM sleep: rapid eye movement sleep

RS: recovery sleep

RT-PCR: reverse transcription polymerase chain reaction

RT-qPCR: real time quantitative polymerase chain reaction

SC: superior colliculus

SCN: suprachiasmatic nucleus

SD: sleep deprivation

SEM: standard error of mean

SPVZ: subparaventricular zone

VIP: vasoactive intestinal peptide

VLPO: ventrolateral preoptic nucleus

VPAC1: vasoactive intestinal peptide/pituitary adenylate cyclase activating polypeptide receptor-1

ZT: Zeitgeber time

1. Abstract

The photopigment melanopsin plays an important role in all three processes known to regulate sleep: the circadian and homeostatic processes and the direct effects of light - results mainly relying on electrophysiological analysis. We therefore examined mRNA expression levels of melanopsin (Opn4) and retinal pituitary adenylate cyclase activating polypeptide (PACAP), a neurotransmitter of the retinohypothalamic tract, which is thought to relay the melanopsin mediated signals to the brain. MRNA expression levels were determined in dependence of light, darkness and circadian and homeostatic processes in nocturnal and diurnal rodents using RT-qPCR.

My results show that melanopsin mRNA concentrations follow a circadian rhythm in nocturnal (C57BL/6 mouse and albino Wistar rat) and diurnal species (Arvicanthis ansorgei). In mice, the PACAP mRNA expression was similarly affected and a comparable tendency was observed in Arvicanthis ansorgei. The phase relationship between both circadian rhythms - Opn4 and PACAP - suggests that PACAP is involved in conveying melanopsin-based photic input to the brain. In all species studied, the respective peaks of Opn4 and PACAP occurred in the activity phases of each animal. In the mouse, the highest melanopsin mRNA expression level was at the end of its activity phase (ZT21) whereas in Arvicanthis ansorgei the peak level occurred at the beginning of the same (ZT0-3). Arvicanthis ansorgei´s melanopsin expression levels were inverted when compared to the albino Wistar rat. Here, the peak was detected at the light-dark transition which is likewise considered as the beginning of its activity.

In addition, I could show that melanopsin mRNA levels increased under sleep deprivation in mice and Arvicanthis ansorgei, returning to control values during recovery sleep. Again, the same pattern could be demonstrated for retinal PACAP mRNA concentration in Arvicanthis ansorgei. Thus, melanopsin could be established as a sleep homeostatic factor, providing a new concept in the field of sleep research.

Finally, I could show that continuous exposure to light or darkness for three days does not influence melanopsin expression in mice or Arvicanthis ansorgei. The findings suggest that Opn4 expression is not regulated by light and dark in these animal models. These results are in contrast to results obtained in albino rats. However, the absence or presence of pigments might have an unrecognized impact.

The current work improves our understanding of the mechanisms regulating melanopsin expression and provides significant clues to further understand the effect of light on sleep, alertness and associated behaviors.

Abstrakt

Das Photopigment Melanopsin spielt eine wichtige Rolle in den drei bekannten Prozessen, die an der Regulation von Schlaf beteiligt sind: Den circadianen- und homöostatischen Prozessen und den direkten Effekten von Licht. Da diese Erkenntnisse vor allem auf elektrophysiologischen Daten basieren, haben wir die mRNA Expression von Melanopsin (Opn4) und retinalem pituitary adenylate cyclase activating polypeptide (PACAP) untersucht, um diese Ergebnisse weiter zu erhärten. PACAP wurde untersucht, da es sich um einen Neurotransmitter des retinohypothalamischen Traktes handelt, der die von Melanopsin aufgenommenen Lichtsignale an das Hirn weiterleitet. Die mRNA Expression wurde in Abhängigkeit von Licht, Dunkelheit und circadianen und homöostatischen Prozessen in nachtaktiven und tagaktiven Nagern durch RT-qPCR bestimmt.

Meine Ergebnisse zeigen, dass die Melanopsin mRNA sowohl in nachtaktiven (C57BL/6 Maus und Albino Wistar Ratte), als auch in tagaktiven (Arvicanthis ansorgei) Tiermodellen einem circadianen Rhythmus folgt. In der Maus wies die PACAP mRNA ein der Melanopsin Expression ähnliches Muster auf und in Arvicanthis ansorgei war eine vergleichbare Tendenz zu beobachten. Das Verhältnis zwischen den circadianen Phasen von Opn4 und PACAP weist darauf hin, dass PACAP an der Melanopsin abhängigen Lichtübertragung zum Gehirn teilnimmt. In allen untersuchten Arten wurde das höchste Expressionsniveau während der Aktivitätsperiode des jeweiligen Tiermodells beobachtet. In der Maus war das Maximum der Expression am Ende ihrer Aktivitätsperiode (ZT21), wohingegen das Maximum in Arvicanthis ansorgei am Anfang der Aktivitätsperiode lag (ZT0-3). Das Melanopsin Expressionsniveau war im Vergleich zu den nachtaktiven Albino Wistar Ratten spiegelverkehrt. Hier zeigte sich ein Expressionshöhepunkt am Übergang von Licht zu Dunkelheit, der ebenfalls mit dem Beginn der Aktivitätsperiode korreliert.

Schlafentzug induziert sowohl in Arvicanthis ansorgei, als auch in der Maus die Expression der Melanopsin mRNA und der anschließende Erholungsschlaf bewirkt das Abfallen auf ein normales Expressionsniveau. Die PACAP mRNA in Arvicanthis ansorgei zeigte ein ähnliches Muster. Die Ergebnisse deuten auf Melanopsin als Schlaf-homöostatischen Faktor hin - Hinweis auf ein neues Konzept in der Schlafforschung.

Schließlich konnte ich zeigen, dass eine kontinuierliche Licht- oder Dunkelexposition die Melanopsin mRNA Expression weder in der Maus noch in Arvicanthis ansorgei beeinflusst. Opn4 wird folglich in den untersuchten Tiermodellen nicht durch Licht oder Dunkelheit reguliert. Die Ergebnisse stehen im Gegensatz zu Ergebnissen in der Albino Ratte, eine Diskrepanz die darin begründet sein könnte, dass eine fehlende Pigmentierung einen erheblichen Einfluss hat.

Die vorliegende Arbeit erweitert unser Verständnis der Regulationsmechanismen von Melanopsin und gibt wichtige Hinweise zum Verständnis der Effekte von Licht auf Schlaf und dem damit verbundenen Verhalten.

2. Introduction

2.1 Sleep

Sleep is a behavioral state characterized by reduction or absence of consciousness in which we as humans spend about one third of our lifetime. It is defined by electroencephalographic (EEG) criteria and can be divided in non-rapid eye movement (NREM) sleep and rapid eye movement (REM) sleep. NREM sleep is characterized by a slowing of cerebral activity in the EEG, a decrease of body temperature, slowing of breathing, a diminished muscle tonus. EEG criteria allow for differentiating three stages of NREM sleep in humans: stages one to three. The higher the stage the deeper sleep is. Stages one and two show a slowing of the EEG with K-complexes and spindles appearing in stage two, stage three is defined by delta waves. In contrast, during REM sleep, muscle tonus is ablated and dreaming is associated with this vigilance state. It is characterized by rapid waves in the EEG and occurrence of rapid ocular movements. Sleep is divided into sleep cycles with alternating NREM and REM sleep phases. After falling asleep humans usually experience three stages of NREM sleep followed by REM sleep. These four stages form a complete sleep cycle that lasts about 90 minutes. During one night four to five sleep cycles can be observed with NREM sleep predominating in the beginning of the night and REM sleep predominating at the end of the night. (1)

Mice on the other hand spend around half of their life asleep (10 to 12 hours / 24 hours), (2, 3). Sleep in rodents is also defined by EEG criteria and, like in humans, is divided into REM and NREM sleep. However, conventionally sleep researchers consider only one stage of NREM sleep which is defined by the presence of delta waves in mice. Like in humans, REM sleep shows an absence of muscle tonus and rapid eye movements. Mice show much shorter sleep cycles than humans. One cycle lasts nine to twelve minutes and can even be shorter (3). Over 24 hours mice show about 48 to 86 minutes of paradoxical sleep and 570 to 788 minutes of NREM sleep depending on the genotype (4).

Sleep is essential to life, an observation true for all mammals, all birds and many reptiles, amphibians and fish. However, up to this day it is still not well understood why

we spend so much time asleep. One of the main functions of sleep is resuscitation and resting of the brain and the body. Resting is essential from a somatic as well as from a psychological perspective and has major effects on physiology, cognition and mood.

The importance of sleep has been studied by many groups by looking at the effects of sleep deprivation in animal studies and in humans. First of all, sleep seems to have an anabolic effect. Benington and Heller have indicated that brain glycogen is depleted during waking and restored during sleep (5). Animal trials have also shown that sleep deprivation causes weight loss even though food intake is higher and a decrease in body temperature (6). Total sleep deprivations of about two to three weeks or paradoxical sleep deprivations of about four to six weeks resulted in death of the rat (6).

In humans it has been shown that the altered metabolisms caused by sleep deprivation relates to lower levels of leptin and higher levels of ghrelin. Both hormones are involved in regulation of food uptake by inducing (ghrelin) or reducing (leptin) the feeling of hunger and appetite. The deregulation of the hormones through sleep deprivation also has the capacity to activate the hypocretin system responsible for arousal, consummatory behavior and energy expenditure – a sleep adverse physiological state which might augment the metabolic effects of sleep deprivation and result in a vicious cycle causing sleep perturbations (7, 8). The increased food uptake makes sleep deprivation a risk factor for obesity and accounts for a deregulated carbohydrate metabolism (9) which is considered a risk factor for the development of diabetes (10). Furthermore, the deregulation of the hypocretin system and its effect on cardiac regulation at times of sleep deprivation and sleep perturbations explains that hypertension is correlated with sleep deprivation (11).

In addition, sleep deprivation seems to have major effects on the immune system. Animal studies are still controversially discussed as to whether there is a failure of the immune system following sleep deprivation but there is evidence that shows in this direction (6, 12). In humans, sleep deprivation affects plasma levels of cytokines or immunocomplexes (13) and reduces the number and activity of natural killer cells (14). In addition, upon sleep deprivation plasma cortisol levels are elevated which are known to affect the immune system (15).

Finally, sleep has major neurological and psychological effects. It has been shown that humans under sleep deprivation perform poorly in psychomotor vigilance tests (16).

Diminished vigilance in turn leads to diminished cognitive functions (17) manifested as an impairment of memory consolidation (18, 19) and a diminished plasticity of the brain (20). Haack et al. could show in their study that sleep restrictions lead to a decline in sociability and fatigue ratings, characterized by diminished optimism and increased tiredness (21). Thus, sleep or even more so the lack of sleep plays an important role in mood.

2.2 The Model of Sleep Regulation

Today it is quite well understood that the structure of sleep and sleep timing is influenced by two different mechanisms: The homeostatic and the circadian process (22), (Figure 1). The homeostatic process monitors sleep-need which increases as a function of time spent awake and decreases exponentially during sleep (23). Delta power increases after sleep deprivation and decreases during sleep (24, 25). Slow wave activity or delta power (0,5/0,75-4,5 Hz or 1–4 Hz) in the EEG is therefore a marker for sleep need (23, 26, 27). In contrast, the circadian system is an inner biological clock of about 24 hours (hence the term circa/about, dies/day), a self-sustained oscillation generated in the suprachiasmatic nucleus (SCN) of the hypothalamus in mammals which governs a physiological and behavioral adaption to the day-night cycle (23). This inner clock functions on its own but is only instrumental if it is synchronized to solar time. This process is called photoentrainment. Entrainment of the circadian cycle relies on so called Zeitgebers which are social and environmental factors contributing to the synchronization. Among the Zeitgebers light is the most important one, however, food intake or social behavior additionally contribute to entrainment of the circadian rhythm and are able to compensate in the absence of light (28). In the laboratory, time is described by Zeitgeber Time (ZT), with ZT0 as light onset and ZT12 as dark onset.

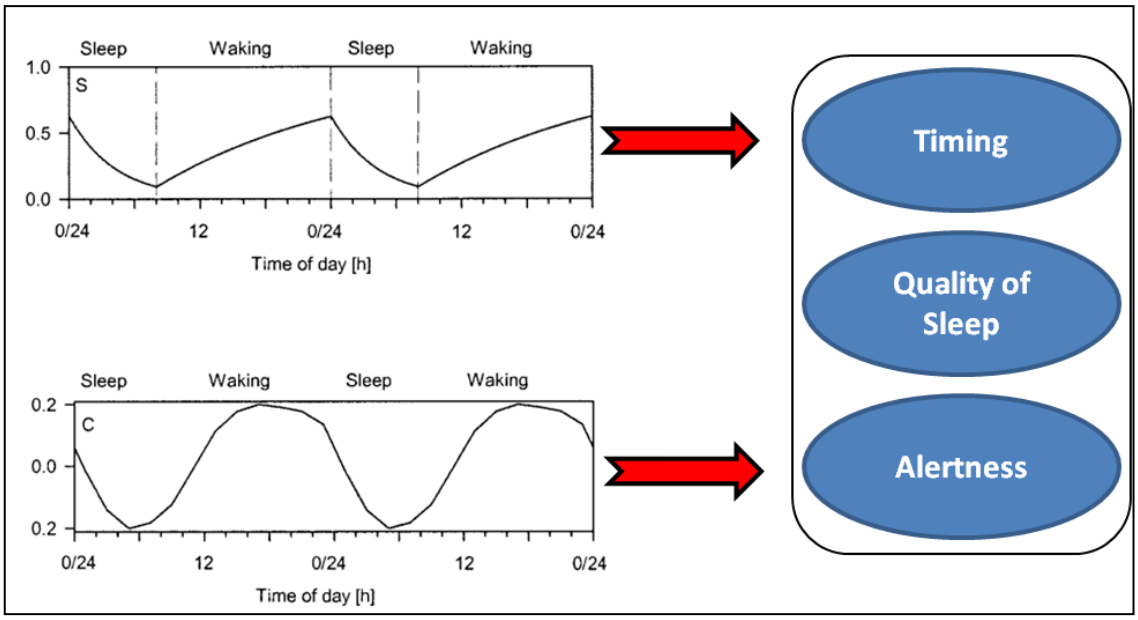

Figure 1: Two process model of sleep regulation, adapted from (22, 23): The homeostatic process (process S, figure at top) describes an increase of sleep need during the time spent awake and a decrease of sleep need while asleep. The circadian process (process C, figure at bottom) represents an inner biological clock of about 24 hours that describes the sleep-wake distribution over the 24 hour cycle. These two processes regulate the timing and quality of sleep and alertness.

2.3 The Photoreceptor Melanopsin

Research in the field of photoreception has led to the discovery of a new photoreceptor: Melanopsin (Opn4) (29) – a photoreceptor that is expressed in a subset (1-2%) of retinal ganglion cells residing in the ganglion cell layer of the retina (30). Due to the expression of the photoreceptor melanopsin these retinal ganglion cells become intrinsically photosensitive (31) and are therefore referred to as intrinsically photosensitive retinal ganglion cells (ipRGCs) (Figure 2).

Different subtypes of ipRGCs have been distinguished based on their morphology as well as their physiology. Up to now five cell types have been identified (M1-M5) with the M1 cells relying primarily on melanopsin phototransduction whereas the other cells have a smaller intrinsic response. Melanopsin cells also form synapses with amacrine and horizontal cells and can therefore receive signals from rods and cones (32). Melanopsin´s sensitivity to light is highest at 460-480nm (33) which corresponds to the blue spectrum of light.

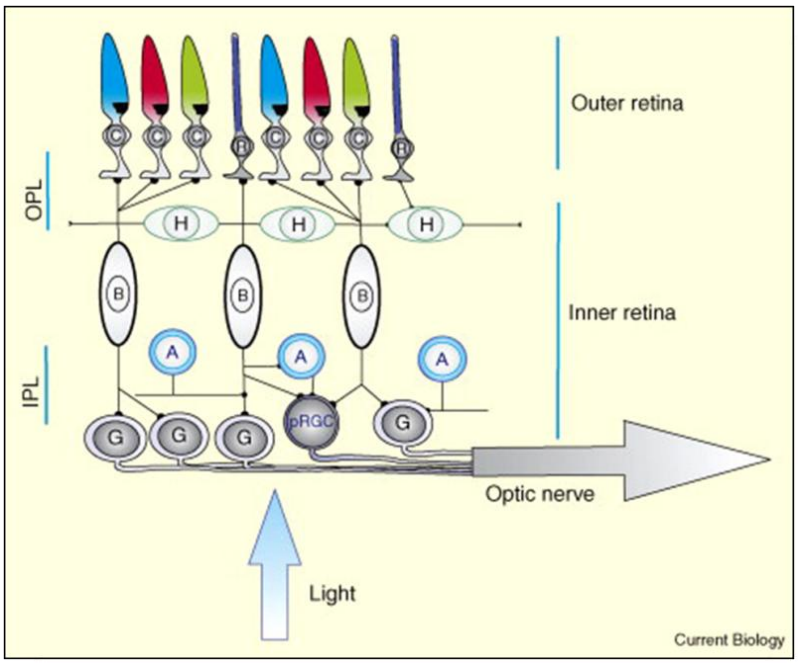

Figure 2: Light detection in the vertebrate retina: Through activation of the pRGCs which are found in the retinal ganglion cell layer, non–visual light information is transmitted to the brain via the optic nerve. Activation of the rod and cone photoreceptors allows for visual light information to be transmitted via the bipolar and amacrine cells to the retinal ganglion cell layer. The axons of the retinal ganglion cell layer converge to the optic nerve and reach the brain. R=rods; C=cones; H=horizontal cells; B=bipolar cells; A=amacrine cells; G=ganglion cells; pRGCs=photosensitive retinal ganglion cells; OPL=outer plexiform layer; IPL=inner plexiform layer (34).

The discovery of ipRGCs and their photoreceptor melanopsin provided the missing link between the Zeitgeber light and photoentrainment. In addition, it extended the physiological role of light. Light is not only responsible for image forming but is an important trigger of non-visual light information.

The melanopsin receptor is a sevenfold transmembrane receptor that consists of an opsin protein coupled to the chromophore 11-cis retinaldehyde (Figure 3). Absorption of light initiates a phototransduction cascade which is triggered by isomerization of 11-cis retinal to all-trans retinal (35). Structural changes of melanopsin in response to the isomerization activate a G Protein which in turn induces the activation of a phospholipase C (36). This activation is followed by an as yet not well understood signaling cascade resulting in the opening of a cation channel followed by depolarization of the membrane. These assumptions are based on the analogy to the invertebrate phototransduction as melanopsin resembles more the invertebrate than with the vertebrate system (37, 38).

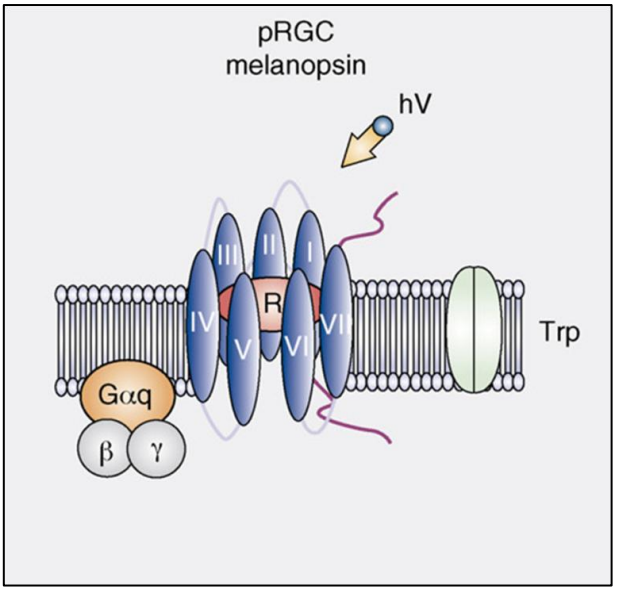

Figure 3: The photoreceptor melanopsin: The opsin protein is a sevenfold trans-membrane receptor that is activated by photons (hV). Structural changes of melanopsin lead to the activation of a G-protein. A not well known signaling cascade leads in turn to the opening of cation channels. Trp=transient receptor potential, R=receptor. (39)

The discovery of melanopsin has opened an entirely new field of research on non-visual light information. The development of melanopsin knockout mice (40, 41) as well as the examination of rodless/coneless mice has enabled researchers to understand the functions of the melanopsinergic system and the crosstalk of image forming and non-image forming information. Today it is well accepted that one important function of melanopsin is that of a signal transducer to enforce photoentrainment acting independently from rods and cones (42, 43). However, the function of melanopsin goes far beyond that of photoentrainment. It also plays an important role in the regulation of the pupillary light reflex (PLR) (33) especially in bright light (44) and in the suppression of pineal melatonin release (45). Studies in humans exposed to short wave length light indicate that melanopsin plays a role in the regulation of heart rate, body temperature and alertness (46). Ablation of ipRGCs in mice has shown an even greater deficit in non-image forming effects than it has been shown in melanopsin knockout mice which suggests a cross talk between the image forming cones and rods and ipRGCs (47). The group even goes as far as to say that “melanopsin cells are the principal conduits for rod–cone input to non-image-forming vision” (47). Melanopsin also acts as a signal transducer to thalamic vision, to neuroendocrine systems and seems to influence

anxiety (32). Recently, another important activity of melanopsin has been identified. Melanopsin not only plays an important role in circadian entrainment but also in the homeostatic process of sleep by conveying direct light signals to the brain which determine the occurrence and quality of sleep (48), (Figure 4).

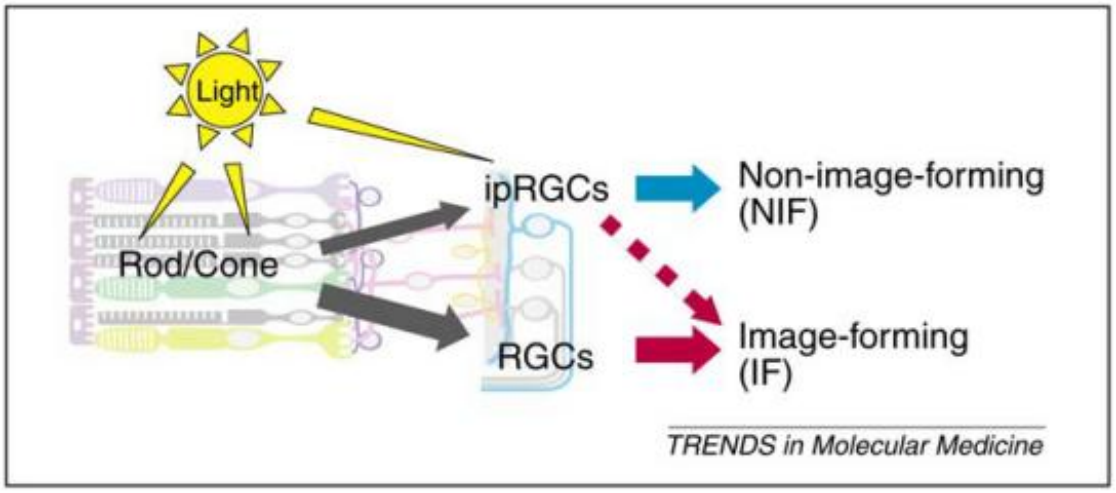

Figure 4: Image–forming and non–image forming light information: Light activates the rod and cone photoreceptors and the ipRGCs (intrinsically photosensitive ganglion cells). The rods and cones transmit image forming or visual light information via the retinal ganglion cells (RGCs) but also non – image forming information via the ipRGCs. A transmission of image forming information via the ipRGCs is also in discussion. The direct activation of the ipRGCs allows non – image forming information to be transmitted to the brain without the need of the rods or cones (37).

2.4 The Retinohypothalamic Tract

Signals initiated by melanopsin are relayed to the brain through the retinohypothalamic tract (RHT). The RHT is a monosynaptic pathway which originates in the ipRGCs and projects to the hypothalamus via the optic nerve and to several other areas where the ipRGCs convey non visual light effects. The main projections are to the SCN, to the intergeniculate leaflet (IGL), and the olivary pretectal nucleus (OPN). Among others there are projections to the lateral hypothalamus (LH), the subparaventrivular zone (SPVZ), the ventrolateral preoptic nucleus (VLPO) and the superior colliculus (SC) (49, 50), (Figure 5).

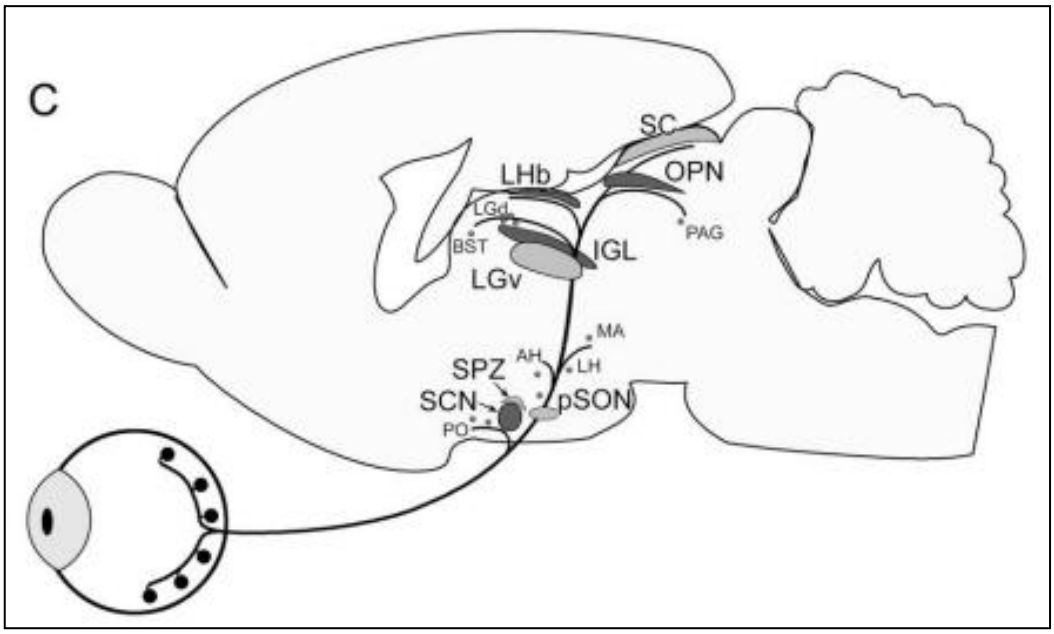

Figure 5: Axonal projections of the intrinsically photosensitive retinal ganglion cells**:** SCN=suprachiasmatic nucleus, SPZ=subparaventricular zone; pSON=peri-supraoptic nucleus; PO=preoptic area; AH=anterior hypothalamic nucleus; LH=lateral hypothalamus; MA=medial amygdaloid nucleus; LGv=lateral geniculate nucleus ventral division; LGd=lateral geniculate nucleus dorsal division; IGL=intergeniculate leaflet; OPN=olivary pretectal nucleus; SC=superior colliculus; LHb=lateral habenula; PA=periaqueductal grey; BST=bed nucleus of the stria terminalis (50).

It has been shown that the RHT is implicated in entraining the circadian rhythm through the projections to the SCN (51, 52).The projections to the OPN (53, 54) indicate a role in the pupillary light reflex.

The functional role of the other regions remains to be further elucidated. The SPVZ is connected to the SCN and plays a role in circadian rhythms and in the photic regulation of sleep. The IGL is interconnected with the SCN and the OPN. It plays a role in photic and non-photic phase shifting of the circadian rhythm. The VLPO is involved in the regulation of sleep, particularly in the switch from waking to sleep (55). The superficial layer of the superior colliculus plays a role in orienting behaviors of the eyes and other organs (56), the LH is implication in energy homeostasis (38). New data show that melanopsin might have influence on the visual system as there is an activation of the lateral geniculate nucleus in rodless/coneless mice (57).

All these effects represent non-visual effects of light that are or might be mediated by melanopsin. The effects reach the brain via the RHT where the pituitary adenylate

cyclase activating polypeptide (PACAP) acts as a co-neurotransmitter with glutamate (58, 59) modulating glutamate induced effects (60). (Figure 6)

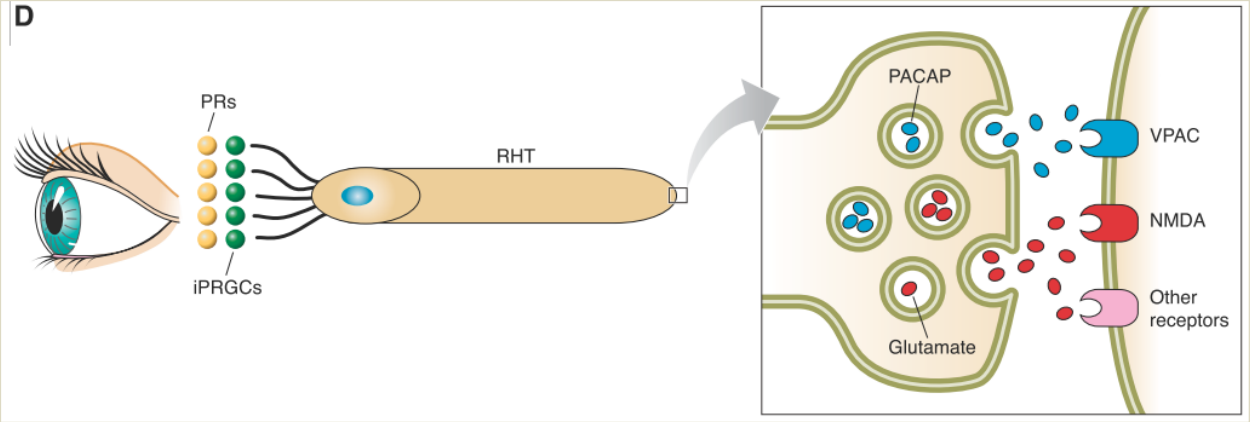

Figure 6: The retinohypothalamic tract (RHT) originating in the intrinsically photosensitive retinal ganglion cells (ipRGCs) and projecting to the brain. PR=photoreceptor; VPAC=VIP and PACAP receptor; NMDA=glutamate receptor (61)

2.5 Pituitary Adenylate Cyclase Activating Polypeptide

PACAP is a neuropeptide which functions as a neurotransmitter and neuromodulator and which is thought to be involved in relaying melanopsin mediated signals via the RHT. It exists in the form of two active peptides: PACAP38 and its truncated PACAP27 form. As the name indicates it activates the adenylate cyclase in different tissues which induces cyclic adenosine monophosphate (cAMP) production and therefore activation of phospolipase C (PLC). Another molecule that can be activated is inositol trisphosphate (IP3). PACAP receptors can be divided into type1 and type 2 receptors. The type 1 receptor is called pituitary adenylate cyclase activating polypeptide receptor-1 (PAC1); the type 2 receptors consist of vasoactive intestinal peptide/pituitary adenylate cyclase activating polypeptide receptor-1 (VPAC1) and VPAC2 receptors. The PAC1 receptor is specific for PACAP, the VPAC receptors bind PACAP and vasoactive intestinal peptide (VIP). The two active forms of PACAP show similar affinities to all PACAP receptors however with different consequences on the transduction signal. PACAP38 has the same effect on cAMP production as PACAP27 when coupled to the PAC1 or to the

VPAC2 receptor but shows a greater induction of cAMP production than PACAP27 when coupled to the VPAC1 receptor (62).

PACAP is widely expressed in the central nervous system and in peripheral tissues. It can also be found in the eye of the rat where it is present in amacrine cells (inner nuclear layer), in the inner plexiform layer, in the nerve fiber layer, in the retinal papilla and in retinal ganglion cells (62). In the rabbit it is also found in fibers innervating the iris sphincters and in the ciliary ganglia (63).

Interestingly, melanopsin is exclusively present in retinal ganglion cells containing PACAP (64). Therefore PACAP is a marker of the RHT and an important factor to distinguish ipRGCs from normal RGCs. PACAP therefore plays an important role in non–image forming effects of light.

PACAP has a multitude of functions like the control of neurotransmitter release, vasodilation, bronchodilation, increase in histamine and insulin secretion and the regulation of energy balance and feeding (62). Its role in regulating melatonin release via the SCN and in entraining circadian rhythms has been clearly established (60). In the regulation of sleep it plays a role in REM sleep induction. PACAP administered in the oral pontine reticular nucleus enhanced REM sleep in the rat (65).

Regulation of PACAP expression might be more finely tuned than its co-transmitter glutamate. In addition, data in the literature (see below) is contradictory as to whether PACAP regulation is influenced by light or rather has its own circadian rhythm. Therefore it is of great interest to examine PACAP mRNA expression under the same experimental conditions as used for the examination of melanopsin mRNA expression in order to better understand the downstream mechanisms of the non-visual effects of light.

2.6 Melanopsin and Sleep

To date, it is well accepted that melanopsin is the phototransducer conveying the signal of the Zeitgeber light to enforce photoentrainment. The link between melanopsin and the circadian system has therefore been clearly established.

Tsai et al. discovered a second mode of action of melanopsin, providing the evidence of a link between melanopsin transmitted light signals and sleep homeostasis (48). The study showed that EEG delta power, a reliable marker of sleep need and of the sleep homeostatic process (23), is decreased in melanopsin knockout mice. Sleep deprivation was conducted to challenge the sleep homeostatic process. The decreased EEG delta activity reached after sleep deprivation in the absence of melanopsin confirms that the photopigment affects sleep homeostasis. Thus, melanopsin transduces signals to the brain which directly affect the homeostasis of sleep and determines the occurrence and quality of sleep.

It was also observed that exposure to light promptly induced sleep in nocturnal animals during the active phase (darkness). Conversely, darkness induced waking during the sleeping phase. These effects are attenuated in melanopsin knockout mice. Thus, a light pulse provoked sleep in wild type mice, but failed to induce sleep in melanopsin knockout mice. Darkness on the other hand induced alertness in nocturnal rodents which was attenuated in melanopsin knockout mice (48, 66, 67). Photic input activates the VLPO (67) and more specifically, the “sleep promoting galaninergic neurons of the VLPO” (48) suggesting that neuronal pathways activated by melanopsin convey these signals. Tsai et al. indicate that these direct effects of light mediated by melanopsin predominate in the subjective dark period showing an interaction with the circadian component (48). Using various light-dark exposures the study demonstrates that melanopsin-dependent light sustained promoting effects on sleep in nocturnal species.

These observations suggest that it might be necessary to add a third independent pathway that converges with the other two to the current model of sleep regulation in saying that the direct influence of light on sleep and alertness represents another key regulatory mechanism of sleep, independent of the circadian component and that melanopsin plays a role in this regulatory process. We have recently discussed the direct effects of light in a review of the literature: “Non-circadian direct effects of light on sleep and alertness: lessons from transgenic mouse models” (68), (Figure 7).

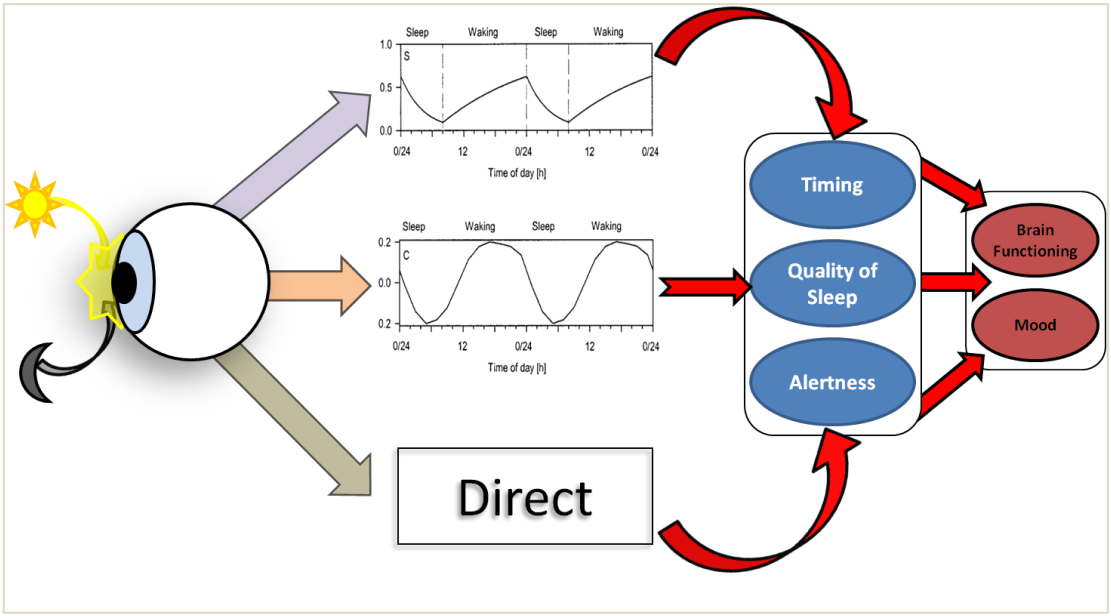

Figure 7: New model of sleep regulation: Sleep is regulated by the homeostatic process, the circadian process and by a third independent process: the direct effects of light. These three processes influence timing and quality of sleep as well as alertness. Sleep in turn has an effects on mood and brain functioning (68). Adapted from (23)

Present studies of melanopsin regulation

To date, few studies of melanopsin expression in dependence of the circadian cycle have been performed. Circadian experiments were mostly conducted in albino rats. Several studies found a circadian regulation of melanopsin messenger ribonucleic acid (mRNA) over the 24 hour cycle (69-72). The peak is shortly after the transition of light to dark at ZT14 or 16. Hannibal and his group also examined protein levels. The Western blot experiments showed highest protein levels between ZT10-12 and lower levels at ZT24 (69). This would mean that transcription from mRNA to proteins would take about 20 hours which is very unlikely. Recent experiments in pigmented Brown Norwegian rats show a similar circadian regulation as in albino rats with a mRNA peak at about ZT13/14 and a protein peak between ZT20-24 (72). These results seem more in line with current knowledge on translation, yet still leave a minimum six hour difference between the mRNA and the protein expression. One group examined the number of melanopsin-expressing cells through immunohistochemical labeling in pigmented C3H/He mice and could show that cell numbers (melanopsin protein) were highest at ZT23 and lowest at ZT3 (73).

These studies also examined the direct effects of light on Opn4 mRNA expression. When RCS/N rats were exposed to darkness during 48 hours one study showed a circadian melanopsin expression comparable to the LD experiment (71), two other studies showed a significant increase in melanopsin mRNA levels after only two days of DD exposure in pink-eyed animals (69, 70) and a significant decrease of melanopsin levels after only two days of LL exposure with melanopsin almost undetectable after three days of LL (69, 74). González-Menéndez et al. also showed that the circadian oscillation seen under LD conditions flattened after six days of DD in the mouse. This finding was interpreted as evidence that the daily fluctuation of Opn4 expression is regulated by light/dark transitions and not by an endogenous circadian rhythm. However the cell numbers were not compared to those under LD condition (73). Mathes et al. could also show that the circadian rhythm was abolished after two days of DD exposure in albino rats (70). Conversely, the most recent experiments in the Brown Norwegian Rat showed that three days of darkness or light do not alter Opn4 mRNA or protein expression. Solely this latter study investigated the influence of prolonged exposures to continuous light or dark in adult animals. After 21 days of continuous light mRNA levels were still not affected, however, melanopsin protein levels showed a significant decrease. After 21 days of continuous darkness Opn4 mRNA levels did not change and the protein only increased to a level it would normally have at the end of the night during a LD cycle. Opn4 mRNA maintained a circadian rhythm after one day in DD but not the protein (72). It was concluded that melanopsin mRNA has an endogenous circadian rhythm but that the protein is regulated by light or darkness.

Present studies of PACAP regulation

The effect of PACAP on circadian rhythms has been extensively studied in the past. PACAP is seen as a retinal messenger to the SCN, co-transmitting the light information received by the melanopsinergic cells (75). It has been shown that PACAP can phase shift the circadian rhythm and the absence of PACAP receptors change the effect of light on the circadian rhythm (75). However there are not many studies examining the circadian regulation of PACAP in the retina. Fukuhara et al. examined the circadian rhythm of the polypeptide in the SCN during a LD and DD rhythm in Wistar rats using a sandwich enzyme immunoassay with monoclonal antibodies. As PACAP is synthesized

in the retinal ganglion cells which terminate in the SCN, it was assumed that they represent the retinal PACAP expression. PACAP decreased after light onset from ZT0 to ZT8 to re-increase and reach a peak at ZT12. This rhythm becomes flat under DD exposure with a stable level of PACAP (76) which demonstrates the importance of light in the rhythm of PACAP. The other brain regions in the brain examined (cortex, periventricular nucleus) did not show a day-night variation. Lee et al. showed that there is a difference between PACAP amount in the SCN between ZT6 and ZT18 with the PACAP amount being more important at ZT18 in Long-Evans male rats (77). Another study also examined PACAP protein using an immunoassay in the chicken retina and other parts of the chicken brain (diencephalon, telencephalon, brain stem etc.) but not the SCN. This study showed a daily variation with a decrease of PACAP, beginning at lights on, reaching a trough after six to nine hours, to then re-increase when the light was off, reaching a peak at ZT0/24. This rhythm was maintained under DD conditions which is contradictory to the results obtained by Fukuhara et al (78). Daily variations with two peaks of PACAP mRNA of receptors (PAC1-R and VPAC-2R) in the SCN were also observed (79, 80) which might explain the effect of PACAP injection only at certain time points.

Animal Models

Given the complexity of the sleep processes the selection of the animal model seems crucial. For a variety of reason which include practicability and the potential of genetic manipulations mice are the animals of choice. The development of the melanopsin knockout mouse in a mixed background strain (C57BL/6–129/Sv) explains why a lot of studies were performed with mice of mixed genetic background (40, 41). However, to minimize the influence of the genetic background of the animal, our group backcrossed the colony on a C57/BL6 background for proper control (C57BL/6). The disadvantage of mice lies in the small retinas which only allow for small sample sizes limiting the number of analysis. For this reason and also because chronobiological studies are often performed in rats, a lot of animal studies had been performed in rats – mostly albino rats as these are the most widely used laboratory strains. It has yet to be fully elucidated whether the lack of eye pigmentation has any effect on non-visual light transmission. Regardless of the genetic variability of these animal models, however, one has to keep

in mind that mice and rats are nocturnal animals and results obtained from these studies might have limited predictive quality to humans. The discovery of the diurnal rat Arvicanthis ansorgei (81) and the habituation to laboratory conditions of this animal gives the opportunity for studies in a small diurnal animal and although mice models will still be very useful, studies in this model might give important new insights and opens up new perspectives for translational research. Arvicanthis ansorgei has for some time been confounded with Arvicanthis niloticus. It probably has a longer life expectancy than A. niloticus (81) which is between two to six years (http://animaldiversity.ummz.umich.edu/site/accounts/information/Arvicanthis_niloticus.html). The coat color is grey, the size variable (81).

2.7 Questions Addressed in this Thesis

It was the intention of my thesis to analyze the effect of the three processes, implicated in the regulation of sleep, on retinal expression levels of melanopsin and PACAP mRNA in a nocturnal mouse model and diurnal rat model. These data would corroborate present findings observed in knockout mouse studies and would significantly improve our understanding of the role of melanopsin in the processes.

1. Examination of melanopsin and PACAP expression levels in dependence of the circadian cycle

   In order to establish the three process model in the mouse it was crucial to first establish a reliable assay of melanopsin mRNA expression levels in dependence of the circadian cycle in the nocturnal mouse which includes the examination of effect sizes and the influences of gender on expression levels. The assay had to be validated in a rat model, as in this model our data could be compared to published mRNA levels. To better understand downstream processes it was also interesting to look at the regulation of PACAP mRNA. Furthermore, in order to study the switch between nocturnal animals and diurnal animals, it was interesting to study a diurnal rat species. One would assume that mRNA expression levels of nocturnal and diurnal animals were inversed.

2. Examination of melanopsin and PACAP expression in dependence of the homeostatic process

If melanopsin is a sleep homeostatic factor, a finding based on studies in melanopsin knockout mice (48), the assumption would be that melanopsin mRNA expression is regulated by sleep deprivation. With a reliable assay in hand these studies could be performed in the mouse and diurnal rat model. It was interesting to study a diurnal rat species in order to study the switch between nocturnal and diurnal animals. Furthermore to better understand downstream processes it was also interesting to look at the regulation of PACAP mRNA.

3. Examination of melanopsin expression in dependence of light or dark exposure

If the theory of our group is correct that light directly modulates sleep one would speculate that the expression of melanopsin mRNA was also modulated directly by prolonged light or darkness exposure in the nocturnal mouse model or the diurnal rat model, respectively. One would further assume that expression levels of nocturnal and diurnal animals were inversed. However, recent results suggest that mRNA is not regulated by light in pigmented animals. Thus, given the controversial results described above, it was important to perform light experiments in the nocturnal mouse and the diurnal rat model.

3. Materials and Methods

3.1 Animals and Animal Facility

Animals were kept in the animal facility, the chronobiotron (Figure 8), at the Institut des Neurosciences Cellulaires et Integratives (INCI) in Strasbourg. The chronobiotron is an outstanding animal facility devoted to the study of sleep and chronobiology in rodents. It houses transgenic animals as well as rodents needed for the study of rhythms or torpor (Siberian, Syrian and European hamsters, transgenic mouse models, Wistar rats) including also Arvicanthis ansorgei, a diurnal rodent that has been acclimated and maintained under laboratory conditions for 15 years. The facility controls all Zeitgebers (light, temperature, humidity, noise, feeding). The main synchronizer (Zeitgeber) of the circadian rhythm is light. The day is therefore divided in Zeitgeber Time (ZT). ZT0 is the time of light onset, ZT12 the time of dark onset. The most usual light cycle conditions in chronobiology are 12 hours of light followed by 12 hours of darkness (LD 12:12). The intensity of the polychromatic light in the chronobiotron is 200-250 lux. Animals have ad libitum access to food and water, temperature is kept constant at 23,5°C $\pm$ 0,5°C and humidity and noise are also controlled.

Figure 8: Chronobiotron: The animal facility at INCI in Strasbourg devoted to the study of sleep and chronobiology in rodents. The facility controls all Zeitgebers: light, temperature, humidity, noise, feeding.

For a variety of reasons which include practicability and the potential of genetic manipulations, mice are the animals of choice to study sleep. The development of a melanopsin knockout mouse in a mixed background strain (C57BL/6–129/Sv) (40, 41) explains why a lot of studies were performed with mice of mixed genetic background. To minimize the influence of the genetic background of the animals however, studies in mice of pure genetic background for proper control were needed. The group in Strasburg performed sleep studies in C57BL/6–129/Sv mice and later also in the backcross strain. It was therefore interesting to examine whether Opn4 mRNA expression was influenced by the genetic background. As a nocturnal animal model C57BL/6–129/Sv mixed background melanopsin wild type mice or C57BL/6 backcross Opn4 wild type mice from the INCI breeding facility were used. Sample size and availability required experiments in males and females. Mice aged two to four months were used.

Regardless of the genetic variability of these animal models, however, one has to keep in mind that mice and rats are nocturnal animals and results obtained from these studies might be different or inversed in humans. The discovery of the sub-Saharan diurnal rat Arvicanthis ansorgei (81) and the habituation to laboratory conditions of this animal gives the opportunity for studies in a small diurnal animal and although mice models will remain very useful, studies in this model might give important new insights and opens up new perspectives for translational research. Therefore **Arvicanthis ansorgei**, maintained and reproduced in the breeding facility of the INCI since 15 years, was used as a diurnal animal model. The INCI sleep team characterized sleep in Arvicanthis ansorgei and confirmed the diurnality through EEG recordings (unpublished data). The animals were nine months to one year in all experimental conditions except for the circadian cycle where animals were aged one to two years. Males and females were used in the circadian and light experiments for reasons of availability. Only males were used in the homeostasis experiments. For the 12 hour sleep deprivation the animals were subjected to a reversed cycle (light on at ZT12, light off at ZT0) during three weeks before being sacrificed.

Animal treatment and experimentation adhered to the Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 86-23, revised 1985) and the French Department of Veterinary Science, Ministry of Agriculture (licence no. 67–132, Dominique Ciocca).

3.2 Study Protocols

In order to study melanopsin and PACAP mRNA expression in a nocturnal and a diurnal animal model different study protocols were used. These are explained in the following.

3.2.1 Circadian Melanopsin and PACAP mRNA Expression

Time points of sacrifice were chosen according to availability of the animals, without more than four ZTs between two time points as is tradition in the field of chronobiology and with sacrifices over more than 24 hours when possible.

3.2.1.1 Nocturnal Animal Model

Two months old C57BL/6 Opn4 wild type mice were sacrificed at ZT0, 3, 6, 9, 12, 15, 18, 21, 24 and 3 (the next day) under LD 12:12 conditions (n=6/time point; 3 males and 3 females/time point), (Figure 9).

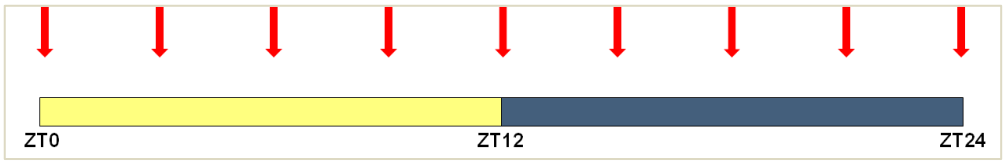

Figure 9: Schema: Circadian cycle mouse: LD 12:12. Yellow=lights on. Blue=lights off. Red arrows indicate time points of sacrifice. ZT=Zeitgeber Time. (n=6 males and females/time point)

A 12:12 LD rhythm in rats was also done in order to validate our method. Male albino Wistar rats (150-200g, aged about two months) were sacrificed at ZT6; 10; 14; 18; 22 and 2 (n=6 males/time point).

3.2.1.2 Diurnal Animal Model

Arvicanthis ansorgei rats aged one to two years were sacrificed at ZT0, 4, 8, 12, 16 and 20 under LD 12:12 conditions (n=4/time point; 2 males and 2 females), (Figure 10).

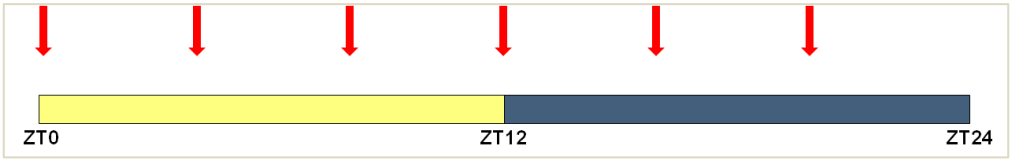

Figure 10: Schema: Circadian cycle Arvicanthis ansorgei: LD 12:12. Yellow=lights on. Blue=lights off. Red arrows indicate time points of sacrifice. ZT=Zeitgeber Time. (n=4 males and females/time point)

3.2.2 Melanopsin and PACAP mRNA Expression and Sleep Homeostasis

Time points of sacrifice and time of sleep deprivation and recovery sleep were defined according to unpublished EEG delta power data the INCI sleep research group and the Tsai publication (48). Gentle handling was chosen for the sleep deprivation as this is by far the least stress inducing method. Toys are given to the animals in order to stimulate them and they are gently nudged when they risk falling asleep. Another method is the slowly moving sleep deprivation wheel which forces the animals to exercise and induces stress.

3.2.2.1 Nocturnal Animal Model

Two to four months old female C57BL/6-129/Sv and C57BL/6 Opn4 wild type mice under 12:12 LD conditions were deprived of sleep by gentle handling during the habitual resting period (daytime). A sleep deprivation (SD) of six hours was done. Animals were deprived of sleep from ZT0 to 6, the remaining animals were allowed to sleep (recovery sleep) from ZT6 to 9. The two genetic backgrounds were evenly distributed (n=3-4) and sacrificed at ZT0, 3 and 6 during SD and at ZT7,5 and 9 during recovery sleep (Figure 11).

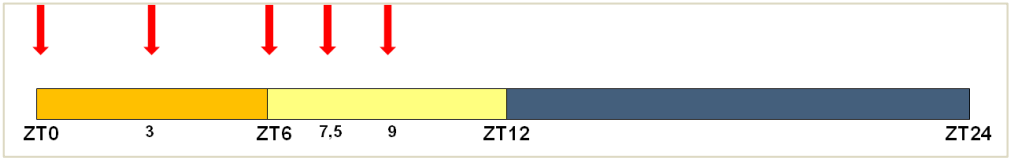

Figure 11: Schema: Sleep deprivation of six hours and recovery sleep in C57BL/6-129/Sv and in C57BL/6 female mice. Yellow=lights on. Blue=lights off. Orange=SD. Red arrows indicate time points of sacrifice. ZT=Zeitgeber Time. (n=3–4 females/time point)

Control experiment: C57BL/6 and C57BL6-129/Sv males and females, aged as above, evenly distributed according to sex and genetic background were sacrificed under 12:12 LD conditions at the same time points as in the sleep deprivation experiment without depriving the animals of sleep: ZT3; 6; 7,5; 9 (n=10-12/time point; 6 males and 4-6 females) (Figure 12).

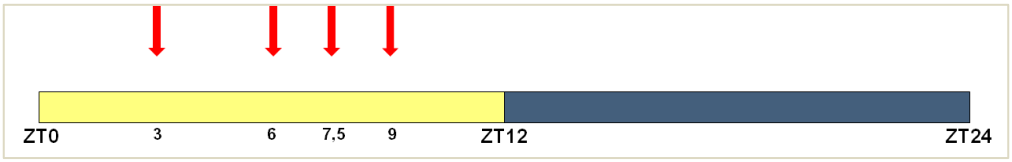

Figure 12: Schema: Control of the six hour sleep deprivation experiment in C57BL/6 and C57BL/6-129/Sv mice. Yellow=lights on. Blue=lights off. Red arrows indicate time points of sacrifice. ZT=Zeitgeber Time. (n=10-12 males and females/time point)

3.2.2.2 Diurnal Animal Model

Nine months to one year old Arvicanthis ansorgei males under LD 12:12 conditions were deprived of sleep by gentle handling during the subjective night. A SD of six, nine and twelve hours was done. In the SD of six hours animals were deprived of sleep from ZT12 to ZT18 and left to sleep from ZT18 to ZT21. N=3 males per time point were sacrificed at ZT12, 15, 18, 19,5 and 21 (Figure 13). In the SD of nine hours, animals were deprived of sleep from ZT12 to ZT21 and left to sleep from ZT21 to ZT24. N=3 males per time point were sacrificed at ZT12, 18, 21, 22,5 and 24 (Figure 15). In the SD of 12 hours animals were deprived of sleep during the subjective night from ZT12 to ZT24 and left to sleep during the following subjective day from ZT0/24 to ZT3. N=4 males were sacrificed per time point at ZT12, 18, 21, 24, 1,5 and 3 (Figure 16).

Control experiment: Arvicanthis ansorgei males, aged as above, under 12:12 LD conditions were sacrificed at the same time points as in the six hour and twelve hour sleep deprivation experiments without depriving the animals of sleep. N=3-8 male animals per time point were sacrificed at ZT15; 18; 19,5; 21 in a first experiment, at ZT18, 21, 24, 1,5 and 3 in a second experiment (Figures 14 and 17).

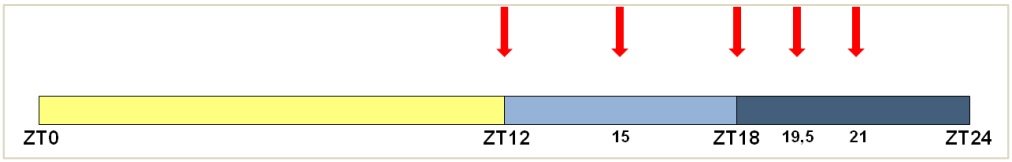

Figure 13: Schema: Sleep Deprivation of six hours and recovery sleep in Arvicanthis ansorgei. Yellow=lights on. Blue=lights off. Light blue=SD. Red arrows indicate time points of sacrifice. ZT=Zeitgeber Time. (n=3 males/time point)

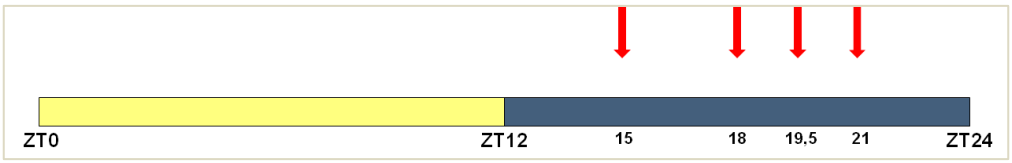

Figure 14: Schema: Control of the sleep deprivation experiment of six hours in Arvicanthis ansorgei. Yellow=lights on, Blue=lights off. Red arrows indicate time points of sacrifice. ZT=Zeitgeber Time. (n=3-6 males/time point)

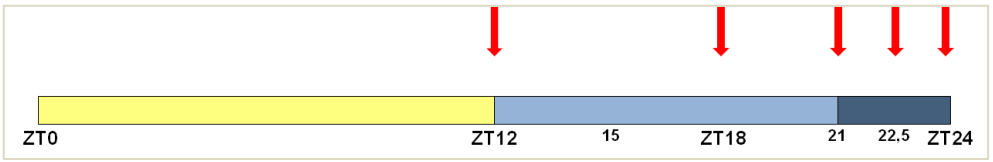

Figure 15: Schema: Sleep deprivation of nine hours and recovery sleep in Arvicanthis ansorgei. Yellow=lights on, Blue=lights off, Light blue=SD. Red arrows indicate time points of sacrifice. ZT=Zeitgeber Time. (n=3 males/time point)

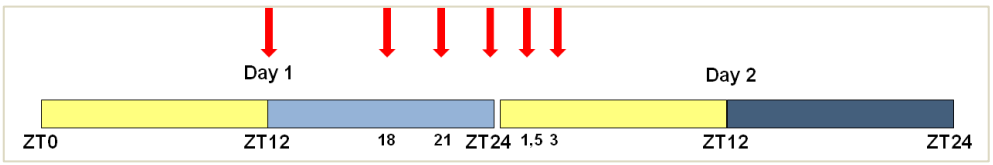

Figure 16: Schema: Sleep deprivation of 12 hours in Arvicanthis ansorgei. Yellow=lights on, Blue=lights off. Light blue=SD. Red arrows indicate time points of sacrifice. ZT=Zeitgeber Time. (n=4 males/time point)

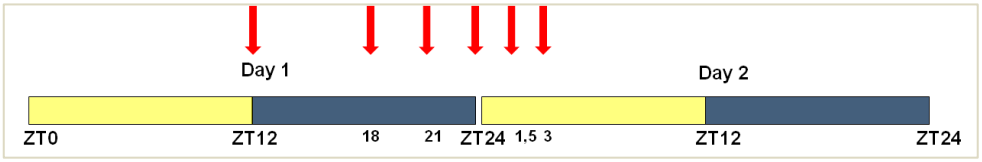

Figure 17: Schema: Control of sleep deprivation of 12 hours in Arvicanthis ansorgei. Yellow=lights on. Blue=lights off. Red arrows indicate time points of sacrifice. ZT=Zeitgeber Time. (n=3-4 males/time point)

3.2.3 Melanopsin mRNA Expression under Direct Influences of Light

The light experiments were constructed according to the original publication of Hannibal's group which showed a significant effect on melanopsin mRNA expression after only three days of light or dark exposure.

Four months old C57BL/6 wild type mice and one year old Arvicanthis ansorgei are kept under LD 12:12 conditions and then subjected to three days of continuous darkness (DD 12:12) or three days of continuous light (LL 12:12). Animals are sacrificed at ZT6 under LD 12:12 conditions (control animals), the remaining animals are then exposed to three days of light or darkness and sacrificed on the fourth day at Circadian Time (CT) 6 (Figure18 and 19). When animals are submitted to three days of darkness or three days of light, light is not a Zeitgeber anymore and the circadian rhythm progressively shifts. Time is therefore not measured in Zeitgeber Time anymore but in CT taking the internal circadian clock into account. Under DD conditions the internal clock of C57BL/6 mice is about 23,33 hours ($\tau = 23{,}33\ hours$). Therefore one can see a phase advance under DD conditions. After three days of darkness a ZT6 in mice becomes a CT5 (data from the INCI laboratory), Arvicanthis ansorgei on the other hand does not shift: ZT6 = CT6 ($\tau = 24 \pm 0{,}1\ hours$) (81). Continuous light exposure delays the phase. After three days of light exposure a ZT6 in mice becomes a CT9 ($\tau = 25\ hours\ in\ LL$) (data from the INCI laboratory), in Arvicanthis ansorgei a ZT6 becomes a CT8,5 ($\tau = 24{,}6 \pm 0{,}1\ hours\ in\ LL$) (81). N=6 animals (3 males and 3 females) were sacrificed per time point.

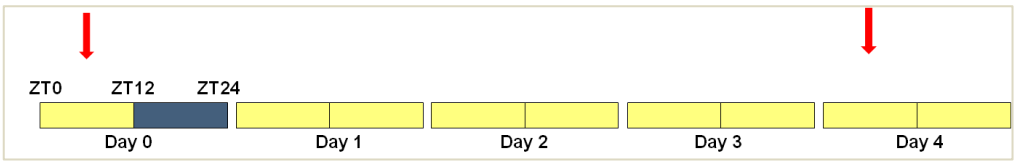

Figure 18: Schema: Exposure to three days of light in C57BL/6 mice and Arvicanthis ansorgei. LL 12:12. Yellow=light on. Blue=lights off. Red arrows indicate time points of sacrifice. ZT=Zeitgeber Time. (n=6/time point; 3 males and 3 females/time point)

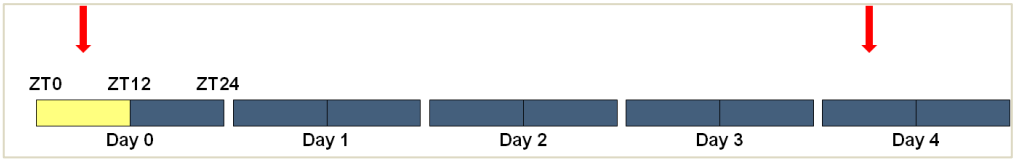

Figure 19: Schema: Exposure to three days of darkness in C57BL/6 mice and in Arvicanthis ansorgei. DD 12:12. Yellow=light on. Blue=lights off. Red arrows indicate time points of sacrifice. ZT=Zeitgeber Time. (n=6/time point: 3 males and 3 females/time point)

3.3 Tissue Extraction

3.3.1 Method of Sacrifice

CO2: Animals were sacrificed by carbon dioxide (CO2) inhalation in order to obtain retinas. Hypoxemia was induced by slowly filling a chamber with CO2 at an emission rate of 10-20% of the volume of the chamber per minute until the CO2 concentration was 100%.

Pentobarbital: For the sleep deprivation experiments, the goal of the experiments was to study retinal melanopsin and PACAP expression and to perform brain immunohistochemistry in the same mice. Animals were sacrificed with an intraperitoneal injection of Pentobarbital (54mg/ml) of about 6 to 7ml/kg. (→324mg/kg). Retinas were extracted and then perfusion started in order to perfuse the brains for **experiments not treated in this thesis**.

3.3.2 Extraction of Retina

Retinas were extracted by opening the eyeball, extracting the crystalline body and then extracting the retina. Retinas were quick frozen in liquid nitrogen and immediately transferred to -80°C until further use.

3.4 Molecular Biology

3.4.1 mRNA Extraction

Extraction of mRNA was done using the Qiagen RNeasy Kit. One retina was used for extraction. For mouse retina 350µl RLT buffer was used, for Arvicanthis retina and

Wistar rat 600µl. All samples were treated with DNAse. The Qiagen protocol was slightly modified: The first centrifugation step was eliminated. (3 min, supernatant extraction). All the centrifugation steps were done for at least one minute instead of 15 seconds. Total mRNA was measured at 280nm and the purity evaluated by measurement of the A260/A280 ratio. The mRNAs were stocked at -80°C or directly used in quantitative PCR.

3.4.2 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

For reverse transcription polymerase chain reaction (RT-PCR) the Kit from Applied Biosystems “high capacity RNA–to–complementary ribonucleic acid (cDNA) master mix” was used.

Melanopsin:

For the **C57BL/6 or C57BL/6-129/Sv** mouse mRNA 500ng of mRNA and 4µl of the mix in a final volume of 20µl was used, for **Arvicanthis ansorgei** and the **albino Wistar rat** 1µg of mRNA and 8µl of the mix in a final volume of 40µl were used.

PACAP:

For the **C57BL/6 or C57BL/6-129/Sv** mouse as well as **Arvicanthis ansorgei** and **Wistar rat** 300ng mRNA and 4µl mix in a final volume of 20µl were used.

The thermocycler was programmed as follows: 5 minutes at 25°C, 30 minutes at 42°C, 5 minutes at 85°C, hold at 4°C.

3.4.3 Real Time Quantitative Polymerase Chain Reaction (RT-qPCR)

For each gene amplification by real time quantitative chain reaction (RT-qPCR), previous experiments were performed to determine the optimal conditions of amplification.

Melanopsin:

To examine the melanopsin mRNA expression in **C57BL/6 and C57BL/6-129/Sv mice** the kit Applied Biosystems TaqMan Gene Expression Master Mix was used for real time quantitative polymerase chain reaction (RT-qPCR). Actin was used as a reference.

2,5µl cDNA, 10µl Master Mix and 1µl TaqMan Assay Primer (melanopsin Mm00443523_m1, actin Mm00619135_m1, 900 nM in final concentration) in a final volume of 20µl were used. Each gene in every sample was analyzed in triplicates.

For the TaqMan amplification the machine was programmed to do a relative quantification as follows: 2 minutes at 50°C, 10 minutes at 95°C, then 40 cycles of first 15 seconds 95°C, then 1 minute at 60°C.

To examine the melanopsin mRNA expression in **Arvicanthis ansorgei** an Applied Biosystems SYBR-Green master mix was used. Actin was used as a reference. The following primers were used: Melanopsin forward 5´- CAGGGATGCTGGGCAATCT - 3´, melanopsin reverse 5´-GTGTCCGCAGGCCTCTGTT–3´, actin forward 5´-CTGCTGCATCCTCTTCCTCTCT–3´, actin reverse 5´-CCACAGGATTCCATACCCAAA–5´. 2,5µl cDNA, 10µl Master Mix and the forward and reverse primers at 900nM in a final volume of 20µl were used. Each gene in every sample was analyzed in triplicates.

For the SYBR-Green amplification the machine was programmed to do an absolute amplification as follows: 10 minutes at 95°C then 40 cycles of first 15 seconds at 95°C, then 1 minute at 60°C.

To examine the melanopsin mRNA expression in the albino **Wistar rat** the kit Applied Biosystems TaqMan Gene Expression Master Mix was used for real time quantitative polymerase chain reaction (RT-qPCR). Actin was used as a reference. 2,5µl cDNA, 10µl Master Mix and 1µl TaqMan Assay Primer (melanopsin Rn00593931_m1, actin Mm00619135_m1, 900 nM in final concentration) in a final volume of 20µl were used. Each gene in every sample was analyzed in triplicates.

For the TaqMan amplification: see above.

<u>PACAP:</u>

To examine the PACAP mRNA expression in **C57BL/6 and C57BL/6-129/Sv mice** and **Arvicanthis ansorgei** a Applied Biosystems SYBR-Green master mix was used. Actin was used as a reference. We used the following primers: PACAP forward 5´-TGGTGTATGGGATAATAATGC-3´, PACAP reverse 5´-GTCGTAAGCCTCGTCTTCT-

3´. 2,0µl cDNA, 10µl Master Mix and the forward and reverse primers at 900nM in a final volume of 20µl were used. Each gene in every sample was analyzed in triplicates.

For the SYBR-Green amplification the machine was programmed to do an absolute amplification as follows: 10 minutes at 95°C, then 40 cycles of first 15 seconds at 95°C, then 1 minute at 60°C.

Analysis of Data:

For each animal and each gene three Cycle thresholds (Ct) were obtained. The means and the standard error of means (SEMs) of these cycle times were calculated. Cts were eliminated when the SEM was over 0,2 (allowed to still keep a duplicate), which is within the accepted range (SEMs can reach up to 0,4 (82)). An amplification factor (amp) corresponding to the slope of the dilution curve was obtained. This factor was applied to every mean of the triplicates:

$$Rnorm = \frac{amp\ e^{-(\bar{X}\ Ct\ gene\ -\bar{X}\ Ct\ internal\ control)}}{amp\ e^{-(\bar{X}\ Ct\ ref\ -\bar{X}\ Ct\ internal\ control)}}$$

Rnorm: normalized ratio

Amp: Amplification factor

Ref: Gene of reference (Actin)

3.5 Statistics

The number of animals was chosen based on previous similar experiments performed in the laboratory. The experimental approach was exploratory as there was no data available on melanopsin and PACAP mRNA expression under sleep deprivation conditions. Even for the circadian expression or light/dark conditions of melanopsin/PACAP we had only a couple of controversial studies, if none for our models (Arvicanthis ansorgei, C57BL/6, C57BL/6-129/Sv). Furthermore, the minimum number of animals used in the couple of published studies was mostly similar to the number used here. Finally, our main criteria to determine the number of needed animals for reaching statistical power, was mostly based on similar experiments performed by

our team or others in the field. Actual sample sizes in the results described below were smaller than the initial number of animals because the efficiency of the RNA extraction was insufficient given the quantity of material. The difference in numbers per time point in the sleep deprivation experiments is due to cumulating different sleep deprivation and control experiments.

For the circadian rhythms the normalized ratios obtained in the data analysis (see above) were presented in percentage using the mean of all the data as 100%. The means and ±SEM were calculated for every ZT time point. Circadian changes in melanopsin and PACAP were tested by nonlinear regression analysis fitting a sine function (f=y0+a*sin(2*pi*x/b+c). A sine function was chosen as this is the gold standard to represent circadian data. Males and females were presented in a scatter plot (males blue, females red) in order to show that mRNA expression is sex independent. C57BL/6 and C57BL/6-129/Sv mice were also presented in a scatter plot to show that there are no differences between the two genetic backgrounds. In addition differences between males and females and between the two genetic backgrounds expression were tested by factorial analysis of variance (ANOVA) in the program STATISTICA. P<0,05 were considered statistically significant. The nonlinear regression analyses were performed using SigmaPlot.

In order to analyze the sleep deprivation experiments the normalized ratios obtained in the data analysis (see above) were presented in percentage and ZT12 was defined as 100%. All means ±SEM were calculated in reference to ZT12 as this was considered the baseline and differences to the baseline were examined. Induction of mRNA expression by sleep deprivation was tested by factorial ANOVA, followed by post-hoc tests (Tukey HSD) if the ANOVA analysis was statistically significant (p <0,05), even though these tests have their limits in small numbers. An equivalent that examines the same question for small numbers does not exist. Analyses were done with the program STATISTICA. Peak transform functions (f=y0+a/((1+exp(p*(t1-x)))*(1+exp(p*(x-t2))))) were fitted to the sleep deprivation data using the program SigmaPlot. The transform functions were used as this is a valid method to demonstrate the increase of the mRNA expression.

In the light experiments, the normalized ratios obtained in the data analysis (see above), were presented in percentage with the baseline (before light exposure) being

considered as 100%. Values are mean±SEM. Mann–Whitney U tests were applied as a valid test to compare two groups of small size. Results were considered significant when $p<0,05$ and analyses done with STATISTICA.

4. Results

In order to assess melanopsin and PACAP expression, mRNA levels were determined under habitual animal housing conditions, sleep deprivations or continuous light or dark exposures. Retinal mRNA was isolated at certain time points followed by cDNA transcription and RT-qPCR. Actin mRNA served as a reference. ZT0 was the time of light onset, ZT12 the time of dark onset. Relative mRNA levels were depicted in a diagram.

4.1 Circadian Melanopsin and PACAP mRNA Expression

For all circadian experiments normalized ratios obtained in the data analysis (see above) were presented in percentage using the mean of all data as 100%. Relative mRNA levels were depicted in a diagram and a sine wave was adjusted.

Circadian Melanopsin Expression in the Nocturnal C57BL/6 Mouse and in the Diurnal Arvicanthis ansorgei

In order to assess melanopsin expression during the circadian cycle, relative mRNA levels were determined in **C57BL/6 mice** over 27 hours. Retinal mRNA was isolated in 3 hour intervals and showed a circadian rhythm as the sine wave that was adjusted had a significant cycle over 24 hours (p=0,026). Melanopsin mRNA decreased in the resting phase (light period) until it reached a nadir at ZT9 and then re-increased during the active phase (subjective dark period) until it reached a peak at ZT21. The difference between the nadir and the peak of the sine wave was about 20% (Figure 20). Plotting males and females in a scatter plot did not show a difference between males and females (Figure 21). In addition, factorial ANOVA over 24 hours between males and females did not show a statistically significant difference (p=0,36). Therefore males and females were cumulated.

Likewise, relative quantification of melanopsin mRNA in **Arvicanthis ansorgei** over 20 hours with analysis of mRNA in four hour intervals showed a circadian rhythm: A sine wave with a period of 24 hours was adjusted which showed a significant cycle (p=0,0083). As in the mouse model, melanopsin mRNA peaked during the active phase

(light period) between ZT0 and 3, decreased to reach a nadir at ZT16 and then re-increased towards ZT20. The amplitude of the rhythm (difference between the peak and the trough of the sine wave) was about 50% (Figure 20). Plotting males and females in a scatter plot did not show a difference between males and females (Figure 22). In addition factorial ANOVA over 20 hours between males and females did not show a statistically significant difference (p=0,54). Therefore males and females were cumulated.

These data suggest that melanopsin mRNA in the nocturnal mouse is regulated by a circadian rhythm that is phase shifted by about four hours in comparison to the diurnal animal model Arvicanthis ansorgei. The highest expression levels of mRNA were found in the activity phase of each species: In the nocturnal mouse the peak was found at the end of the dark phase, in the diurnal Arvicanthis ansorgei the peak was situated at the beginning of the light phase.

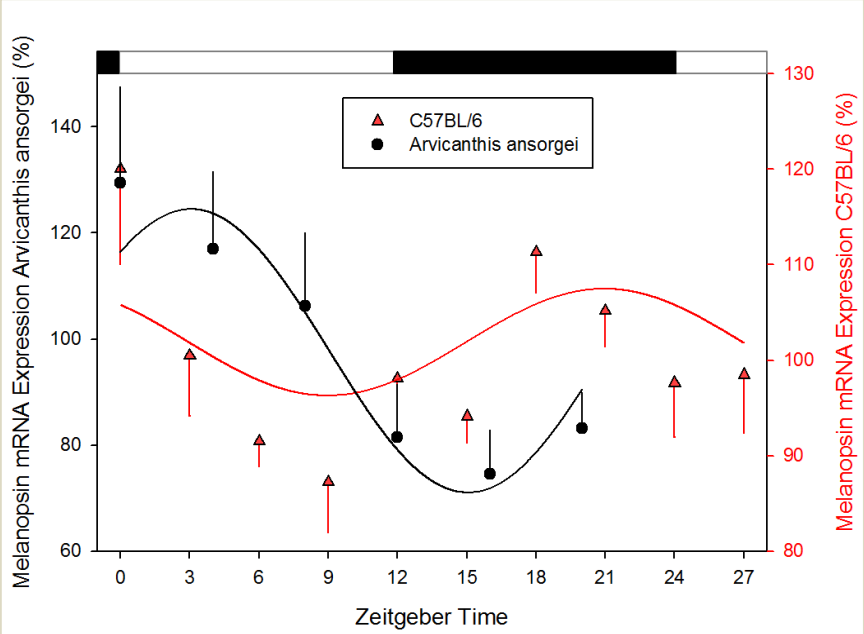

Figure 20: Melanopsin mRNA shows a circadian rythm in the C57BL/6 mice (red) and in Arvicanthis ansorgei (black). Normalized ratios obtained in the data analysis (see above) were presented in percentage using the mean of all the data as 100%. Sine waves were adjusted: Mouse p=0,026, Arvicanthis p=0,0083. Values are mean±SEM. (C57BL/6 mice n=4-6; Arvicanthis ansorgei n=2-4)

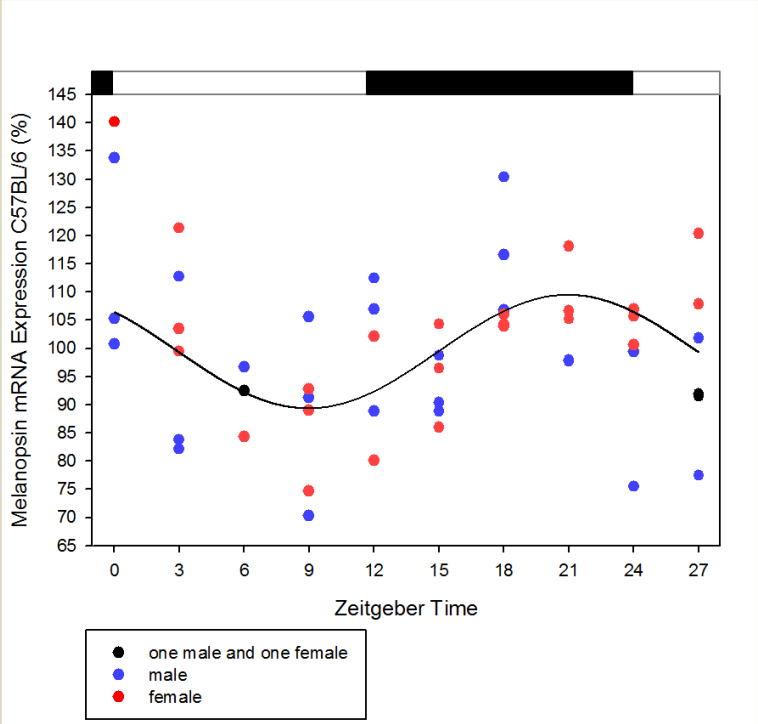

Figure 21: Melanopsin mRNA expression is sex independent in C57BL/6 mice. The scatter plot depicts melanopsin mRNA levels. Normalized ratios obtained in the data analysis (see above) were presented in percentage using the mean of all the data as 100%. A sine wave was adjusted: p=0,026. (n=4-6)

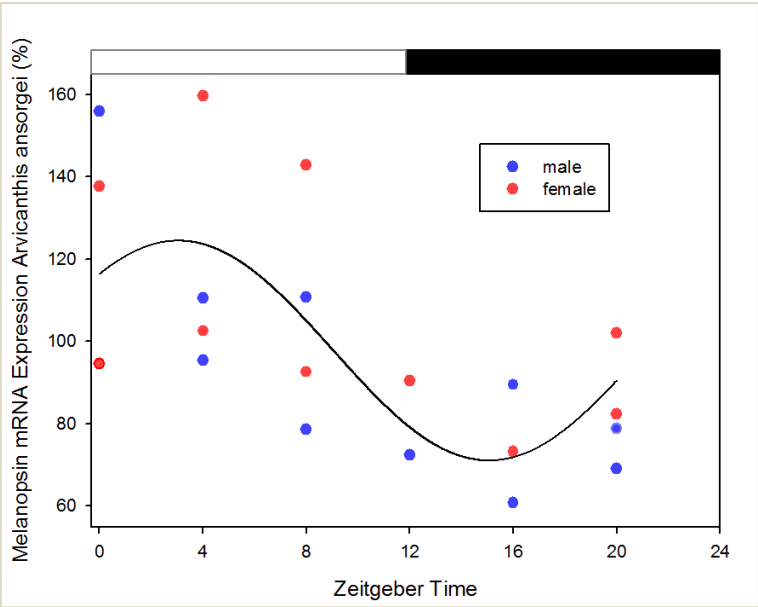

Figure 22: Melanopsin mRNA expression is sex independent in Arvicanthis ansorgei. The scatter plot depicts melanopsin mRNA levels. Normalized ratios obtained in the data analysis (see above) were presented in percentage using the mean of all the data as 100%. A sine wave was adjusted: p=0,0083. (n=2-4)

Circadian PACAP Expression in the Mouse and in Arvicanthis ansorgei

In order to assess whether expression levels of the neurotransmitter PACAP also follow a circadian cycle, PACAP mRNA levels were determined concurrently. In **C57BL/6** mice PACAP mRNA levels paralleled melanopsin levels indicating that PACAP expression is also governed by the circadian cycle. The mRNA level decreased until it reached a nadir at ZT7–8, to then re-increase reaching a peak at ZT21. Fitting a sine wave showed that like melanopsin mRNA, PACAP mRNA significantly changed over the 24 hour cycle (p=0,0019). The amplitude of the circadian rhythm (fitted sine wave) was about 60% (Figure 23). Plotting males and females in a scatter plot did not show a difference between the sexes (Figure 25). In addition factorial ANOVA between males and females did not show a statistically significant difference (p=0,31). Therefore males and females were cumulated.

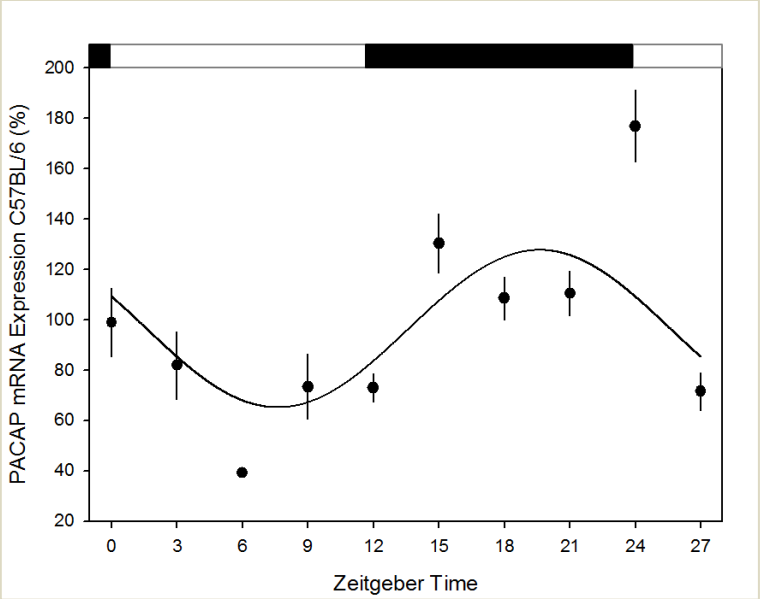

Figure 23: PACAP mRNA shows a circadian expression in C57BL/6 mice. Normalized ratios obtained in the data analysis (see above) were presented in percentage using the mean of all data as 100%. A sine wave was adjusted: p=0,0019. Values are mean ±SEM. (n=3-6, except ZT6: n=1)

Determined PACAP mRNA levels in the **Arvicanthis ansorgei** model were not as conclusive as in the mouse model. PACAP mRNA in Arvicanthis ansorgei could not be analyzed by adjusting a sine wave as the sine wave was not significant (p=0,078). Therefore the results were plotted in a histogram in comparison to melanopsin mRNA

(Figure 24). There was a tendency that showed a similar regulation of PACAP and melanopsin mRNA with highest expression levels at ZT0, with a decrease towards ZT16. At ZT12 however, there seemed to be an increase in PACAP mRNA expression which was not present in the melanopsin experiments. Analysis of variance between melanopsin and PACAP did not show a significant difference between the two genes which is in favor of a similar rhythm (p=0,84). Plotting males and females in a scatter plot did not show a difference between the sexes (Figure 26). In addition factorial ANOVA between males and females did not show a statistically significant difference (p=0,18). Therefore males and females were cumulated.

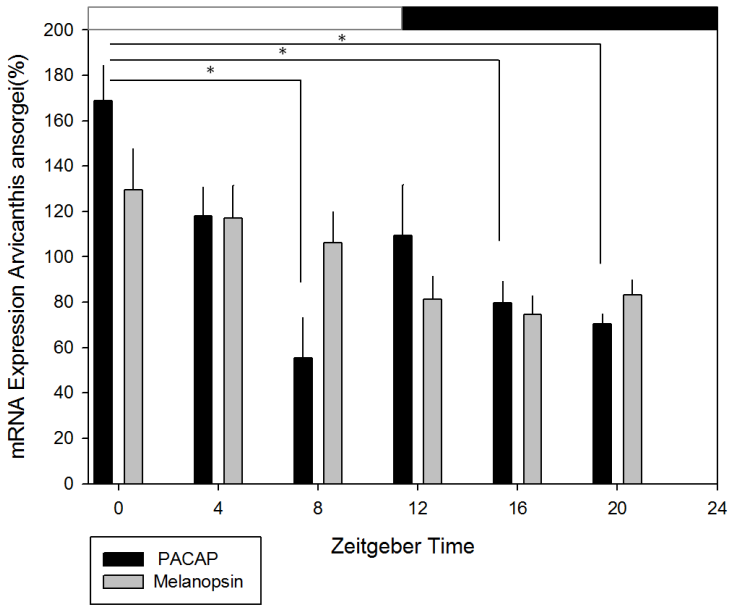

Figure 24: PACAP mRNA in Arvicanthis ansorgei shows a tendency to circadian mRNA expression when compared to melanopsin mRNA expression as analysis of variance did not show a significant difference between melanopsin and PACAP mRNA expression (p=0,84). Mann-Whitney U tests were used to study the difference between PACAP mRNA expression between certain time points: ZT0 and ZT8, ZT0 and ZT16, ZT0 and ZT20. There was a significant difference between the time points studied. Values are mean±SEM. (n=3-4)

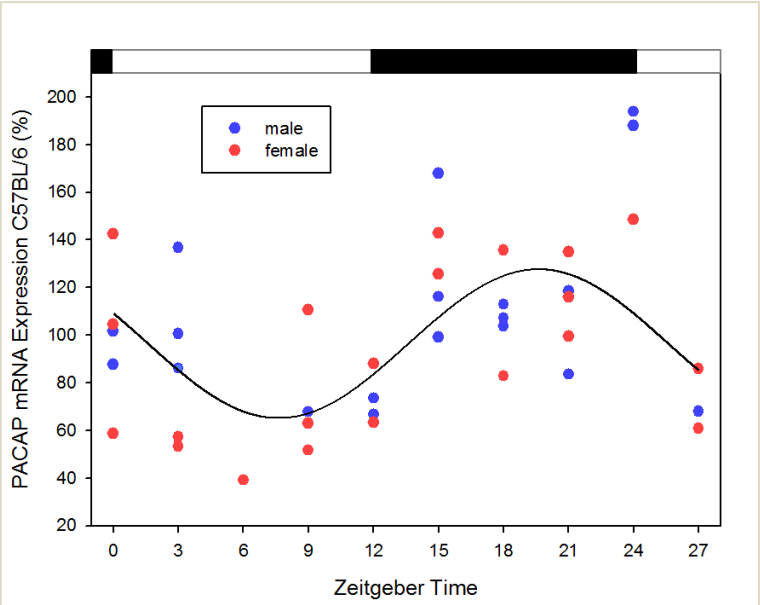

Figure 25: PACAP mRNA expression is sex independent in C57BL/6 mice. The scatter plot depicts PACAP mRNA levels. Normalized ratios obtained in the data analysis (see above) were presented in percentage using the mean of all data as 100%. A sine wave was adjusted: p=0,0019. (n=3-6, except ZT6: n=1)

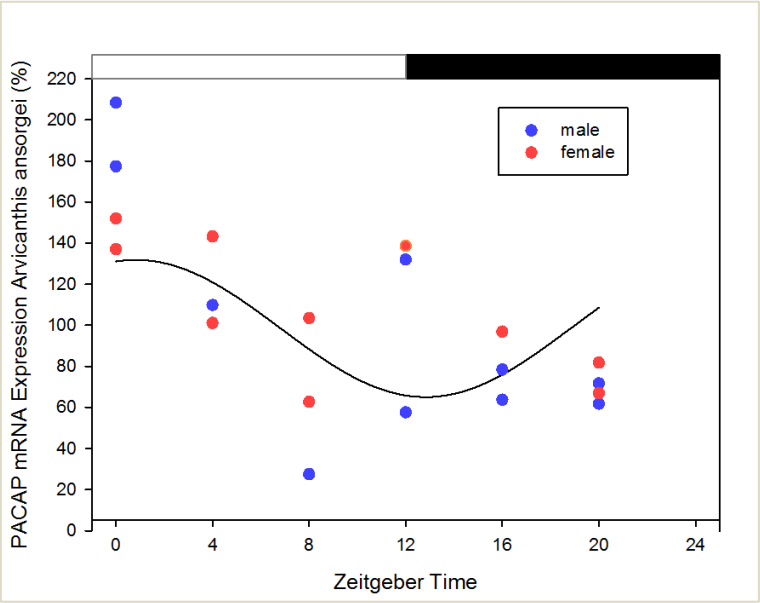

Figure 26: PACAP mRNA expression is sex independent in Arvicanthis ansorgei. The scatter plot depicts PACAP mRNA levels. Normalized ratios obtained in the data analysis (see above) were presented in percentage using the mean of all data as 100%.The sine wave adjusted was not significant: p=0,078. (n=3-4)

Circadian Melanopsin Expression in the Albino Wistar Rat

We repeated the previous experiments with a third animal model, the albino Wistar rat. Melanopsin mRNA expression levels in albino Wistar rats in dependence of the circadian cycle have been previously described in the literature. The validation seemed necessary as the obtained data in the mouse did not correspond to mRNA expression data found in the literature (69, 71, 72). Our data obtained in the rat showed that relative melanopsin mRNA levels increased from a nadir at ZT2 until it reached a peak at ZT10–12. After the peak melanopsin mRNA decreased again until ZT22. Fitting a sine wave showed that melanopsin mRNA levels changed significantly over the 24 hour cycle (p=0,0063). The difference between the nadir and the peak of the curve was about 60% (Figure 27). The peak is similar to data of melanopsin rhythms in the literature: ZT12-14 (69), ZT14 (71), ZT11-15 (72).

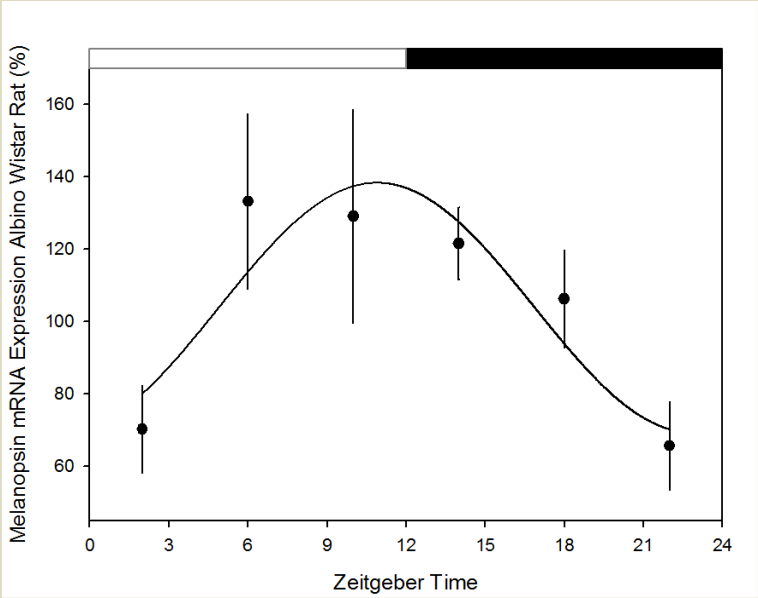

Figure 27: Melanopsin mRNA shows a circadian expression in albino Wistar rats. Normalized ratios obtained in the data analysis (see above) were presented in percentage using the mean of all data as 100% A sine wave was adjusted: p=0,0063. Values are mean ±SEM. (n=5-6)

4.2 Sleep Homeostasis and Melanopsin and PACAP mRNA Expression

In sleep the homeostatic process is governed by sleep need. Accordingly, sleep deprivation increases sleep need and analysis of expression levels under the condition of sleep need allow for identification of genes involved in homeostasis. Therefore, sleep deprivation in the animal models studied, mouse and Arvicanthis ansorgei, should allow analysis of the homeostatic effect on melanopsin and PACAP mRNA expression.

In order to assess the influence of the sleep homeostatic process on melanopsin and PACAP expression, the time course of melanopsin and PACAP mRNAs was determined during sleep deprivation and recovery sleep (sleep homeostasis) in C57BL/6 and C57BL/6-129/Sv mice and in Arvicanthis ansorgei under normal LD conditions. The intervals of time points were chosen according to EEG delta power analysis after sleep deprivation from EEG data from the INCI laboratory (unpublished data). Normalized ratios obtained in the data analysis (see above) were presented in percentage using ZT12 as 100%. Relative mRNA levels were depicted in a diagram.

Sleep Homeostatic Melanopsin Expression in the Mouse

In order to elucidate whether melanopsin mRNA expression levels were affected by sleep homeostasis in mice, we analyzed mRNA levels at different time points across a six hour sleep deprivation and during recovery sleep. C57BL/6 and C57BL/6-129/Sv female mice were deprived of sleep for 6 hours and then allowed to sleep for three hours. The mRNA was examined in 3 hour time intervals during the sleep deprivation and 1,5 hour intervals during the recovery sleep. The control group was kept under normal housing conditions and sacrificed at the same ZT points as in the SD experiment.

Melanopsin mRNA expression was studied in female mice under sleep deprivation during the habitual resting period (daytime/light) in comparison to control mice that were not deprived of sleep (sacrifice at the same time points as the sleep deprived mice). Relative mRNA levels were determined and plotted as described above. As shown in Figure 28 already three hours of sleep deprivation caused a significant increase in

melanopsin mRNA expression in female mice when compared to the control group. The increase stayed significant after six hours and was reversed when animals were allowed to rest. Analysis of variance was significant between melanopsin mRNA expression in female mice after sleep deprivation and the control group ($p<0,01$). Post-hoc Tukey HSD test showed a significant increase after 3 and 6 hours of sleep deprivation in comparison to the control group ($p=0,0002$ and $p=0,002$, respectively). During the recovery sleep mRNA levels decreased to return to control levels. There was no significant difference between the recovery sleep group and the control group.

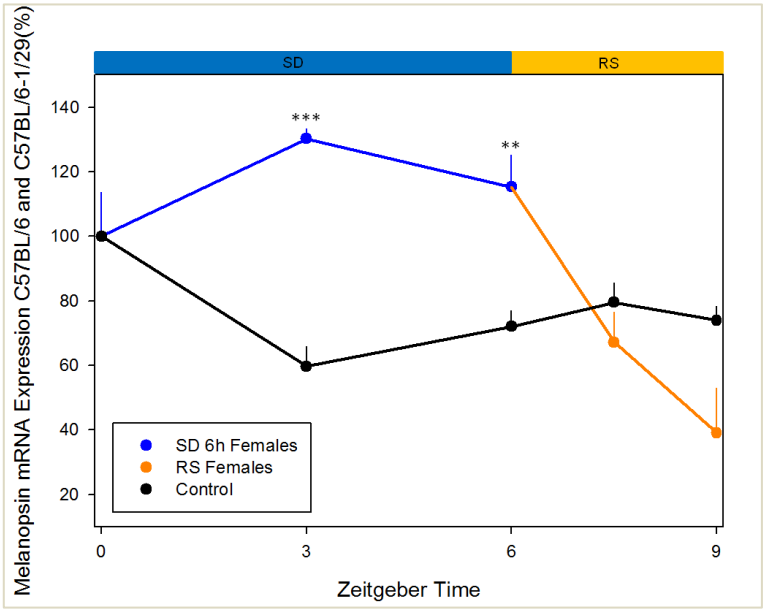

Figure 28: Sleep deprivation (SD) in mice induces an increase of melanopsin mRNA expression after 3 and 6 hours of SD. Recovery sleep (RS) allows mRNA levels to return to control values. The control group shows the same circadian rhythm as described in the circadian experiments. Normalized ratios obtained in the data analysis (see above) were presented in percentage using ZT12 as 100%. Values are mean ±SEM. Mice from the two genetic backgrounds were pooled. (SD n=3-4 except for ZT3 n=2; control n=7-10)

Above it was demonstrated that melanopsin mRNA expression is not influenced by gender. Here, we demonstrate that the genetic background does not affect melanopsin mRNA expression. To determine whether the two genetic backgrounds can be pooled, Opn4 mRNA levels of C57BL/6 and C57BL/6-129/Sv mice were compared in the control condition of the sleep deprivation experiment. The animals were exposed to baseline LD 12:12 conditions and sacrificed at 3 hour intervals from ZT0 to ZT6 and at 1,5 hour intervals from ZT6 to ZT9. A sine wave was adjusted ($p=0,004$) which shows the

circadian melanopsin mRNA regulation during the first 12 hours of the cycle with a nadir at ZT5. These results are comparable to the circadian experiments described above. The scatter plot did not show differences between the two genetic backgrounds (Figure 29). Similarly, factorial ANOVA did not show any significance for the factor genetic background (p=0,76) nor the factor sex (p=0,84) as expected from the results described above. Therefore male and female C57BL/6 and C57BL/6-129/Sv mice were cumulated in the control experiment. Female C57BL/6 C57BL/6-129/Sv mice in the sleep deprivation group were also cumulated.

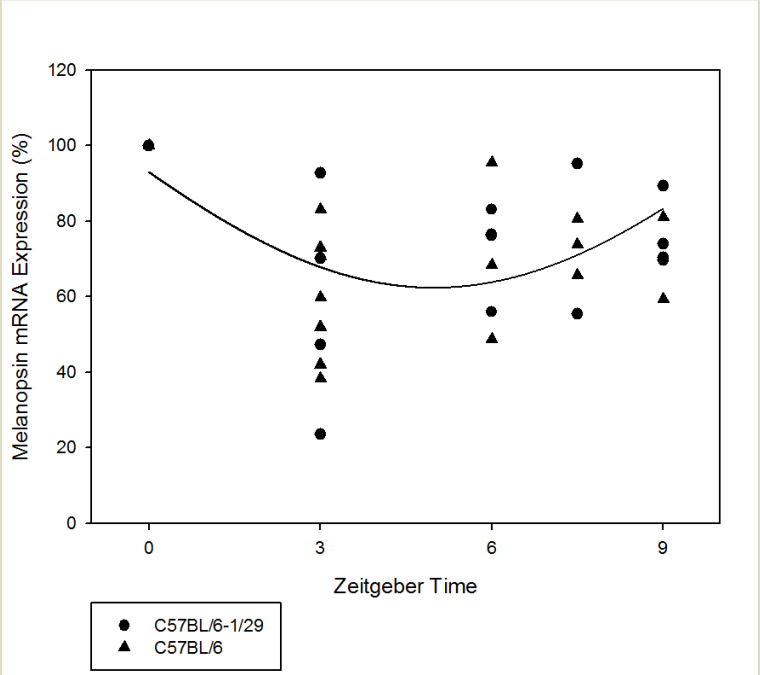

Figure 29: Melanopsin mRNA expression is genetic background independent in C57BL/6 and C57BL/6-129/Sv mice. The scatter represents mRNA levels in the control group of the sleep deprivation experiment. A sine wave was adjusted: p=0,004. The control group shows the same circadian rhythm as described in the circadian experiments (see above). Normalized ratios obtained in the data analysis (see above) were presented in percentage using ZT12 as 100%. Values are mean ±SEM. (n=7-10)

Sleep Homeostatic Melanopsin Expression in Arvicanthis ansorgei

In order to assess the effect of sleep deprivation on melanopsin expression in a diurnal model, melanopsin mRNA levels were determined in Arvicanthis ansorgei deprived of sleep during the habitual resting period (nighttime/dark) in comparison to control animals that were not deprived of sleep. In this experiment the group of sleep deprived

animals was divided in three cohorts, experiencing an increasing time of sleep deprivation (3, 6 and 9 hours) and then left to sleep for 3 hours. The animals were sacrificed in intervals of 3 hours during the sleep deprivation and 1,5 hours during the recovery sleep. Animals in the control group were sacrificed at the same time points. Relative mRNA levels were determined as described above. Factorial ANOVA between mRNA expression in the sleep deprived group and the control group was significant ($p<0,001$). Post-hoc Tukey HSD tests showed a significant increase after six hours of sleep deprivation (ZT 18) ($p=0,0048$) and after nine hours (ZT 21) ($p=0,00013$) in comparison to the control group (Figure 30). However, after a sleep deprivation of 12 hours (ZT24) melanopsin mRNA did not increase any further when compared to the control group - in favor of a saturation of the homeostatic process. During the recovery sleep after the six, nine and twelve hour sleep deprivations melanopsin mRNA levels decreased to rejoin control values with no significant difference between the recovery sleep group and the control group.

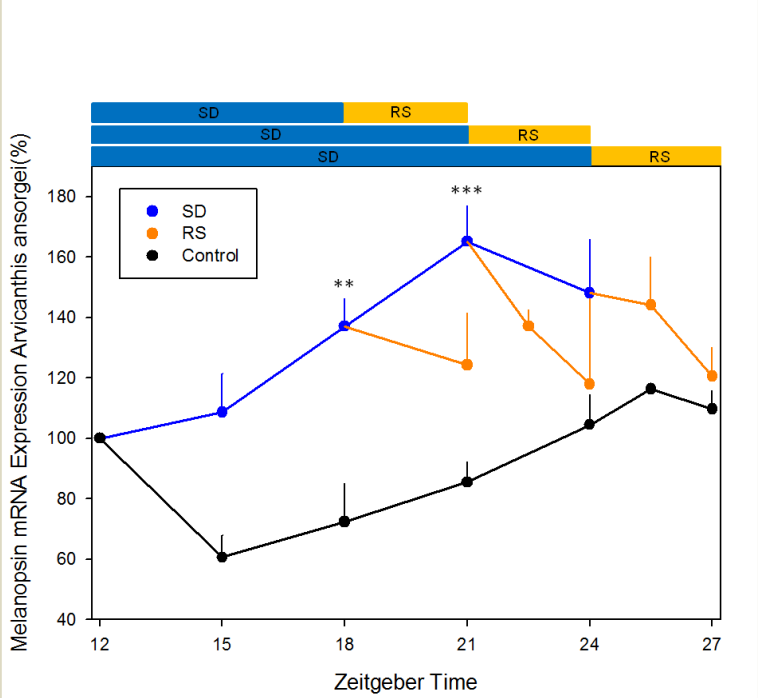

Figure 30: Sleep deprivation (SD) in Arvicanthis ansorgei induces an increase of melanopsin mRNA expression after 6 and 9 hours of SD. After 12 hours the difference is not significant anymore and recovery sleep (RS) allows mRNA levels to rejoin the level of the circadian rhythm. The control group shows the same circadian rhythm as described in the circadian experiments. Normalized ratios obtained in the data analysis (see above) were presented in percentage using ZT12 as 100%. Values are mean ±SEM. (n=3-9)

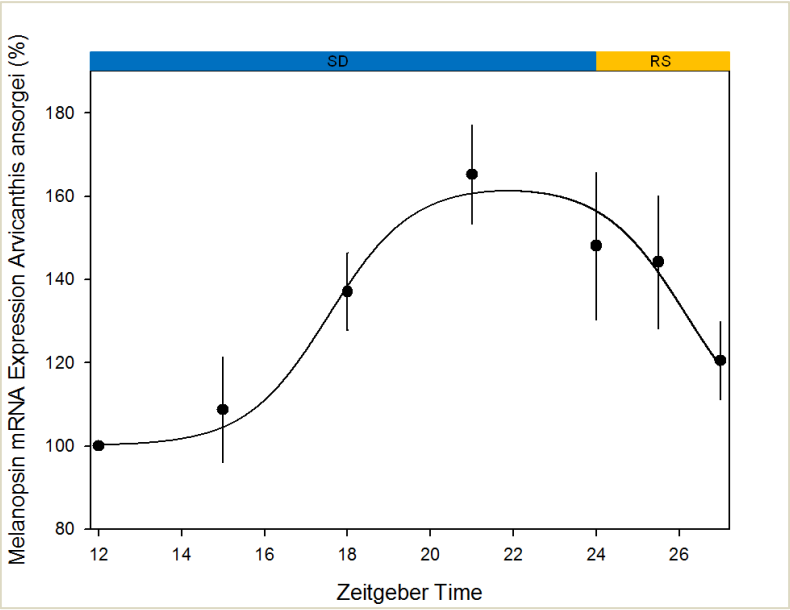

Figure 31: The peak transform function (p=0,0001) shows an increase of melanopsin mRNA during the nine hour sleep deprivation, a saturation of the curve after 12 hours of sleep deprivation and a decrease of melanopsin mRNA expression during recovery sleep. Normalized ratios obtained in the data analysis (see above) were presented in percentage using ZT12 as 100%. Values are mean ±SEM. (n=3-6)

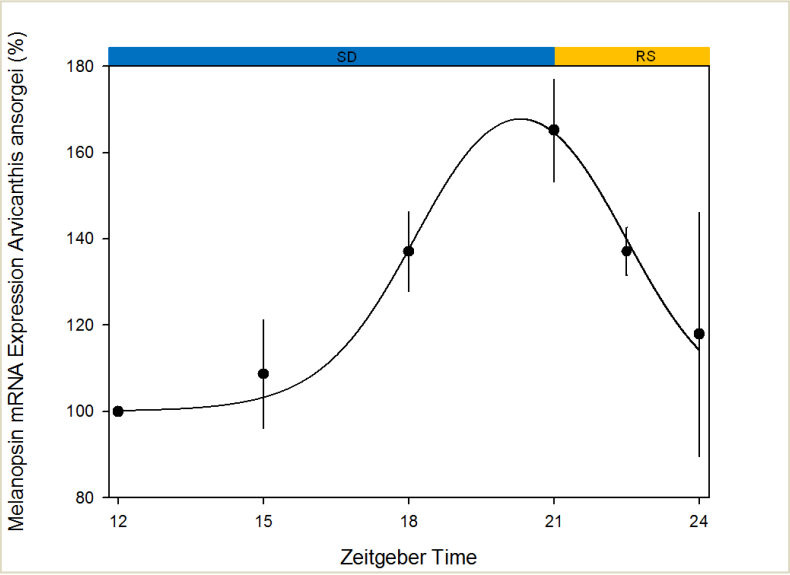

Figure 32: The peak transform function (p=0,0005) shows an increase of melanopsin mRNA during the nine hour sleep deprivation, and a decrease of melanopsin mRNA expression during recovery sleep. Normalized ratios obtained in the data analysis (see above) were presented in percentage using ZT12 as 100%. Values are mean ±SEM. (n=3-6)

In order to illustrate the process of an increase of melanopsin expression during nine hours of sleep deprivation (until ZT21) with a saturation of the process after 12 hours

(ZT24) and a decrease of melanopsin mRNA during recovery sleep a peak transform function(p=0,0001) was adjusted to the data (Figure 31). A peak transform (p=0,0005) function was also adjusted to the data of the nine hour sleep deprivation (until ZT21) to illustrate the increase of melanopsin expression during sleep deprivation and the decrease during recovery sleep (Figure 32).

The control group showed the same circadian rhythm as was already shown in the circadian experiments (see above).

Sleep Homeostatic PACAP Expression in Arvicanthis ansorgei

PACAP mRNA expression in Arvicanthis ansorgei was determined using the same samples as for melanopsin mRNA expression. Animals were deprived of sleep during the habitual resting period (nighttime/dark) in comparison to control animals that were not deprived of sleep.

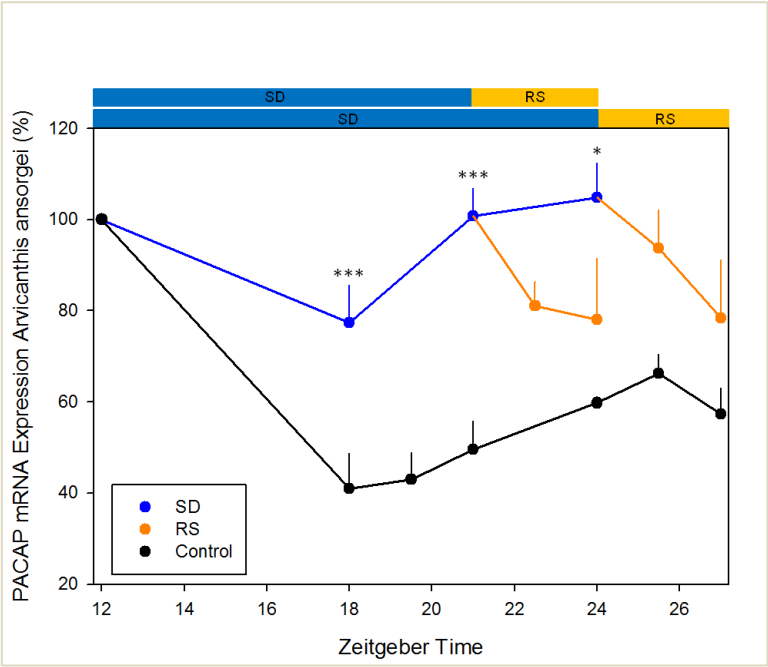

Figure 33: Sleep deprivation (SD) in Arvicanthis ansorgei shows a significant increase of PACAP mRNA levels compared to the control group after 6, 9 and 12 hours of SD. The recovery sleep (RS) allows mRNA levels to rejoin the level of the circadian rhythm. The control group shows a circadian rhythm as described in the circadian experiments. Normalized ratios obtained in the data analysis (see above) were presented in percentage using ZT12 as 100%. Values are mean ±SEM. (n=3-10)

Factorial ANOVA between the PACAP mRNA expression in the sleep deprivation group and the control group was significant ($p<0,001$). PACAP mRNA increased significantly after six, nine and twelve hours of sleep deprivation and decreased to rejoin the control levels during the recovery sleep after the nine hour and the twelve hour sleep deprivations (post-hoc Tukey HSD test at ZT18 ($p=0,0001$), ZT21 ($p=0,0002$) and ZT24 ($p=0,02$), (Figure 33). Differences between the ZT times of the recovery sleep group and the control group were not significant.

The control group followed the same circadian rhythm as was already found in the circadian experiments (see above).

4.3 Sustained Light/Dark Effects of Light on Melanopsin mRNA Expression

In order to assess direct influences of light on melanopsin, mRNA expression levels were determined at baseline and after three days of either constant light or constant dark exposure in C57BL/6 mice. Normalized ratios obtained in the data analysis (see above) were presented in percentage using the mean of the baseline experiment as 100%. Males and females were cumulated as experiments above did not show a gender difference of melanopsin mRNA expression.

The light experiments were designed based on Hannibal´s first publication which showed a significant effect on melanopsin mRNA expression after only three days of light or dark exposure in albino Wistar rats (69).

Three days of constant light exposure

C57BL/6 mice and Arvicanthis ansorgei are kept under LD 12:12 conditions and then subjected to three days of continuous light (LL 12:12). The control animals were sacrificed at ZT6 under LD 12:12 conditions. The remaining animals were subjected to three days of light and were sacrificed at CT6 on the fourth day. In order to compare the two groups Mann Whitney U tests were used.

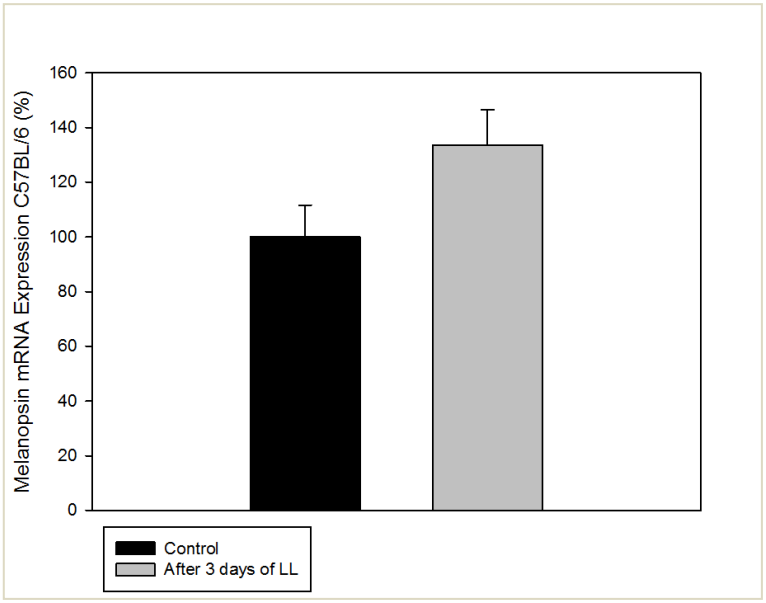

Figure 34: Exposure to three days of constant light does not influence the expression of melanopsin mRNA (p=0,14) in C57BL/6 mice. The mean of normalized ratios ±SEM obtained in the data analysis (see above) were presented in percentage using the baseline as 100%. Values are mean ±SEM. (n=5)

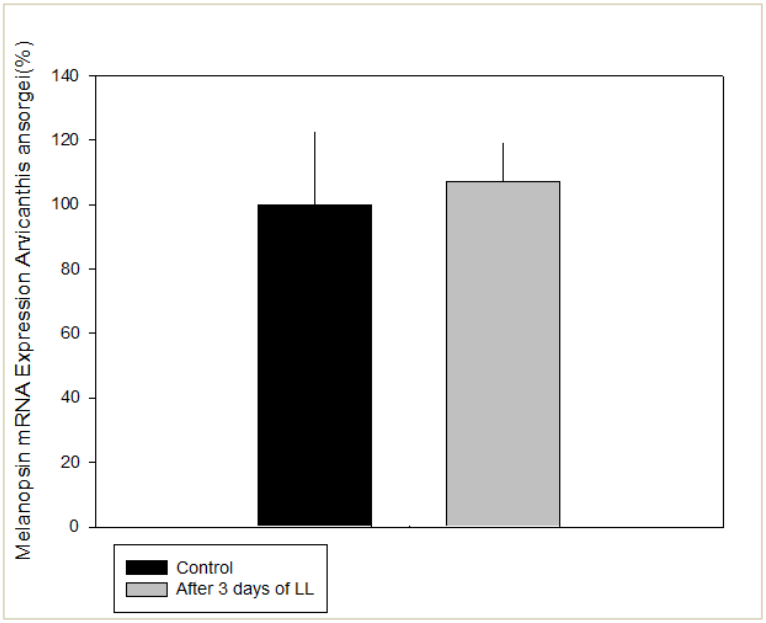

Figure 35: Exposure to three days of constant light does not influence the expression of melanopsin mRNA (p=0,6) in Arvicanthis ansorgei. The mean of normalized ratios ±SEM obtained in the data analysis (see above) were presented in percentage using the baseline as 100%. Values are mean ±SEM. (n=5)

Neither the nocturnal mouse (p=0,14), (Figure 34) nor the diurnal Arvicanthis ansorgei (p=0,6), (Figure 35) showed a significant difference in melanopsin mRNA expression levels after exposure to three days of light.

Three days of constant darkness

C57BL/6 mice and Arvicanthis ansorgei were kept under LD 12:12 conditions and then subjected to three days of only darkness (DD 12:12). Control animals were sacrificed at ZT6 under LD 12:12 conditions. The remaining animals were then exposed to three days of continuous darkness and sacrificed on the fourth day at CT6. In order to compare the two groups Mann Whitney U tests were used. As in the constant light experiment neither the nocturnal mouse (p=0,25), (Figure 36) nor the diurnal Arvicanthis ansorgei (p=0,46), (Figure 37) showed a significant difference in melanopsin mRNA expression levels after exposure to three days of darkness.

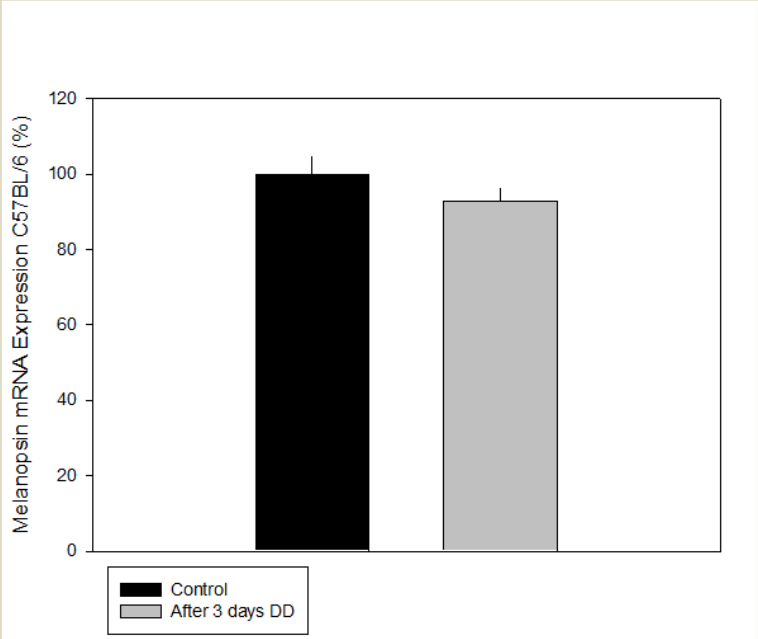

Figure 36: Exposure to three days of constant darkness does not influence the expression of melanopsin mRNA (p=0,25) in C57BL/6 mice. The mean of normalized ratios ±SEM obtained in the data analysis (see above) were presented in percentage using the baseline as 100%. Values are mean ±SEM. (n=5)

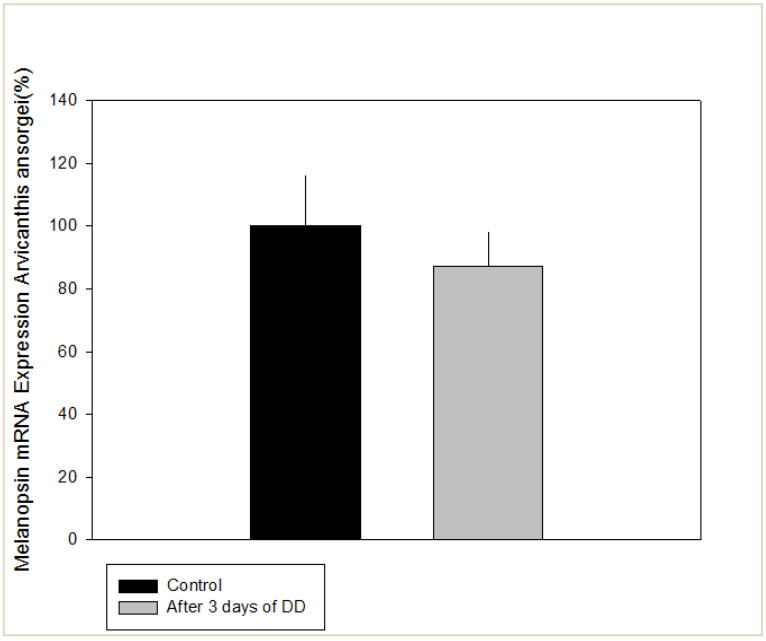

Figure 37: Exposure to three days of constant darkness does not influence the expression of melanopsin mRNA (p=0,54) in Arvicanthis ansorgei. The mean of normalized ratios ±SEM obtained in the data analysis (see above) were presented in percentage using the baseline as 100%. Values are mean ±SEM. (n=5-6)

5. Discussion

Since the discovery of melanopsin many studies in humans and in animal models have given evidence of the importance of this photopigment in non visual light signaling such as melatonin suppression, pupillary light reflex or the regulation of sleep. The involvement of melanopsin in sleep regulation has been demonstrated by using a variety of techniques including behavioral and electrophysiological analysis in wild type and knockout mice and melanopsin expression analysis in rat or mice animal models. Thus, the two main processes which are today known to regulate sleep - the circadian and the homeostatic process - are affected by melanopsin (42, 43, 48). Signals initiated by melanopsin are relayed to the brain through the RHT. The neurotransmitter PACAP could therefore play a key role in conveying melanopsin-dependent light information to the brain. The INCI group and others have recently obtained data indicating direct effects of light on sleep (48, 66-68). In nocturnal mice, light acts like a switch to induce sleep while darkness immediately results in wakefulness. Unpublished data indicate that the melanopsin-dependent direct effects of light account for about 30% of the daily distribution of sleep and wake. Therefore, the INCI group proposes a triforked mechanistic model for the regulation of sleep which includes the direct effect of light on sleep and in turn on mood and cognition (83, 84), (Figure 38).

Therefore, we performed expression analysis studies to elucidate how the different processes modulate Opn4 and PACAP mRNA expression. If melanopsin is involved in the processes one would assume that the expression levels would reflect the biological requirements. This study was first conducted in nocturnal mice to revisit the role of melanopsin in sleep and to fill the knowledge gap between behavior and molecular analysis. An important further corroboration was the analysis of melanopsin expression levels in the diurnal animal model (Arvicanthis ansorgei) as one would expect that the expression pattern would reflect the respective biological behavior.

5.1 Strengths and Limitations of the Study

A strength of the studies described in this thesis is the fact that they were conducted in an environment – the chronobiotron – which allows to control most environmental

parameters and therefore eliminates many biases, especially factors affecting sleep, circadian rhythms and photoreception like temperature changes, noise differences or light intensity differences. All animals grow up in the same environment and the mice display the same genetic background. Therefore the inclusion of smaller groups of animals is accepted in the field. The relatively small number of animals used in some of the present experiments - a possible limitation of the study - can also be explained by the availability of the animals, especially Arvicanthis ansorgei which is a species difficult to breed as its habituation to laboratory conditions is relatively recent. Sleep and behavior studies are essentially performed in males. Most of the experiments described here were done in males and females which allow addressing the questions concerning gender differences. Males and females were plotted in scatter plots to show that the expression levels do not differ between the sexes. Some drawbacks of the sleep homeostatic experiments were that the experiments in Arvicanthis ansorgei were only done in males and the sleep deprivation experiment in mice only in females. It would be interesting to complete the gender analysis in the future.

The study described above also showed that there is no difference between the genetic backgrounds used which justifies cumulating C57BL/6 and C57BL/6-129/Sv mice. In addition analysis of EEG sleep data did not show a difference between the two genetic backgrounds (unpublished data). Moreover, the fact that our observations were validated in a rich genetic environment (C57BL/6-129/Sv) as well as in a controlled genetic background (C57BL/6) and are therefore independent of the genetic environment, strengthens the findings.

Similarly, another great strength of the study presented, is the fact that three different species were studied which allows a comparison between species and between nocturnal and diurnal models.

Moreover, the analysis of melanopsin and PACAP mRNA expression at different time points in several sleep deprivation experiments i.e. as a course of the homeostatic process strengthens the establishment of a relationship between melanopsin and sleep homeostasis.

In all circadian experiments sine waves were adjusted as this is the statistical test used in the literature when studying circadian rhythms. ANOVA was often used as an additional method as this is the method used in the literature even with small animal

numbers and because a comparable test for small numbers does not exist. For post-hoc tests the Tukey HSD test was chosen as it is more conservative than for example the Fisher test. For statistical analysis of the light experiments Mann-Whitney U tests were used as this is an adequate test to compare two groups of small numbers.

5.2 Circadian Regulation of Melanopsin and PACAP mRNA

Melanopsin mRNA expression parallels the circadian rhythm

Opn4 expression followed a circadian rhythm in C57BL/6 mice, Arvicanthis ansorgei and in albino Wistar rats. The amplitude of the rhythm in mice was about 20%, in Arvicanthis ansorgei about 60%. In albino Wistar rats it was about 60% which is comparable to the results found in the literature in rats (70% (71), 60% (70)) except for Hannibal´s publication (300% (69)).

In both mouse and Arvicanthis ansorgei, melanopsin mRNA expression peaked during their activity phase.

In the **C57BL/6 mouse**, this was at ZT 21 which is three hours before the dark-light transition which corresponds to the time of dawn in their natural environment. It is noteworthy that unlike sunlight which displays an evenly dispersed emission spectrum from red to blue light, the spectrum of dawn has a maximum between 400 and 500nm (85, 86) which correspond to the absorption maximum of melanopsin. To my knowledge no other group has studied melanopsin mRNA expression in the mouse to this date. One group studied melanopsin protein expression over the circadian cycle in the C3H/He mouse using immunohistochemistry and could show that the protein peak is at about ZT23 (73). This would mean a protein peak about two hours after the mRNA peak we found which is coherent with the current knowledge on protein translation.

The daily melanopsin mRNA expression obtained in the mouse was unexpected as it was different from data published in rats and one would expect two nocturnal models to have a similar expression profile. Studies in the nocturnal rat show a peak at the day-night transition (dusk) between ZT12-14. Therefore, additional studies were performed in the **albino Wistar rat**. In this latter species we demonstrated that peak melanopsin

mRNA levels were comparable to results obtained in the other groups with a peak situated between ZT10 and 12. (ZT12-14 in albino Wistar rats (69); ZT 14 in tan hooded pink eyed RCS/N-rdy+ rats (87), (71); ZT 12 in albino Sprague-Dawley rats (70)). Interestingly, the time point of the peak expression does not differ in Brown Norwegian rats (ZT13-14 (72)) suggesting that the daily expression of melanopsin is not affected by the lack or presence of pigmentation. The protein peak seems to be at least six hours after the mRNA peak which is a more coherent result than the previous report of a delay of at least 20 hours in the albino Wistar rat (69, 72). Still, it remains to be clarified why the translation in rats would be 6 hours when it is probably only two hours in the mouse.

In the diurnal **Arvicanthis ansorgei** which is pigmented, the peak of melanopsin mRNA expression was between ZT0 and 3 which is at the transition from dark to light (dawn). This peak is inverted to the peak in the nocturnal pink-eyed (see results above) or pigmented (72) rat which is at the light-dark transition and three to six hours later than the pigmented nocturnal mouse. In light of the natural environment of Arvicanthis ansorgei which is the desert, one could assume that the peak activity of this rat would be in the early morning or evening. Indeed, Arvicanthis shows crepuscular behavior (data of the INCI group) with peak activity at the dark-light or the light-dark transition with sleep bouts over the 24 hours. However, data obtained in the group indicate that the rodent is still clearly diurnal as the mouse is nocturnal when hypnograms of Arvicanthis ansorgei and C57BL/6 mice are compared. Nocturnal and diurnal behavior in rodents is not as consolidated as is for example the diurnal behavior in humans. Mice show less crepuscular behavior than Arvicanthis ansorgei but also sleep during their active phase. Therefore Arvicanthis seems to be a good diurnal model to study sleep and results from these studies might be translatable to humans. To date this is the first study of melanopsin mRNA expression in a diurnal rodent. As the peak mRNA expression seems inverse to the peak seen in pink-eyed or pigmented rats this might be a clue as to where the switch between diurnal and nocturnal animals takes place. However, species difference also seems to play a major role which becomes clear when looking at the results obtained in the mouse which is also a nocturnal species but shows a peak shifted in comparison to the nocturnal rat.

To date, there is no explanation why the expression pattern of different nocturnal animal models differs. It would be interesting to understand why the circadian rhythm of melanopsin expression is shifted of about 7 to 9 hours between mice and rats. A

possibility is that the peak of expression at dawn and dusk respectively matches different activity peaks during the circadian cycle although they have the same time periods of activity.

PACAP mRNA expression parallels the circadian rhythm

The mRNA levels of the neurotransmitter PACAP paralleled the expression pattern of melanopsin in C57BL/6 mice, suggesting an involvement of PACAP in signal transmission. A nadir was found at ZT7-8, a peak at ZT21 with an amplitude of the rhythm of about 50% of the 24 hour mean average level. Therefore, the phase of both rhythms, Opn4 and PACAP are identical. In Arvicanthis ansorgei, the correlation between PACAP and melanopsin was not as clear. There was a trend towards a relationship of melanopsin and PACAP expression (similar pattern of PACAP and melanopsin mRNA expression) even though a sine wave could not be adjusted in Arvicanthis ansorgei. It has to be kept in mind that PACAP is not only present in the retinal ganglion cells but also in amacrine cells (inner nuclear layer), in the inner plexiform layer, in the nerve fiber layer and in the retinal papilla of the rat eye (62). Thus, the PACAP mRNA signal is potentially diluted.

To my knowledge there is no data in the literature concerning PACAP expression in the retina and little in areas receiving innervation from the RHT, such as the SCN. PACAP is seen as a retinal messenger to the SCN, co-transmitting light information with glutamate to the brain via melanopsinergic cells (75). Fukuhara and colleges showed in the Wistar rat that there is a circadian rhythm of the polypeptide in the SCN during a 12:12 LD cycle with a peak at about ZT 12. This rhythm becomes flat under DD exposure (76) which demonstrates the importance of light in the PACAP rhythm and suggests that melanopsin activation might be required for PACAP modulation. However, in the chicken, a circadian rhythmic expression of PACAP is observed that is conserved in DD (78), suggesting that light influence on PACAP regulation might differ between mammals and birds, raising the question whether melanopsin-based photo-detection recruits different signal pathways. To compare the aforementioned data from the literature with our results, one should take into account that PACAP is studied on different levels: We studied mRNA in the retina whereas the other groups studied the polypeptide in the SCN. In addition, studies were conducted in different animal models:

diurnal chicken and nocturnal Wistar rat in the literature and diurnal Arvicanthis ansorgei and nocturnal mouse in the above presented studies. Altogether, the data in rodents suggest that PACAP expression peaks at the time of maximal activity whereas in birds (chicken) it peaks during the resting period. Indeed, the fact that PACAP follows melanopsin mRNA expression, suggests that this peptide plays a central role in Opn4-based signaling to the brain.

Finally, to summarize, in both models melanopsin expression is highest when the animals are active and have their eyes open to collect light and when there is a physiological need for transmission of the signal to the brain. It will be most interesting to dissect the signaling cascade responsible for Opn4 transcription. Both melanopsin and PACAP will have to be further studied on the protein level in order to assure that induction of mRNA expression translates to increased protein levels. In this context it will also be very interesting to determine the transcription/translation kinetics and half life of the proteins as peak levels in mRNA expression might not be completely congruent with protein expression. However our results indicate clearly that there is a circadian regulation of melanopsin and PACAP mRNA in the C57BL/6 mouse. In the diurnal Arvicanthis ansorgei there is equally a clear circadian rhythm of melanopsin mRNA expression and preliminary results indicate that PACAP is regulated in a similar fashion. It remains to be determined whether the circadian modulation of Opn4 and PACAP depend on retinal mechanisms and retinal clock and/or the master clock located in the SCN. This might be evaluated in transgenic mice lacking a functional pacemaker that are available in the laboratory. Moreover, the circadian profile of melanopsin and PACAP suggested in Arvicanthis ansorgei needs to be confirmed with a larger number of animals. Additionally, given the contradictory results of PACAP expression under DD in the literature, it would be interesting to monitor PACAP mRNA levels under DD conditions in our animal models. All in all, our results might lead to a better understanding of the switch between a nocturnal and a diurnal rodent: Opn4 might participate in the mechanisms responsible for the inverse effects of light underlying nocturnality and diurnality. The present circadian profiles give room for questions as to the physiological significance of a circadian modulation of a photopigment. A peak of melanopsin mRNA expression during the activity period could mean that the protein is needed at this time of the day for a better transmission of non-visual effects of light to the brain as for example the pupillary light reflex.

5.3 Sleep Homeostasis Affects Melanopsin and PACAP mRNA Expression

In addition to the circadian rhythm, the present data indicate that melanopsin mRNA increased with time spent awake i.e. when sleep need rose in both animal models. Moreover, melanopsin mRNA expression returned to normal levels during the recovery sleep. These patterns describe a process of homeostatic regulation.

In **mice** a short sleep deprivation of only three hours already induced a significant increase of melanopsin mRNA which stayed at comparable levels after a longer (six hours) SD. The increase of mRNA expression is always in comparison to a control group and never a comparison between two sleep deprivation time points. This suggests that the homeostatic up regulation of melanopsin mRNA reaches a maximum after a relatively short period of time spent awake. A photopigment might not need to be regulated above a certain level to avoid an overstimulation of non-visual brain functions. Indeed, adverse effects such as headaches or nauseas have been reported in consequence of an excess of light irradiance. The sleep deprivation experiment was only done in females and will have to be completed by experiments in males. However, as mood disorders are more frequent in women than in men, our data is relevant. All in all the results show that the homeostatic process influences melanopsin mRNA expression in mice.

The results in **Arvicanthis ansorgei** show that, as in the nocturnal rodent, melanopsin mRNA expression follows the homeostatic process. The increase of melanopsin mRNA is significant after six hours of sleep deprivation which is three hours later than in the mouse. This is consistent with the observation (data not shown and not yet published) that Arvicanthis ansorgei - a larger animal - is more resistant to sleep deprivation (slower build up of sleep pressure). The increase stayed significant at nine hours of SD. However, after 12 hours of sleep deprivation, the Opn4 mRNA level did not differ significantly from control values that peak at the same ZT due to the circadian drive.

This again suggests that even under circadian and homeostatic pressure, melanopsin cannot be up regulated above certain levels.

When the animals were allowed to rest, melanopsin mRNA levels return to circadian expression levels. This regulation shows a clear homeostatic influence on melanopsin mRNA expression.

PACAP mRNA in Arvicanthis ansorgei shows the same increase as melanopsin mRNA suggesting that the sleep homeostatic process regulates the Opn4-based signaling to the brain to modulate the non-visual function as a function of time spent awake. This also suggests that a melanopsin up regulation translates in PACAP transmission of light information. Contrarily, to the hypothesis proposed by Josza et al. (78), retinal PACAP seems not only to be regulated by the circadian drive but also by other mechanisms such as the sleep homeostatic process.

An interesting aspect is the identical effect of sleep deprivations in nocturnal and in diurnal species. There is no inverse response to the sleep homeostatic process. In both cases, the sleep deprivations were performed during the resting period of the animal which corresponds to the light phase and dark phase in nocturnal and diurnal animals, respectively. It would be interesting to repeat these experiments under different light/dark conditions to determine whether this might influence the homeostatic regulation. However, the current findings demonstrate that the sleep homeostatic regulation of melanopsin and retinal PACAP is independent of nocturnality or diurnality.

Sleep deprivations were done through minimal amounts of gentle handling which is much less stressful than sleep deprivation through forced locomotion in a continuously turning wheel. However, the fact that sleep deprivation might also induce stress and that the effects seen might be in correlation with stress have to be taken into consideration. Sleep deprivation raises cortisol levels in rat pups (88) and evening cortisol levels in humans (89). Tobler et al. studied plasma corticosterone levels in the rat after 21,5 hours of sleep deprivation by forced locomotion showed an increase of corticosterone levels, however this increase was not significant in comparison to control groups (90). In absence of cortisol measurements, it is not possible to rule out that melanopsin and PACAP mRNA expression might be influenced by mechanisms related to stress. It seems however unlikely that stress influences a photopigment.

Melanopsin has been shown to affect the sleep homeostatic response. (48). Here we demonstrate that the sleep homeostat influences melanopsin expression. This reciprocal relationship defines melanopsin as a sleep homeostatic factor (91). The observation that the photopigment is upregulated implies that the quantity of the protein determines the quantity of light transmission. Therefore, sleep deprivation might increase the sensibility to the non-image forming effects of light. Alternatively, unknown factors induced by the forced alertness might be responsible for the induction of melanopsin expression, pretending a biological need and thus rendering the animals susceptible to light. This is particularly relevant since the alerting effect of light, reinforced by melanopsin up regulation, might help resist to sleep deprivation by maintaining a high level of alertness. Why this effect is not inversed in diurnal and nocturnal animals raises many questions. A photopigment as a sleep factor is a very surprising and interesting finding. The underlying mechanisms are not understood and will have to be investigated. Anatomic experiments exploring the neuronal circuits implicated will be very interesting in the future. The effect of sleep deprivation on cFos expression (an early transcription factor and marker of neuronal activation) in regions involved in sleep homeostatic regulation is one of the aspects that would be interesting to investigate.

It has been shown that an increase of sleep-need correlates with a decrease of mood and alertness (83). Some depressive patients (in particular patients with seasonal affective disorders) show a change in sleep structure and a decrease of the homeostatic sleep drive (EEG delta power) (83). Showing that sleep homeostasis affects melanopsin, opens new perspectives in the field of understanding of mood disorders and the interactions between light, sleep disturbances and mood disorders. The efficiency of sleep deprivation as a therapeutic tool in depression (83) might be explained by our model. As we have shown, sleep deprivation influences the expression of melanopsin. Therefore the projection of light to the brain might be altered through the up-regulation of the photoreceptor and this might in turn influence mood. The understanding of these mechanisms opens up new avenues for clinical indications of light therapy.

5.4 Sustained Light/Dark Effects on Melanopsin mRNA Expression

Exposure to three days of light and three days of darkness did not change melanopsin mRNA levels in the C57BL/6 mouse nor in Arvicanthis ansorgei. This is an astonishing finding when results are compared to results in the albino rat in the literature. Hannibal's group showed that melanopsin mRNA expression increased significantly in albino Wistar rats after only two days of dark and decreased dramatically after three days of light (69). This regulation was observed at the mRNA level by RT-qPCR and also confirmed by anatomic experiments which show a clear increase of melanopsin immunoreactivity after exposure to darkness and a decrease after exposure to light. The group of Spessert also reported an increase of melanopsin mRNA expression in albino rats (Sprague-Dawley) after 2 days of darkness and a decrease after 2 days of light when pooling the retinas of all time points taken (70). However, this dark-induced melanopsin mRNA expression was not observed by Tosini´s group who did not show an increase of melanopsin mRNA expression after 2 days in darkness in male tan hooded pink eyed RCS/N-rdy+ rats (87), (71). Additionally, in non-pigmented animals, a report suggests that the sensitivity of the system depends on prior lighting conditions. Light exposure desensitized ipRGC in Sprague-Dawley rats, dark exposure on the other hand sensitized the cells to light flashes (92). Conversely, a study in pigmented mice showed that light or darkness does not influence melanopsin expression (74). These findings were recently further corroborated by Hannibal´s study in Brown Norwegian rats (pigmented) which did not show a change in melanopsin mRNA expression after three days of light or dark exposure (72). Hannibal´s group pursued the study up to a prolonged continuous light or dark exposure (21 days) which showed that melanopsin mRNA was not affected by such extreme conditions in pigmented rats. Protein levels on the other hand, studied by western blot analysis, decreased significantly after 21 days of light exposure but did not significantly change after 21 days of dark exposure. The authors concluded that in pigmented animals melanopsin is regulated by an endogenous circadian rhythm at the mRNA level and by light at the protein level (72), an hypothesis that seems unlikely. Another study also suggested that melanopsin protein is regulated by light and not by an endogenous circadian rhythm (73).

In the same line, an interesting finding was that actimetry studies showed that the duration of prolonged exposure to darkness had an effect on the magnitude of the

phase shift induced by light in certain species like the Wistar rat or the Syrian hamster but not in C57BL/6 mice nor in Arvicanthis niloticus (93). These results are interesting as Arvicanthis niloticus is closely related to Arvicanthis ansorgei where we could not show a difference of melanopsin expression after DD exposure as we could not show a difference in mice after dark exposure. Our findings corroborate the finding that dark exposure does not have an influence on melanopsin expression in these animals. Further experiments in albino as well as experiments in pigmented animals will have to be conducted to clarify the controversial influence of light on melanopsin expression. If the time duration of light exposure does not affect melanopsin expression, it might be interesting to raise light intensities (72) in future experiments.

Light is more toxic to the retinas of albino rats than of pigmented rats (72) which could explain the decrease of melanopsin in albino rats after prolonged light exposure. Hannibal´s theory concerning the missing effect of darkness on melanopsin protein expression in the pigmented rat is a possible constant suppression of melanopsin levels in the albino retina. Constant DD exposure can only increase protein to the level it can obtain at the end of a normal LD exposure (72).

Concerning the study of the direct effects of light one has however to keep in mind that the experiments described above study sustained direct effects of light (exposure during three days) and not the immediate effects of light on vigilance seen in melanopsin knockout studies where light or dark exposure induces an immediate change of the vigilance state. The direct effects of light that regulate sleep are immediate effects which do not leave enough time to up-or down-regulate a protein. However, unpublished data of the INCI group also shows that melanopsin plays a role in sustained direct effects of light. One also has to consider that a prolonged exposure to light or dark affects activity and behavior of animals and it might not be possible to discriminate between light and stress factors. In addition, the lack of modulation of melanopsin expression does not rule out the possibility that light affects the sensitivity of the system at a different level especially within downstream processes or the conformation of the protein.

Finally, taking all studies into consideration, the results suggest that light influences melanopsin expression depending on the pigmentation of the animals.

5.5 Relevance of the Findings in the Context of Seasonal Affective Disorder

Our data did not provide any evidence that prolonged exposure to light or darkness directly induces the expression of melanopsin and thereby modulates sleep in the animal models studied. Further experiments will be needed to clarify this issue in pigmented animals as this concept might help to explain the physiopathology of seasonal affective disorders (SADs) in which patients show signs of sadness and anxiety especially in seasons with a short photo-period and decreased light intensity. SAD patients show less delta power and less sleep efficiency in winter than in summer (94). One explanation could be that the different light levels in winter and summer could affect alertness as well as sleep homeostasis and therefore mood. Regardless of the mechanism underlying the effect, light therapy in SAD patients but also in patients with other depressive disorders, used alone or as a complementary therapy to medication, has been shown to be effective (95). Understanding the non-circadian influence of light on sleep may lead to the understanding of the antidepressant effect of light therapy. Light experiments under more physiological conditions might be interesting. Perhaps different photoperiods or chronic higher light intensities as experienced in summer have an influence on melanopsin expression in humans.

The role of light therapy as an efficient treatment for depression through a phase advance effect of the circadian rhythm has been well documented. However, this effect on the biological clock does not explain all of the anti-depressive effects of light. Specifically in seasonal affective disorder (SAD), the homeostatic process of sleep seems to be altered. According to our findings, we would hypothesize that light therapy will have a greater influence on melanopsin and sleep homeostasis in SAD patients as compared to controls, as indeed a melanopsin polymorphism has been suggested to be associated to SAD (96). Demonstrating that light affects melanopsin and subsequently improves mood through a modulation of the homeostatic process could bring a new pathophysiological mechanism to the field of mood disorder and light therapy (97). However, it is also possible that melanopsin is regulated by internal processes such as the circadian and sleep homeostatic processes but that there is no direct influence of light. This is in favor of an intrinsic higher reactivity of the photoreceptor at certain times of the day and a higher sensitivity to light under sleep deprivation conditions.

5.6 Relevance of the Findings in the Context of Sleep/Wake Regulation and Perspectives

Melanopsin plays a key role in modulating the circadian and homeostatic processes as well as the direct non-circadian input of light to sleep. Here, we show that the circadian and homeostatic drive affect melanopsin expression and to some extent PACAP expression. Surprisingly, light did not directly influence melanopsin expression. Given that light and darkness provoke immediate sleep/wake responses, it seems plausible that these responses were not reflected in the expression levels of melanopsin. After all it usually takes minutes to hours to produce a fully functional protein. The irresponsiveness to light does not necessarily argue against the role of melanopsin as a mediator, it merely reflects the biological requirements which demand spontaneous reactions. However, more recent data from the group show that sustained direct effects of light persistently affect sleep and alertness, implying that more experiments are needed, especially to determine whether light could affect the melanopsin transduction pathways (Figure 38).

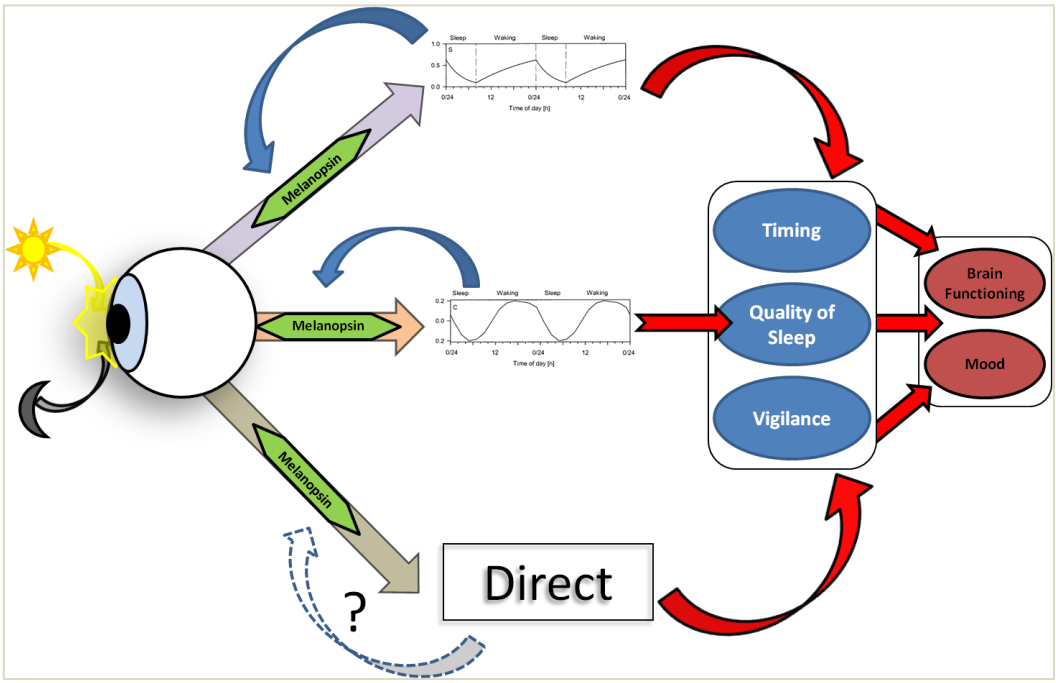

Figure 38: Model of sleep regulation focusing on melanopsin as it is addressed in this thesis. Melanopsin has an influence on the homeostatic and circadian processes and vice versa. It also has an influence on the direct effects of light. The influence of light on melanopsin could not be shown.

It remains to investigate the mechanisms by which melanopsin expression is so strongly regulated, especially at the transcriptional level by analyzing the promoter region. In the mouse about 1000 base pairs upstream of the start codon were subjected to sequence analysis. The analysis revealed the presence of binding sites for 44 regulatory factors including transcription factors with several binding sites for AP-1, NF-1, Sp-1 or Oct-1. Oct-1 e.g. is thought to function as a sensor for metabolic and stress signals (98) and thus might provide the link to the circadian cycle.

Furthermore, studies of melanopsin in humans will be a very interesting approach in the future. Our findings suggest that melanopsin and PACAP expression are related to activity levels and sleep need. This in turn suggests that the efficiency of melanopsin phototransduction adapts to the physiological needs allowing a synchronization of the reactivity to light at the appropriate time of day or a greater sensitivity to light under sleep deprivation conditions. It remains to further understand how this applies to the daily fluctuations of the alerting effects of light in human. Melanopsin expression levels could be indirectly studied by pupillometry, especially with sustained blue light exposure and long response analysis. These studies might enable us to prove the involvement of melanopsin in sleep homeostasis and sleep/wake regulation in humans and to translate the proposed three process model to humans. Light plays an important role in our society and a better understanding of these mechanisms could have a great impact on lighting strategies. Most interestingly, the importance of blue light has already been corroborated in different studies. Blue light has a great impact on brain function, cognition and performance (27, 46) as for example sustained attention tasks (99). As melanopsin has its greatest sensitivity in the blue light spectrum, this confirms the importance of melanopsin as a mediator of non-visual light function in humans. Light also has an acute alerting effect in humans and a circadian impact in shifting the rhythm (100). People who work at night might preserve a higher vigilance under blue enriched light conditions or less intense light might be indicated under sleep deprivation conditions.

In summary this is a very challenging field of research that might have a great impact on the understanding of the complexity of phototransduction and its influence on vigilance. This thesis gives further evidence of the functional role of melanopsin and might ultimately lead to a better understanding of the mechanisms underlying the regulation of sleep - a state of conscience that is as yet so poorly understood.

6. References

1. Billiard M, Dauvilliers Y. Sommeil normal. In: Les troubles du sommeil. Elsevier Masson. 2e édition. Elsevier Masson, 2012:3-9
2. Valatx JL, Bugat R, Jouvet M. Genetic Studies of Sleep in Mice. Nature 1972;238:226-7.
3. McNamara P, Capellini I, Harris E, Nunn CL, Barton RA, Preston B. The Phylogeny of Sleep Database: A New Resource for Sleep Scientists. Open Sleep J 2008;1:11-4.
4. Tafti M, Franken P, Kitahama K, Malafosse A, Jouvet M, Valatx JL. Localization of candidate genomic regions influencing paradoxical sleep in mice. Neuroreport 1997;8:3755-8.
5. Benington JH, Craig Heller H. Restoration of brain energy metabolism as the function of sleep. Progress in Neurobiology 1995;45:347-60.
6. Rechtschaffen A, Bergmann BM. Sleep deprivation in the rat: an update of the 1989 paper. Sleep 2002;25:18-24.
7. Van Cauter E, Spiegel K, Tasali E, Leproult R. Metabolic consequences of sleep and sleep loss. Sleep Medicine 2008;9, Supplement 1:S23-S8.
8. Adamantidis A, de Lecea L. Sleep and metabolism: shared circuits, new connections. Trends in Endocrinology & Metabolism 2008;19:362-70.
9. Hanlon EC, Van Cauter E. Quantification of sleep behavior and of its impact on the cross-talk between the brain and peripheral metabolism. Proceedings of the National Academy of Sciences 2011;108(Supplement 3):15609-16.
10. Knutson KL. Impact of sleep and sleep loss on glucose homeostasis and appetite regulation. Sleep Med Clin 2007;2:187-97.
11. Knutson KL. Sleep duration and cardiometabolic risk: A review of the epidemiologic evidence. Best Practice & Research Clinical Endocrinology & Metabolism 2010;24:731-43.
12. Everson CA, Toth LA. Systemic bacterial invasion induced by sleep deprivation. American Journal of Physiology - Regulatory, Integrative and Comparative Physiology 2000;278:R905-R16.
13. Foster RG, Wulff K. The rhythm of rest and excess. Nat Rev Neurosci 2005;6:407-14.

14. Irwin M, McClintick J, Costlow C, Fortner M, White J, Gillin J. Partial night sleep deprivation reduces natural killer and cellular immune responses in humans. The FASEB Journal 1996;10:643-53.
15. Leproult R, Copinschi G, Buxton O, Van Cauter E. Sleep loss results in an elevation of cortisol levels the next evening. Sleep 1997;20:865-70.
16. Lim J, Dinges DF. Sleep Deprivation and Vigilant Attention. Annals of the New York Academy of Sciences 2008;1129:305-22.
17. Van Dongen HPA, Maislin G, Mullington J, Dinges D. The cumulative cost of additional wakefulness: dose-response effects on neurobehavioral functions and sleep physiology from chronic sleep restriction and total sleep deprivation. Sleep 2003;26:117-26.
18. Marshall L, Molle M, Hallschmid M, Born J. Transcranial direct current stimulation during sleep improves declarative memory. J Neurosci 2004;24:9985-92.
19. Wamsley EJ, Stickgold R. Memory, Sleep and Dreaming: Experiencing Consolidation. Sleep Med Clin 2011;6:97-108.
20. Meerlo P, Mistlberger RE, Jacobs BL, Craig Heller H, McGinty D. New neurons in the adult brain: The role of sleep and consequences of sleep loss. Sleep Medicine Reviews 2009;13:187-94.
21. Haack M, Mullington JM. Sustained sleep restriction reduces emotional and physical well-being. Pain 2005;119:56-64.
22. Borbély AA. A two process model of sleep regulation. Human neurobiology 1982;1:195-204.
23. Achermann P. The two-process model of sleep regulation revisited. Aviation, space, and environmental medicine 2004;75(3 Suppl):A37-A43.
24. Webb WB, Agnew HW, Jr. Stage 4 sleep: influence of time course variables. Science 1971;174:1354-6.
25. Borbely AA, Baumann F, Brandeis D, Strauch I, Lehmann D. Sleep deprivation: effect on sleep stages and EEG power density in man. Electroencephalogr Clin Neurophysiol 1981;51:483-95.
26. Franken P, Chollet D, Tafti M. The Homeostatic Regulation of Sleep Need Is under Genetic Control. The Journal of Neuroscience 2001;21:2610-21.
27. Dijk D-J, Archer SN. Circadian and Homeostatic Regulation of Human Sleep and Cognitive Performance and Its Modulation by PERIOD3. Sleep Medicine Clinics 2009;4:111-25.

28. Schulz P, Steimer T. Neurobiology of Circadian Systems. CNS Drugs 2009;23:3-13.
29. Provencio I, Jiang G, De Grip WJ, Hayes WP, Rollag MD. Melanopsin: An opsin in melanophores, brain, and eye. Proceedings of the National Academy of Sciences 1998;95:340-5.
30. Gooley JJ, Lu J, Chou TC, Scammell TE, Saper CB. Melanopsin in cells of origin of the retinohypothalamic tract. Nature Neuroscience 2001;4:1165.
31. Berson D. Phototransduction in ganglion-cell photoreceptors. Pflügers Archiv European Journal of Physiology 2007;454:849-55.
32. Schmidt TM, Do MTH, Dacey D, Lucas R, Hattar S, Matynia A. Melanopsin-Positive Intrinsically Photosensitive Retinal Ganglion Cells: From Form to Function. The Journal of Neuroscience 2011;31:16094-101.
33. Lucas RJ, Douglas RH, Foster RG. Characterization of an ocular photopigment capable of driving pupillary constriction in mice. Nat Neurosci 2001;4:621-6.
34. Foster RG, Hankins MW. Circadian vision. Current Biology 2007;17:R746-R51.
35. Walker MT, Brown RL, Cronin TW, Robinson PR. Photochemistry of retinal chromophore in mouse melanopsin. Proceedings of the National Academy of Sciences 2008;105:8861-5.
36. Graham DM, Wong KY, Shapiro P, Frederick C, Pattabiraman K, Berson DM. Melanopsin Ganglion Cells Use a Membrane-Associated Rhabdomeric Phototransduction Cascade. Journal of Neurophysiology 2008;99:2522-32.
37. Hatori M, Panda S. The emerging roles of melanopsin in behavioral adaptation to light. Trends in Molecular Medicine 2010;16:435-46.
38. Do MTH, Yau K-W. Intrinsically Photosensitive Retinal Ganglion Cells. Physiological Reviews 2010;90:1547-81.
39. Hankins MW, Peirson SN, Foster RG. Melanopsin: an exciting photopigment. Trends in Neurosciences 2008;31:27-36.
40. Ruby NF, Brennan TJ, Xie X, Cao V, Franken P, Heller HC, O'Hara BF. Role of Melanopsin in Circadian Responses to Light. Science 2002;298:2211-3.
41. Panda S, Sato TK, Castrucci AM, Rollag MD, DeGrip WJ, Hogenesch JB, Provencio I, Kay SA. Melanopsin (Opn4) Requirement for Normal Light-Induced Circadian Phase Shifting. Science 2002;298:2213-6.

42. Freedman MS, Lucas RJ, Soni B, von Schantz M, Munoz M, David-Gray Z, Foster R. Regulation of mammalian circadian behavior by non-rod, non-cone, ocular photoreceptors. Science 1999;284:502-4.

43. Foster RG, Provencio I, Hudson D, Fiske S, Grip W, Menaker M. Circadian photoreception in the retinally degenerate mouse (rd/rd). Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology. 1991;169:39-50.

44. Lucas RJ, Hattar S, Takao M, Berson DM, Foster RG, Yau K-W. Diminished Pupillary Light Reflex at High Irradiances in Melanopsin-Knockout Mice. Science 2003;299:245-7.

45. Lucas RJ, Freedman MS, Muñoz M, Garcia-Fernández J-M, Foster RG. Regulation of the Mammalian Pineal by Non-rod, Non-cone, Ocular Photoreceptors. Science 1999;284:505-7.

46. Cajochen C, Münch M, Kobialka S, Kräuchi K, Steiner R, Oelhafen P, Orgül S, Wirz-Justice A. High Sensitivity of Human Melatonin, Alertness, Thermoregulation, and Heart Rate to Short Wavelength Light. Journal of Clinical Endocrinology & Metabolism 2005;90:1311-6.

47. Guler AD, Ecker JL, Lall GS, Haq S, Altimus CM, Liao H-W, Barnard AR, Cahill H, Badea TC, Zhao H, Hankins MW, Berson DM, Lucas RJ, Yau K-W, Hattar S. Melanopsin cells are the principal conduits for rod-cone input to non-image-forming vision. Nature 2008;453:102-5.

48. Tsai JW, Hannibal J, Hagiwara G, Colas D, Ruppert E, Ruby NF, Heller HC, Franken P, Bourgin P. Melanopsin as a sleep modulator: circadian gating of the direct effects of light on sleep and altered sleep homeostasis in Opn4(-/-) mice. PLoS Biol 2009;7:e1000125.

49. Hannibal J, Fahrenkrug J. Target areas innervated by PACAP-immunoreactive retinal ganglion cells. Cell and tissue research 2004;316:99.

50. Hattar S, Kumar M, Park A, Tong P, Tung J, Yau K-W, Berson DM. Central projections of melanopsin-expressing retinal ganglion cells in the mouse. The Journal of Comparative Neurology 2006;497:326-49.

51. Berson DM, Dunn FA, Takao M. Phototransduction by Retinal Ganglion Cells That Set the Circadian Clock. Science 2002;295:1070-3.

52. Hirota T, Fukada Y. Resetting Mechanism of Central and Peripheral Circadian Clocks in Mammals. Zoological Science 2004;21:359-68.

53. Clarke RJ, Ikeda H. Luminance and darkness detectors in the olivary and posterior pretectal nuclei and their relationship to the pupillary light reflex in the rat. Experimental brain research 1985;57:224.
54. Trejo LJ, Cicerone CM. Cells in the pretectal olivary nucleus are in the pathway for the direct light reflex of the pupil in the rat. Brain Research 1984;300:49-62.
55. Gallopin T, Fort P, Eggermann E, Cauli B, Luppi PH, Rossier J, Audinat E, Muhlethaler M, Serafin M. Identification of sleep-promoting neurons in vitro. Nature 2000;404:992-5.
56. Isa T. Intrinsic processing in the mammalian superior colliculus. Current Opinion in Neurobiology 2002;12:668-77.
57. Brown TM, Gias C, Hatori M, Keding SR, Semo Ma, Coffey PJ, Gigg J, Piggins HD, Panda S, Lucas RJ. Melanopsin Contributions to Irradiance Coding in the Thalamo-Cortical Visual System. PLoS Biol 2010;8:e1000558.
58. Hannibal J, Moller M, Ottersen OP, Fahrenkrug J. PACAP and glutamate are co-stored in the retinohypothalamic tract. The Journal of Comparative Neurology 2000;418:147-55.
59. Hannibal J, Vrang N, Card JP, Fahrenkrug J. Light-Dependent Induction of cFos during Subjective Day and Night in PACAP-Containing Ganglion Cells of the Retinohypothalamic Tract. Journal of Biological Rhythms 2001;16:457-70.
60. Hannibal J. Roles of PACAP-Containing Retinal Ganglion Cells in Circadian Timing. In: Kwang WJ, editor. International Review of Cytology: Academic Press; 2006:1-39.
61. Golombek DA, Rosenstein RE. Physiology of circadian entrainment. Physiol Rev 2010;90:1063-102.
62. Vaudry D, Falluel-Morel A, Bourgault S, Basille M, Burel D, Wurtz O, Fournier A, Chow BKC, Hashimoto H, Galas L, Vaudry H. Pituitary Adenylate Cyclase-Activating Polypeptide and Its Receptors: 20 Years after the Discovery. Pharmacological Reviews 2009;61:283-357.
63. Wang ZY, Alm P, Håkanson R. Distribution and effects of pituitary adenylate cyclase-activating peptide in the rabbit eye. Neuroscience 1995;69:297-308.
64. Hannibal J, Hindersson P, Knudsen SM, Georg B, Fahrenkrug J. The Photopigment Melanopsin Is Exclusively Present in Pituitary Adenylate Cyclase-Activating Polypeptide-Containing Retinal Ganglion Cells of the Retinohypothalamic Tract. The Journal of Neuroscience 2002;22:RC191.

65. Ahnaou A, Laporte AM, Ballet S, Escourrou P, Hamon M, Adrien J, Bourgin P. Muscarinic and PACAP receptor interactions at pontine level in the rat: significance for REM sleep regulation. Eur J Neurosci 2000;12:4496-504.
66. Altimus CM, Güler AD, Villa KL, McNeill DS, LeGates TA, Hattar S. Rods-cones and melanopsin detect light and dark to modulate sleep independent of image formation. Proceedings of the National Academy of Sciences 2008;105:19998-20003.
67. Lupi D, Oster H, Thompson S, Foster RG. The acute light-induction of sleep is mediated by OPN4-based photoreception. Nat Neurosci 2008;11:1068-73.
68. Hubbard J, Ruppert E, Gropp C-M, Bourgin P. Non-circadian direct effects of light on sleep and alertness: Lessons from transgenic mouse models. Sleep Medicine Reviews 2013. (Epub ahead of print) pii: S1087-0792(13)00002-6. doi:10.1016/j.smrv.2012.12.004
69. Hannibal J, Georg B, Hindersson P, Fahrenkrug J. Light and darkness regulate melanopsin in the retinal ganglion cells of the albino Wistar rat. J Mol Neurosci 2005;27:147-55.
70. Mathes A, Engel L, Holthues H, Wolloscheck T, Spessert R. Daily profile in melanopsin transcripts depends on seasonal lighting conditions in the rat retina. J Neuroendocrinol 2007;19:952-7.
71. Sakamoto K, Liu C, Tosini G. Classical photoreceptors regulate melanopsin mRNA levels in the rat retina. J Neurosci 2004;24:9693-7.
72. Hannibal J, Georg B, Fahrenkrug J. Differential expression of melanopsin mRNA and protein in Brown Norwegian rats. Exp Eye Res 2013;106:55-63.
73. Gonzalez-Menendez I, Contreras F, Cernuda-Cernuda R, Garcia-Fernandez JM. Daily rhythm of melanopsin-expressing cells in the mouse retina. Frontiers in Cellular Neuroscience 2009;3.
74. González-Menéndez I, Contreras F, Cernuda-Cernuda R, Provencio I, García-Fernández JM. Postnatal Development and Functional Adaptations of the Melanopsin Photoreceptive System in the Albino Mouse Retina. Investigative Ophthalmology & Visual Science 2010;51:4840-7.
75. Fahrenkrug J. PACAP—A Multifacetted Neuropeptide. Chronobiology International: The Journal of Biological & Medical Rhythm Research 2006;23:53-61.
76. Fukuhara C, Suzuki N, Matsumoto Y, Nakayama Y, Aoki K, Tsujimoto G, Inouye S-IT, Masuo Y. Day-night variation of pituitary adenylate cyclase-activating polypeptide

(PACAP) level in the rat suprachiasmatic nucleus. Neuroscience Letters 1997;229:49-52.

77. Lee JE, Zamdborg L, Southey BR, Atkins N, Mitchell JW, Li M, Gillette, Martha U, Kelleher NL, Sweedler JV. Quantitative Peptidomics for Discovery of Circadian-Related Peptides from the Rat Suprachiasmatic Nucleus. Journal of Proteome Research 2012;12:585-93.

78. Józsa R, Somogyvári-Vigh A, Reglödi D, Hollósy T, Arimura A. Distribution and daily variations of PACAP in the chicken brain. Peptides 2001;22:1371-7.

79. Cagampang FRA, Piggins HD, Sheward WJ, Harmar AJ, Coen CW. Circadian changes in PACAP type 1 (PAC1) receptor mRNA in the rat suprachiasmatic and supraoptic nuclei. Brain Research 1998;813:218-22.

80. Shinohara K, Funabashi T, Kimura F. Temporal profiles of vasoactive intestinal polypeptide precursor mRNA and its receptor mRNA in the rat suprachiasmatic nucleus. Molecular Brain Research 1999;63:262-7.

81. Challet E, Pitrosky B, Sicard B, Malan A, Pévet P. Circadian Organization in a Diurnal Rodent, Arvicanthis ansorgei Thomas 1910: Chronotypes, Responses to Constant Lighting Conditions, and Photoperiodic Changes. Journal of Biological Rhythms 2002;17:52-64.

82. Karlen Y, McNair A, Perseguers S, Mazza C, Mermod N. Statistical significance of quantitative PCR. BMC Bioinformatics 2007;8:131.

83. Wirz-Justice A, Van den Hoofdakker RH. Sleep deprivation in depression: what do we know, where do we go? Biological Psychiatry 1999;46:445-53.

84. Saper CB, Cano G, Scammell TE. Homeostatic, circadian, and emotional regulation of sleep. J Comp Neurol 2005;493:92-8.

85. Roenneberg T, Foster RG. Twilight Times: Light and the Circadian System. Photochemistry and Photobiology 1997;66:549-61.

86. Zordan M, Osterwalder N, Rosato E, Costa R. Extra Ocular Photic Entrainment in Drosophila Melanogaster. Journal of Neurogenetics. 2001;15:97-116.

87. Strain report. Rat Genome Database of the Medical College of Wisconsin. (Accessed July 14, 2013 at http://rgd.mcw.edu/rgdweb/report/strain/main.html?id=68122)

88. Hairston IS, Ruby NF, Brooke S, Peyron C, Denning DP, Heller HC, Sapolsky RM. Sleep deprivation elevates plasma corticosterone levels in neonatal rats. Neuroscience Letters 2001;315:29-32.

89. Spiegel K, Leproult R, Van Cauter E. Impact of sleep debt on metabolic and endocrine function. The Lancet 1999;354:1435-9.
90. Tobler I, Murison R, Ursin R, Ursin H, Borbély AA. The effect of sleep deprivation and recovery sleep on plasma corticosterone in the rat. Neuroscience Letters 1983;35:297-300.
91. Jouvet M. Neuromediators and hypnogenic factors. Revue neurologique 1984;140:389-400.
92. Wong KY, Dunn FA, Berson DM. Photoreceptor Adaptation in Intrinsically Photosensitive Retinal Ganglion Cells. Neuron 2005;48:1001-10.
93. Refinetti R. Enhanced circadian photoresponsiveness after prolonged dark adaptation in seven species of diurnal and nocturnal rodents. Physiology & behavior 2007;90:431-7.
94. Anderson JL, Rosen LN, Mendelson WB, Jacobsen FM, Skwerer RG, Joseph-Vanderpool JR, Duncan CC, Wehr TA, Rosenthal NE. Sleep in fall/winter seasonal affective disorder: effects of light and changing seasons. J Psychosom Res 1994;38:323-37.
95. Even C, Schröder CM, Friedman S, Rouillon F. Efficacy of light therapy in nonseasonal depression: A systematic review. Journal of Affective Disorders 2008;108:11-23.
96. Roecklein KA, Wong PM, Franzen PL, Hasler BP, Wood-Vasey WM, Nimgaonkar VL, Miller MA, Kepreos KM, Ferrell RE, Manuck SB. Melanopsin Gene Variations Interact With Season to Predict Sleep Onset and Chronotype. Chronobiology International 2012;29:1036-47.
97. Stephenson KM, Schroder CM, Bertschy G, Bourgin P. Complex interaction of circadian and non-circadian effects of light on mood: Shedding new light on an old story. Sleep Med Rev 2012;16:445-54.
98. Wang P, Jin T. Oct-1 functions as a sensor for metabolic and stress signals. Islets 2010;2:46-8.
99. Chellappa S. Can light make us bright? Effects of light on cognition and sleep. Progress in Brain Research 2011;190:119.
100. Dijk D-J, Archer SN. Light, Sleep, and Circadian Rhythms: Together Again. PLoS Biol 2009;7:e1000145.

Eidesstattliche Versicherung

„Ich, Claire-Marie Gropp, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „Regulation of Melanopsin and PACAP mRNA by Circadian and Homeostatic Processes and by Direct Effects of Light“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren beruhen, sind als solche in korrekter Zitierung (siehe „Uniform Requirements for Manuscripts (URM)“ des ICMJE -www.icmje.org) kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) entsprechen den URM (s.o) und werden von mir verantwortet.

Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Betreuer/in, angegeben sind. Sämtliche Publikationen, die aus dieser Dissertation hervorgegangen sind und bei denen ich Autor bin, entsprechen den URM (s.o) und werden von mir verantwortet.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§156,161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

Datum Unterschrift

Curriculum Vitae Claire-Marie Gropp

"Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht."

List of Publications

- Hubbard J, Ruppert E, Gropp C-M, Bourgin P (2013). Non-circadian direct effects of light on sleep and alertness: Lessons from transgenic mouse models. Sleep Medicine Reviews 2013; (Epub ahead of print) pii: S1087-0792(13)00002-6. doi:10.1016/j.smrv.2012.12.004
- Hubbard J, Ruppert E, Allemann C, Choteau L, Gropp C-M, Calvel L, Challet E, Bourgin P. A novel diurnal model, Arvicanthis ansorgei: validation and characterization of sleep regulatory mechanisms. Oral presentation at the 21st Congress of the European Sleep Research Society 2012 in Paris. Abstract book p.54 (O154)
- Calvel L, Hubbard J, Choteau L, Gropp C-M, Ruppert E, Bourgin P. The non-circadian effects of light influence depression–like behavior in mice via melanopsin-based pathways. Poster presentation at the 21st European Sleep Research Society 2012 in Paris. Abstract book p.106 (P622)
- Calvel L, Hubbard J, Choteau L, Cezarzyk C, Gropp C-M, Ruppert E, Bourgin P. Effets antidépresseur de la lumière: la mélanopsine, un médiateur influencent l´homéostasie de sommeil et des troubles de l´humeur. Poster presentation at the French sleep congress in Strasbourg 2011. Abstract book p. 85 (P97).

Acknowledgments

I would like to express my deep gratitude to Professor Patrice BOURGIN, my research supervisor, to have given me the great opportunity to work in his laboratory and to do this thesis, for his kindness, competence, support and readiness to welcome me in his team as well as for his scientific guidance.

I would also like to offer special thanks to Professor Heidi DANKER-HOPFE, supervisor of this thesis, who accepted to support this work, for her constructive suggestions, her useful criticism, her support and her willingness to give her time and to encourage the progress of this work.

I would like to particularly thank Ludivine Robin who accompanied my first steps in the laboratory and without whose help this thesis would not have been possible. Thank you for the productive discussions and the help in the day to day laboratory work.

I am grateful for the assistance given by the rest of the INCI research team 4, with particular thanks to Jeffrey Hubbard and Laurent Calvel. I wish to extend my thanks to the rest of the members of the INCI laboratory for their advice and help.

My grateful thanks to André Malan for his help with the statistical analyses and to Dominique Ciocca, head of the chronobiotron, for her dynamism and readiness to help and to Sophie Reibel, veterinarian, for her help with the colonies and her readiness to answer my questions.

I wish to acknowledge the help provided by the team at the university hospital of Strasbourg. I would like to especially thank Dr. Elisabeth Ruppert who is also part of the INCI team for her help with the experiments, her kindness, advice and support as well as Dr. Ulker Kilic-Huck for her advice and considerateness making me feel at home in the medical team. I wish also to thank Dr. Carmen Schröder for her constructive suggestions and kindness.

Finally, I would like to thank my loved ones for their support, advice and affection. Without their help this thesis would not have been possible.