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The melanopsin-dependent direct non-circadian effects of light: A third principal mechanism for the regulation of sleep and wake

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To my parents, my brother

And my wife, Ilona

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Abstract in English

Sleep disorders affect between 15-30% of the general population representing a major health problem. In the absence of sufficiently effective treatments, it is necessary to better understand the pathophysiology of the regulation of sleep and waking. Over the past three decades, the mechanisms of sleep regulation have been conceptualized as a two process model; circadian and homeostatic (Borbély 1982). The circadian process (C) is generated by an endogenous clock whose period is approximately 24 hours and is located in the suprachiasmatic nuclei (SCN, master clock). The homeostatic process (S) is characterized by an increase in the level of sleep pressure as a function of time spent awake, and its subsequent decrease with sleep. In addition light is known to play a fundamental role in the regulation of sleep and behavior via two distinct types of non-visual effects; indirect through the resynchronization of the clock (well-characterized mechanisms), and direct, acting on the expression of sleep and wakefulness via mechanisms independent of the circadian system that remain poorly understood. These non-visual effects are mediated by melanopsin (*Opn4*), a photopigment expressed in a subpopulation of intrinsically photosensitive ganglion cells of the retina, but also by both rods and cones. In recent years, research into the role of melanopsin has led several research teams to revisit the non-visual effects of light. Previously our group showed that by using through a transgenic mouse model with invalidation of the melanopsin gene (*Opn4*$^{-/-}$), the direct non-circadian effects of light interact with process C to influence sleep and wakefulness. Analysis of EEG delta power in *Opn4*$^{-/-}$ mice also suggested that melanopsin may be a homeostatic sleep factor (Tsai, Hannibal et al. 2009), yet needed to be further established.

The focus of this doctoral thesis was to demonstrate that the non-circadian effects of light on sleep represent a major mechanism capable of regulating vigilance states, and that it can contribute significantly to the daily distribution of sleep and waking. Specifically, we addressed the following three areas:

1) **The direct effects of light (mediated principally by *Opn4*-based phototransduction) represent a regulatory mechanism capable of maintaining an organization of sleep and waking synchronized with the light/dark cycle in the absence of a circadian pacemaker**.

2) **The characterization of *Arvicanthis ansorgei* as a novel diurnal rodent model for the study of sleep and validation of the non-circadian direct effects of light.**

3) **A demonstration that light and melanopsin influences NREM sleep homeostasis in both diurnal and nocturnal rodents**

Results from these three projects yielded the following conclusions. (I) Whereas the primary function of the SCN was thought to be almost exclusively to be a central circadian pacemaker, our findings allow for its reclassification as a relay system for approximately 50% of the melanopsin-dependent non-circadian direct effects of light. (II) The sleep-wake cycle was considered mostly determined by the circadian clock, entrained by light. Our results demonstrate that the melanopsin-dependent direct photic input to sleep across a normal 24-hour day (12hL:12hD light-dark cycle) determines approximately 1/3 of the daily sleep and wake distribution and allows mice to maintain a functional sleep-wake cycle even in the absence of a circadian drive. (III) The diurnal rodent, arvicanthis ansorgei, is a valid model for studying the regulatory mechanisms of sleep, including the direct effects of light, and will help to create a viable translational model between laboratory rodents and basic human research for the study of sleep. (IV) The homeostatic response characterized by the increase in EEG delta as a function of time spent awake, was dramatically altered in the absence of melanopsin, and independent of genetic background, as well as present in a diurnal rodent model. This finding establishes the proof of concept that a photopigment, melanopsin, can affect sleep homeostasis. Furthermore these results indicate the presence of the first biological link between light and sleep homeostasis and completion of the experimental analysis will allow us to further understand the light-melanopsin-sleep homeostasis link.

The work in this doctoral thesis examines from several directions, the accepted 2-process model for the regulation of sleep and wake, proposing a third mechanism both independent yet interrelated. These data, suggest a more complex role for light which may affect sleep and waking from multiple aspects: indirectly on the circadian drive, and directly on the expression of sleep and waking and through modulation of the sleep homeostat. In essence these results encourage a reevaluation on the role of light in mammalian physiology. Additionally, with further validation of this model in humans, this could provide a new conceptual framework for understanding the disturbances associated with sleep disorders, as well as the way we in our individual daily lives artificially expose ourselves to light and darkness.

Résumé en français

Les troubles du sommeil affectent 15% à 30% de la population générale représentant un enjeu majeur de santé publique. En l'absence de thérapeutiques suffisamment efficaces, il est nécessaire de mieux comprendre leur physiopathologie ce qui repose sur une meilleure connaissance des régulations du sommeil. Au cours des trois dernières décennies et jusqu'à aujourd'hui, ces mécanismes de régulation du sommeil ont été conceptualisés sous la forme d'un modèle à deux processus ; circadien et homéostasique (Borbély 1982). Le processus circadien (C) est généré par une horloge endogène dont la période est d´environ 24 heures et qui est localisée dans les noyaux suprachiasmatiques (NSC, horloge principale). Le processus homéostasique (S) est caractérisé par l'augmentation du niveau de pression de sommeil en fonction du temps passé éveillé et sa diminution avec le sommeil. Ce processus est évalué en réalisant des privations de sommeil, la quantité de sommeil perdu étant récupérée lors du rebond de sommeil, ce phénomène étant au mieux caractérisé par la mesure de la puissance spectrale delta de l'EEG.

La lumière joue un rôle fondamental dans la régulation du sommeil et des comportements en exerçant deux types d'effets non-visuels; indirect en resynchronisant l'horloge (mécanismes bien caractérisés), et directs agissant sur l'expression du sommeil et de la veille via des mécanismes qui restent mal compris et indépendants du système circadien. Ces effets non-visuels sont médiés par la mélanopsine (*Opn4*), un photopigment exprimé dans une sous population de cellules ganglionnaires de la rétine, leur procurant une photosensibilité intrinsèque, mais aussi par les cônes et bâtonnets. La caractérisation des processus de phototransduction liés à la mélanopsine au cours de ces dernières années a permis de revisiter les effets non-visuels de la lumière. Notre équipe a ainsi montré grâce à un modèle de souris transgénique avec invalidation du gène de la mélanopsine (*Opn4*$^{-/-}$) que les effets directs non-circadiens de la lumière interagissent avec les processus C et S pour influencer la veille et le sommeil. L'analyse de la puissance EEG delta de souris *Opn4*$^{-/-}$ suggérait également que la mélanopsine puisse constituer un facteur homéostasique de sommeil (Tsai et al., 2009). Ces résultats ouvrent des perspectives importantes pour améliorer notre compréhension des régulations du sommeil et ont été discutés dans un travail de synthèse (Hubbard et al., soumis, *sleep medicine reviews*, 2012).

L'objectif de ce travail de thèse a été de démontrer que les effets non-circadiens de la lumière sur le sommeil constituent un mécanisme majeur de régulation des états de vigilance pouvant contribuer significativement à la distribution journalière veille sommeil. Plus spécifiquement, nous avons abordé les trois axes suivants 1) Les effets directs de la lumière constituent-ils un mécanisme capable de maintenir une organisation temporelle veille sommeil synchronisée avec le cycle de lumière obscurité en l'absence de pacemaker circadien. En répondant à cette question nous proposons aussi de démontrer si les NSCs au-delà de leur fonction d'horloge, constituent une structure de relai de ces effets. En effet, l'analyse de modèles de souris sans horloge centrale permet de répondre à ces deux questions en parallèle. 2) caractériser un nouveau modèle de rongeur diurne, arvicanthis ansorgei pour l'étude du sommeil et déterminer si les effets non-circadiens directs de la lumière sont conservés et inversés chez les espèces diurnes. 3) démontrer que la lumière influence l'homéostat de sommeil via la mélanopsine chez le rongeur diurne et nocturne.

Pour réaliser ce travail, nous avons utilisé les modèles suivants : souris sans mélanopsine (*Opn4*$^{-/-}$) ; souris sans pacemaker circadien, obtenues soit par lésion électrolytique des NSCs, soit par invalidation de l'horloge par transgénèse (*Synaptotagmin10*$^{Cre/Cre}$, *Bmal1*$^{fl/-}$) ; Arvicanthis Ansorgei, un rongeur diurne provenant d'Afrique Subsaharienne et maintenu au laboratoire depuis une quinzaine d'années. L'activité locomotrice (actimétrie), le sommeil et l'EEG (puissance spectrale) des animaux ont été analysés (après implantation d'un set classique d'électrodes sous anesthésie et après une période d'habituation aux conditions d'enregistrement) dans différentes conditions de lumière obscurité. Concernant les lésions des NSCs, l'intégrité des structures avoisinantes ainsi que des projections rétiniennes vers les structures cérébrales autres que les NSCs ont été vérifiées par anatomie (injection à la fin du protocole d'un marqueur rétrograde, la sous-unité B de la toxine cholérique, dans la chambre postérieure de l'œil et immun-marquage post-mortem).

1er volet) Effets directs de la lumière médiés par la mélanopsine : un mécanisme suffisant pour maintenir une organisation journalière veille sommeil en fonction du cycle de lumière/obscurité en l'absence de pacemaker circadien (Hubbard et al, prêt pour soumission)

Il est généralement admis que le processus circadien est principalement responsable de l'organisation journalière de la veille et du sommeil. En l'absence d'horloge centrale, le rythme d'activité locomotrice reste cependant partiellement synchronisé avec le cycle de lumière obscurité, un phénomène mal caractérisé et suggérant l'existence d'un mécanisme supplémentaire indépendant de l'horloge et qui reste à identifier. Les cellules ganglionnaires intrinsèquement photosensibles de la rétine (ipRGCs) exprimant la mélanopsine, relaient les « informations non-visuelles » à des nombreuses structures cérébrales. L'activation des NSCs par la lumière, mise en relation avec l'entrainement de l'horloge, ne permets pas d'exclure que les NSCs, au-delà de leur rôle d'horloge, influencent le sommeil en relayant les effets directs de la lumière. Ces deux questions ont été abordées en parallèle en étudiant d'une part des animaux rendus arythmiques après lésion des NSCs ; d'autre part des souris transgéniques également arythmiques et présentant une invalidation de l'horloge mais des NSCs intacts (invalidation d'un gène, la *synaptotagmine 10*, fortement exprimé dans les NSCs et invalidation restreinte aux NSCs d'un gène d'horloge *Bmal1* ; *Synaptotagmin10*$^{Cre/Cre}$*, Bmal1*$^{fl/-}$).

Les résultats montrent que chez la souris sauvage la lumière induit le sommeil et l'obscurité l'éveil, ces effets étant étudiés en réponse à des pulses d'une heure de lumière et d'obscurité respectivement. Cette réactivité est atténuée de moitié chez les animaux sans mélanopsine ou sans pacemaker circadien, et complètement abolie en l'absence des deux. L'analyse d'un rythme ultradien lumière/obscurité de 1hL:1hD appliqué pendant 24 heures permettent d'étudier cette réactivité en fonction du moment de la journée. En l'absence de mélanopsine, ces modifications sont essentiellement observées pendant la période nocturne (ZT12-ZT24) (**Figure 0.1**). La qualité de l'éveil induit par l'obscurité a été évaluée en mesurant les rythmes EEG thêta et gamma, qui représentent des corrélats électrophysiologiques de vigilance et du fonctionnement cognitif. La stimulation de ces rythmes par l'application de pulses d'obscurité nécessitait la conservation des NSCs.

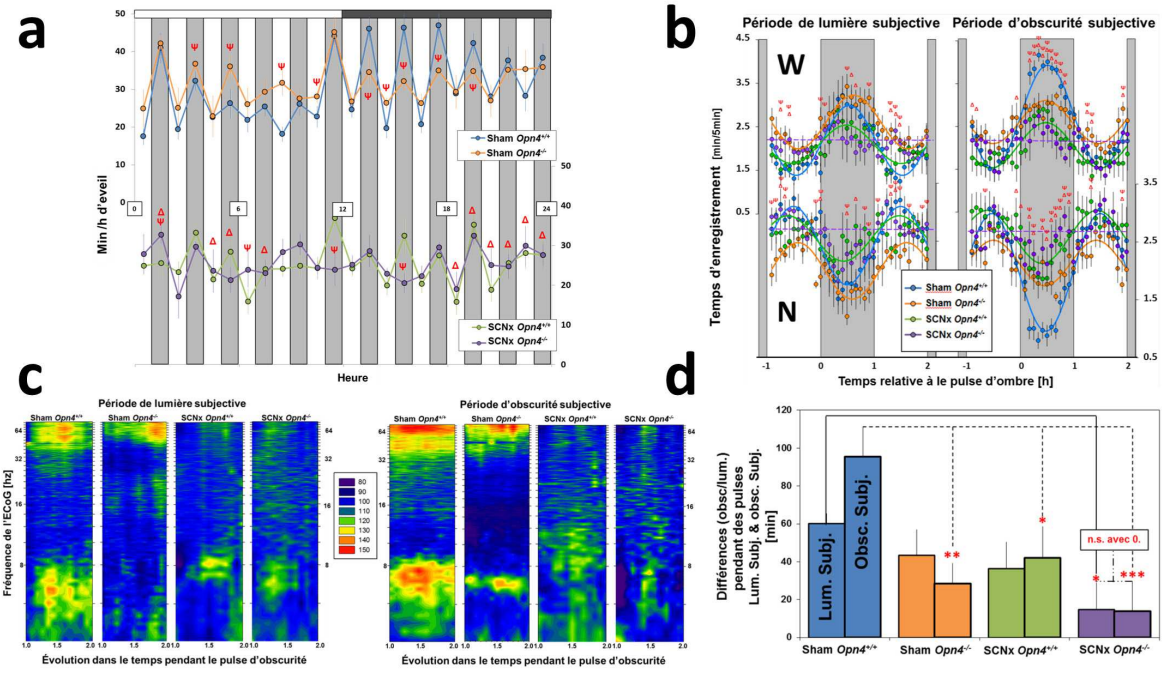

Figure 0.1- Effets directs d'un cycle ultradien de lumière obscurité (1h :1h) sur le sommeil et l'éveil chez des souris sans SCNs et/ou sans mélanopsine

(**a**) Durées moyennes de l'éveil par heure d'enregistrement. Les pulses de lumière atténuent les taux d'éveil alors que les pulses d'obscurité ont un effet éveillant. Cette réactivité est plus marquée pendant la période subjective d'obscurité que pendant la période subjective de lumière. Cette réactivité est considérablement diminuée chez les souris *Opn4*$^{-/-}$ et cette absence de réactivité est maximum pendant la période subjective d'obscurité (suggérant que la mélanopsine est le principal médiateur de ces effets la nuit et les cônes et bâtonnets la journée. Les animaux *SCNx* ont une nette diminution de réactivité qui est même abolie chez les souris SCNx-*Opn4*$^{-/-}$. (**b**) Profil temporel moyen de la réactivité de l'éveil (W, haut) et du sommeil lent (NREM, bas) aux cycles de lumière/obscurité pendant la période subjective de lumière (gauche) et la période subjective d'obscurité (droite). Valeurs moyennes (+/- sem) par intervalles de 5 min pendant l'heure précédent, durant et suivant les 6 pulses d'obscurité administrés pendant la période subjective de lumière et d'obscurité, respectivement. (c) Décours temporel du profil spectral (heat map) de l'EEG à l'éveil moyennés pendant les 6 pulse d'obscurité (moyenne) pendant la période subjective de lumière (gauche) et d'obscurité (droite). La densité de puissance spectrale de l'EEG est exprimée en pourcentage de la puissance spectrale de l'éveil pour chaque animal en condition baseline (période de lumière = 100%). Les couleurs chaudes représentent une augmentation et les couleurs les plus froides

une diminution par rapport à l'activité EEG en baseline. Les profils de puissance spectrales relatives sont calculés sur des périodes de 10 min d'éveil par en augmentant par pas de 5 min (13 spectres/hr). (d) Les différences de temps passé éveillé entre un pulse d'obscurité et les pulses de lumière adjacents (précédant et suivant) sont quantifiés et moyennés pour les 6 pulses d'obscurité pendant la période subjective de lumière (gauche) et pendant la période subjective d'obscurité (droite). Moyennes +/- sem (en min). Statistiques, voir chapitre 5.

De manière similaire, la répartition de la veille et du sommeil au cours du cycle standard de 12h de lumière 12h d'obscurité était diminuée de moitié en l'absence de mélanopsine ou des NSCs et aucun « rythme veille sommeil » n'était détectable en l'absence des deux. De même l'expression des rythmes EEG thêta et gamma pendant la période d'obscurité était significativement diminuée en l'absence des NSCs, suggérant un rôle important de cette structure dans la genèse de ces rythmes (**Figure 0.2**).

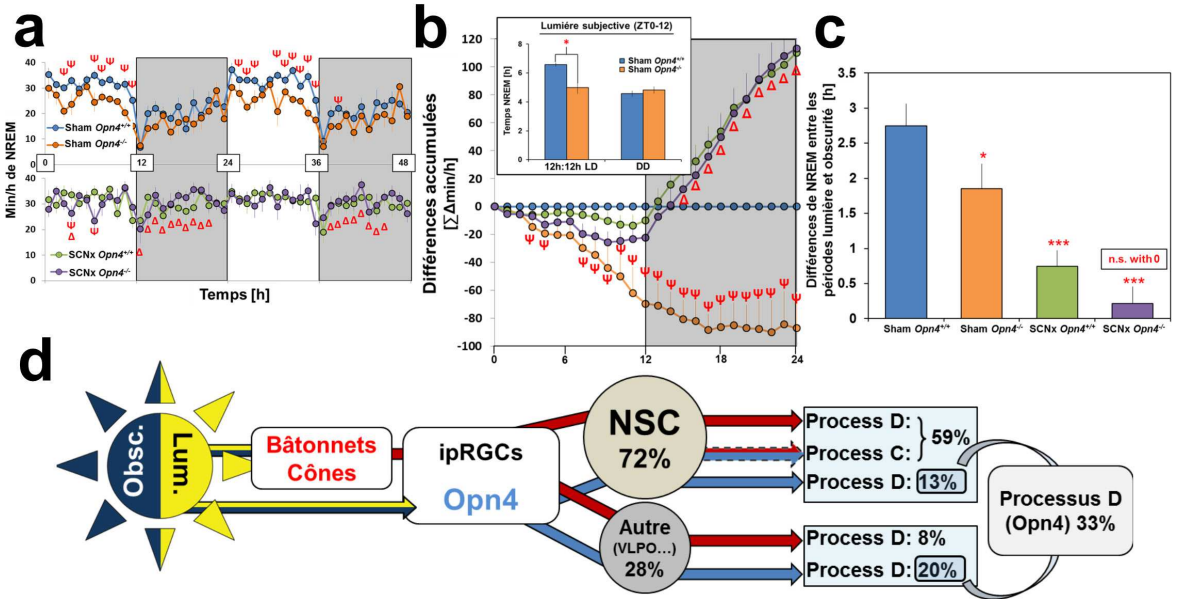

Figure 0.2- Distribution journalière du sommeil en l'absence d'horloge centrale et/ou de mélanopsine et modélisation des voies cellulaires médiant les différents effets de la lumière sur le cycle circadien veille-sommeil

(**a**) Le sommeil lent est diminué pendant la période de lumière en l'absence de mélanopsine (souris *Sham Opn4*$^{-/-}$). La distribution journalière jour/nuit du sommeil est très nettement atténuée en l'absence de NSCs (*SCNx*) et complètement abolie en l'absence des deux (*SCNx-Opn4*$^{-/-}$). (**b**) Les différences accumulées (moyenne sur 2 jours) entre

groupes de souris montrent que les souris *Opn4*$^{-/-}$ perdent environ une heure de NREM pendant la période de lumière. Les valeurs moyennes sont exprimées en comparaison au groupe contrôle (*Sham- Opn4*$^{+/+}$*)*. Encadré : En présence ou en l'absence de lumière (obscurité pendant 24 hr), les souris *Opn4*$^{-/-}$ présentent pendant la période subjective de lumière les mêmes taux de sommeil que les animaux contrôles mis en obscurité : ceci confirme que la différence génotypique observée en a) et b) résulte d'un effet hypnogène de la lumière médiée par la mélanopsine. (**c**) Distribution jour/nuit du sommeil lent (exprimée / hr : valeur de jour – valeur de nuit) dans les différents groupes. Les souris sans horloge centrale (*SCNx)* conservent un cycle journalier veille sommeil grâce aux effets directs de la lumière/obscurité médiés par la mélanopsine. (**d**) Modèle représentant les différentes voies par lesquelles le cycle quotidien de lumière/obscurité module l'organisation temporelle de 24 heures du cycle veille sommeil. Statistiques, voir chapitre 5.

Nous avons ensuite confirmé ces données chez des souris *Synaptotagmin10*$^{Cre/Cre}$, *Bmal1* $^{fl/-}$. Les résultats préliminaires montrent une distribution veille sommeil qui reste synchronisée avec le cycle de lumière obscurité chez ces souris sans pacemaker circadien mais avec des NSCs intacts (**Figure 0.3**).

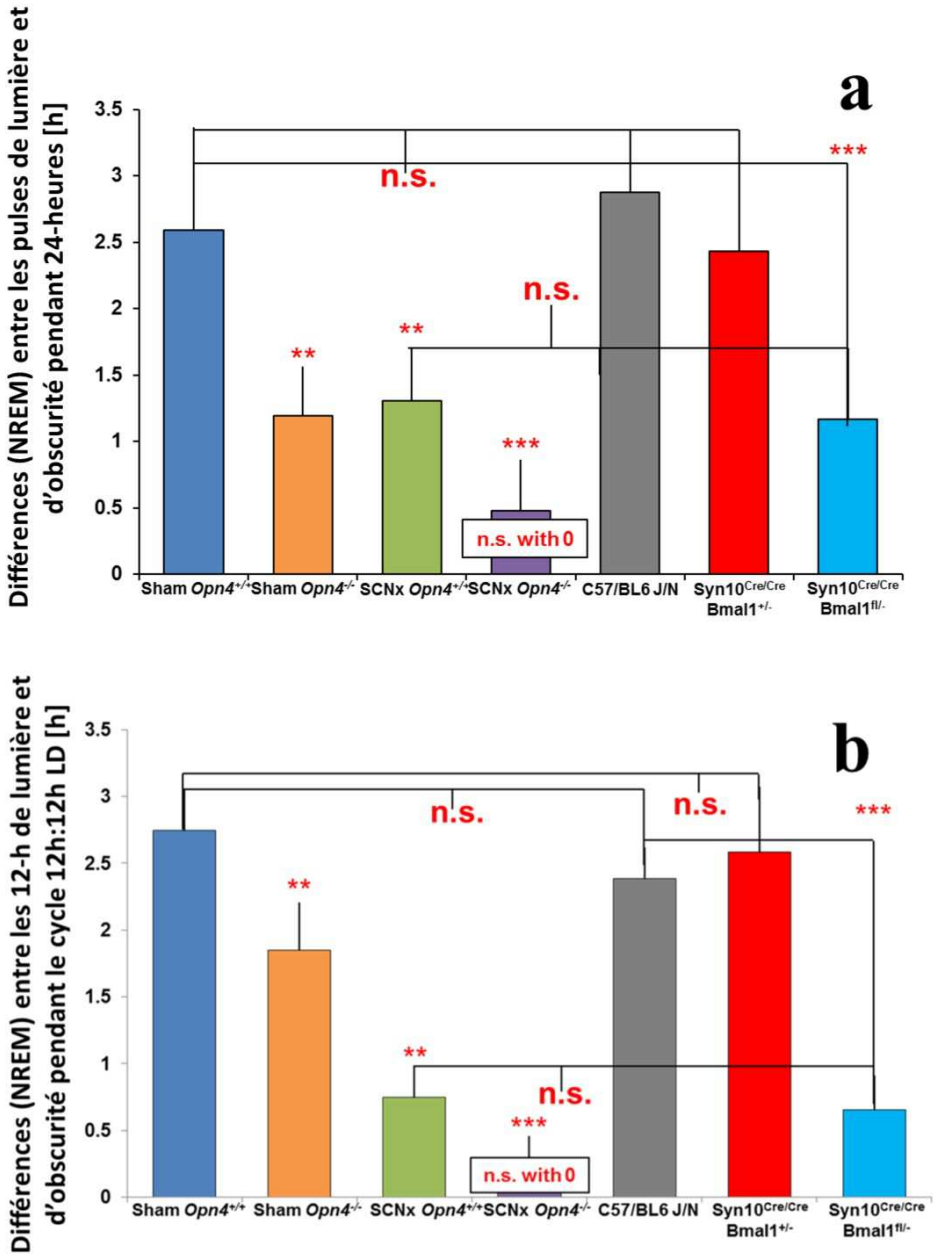

Figure 0.3- Quantification des effets directs de la lumière sur le sommeil en l'absence de mélanopsine, de noyaux suprachiasmatiques ou d'horloge circadienne.

Temps de sommeil lent : différences entre les périodes de lumière et d'obscurité lors d'un cycle ultradien 1h:1h (a) et standard 12h:12h (b), chez des souris Opn4$^{-/-}$ avec ou sans lésion des NSCs et Syn10$^{cre/cre}$Bmal$^{fl/-}$ et leurs contrôles.

(**a**) L'effet d'un cycle ultradien de lumière obscurité sur les taux de sommeil est significativement diminué (environ de moitié) en l'absence de mélanopsine (*Opn4*$^{-/-}$) ou de

NSCs (SCNx) et est aboli en l'absence des deux (*SCNx Opn4*$^{-/-}$). Il est également diminué significativement et dans les mêmes proportions, de moitié, en l'absence d'horloge circadienne, mais avec conservation des NSCs (*Syn10*$^{cre/cre}$ *Bmal1*$^{fl/-}$).

(**b**) Lors d'un cycle habituel de lumière obscurité de 12hL:12hD, l'amplitude du cycle veille sommeil est significativement diminuée chez des souris sans mélanopsine (*Opn4*$^{-/-}$) ou sans NSCs (*SCNx*) et en l'absence des deux aucun cycle veille sommeil ne peut être observé. Le cycle veille sommeil est significativement atténué chez des animaux transgéniques sans horloge circadienne, dans des proportions comparables à celles observées en l'absence de NSCs.

Dans les deux conditions (a et b), les effets du cycle de lumière obscurité n'étaient pas affectés chez les souris contrôles *Syn10*$^{cre/cre}$*Bmal*$^{+/-}$ (animaux rythmiques) en comparaison à des souris sauvages.

La cohérence des résultats obtenus par les deux approches complémentaires, lésionnelle et transgénique, conforte nos résultats et nous permets de conclure que la régulation photique directe du sommeil médiée par la mélanopsine contribue pour un tiers à l'organisation journalière de la veille et du sommeil, constituant ainsi un mécanisme majeur de régulation.

2 éme volet) validation *d'arvicanthis ansorgei* comme modèle diurne d'étude du sommeil et conservation des effets directs de la lumière sur le sommeil chez le rongeur diurne (Hubbard et al, en préparation)

Aujourd'hui notre compréhension de la neurobiologie du sommeil résulte essentiellement de données obtenues chez la souris et le rat, c'est-à-dire chez des rongeurs nocturnes. Pourtant, l'organisation circadienne de la veille et du sommeil de ces animaux est inversée par rapport à celle de l'homme. Contrairement au rythme circadien d'activité locomotrice, le sommeil des rongeurs diurnes n'a jamais été spécifiquement étudié. L'analyse de l'EEG, EMG, EOG et de la vidéo a permis de caractériser les états de vigilance de ces animaux.

Les résultats montrent que les caractéristiques de l'éveil, du sommeil lent et du sommeil paradoxal sont similaires à celles décrites chez les rongeurs nocturnes; en particulier les rythmes EEG (puissance spectrale) (delta-sommeil lent ; Thêta - éveil et sommeil paradoxal). L'organisation circadienne est inversée par rapport à celle des rongeurs nocturnes et la régulation homéostasique du sommeil est conservée puisqu'un rebond de sommeil lent et de puissance EEG delta est observé en fonction de la durée de la privation de sommeil. L'analyse du rythme ultradien de lumière et obscurité de 1h:1h montre que les effets directs non-circadiens de la lumière sont d'amplitude similaire mais inversés par rapport à ceux observés chez la souris (**Figure 0.4**).

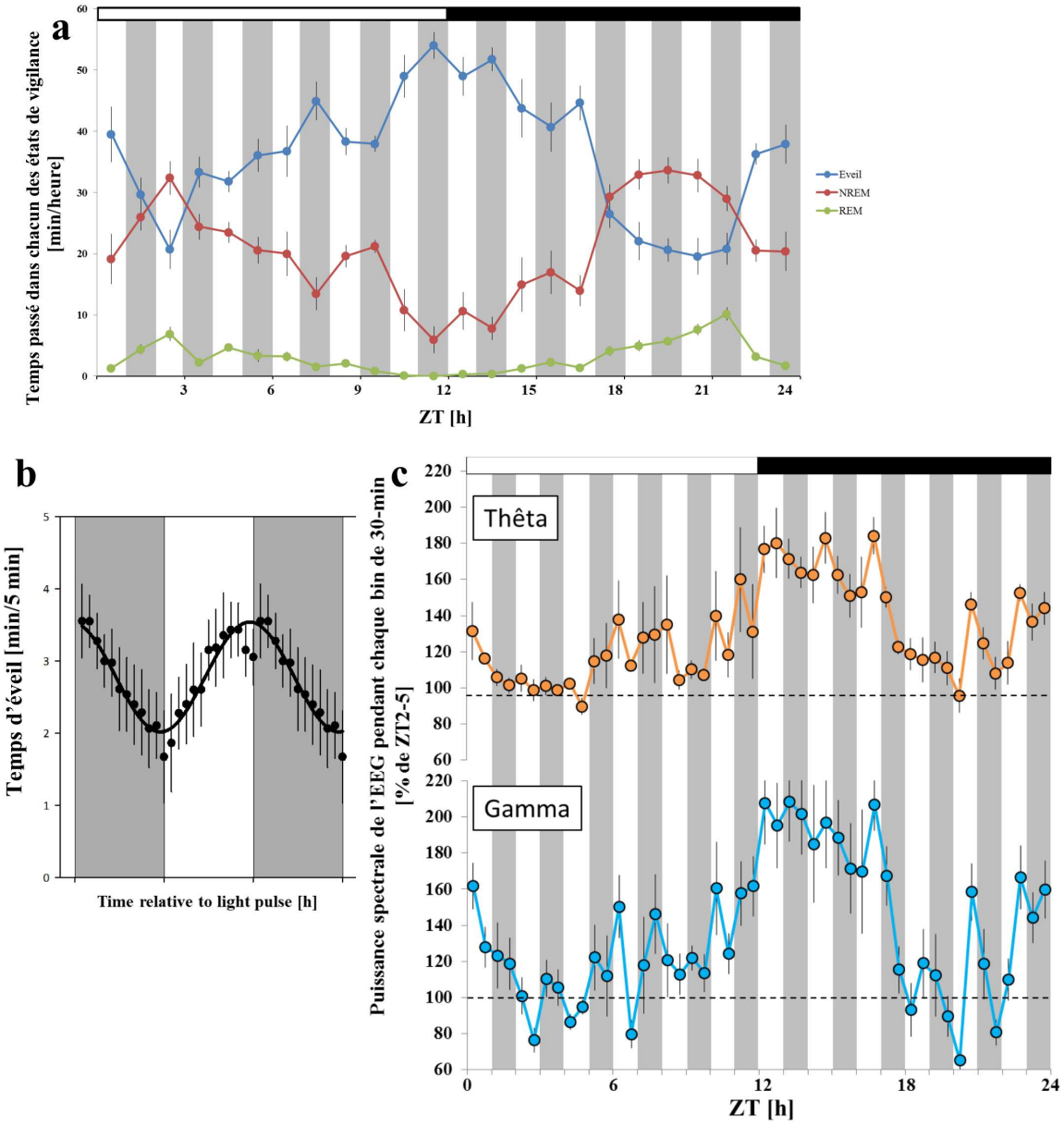

Figure 0.4- Effet éveillant de la lumière lors d'un cycle ultradien de lumière obscurité (1h :1h) administré pendant 24 heures.

(**a**) Temps passé dans chacun des états de vigilance (éveil, sommeil lent ou NREM et sommeil paradoxal ou REM) lors d'un cycle ultradien 1h:1h de lumière obscurité, mesurée par le nombre de minutes par heure (moyenne +/- s.e.m). (**b**) Cinétique des changements induits par le cycle ultradien de lumière obscurité. Les valeurs représentent le temps passé éveillé par intervalles de 5 min pendant l'heure précédent, suivant et les 12 pulses de lumière administrés. (**c**) Effets du cycle ultradien de lumière sur les puissances spectrales EEG thêta et gamma, corrélats électrophysiologiques de la vigilance et du fonctionnement cognitif. Noter l'augmentation de l'activité EEG thêta et gamma durant la seconde moitié des pulses de lumière administrés pendant la période d'obscurité subjective.

La régulation photique directe du sommeil est donc conservée chez le rongeur diurne mais inversée puisque la lumière exerce un effet éveillant comme cela est observé chez l'homme. Cette première caractérisation du sommeil d'un rongeur diurne et la validation d'Arvicanthis ansorgei comme modèle d'étude du sommeil ouvre des perspectives très importantes dans une démarche de recherche translationnelle, et à long terme pour comprendre les mécanismes de la diurnalité-nocturnalité.

3ème volet) Démontrer que la lumière influence l'homeostat de sommeil via la mélanopsine chez le rongeur diurne et nocturne (Hubbard et al., en préparation)

La régulation homéostasique du sommeil est atténuée de 50% chez des souris sans mélanopsine suggérant que ce photopigment puisse être un facteur homéostasique de sommeil. Ce résultat original nécessite d'être formellement établi. Pour ceci, nous avons réalisé des privations de sommeil des différentes durée (1.5hr, 3h, 6h, 9h ; courbe dose réponse) et dans différentes conditions d'exposition lumineuse pour évaluer l'influence de la lumière sur l'homéostat de sommeil. Ces expériences ont été réalisées chez des animaux avec ou sans mélanopsine et chez Arvicanthis Ansorgei. Les principales variables analysées ont été le rebond de sommeil et l'activité EEG delta (0.75-4 Hz) qui constitue le marqueur le plus fiable de la pression homéostatique de sommeil. L'augmentation d'activité EEG delta en réponse à une privation de sommeil est significativement diminuée en l'absence de mélanopsine et la différence de réponse entre les souris sauvages et Opn4$^{-/-}$ est proportionnelle à la durée de la privation de sommeil (**Figure 0.5**).

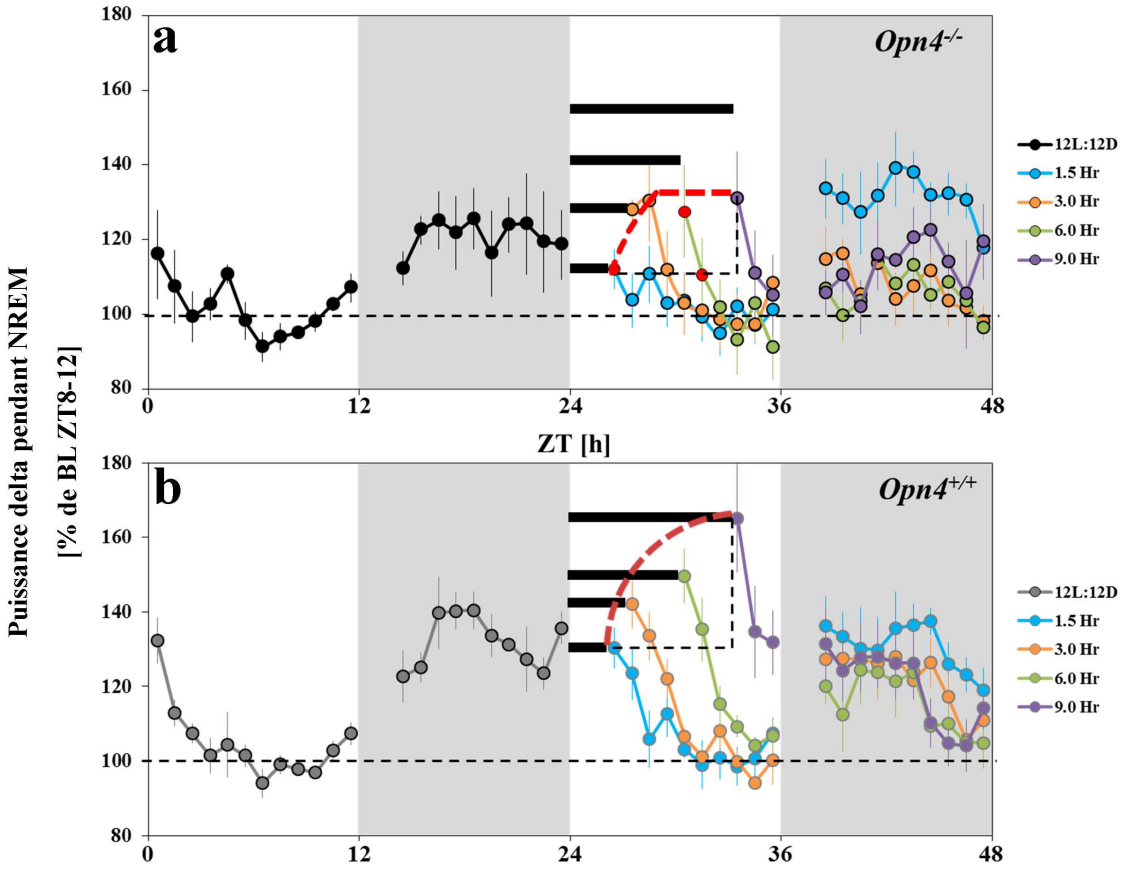

Figure 0.5: Activité EEG delta en sommeil lent chez les souris Opn4$^{+/+}$ and Opn4$^{-/-}$ en réponse à des privations sommeil de durée variable

a) Le pic de puissance EEG delta observé après privation de sommeil est significativement et sévèrement diminué en l'absence de mélanopsine (*Opn4*$^{-/-}$). (**b**). La puissance EEG delta augmente proportionnellement au temps passé éveillé chez les souris sauvages (*Opn4*$^{+/+}$) (courbe dose réponse). (La courbe dose réponse n'est plus observée et les résultats suggèrent une saturation du processus homéostasique puisque le pic d'activité EEG delta n'augmente plus lorsque la privation de sommeil est plus longue que 3 heures. La puissance delta est mesurée en tant que valeur normalisée par rapport à la période de référence, période pendant laquelle le besoin de sommeil au cours du nycthémère est le plus faible, (ZT8-12).

L'analyse des différentes conditions expérimentales est en cours et permettra d'établir une preuve de concept du lien entre la lumière, la mélanopsine et l'homéostasie du sommeil apportant ainsi la première démonstration d'un lien biologique entre la lumière et l'homéostasie du sommeil.

Perspectives

Ce travail a permis de démontrer que la mélanopsine et les effets directs non-circadiens de la lumière représentent un mécanisme majeur de régulation du sommeil chez les espèces diurnes et nocturnes. Ces données vont encourager de nouvelles voies de recherche pour réévaluer le rôle de la lumière sur la physiologie des mammifères et en particulier chez l'homme. Elles remettent en question le modèle de régulation du sommeil à 2-processus, nous incitant à proposer un nouveau modèle incluant une troisième composante. Nous pourrons ainsi proposer un nouveau cadre conceptuel de régulation du sommeil indispensable à une meilleure compréhension de la physiopathologie des troubles du sommeil afin de pouvoir développer à long terme de nouvelles stratégies thérapeutiques.

Glossary of Terms

3V	third ventricle
5HT	**s**erotonin
μV^2	microvolt-squared
aDTA	ablated diphtheria toxin A
AVP	arginine vasopressin
BMAL1	brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like
BSL	baseline
CA1-CA3	cornu ammonis area 1 and 3
CLOCK	circadian locomotor output cycles kaput
CNGA3	cyclic nucleotide-gated channel alpha 3
CRY1-2	cryptochrome 1 and 2
CTBCtB	cholera toxin subunit B
D	dark
DAPI	4',6-diamidino-2-phenylindole
DD	constant darkness or Dark-Dark
DFT	discrete Fourier transformation
DGL	**d**orsal geniculate leaflet
DP	dark pulse
ECoG	electrocorticogram
EEG	electroencephalogram (used interchangeably when discussing ECoG in animals)
EMG	electromyogram
EOG	electrooculogram
FFT	fast Fourier transform
GABA	gamma-aminobutyric acid

GNAT1	guanine nucleotide-binding protein subunit alpha-1
HIS	histamine
Hz	hertz
IGL	intergeniculate leaflet
IPRGC	intrinsically photosensitive retinal ganglion cells
KO	knock-out
L	light
L-CONE	long-cone
LC	locus coeruleus
LD	light-dark
LH	lateral hypothalamus
LP	light pulse
M-CONE	medium-cone
N3	NREM sleep stage 3
NA	noradrenaline
NIF	non-image forming
NREM	non-rapid eye movement sleep
OC	optic chiasm
OPN4	**o**psin 4 or melanopsin
OPT	olivary pretectal nucleus
PER1-2	period 1 and 2
PET	positron emission tomography
PGO	ponto-geniculo-occipital
PPT	pedunculopontine tegmentum
PRF	pontine reticular formation
PROCESS C	circadian process of sleep regulation
PROCESS S	homeostatic process of sleep regulation

PS	paradoxical sleep (REM)
rd/rd cl	rodless/coneless
REC	recovery following sleep deprivation
REM	rapid eye movement sleep
RHT	retinohypothalamic tract
S-CONE	short-cone
SAD	**s**easonal affective disorder
SC	superior colliculus
SCG	superior cervical ganglion
SCN	suprachiasmatic nucleus
SCNx	suprachiasmatic nuclei lesion
SD	sleep deprivation
SPVZ	subparaventricular zone
SWS	slow-wave sleep
SYN10	synaptotagmin-10
TMN	tuberomammillary nucleus
VGL	ventral geniculate leaflet
VLPO	ventrolateral preoptic area
WT	wild-type
ZT	zeitgeiber-time

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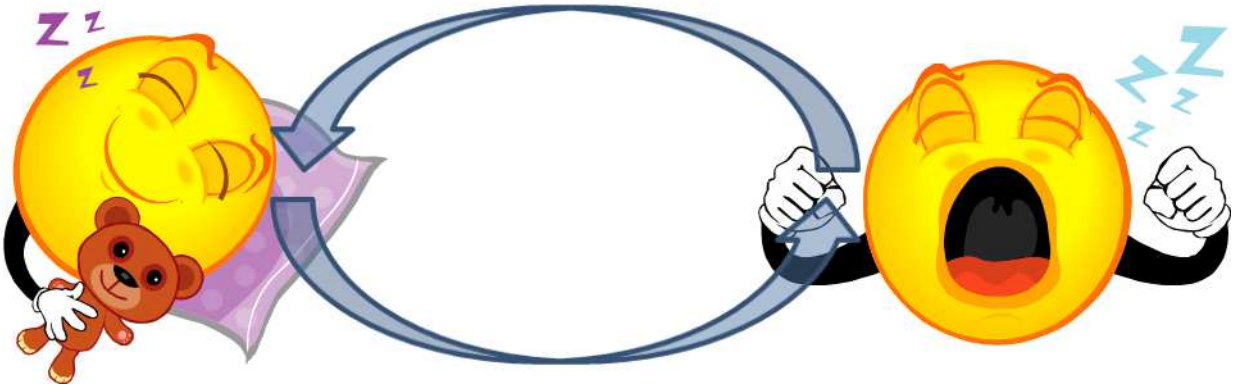
Part I: Theoretical Background



Chapter 1- Sleep

1.1 - Introduction

1.1.1 Background

Sleep is an ever present process in the existence of most organisms, occupying a significant portion of their lives. In humans, sleep accounts for a large amount of the 24-hour period; the average adult will spend approximately one third of their time sleeping. From the smallest organisms, such as the *Caenorhabditis Elegans,* a nematode smaller than a centimeter (Dabbish and Raizen 2011; Schwarz, Lewandrowski et al. 2011), that shows clear periods of metabolic resting, to the blue whale, the largest animal on the planet, who can rest only one brain hemisphere at a time while keeping the other half conscious, and every organism in between; all of them sleep or rest. Some animals have functional adaptations, such as the horse which sleeps standing up, or certain insects which retract their antennae in order to reduce the amount of external stimuli they experience. In essence, sleep and resting, though incredibly varied amongst many animal species, always serves the same purpose, a restoration and regulation of the animal's internal processes so that they may better interpret and adapt to their surrounding environment when they are awake.

1.1.2 A brief history of sleep research

Since the advent of the human species it's likely that people were aware of the phenomenon of sleep, the idea that at some period during the day fatigue would set in and the body would become unconscious. As early as the Classical period in Greece, philosophers were engaged in rhetoric and discussion about the topic. Around 350 BCE, Aristotle proselytized that sleep was "an inhibition of sense perception", removing the person from their interaction with the physical world. However, it was not until the 20th century that the idea of researching and observing sleep phenomena became a subject for scientific inquiry. The first major contribution came in 1913 from French psychologist Henri Pieron who with his text, "Le Probleme Physiologique Du Sommeil" described the physiological aspects of sleep he had seen in his patients. In 1929 the most important scientific breakthrough for studying sleep was invented when German psychiatrist Hans Berger attached the first electrodes to a human scalp. Though these initial

electroencephalographic (EEG) recordings were crude by today's standards, differences between waveforms were easily identifiable (**Fig. 1.1**) (Berger 1929). This was followed by Loomis and colleagues who in 1937 noticed significant differences between waking and sleeping brains (Davis, Davis et al. 1937), and soon after the development of new tools for measurement. By the 1930s and 40s, scientists had begun experimenting in cats, with not only EEG recording but artificial induction of sleep through electrical stimulation of the reticular formation (Moruzzi and Magoun 1949). During this time Nathaniel Kleitman, a physiologist at the University of Chicago, began investigating biological rhythms and sleep deprivation, giving birth to an entirely new research field.

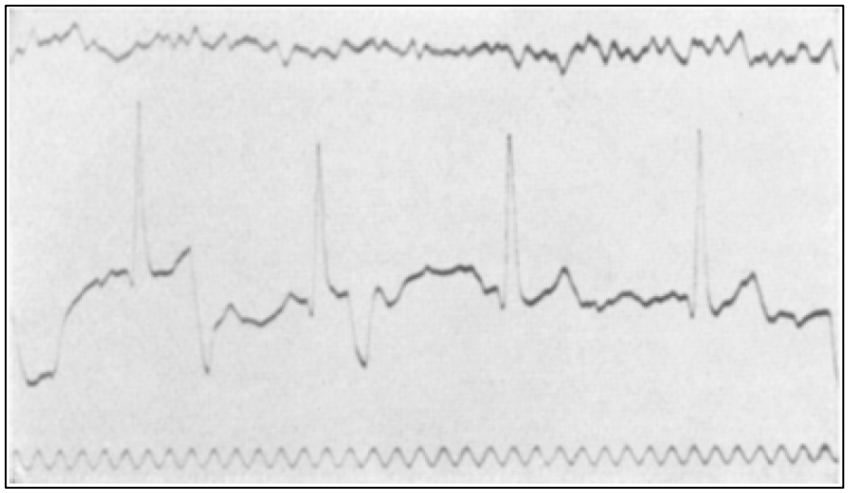


Figure 1.1: An early EEG recording from Hans Berger (Berger 1929)

With the establishment of sleep specific brain activity across multiple mammalian species, the next step was to understand how the sleep-wake cycle was generated and regulated. Soon after, rapid-eye movement (REM) sleep was discovered when a student of Kleitman, Eugene Aserinksy, passed hours observing subjects and began noticing oscillating eye movements constrained to certain times during their sleeping period (Aserinsky and Kleitman 1953). Along with another student, William Dement, REM sleep was defined. Several years later Jouvet, a researcher in Lyon, began studying sleep in cats using EEG and noticed these "REM periods" characterized by a desynchronized EEG, more similar to waking in contrast to normal sleep EEG, when the animal was behaviorally sleeping and termed them "paradoxical" (Jouvet and Jouvet 1963; Jouvet, Jouvet et al.

1963; Mouret, Jeannerod et al. 1963). Subsequently, Dement and colleagues identified the relationship between REM sleep and dreaming, giving rise to a greater understanding of both. In 1962, more extreme research into biological rhythms began, starting with Siffre who lived underground without light to study its effects (Siffre 1963), and continued with Aschoff and Wever, who observed research subjects placed in sealed chambers (Aschoff, Gerecke et al. 1967). In the 1970s the discovery of the suprachiasmatic nucleus, a central biological clock located in the hypothalamus led to the study of internal biological rhythms (Moore and Eichler 1972).

In subsequent years, a variety of sleep problems would be discovered and examined, starting with Gastaut, who first identified sleep apneas (Gastaut, Tassinari et al. 1965). Numerous disorders were identified from the extremely rare, such as Fatal Familial Insomnia in 1986, a disease linked with genetic factors (Lugaresi, Medori et al. 1986; Medori, Tritschler et al. 1992), to less rare (narcolepsy), or highly frequent, such as restless leg syndrome or the sleep respiratory syndromes. For example, narcolepsy, first observed and studied in the 19th century, was not fully explained until the discovery of hypocretin in 1998 (de Lecea, Kilduff et al. 1998), changing interest towards the examination of the underlying mechanisms of this disease. In the 21st century, research in sleep continues to grow exponentially, achieving a higher place within the domain of neuroscience, and involving a variety of other scientific disciplines including but not limited to, genetics, molecular biology, internal medicine, pulmonology, neurology, and psychiatry. Currently there are thousands of scientists in hundreds of clinical treatment and research centers devoted exclusively to the study of sleep, and the field continues to grow.

1.2 - The EEG

1.2.1 The EEG

Electroencephalography or EEG is the method for assessing electrical activity produced in the brain in human beings, which is achieved by placing electrodes on the surface of the scalp in specific configurations. The signal itself is a by-product of communication between pyramidal cells, producing electrical activity which can then be amplified and analyzed. As millions of consecutive cells are aligned in the same direction, a signal propagation wave is produced, eventually reaching the scalp where the metal contained within the electrodes, usually Au or AgCl, will push or pull the ions and generate a voltage signal. Electrical signals coming from the electrode are then transmitted to an amplifier which will allow for signal analysis and facilitate its interpretation. Oscillatory neural activity exists between thalamus and cortex, wherein the thalamus gates information to the cortex as well as providing reciprocal feedback. This transfer of information between thalamic neurons and pyramidal cells in the cortex creates the thalamocortical loop and is the basis for the propagating electrical activity recorded by the EEG. This will be altered depending on the wake or sleep state of the brain. A schema of this interaction can be seen in **Fig. 1.2**.

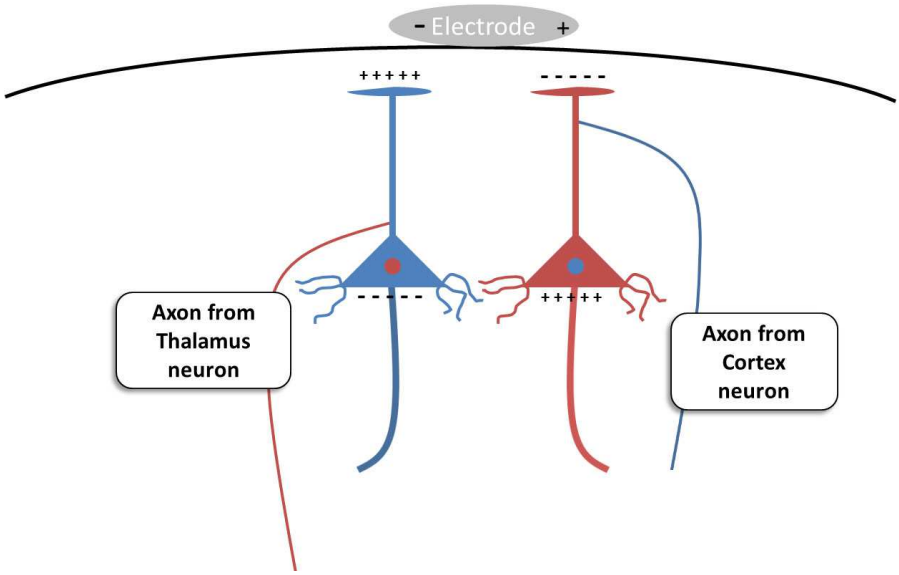


Figure 1.2: Schema of the thalamocortical loop

The signal which is seen by the observer is normally a subtraction between the surface electrode and a reference area of the body (in humans the contralateral mastoid process, for example). The EEG is an incredibly important device for evaluating a variety of neurological disorders. EEGs are vital to the field of epileptology where they are used to identify seizures, in conjunction with neuroimaging, and neuropsychological assessment, to determine the best course of action for a patient. However, in regards to the subject of this dissertation it is the use of EEG to examine sleep and wake that is most pertinent.

1.2.2 EEG as a tool for sleep

As previously stated, a single EEG is a sum of the depolarization of each cell, measured in voltage. However, these signals can be radically different, and have therefore been categorized into different subgroups to represent diverse stages of sleep and alertness. Categories are determined by the amplitude, or height of the signal, and the frequency at which it repeats. Each waking or sleep stage has specific features in the EEG which under optimal circumstances make identification relatively simple.

Sleep stages in human beings, as defined by the American Academy of Sleep Medicine, consist of wake, NREM stage 1-3, and REM (paradoxical) sleep. Though NREM stage 1-3 are all generally described as non-rapid eye movement sleep, changes within these stages on the EEG can be quite striking. An EEG of a person who is awake will show desynchronized waveforms. However, this stage is still dominated by specific frequencies, principally alpha (8-12 Hz) and beta (13-30 Hz). As a person becomes drowsy they enter into the first stage of NREM sleep, which can also be thought of as a transitional stage between conscious and unconscious states. This stage is classified based upon the continued presence of alpha waves and inclusion of theta (4-7 Hz). This is followed by stage 2 sleep, defined by the appearance of unique waveforms, known as spindles and k-complexes. Their function is still unknown yet possible functions have been suggested (Tononi and Cirelli 2006; Cash, Halgren et al. 2009), and it is still unclear as to whether they exist in other animals which display similar sleep patterns to human beings. In addition, the functional significance of sleep spindles also remains unclear though some have suggested a relationship with cognition. K-complexes, which are large negative

spikes in EEG activity, are topographically different, and normally follow a group of sleep spindles.

The final stage of NREM sleep is called stage 3, or slow-wave-sleep, due to the presence of low frequency delta waves (0.5-4 Hz) which are highly synchronized. This type of sleep is the primary component of the restorative part of the sleep/wake cycle and will be discussed further in section **1.3.3**.

In contrast to NREM, REM sleep is characterized by a desynchronized EEG, more similar to waking, though with complete muscle atonia and rapid eye movements. During this stage of sleep, rapid low-voltage, desynchronized EEG activity is evident, as well as activation in region-specific brain areas such as the visual cortex and those important to memory such as the hippocampus, evidenced by the propensity of theta and gamma oscillations.

1.3 - The Two-Process Model of Sleep Regulation

1.3.1 Two separate but connected processes

The regulation of the sleep and wake cycle is classically thought to be under the control of 2 independent mechanisms: homeostatic (process S) and circadian (process C). Process S can be described as an increase in the pressure to sleep as a function of time spent awake. The circadian process describes a regulatory mechanism under the control of a central clock, which lasts approximately 24-hours. The interaction of these two processes determines the timing and quality of sleep and alertness. First described in 1982 by Borbély, this model has driven both the clinical and fundamental sleep research fields for the past three decades (Borbely 1982). **Fig. 1.3** summarizes the two models.

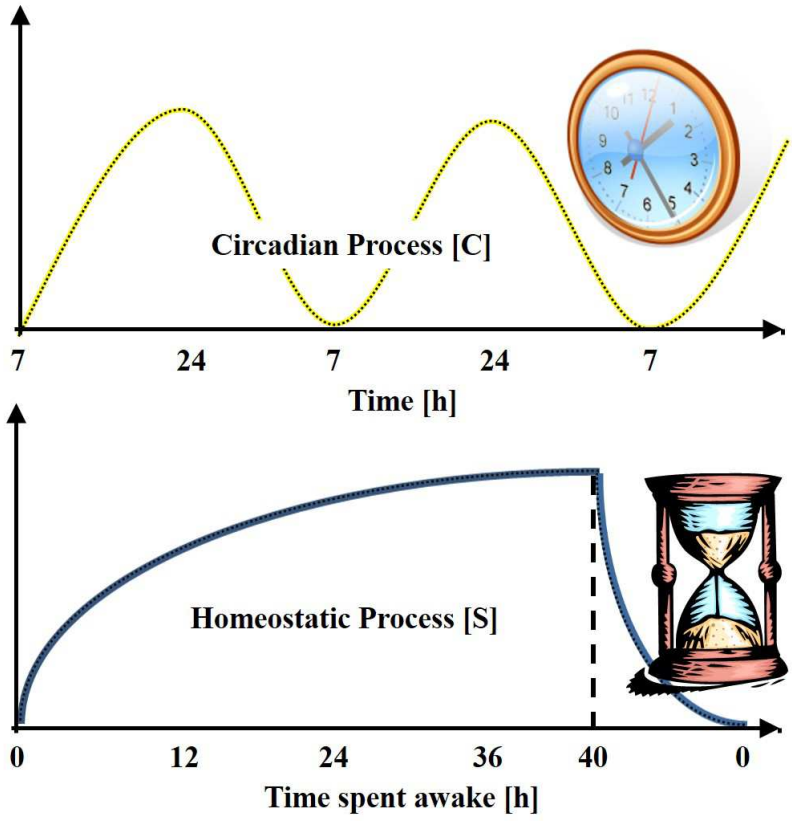


Figure 1.3: The two-process model of sleep and wake regulation

1.3.2 The circadian process

The term “circadian”, is derived from two Latin words: *circa*, meaning “approximately”, and *dies*, or “day”. The adaptation to this 24-clock is consistent among nearly all plant and animal life. In mammals this is under the direct regulation of the suprachiasmatic nucleus, controlled principally by light; synchronizing the expression of the clock, based on a feed-back loop controlling the 24-hour expression of clock genes. These markers were shown to exist in a variety of species including plants, drosophila, and mammals, leading to extensive characterization of the system in laboratory animals (Vitaterna, King et al. 1994). The timing of the clock is a result of the regulation of specific proteins: *CRY1-2*, *CLOCK*, *BMAL1*, *PER1-2*, and others.

There is strong evidence supporting the claim that the SCN is the central biological clock, regulating the sleep and wake cycle, at least in terms of 24-hour distribution under light and darkness, coming from studies where the effect of removing these nuclei through electrolytic lesion, was examined. These results indicated that complete removal of this structure caused the animal to completely desynchronize its circadian rhythm, with sleep and waking more evenly distributed across the 24-hour period regardless of the amount of light (Moore and Eichler 1972; Ibuka and Kawamura 1975).

Additionally, light via the SCN can coordinate the release of melatonin, a hormone produced by the pineal gland, critical for the timing of sleep. Melatonin exerts a strong hypnotic effect and synchronizes the clock through the SCN. When the SCN is activated by light, neurons send information to the preautonomic neurons of the paraventricular nucleus (PVN), the sympathetic preganglionic neurons in the spinal cord, and noradrenergic neurons in the superior cervical ganglia (SCG), located in the lateral horn of the spinal cord, in turn projecting to the pineal gland (**Fig. 1.4**). A decrease in the amount of light promotes the production of the hormone, whereas exposure will suppress it.

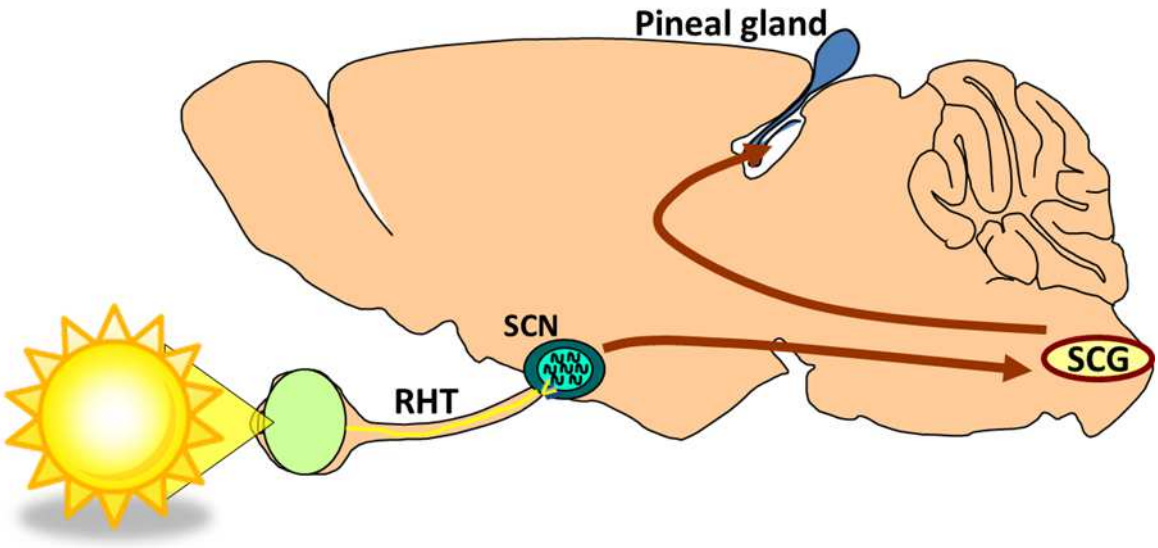


Figure 1.4: SCN control of the pineal gland and secretion of melatonin in rodents

1.3.3 The homeostatic mechanism of sleep regulation

The homeostatic regulation of sleep describes the increase in sleep pressure as a function of time spent awake, and the release of this pressure with sleep onset. Studies to examine this process involves sleep deprivation protocols In human beings and other diurnal mammals, this means keeping them awake during the night, contrasted with most laboratory animals which are nocturnal thus requiring a deprivation during the day. In human beings the threshold for deprivation without the use of any chemical stimulants is likely around 250 hours, though experiments examining this were not well-controlled and performed several decades ago. Laboratory rats have been shown to expire within 32 days of starting a sleep deprivation (Everson, Bergmann et al. 1989).

EEG observation of the homeostatic process can be seen in the increased propensity of delta wave activity (0.5-4 Hz) during NREM sleep. Recent evidence has indicated a genetic component may contribute in some part to the changes observed (Franken, Chollet et al. 2001).

1.3.4 Mathematical modeling of the 2-process model

In order to remove some of the abstraction of the theory, several attempts have been made to mathematically quantify the 2-process model. In 1999, a review by Borbély and Achermann, put forth several equations to describe these two phenomena, illustrated below in **Fig. 1.5**.

Process S

$$S_t = \begin{Bmatrix} d\,S_{t-1}\,;d = e^{\frac{-\Delta t}{\tau_d}}\,(sleep) \\ 1 - r(1 - S_{t-1});r = e^{\frac{-\Delta t}{\tau_r}}\,(wake) \end{Bmatrix}$$

Process C

$$C = A\begin{Bmatrix} 0.97\sin\left[\omega\,(t-t_0)\right] + 0.22\sin\left[2\omega\,(t-t_0)\right] + 0.07\sin\left[3\omega\,(t-t_0)\right] \\ +0.03\sin\left[4\omega\,(t-t_0)\right] + 0.001\sin\left[5\omega\,(t-t_0)\right] \end{Bmatrix}$$

$$\omega = \frac{2\pi}{\tau}$$

Figure 1.5: Proposed mathematical equations to quantify the 2-process model from (Borbely and Achermann 1999)

S: homeostatic process, increasing during waking, decreasing during sleep; d: decay factor of S; r: rise factor of S; td, tr: time constants; Dt: time step. Note: S is described by a recursive formulation (iteration); time steps indicated by indices t, t – 1. C: circadian process independent of sleep and waking A: amplitude of skewed sine wave (sign determines direction of skewing); t: time; t: period of C; t0: defines the circadian phase at the beginning of the simulation.

1.4 - Sleep in humans

1.4.1 Background

Sleep in humans is present during the resting part of the 24-hour cycle and consists of several cycles, each comprised of several different sleep stages (as discussed before in section 1.2.2) identifiable upon visual inspection of the EEG. Each sleep cycle persists for around 90 minutes containing both NREM (Stage 1-3) and REM components. NREM stages 1-3 are named in accordance to their profundity, with stage 1 the shallowest and therefore having electrical activity of a higher frequency, and stage 3, the deepest sleep and slowest wave frequency, or higher EEG synchronization. REM sleep is dominated by high frequency waves of low amplitude and resembles a waking state, yet with complete muscle atonia. The overall architecture during a night of sleep is dominated by slow-wave sleep (stage N3) at the beginning, with REM consolidated closer to the end. A normal night of sleep will consist of around five cycles though this number can increase or decrease depending on the length of time spent asleep.

1.4.2 Sleep distribution

Sleep stages do not always progress directly from stage 1-3 though, normally in order to reach the deepest levels of sleep, the person will pass from wake, to stage 1, 2, etc. In most cases deeper sleep will be consolidated to the beginning of the night, with bouts of REM and an increase in light sleep nearer to the end of the sleep period. When examining sleep, distribution is shown as a hypnogram, a graphic representation of the underlining periods of transition from one stage to another across the night. A generalized hypnogram is presented below of a normal night of sleep in a healthy adult (**Fig. 1.6**)

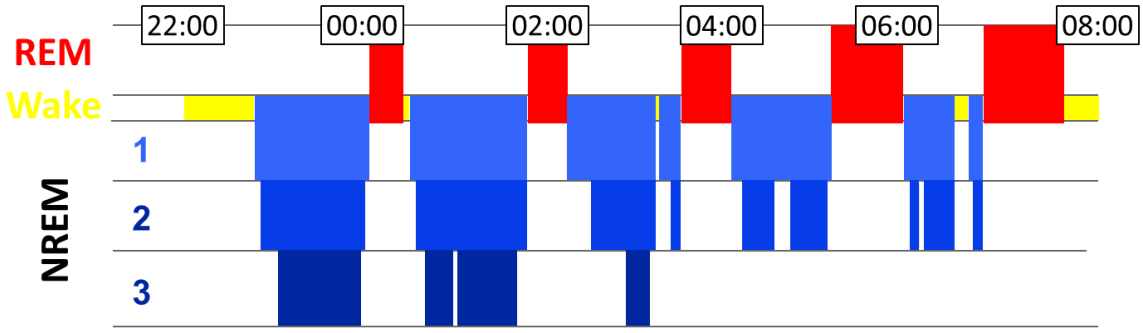


Figure 1.6: Hypnogram of healthy adult subject (NREM stages are listed in varying shades of blue)

1.4.3 Power spectrum analysis

Sleep can be scored directly using standardized criteria to determine each sleep stage, however to truly parse apart the signal and look at the composition of the EEG, a Fourier transformation is normally used. Applying this equation (see **Fig. 1.7**) to the EEG allows one to perform a power analysis which can isolate for example in 0.25 Hz groups, the contribution of each frequency to the overall signal. A Fourier transform takes x(t) (time) as a sum (represented by an integral) of an infinite number of waves ($e^{j2\pi ft}$. In essence it is the transformation of a "time-domain" into a "frequency domain", creating sinusoids for each 0.25 Hz frequency.

$$x(t) = a_0 + \sum_{k=1}^{\infty}\left(a_k \cos\left(2\pi k f_0 t\right) + b_k \sin\left(2\pi k f_0 t\right)\right)$$

Figure 1.7: Continuous and periodic EEG signal calculation

The amplitude of the resulting wave is called the "power spectrum", and expressed as (μV^2/0.25 Hz), for example. Once this calculation is completed, sleep can be assessed according the percentage of each Hz of the total power of the signal. This allows direct observation of the spectral components of each sleep stage. For example, SWS during stage 3 would be dominated by delta activity. **Fig. 1.8** shows a clinical example with EEG signals coming from the central and frontal areas of a patient, producing a similar wave profile during stage 3, for the frequency range 0.25-25 Hz.

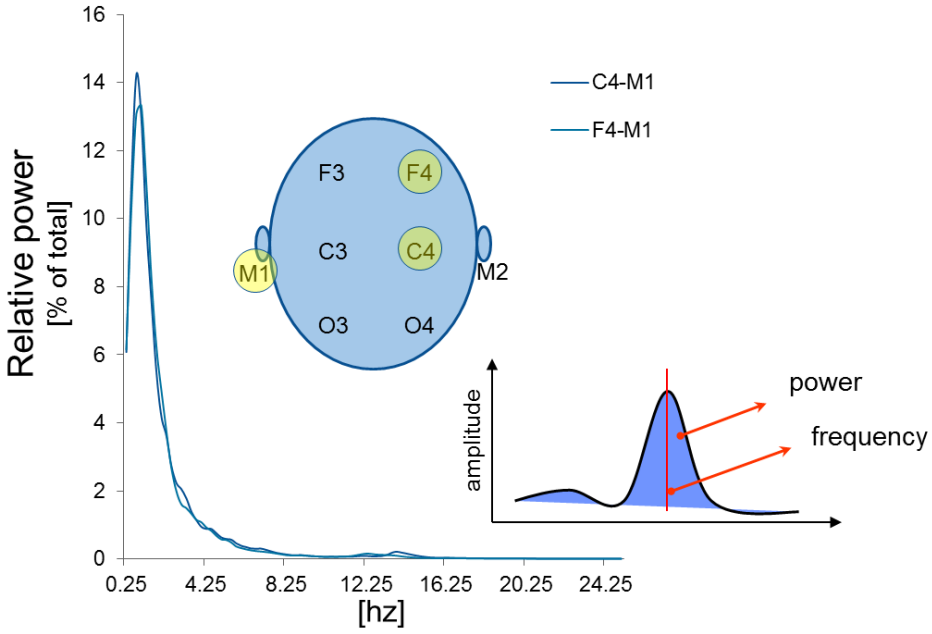


Figure 1.8: Example of the power spectrum of a clinical subject during N3 (personal data)

1.5 - Sleep in rodents

1.5.1 Background

Though sleep in humans has been extensively studied in both a clinical and fundamental context, a large number of scientific projects are still performed at the basic level in laboratory rodents. Although only considered a valid model for study after the confirmation of the sleep and wake mechanisms in other mammals such as cats and rabbits and monkeys, most research today is performed with laboratory rodents. As with other research domains, the use of rodents, and specifically mice, became increasingly important due to the availability of transgenic models. Currently, a large number of labs perform research in sleep solely on rodents.

1.5.2 An appropriate model to study sleep?

If you were to observe a mouse sleeping, several things would be noticeable immediately. Mice sleep mostly during the day because they are nocturnal, and secondly that it does not sleep for an extended period of time. Taking a 24-hour snapshot of its sleep/wake cycle in the form of a hypnogram would look like the example below (**Fig. 1.9**), with vigilance states simply split into wake, slow-wave sleep (SWS) and REM. Sleep cycles in rodents tend to be extremely brief, usually only lasting a few minutes, before reawakening.

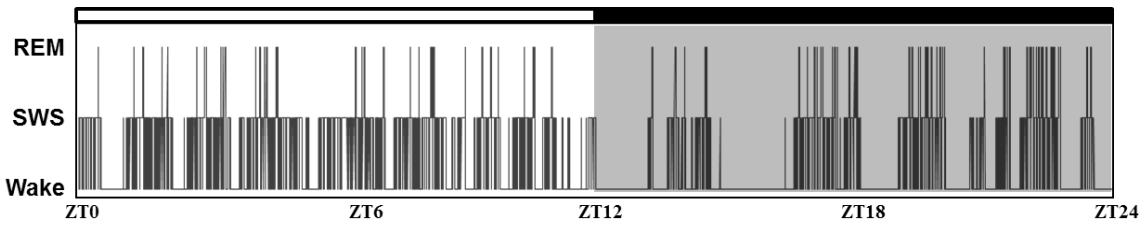


Figure 1.9: Hypnogram for 24-hours in a normal mouse (personal data)

1.5.3 Recording and scoring sleep in rodents

Quantitative sleep studies in rodents operate more or less on the same principles as human beings. However, the electrode implantation is less extensive and more invasive. Unlike in humans, EEGs in mice are implanted underneath the skull and directly on to the cortex and thus these signals are referred to as electrocorticograms (ECoG). This creates a different signal as the electrical output of the brain is not diffused by any bone matter. Standard implantation to record sleep in mice is a 5-channel chip attached to the head using dental cement with 3 ECoGs, and two EMGs placed in the muscles of the neck to record body movement, and in some experiments the recording of ocular movements (EOG). Throughout this thesis ECoG will be referred to as EEG.

Scoring sleep in rodents is based on specific changes in the EEG signal, in terms of dominant frequency, as well as differences in the EMG, or EOG. The scoring window is generally lower than in human beings, though a smaller window increases the accuracy of the power spectrum calculation (Franken, Malafosse et al. 1998).

Wake or vigilance in mice is dominated by theta (6-10 Hz) and gamma (40-70 Hz) ECoG activities, ECoG correlates of cognition and alertness, respectively, which originate in the CA1-CA3 cell layers of the hippocampus (Chrobak and Buzsaki 1998; Montgomery, Sirota et al. 2008). SWS (NREM) represents the majority of the rodent resting period and is dominated by delta frequencies with long rhythmic oscillations that are high in amplitude. REM, is similar to humans and can be characterized by rhythmic rapid eye movements, and muscle atonia, as evidenced on the EMG. Furthermore, REM sleep is dominated by desynchronized theta activity.

1.5.4 The neuronal networks responsible for sleep and waking

Different structures within the brain are responsible for the induction of wake and sleep as well as the balance and regulation therein. To change from a sleeping state to awaking state, certain neurons must be activated in conjunction with the synthesis and release of specific neurotransmitters. The various brain structures involved in the regulation of sleep and wake are complex and require comprehensive explanations; however given the focus of this dissertation is not on neuroanatomical systems, it is only briefly discussed.

Key to understanding this process was the discovery of the reticular activating system, a region of the brainstem tegmentum, which arouses the animal if stimulated, and specifically a subset of cholinergic nuclei which project to thalamocortical neurons involved in sleep/wake regulation. In addition, the main wake promoting systems are represented by the noradrenalinergic (NA; locus coeruleus), serotoninergic (5-HT; dorsal and median raphe), and histaminergic (His; tubero-mammillary nucleus, TMN), neurons. The hypocretinergic system, as mentioned before, is also involved in promoting wakefulness.

The majority of the control of NREM sleep rests within the hypothalamus including the VLPO. It is known that lesions in this region can severely disrupt sleep in both humans and laboratory animals, for example, causing insomnia (Von Economo 1923). The transition from waking to NREM sleep, and vice-versa, is based on a reciprocal inhibitory interaction between "sleep-promoting neurons" of the ventrolateral preoptic area (VLPO) that contain GABAergic cells and co-express galanine, and the wake-promoting systems (NA, His, 5-HT, Hcrt). NREM sleep depends on thalamocortical interactions or feedback loops that will control cortical synchronicity as measured at the level of the EEG.

For several decades the structures involved in REM sleep regulation were thought to be mainly located within the brainstem, dependent on an interaction between "REM on" and "REM off" structures, respectively activated and silent during REM sleep (Pace-Schott and Hobson 2002). According to this model of reciprocal interaction, the level of activity of PRF (pontine reticular formation) neurons, which are under control of these on/off components, is critical for REM sleep generation. Based on more recent data, other researchers, especially the group of Lyon, propose an updated integrated model. This hypothesizes that transitions from NREM to REM are due to the stimulation of the GABAergic neurons comprising melanin concentrating hormone (MCH) in REM-on contained in the ventrolateral periaqueductal gray, the dorsal paragigantocellular reticular nucleus, and the posterior hypothalamus. Conversely, REM episodes terminate with the inhibition of these neurons by using a REM-gating system composed of these GABAergic neurons of the ventrolateral periaqueductal gray, and other systems involved in waking such as those containing noradrenergic and hypocretinergic neurons. Serotonergic and monoaminergic neurotransmitter levels rapidly decrease during REM, yet cholinergic neurotransmission increases to levels seen in a waking brain. REM sleep is also characterized by rapid eye movements, generated at the pontine level, whose signal from

the pons is relayed directly to the thalamus, which is then sent to the occipital cortex, referred to as ponto-geniculo-occipital (PGO) waves. Currently, the function of these eye movements is unknown as they require no visual stimulus as in wake.

1.5.5 Conservation of the 2-process model in rodents

The circadian function in rodents has been studied with a variety of methods. Principle among them is observation based on actimetric recordings, measurements of locomotor activity during a given period. Since it is non-invasive, measurements have been taken for months or years at a time, identifying seasonal activity patterns and other similar behaviors. In addition, recording internal body temperature gives a clear pattern of variability depending on time of day. From the perspective of sleep, simple distribution of the cycle as seen previously (**Fig. 1.9**) is confirmation of a time-of-day organization. In rodents this cycle is approximately 24 hours, though there are slight changes depending on the species.

The homeostatic process can be challenged in rodents through sleep deprivation. Though initially this involved forcing the animal to run on a moving wheel, a less aggressive method known as “gentle handling”, is now used. However automated systems such as the multiple platforms technique are also implemented at certain labs. By forcing the animal to stay awake, researchers can increase the amount of homeostatic sleep pressure and then observe the response in the delta power. Delta (0.75-4 Hz) power is the most reliable marker for sleep need (Franken, Dijk et al. 1991) and increases are noted in the absence of sleep, with decreases following sleep bouts, thus the level of delta power is considered to reflect overall sleep need at a given time point. Human studies have confirmed this is a valid method, for testing the limits of the homeostatic sleep system. In a clinical lab, sleep deprivation may continue for 20-30 hours however, with mice, a sleep deprivation of just 6-hours or less at resting phase onset (light) has proven effective at eliciting a delta power response. Through comparison of the delta power from 24-hour period before the sleep deprivation, a strong drive from the homeostat can be seen, followed by a short recovery period lowering the delta power to its previous levels (**Fig. 1.10**).

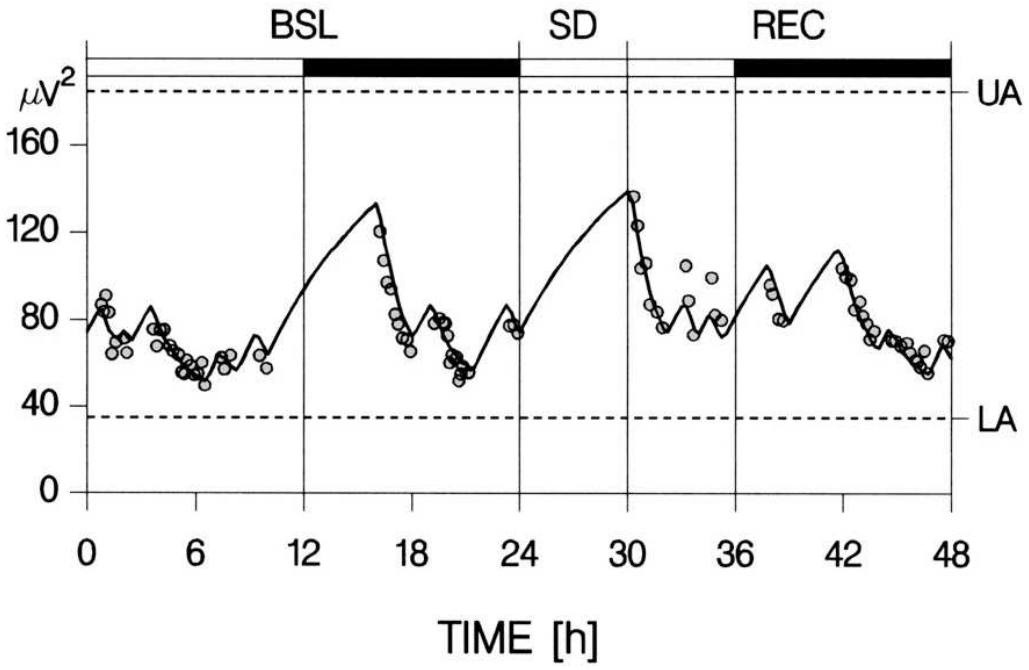


Figure 1.10: Delta power during 24-hours of baseline followed by a 6-hour sleep deprivation and recovery in a mouse adapted from Franken et al., 2001 (Franken, Chollet et al. 2001)

Chapter 2 - Light

2.1 - Introduction

2.1.1 Background

Once every twenty-four hours the planet Earth completes a single rotation along its axis, crucial for the survival of all life. The most essential feature of this rotation is the exposure to light during certain times, and darkness at others. Given the latitudinal position and time of year, the amount time spent in light or dark can shift drastically. Yet so immense is the impact that the evolution of thousands of species and their adaptation to certain behaviors is a result of this process. Light is responsible for vision, certain wavelengths for heating, and entrainment of the central biological clock. Animals need light to determine their feeding and reproductive cycles, through activation and stimulation of certain areas of the brain. In human beings, we use light to modify our natural environment to adapt to changing conditions. Light will affect cardiac rhythm, core body temperature, cognitive processing and alertness. Light changes sleep by promoting wake through the suppression of melatonin, a hormone secreted by the pineal gland, or change mood states, such as in people suffering from Seasonal Affective Disorder (Vandewalle, Gais et al. 2007).

2.1.2 Light and the brain

Light enters the brain via the eye, which is composed of several key structures: the pupil, the iris, and the retina. The pupil controls the amount of the light able to pass into the eye. The iris, a circular structure surrounding the pupil will expand or contract depending on the requirements, meaning the further the pupil expands, the higher the quantity of light can enter, and inversely as it contracts, the lower. After light has entered the pupil it passes through the interior chamber of the eye to the retina, a thin membrane of cells on the outer wall, sensitive to changes in light (**see Fig. 2.1**). At this point, light, both visual and non-visual, activates specific subtypes of cells within the retina and transmits this information to various areas of the brain to be interpreted.

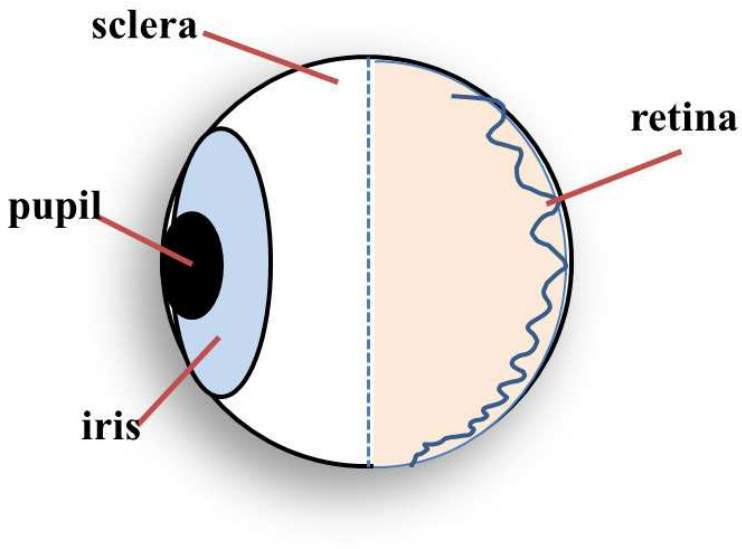


Figure 2.1: Model of the human eye

2.2 - The Retina and Visual Light

2.2.1 Retina

The existence of the retina and its structure has been known for many years and was clearly illustrated in 1900 by famed anatomist Santiago Ramon y Cajal. The mammalian retina is composed of distinct types of cells which serve a variety of functions, principally the interpretation of visual and non-visual light produced by the environment. Visual light, composed of wavelengths within the observable electromagnetic spectrum, activates specialized cells in the outer layer called rods and cones (**Fig. 2.2**).

Rods, named for their elongated cylindrical shape, are extremely light sensitive cells important for vision, also known as scotopic vision, in environments with extremely low luminosity. The cell itself is composed of an inner segment, consisting of the organelles and nucleus, an outer segment, which functions as the light absorption area, and a synaptic terminal to transmit information to bipolar neurons or horizontal cells.

Color vision is constructed based on the reactivity of cone cells sensitive to specific wavelengths, and functions most efficiently under high luminosity. Though the basic cellular structure is nearly identical to rods, their physical shape is drastically different due to the requirements of photosensitivity. Several subtypes of cones are present: (i) the short-wavelength (S-cone), which is sensitive to blue, (ii) a medium-wavelength (M-cone), sensitive to green, and (iii) a long-wavelength (L-cone), sensitive to red, which makes up the majority of light entering the eye.

Thus it is a combination of photosensitive information from these cells which produces the interpretation of color. However, the distribution and density of rods and cones can vary widely depending on the animal, from over one hundred million in human beings, to far less in species of birds and reptiles. Following synaptic transmission to the bipolar and amacrine cells, which help to organize information from rods and cones, visual light, activates the retinal ganglion cells, neurons containing photo-opsins, which in turn communicate to the brain via the retinohypothalamic tract.

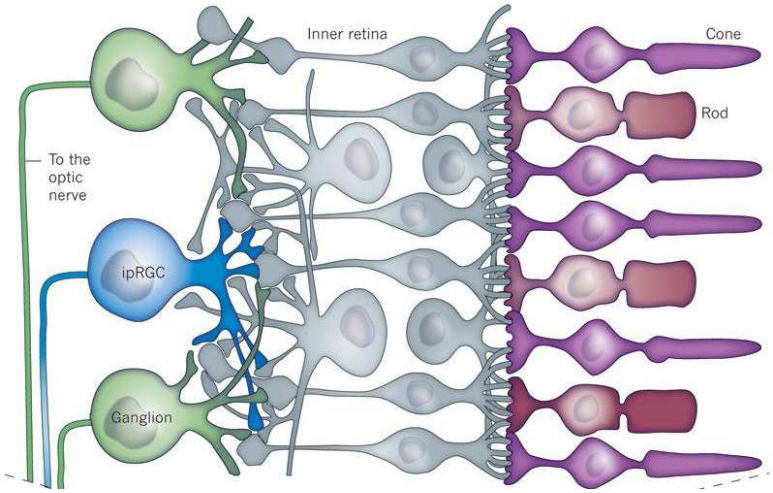


Figure 2.2: Simplified schematic of the mammalian retina outlining location of cell types and interrelation – Lok, 2011 (Lok 2011)

2.2.2 Visual light

Visual light is extremely important for the way organisms interact with their environment, and varies depending on the species. Light itself is only one small part of the overall electromagnetic spectrum, which includes radio and microwaves, as well as more exotic types of energy. Human beings have a relatively limited visual spectrum which they directly view (**Fig. 2.3**) ranging from 380nm (violet) to 740nm (red), and a frequency of 405 THz to 790 THz. Outside of this spectrum of light on either side are ultraviolet (10-~380nm) and infrared (750-1000nm). Certain species of insects and birds are able to see in the ultraviolet spectrum due to adaptations for discerning certain contrasting objects. Certain damages to cone structure in the retina in humans can cause color blindness which removes the ability to perceive certain colors within the visible spectrum, a disease with a prevalence as high as 5% in the general population.

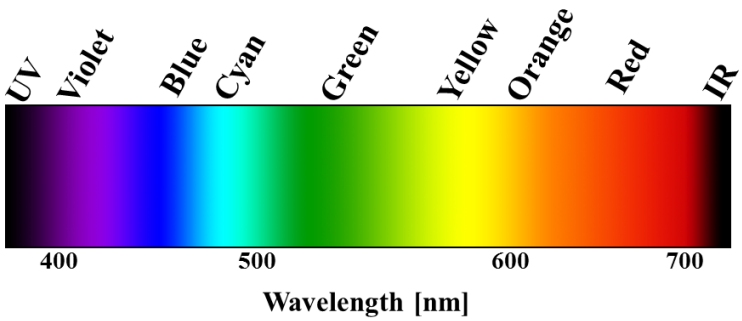


Figure 2.3: Visible spectrum of light for the human eye surrounded by UV and IR bands.

2.3 - Non-visual Light and Melanopsin

2.3.1 Non-visual light

For many years, non-visual light was not considered substantial in its influence upon key regulatory systems in the brain, and the assumption was that previously known photoreceptors involved in vision were responsible for processes such as circadian regulation. Thus these subsets of intrinsically photosensitive retinal ganglion cells (ipRGCs), which are now known to specifically transmit this information, were largely ignored upon their initial discovery. Though first identified in 1923 by Clyde Keeler, it was not until the end of the 20th century that their significance was truly understood. The principle finding in 1991 by Foster et al., was that this highly specialized cell was directly responsible for sending information to a variety of brain areas responsible for different functions (Foster, Provencio et al. 1991). It was shown that non-visual light mediated the circadian cycle through activation of the SCN, and additionally, controlled the pupillary light reflex, a function of the olivary pretectum nucleus (OPT). Furthermore, the function of melatonin, critical for inducing sleep, was altered depending on changes to environmental non-visual light. However, it was the discovery of melanopsin (*Opn4*), a new photopigment within these cells, in 2000 by Provencio and colleagues, which showed the most promise for scientific advancement (Provencio, Rodriguez et al. 2000).

2.3.2 Melanopsin

Opn4, is a photopigment whose existence was initially suggested by the group of Foster, who in 1999 identified a continued response to light in mice which possessed neither rods nor cones (Lucas, Freedman et al. 1999). These mice when placed under different light and dark conditions were still able to entrain their circadian cycle despite a total lack of visual input. Following this research axis, Berson underlined that these photosensitive cells were completely separate and were able to depolarize in response to light even when synaptic transmissions from rods and cones were blocked (Berson 2007). Later this same team would identify the sensitivity of Opn4 by using various levels of light and darkness to describe its responsiveness. Critical to understanding the contribution of this non-visual melanopsin-mediated information was to identify the areas of the brain

being targeted by projections from the retinohypothalamic tract (RHT). Groups lead by Saper and Hannibal (Gooley, Lu et al. 2001; Hannibal and Fahrenkrug 2004) demonstrated that these melanopsin ganglion cells projected directly via the RHT to the SCN and other areas of the brain, thus confirming the initial experiments by Foster. Several years later researchers identified the absorption peak of the photopigment as centered to the blue light (460-480nm), close to the visual spectrum of the S-cones, suggesting wavelength specific adaptation of the system (**Fig. 2.4**). Use of a rodless-coneless animal was extremely important in proving the existence of Opn4, however it was not until the use of melanopsin knock-out mice that the contributions were specifically elucidated. In 2008 and 2009, concurrent research studies using this model from Tsai et al. and Altimus et al, and Lupi et al., identified a direct non-circadian influence of non-visual light on sleep and alertness, mediated by Opn4 (Altimus, Guler et al. 2008; Lupi, Oster et al. 2008; Tsai, Hannibal et al. 2009). These findings will be more rigorously discussed in a review presented in chapter 3.

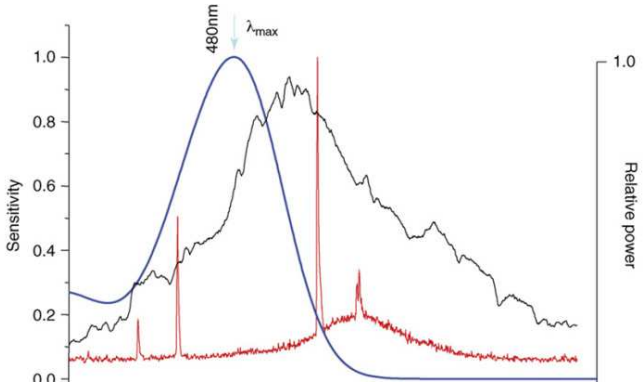


Figure 2.4: Melanopsin photopigment absorption peak adapted from Hankins et al. (Hankins, Peirson et al. 2008)

These ipRGCs though extremely small in number, accounting for less than 3% of mammalian ganglion cells, have now been highly implicated in a variety of regulatory processes. As previously discussed, melanopsin is able to directly control circadian rhythms through modulation of photic phases (Panda, Sato et al. 2002; Ruby, Brennan et al. 2002).

2.3.5 Innervations from the retina to the brain

Signals coming from the retina are transduced down the RHT, specifically innervating several cerebral structures (summarized in **Fig. 2.5**). However not all areas are innervated evenly. The areas of highest innervation are the SCN in the hypothalamus, the intergeniculate leaflet, and the olivary pretectum, responsible for circadian synchronization, interpretation of photic information, and the pupillary reflex, respectively. To a lesser extent is the VLPO in the hypothalamus, an area with a high concentration of sleep-promoting (galanin-containing GABAergic) neurons. An additional area of high innervation is the superior colliculus, which has a variety of functions including control of eye movements, and sleep regulation. The weakest projections are seen to the dorsal lateral geniculate nucleus, which functions mainly as a visual relay, and the subparaventricular zone (SPVZ), involved in regulating body temperature and food-energy intake (Hattar, Liao et al. 2002; Hannibal and Fahrenkrug 2004).

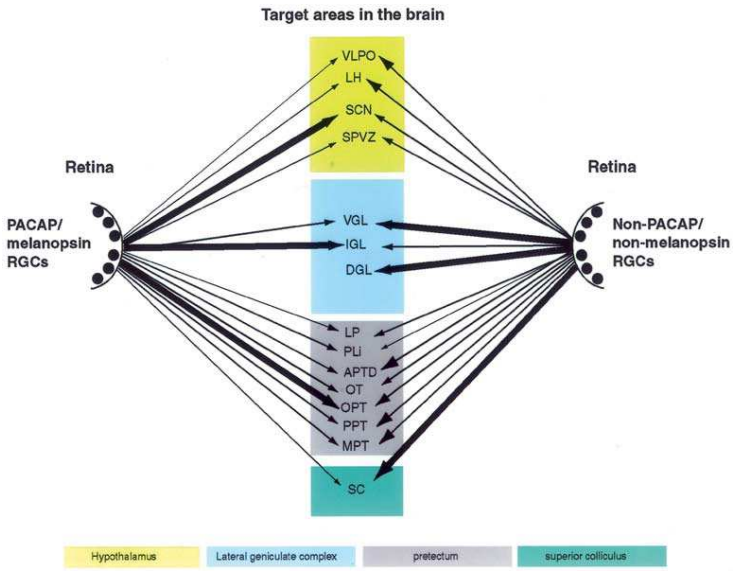
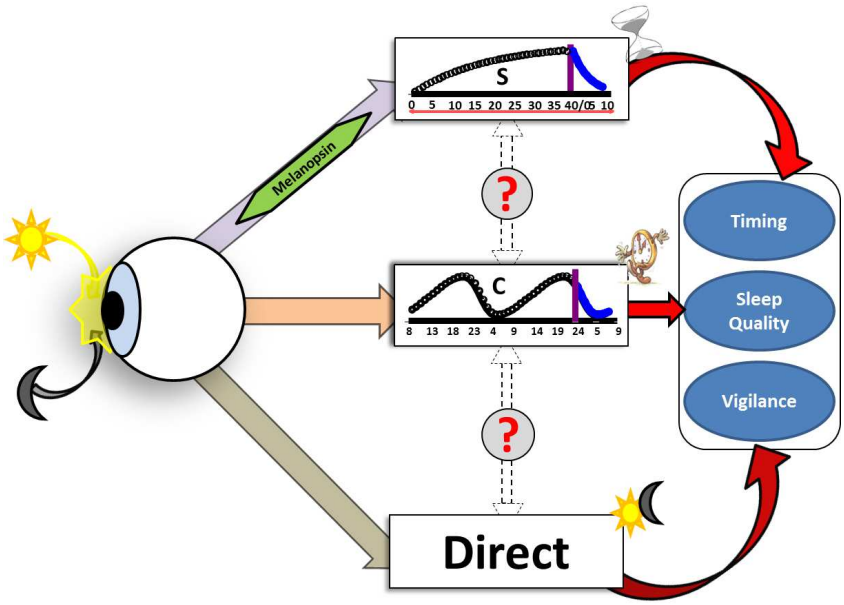


Figure 2.5: retinal targets to different brain areas, adapted from Hannibal and Fahrenkrug (Hannibal and Fahrenkrug 2004).



Chapter 3 - Article 1 (Review): Non-circadian direct effects of light on sleep and alertness: lessons from transgenic mouse models

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THEORETICAL REVIEW

Non-circadian direct effects of light on sleep and alertness: Lessons from transgenic mouse models

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SUMMARY

Light exerts a strong non-visual influence on human physiology and behavior. Additionally light is known to affect sleep indirectly through the phase shifting of circadian rhythms, and directly, promoting alertness in humans and sleep in nocturnal species. Little attention has been paid to the direct non-image-forming influence of light until recently with the discovery and emerging knowledge on melanopsin, a photopigment which is maximally sensitive to the blue spectrum of light and expressed in a subset of intrinsically photosensitive retinal ganglion cells. Indeed, the development of transgenic mouse models targeting different phototransduction pathways has allowed researchers to decipher the mechanisms by which mammals adapt sleep to their light environment. This review summarizes the novel concepts and discrepancies from recent publications relating to the non-circadian effects of light on sleep and waking. Specifically, we discuss whether darkness, in addition to light, affects their quality. Furthermore, we seek to understand whether longer sustained periods of light exposure can influence sleep, if the direct photic regulation depends on time of day, and whether this affects the homeostatic sleep process. Moreover, the neural pathways by which light exerts a direct influence on sleep will be discussed including the respective role of rods/cones and melanopsin. Finally, we suggest that light weighs on the components of the flip-flop switch model to induce respectively sleep or waking, in nocturnal and diurnal animals. Taken this data into account we therefore propose a novel model of sleep regulation based on 3 processes; the direct photic regulation interacting with the circadian and homeostatic drives to determine the timing and quality of sleep and waking. An outlook of promising clinical and non-clinical applications of these findings will be considered as well as directions for future animal and human research.

Introduction

Exposed to the terrestrial light–dark cycle and required to adapt to permanent changes in ambient light, all human beings experience the powerful influence of light. Since the emergence of our self-awareness, the relationship between mankind and light has always been prevalent, and the sun, our principal source, has become a major component of our shared cultural history. In numerous societies the deification of the sun is evident: from the Mayan pyramids of Mesoamerica, to the cathedrals of medieval Europe, deliberately constructed to bathe their worshipers in light. In fact, religious iconography often involves reference to the divinity of light. It has become a symbol of knowledge, the root of the word "enlightenment", and a way of describing intelligence (bright). In its absence we believe we are lost; sightless from awareness and development. Light thus simultaneously serves as our agent of progress and our continued emancipation as a species.

Light affects a broad range of physiological parameters and behavior,[1] including sleep and alertness,[2] mood,[3,4] and cognition,[5,6] both indirectly, through the phase shifting of circadian rhythms, and directly, in a circadian independent fashion. Therefore, in addition to aligning sleep and waking with time of day, light also acutely promotes alertness in humans, and sleep in nocturnal species.[7–9] Whereas the indirect influence through the entrainment of the circadian timekeeping system to the ambient light–dark cycle was the main focus for the last several decades, the direct effects of light have received little attention from chronobiologists, who commonly referred to it as a "masking effect",[10,11] shrouding it in the evaluation of circadian rhythms.

Light enters the organism through the eyes and characterization over the last two decades of the pathways by which photic information is conveyed to the brain was crucial for understanding its non-visual effects. Rods and cones are essential for animals to generate an image of the world, yet vision is not their only function. They also provide a measurement of light irradiance and contribute to non-

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Abbreviations

5-HT	serotonin
ADTA	ablated diphtheria toxin-A
CNGA3	cyclic nucleotide-gated channel alpha 3
EEG	electroencephalogram
GNAT1	guanine nucleotide-binding protein subunit alpha-1
IGL	intergeniculate leaflet
ipRGCs	intrinsically photosensitive retinal ganglion cells
KO	knockout
LD	light–dark
LH	lateral hypothalamus
NREM	non-rapid eye movement sleep
OPN	olivary pretectal nucleus
Opn4	melanopsin
OPT	olivary pretectum
RD/CL	rodless/coneless
REM	rapid eye movement sleep
RGC	retinal ganglion cell
SC	superior colliculus
SCN	suprachiasmatic nuclei
SPVZ	subparaventricular zone
VLPO	ventrolateral preoptic nucleus
WT	wild type
ZT	zeitgeiber time

visual functions such as photoentrainment and pupillary constriction.[12,13] As early as 1984, Takahashi and colleagues noted the presence of an unusual spectral sensitivity in the photoreceptive cells of hamsters, which affected circadian rhythms and seemed separate from the visual system.[14] The first evidence of a non-visual light system impacting physiology in humans was shown in blind patients wherein suppression of melatonin was seen following exposure to light,[15] later confirmed by others.[16–18] This observation that photoentrainment persists in the absence of rods and cones, was further demonstrated in animals and served as a critical step toward the discovery of a third component, melanopsin.[19,20] Melanopsin (*Opn4*)[21] is a photopigment crucial for irradiance detection and particularly sensitive to the blue spectrum of light (peak at 460–480 nm). This protein is expressed in a subset (1–2%) of retinal ganglion cells termed intrinsically photosensitive (ipRGCs), and is tightly linked to the non-visual functions of light.[22–24] These ipRGCs integrate the irradiance signal from both rods and cones and melanopsin-based pathways, transferring light information to various areas of the brain and representing the essential conduit for non-visual functions.[25,26] However, as previously stated most characterization of the non-visual functions of light has focused on output, such as circadian entrainment or constriction of the pupil.[12,13] In humans the direct (non-circadian non-image-forming, referred to subsequently as "direct") effects of light when administered at night, especially short wavelengths, have been shown to affect vigilance, attention, and waking electroencephalogram (EEG),[27] suggesting that the visual photopic system is not the primary photoreceptor system mediating these responses to light. Until recently, little was known about the significance of photoreceptors in regards to their mediation of the direct effects of light on sleep, in part because these photic inputs were difficult to distinguish from the influencing effects of the visual or circadian processes. Over the last few years, the discovery of melanopsin and subsequent development of transgenic models targeting the phototransduction pathways has allowed researchers to revisit the effects of light on behavior. Several groups[28–30] recently demonstrated that alteration of the light input to the brain by genetic ablation or inhibition of phototransduction components (rods and cones, *Opn4*, or ipRGCs) severely affects both sleep and waking. These data not only reveal a deeper and more complex role of light than was previously thought, but raises critical questions on how the direct effects of light interact with the circadian and homeostatic processes to determine the timing and quality of sleep and waking.

The mirrored effects of light and darkness are mediated through melanopsin and rod/cone-based phototransduction: a proof of concept

In order to quantify the acute sleep photic regulation and to determine the respective contribution of rods/cones and melanopsin-based pathways, three teams independently analyzed sleep in response to a light or dark pulse using different transgenic mouse models. To compare the results between studies, we normalized the data by setting the sleep response to a light pulse at 100% for the respective controls (wild type; WT) of each of the different transgenic mouse models, and by analyzing the same duration of pulse (only the first hour; Fig. 1). This assessment is appropriate given the comparable parameters of the light pulses used by each of the groups. All three studies used similar lighting intensities and spectral compositions and administered the light pulse at approximately the same zeitgeiber time (ZT).

Acute influence of light on sleep and waking

Lupi and coll.[29] and Tsai and coll.[30] used identical lengths for the pulse though with a different starting point, ZT 16 versus ZT 15 respectively, as compared to Altimus and coll.,[28] who used a slightly longer pulse beginning at ZT 14, exposing the mice for 3 h. A light pulse given during the habitual dark period readily induces sleep at the expense of wakefulness. These increases concern both non-rapid eye movement (NREM) and rapid eye movement (REM) sleep,

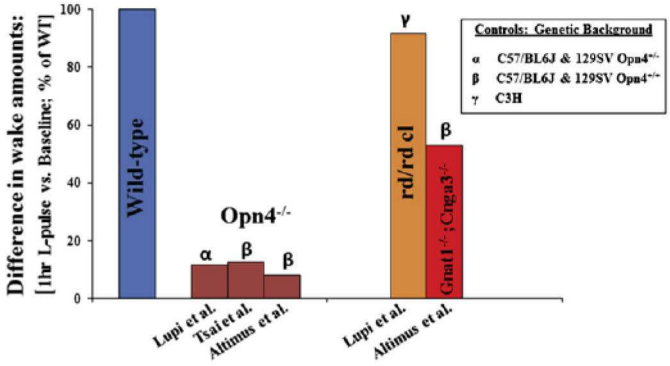

Fig. 1. Differences between studies in wake amounts during a 1-h light pulse administered at the same ZT (early part of the dark phase). Effect of a 1-h light pulse administered at ZT14, ZT15, or ZT16 on wake suppression in different transgenic mouse models (maintained under 12-h: 12-h light–dark cycle; 12 hL:12 hD). The data were extrapolated from different studies[28–30] and expressed as a deviation from their respective controls (symbols, α, β, γ, indicate the genetic background used in each of the studies) that are normalized to 100%. No s.e.m. is given nor statistics done due to the availability of the data extrapolated. Opn4$^{+/+}$ represents the WT control against Opn4$^{-/-}$, however Lupi et al. used heterozygous Opn4$^{+/-}$ animals as controls. Opn4$^{-/-}$ melanopsin knockout; rd/rd cl: rodless coneless; Gnat1$^{-/-}$; Cnga3$^{-/-}$: mutations in the rod transducin gene (Gnat1) and the cone cyclic nucleotide-gated channel gene (Cnga3) resulting in intact melanopsin-based photoreception but lack of ability of both rods and cones to detect light.[12] Wake was chosen as the output measure as Altimus et al. categorized sleep as a whole and did not distinguish between NREM and REM sleep, and though a 3-h light pulse was given during this experiment, only data from the first hour were used so as to compare with the other two studies. Percentages for Altimus have been converted into minutes/hours.

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although it is difficult to determine to what extent this is the case in Altimus and coll., as they opted to include NREM and REM sleep together rather than separately. In all three studies the light pulse failed to induce either NREM or REM sleep in melanopsin deficient mice (*Opn4*$^{-/-}$), a finding consistent with the lack of sleep induction observed in animals lacking ipRGCs.[28] The ipRGCs in these mice are selectively ablated using specific expression of an attenuated version of the diphtheria toxin-A (*aDTA*) subunit, under the control of the melanopsin locus (*Opn4*$^{aDTA/aDTA}$).[25] Whether the photo-induced REM sleep promotion results from the activation of *REM* on neuronal networks by light and ipRGCs, or from an ultradian regulation[31] as a consequence of an increase in NREM sleep remains however to be determined.

The *Opn4*-based pathways are mediating the direct photic input to sleep but can the visual photopic system also contribute to this photic regulation? Altimus and coll.[28] reported an attenuated sleep-promoting effect of light in mice expressing rods and cones which lack the ability to detect light due to mutations in both the rod transducin gene (*Gnat1*$^{-/-}$) and the cone cyclic nucleotide-gated channel gene (*Cnga3*$^{-/-}$). The time-course analysis of these mice indicates that rods/cones might also play a role in the initial response of this effect. However, when the sleep amounts during the light pulse are compared to baseline values using a larger window centered around this ZT, our group observed a complete lack of response in *Opn4*$^{-/-}$.[30] This is in line with the conservation of the light pulse-induced NREM–REM sleep promotion observed in rodless/coneless (*rd/rd cl*) mice.[29] This latter model was kept on a *C3H* genetic strain which produces melatonin, a sleep-promoting hormone released by the pineal gland, contrary to the majority of laboratory mice strains. The observation in both melatonin proficient and deficient mice, suggests that the direct effects of light are not only a mechanism counteracting the lack of melatonin, but also a significant mechanism conserved across a large range of species.

Finally, while it was previously assumed that only the rod–cone system was mediating these direct effects of light, overall the data from these transgenic mouse models provide a proof of concept that melanopsin plays a major role, at least at the time of the day studied (early part of the dark phase). Both input from *Opn4* and rods and cones are mediated by the ipRGCs that represent the principle conduit in mediating the photic input to sleep and waking. The influence of the time of day on the respective contribution of rod/cone- and *Opn4*-based photic regulation will be discussed in a subsequent section.

Dark versus light: dark exerts an alerting effect

Historically, night and darkness in general were negatively characterized; times without sunlight during which humans and animals remained in their homes, waiting for sunrise. For example Akhenaten states that light makes life possible whilst darkness symbolizes death. Light gives rise to vision and is positively rewarding in humans. Thus, the majority of research has focused on the effect of light, with less attention paid to the exploration of a possible role for darkness. However, a large number of animal species spend as much time under darkness as under sunlight, and for numerous scientific fields, consequences arising from the absence of a stimulus remain as pertinent as what comes from its presence. Nocturnal rodents such as the rats are awoken by darkness, stirring them from sleep, to forage for food.[7] Similarly, in mice the administration of a dark pulse during early daytime awakens the animal at the cost of sleep. Both Tsai and colleagues[30] and Altimus and colleagues[28] found the wake-promoting effect of the dark pulse to not be significant in the absence of melanopsin, although it tended to be slightly attenuated. Conversely, the dark-induced waking response was decreased in mice lacking efficient rod/cone phototransduction, and abolished in ipRGC-deficient mice, suggesting that wake promotion is preferentially mediated by the classical photoreceptors at this time of the day (ZT2-5). Tsai and coll.[30] evaluated the response of EEG rhythms to dark pulses, examining spectral bands known to be specific rodent correlates of exploratory behavior and alertness.[32,33] Concomitant to wake induction, light-to-dark transitions induced a rapid and prolonged induction in EEG theta (7–10 Hz) and gamma (40–70 Hz) activity that were delayed and reduced in the absence of melanopsin, although not completely suppressed. Altogether, these data demonstrate that the ipRGCs can mediate an *OFF signal* to the brain and convey a *light OFF* response coming from rods/cones and melanopsin at early daytime (i.e., at a time when both the circadian and homeostatic sleep pressure is high in mice). These findings also suggest that rods and cones are primarily involved in promoting the wake state, whereas melanopsin might be preferentially mediating the EEG correlates of cognitive processes associated to waking, suggesting a higher level of alertness. Thus, the direction of future research should include examination of the influence of darkness on cognitive processes with the potential for translation to human research and clinical practice, further deciphering the mechanisms underlying the alerting effect of light.

The non-circadian effects of light and darkness depend on time of day

Spontaneously the three teams previously mentioned,[28–30] applied light or dark pulses in mice at similar ZTs because, without consulting one another, they believed certain hours during the day would exist when the effect of these respective pulses would be maximum. However, what would have been their conclusion if they would have applied the pulses at other times of day? To address this question Altimus et al.,[28] and Tsai et al.[30] applied repetitive, consecutive, and short light–dark pulses, creating an ultradian cycle and likely the most appropriate way of assessing this question.[28,30]

In animals, most ultradian studies have used a 3.5 hL:3.5 hD cycle though at varying lengths. However, others have found 3 hL:3 hD to be affective as well, though this will eventually entrain to a 24-h clock.[34] Regarding the effect of the ultradian cycle on sleep in nocturnal rodents, though the dark-induced wake and light-induced sleep was shown to be effective on a repetitive schedule at early stages,[35] little research has been conducted until recently due to the lack of appropriate models to study this photic regulation.

Both Hattar's group and ours attempt to answer this question through use of an ultradian light–dark cycle. The first group uses an alternating period of 3.5 h of light followed by 3.5 h of dark, repeating 12 times for a total of 84 h.[28] The advantage of this protocol fits to 21 or 28 h, and is misaligned with the endogenous period of the clock, close to 24 h, inevitably forcing the animal out of its normal circadian rhythm. Under this condition, the chronic light pulses consistently decreased the wheel-running activity during dark-to-light transitions, demonstrating that activity can be recurrently suppressed by light. In contrast, once the first two cycles had passed, there were not significant increases in the amount of sleep as a result of light–dark transitions. Altimus and colleagues suggested that the threshold for sleep induction by light is higher than the inhibition of wheel-running activity.[28] One explanation proposed by the authors is that sleep control centers, such as the ventrolateral preoptic nucleus (VLPO), receive weaker input from the ipRGCs during light activation, in contrast with circadian centers (suprachiasmatic nucleus; SCN).[36] However, this 3.5 hL:3.5 hD cycle creates repetitive conflict between direct photic regulation and the circadian drive making interpretation of this effect difficult. Thus it is more probable that the direct effect of light needs to

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overcome radically different thresholds from one cycle to the next, of the circadian and homeostatic drives.

This limit was overcome by our group[30] who opted for a repeating cycle of 1-h light and dark pulses over 24-h respecting the endogenous period of the animal (i.e., rhythmicity with a minimum of phase delay), in order to examine the interaction between the circadian drive and the acute effects of light. In WT mice under these conditions, 1-h pulses of light and darkness strongly induced sleep and wakefulness, respectively, with slight modulations across the day. After the first three light–dark (LD) cycles, mice appeared capable of anticipating dark onset, evidenced by increases in wakefulness preceding the pulse. One could question the efficacy and appropriateness of this protocol as light given at a particular hour might affect subsequent responses and that animals could entrain their sleep-wake organization to this ultradian schedule. This important question should be addressed in the future, applying ultradian cycles for longer periods of time (i.e., several days), and should be evaluated additionally in humans. The light-induced sleep response was abolished during the dark phase in *Opn4*$^{-/-}$ mice, although under the light phase the response was unattenuated. Therefore, it seems that the direct photic regulation of sleep depends on a balance between melanopsin that appears to be most prominent mediator during the subjective dark phase (biological night; second 12-h of the 1 hL:1 hD cycle), whereas during the subjective light phase (biological day; first 12-h of the 1 hL:1 hD cycle) rods and cones contribute largely to this effect. This is clearly illustrated when the differences between dark and light pulses are examined across this 1 hL:1 hD cycle (as shown in Fig. 2). These findings should be confirmed by applying this protocol to the transgenic mouse models mentioned above.

Previously modulation of the retinal output was studied, specifically the differences in the electrical coupling between rods and cones, which in these structures created higher photosensitivity to darkness.[37] Conversely, Barnard et al. found that higher cone sensitivity was present in the (subjective) light period, owing this to a circadian mediated change requiring melanopsin to be present.[38] Measurement of *Opn4* mRNA levels have been shown to vary across the day as a consequence of both light- and circadian-dependent modulation, with the highest expression at the transition from light to dark [39,40; personal data not shown]. Further investigation into these results is necessary as differences in the levels of protein may indicate an altered sensitivity to light. In essence, this may demonstrate a circadian gating function of the SCN for the direct photic regulation of sleep and waking, opening new avenues in both human and fundamental animal research, already underway at several laboratories.

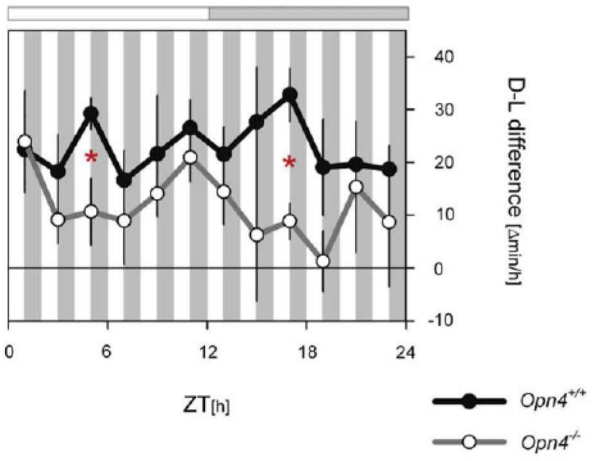

Fig. 2. Time of day influence on the direct photic input to sleep and waking. Dark-light difference in Opn4$^{-/-}$ and Opn4$^{+/+}$ under a 1 hL:1 hD cycle for 24-h. Data represent the change in wake amounts (min) between 1 h dark and 1 h light pulses for each cycle across the 24-h of the 1 hL:1 hD ultradian cycle. Vertical lines represent s.e.m. Data were extrapolated from Tsai et al.[30] ANOVA denotes higher D–L differences in Opn4$^{+/+}$ than in mice lacking melanopsin ($p < 0.05$), asterisks denote significant genotype differences ($p < 0.05$; post hoc *t*-tests).

A sustained role for the direct effects of light

The direct effects of light are continuously referred to as acute. However, when examining the effect of longer pulses as in Altimus et al.,[28] the photic regulation of sleep is conserved during the second and third hour, clearly suggesting the existence of a sustained effect of light or dark over time. When these results are studied in more detail, the last 2 h of the dark or light pulses across the different transgenic models used, we can observe an attenuation of the light/dark effects (in *Opn4*$^{-/-}$) or their absence (in *aDTA*), indicating that the acute effects of light and dark would be sustained by *Opn4* and rod/cone-mediated, and relayed solely by ipRGCs.

However, if the photic regulation of sleep can be sustained for longer periods of time, sleep and waking under normal exposure of light and dark should be affected in mice with genetic alteration of their photoreception components. Thus with these models the sustained effects of light should be revealed. Lupi et al.,[29] as well as Altimus et al.[28] found no significant genotype differences between any of their groups, something they anticipated. However, when analyzing in more detail the 12-h light and dark periods, Tsai et al.[30] observed that *Opn4*$^{-/-}$ mice display an accumulated 1-h loss of NREM sleep during the light period, not observed during the dark phase. This loss was due to shortened waking bouts suggesting a similar mechanism to the acute effect of light (i.e., an inability of the mice lacking melanopsin to return to sleep in the presence of light). Using this type of analysis in these other transgenic models may more clearly illustrate this process. As it stands it is still unknown whether this direct effect of light might contribute to the partitioning of sleep and waking within the light and dark periods under normal conditions. With this initial data from these studies it is evident that the sustained direct effects need to be researched further in order to provide greater comprehension of the mechanisms at work. This relationship of sustained exposure of light and dark to sleep and wake has numerous applications, not the least of which is the way in which we adhere to artificial societal lighting conditions.

Direct effects of light and darkness rather than masking effect of light

For a number of years the direct influence of light on sleep and alertness was poorly understood and considered minor by most researchers. Chronobiologists referred to it as a "masking" effect, wherein the locomotor activity in a nocturnal laboratory rodent, such as the mouse or rat, was suppressed under light, which hid underlying changes in their circadian rhythm.[11,41] The masking effect is defined as a change in the normal rhythm of the animal due to environmental stimuli which persists solely during exposure to these stimuli. If a stimulus is given when an animal is at rest, causing awakening with high activity, it is thought of as "positive" masking. Conversely, "negative" masking refers to a stimulus that causes the animal to cease its activity, such as shifting the onset of darkness forward artificially.[10,11] For these researchers the masking effect is specifically considered to be an expression of changes in circadian rhythm, taking into account only locomotor activity or its cessation, and rarely sleep and wake, as measured with EEG. It is known that light is able to acutely affect a variety of physiological mechanisms.[1] Animal studies on the direct effects of light, as described previously, clearly and consistently demonstrate acute

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sleep promotion by light, and an acute waking (or alerting) induction by darkness. Moreover, the observation that a lack of melanopsin induces a sleep loss during the light phase reveals that the direct effects of light contribute to the sleep-wake organization without affecting the circadian sleep-wake rhythm. Thus, this process should not be restrictively referred to as "masking" but considered rather as an independent regulatory mechanism more appropriately termed the "direct effects" of light and darkness.

The unexpected discovery that a photopigment can affect sleep homeostasis

In the model of sleep regulation established by Borbely and Achermann[42,43] it is well understood that two-processes (circadian and homeostatic) influence the regulation of sleep. The homeostatic process monitors sleep need which increases as a function of time spent awake and decreases exponentially during sleep.[44] In order to determine sleep need, researchers examine slow wave activity or delta power (0.5—4.5 Hz) through power spectrum analysis.[44] Rationally the link between light and sleep homeostasis has never been established, however a surprise finding by Tsai et al.[30] demonstrated that *Opn4*$^{-/-}$ mice showed lower levels of delta power compared to *Opn4*$^{+/+}$ mice even though *Opn4*$^{-/-}$ mice slept 1 h less over 24 h.[30] Delta power was especially reduced in the initial part of the dark phase of the 12 hL:12 hD schedule with an exponential decrease after activity peak which corresponds to the time when mice are most active and therefore show an increase of homeostatic pressure. Under the 1 hL:1 hD schedule the same difference between the subjective light and dark phase was observed and thus cannot be explained by light influence. The influence of melanopsin on the sleep homeostat was confirmed by a sleep deprivation experiment. After a 6-h sleep deprivation *Opn4*$^{-/-}$ mice showed significantly lower levels of delta power during NREM sleep following the sleep deprivation in comparison to *Opn4*$^{+/+}$ mice. Without melanopsin, the amount of sleep pressure, as measured by NREM delta power, reached after sleep deprivation is significantly lower as compared to their wild-type littermates. Indeed, the decrease of delta power seems to be greater than in a panel of six inbred strains of mice tested,[44] indicating the importance of melanopsin in the homeostatic process. These findings give rise to many questions as to the interactions with ZT or different lighting conditions, the influence of rods and cones on the process, or the physiological mechanisms behind the transmission of photic information. Continued studies in other models such as rodless/coneless mice and in mice without melanopsinergic cells (aDTA) might lead to a better understanding of this process. A confirmation of this experiment in a backcross strain will also be needed. In human beings it is speculated that the highest point of homeostatic sleep pressure is also the peak where the alerting effects of light are strongest.[2] However, recently it has been shown that both the efficacy of the acute effects of light is dependent on the circadian rhythm of the individual[6] and the phase shifting effect may depend on sleep pressure.[45] Furthermore, relationships between polymorphisms in the Per3 gene and sleep need suggest another potential impact on the relationship between light and sleep homeostasis.[46] Given the nature of these findings, it is therefore essential that this underlying relationship be examined more thoroughly; hence the continued research underway at several laboratories.

Neuronal pathways relaying non-circadian light information

The neurophysiological underpinnings that account for the light-induced response are not yet fully comprehended. The

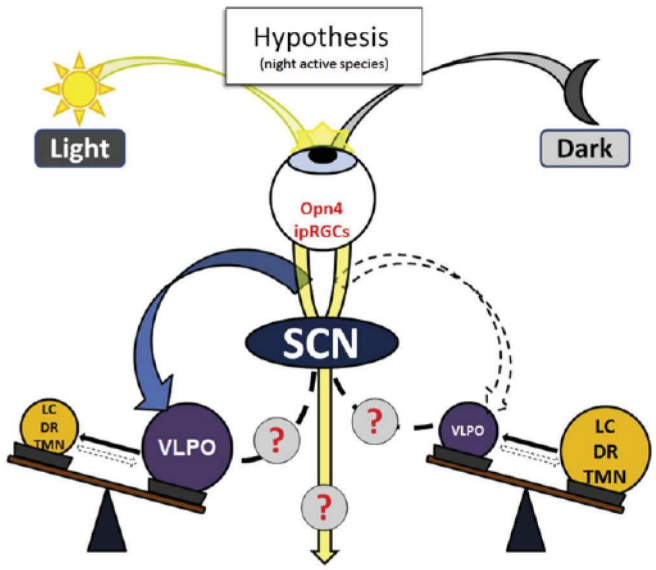

Fig. 3. A model in which the direct effects of light and darkness impinge on the balance of the flip-flop switch to promote sleep or waking. Here, the model is described for nocturnal rodents, with the influence of light and darkness on the flip-flop switch being reversed in diurnal species. The flip-flop switch corresponds to a reciprocal inhibitory interaction between sleep- (VLPO) and wake-promoting brain regions. Light activates the "sleep-promoting neurons" (GABAergic and galaninergic) of the VLPO that in turn inhibits the amine-mediated arousal systems. In darkness, the lack of activation of the VLPO disinhibits the arousal-promoting regions. It remains to be determined whether light and darkness might influence the wake-promoting systems, as previously suggested in humans (57). Furthermore ("?"), it is not yet known whether the SCN, beyond its role as a clock, might also relay the direct non-circadian effects of light and darkness, transferring this information to neuronal networks controlling sleep and waking, such as the VLPO. Full arrows: active interactions, dashed arrows: inactive interactions. Abbreviations: LC: locus coeruleus (noradrenaline); DR: dorsal raphe (5-HT); TMN: tuberomammillary nucleus (histamine).

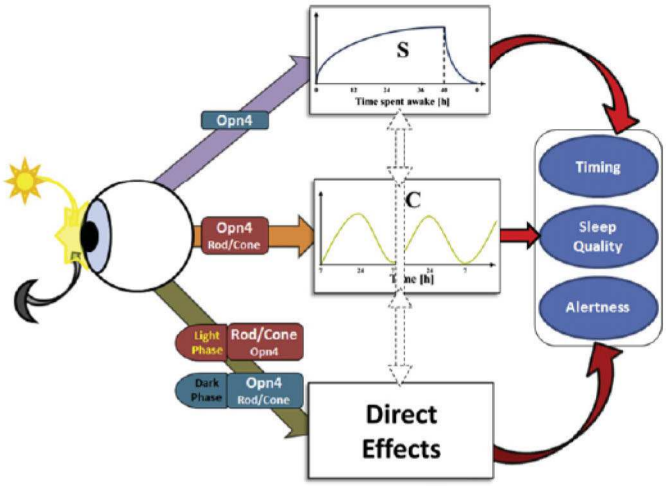

Fig. 4. Schema proposing a 3-process model for sleep and wake regulation. Light and darkness exert a more complex role through a direct acute and sustained influence on the expression of sleep and waking as well as indirectly through the entrainment of the circadian timing system. Additionally, the photopigment melanopsin influences the sleep homeostatic drive. During the light phase the direct photic input to sleep and waking is primarily mediated by rods and cones and to a lesser extent by melanopsin. Conversely during the dark phase, melanopsin is the main mediator of the direct effects exerted by light and dark on sleep and waking. Opn4 and rods/cones compensate for one another to maintain a powerful direct non-circadian influence of light and dark across the daily cycle of 24 h. The way in which the circadian, homeostatic, and direct photic regulatory mechanisms interact to determine the timing and quality of sleep and alertness, needs to be more fully understood.

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findings we have previously discussed confirmed that the ipRGCs convey non-visual direct light information, transmitted by the classical photoreceptors, as well as melanopsin to the brain.[25,26,28–30] The ipRGCs send monosynaptic projections to the suprachiasmatic nucleus (SCN) and the intergeniculate leaflet (IGL) for entraining circadian rhythm and to the olivary pretectal nucleus (OPT) for influencing the pupillary light reflex.[47] In addition, these cells also project to the VLPO (as well as having indirect secondary afferents from the SCN), the lateral hypothalamus (LH), the subparaventricular zone (SPVZ), and the superior colliculus (SC).[36,48] The role of the VLPO in sleep is well established through a cluster of galaninergic neurons characterized as "sleep-promoting neurons".[49] The SC-pretectum has been suggested to mediate the direct effects of light, yet the assumption is based on larger lesioning experiments[50,51] and this structure has not proven to be so crucial for sleep regulation.[52–54]

To assess whether some of these structures might be the anatomical targets by which ipRGCs interact with the sleep-wake regulatory systems, Tsai et al.,[30] and Lupi et al.,[29] compared neuronal activation of the VLPO, the SC, and the SCN in response to nocturnal light exposure in *Opn4*$^{-/-}$ and *Opn4*$^{+/-}$ mice. To accomplish this c-Fos was used as a marker of neuronal activity in response to the administration of a light pulse administered at a ZT demonstrated to have a light-induced promotion mediated by melanopsin. Fos activation in the SCN was attenuated as previously described[55,56] but not absent.[29,30] This has been associated to a reduction in circadian rhythms phase shifting but does not exclude the possibility that this diminished activation of SCN neurons might also lessen the direct effects of light on sleep and waking in *Opn4*$^{-/-}$ mice. Studies using mice with invalidation of the central clock should further determine whether the SCN might be a relay for the direct photic regulation of sleep and waking. Tsai et al. and Lupi et al. also reported a photic activation of the SC, the VLPO[29] and even more specifically of the "sleep-promoting galaninergic neurons" it contains.[30] This question was also addressed in human studies using fMRI and light[57–60] reporting alerting effects of blue light pulses on cognitive task performance in lab studies.[58,61] Interestingly there were region-specific time courses, such as enhanced responses in the posterior thalamus including the pulvinar nucleus, implicated in the regulation of visual attention and alertness. This suggests that light may modulate activity of subcortical structures involved in alertness, thereby promoting cortical activity in networks involved in ongoing non-visual cognitive processes. Moreover, Perrin and coll., reported a decreased response to light in the hypothalamic region in proportion to previous exposure to light.[57] As spatial resolution of PET scanning does not allow the authors to specify the nuclei in the deactivated area, they surmised that the latter correspond to the SCN and other hypothalamic structures involved in the non-image-forming responses in rodents, such as the subparaventricular zone or the VLPO. In a latter study they observed a blue light-induced increased response in a bilateral area of the brainstem consistent with activation of the locus coeruleus, a key structure for waking containing noradrenergic neurons.[59] These observations, inversed to Lupi et al. and ours,[29,30] are altogether coherent given the nocturnality of mice and diurnality of humans.

The current model proposed for sleep and wake transitions is based on a reciprocal inhibitory interaction of two neuronal assemblies. The VLPO inhibits the ascending arousal regions and is in turn inhibited by them, referred to as "flip-flop switch".[62] Based on all the available data, from both animal and human research, we propose a model wherein light directly impinges on the balance of the flip-flop switch on VLPO sleep-active neurons in nocturnal animals, shifting the reciprocal inhibitory interaction toward sleep promotion. Conversely, a lack of VLPO activation possibly associated with light-induced activation of the locus coeruleus (LC)[57] and hypothetically other wake-promoting systems (raphe nuclei-5-HT; tuberomamillary nucleus–histamine) shift toward arousal in diurnal species (Fig. 3). Finally, it remains to be determined whether the SCN, beyond its clock function, may also influence sleep and waking through relaying these direct effects of light.

Conclusion: toward a three-process model?

The data examined in this review, principally from the findings in transgenic mouse models, present a strong foundation for the introduction of a new key regulatory mechanism of sleep and alertness. This suggests that darkness exerts a similar function in night active species as the alerting effect of light observed in diurnal species. These inverted effects of light and dark between diurnal and nocturnal species might represent a necessary system of evolution that allows animals to be awake at appropriate times to search for food and avoid predators. These data have changed the way in which we view the role of light and darkness, establishing that light/dark inputs continuously (acute and sustained effects) shapes the temporal organization of sleep and waking through an interaction with the circadian and homeostatic processes, and possibly by impinging on the flip-flop switch. In essence, this represents a movement away from the classical 2-process model, to one including a more complex role for photic regulation. With this consideration we can therefore propose a theoretical three-process model of sleep regulation which takes into account these novel concepts (Fig. 4). Accordingly in our proposed model, the direct photic regulation of sleep interacts with the circadian and homeostatic processes to determine the timing and quality of sleep and alertness. These data urge for a strong reexamination on the role of light in mammalian physiology and behavior. If confirmed in humans, this three-process model would provide a useful framework to further understand sleep disorders and sleep disturbances associated with neuropsychiatric disorders. Moreover, it will encourage the reassessment for the possible applications of light therapy, beyond their current use. The implications are far-reaching and may help us eventually with improving light implementation within our society.

Practice points

These transgenic models have identified that:

1) light and dark actively exert direct non-circadian effects in mirror between nocturnal and diurnal species.
2) these effects depend on time of day, mainly melanopsin-based phototransduction during the dark phase and rods/cones pathways during the light phase (daytime).
3) melanopsin-based phototransduction exerts a sustained continuous effect on sleep and waking.
4) light and dark, through ipRGCs, influence several brains areas, including some involved in the flip-flop switch model.
5) melanopsin affects the sleep homeostat providing a biological link between light and sleep homeostasis.
6) the direct effects of light represent a third key regulatory mechanism interacting with the circadian and homeostatic drive to shape the temporal organization of sleep and waking (three-process model).

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Research agenda

Future studies need to more comprehensively:

1) identify the mechanisms and neurobiological network underlying these non-circadian direct effects of light and darkness.
2) use clock-disabled animals to determine the more precise contribution of the direct effects of light/dark to the daily sleep-wake cycle in the absence of a circadian drive.
3) revisit the possible role of light on sleep homeostasis through melanopsin.
4) validate the proposed three-process model in humans.

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* The most important references are denoted by an asterisk.

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Part II: Research Projects

Introduction to research projects

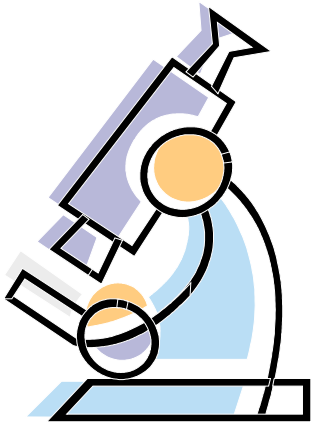
The classical 2-process model describing the regulation of sleep and waking has been robustly studied in a variety of species for over three decades, and their functions are well known at this point. However, given what is now known about the presence of non-visual light and retinal biology, in addition to the data reviewed in the previous chapter (Altimus, Guler et al. 2008; Lupi, Oster et al. 2008; Tsai, Hannibal et al. 2009), begs the question as to whether an auxiliary process might exist, one functionally different from the previous two yet strongly involved in an interconnected relationship. This non-circadian direct effect of light thus bears further exploration given the fortitude such information brings to the field of chronobiology and sleep research. Consequently, the research projects presented in this dissertation were specifically designed to examine these questions from several congruent axes.

The first research project dealt specifically with the comprehension of the underlying neurobiological pathways involved in diffusion of these direct effects to various areas of the brain involved in sleep and waking. To parse apart these pathways and the transmission of this melanopsin-based non-circadian light information, mice lacking both *Opn4* and with an electrolytic lesion of the central circadian clock, were compared for differences with their controls under a variety of light-dark conditions. In addition, a mutant mouse possessing a disrupted central clock ($Syt10^{Cre/Cre}Bmal1^{fl/-}$), yet with intact cellular structures, was used as a complementary approach to the SCN lesion. Indeed, inhibition of the clock in two ways, through transgenes and lesion of the SCN will allow controlling for the limitations of each approach. Finally, the overarching goal was to not only identify the pathways responsible but to determine the proportional contribution of the direct effects of light on the sleep wake distribution under habitual environmental settings.

As the University of Strasbourg is one of the only laboratories to possess a diurnal rodent model for studying chronobiology, *Arvicanthis ansorgei*, a second project was designed to demonstrate that these direct effects of light exist across multiple species, even if the interpretation of light and dark is inverted. Primarily this required modification of the EEG system for use with a larger more active rodent, as well as verification of the vigilance states present. This approach used a similar protocol as with the mice, though slightly modified due to the diurnality of the species. The objective of this project was not

only to validate the existence of the direct effects of light in a separate species, but indeed on a long-term perspective, to understand more clearly the inherent differences which determine nocturnality vs. diurnality. Furthermore, it was in search of a stronger translational model to human research, available for manipulation within controlled laboratory settings.

Building upon a surprising result found by our group (Tsai et al., 2009) a third project sought to examine a biological link, the first ever seen, between sleep homeostasis and light via melanopsin. A deprivation protocol was designed around a dose-response curve to elicit a clear model of the homeostatic process. Analysis of the delta (0.75-4 Hz) power spectrum gave a clear impression of the changes between these experiments. Genetically ablated *Opn4* mice were used in conjunction with their controls to quantify this difference, in addition to an inverted, yet identical procedure for arvicanthis ansorgei. Isolation of this association is vital to revealing the fundamental information gating of non-circadian light on the accumulation of and release of sleep pressure.



Chapter 4 - Laboratory techniques and materials

4.1 - Laboratory Animals

4.1.1 Opn4 Gene-ablated mice

Experimental studies on the melanopsin-mediated direct effects of light and sleep homeostasis in mice were realized through the use of Opn4 genetically ablated mice. The mutant mice were initially generated by Jackson laboratory. Gene disruption was made using a 38-bp fragment corresponding to a segment of the protein-coding region that was replaced by an IRES-lacZ reporter and neomycin resistance cassette (IRES-*lacZ-neo*). This mutation was designed to produce a loss-of-function mutation by deletion of amino acids 116 to 128, as well as insertion of the 6.9-kb reporter/resistance cassette. After electroporation into mouse embryonic stem cells of strain 129P2/OlaHsd, selecting those with G418 resistance, the cells were injected into blastocysts from a C57BL/6 strain. These chimeric mice were then crossbred with wild-type C57BL/6 producing the first generation heterozygotes. The mice were initially transferred from Stanford University, and the colony was extended at our facility in Strasbourg. $Opn4^{-/-}$ mice from two different genetic backgrounds (C57/Bl6&129P2 and C57/Bl6 backcross) were studied during the course the doctoral program. The majority of the experiments were performed on $Opn4^{-/-}$C57/Bl6&129P2 strain due to the availability of mixed genetic background mice at the start of the PhD. To control for genetic heterogeneity (C57/Bl6 and 129P2) the experiments were performed on male littermate homozygous offspring ($Opn4^{+/+}$ & $Opn4^{-/-}$) obtained from cross-breeding heterozygous ($Opn4^{+/-}$ x $Opn4^{+/-}$) males and females. In addition, a second colony of melanopsin KO mice with a homogeneous C57/Bl6 genetic background was developed. The backcross began at Stanford University, and subsequently continued in Strasbourg, arriving at 10 backcross generations by the end of the PhD. At all times prior to experimentation mice were kept under environmentally stable conditions (12hL:12hD; 25 ± 0.5° C, with food and water ad libitum.

4.1.2 The synaptotagmin10-Cre Bmal1 conditional KO mice (conditional deletion of the clock gene Bmal1 using the Syt10Cre driver)

The second transgenic mouse model included in the first experimental study was composed of Synaptotagmin10-Cre ($Syt10^{Cre}$) Bmal1 conditional knock-out ($Bmal^{fl/-}$) mice coming directly from the Max Planck Institute for Genetics in Göttingen, Germany. These

mice were used specifically for transgenic modifications which invalidated their circadian clock in the suprachiasmatic nucleus. Here, we briefly describe the generation of this model; for details see Husse et al (Husse, Zhou et al. 2011). Based on the Allen Brain Atlas (Lein et al., 2007) our collaborators from the Göttingen identified Synaptotagmin10 (Syt10) as a gene strongly expressed in the SCN with relatively few auxiliary expression sites in the CNS, even during development. The generation of a $Syt10^{Cre}$ driver line enables SCN targeting without targeting of peripheral, non-neuronal clocks. The exon 1 of the Syt10 gene was replaced by a Cre knock-in cassette, and after cloning of the $Syt10^{Cre}$ vector, the targeted clones were then injected into blastocysts of C57/Bl6 mice. These chimeric mice were then bred using wild-type C57/Bl6 to produce F_1, followed by continued breeding to produce a colony by back-crossing to C57/Bl6. $Syt10^{Cre}$ were then crossed with $Bmal1^{fl/fl}$ to disable Bmal1 expression, solely in the SCN. Depending on the dosage of the Cre recombinase, mice with phenotypes ranging from minimal circadian perturbation to complete arrythmicity were obtained, confirming the usefulness of the $Syt10^{Cre}$ driver line. These $Syt10^{Cr/Cree}$ $Bmal1^{fl/-}$ mice retained arrythmicity (locomotor activity, time course quantification of clock genes expression in the SCN) consistent with a disabled SCN. The controls used in this study were $Syt10^{Cre/Cre}$ $Bmal1^{+/-}$ mice that are rhythmic and allow us to control for *Bmal1* and *Syt10* gene expression (from our collaborators, data not shown). The genetic background of these mice was C57/Bl6 & C57/Bl6n, thus C57/Bl6 mice were used as an additional control for this study.

4.1.3 Arvicanthis Ansorgei

The Sudanian Grass Rat (*arvicanthis ansorgei*) is a diurnal rodent from the northern African grasslands. Though not extensively used in a laboratory setting, breeding facilities and previous research studies performed by the institute made this animal model available (Challet, Pitrosky et al. 2002). The laboratory colony began in 1998 using trapped animals from southern Mali, initially 10 males and 15 females. To confirm the species was correctly identified, karyotipic analysis was performed on colony animals. Arvicanthis are closer in size to laboratory rats and as such are at least 5-10 fold higher in weight as compared to mice (150-250g vs. 25-35g). All animals were maintained in 12:12 light-dark (LD) cycle under constant temperature of 22 ± 1 °C, with food and water ad libitum.

4.2 - Methods

4.2.1 PCR Genotyping

Genotyping for Opn4 mice was performed using a standard PCR protocol. Before PCR DNA concentration was determined using a standard spectrometer (BioRad). Primers used were:

Forward- Mel4: 5'– TCA TCA ACC TCG CAG TCA GC –3';

Reverse- Mel2: 5'– CAA AGA CAG CCC CGC AGA AG –3';

Forward-TodoNeo1: 5'–CCG CTT TTC TGG ATT CAT CGA C–3'.

PCR consisted of 30 cycles: 95°C for 30s, 60°C for 30s, 72°C for 1 min. WT band was seen at 290 bp, mutant band at 950 bp.

Genotyping of Syt10 mice was performed using the following protocol: 38 cycles with an annealing temperature of 65˚C using the primers-

Forward-5′-AGA CCT GGC AGC AGC GTC CGT TGG-3′;

Reverse-5′-AAG ATA AGC TCC AGC CAG GAA GTC-3′,

and for the KI Reverse-5′-GGC GAG GCA GG CCA GAT CTC CTG TG-3′

For WT, band was located at 426 bp, and for mutants, 538 bp. Bands were separated and run on a 1.5% agarose gel (Husse, Zhou et al. 2011).

4.2.2 Protein quantification through western blot

Western blot procedures were used to determine amount of the melanopsin protein in mice retinas at different time points across the day. For all western blots a standard extraction protocol was used consisting of lysis buffer: 100 µL (120 mg Tris Base 20mM, 435 mg NaCl 15mM, 500 µL Triton 1%, 18.6 mg EDTA 1mM) and16 µL protease inhibitor cocktail (Invitrogen). The lysates were then placed upon polyacrylamide gel for electrophoresis and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Boston, MA). Reaction was then blocked with 5% skim milk and membranes were incubated overnight in a cold room at 4˚ C with either: PA1-781 (1:1000, Affinity), ab65679 (1:4000, Abcam), UF006

(1:1000 Provencio), or D-18 (1:1000, Santa Cruz Biotechnologies), primary antibodies. Following this, the membranes were incubated with secondary antibodies, and then after exposition of the membranes to photoreactive film, the Opn4 protein was expected at ~53 and ~85 kDa (glycosylated form of the protein) via chemiluminescence (General Electric). Unfortunately after performing several experiments attempting to optimize the protocol conditions, the results were not convincing enough to pursue due to specificity/sensibility of the different antibodies and low level of expression of the protein in the tissue. Additionally, other teams have failed to obtain relevant and reproducible results from melanopsin western blot.

4.2.3 Surgery:

All surgical procedures were performed under deep anesthesia delivered intraperitoneally with Nembutal (68 mg/kg; University IRB-approved).

4.2.3.1 Lesion of the suprachiasmatic nuclei

Under pentobarbital anesthesia removal of the two SCNs was achieved using radio frequency lesions, according to published protocols (Easton, Meerlo et al. 2004). Lesions were achieved by heating the (250µm) tip of a Radionics (Burlington, MA) TCZ electrode to 55°C for 20 sec by passing RF current from a RFG-4 lesion generator (Radionics). Our group previously refined the various parameters to that we are able to create minimal lesions that spared surrounding brain structures. Briefly speaking, the lesions were performed stereotaxically (Kopf Instrument) with electrodes (0.3mm in diameter) introduced at the following coordinates (stereotaxic coordinates from zero ear bar, nose at +5°: lateral: +/-0.2 mm; antero-posterior: +3.4 and +3.6 mm; dorso-ventral: +0.95 mm; (Paxinos and Franklin 2001)) (Radionics Lesion Generator System) corresponding to the two SCN nuclei. Arrythmicity was confirmed by actimetry recordings under 12-12 LD cycle (10 days) and constant darkness (10 days), and effectiveness of lesions was assessed by periodogram analysis of locomotor activity (ClockLab, Actimetrics, Wilmette, IL, USA). Following the experiments lesions were verified histologically by performing Nissl staining on coronal brain sections.

4.2.3.2 EEG implantation

All animals underwent identical EEG implantation procedures, with the only exception being the size of the chip for arvicanthis. The chip used for these animals was more robust due to their increased size and activity as compared to mice.

Adult male mice and arvicanthis were implanted with a classical set of electrodes including two EEG, one reference, and two EMG electrodes at an age of 10-12 weeks at the time of surgery. Two gold-plated wires were inserted into the neck muscle tissue to record electromyogram (EMG) and two EEG electrodes were implanted on the dura skull over the right frontal and parietal cortex, respectively. In mice the electrodes were positioned in the frontal: 1.7mm lateral to midline, 1.5mm anterior to bregma, and parietal: 1.7mm lateral to midline, 1.0mm anterior to lambda.

In a subset of arvicanthis, EOG electrodes were implanted under the surface of the skin to record eye movements in order to characterize REM sleep. The five (or seven in case of EOG) electrodes were soldered to a connector and cemented to the skull. Each animal was housed in an individual cage with water and food provided ad libitum. After recovery from surgery (4-7 days) mice were connected to a swivel contact (6/12-Channel, Plastics One) through recording leads and allotted at least one week for cable adaptation. EEG, EMG and EOG signals were amplified, filtered, and their signals analog-to-digital converted and stored at 256 Hz (64-channel bipolar amplifier, Micromed France, SystemPLUS Evolution version1092).

All animals were given a 48-hour baseline assessment under 12h:12h light-dark conditions (150-200 lux- white fluorescent lights measured at the bottom of the cage using a lux meter). Following baseline, continuous sleep recordings were taken under a variety of experimental conditions. A minimum of 14 days under 12h:12h LD was used to habituate animals to their baseline condition before proceeding to the next recording condition. Prior to each experiment, a 24-hour period was assessed to ensure that the sleep wake distribution of the animals had returned to baseline levels. Sleep deprivations were performed by gentle handling (Bourgin, Fabre et al. 2007).

4.2.4 Quantitative analysis of vigilance states

The behavioral states wakefulness (W), rapid eye movement sleep (REMS), and non-REM sleep (NREMS) were visually assigned for consecutive 4s epochs, either as Wake, NREM, or REM. In arvicanthis, EOG and a video capture system was used to verify the accuracy of the scoring with physiological behavior. All recordings were scored every 4-sec based on visual inspection of the EEG and EMG, as described previously (Bourgin, Fabre et al. 2007)and without knowledge of genetic background. If epochs contained signal artifacts they were included in the analysis for state amounts, yet excluded for power spectrum (see next). Amounts spent in each vigilance state were calculated in 5 and 30 min, and 1-, 12-, and 24h intervals.

4.2.5 Power spectrum analysis of the EEG

Once scoring was completed an average spectral profile was constructed using the entire experimental period, excluding epochs marked as artifacts. All EEG signals were converted using a Discrete-Fourier Transformation (DFT) giving a power spectra between 0 and 128 Hz (0.25Hz resolution), and using a rectangular calculation with an overlapping window of 4-sec. The frequency range 49-51 Hz was omitted from the analysis due to power-line artifacts in some of the recordings. For each vigilance state and animal, an individual EEG spectral profile was constructed, using a mean average of all 4s epochs.

For all NREM sleep epochs, any time-dependent changes in EEG power for the delta (0.75-4Hz) band were examined. For epochs scored as wake, both theta (6-10 Hz) and gamma (40-70 Hz) bands were measured as EEG markers of alertness. To normalize for delta power during NREM sleep the last 4h of the (subjective) light period, the lowest period of homeostatic sleep pressure, was used and all values measured against it in mice. Spectral profiles of theta and gamma were calculated using an overlapping 10 min. window of 5-min. increments, yielding 13/hour, or for hourly values, were normalized against the lowest period of gamma and theta during the day (i.e. the least alert period). Custom programs were written for analysis in Pascal and then transformed into Visual C++.

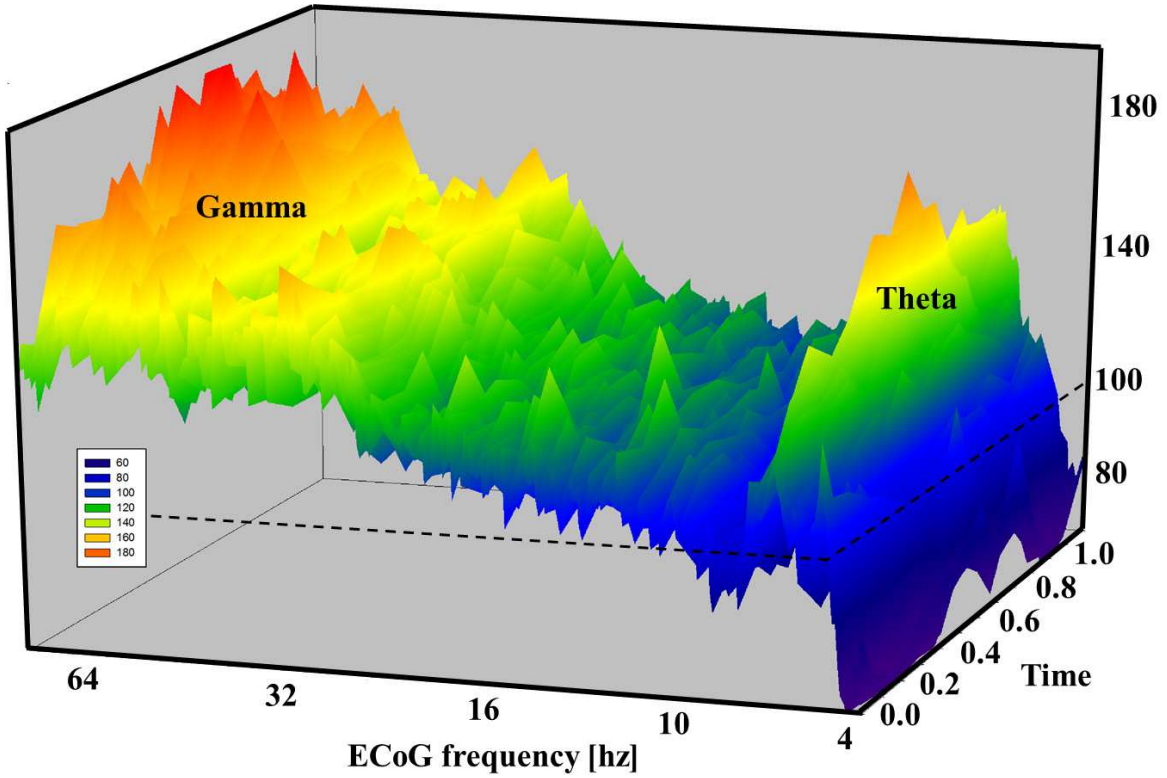
4.2.6 Actimetric recordings

Locomotor activity recording was performed using either single-wheel cages or infrared motion capture, simultaneous or not to EEG recording. When the animals were in individual cages equipped with wheels (diameter of 22cm), locomotor activity was measured based on the number of wheel turns, recorded via a standard actimetry system (DataportDP24, Minimitter). Animals were recorded for at least 10 days for each of the light/dark exposure to establish clear patterns of activity. Infrared motion detectors were used for EEG sleep recording periods, as a wheel would be prohibitive due to the data cable attached to the animal. Following acquisition, data was organized into 10 5-minute bouts of locomotor activity using software (VitalView, Minimitter). Actograms and chi-squared analysis were performed using Clocklab (Actimetrics) following data transformation via Matlab.

4.3 - Anatomy

Once experimental protocols were completed, animals were deeply anesthetized with pentobarbital and perfused with heparin/NaCl followed by transcardial fixation for 15 minutes with 4% paraformaldehyde in PBS, pH 7.4 for in situ hybridization and immunohistochemistry. After dehydration in 30 % sucrose for 48 hours, the brains were frozen and cut in a cryostat.

To prepare tissues for immunohistochemistry or in-situ hybridization, samples were removed from -80˚ C storage containers and placed in a standard cryostat at -18˚ C (Leica). Slices were then placed in a free-floating buffer solution for later use. Immunohistological and in-situ hybridizations were performed by other members of the research team, Elisabeth Ruppert and Ludivine Choteau.



Chapter 5 - Melanopsin-based photic regulation maintains a sleep-wake cycle in mice lacking a functional circadian pacemaker

Melanopsin-based direct photic regulation maintains a sleep-wake cycle in arrhythmic mice

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Main Methods (300 max): 222

Main Figure count : 3

Figure Legend (300 per fig/800 max): Fig 1 (259), Fig 2 (233), Fig 3 (218).

Supplementary Table count : 1

Supplementary Figure count: 12

Reference count (no more than 30): 2

5.1 - Abstract

Regulation of sleep and waking is classically defined as a two-process model, consisting of circadian (C) and homeostatic (S) components[1]. Light entrains process C aligning sleep and waking with time of day, and additionally exerts a direct effect promoting alertness in diurnal and wake in nocturnal, respectively[2-5]. Melanopsin (*Opn4*)-containing intrinsically photosensitive retinal ganglion cells (ipRGCs) are essential to relaying non-image-forming light (NIF) and convey this information to various areas of the brain including the master clock, the suprachiasmatic nucleus (SCN), and others[6-10]. However, what is currently unknown is whether *Opn4*-dependent direct photic regulation could also shape the sleep/wake pattern consistent with the external light-dark cycle. Here we establish that the *Opn4*-dependent direct non-circadian effects of light contribute to one third of the temporal organization of sleep and waking of mice under a normal light/dark cycle (12hL:12hD). We analyzed sleep and the EEG under various light/dark regimens in mice lacking *Opn4*, SCNs, or both, as well as mice lacking a functional clock through transgenesis ($Syn10^{Cre/Cre}Bmal^{fl/-}$). Acute light/dark effects and the temporal organization of sleep and waking under a standard 12hL:12hD schedule, were dramatically decreased in SCN-lesioned or $Opn4^{-/-}$ mice, and completely flattened in those lacking both. Clock-disabled transgenic $Syn10^{Cre/Cre}Bmal1^{fl/-}$ mice reacted similarly to SCN-lesioned (*SCNx*) *wild-type* animals. This study demonstrates for the first time, at least in nocturnal animals, that the *Opn4*-dependent direct effects of light partition sleep and waking within the daily light/dark cycle in the absence of circadian drive. Additionally, the SCN, beyond its primary function as the central clock, is one of several structures relaying these effects. These findings challenge the accepted 2-process model of sleep/wake regulation and calls for a reassessment for the role of light on not only mammalian, but specifically human physiology.

5.2 – Results

To examine the acute effects of light and darkness on sleep and waking across the day we exposed seven groups of mice to an ultradian 1-hour alternating light-dark cycle (1hL:1hD) over 24-hours and analyzed via EEG, their waking and sleep states. This allowed us to determine the direct photic input to sleep and the respective contribution of different photic pathways as arrhythmic mice such as *SCNx* and *Syn10*$^{Cre/Cre}$*Bmal1*$^{fl/-}$ do not interpret zeitgeiber time (ZT). Under this condition in control animals (*Sham Opn4*$^{+/+}$), 1-hour periods of light readily induced NREM sleep whereas dark pulses promoted wakefulness (Fig. 5.1a, Supplementary Table 5.1). Dark to light transitions also favored REM sleep (Supplementary Fig. 5.1a, b). This reactivity to light and dark was significantly higher ($P < 0.001$) during subjective dark (ZT13-ZT24) as compared to subjective light (ZT1-12). When all light/dark induced changes in wake and NREM sleep were averaged and parsed into 5-minute bouts, a clear pattern of responsiveness emerged (Fig 5.1b; for time course over 24-hours see Supplementary Fig. 5.2). *Sham Opn4*$^{-/-}$ showed similar reactivity to *Sham Opn4*$^{+/+}$ during the subjective light period but failed to respond as efficiently during the subjective dark period. In the absence of SCNs, the effects of the light and dark pulses were decreased by half, demonstrating that the SCN, independent of its clock function, exerts a strong acute influence on sleep and waking by relaying a large amount (approximately 50%), though not all, non-visual, non-circadian light information. Removal of both melanopsin and the SCNs, almost completely abolishes these responses with, over 24-hours, [18% (respectively 14% during subjective dark and 25% during subjective light phase)] of this acute reactivity to light mediated by rods/cones through brain relays exterior to the SCN (Fig. 5.1c). Acute light pulses also promoted REM sleep, and similarly to NREM, this effect was also dependent on the presence of both *Opn4* and the SCNs (Supplementary Fig. 5.1a, b). Darkness promoted waking, but this wake-enhancing effect was associated with a rapid and prolonged induction over the course of all 1h dark pulses in EEG theta (7–10 Hz) and gamma (40–70 Hz) activity (Supplementary Fig. 5.3a,b and Supplementary Fig. 5.4), (i.e. EEG correlates of alertness and exploratory behavior in rodents[4,11,12]). *Syn10*$^{Cre/Cre}$*Bmal1*$^{fl/-}$ mice showed a nearly identical lack of theta and gamma induction as compared to the *SCNx* animals. *Syn10*$^{Cre/Cre}$*Bmal1*$^{+/-}$ were similar to both *Sham Opn4*$^{+/+}$ and C57/B6, reinforcing the reliability of this control group. This alerting effect of darkness differed between genotypes and suggests that the SCN has a critical role in mediating the acute alerting effect of darkness.

Given the significance of the aforementioned acute effects of light, we assessed whether this would influence the organism for longer sustained periods, specifically if *Opn4*-based direct photic information could significantly contribute to the temporal organization of sleep and waking within the light-dark cycle. Thus we quantified the proportions of sleep and waking and their distribution across 48-hours under a 12hL:12hD cycle. The dynamics in hourly amounts of NREM sleep (Fig. 5.2a and Supplementary Table 5.1) showed a clear genotype difference, with *Sham Opn4*$^{-/-}$ mice displaying an accumulated NREM sleep deficit of approximately 1-hour, lost exclusively during the 12h light period, as we previously suggested[4] (Supplementary Fig. 5.5a). *Syn10*$^{Cre/Cre}$*Bmal1*$^{fl/-}$ showed similar reactivity to SCNx animals, consistent with previous activity recordings in these animals (Supplementary Fig. 5.5b)[13]. Time course and total sleep amounts for *Syn10*$^{Cre/Cre}$*Bmal1*$^{+/-}$ were nearly identical to both *Sham Opn4*$^{+/+}$ and C57/B6 mice. To confirm that this genotype difference was due solely to a loss of the direct component of the *Opn4* photic input and not related to a weakened circadian drive, we performed a complimentary experiment placing both *Sham Opn4*$^{+/+}$ and *Opn4*$^{-/-}$ mice under constant darkness (DD) for 24-hours. During the first 12 hours the amounts of NREM, REM, and wake in both groups under DD were similar to those of *Sham Opn4*$^{-/-}$ mice under a 12hL:12hD cycle (i.e. decreased as compared with *Sham Opn4*$^{+/+}$ under the same condition; NREM- Fig. 2b and REM/wake- Supplementary Fig. 5.6) confirming that the 1-hour sleep loss and subsequent attenuation of the daily sleep-wake cycle results is an *Opn4*-mediated direct photic regulation. The capacity of light to induce sleep was decreased but not abolished in mice lacking SCNs suggesting that the sustained sleep-promoting effect of light for longer periods is mediated partly by the SCN, yet also by other brain relays. The *SCNx* animal groups, due to missing circadian input, lacked the reduction in sleep observed in control animals during the dark period and their daily sleep-wake cycle was flattened (Fig. 5.2a, Supplementary Fig. 5.5a). *Syn10*$^{Cre/Cre}$*Bmal*$^{fl/-}$ mice displayed similar reactivity patterns to *SCNx Opn4*$^{+/+}$. Arrythmicity of this animal was verified by placing animals under DD and scoring their vigilance states (Fig. 5.2c), which showed no differences in NREM between subjective light and dark periods. In *SCNx Opn4*$^{+/+}$ a significant portion (28%) of the daily sleep/wake distribution was still entrained by light and dark phases (Fig. 5.2d), which indicates a direct sleep photic input relayed by brain areas external to the SCN. The role of the SCN remains predominant, supporting 72% of the sleep/wake cycle largely through its function as a clock, but also in relaying the direct effects of light. Nevertheless, in animals lacking both *Opn4* and SCNs the sleep/wake cycle is flat with a non-significant difference in NREM between light and dark periods representing 8% of that observed in

control animals (Fig. 5.2d). Through measurement of these inter-group differences in NREM sleep in comparison to one another we arrive at a modelization of the relative contributions of each system for the transmission of the direct effects of light (Fig 5.2e). Thus, these direct *Opn4*-mediated effects of light are responsible for 1/3 of the distribution of wake and sleep across a normal 24-hour light-dark cycle, distributed between the SCN (13%) and other extra-SCN pathways such as the VLPO (20%).

Interestingly, the light-dark difference in REM sleep amount was not affected by genotype in either *Sham* or *SCNx* mice, suggesting that regardless of its effects on NREM sleep, the direct *Opn4*-mediated light information poorly influences the 24-hour REM sleep cycle (Supplementary Fig. 5.7; Supplementary Fig. 5.8). EEG theta and gamma partition during wake was significantly attenuated during the dark period in the absence of melanopsin and was annulled in both *SCNx* groups (Supplementary Fig. 5.9), a finding that suggests the alerting effect of darkness in nocturnal animals is mostly mediated by the SCN, a structure known to influence, albeit indirectly, hippocampal activity and possibly theta/gamma rhythms generators[5,14]. This lack of induction in theta and gamma was also seen in $Syn10^{Cre/Cre}Bmal^{fl/-}$ mice, though to a lesser extent (Supplementary Fig. 5.10). Currently, there is no proof of a direct link between the alerting effects of light and *Opn4* in humans, although several studies comparing the effects of green (555nm) and blue (480nm) light suggest this relationship (for review see[15]). Our findings provide compelling supportive evidence that humans, like mice, use *Opn4*-based pathways relayed by the SCN to provide a non-circadian acute, but also a sustained alerting effect of light.

Post-mortem analysis of arginine vasopressin (AVP) and 4',6-diamidino-2-phenylindole (DAPI) via immunostaining in lesioned mice confirmed the preservation of areas surrounding the SCN in comparison to their *Sham* controls (Supplementary Fig 5.11a-d). Furthermore, detailed analysis using Cholera-Toxin B (CtB) retrograde fiber tracing, showed that retinal projections were conserved in similar proportions to those observed in Sham mice, both in highly innervated brain areas such as the ventral geniculate leaflet nucleus or the superior colliculus and in other areas less innervated but critically relevant for sleep regulation such as the VLPO (Supplementary Fig 5.11e-h). Additionally, the amplitude the direct effects of light observed in *SCNx* animals was not attenuated, implying the conservation of retinal projections innervating brains areas outside the SCN. The $Syn10^{Cre/Cre}Bmal^{fl/-}$ mice showed similar reactivity to light and dark pulses as *SCNx* $Opn4^{+/+}$ animals, confirming that effects must be a result of information passing external to it. These clock-disabled mutants

still possess the physical structure of the SCN, yet this lack of difference, suggests an anatomically dysfunctional system not only for the circadian, but also the direct effects of light. Based upon results from our previous studies and others we subjected *Syn10*$^{Cre/Cre}$*Bmal*$^{fl/-}$ mice and their controls (*Syn10*$^{Cre/Cre}$*Bmal*$^{+/-}$ and *C57/B6 WT*) to a one-hour light pulse administered during the dark period (ZT15-16)[4,16,17]. Interestingly c-Fos immunoreactivity was severely diminished in *Syn10*$^{Cre/Cre}$*Bmal*$^{fl/-}$ following the pulse and was conserved to the retino-recipient level when compared to their controls (Fig 3a-d, h). Furthermore, no AVP activation was seen in the SCN in the mutants (Fig 3e-g). This suggests that the interpretation of the direct effects of light is possible without a functioning SCN though is severely reduced. Moreover, disruption of clock gene expression, in this case *Bmal1,* seems to disable the functionality of certain light information cells in the SCN, or impede transmission to more posterior areas which project to areas of the brain involved in sleep and wake regulation.

5.3 - Discussion

One can argue that animal studies, such as the present one, do not control for sleep homeostasis. However, animal research controls for environmental and circadian zeitgeibers, genetic background, and most major advancements in chronobiology come from research that did not control specifically for process S. When deciphering the acute photic effects under a 1hL:1hD schedule, we can assume that within the time frame of a 1hr pulse, there is not enough time for the animal to build up sleep homeostatic pressure importantly. This can be compared to multiple nap protocol designed to study sleep regulation in human subjects with low sleep pressure[15]. The time-course for building up sleep pressure in mice requires several hours, leading the rodent to display recurrent naps over the course of a 24-hour day[18]. Thus, the dynamics of the homeostatic process are long enough that any effects of the 1-hour light/dark pulses are minute so as to not significantly hinder the sleep balance between the 12-hour light and dark phases.

The SCN promotes alertness and allows for the maintenance of a daily sleep-wake organization in case of weakened circadian rhythms. However, the direct effects of light now seem critical for adaptation to the light/dark cycle, characteristic of life on earth. This may explain why the direct photic input to sleep and waking is based on two phototransduction systems, melanopsin and the rods/cones, which compensate for one another in case of failure in either of the two signaling pathways. The SCN was treated for years as having one function to serve as the master circadian pacemaker in the body; however these data indicate it has an important role in the transmission of the direct effects of light. Furthermore, until now the role of the direct influence of light on sleep and waking was thought to be negligible, remaining confined to acute transitory effects. The current study demonstrates that direct photic influence corresponds to this unidentified mechanism, capable of compensating for the circadian drive to preserve a synchronization of sleep and waking with the external light/dark cycle in the absence of a functioning clock. These findings challenge the classical two-process model of sleep regulation and the way in which animals interpret light in their environment. Additionally, this calls for a reassessment for the role of light on not only mammalian, but human physiology and paves the way for the development of new concepts for better usage of societal lighting and to tackle sleep disorders and other neuropsychiatric illnesses.

5.4 – Methods Summary

Sleep and the EEG were analyzed under various lighting schedules in mice possessing a targeted disruption of the gene responsible for melanopsin (*Opn4*$^{-/-}$; see Ruby et al.[17]). A subset of mice (*Opn4*$^{-/-}$, n=8) and their littermate controls (*Opn4*$^{+/+}$, n=10) were radio frequency lesioned around the area of the suprachiasmatic nucleus, before electrode implantation for sleep recording[19]. The vigilance states for each 4-s epoch were determined to be waking, REM sleep, or NREM sleep, by visually assessing both the EEG and EMG signals without knowledge of the genotype or SCN condition according to standard criteria[20]. This allowed us to piece apart the direct effects of light from the circadian influence, as well as estimate the respective contribution of *Opn4* versus rod/cone-based phototransduction. Locomotor activity recording (actimetry) confirmed the efficiency of the lesion as all SCN-lesioned animals were arrhythmic under constant darkness (data not shown). The lack of auxiliary damage to structures surrounding the SCN and the integrity of the innervation provided by the RGCs to the remaining brain areas was confirmed by post-mortem anatomical study (Supplementary Fig. 5.11). Additionally we studied a novel mouse model *Syn10*$^{Cre/Cre}$*Bmal1*$^{fl/-}$ which contains intact anatomical structures while possessing a disabled central clock[13]. The use of complimentary approaches, based on invalidation of the clock through lesioning of transgenesis allowed us to control for the limits inherent to each model.

5.5 - Figures

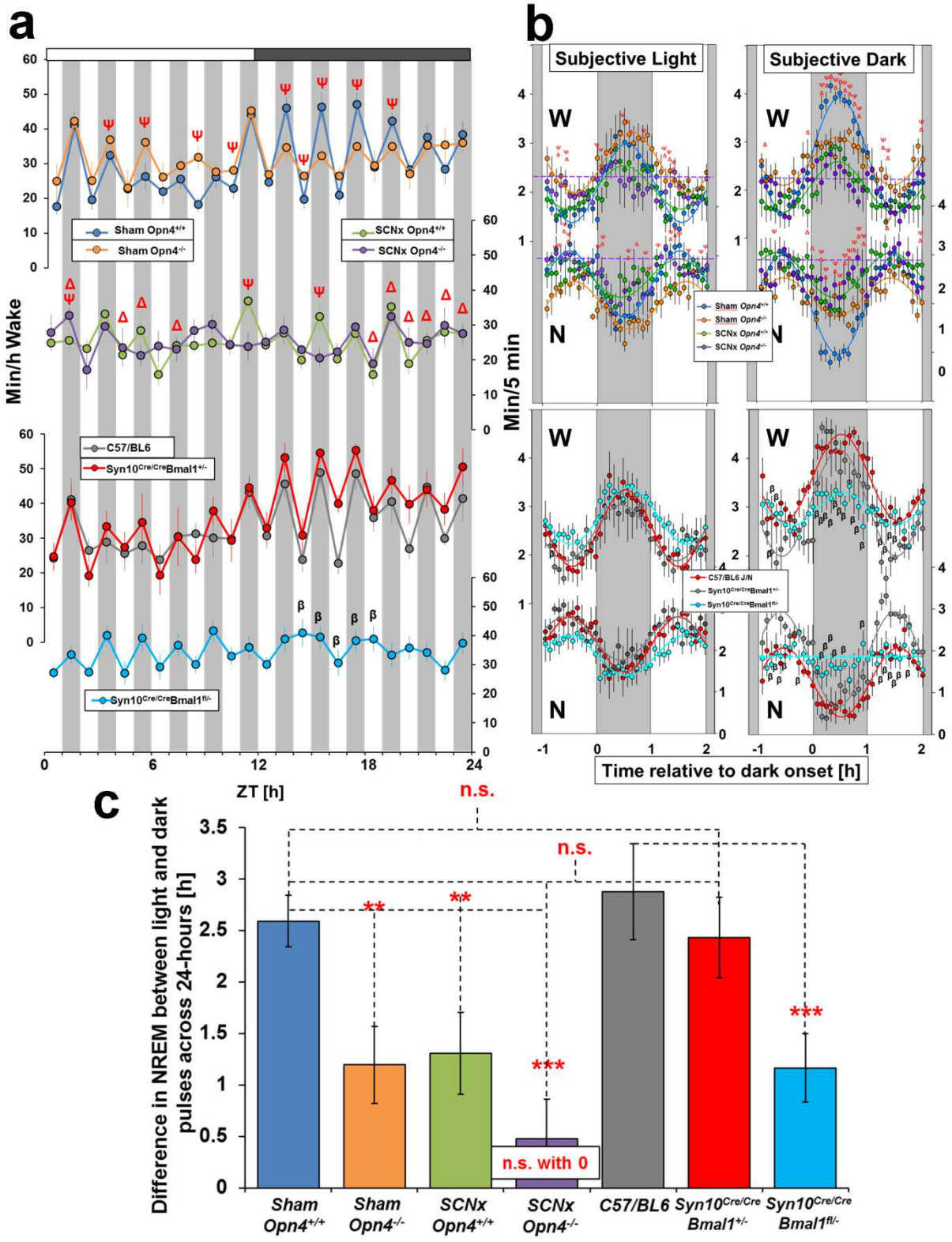


Figure 5.1: Direct effects of repeated 1-hour light and 1-hour dark pulses on sleep and waking under a 24-hour 1hL:1hD ultradian cycle

(a) Hourly mean values of wake during 1hL:1hD cycle. Top white and grey horizontal bars mark the subjective light and dark periods. Hourly light pulses suppressed wakefulness whereas dark pulses induced the opposite. This effect was greater during subjective dark and significantly attenuated in *Syn10*$^{Cre/Cre}$*Bmal1*$^{fl/-}$ mice, similar to *SCNx Opn4*$^{+/+}$mice. (b) Average time-course for LD-induced changes in wake, NREM, and REM during the 12-h subjective light and dark periods in *Sham* and *SCNx Opn4*$^{+/+}$ and *Opn*$^{-/-}$ and *Syn10*$^{Cre/Cre}$*Bmal1*$^{fl/-}$ and their controls. Values represent means (± s.e.m.) over 5-min intervals in the hour preceding, during, and following the six 1-h dark pulses. Sine waves were calculated to show best fits for the data points. Reactivity was flat during subjective dark for NREM in *Syn10*$^{Cre/Cre}$*Bmal1*$^{fl/-}$ and is represented with a dashed line. A three-way ANOVA with factors “genotype”, “time of day” (subjective light versus subjective dark period), and “time course” (5-min values) revealed that for wake (W), NREM (N), and REM (R), time course was significantly affected by genotype (W: $P = 0.004$; N: $P < 0.0001$) and genotype (W: $P = 0.003$; N: $P = 0.028$) (interactions: genotype x time of day W: $P < 0.001$, N: $P = 0.02$; genotype x time course: W: $P < 0.001$ and N: $P = 0.001$). Red phi symbols denote significant genotype differences between *Syn10*$^{Cre/Cre}$*Bmal1*$^{fl/-}$ and *C57/Bl6* wild-type controls. (c) NREM differences between the two surrounding light pulses and inclusive dark pulse were quantified and averaged across the 24-hour period. All values shown are mean ± s.e.m. (C57/Bl6 n=7; *Syn10*$^{Cre/Cre}$*Bmal1*$^{+/-}$ n=6; *Syn10*$^{Cre/Cre}$*Bmal1*$^{fl/-}$ n=8)

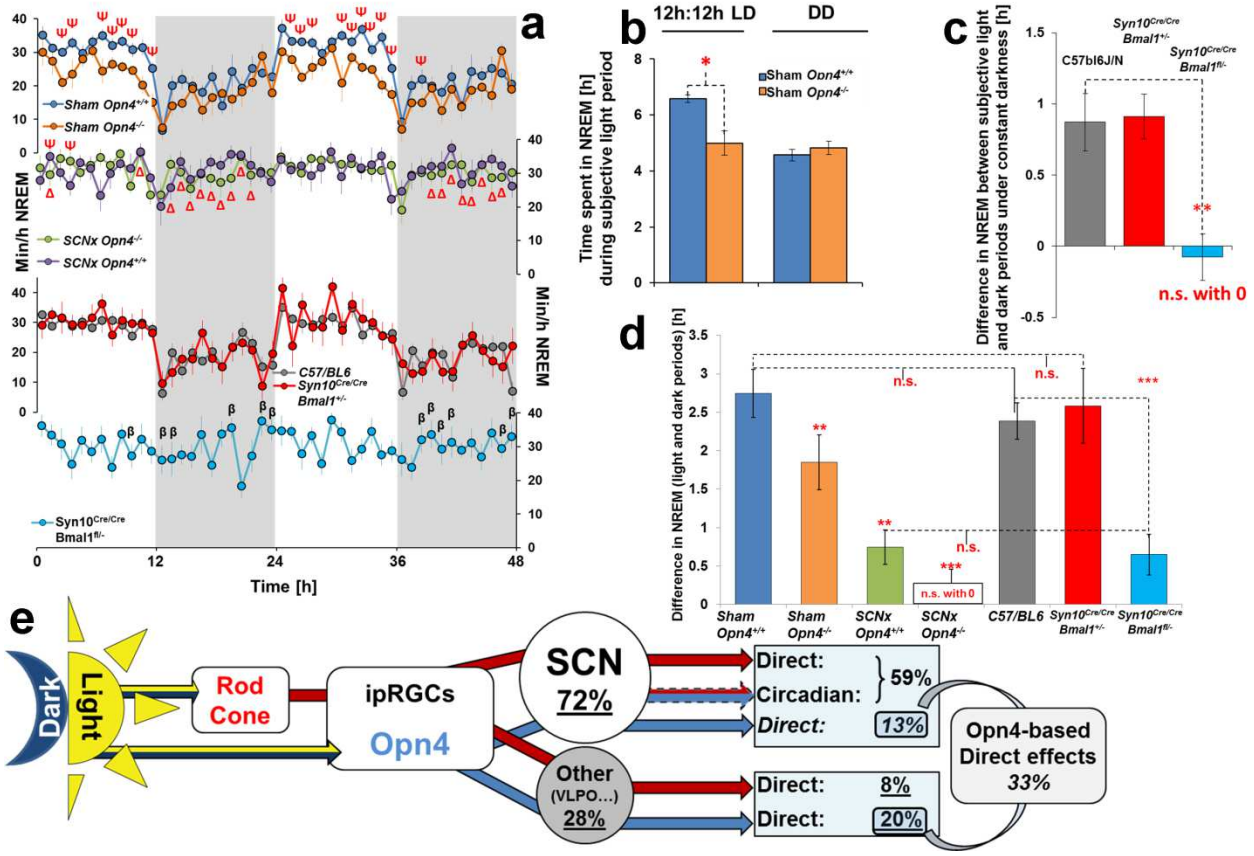


Figure 5.2: Time course of NREM sleep under a standard 12hL:12hD cycle

(a) Min/h NREM. *Sham Opn4*$^{-/-}$, genotype differences that were abolished in the *SCNx* animals, as was the NREM circadian distribution. *Syn10*$^{Cre/Cre}$*Bmal1*$^{fl/-}$ showed similar reactivity to their wild-type controls during the light period, yet diverged greatly during dark. (b) Both *Sham Opn4*$^{+/+}$ and *Opn4*$^{-/-}$ exposed to DD show similar NREM levels during subjective light period as *Sham Opn4*$^{-/-}$ under 12hL:12hD. (c) Light-Dark difference in NREM between subjective light and subjective dark under 24-hours of DD in *Syn10*$^{Cre/Cre}$*Bmal1*$^{fl/-}$ and their controls. These animals are not significant with zero under DD. (d) Differences in NREM between the light and dark periods showed significant changes depending on genotype and/or SCN condition. *SCNx Opn4*$^{-/-}$ L-D difference in NREM was not significantly different from zero. *Syn10*$^{Cre/Cre}$*Bmal1*$^{fl/-}$ mice were not significantly different in terms of reactivity from arrhythmic *SCNx Opn4*$^{+/+}$. (e) Model representing the different pathways by which the daily light/dark cycle shapes the 24-hour temporal organization of sleep and waking. The calculations are based upon inter-group differences according to data in c and allow to estimate the relative contributions of the melanopsin and rods/cones encoding pathways as well as the relative contributions of SCN and extra-SCN relays to

mediate the direct photic input to sleep and waking (for detailed calculations see supplementary methods). Values represent mean ± SEM (for both days *Sham Opn4*$^{+/+}$ n=9; *Sham Opn4*$^{-/-}$ n=7; *SCNx Opn4*$^{+/+}$ n=10; *SCNx Opn4*$^{-/-}$ n=8; *C57/Bl6* n=7; *Syn10*$^{Cre/Cre}$*Bmal1*$^{+/-}$ n=7; *Syn10*$^{Cre/Cre}$*Bmal1*$^{fl/-}$ n=10).

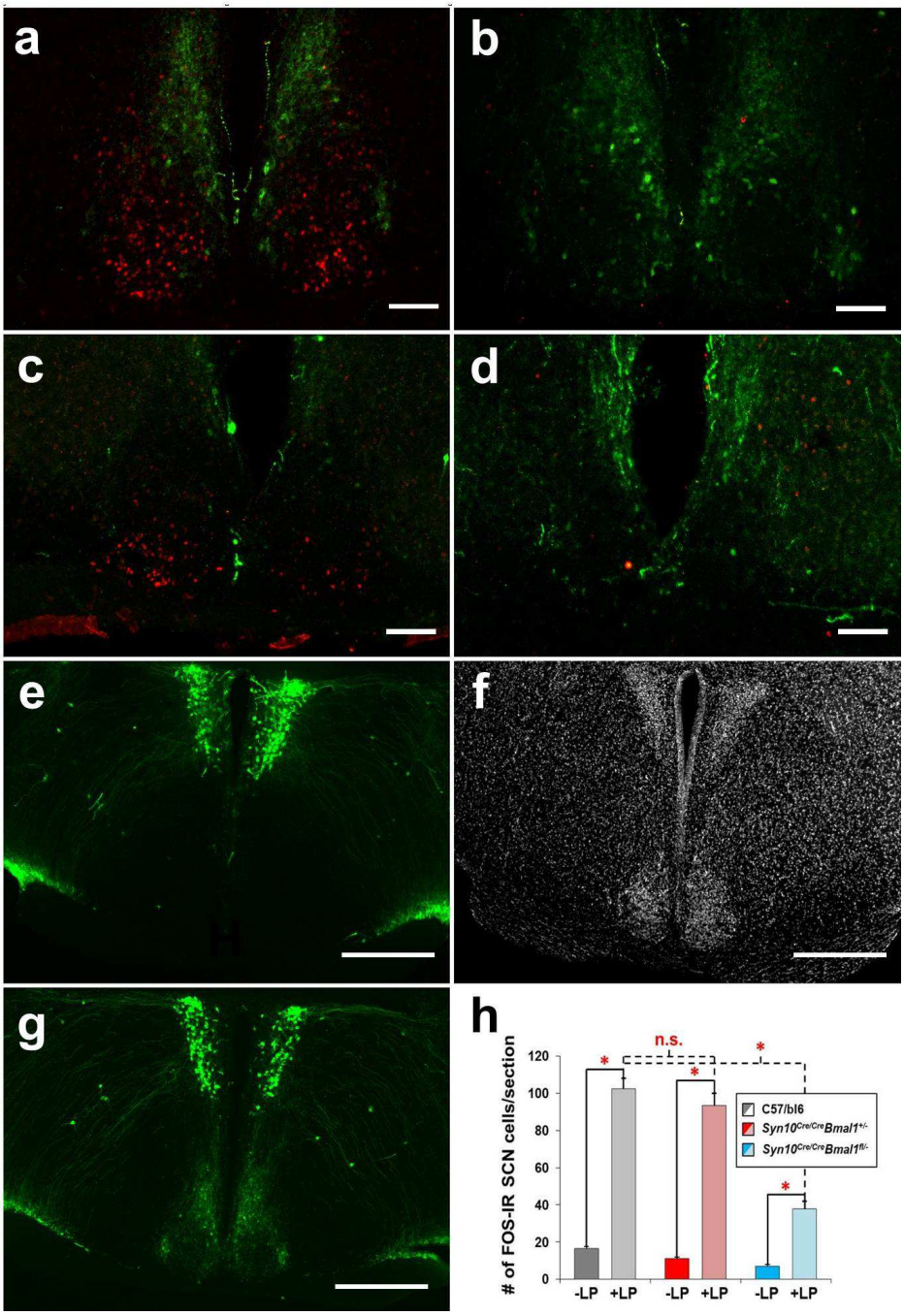


Figure 5.3: Effects of a 1-h light pulse on c-Fos immunoreactivity in the SCN in *Syn10$^{Cre/Cre}$Bmal1$^{+/-}$* and *Syn10$^{Cre/Cre}$Bmal1$^{fl/-}$*mice

Effects of the LP administered during the habitual dark period (ZT15-16) on c-Fos immunoreactivity in the SCN of *Syn10$^{Cre/Cre}$Bmal1$^{+/-}$* and *Syn10$^{Cre/Cre}$Bmal1$^{fl/}$* mice (a-d). Few c-Fos immunoreactive cells (labeled red) are found in the dorsomedial (arginine-vasopressine [AVP]-containing SCN (labeled green) in control (no LP) animals (b and d). Light induced c-Fos in the retino-recipient zone of the SCN in WT (data not shown), in *Syn10$^{Cre/Cre}$Bmal1$^{+/-}$* (a) and to a lesser extend in the *Syn10$^{Cre/Cre}$Bmal1$^{fl/}$* mice (c).

Syn10$^{Cre/Cre}$*Bmal1*$^{fl/}$ mice show a dysfunctional SCN (a-g). AVP -staining of the SCN is preserved in *Syn10*$^{Cre/Cre}$*Bma1l*$^{+/-}$ mice (a,b,g) and comparable of that found in WT animals (data not shown). Mutant mice do not show AVP expression in the SCN, whereas it remains unaltered in adjacent regions (c-e). The SCN is structurally intact as shown by DAPI-staining (grey) (f). Scale bar in a-d indicate 100µm, and in e-g 500µm.Histogram represents the number of c-Fos immunoreactive neurons per SCN section in control WT, *Syn10*$^{Cre/Cre}$*Bma1l*$^{+/-,}$ and *Syn10*$^{Cre/Cre}$*Bmal*$^{fl/}$ mice after a 1h LP (mean ±sem) (h). In mice lacking a functional SCN an induction was observed though was significantly reduced (63%) (Two-way ANOVA, light pulse effect: $p<0.001$; an asterisk (*) indicates post hoc Fisher PLSD: $p<0.05$. No significant differences were observed between the two control genotypes. A two-way ANOVA, the star indicates light pulse (genotype interaction: $p<0.001$).

Supplementary Figures

Schedule	Time Spent	Condition	*Opn4* Genotype	Waking (h)	NREMS (h)	REMS (min)
LD 12:12	12-h light period	*Sham*	-/-	5.48±0.48	5.23±0.45	79.9±3.3
			+/+	4.14±0.10	6.58±0.14	76.8±3.7
		SCN Lesion	-/-	4.71±0.28	6.24±0.21	63.1±4.6
			+/+	4.50±0.19	6.43±0.16	64.4±2.6
	12-h dark period	*Sham*	-/-	7.80±0.29	3.44±0.27	40.0±3.7
			+/+	7.38±0.30	3.96±0.27	39.9±3.8
		SCN Lesion	-/-	4.90±0.40	6.06±0.30	62.3±6.5
			+/+	5.33±0.21	5.68±0.17	52.1±3.0
	LD difference	*Sham*	-/-	1.57±0.81	1.29±0.49	34.2±4.5
			+/+	3.35±0.20	2.75±0.12	36.0±0.1
		SCN Lesion	-/-	0.19±0.12	0.18±0.28	0.79±1.9
			+/+	0.83±0.03	0.75±0.02	12.3±0.4
	24 h	*Sham*	-/-	13.49±0.61	8.69±0.53	125.8±8.3
			+/+	11.62±0.43	10.44±0.43	116.6±8.3
		SCN Lesion	-/-	9.61±0.68	12.30±0.52	125.4±11.2
			+/+	9.82±0.40	12.11±0.33	116.48±5.7
LD 1:1	*Subjective* 12-h light period	*Sham*	-/-	6.25±0.44	4.79±0.33	56.72±9.0
			+/+	5.30±0.19	5.73±0.22	59.0±7.6
		SCN Lesion	-/-	5.10±0.18	5.87±0.17	52.4±2.2
			+/+	5.12±0.15	6.03±0.14	50.8±2.6
	Subjective 12-h dark period	*Sham*	-/-	6.31±0.31	5.06±0.43	37.7±8.6
			+/+	6.79±0.23	4.50±0.20	39.6±7.6
		SCN Lesion	-/-	5.13±0.22	6.03±0.23	50.1±2.6
			+/+	5.06±0.16	6.11±0.15	49.98±1.5
	24 h	*Sham*	-/-	12.56±0.65	9.86±0.49	94.4±17.4
			+/+	12.08±0.31	10.23±0.32	98.6±14.8
		SCN Lesion	-/-	10.23±0.31	11.9±0.25	102.6±3.3
			+/+	10.18±0.25	12.14±0.25	100.75±3.2
	12 1-h Light periods	*Sham*	-/-	5.49±0.38	5.55±0.38	57.4±12.2
			+/+	4.54±0.16	6.41±0.18	62.3±8.8
		SCN Lesion	-/-	4.83±0.18	6.29±0.16	53.0±4.7
			+/+	4.36±0.22	6.73±0.22	55.2±2.7
	12 1-h Dark periods	*Sham*	-/-	7.07±0.20	4.31±0.19	37.0±12.1
			+/+	7.54±0.24	3.82±0.22	38.6±5.6
		SCN Lesion	-/-	5.41±0.36	5.78±0.33	48.6±2.7
			+/+	5.82±0.26	5.42±0.24	45.6±2.7

Supplementary Table 5.1: Time spent in NREM, REM, and wake under the 12hL:12hD and 1hL:1hD conditions for *Sham* and *SCNx Opn4*$^{-/-}$ and their controls

12hL:12hD - Light vs. Dark 3-way ANOVA genotype x SCN x TIME: genotype x SCN (W: $P = 0.01$, N: $P = 0.002$); SCN x TIME (W: $P = 0.004$, N: $P = 0.003$, R: $P < 0.0001$); genotype x TIME (W: $P < 0.0001$, N: $P < 0.0001$)

24-hr - 2-way ANOVA genotype x SCN: genotype (W: $P = 0.04$, N: $P = 0.04$; SCN $P < 0.0001$)

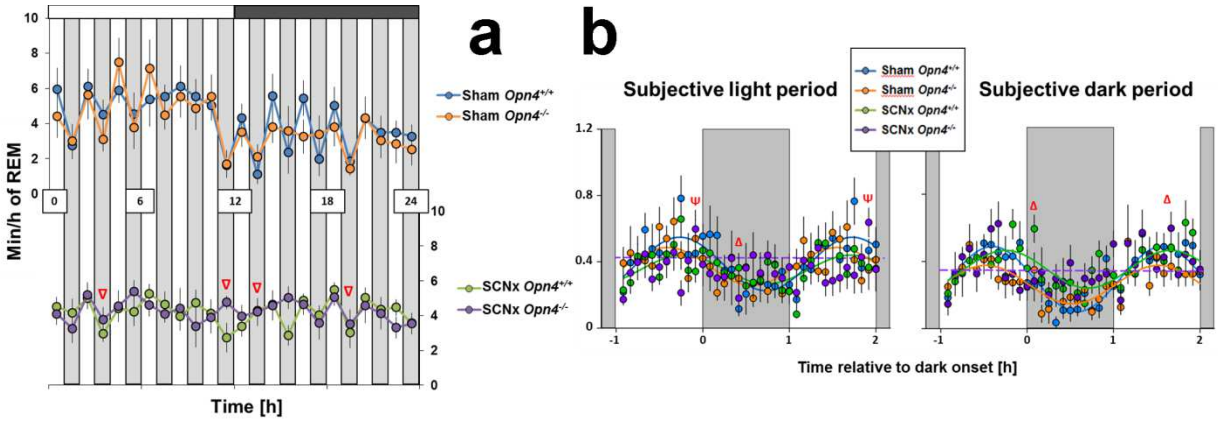
1hL:1hD - Light vs. Dark 3-way ANOVA genotype x SCN x TIME: genotype x SCN x TIME (W: $P = 0.009$, N: $P = 0.015$); SCN x TIME (W: $P = 0.007$, N: $P = 0.04$, R: $P < 0.0001$); genotype x TIME (W: $P = 0.02$, N: $P = 0.007$)

24-hr - 2-way ANOVA genotype x SCN: SCN (W: $P < 0.0001$, N: $P < 0.0001$)

12x L vs. D pulses - 3-way ANOVA genotype x SCN x TIME: SCN x TIME (W: $P = 0.005$, N: $P = 0.008$, R: $P = 0.02$); genotype x TIME (W: $P = 0.01$, N: $P = 0.005$)

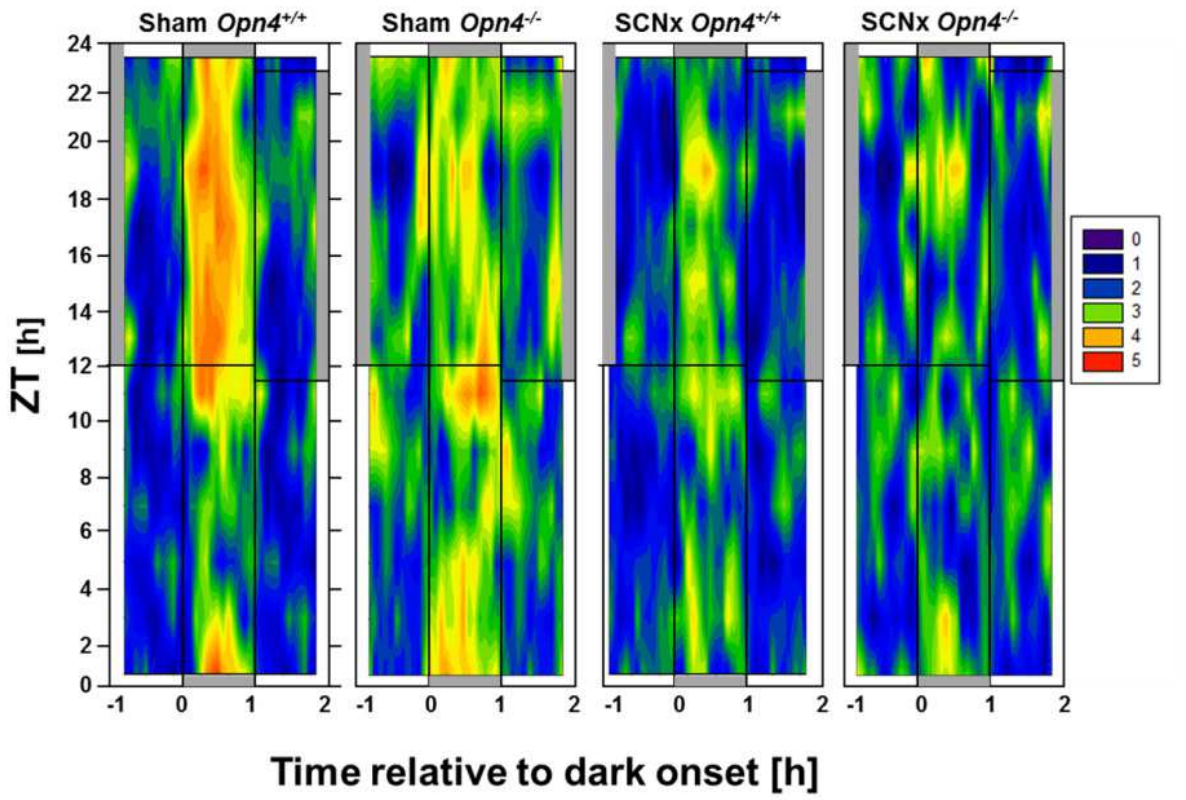
(a) Indicates significant genotype difference (post-hoc $P < 0.05$); (b) Indicates significant SCN difference (post-hoc $P < 0.05$); (c) Indicates significant difference between subjective 12-h D and L cycles (post-hoc $P < 0.05$)

Values represent mean ± s.e.m. (for 12hL:12hD *Sham Opn4*$^{+/+}$ n=9; *Sham Opn4*$^{-/-}$ n=7; *SCNx Opn4*$^{+/+}$ n=10; *SCNx Opn4*$^{-/-}$ n=8 & for LD 1:1 *Sham Opn4*$^{+/+}$ n=10; *Sham Opn4*$^{-/-}$ n=7; *SCNx Opn4*$^{+/+}$ n=10; *SCNx Opn4*$^{-/-}$ n=8)



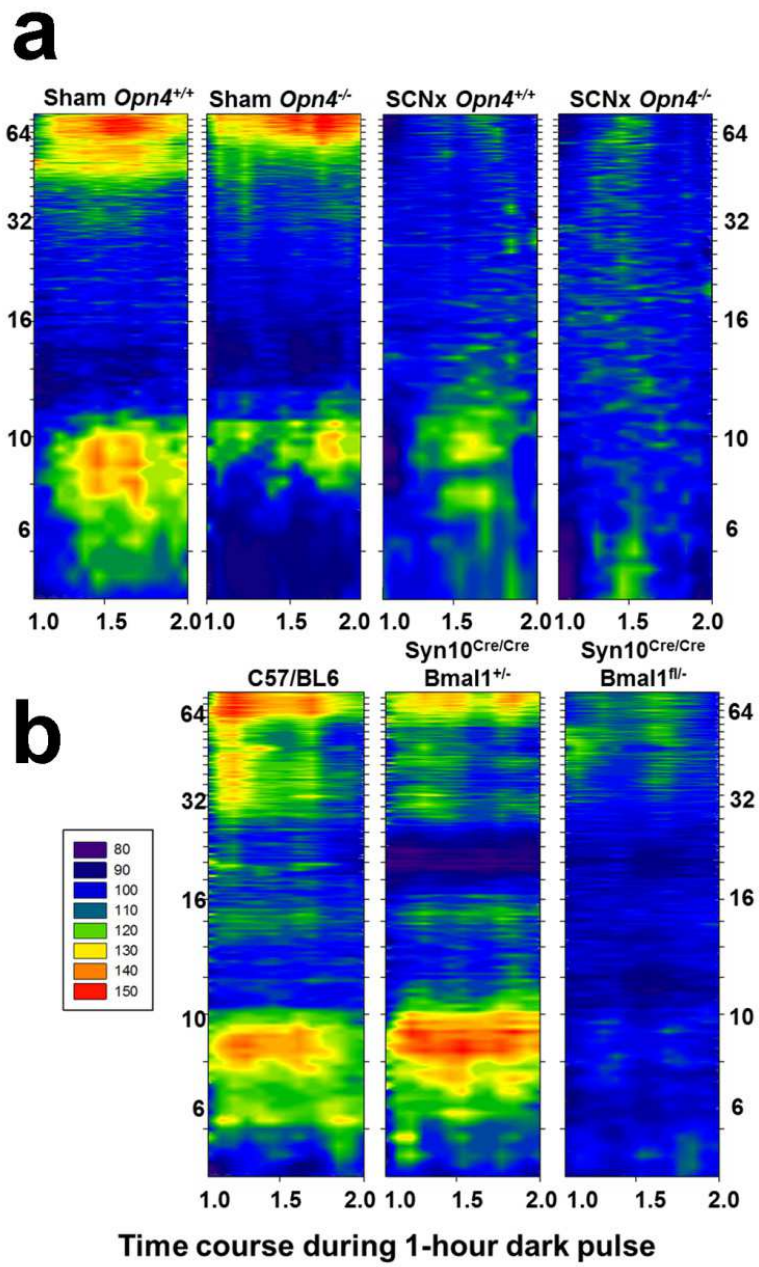
Supplementary Figure 5.1: Total REM during 1hL:1hD cycle

(a) Total REM per hour under 1hL:1hD cycle (compare to Fig. 1a). Intergroup differences were less severe as compared to other vigilance states. A 3-way ANOVA with factors "genotype", "SCN condition", and "time-course", showed significance for SCN condition x time-course ($P < 0.0001$). 2-way ANOVA (SCN condition x genotype), for each hour showed SCN condition significance at certain times over the 24 hour 1hL:1hD cycle. (b) Similar to Supplementary Fig. 2 this shows the kinetic reactivity of REM sleep during the subjective light vs. dark periods of the 1hL:1hD cycle. Sine-waves have been fitted to data when available (dashed lines indicate sine-wave was not able to be calculated). Reactivity for all four groups is relatively the same regardless of time of day. A 4-way ANOVA with factors "genotype", "SCN condition", "time-of-day", and "time-course": time-course x time-of-day x genotype $P = 0.002$; time-course x genotype $P = 0.002$. A 2-way ANOVA (SCN x genotype), for each 5-min bout, showed SCN condition significance ($P < 0.05$) at certain times during the light and dark pulses.



Supplementary Figure 5.2: Time-of-day-dependent changes under 24-hour 1hL:1hD cycle

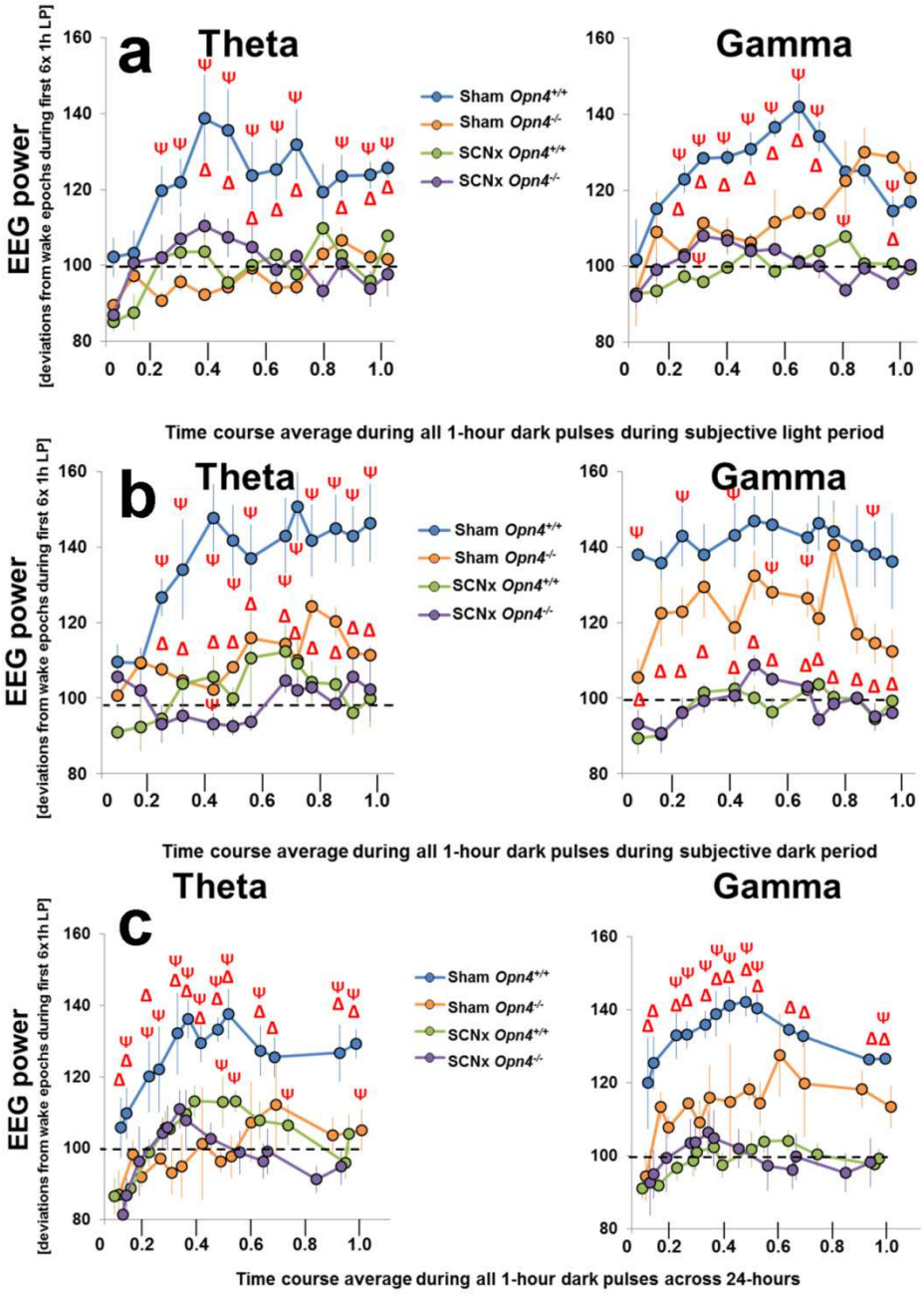
Waking values expressed as minutes/5 minute bout. In Sham Opn4-/-, mice do not have as strong an induction of wake from the dark pulse, especially during the subjective dark period, as compared to *Sham Opn4*$^{+/+}$. *SCNx Opn4*$^{+/+}$ still react to the dark pulse but at a much lower interval, and does not seem to be dependent on circadian time-of-day. *SCNx Opn4*$^{-/-}$ mice are unable to anticipate or react to the dark pulses regardless of ZT. X-axis numbers denote the time before and after the dark pulse (centered).



Supplementary Figure 5.3: Heat map of power spectrum in waking EEG during the 1hL:1hD cycle

averaged over the 6 1-hour dark pulses across the 24-hours of the ultradian cycle, shown as a percentage of individual waking EEG spectra (taken as reference) during the first 6 1-hour light pulses of the 1hL:1hD cycle (=100%). Warmer colors are increases and colder colors, decreases, relative reference EEG waking activity. Note that the dark-induced Theta (7–10 Hz) and gamma (40–70 Hz) EEG activities depend significantly on time of day. (b) C57/Bl6 and *Syn10*$^{Cre/Cre}$*Bmal1*$^{+/-}$ controls showed similar results to Sham animals (a), yet

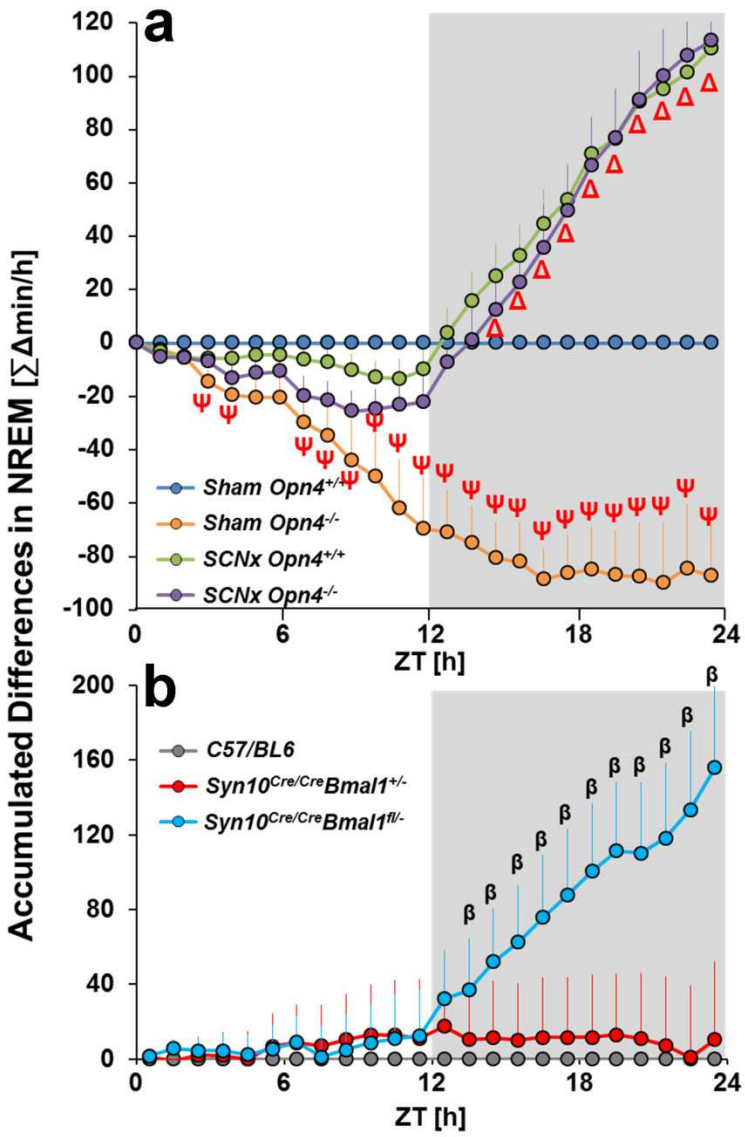
Syn10$^{Cre/Cre}$*Bmal1*$^{fl/-}$ was drastically different, showing very little evidence of an alerting effect from the dark pulses in either subjective period, similar to SCNx animals.



Supplementary Figure 5.4: Theta/Gamma reactivity during the 12 1-hour dark pulses during subjective light and dark periods, and over 24-hours under the 1hL:1hD cycle

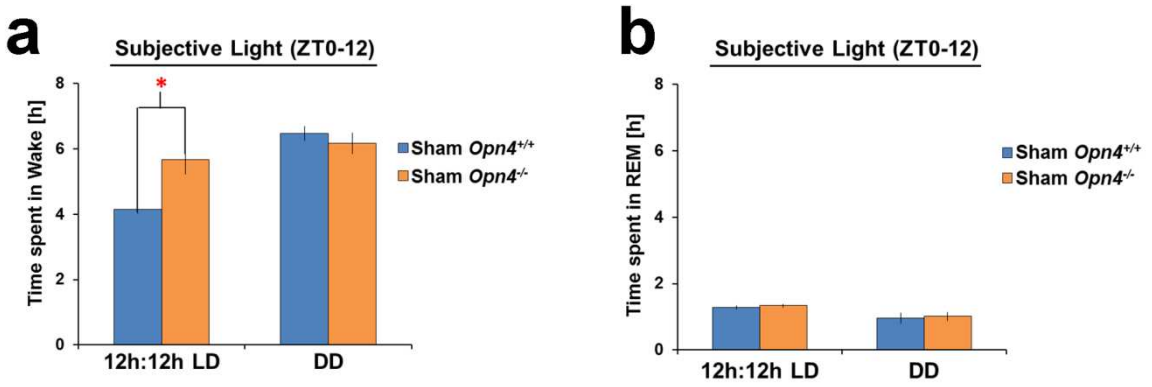
As shown in supplementary figure 5a summarized here for the theta (7–10 Hz) and gamma (40–70 Hz) frequency bands. A delayed and lower activation of both theta and gamma is present in in *Sham Opn4*$^{-/-}$ mice as compared to *Sham Opn4*$^{+/+}$ controls. Subjective Light (a), Subjective Dark (b), and 24-hour (c) are shown here. A 3-way repeated measures ANOVA

with factors “genotype”, “SCN condition”, “time-course”: Theta (Subjective Light: time-course x genotype $P = 0.001$, time-course x genotype x SCN condition $P < 0.001$; Subjective Dark- time-course x genotype $P = 0.01$, time-course x SCN condition $P < 0.0001$, time-course x genotype x SCN condition $P = 0.027$; 24-hours- SCN condition $P = 0.003$, time-course $P = 0.0005$). Gamma (Subjective Light- time-course x genotype $P < 0.001$, time-course x genotype x SCN condition $P < 0.001$; Subjective Dark- time-course x genotype $P = 0.003$, time-course x SCN condition $P < 0.0001$; 24-hours- SCN condition $P < 0.001$, time-course $P < 0.001$.) Post-hoc analysis shows significance for genotype and SCN condition depending on the time during the dark pulses. Psi symbols indicate significant genotype differences and delta SCN condition differences.



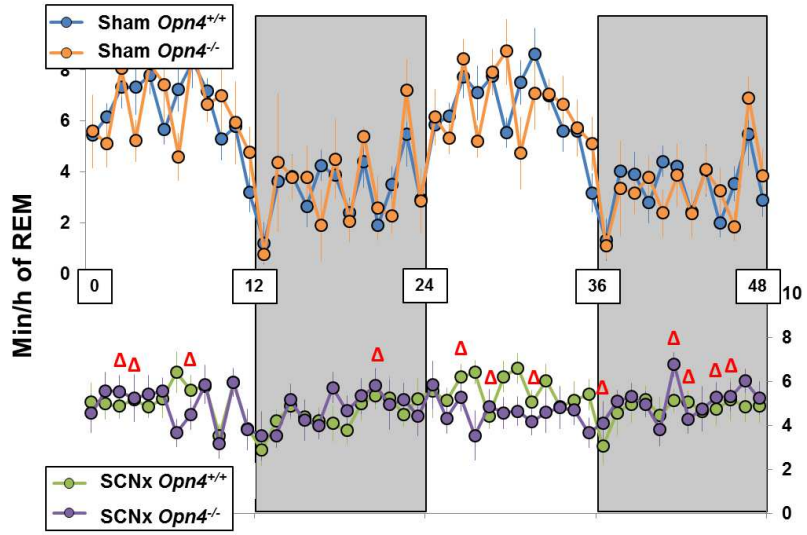
Supplementary Figure 5.5: Dynamics of the accumulated differences (average of 2 baseline days).

(a) *Sham Opn4*$^{-/-}$ mice lose approximately 1-hour of NREM during the 12-hour light period and is significantly attenuated in the absence of SCNs. (b) *Syn10*$^{Cre/Cre}$*Bmal1*$^{+/-}$ and C57/Bl6 behave nearly identically. *Syn10*$^{Cre/Cre}$*Bmal1*$^{fl/-}$ show a similar pattern to *SCNx* animals though at a slightly higher rate during the dark period.



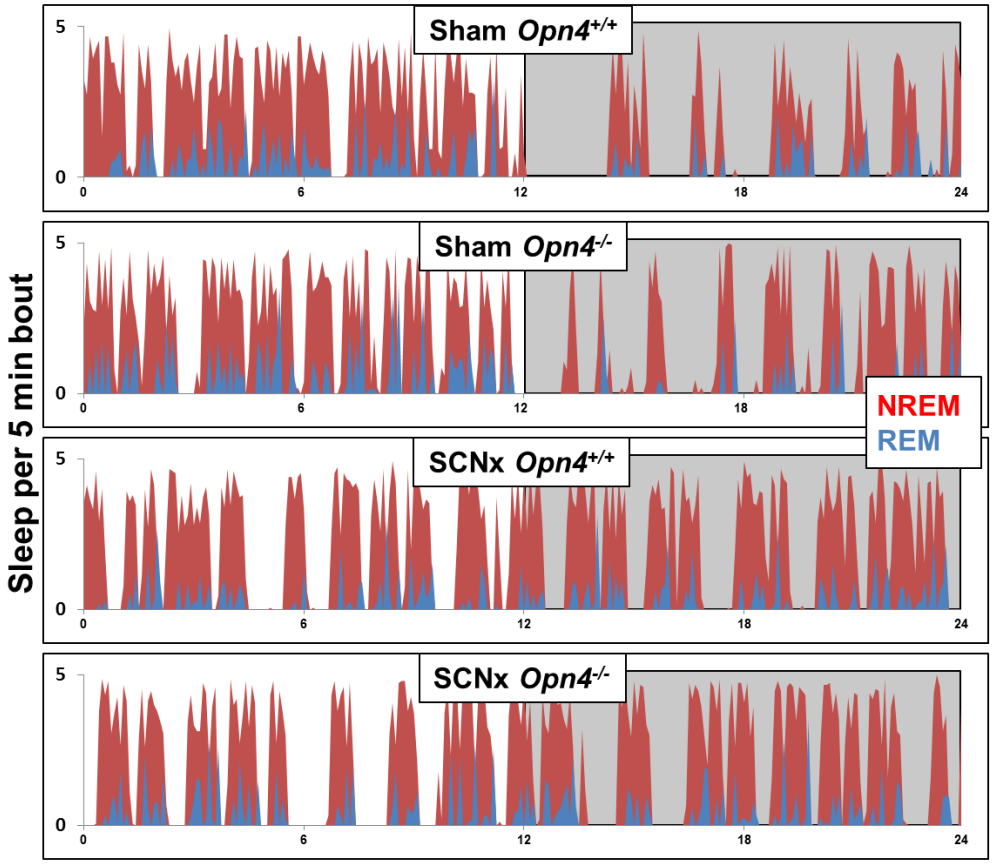
Supplementary Figure 5.6: Wake, and REM per hour during subjective light period-12h:12h LD vs. 24h DD

Comparison of the first 12 hours of the baseline condition with the same period (subjective light) as constant darkness (DD) showed similarities in the two *Sham* genotypes. Under constant darkness both groups behaved as though *Opn4* was not present A 2-way ANOVA with factors "genotype", "light condition" showed high significance ($P = 0.006$) between 12hL:12hD and DD during the subjective light period, but not between genotypes in either case. (*Sham Opn4*$^{+/+}$, n=5; *Sham Opn4*$^{-/-}$, n=7)



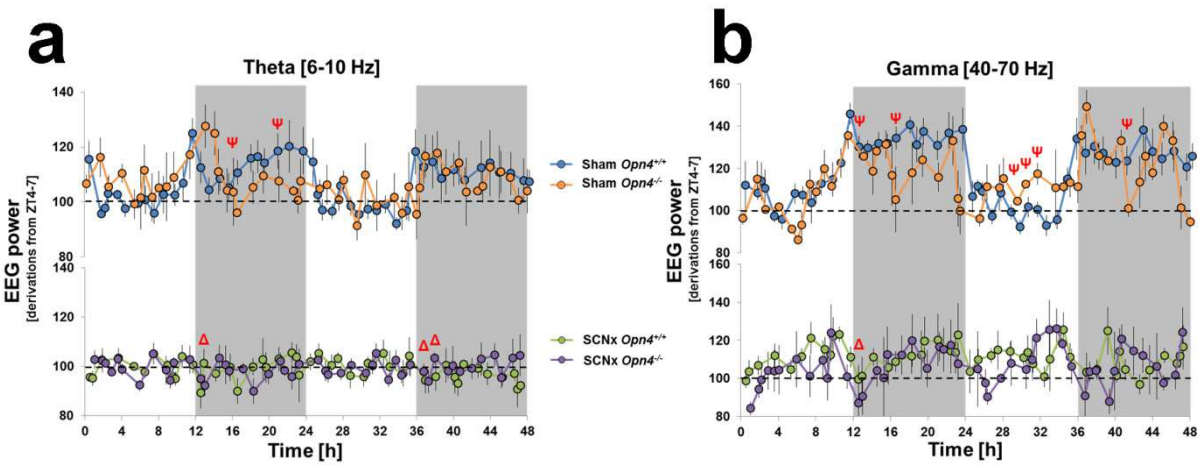
Supplementary Figure 5.7: Total REM during 12hL:12hD

Intergroup differences were less severe during 12hL:12hD as compared to other vigilance states. A 3-way ANOVA with factors genotype, SCN condition, and time course showed significance for "SCN condition", "time-course" ($P < 0.0001$). 2-way ANOVAs (SCN condition x genotype) for each hour showed SCN condition significance ($P < 0.05$) at certain times over the 48-hrs of baseline.



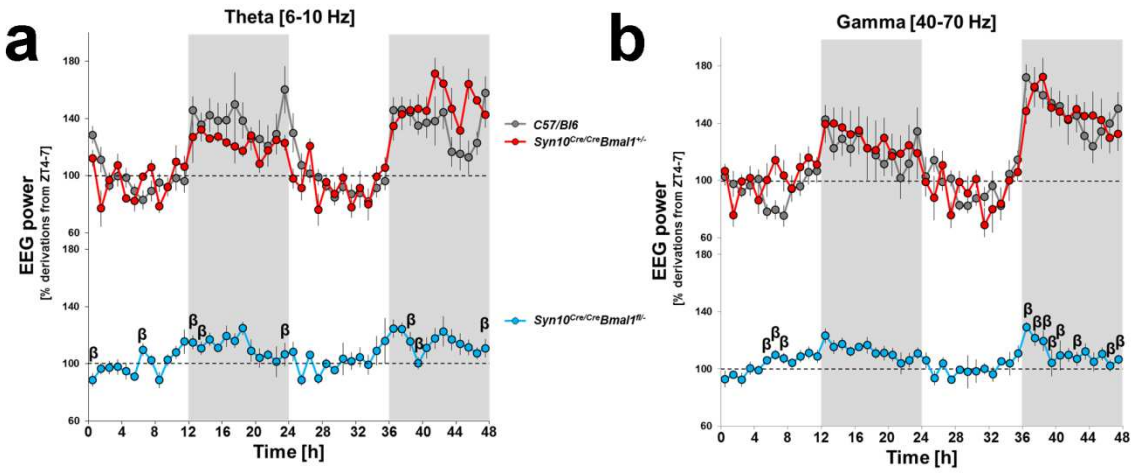
Supplementary Figure 5.8: Example of NREM/REM distribution for each group under 12hL:12hD

Overview of NREM (red) and REM (blue) sleep expressed per 5-min intervals in one animal from each experimental group under a 24-hour 12hL:12hD condition.



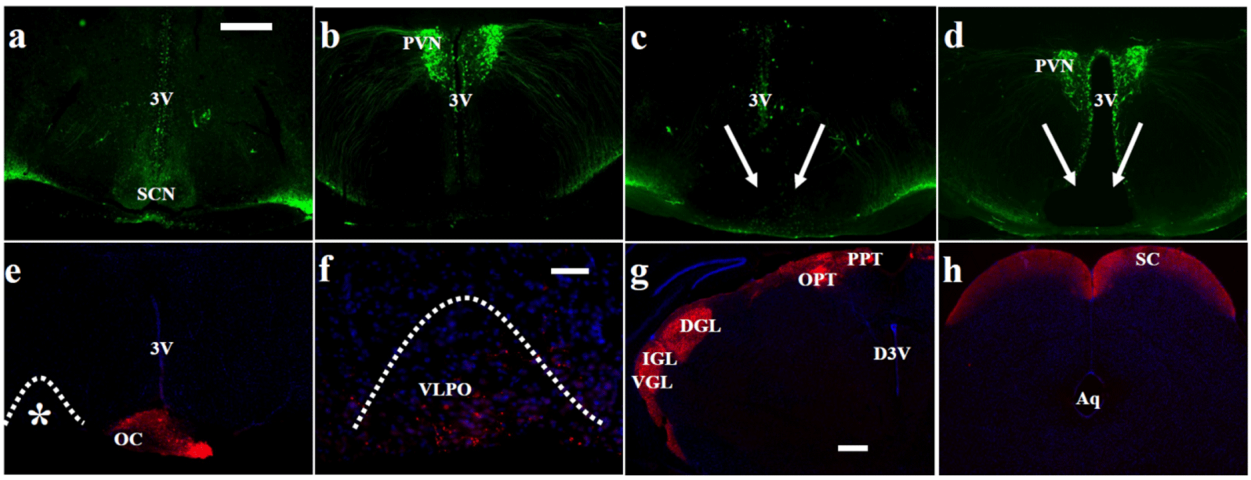
Supplementary Figure 5.9: Theta and Gamma per hour of wake under 12hL:12hD in SCN-lesioned Opn4$^{-/-}$ mice and their controls

A 3-way ANOVA was performed during light and dark to compare SCN condition and genotype effect (genotype x SCN condition x time-course) of changes in theta (a) and gamma (b) across the 48-hours of 12hL:12hD. No significant differences were observed during the subjective light period. During dark significant differences were seen between genotype (theta: $P < 0.001$; gamma: $P = 0.011$) and between SCN condition (theta: $P = 0.002$; gamma: $P < 0.001$). Post-hoc analysis shows significance for genotype and SCN condition depending on the time during the pulse. Psi symbols indicate significant genotype either between *Sham* or *SCNx* animals, and delta represents significant SCN condition differences. EEG power was normalized against the period of lowest power for theta and gamma (ZT4-7 for each day). For day 1 (*Sham Opn4*$^{+/+}$ n=8; *Sham Opn4*$^{-/-}$ n=6; *SCNx Opn4*$^{+/+}$ n=5; *SCNx Opn4*$^{-/-}$ n=5), and day 2 (*Sham Opn4*$^{+/+}$ n=7; *Sham Opn4*$^{-/-}$ n=4; *SCNx Opn4*$^{+/+}$ n=5; *SCNx Opn4*$^{-/-}$ n=5)



Supplementary Figure 5.10: Theta and Gamma per hour of wake under 12hL:12hD in Syn10$^{Cre/Cre}$Bmal1$^{fl/-}$ and their controls

One way ANOVA between WT controls and Syn10$^{Cre/Cre}$Bmal1$^{fl/-}$ were significant ($p<0.05$) ofchanges in theta (a) and gamma (b) during certain hours across 48-hours of 12hL:12hD. Significance is represented by beta symbols. Power spectrum is calculated as in Supp. Fig. 9. For day 1 and 2 (*C57/B6* n=6; *Syn10$^{Cre/Cre}$Bmal1$^{+/-}$* n=5; *Syn10$^{Cre/Cre}$Bmal1$^{fl/-}$* n=8).



Supplementary Figure 5.11: Anatomic control of SCN lesion and preservation of retinal projections to the brain

Top: Coronal sections at the mid (a, c) and caudal (b, d) level of the SCN in a Sham (a-b) and SCN-lesioned mouse (c-d) stained for AVP (green), a marker expressed in the SCN (shell). Note the complete removal of both SCNs, yet small lesion size. Bottom: Distribution of retinal projections to the brain: Staining of cholera toxin subunit b (CtB-red) and DAPI (blue) in an SCN-lesioned mouse at the level of the (e) Optic chiasm; (f) VLPO (Enlargement of VLPO fibers from * in e); (g) geniculate leaflets (DGL, IGL,VGL), and pretectal nuclei (OPT, PPT) and (h) superior colliculus (SC). Retinal fiber tracing via CtB shows an intact system following SCN-lesion with fibers through the optic chiasm reaching areas of lower innervation but critical for sleep regulation such as the VLPO as well as the brain targets known to be densely innervated (DGL, IGL,VGL, OPT, PPT, SC). Scale bars in (a-e) indicate 500µm, in (f) 20µm and in (g-h) 400µm.

5.6 – Materials and Methods

Animals

All experiments were performed on adult male *Opn4*$^{-/-}$ mice and wild-type littermates (as controls), were approved by university animal care facilities and pursuant to local and international rules. To disable the circadian pacemaker we performed under deep anesthesia a state-of-the-art 4-position electrolytic lesion of the SCN as detailed in supplementary material. The same surgical and stereotaxic procedure without injected current was used for Sham control animals. Locomotor activity was recorded using a standard infrared motion detector. All mice were implanted with a classical set of electrodes. Sleep and EEG power spectrum were analyzed and quantified according to standard criteria[20]. After completion of the protocol, the retrograde tracer CTB was injected under anesthesia into the posterior chamber of the eye. After sacrifice and perfusion of the animals three days later, DAPI and immunostaining to AVP and CTB was performed and visualized using a confocal microscope.

Sleep experiments were performed using male *Opn4*$^{-/-}$ *C57/Bl6&129P2* mice, age approximately 2 months at time of implantation. These mice were originally obtained from Deltagen Laboratory, and subsequently bred in the Institute for Cellular and Integrative Neurosciences breeding facility at the University of Strasbourg. Animals were raised under environmentally stable conditions (12hL:12hD; 25 ± 0.5° C, food and water ad libitum) and handled properly in agreement with the ethical guidelines for laboratory animal experimentation in the European Union. All experimental protocols were approved by the appropriate committees at the university and supervised by a veterinarian. Genotyping to verify the genetic background was performed using a standard PCR (primers: Mel4: 5'–GCT CAC TAT ACC CTA GGC AC–3'; Mel2: 5'–GTC CAT GGC TAT GGC TGT CA–3'; TodoNeo1: 5'–CCG CTT TTC TGG ATT CAT CGA C–3'from Integrated DNA Technologies, Inc.), described previously in Ruby, et al.[17]

A complimentary group of transgenic mice were used to verify the effects in absence of a working SCN without lesioning the tissue. Synaptotagmin10-Cre (*Syt10Cre*) *Bmal1* knock-out (*Bmal1*$^{fl/-}$) mice from the Max Planck Institute for Genetics in Göttingen, Germany, were subject to identical photo periods as the *Opn4*. These mice, created with a *Syt10Cre* driver line, individual targeted the SCN. Cloning of the *Syt10Cre* vector was done with replacement of the ATG in exon 1 of the *Syt10* gene using a Cre cassette. A genomic region

of 8.7-kb was then cloned. Targeted clones were then injected into blastocysts of *C57/Bl6* mice and resulting offspring were bred using wild-type *C57/Bl6* to produce F1, followed by continued breeding to produce a colony. *Syt10Cre* were then crossed with $Bmal1^{fl/fl}$ to disable *Bmal1* expression, solely in the SCN.

Lesion of the SCN

A subset of mice ($Opn4^{-/-}$, n=8) and their littermate controls ($Opn4^{+/+}$, n=10) were radio frequency lesioned around the area of the suprachiasmatic nucleus, before electrode implantation as previously described[19]. Lesions were performed under anesthesia by heating the (250µm) tip of a Radionics (Burlington, MA) TCZ electrode to 55°C for 20 sec by passing RF current from a RFG-4 lesion generator (Radionics). The mice were then placed in a stereotaxic instrument (Kopf Instrument), and a standard electric probe was lowered into four points of the SCN region (from zero ear bar, nose at +5°: lateral: +/-0.2 mm; antero-posterior: +3.4 and +3.6 mm; dorso-ventral: +0.95 mm;[21]). A lesion generator (Radionics Lesion Generator System) was then used to control both the temperature and voltage of the probe, for 30 seconds at each lesion site. All other mice underwent Sham lesions for control, where the probe was used but the generator was not activated. All animals then underwent identical ECoG/EMG electrode implantation to the others (described below).

EEG implantation

Before undergoing implantation mice were anesthetized with an intraperitoneal injection of pentobarbital (68 mg/kg; protocol-approved). Male $Opn4^{-/-}$ (n=16) , $Opn4^{+/+}$ (n=19), $Syn10^{Cre/Cre}Bmal1^{fl/-}$ (n=10), $Syn10^{Cre/Cre}Bmal1^{+/-}$ (n=7), and *C57/Bl6* (n=7) mice were implanted with two EEG, one reference, and two EMG electrodes in order to record vigilance states, aged 10-12 weeks. Mice were given a minimum of 14 days to recover from surgery and habituate to the baseline control conditions before any further experiments were carried out. 48 hours was recorded under 12hL:12hD light-dark conditions (white fluorescent lights, 150lux, measured at the bottom of the cage) for use as a baseline measurement and made using commercially available hardware and software (Micromed France, SystemPLUS Evolution version 1092). Following this, other continuous sleep recordings were taken under several experimental conditions: (1) a 1hL:1hD cycle for 24 hours, (2) 24-hour cycle of

constant darkness under red light. All experiments occurred on different days from one another and a minimum of 14 days under 12hL:12hD was used to habituate the animals to the control condition. The 24 hours of recording which preceded each experiment was used to confirm that the mice had returned to baseline, in terms of sleep-wake amounts and architecture had. All mice were recorded simultaneously.

Scoring of sleep and wake state

EEG and EMG signals were amplified, filtered, and analog-to-digital converted to 256Hz. The EEG signal was then modified using a Discrete-Fourier Transform (DFT) to yield a power spectra between 0 and 90 Hz (0.25Hz resolution) using a 4-s window. Any epochs containing EEG artifacts identified and excluded during further analyses. Differences between genotypes in sleep amounts were calculated by averaging time spent in each state over 5-min, and 1-, 12-, and 24-h intervals.

The amplitude of the light-dark dependent changes in wakefulness under the 1h:1h LD schedule were determined using sine-waves, fitted to the individual 5-min values for wakefulness obtained in the 1-h before, during, and after the 1-h dark periods. For each mouse, the 5-min values for the 6 dark-pulses given during the 12-h subjective light period and the 6 dark-pulses given during the 12-h subjective dark period were averaged. Sine-waves were fitted to the average time courses using SigmaPlot (Systat v. 12) with amplitude, phase, y-offset as free parameters and period set to 2 hours. These individual amplitudes for the subjective light and dark period were then further analyzed using a factorial 2-way ANOVA with genotype and SCN condition and the repeated measures for factor light or dark period.

For each vigilance state of the ECoG, an average spectral profile was constructed using all 4-s epochs scored with the same state. The frequency range 49-51 Hz was omitted due to power-line artifacts in some of the recordings. In NREM sleep, time-dependent changes in ECoG power for specific frequency bands, was performed for delta (0.75-4Hz). During wakefulness, theta (6-10Hz) and gamma (40-70Hz) were measured instead. ECoG delta power during NREM sleep was normalized by expressing all values relative to the mean value obtained in the last 4-h of the (subjective) light period, the lowest period of homeostatic

sleep pressure. Profiles were calculated using overlapping 10 min windows of waking at 5-min increments (13/hour).

Detailed Calculations for Schema Figure 2f

Under a standard 12hL:12hD cycle, the intact circadian system is responsible for the majority of regulation of sleep and alertness and is abolished with the removal of the SCN, demonstrated in fig 1A. Melanopsinergic cell transmission under this condition passes both directly through and around the SCN, though at a smaller magnitude than under a non-circadian light/dark regimen.

During the 12hL:12hD cycle a combination of direct and indirect effects of light with or without Opn4 on sleep and alertness is observable. In order to determine the relative contribution of melanopsin and rods/cones through and external to the SCN, the groups must be parsed apart. The total contribution of effects mediated or relayed by the SCN is represented as the normalized difference between *Sham Opn4*$^{+/+}$ and *SCNx Opn4*$^{+/+}$ (72%), which consists of the circadian drive with light entrainment via melanopsin and rod and cones non-circadian and circadian effects of *Opn4* via the SCN and well as the contribution of rods/cones and intrinsic circadian processes. The melanopsin direct non-circadian effect of light passing through the SCN during this condition is represented as the total amount of melanopsin contribution (the difference between the two *Sham Opn4*$^{+/+}$ and *Opn4*$^{-/-}$) subtracted from the difference of melanopsin contribution when the SCN is removed (*SCNx Opn4*$^{+/+}$ and *Opn4*$^{-/-}$) and normalized (13%). The contribution via external pathways from *Opn4* would simply be the difference between the two *SCNx* genotypes (20%). The *SCNx Opn4*$^{-/-}$ group would thus represent rod/cones contributions to the non-circadian extra-SCN response to light. Since further reduction is difficult under these experimental conditions the remainder is assumed to consist of all circadian processes contributions to the regulation of sleep and alertness via the SCN, which is calculated as the difference between the groups lacking *Opn4* (*Sham* and *SCNx*). However, a certain percentage of this remaining 59% represents the direct effects of light on rods/cones via the SCN, which involve the *OPN4* ipRGCs as well. Thus by using a similar ratio as evidenced between melanopsin information via and external to the SCN, we can postulate that the percentage of rods and cones passing information via the SCN is approximately 5% (a ratio of the 8% previously stated above).

Statistical methodology

All statistics were calculated using standard methods with Statistica (Statsoft v. 8) and graphics were generated either in SigmaPlot (Systat, v. 11) or Microsoft Excel (v. 2010). Differences in n-values between certain light/dark regimes were due to signal problems on day of recording. For EEG spectrum analysis some animals were excluded due to the increased number of signal artifacts which allowed for the quantification of sleep and wake distribution, but hampered Fourier signal transformation.

Main Figures

For Figure 1, (b) Sine waves were calculated to show best fits for the data points using SigmaPlot (Systat, v. 11). The lack of reactivity in SCNx Opn4-/- mice is represented with a dashed line (purple). A four-way ANOVA with factors ‘‘genotype,’’ “SCN condition”, ‘time of day’’ (subjective light versus subjective dark period), and ‘‘time course’’ (5-min values) revealed that for wake (W) and NREM (N), time course was significantly affected by SCN condition (W: P=0.004; N: P<0.0001) and genotype (W: P=0.003; N: P=0.028) (interactions: genotype x SCN condition: W: P=0.04; genotype x time course: W: P=0.0005 and N: P=0.00001; SCN condition x time course: W: P<0.0001 and N: P=0.0001). Red tridents denote significant genotype differences, red triangles denote significant SCN differences as compared to Sham Opn4+/+ (p<0.05; post hoc t-tests). Psi symbol represents statistically significant genotype differences either between Sham or SCNx groups. Delta symbols represent SCN-condition differences between Sham Opn4+/+ and SCNx groups. (c) A one-way ANOVA for showed significant differences between $Syn10^{Cre/Cre}Bmal1^{fl/-}$ and their controls ($P = 0.005$) Red asterisks indicate significant differences compared to C57/Bl6 and $Syn10^{Cre/Cre}Bmal1^{+/-}$ (post hoc t-tests; $P < 0.05$, $P < 0.01$, $P < 0.001$ respectively). Comparisons between *SCNx* $Opn4^{+/+}$ and $Syn10^{Cre/Cre}Bmal1^{fl/-}$ showed no significance, as did comparison between *Sham* $Opn4^{+/+}$ and C57/Bl6 and $Syn10^{Cre/Cre}Bmal1^{+/-}$ controls.

For figure 2, (a) A three-way ANOVA (genotype, SCN condition, time-course), showed significance for “SCN condition x genotype” (P = 0.004), “SCN condition x time-course” (P < 0.0001), “genotype x time-course” (P = 0.01), “SCN condition” (P < 0.001), and “genotype” (P = 0.01); post-hoc t-tests showed significant (P < 0.05) genotype differences

(red psi), and SCN differences (red delta). For Syn10Cre/CreBmal1fl/- a two-way ANOVA (genotype, time-course), showed significance for, "genotype x time-course" (P = 0.01), "time" (P < 0.001), and "genotype" (P = 0.007); post-hoc t-tests showed significant (P < 0.05) genotype differences (red phi). Dunnet's test was used for (b). (c) One-way ANOVA (genotype) – $P < 0.05$(d) One-way ANOVA (genotype) - $P = 0.005$, post-hoc $P < 0.05$. € . A two-way ANOVA (genotype, SCN condition), showed significance for "SCN condition" ($P = 0.002$), and "genotype" ($P < 0.001$) [post-hoc t-tests against control group *Sham Opn4*$^{+/+}$ and C57/Bl6 ; $P < 0.05$, $P < 0.01$, $P < 0.001$ respectively, Red asterisks in (c)]. *SCNx Opn4*$^{-/-}$ L-D difference in NREM was not significantly different from zero as determined using One-Sample Signed Rank Test [($P = 0.48$) insert)].

EEG Immunohistochemistry (IHC)

AVP and CTB immunostaining was carried out as described previously[4]. Free-floating slices were heated to ambient temperature before being rinsed with a solution containing PBS and 0,25% of Triton-X-100. Then slices were blocked with a solution containing 0,25% of bovine albumin and 5% of normal donkey serum. Hereafter, slices were incubated overnight at 4°C with two antibodies. SCN was stained for arginin-vasopressin in lesioned and Sham animals to visualize the extent of the lesion with an anti-AVP antibody diluted at 1:2000 (Bachem T-5018 Arg8-Vasopression-Undiluted Antiserum for Immunohistochemestry, Host: Guinea Pig). Anti-cholera toxin B subunit antibody diluted at 1:1000 (Calbiochem© 227040 Anti-Cholera Toxin, B-Subunit, Goat pAb) was used to check whether fibers of the retinohypothalamic tract remained intact and projected to the different cerebral areas. The next day, slices were rinsed (PBS – 0,25% Triton-X-100) before being incubated for 30 minutes with the fluorescent secondary antibodies obtained from the donkey: anti-guinea pig conjugated Cy5 (Donkey Anti-Guinea Pig IgG (H+L)ML* / Jackson ImmunoResearch 706-175-148) diluted at 1:200, and anti-goat conjugated Alexa 555 (Donkey anti –goat IgG (H+L) Invitrogen A21432) diluted at 1:200. Slices then were rinsed (PBS – 0,25% Triton – X – 100) and mounted on slides with DAPI diluted at 1:500 (4',6-diamidino-2-phenylindol dihydrochloride Sigma-Aldrich D9542). Omission of the primary antibody abolished all staining.

Photomicrographs

Expressing neurons and fibers were analyzed using a microscope equipped with appropriate filter settings for detecting Cy5, Alexa 555 and DAPI. Fluorescence images were obtained via a non-confocal microscope (DMRXA2, Leica Microsystems) equipped with Metamorph v 2.1.39 (Olympus, Ballerup, Denmark). Light microscopy images were grabbed with a Leica DC200 camera using Leica DC200 software (Leica, Cambridge, UK). The software program Image J was used to fusion the images and the image editing software Microsoft publisher was used to combine the obtained imaged into plates. Reference of the various brain structures was made according to the Franklin and Paxinos atlas “mouse brain in stereotaxic coordinates” (third edition, 2007).

5.7 - References

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Preface

The study of sleep in fundamental science has, up until this point, been performed almost exclusively in rodents such as mice and rats. Unfortunately, these models are limited by a number of factors, most importantly their nocturnality, with approximately inverted physiological mechanisms to human beings. The direct effects of light seen in these mice, though promising, are difficult to translate into human physiology. Thus, research in a diurnal rodent characteristically similar in its behavior, anatomy, and experimental manipulability, represents an important step between the laboratory and clinical treatment. Diurnal rodent models have been used for a number of years in chronobiology, to study circadian and seasonal activity under various conditions. However, use for the study of sleep was considered, yet never accomplished. Taking advantage of our colony of *arvicanthis ansorgei*, a diurnal grass rat from sub-Saharan Africa, we designed a protocol with the specific intent of phenotyping this animal and identifying the direct effects of light and whether they are inverted as related to other laboratory animals.

Chronobiotron

Chapter 6 - Characterization of the alerting effect of light in arvicanthis ansorgei: validation of a novel diurnal rodent model for sleep study

Characterization of the alerting effect of light in arvicanthis ansorgei: validation of a novel diurnal rodent model for sleep study

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6.1 - Abstract

Our knowledge on sleep neurobiology comes mainly from studies conducted in rat and mice, (i.e. nocturnal rodent species). However, their daily sleep/wake organization is inversed to what is observed in humans. Though studies on locomotor activity, and others examining circadian rhythm have been performed, sleep in diurnal rodents has never been consistently examined. Additionally, recent mounting evidence has shown that the direct effects of light represent a third regulatory mechanism which interacts with the circadian and homeostatic drive to determine the daily sleep/wake organization. Our goal was to phenotype arvicanthis ansorgei, a diurnal rodent regularly used in the field of chronobiology, to observe whether the circadian process and non-circadian direct effects of light are inverted as compared to mice as well as whether the homeostatic process is conserved. Analysis of EEG-video, EMG and EOG recordings allowed characterization of sleep and waking states in these animals. A classical set of electrodes was implanted in male arvicanthis ansorgei (n=14) and after habituation to the recording conditions, were placed under a 12hL:12hD for baseline assessment of the daily cycle, as well as 24-hours under constant darkness for evaluation of the endogenous circadian component. Additionally, to assess the direct effects of light the following conditions were also included: 1-hour light-pulse at ZT15 during the dark period, 1-hour dark-pulsed at ZT3 during the light period, and ultradian 1hL:1hD cycle which lasted for 24 hours. A 6-hour sleep deprivation was performed at dark onset (ZT12) to study the homeostatic process, followed by deprivations of 90 minutes and 9 hours, to elicit a dose response curve. Vigilance states (wake, NREM, and REM) were easily identifiable with Video-EEG, EMG, and EOG recordings, and showed similar electrophysiological characteristics as nocturnal rodents. The time course of sleep and waking across the 12hL:12hD in arvicanthis was relatively inverted to that observed in rats or mice, though with larger amounts of crepuscular activity at light and dark onset. Under constant darkness, animals showed higher amounts of NREM sleep but the same overall distribution across the 24-hour period. The homeostatic process was intact and confirmed by a 6-hour sleep deprivation experiment at ZT12 (inverse to mice), and delta power during sleep recovery was shown to be dependent on length of the deprivation. Under the 1hL:1hD cycle, arvicanthis showed an inverted reactivity to that observed in mice and rats and was characterized by an alerting effect of light and a sleep promoting effect of darkness, with the maximum photic

regulation of sleep during the subjective dark period. The present findings show that arvicanthis is a valid diurnal rodent model for studying the regulatory mechanisms of sleep and suggests that the non-circadian effects of light are inverted as compared to nocturnal rodents. Experiments are under way to decipher the neuronal substrates underlying the switch of direct photic input to sleep between nocturnal and diurnal species, a first step in the understanding the evolutionary changes between nocturnality and diurnality.

6.2 - Introduction

Sleep has been characterized in a large range of species. Historically, animal sleep research involved different type of animals such as cats, though since has been almost abandoned, (experiments and colonies were expensive to maintain, there was a lack of genetic tools and difficulties with extensive research), or even rabbits. Canine models of narcolepsy also yielded important information about the biology of this disease, yet were eventually halted as well. Both flies and zebrafish are very powerful models for genetic tools, although restrictions such as the limited neuronal network of the drosophila, and the aquatic environment of the zebrafish, restrain their interest for research on mammalian physiology and behavior. Therefore, the majority of laboratory sleep research has focused on rodents and today, primarily on mice due to the incredibly powerful genetic tools available, including the transgenics and more recently optogenetics. Through mice research has provided major insights into sleep neurobiology, these animals are nocturnal, underlying the need for a diurnal rodent model as illustrated by *Arvicanthis ansorgei* in the chronobiology field. *Arvicanthis ansorgei* has been extensively analyzed for the study of circadian rhythms, Indeed the first article using this species clearly identified it's circadian organization as being significantly different from similar sized nocturnal species (Challet, Pitrosky et al. 2002). Challet et al, confirmed that of the 108 animals screened, 84% were clearly diurnal, with activity onset slightly before and after (06:20 AM and 07:40 PM, respectively) light-dark transitions. Subsequent studies examined the circadian expression of neuropeptides in the SCN (Dardente, Menet et al. 2004), melatonin output of the pineal gland (Garidou-Boof, Sicard et al. 2005), and resetting of the circadian clock through use of hypocaloric feeding (Mendoza, Gourmelen et al. 2012). These studies taken together confirm a strong, consistent, diurnal circadian rhythm, yet this was never established using EEG recording. To date only two sleep EEG studies exist which specifically examined a diurnal rodent, yet were limited into their characterization of sleep regulatory mechanisms. In 1989, Dijk and Daan, implanted a series of electrodes in a chipmunk *Eutamias sibiricus*, with the purpose of identifying sleep and wake distribution across the 24-hour day (Dijk and Daan 1989). Additionally, a significant increase in the level of delta power was seen following a 24-hour sleep deprivation, as measured through power spectrum. This was further explored in a follow-up study in 1991 by the same group, though with the purposes of examining drug interaction (Dijk, Strijkstra et al. 1991). Though the initial results were promising, an animal which slept

75% during the dark period, further experimentation was stopped due to the isolated research conducted on a biologically poorly understood model, as well as the lack of laboratory colonies and difficulties to realize additional studies. .

It is currently unknown as to the evolutionary and physiological change necessary for an animal to be diurnal or nocturnal. Crucial to this understanding is comprehension of the sleep and alertness inducing areas of the brain and why they would operate in the opposite fashion when exposed to external light and darkness. Induction of sleep or waking is thought to depend on a reciprocal inhibitory interaction between structures within the brain, such as the galanin-containing GABAergic neurons of the ventrolateral preoptic nucleus (VLPO) for sleep, and the tuberomammillary nucleus, locus coeruleus, and raphe nuclei, containing of histaminergic, noradrenergic, and serotoninergic neurons, for arousal (Saper, Cano et al. 2005). Given our observation of c-Fos activation of "sleep promoting galaninergic neurons" neurons of the VLPO in response to light application (Tsai, Hannibal et al. 2009), our hypothesis is thought that this "flip-flop" switch should therefore be inverted in a diurnal species, with light stimulating arousal, and darkness promoting sleep, as is the case in human beings (Münch, Kobialka et al. 2006).

In the current study we sought to address whether arvicanthis is a valid model for studying the regulatory mechanisms of sleep. In addition we aimed to determine whether it is indeed a diurnal rodent based on EEG activity, and whether the direct effects of light are inverted to those we and others recently characterized in nocturnal mice. Finally, our goal is to determine whether these effects inversely alter the balance of the flip-flop switch, as a first step towards understanding the change between nocturnality and diurnality.

6.3 - Results

EEG characterization of sleep and waking in arvicanthis ansorgei

Before examining the regulatory mechanisms of sleep, in arvicanthis ansorgei the first step was to characterize waking, NREM and REM sleep. During waking arvicanthis showed highly desynchronized EEG and high muscle activity on the EMG, as well non-rhythmic eye movements on the EOG. The EEG power spectrum analysis revealed, in comparison to sleep, a higher composition of high frequencies rhythms such as theta and gamma activities, also observed in nocturnal rodents during wake (**Fig. 6.1a, b**). In NREM sleep the animal showed highly synchronized EEG activity, associated with low muscle tone, consistent with a resting state. Additionally no eye movements were observed (**Fig. 6.1c**). The EEG Power spectrum was dominated by delta frequencies (0.75-4 Hz), characteristic EEG rhythm of NREM sleep (**Fig. 6.1d**). REM or rapid-eye-movement sleep, characterized by highly desynchronized EEG activity, coupled with complete muscle atonia, and rapid eye movements every several seconds, circled in red on EOG with highlighted corresponding portions of the EEG (**Fig. 6.1e**). Note the EEG theta-waves characteristic of this state (**Fig. 6.1f**).

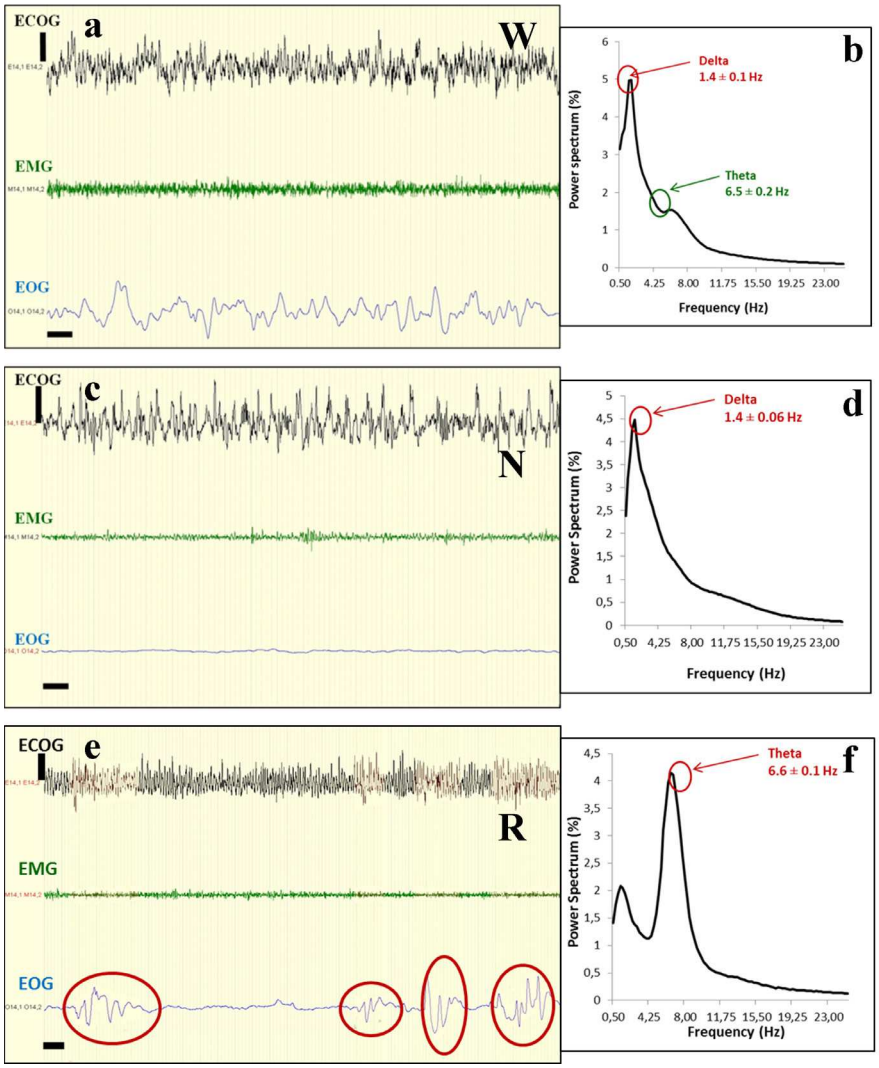


Figure 6.1: EEG characteristics associated with wake and sleep states

(a) Examples of EEG, EOG and EMG recordings: a) during waking. Note the desynchronized EEG pattern high levels of activity under EMG and EOG (b) Power spectrum analysis of all waking epochs during baseline. Graph represents peak relative frequencies of total power. (c) Recordings during NREM show spectral profiles (d) dominated by delta frequencies with a peak of 1.4 ± 0.06 Hz. In REM (e) spectrum is dominated by theta activity (f) with a peak frequency of 6.6 ± 0.1 Hz. Window represents 20 seconds of recording time. Bar: 1 second

Circadian, homeostatic and crepuscular components regulate sleep and waking in arvicanthis

Daily and circadian distribution of sleep and waking in arvicanthis ansorgei

Through previous observation of wheel running activity showing that arvicanthis is mostly active during daytime, the diurnality of the species needed to be verified with daily sleep recordings under a standard 12hL:12hD cycle. During baseline, wake accounted for 62% during the light period compared to 34% in the dark (**Fig. 6.2a**). Conversely, there is also a significant difference of time spent in NREM or REM between the light and dark periods. These values are summarized below in **Fig. 6.3e.** NREM sleep is relatively mirrored to the wake pattern across the 24-hour period. However, REM sleep is almost twice as high during the dark period as compared to the light period, though this may be a function of the REM homeostasis mechanisms, especially from an ultradian driven mechanism dependent on previous time spent in NREM and not on circadian influence (Benington and Heller 1994).

Observation under standard 12hL:12hD cycle suggests a circadian distribution of sleep and waking across the day, yet confirmation in the absence of light remained to be determined, thus the animals were placed under 24-hour constant darkness. Under this condition there was an overall decrease in the amount of waking, a slight increase in NREM, and a significant increase in REM sleep. These differences between 12hL:12hD and DD are possibly due several factors: i.) a decrease in the amplitude of the clock signal; ii.) a sustained direct effect of light; iii.) the absence of light influence on melatonin suppression. Under the constant darkness protocol, data suggest that arvicanthis has a period closer to 12-hours due to crepuscular activity (see below), though displaying an overall daily distribution of sleep and waking relatively similar to baseline conditions (**Fig. 6.2c**). Abrupt changes in wake and sleep are observed at L-D-L transitions and wake amounts are predominantly expressed during the subjective light period (ZT0-ZT12; corresponding to the light period of the preceding days under 12hL:12hD prior to the entry in DD) whereas sleep is prominent during ZT12-ZT24 period, this distribution confirms the circadian organization of sleep and waking.

Crepuscular regulation of sleep and waking

Certain species of animals are incredibly active at light and dark onset, referred to as crepuscular and reactivity under this condition per hour implies crepuscularity rather than diurnal behavior. Remarkably, in arvicanthis ansorgei, two sustained periods of waking are observed under the 12hL:12hD as well as DD, at a time during peaks of locomotor activity are noticed [**Fig 6.2a, 6.2c**, (as illustrated along the right side of **Fig. 6.2c** wherein the animal begins using the wheel several minutes prior to illumination of the cage at 7h00 (ZT0), and extends several minutes passed when the dark period begins at 19h00 (ZT12)]. The consolidated periods of waking are even more sustained beginning at least one hour both before and after changes between light and dark and ending after the light and dark onset. This wake-induced crepuscular regulation is far more pronounced than what is normally seen in laboratory rats and mice. In line with this observation, EEG power spectrum analysis showed clear time points of highly increased theta and gamma frequencies, EEG correlates of cognition and exploratory behavior in rodents (Chrobak and Buzsaki 1998; Montgomery, Sirota et al. 2008) (**Fig 6.2b**). An additional experimental condition that reveals this powerful influence of the crepuscular regulation is the ultradian 1hL:1hD cycle administered for 24 hours (see below). Indeed, whereas consecutive short light and dark pulses are known to dramatically modulate the expression of sleep and waking, the maximum level of waking around ZT0 and ZT12 suggests that the crepuscular activity overrides the 1hL:1hD cycle.

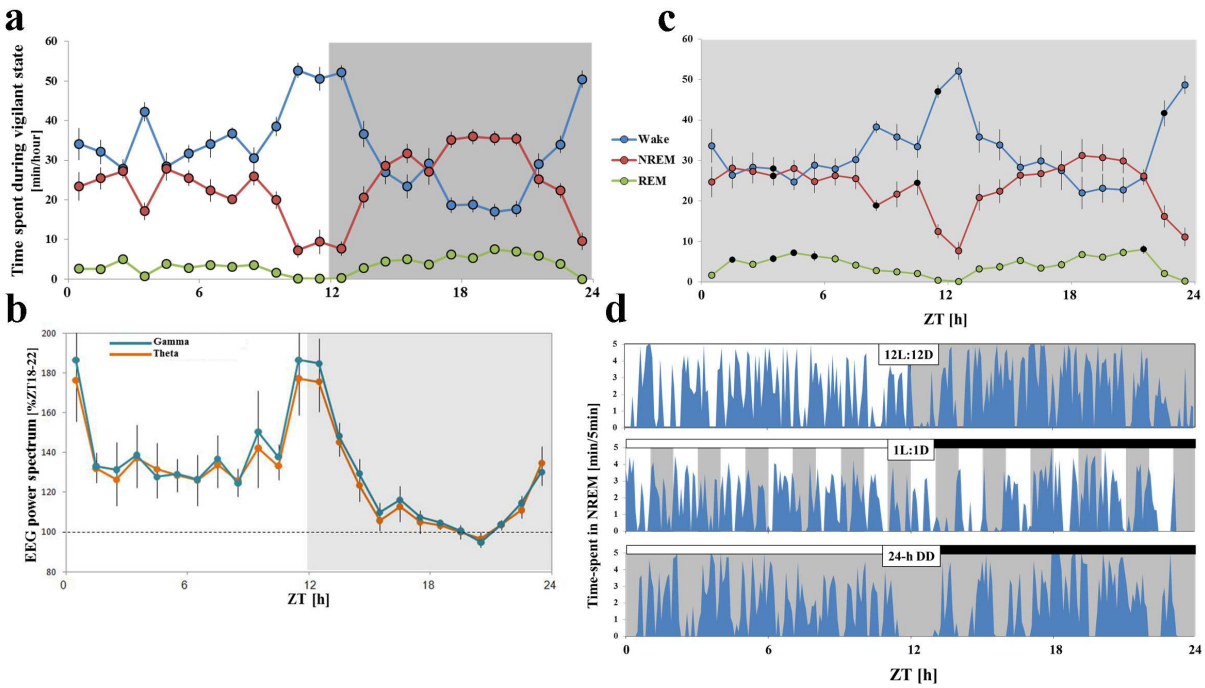


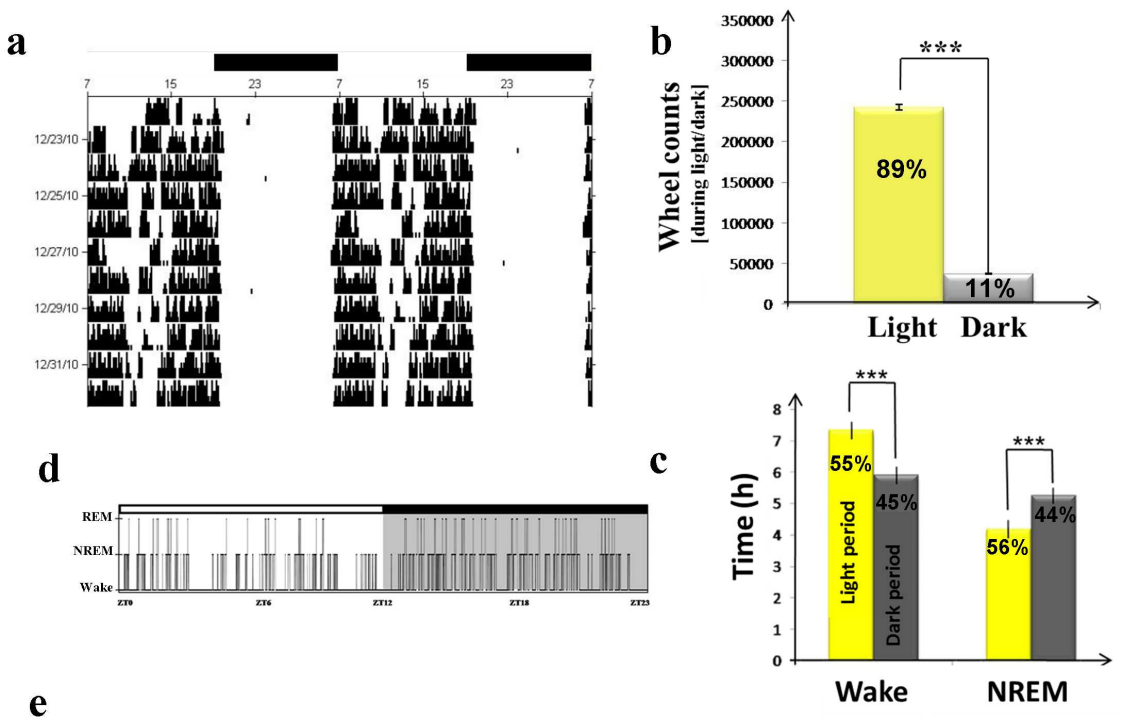
Figure 6.2: Sleep and waking under 24-hours of 12hL:12hD and constant darkness (DD)

(a) Vigilance states represented as number of minutes per hour across a 24-hour 12hL:12hD cycle. All subsequent values are expressed as average ± s.e.m. (b) Theta and gamma EEG power spectrum analysis of waking epochs per hour. (n=11) (c) As in (a) though under 24-hours constant darkness. Time-course of vigilance states under 24-hours of constant darkness expressed as minutes per hour. A one-way ANOVA between subjective light vs. dark was not found to be significant. A repeated measures ANOVA over the course of 24-hours between one day of 12hL:12hD and DD showed significance (W: $p=0.006$; N: $p<0.001$; R: $p<0.001$). Filled black circles represent significant differences after post-hoc analysis in vigilance states as compared to 12hL:12hD (n=8) (d) Example of NREM sleep per 5 minute bouts of an individual arvicanthis under 3 different light/dark conditions: 12hL:12hD, 1hL:1hD, and 24 hours of constant darkness.

Circadian regulation of locomotor activity and sleep and waking

Though arvicanthis ansorgei was previously shown to be a diurnal animal based on wheel running measures of locomotor activity, given the variability sometimes seen in this species confirmation was needed of their activity rhythm prior to EEG implantation. After several weeks under a standard 12hL:12hD cycle a clear pattern of daytime activity (wheel running), with an almost complete cessation during the night period, was observed (**Fig. 6.3a**). Examination of the total number of wheel counts between the light and dark periods (**Fig. 6.3b**) further confirmed this rhythmicity in all animals included in the study. There was a highly significant difference ($p<0.001$), indicating use of the wheel preferentially during the first 12-hours of the day.

However, the circadian organization of sleep and wake as measured with the EEG suggests, as mentioned before, a weak sleep-wake rhythm in contrast to the high amplitude locomotor activity rhythm (**Fig. 6.3c**). Note the dramatic difference of distribution of locomotor activity compared to sleep and wake with light and dark. The main experimental difference between both conditions results from the wheel suggesting that the reward properties of locomotor activity might synchronize the clock.



	Wake [h]	NREM [h]	REM [min]
Light Period	7.4 ± 0.27**	4.1 ± 0.24**	28.2 ± 5.1***
Dark Period	5.9 ± 0.27	5.2 ± 0.25	51.4 ± 6.0
24-hours	13.3 ± 0.41	9.3 ± 0.38	79.6 ± 10.4

Figure 6.3: Comparison of daily wheel-running activity and vigilance state distribution a standard 12h:12h LD cycle

(a) Actimetry example from a single animal, double-plotted and centered at ZT0 (7h00) for a total period of 11 days under standard baseline conditions. (b) Differences for total wheel counts under actimetry recording during the light vs. dark period. Analysis was done using Student T-test and found to be highly significant ($p<0.001$). (c) Differences between light and dark periods in wake and NREM as measured by EEG. Asterisks denote highly significant differences ($p<0.001$) (d) example of a hypnogram in arvicanthis across a normal 24-hour LD cycle (e) Total amounts of each of the vigilance states during baseline (12hL:12hD cycle). One-way ANOVA was performed between light and dark periods under baseline. Asterisks denote significant differences between light and dark periods: Wake $p=0.0038$; NREM $p=0.0081$; REM $p<0.001$). (n=12)

Process S is conserved in arvicanthis ansorgei

EEG delta activity is the most reliable marker of the buildup of homeostatic sleep pressure. Analysis of baseline days revealed an increase in Delta power following short activity periods corresponding to the animal's crepuscular peaks of activity at light and dark onset. This is consistent with the increases seen in gamma and theta, makers for alertness, observed just before these first bouts of NREM sleep. The EEG delta activity exponentially decreased during subsequent NREM rebound following these activity peaks, describing the classical time course of sleep homeostasis. Sleep deprivation is the best method to challenge the sleep homeostat. Due to an overall inversion of the sleep wake architecture in arvicanthis ansorgei as compared to mice, animals were placed under a 6-hour sleep deprivation which began at dark onset (ZT12), whereas sleep deprivation experiments in mice are usually performed at light onset (ZT0). Following the sleep deprivation, animals showed a significant increase in the amount of delta power (**Fig. 6.4a,b**). Sleep rebound was similar to that seen mice under a similar length deprivation, exponentially decreasing to pre-deprivation levels within 4-hours. Sleep deprivations performed for 90 minutes as well as 9-hours showed significant differences in delta power activity during sleep rebound, as compared to the 6-hour condition (**Fig. 6.4c**).

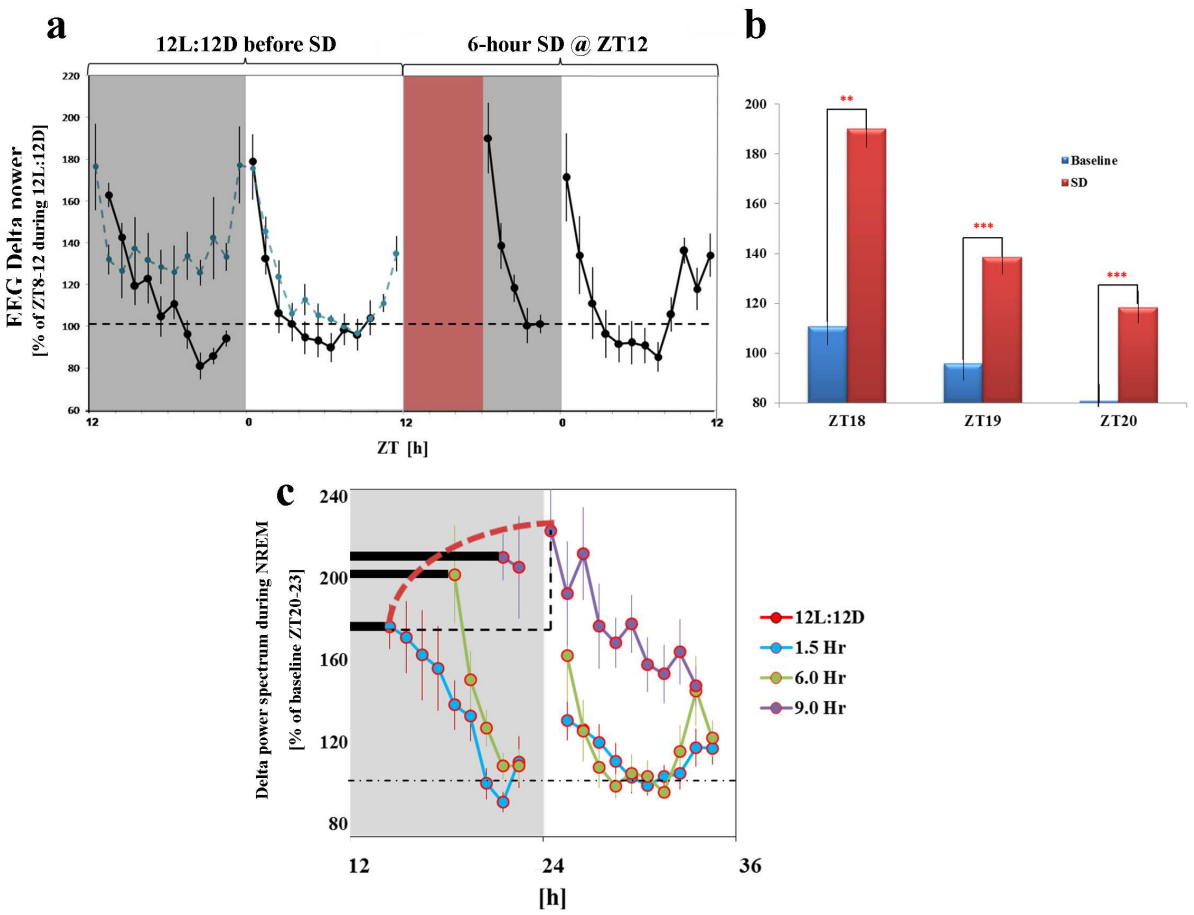


Figure 6.4: EEG delta power under sleep deprivations @ ZT12

(a) EEG delta power expressed as a percentage of ZT16-ZT22 during baseline, determined to consistently be the period with the lowest sleep pressure. 6-hour sleep deprivation is displayed as well as the preceding baseline day. (b) Histograms representing delta power during the three first recovery hours after the sleep deprivation as compared to the same baseline ZT (n=8). (c) Sleep deprivation dose response experiment showing an increase in delta power during recovery with an increase in sleep deprivation length.

The direct effects of light are inverted to nocturnal rodents

The direct effects of light are known to highly effect sleep in nocturnal rodents, as discussed in chapter 3 and 4 (Altimus, Guler et al. 2008; Lupi, Oster et al. 2008; Tsai, Hannibal et al. 2009), thus the next experiment was to confirm the significance of this regulatory mechanism in a diurnal species. Assuming that the neuroanatomical mechanisms required are inverted as compared to mice, exposure to light should awaken the animal, whereas exposure to darkness should induce sleep. A 1-hour dark-pulse was given 3 hours after light onset (ZT3). To remove any possible confounding factors animals were kept under a 12hL:12hD cycle. A significant induction of NREM sleep was seen when compared to the same ZT from the previous day under a baseline condition and consequently a decrease in wake occurred (**Fig. 6.5a**). REM increased slightly, but not significantly as compared to baseline. However, this is in line with what was seen under the 12hL:12hD condition, in that there is less REM during the light period. This confirms that at least around this time during the 24-hour period the animal is susceptible to an induction of sleep by darkness.

To specifically examine whether light could alert the animal in the same way as darkness in mice, a 1-hour light pulse was given 3 hours after dark onset at ZT15. Based on EEG analysis under the baseline condition, the animals are normally asleep at this time. As can be seen in **Fig. 6.6b**, reactivity to the light pulse was almost immediate, with the animal fully awake after 15 minutes. Waking was relatively maintained across the pulse, though the animals did not immediately return to sleep with its cessation. When the EEG spectra was analyzed across the light pulse a strong theta (6-10 Hz) and gamma (40-70 Hz) induction was observed correlating with the maximum period of wake (**Fig. 6.6c,d**). This confirms that the direct effect of light is indeed present and likely modulated by melanopsin, and that it remains inverted to the results seen in mice.

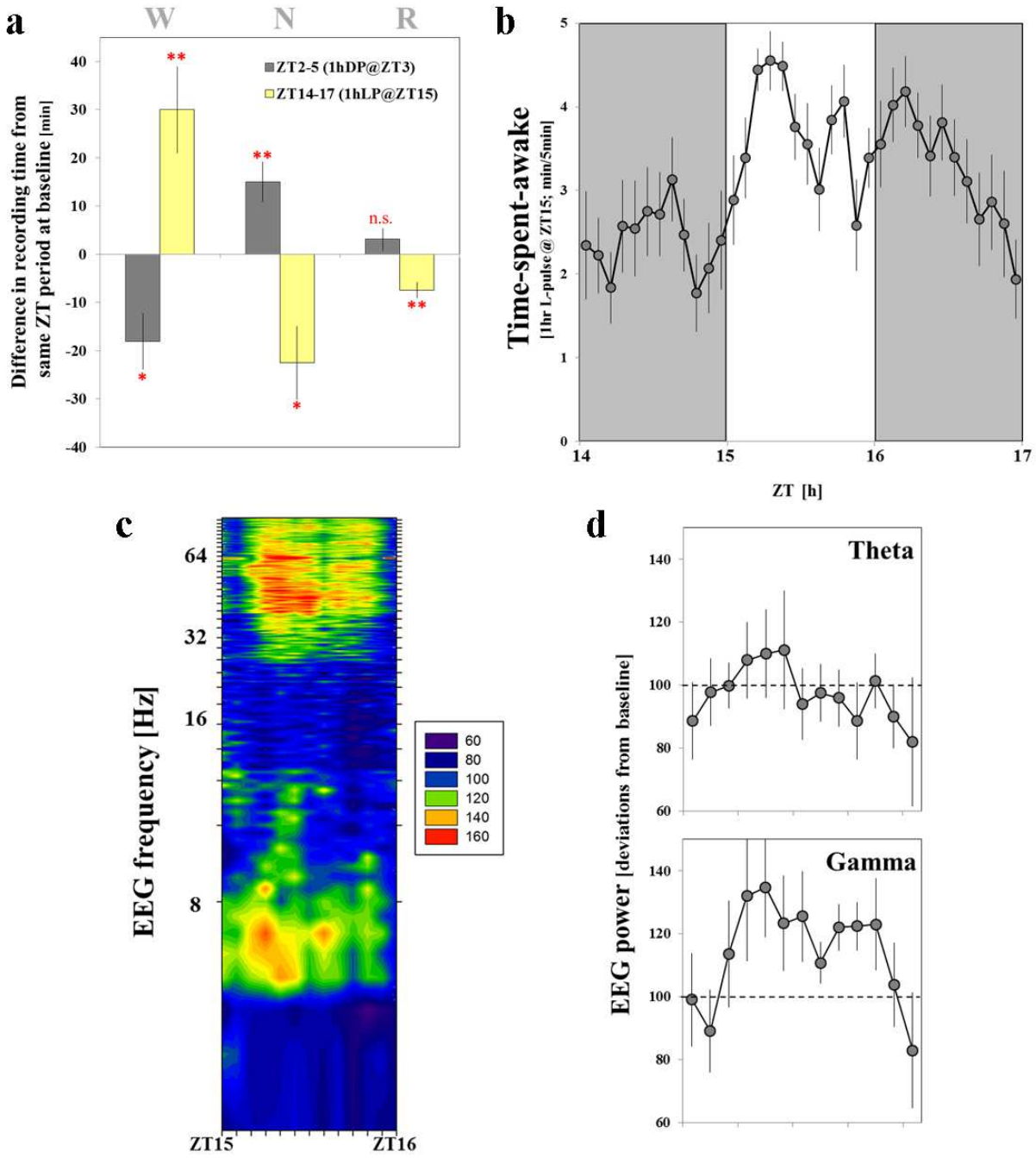


Figure 6.5: The direct effects of a single 1hr LP at ZT15 and 1hr DP at ZT3

(a) Difference in vigilance states during 1-hour dark (grey) and 1-hour light (yellow) pulses as compared to the same ZT and surrounding two hours during the previous day under 12hL:12hD. One-Sample Signed Rank Test confirmed significant changes for all states except REM under dark pulse (wake: DP- $p<0.05$, LP- $p<0.01$; NREM: DP- $p<0.01$, LP- $p<0.01$; REM: DP- n.s., LP- $p<0.01$) (n=11). (b) Time-course of wake during the 1-hour light pulse @ ZT15. Values represent amount of wake per 5 minutes bouts. (n=11) (c) and (d) Induction of EEG theta and gamma activities, EEG correlates of alertness, across a 1-hour light pulse (d) a

repeated measures ANOVA across the dark pulse indicated significant differences for both theta and gamma ($p<0.05$; $n=4$).

The alerting effect of light is dependent on time of day

Following a test of the direct effects via single 1-hour light and dark pulses, animals were placed under an ultradian 1hL:1hD cycle which examined at the effect of light pulses throughout the 24-hour day. This lighting condition was originally designed to study the effects of light under a circadian cycle (Borbely, Huston et al. 1975). Consisting of alternating light and dark pulses over a period of 24-hours, this protocol has shown efficacy in the past as a way of illustrating the time-dependent direct effects of light (Altimus, Guler et al. 2008; Tsai, Hannibal et al. 2009) (**Fig. 6.6a**). When the reactivity is examined during each of the light pulses, wake promotion is almost only observed during the subjective dark period. Another unanticipated finding was that the dark-induced wake promotion was delayed in arvicanthis contrary to what we previously noticed in mice (Tsai, Hannibal et al. 2009)(**Fig. 6.6b**). Further confirmation of this time course dependent alerting effect came from analysis of theta and gamma induction, which was present only during the second half of the one hour pulse of light (**Fig. 6.6c**).

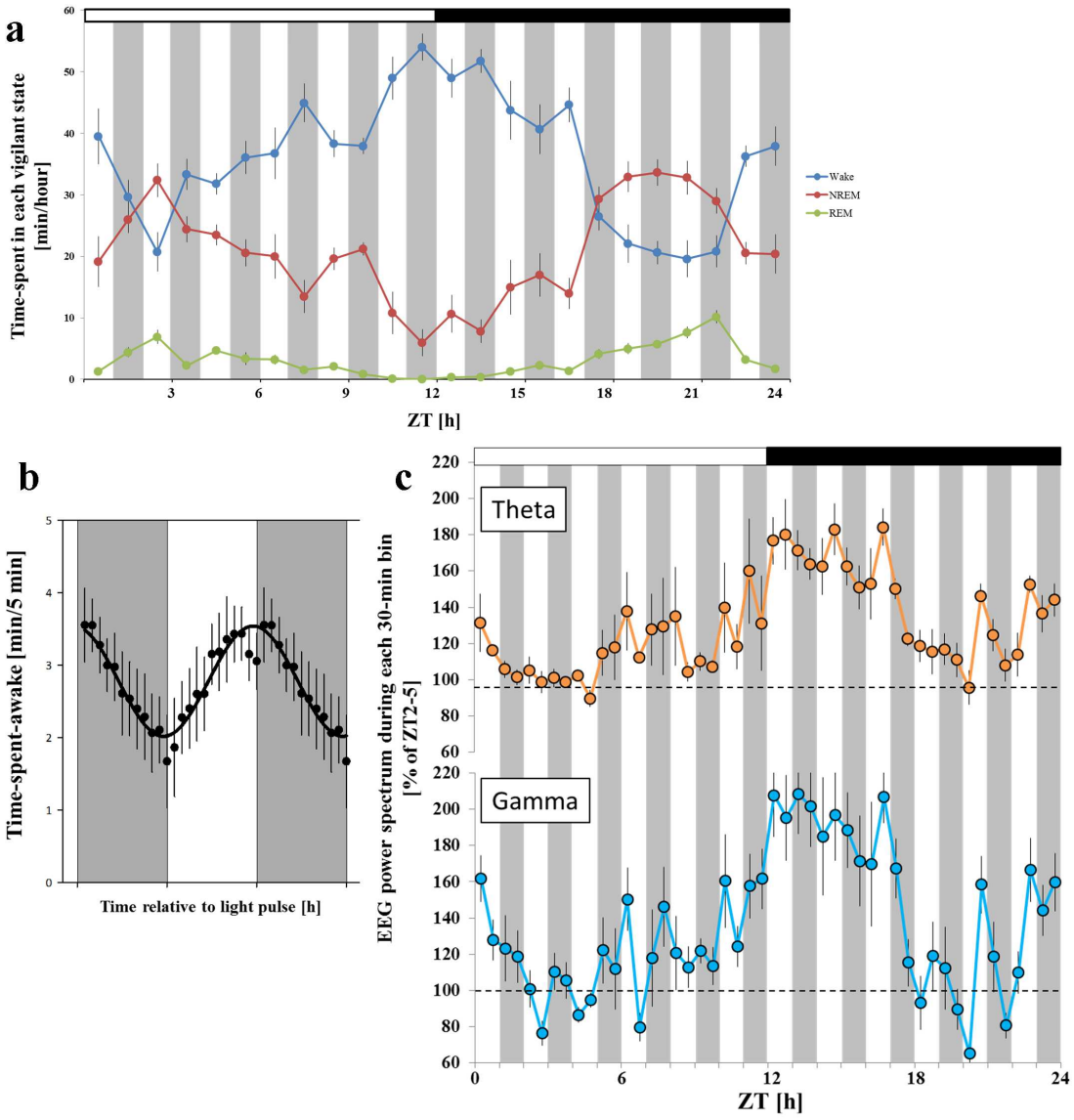


Figure 6.6: The direct effects of 1-hour light and dark pulses during a 1h:1h LD cycle

(a) Time-course of vigilance states under a 1hL:1hD ultradian cycle, measured as number of minutes per hour. (b) Wake reactivity during the subjective dark period (second 12 hours of the 1hL:1hD cycle) of arvicanthis (centered on light pulse). Data represent average of the six 6 light/dark transitions during the subjective dark period expressed as minutes of wake per 5-minute bout. Sine-waves were calculated and superimposed. (n=10) (c) Theta and Gamma progression across the light/dark pulses. Note the increase in alertness during the second half of several consecutive light pulses of the subjective dark phase (n=5). Note the maximum amount of waking around ZT0 and ZT12, as a reflection of the powerful influence of the crepuscular regulation.

6.4 - Discussion

Arvicanthis has proved to be a useful model in chronobiology for the specific study of circadian rhythms, yet EEG was never recorded. Here we characterized sleep and waking as well as sleep regulatory mechanisms in arvicanthis ansorgei. Though not as diurnal, the project nevertheless yielded the assumption that arvicanthis ansorgei represents a novel model for studying the regulatory mechanisms of sleep and alertness, in a translational approach between nocturnal rodents and human research.

Arvicanthis ansorgei is a diurnal rodent whose sleep is regulated by a circadian, crepuscular and homeostatic process

Sleep recording and analysis of rodents has been performed for a number of years on a variety of species, but as of this writing, never on arvicanthis ansorgei. Despite challenges associated with this animal due to its more savage nature within the laboratory environment as compared to rats or mice, the procedure was nevertheless similar. Based on EEG, EOG, EMG, and with video support, arvicanthis ansorgei have clear differences in their vigilance states which are nearly identical to those found in other rodents such as mice and rats (**see chapters 3, 4**). Characteristics seen in each vigilance state based upon power spectrum analysis were extremely similar to those observed in previously studied nocturnal rodents, in addition to the length of sleep and wake bouts. Taking a single arvicanthis under 24h DD, 12hL:12hD, and 1hL:1hD (ultradian LD cycle with respect to a 24-hour entrainment), and comparing the distribution of the NREM sleep across 24-hours yields some differences though the animal remains surprisingly consistent (**Fig. 6.2d**). A strong circadian distribution is observed but at reduced amplitude compared to other rodent species (calculation of the exact period and amplitude would require several consecutive days of EEG recording) (**Fig. 6.2a**), with only slight variations in NREM sleep amount per hour from ZT14 to ZT20. This observation illustrates the value of recording sleep in such an animal. Indeed, most research teams studying this animal previously used running-wheel based actimetry and this technique is known to strongly reinforce the circadian component. With this apparatus a strong circadian pattern of locomotor activity emerges, though surprising our EEG recordings did not reflect this result, displaying a comparatively low amplitude of a circadian sleep-wake rhythm. This

may be a consequence of the diurnality of the animal being weaker in the absence of the physical reward system generated by the wheel. However, the amplitudes of the locomotor activity and the sleep-wake rhythm may simply be dissociative with one another. To remove possible bias created by the wheel, locomotor activity should be recorded using infrared motion detectors which we propose to address in future experiments. Regardless of the reason for these differences, the circadian and diurnal components are well-conserved, being clearly present under a variety of light/dark conditions: DD, 1hL:1hD, and 12hL:12hD baseline. Overall when assessed against nocturnal rodents, the sleep and wake distribution is relatively inverted providing the scientific community with a clear diurnal rodent model for sleep neurobiology.

Homeostatic sleep pressure builds more rapidly

The arvicanthis has been shown in the current study and those previous to be an extremely active animal. Increased activity is known to augment homeostatic pressure in animals (Tobler and Borbely 1986; Dijk, Beersma et al. 1987). The time course of delta activity following the two periods of high activity suggests that sleep pressure builds up faster than in mice or rats, yet this remains to be further established. Given that there are two peaks of activity it may be pertinent to perform a sleep deprivation at light onset (ZT0) as well as dark onset. The assumption was made that as a diurnal animal the most obvious point in time would be at dark onset (ZT12). However given the results which were obtained, we might find similar increases in delta power if we were to shift the sleep deprivation starting point. It is possible that the "gentle handling" required for the sleep deprivation entrains higher activity patterns less research adapted animals in comparison to laboratory rodents, potentially increasing fatigue and thus the rate of sleep pressure buildup. Our complimentary experiment which examined delta power with different sleep deprivations showed a clear dose dependent relationship between the length of the deprivation and the peak of delta activity during sleep rebound. A more complete analysis of all increases in delta in relation to periods of waking needs to be examined to further comprehend the mechanism.

Crepuscular activity overrides the circadian drive

Under standard 12hL:12hD cycles the animals have clear peaks of crepuscular activity which are conserved, albeit altered, when the light condition changes. Several reasons might explain this particular behavior. Primarily one must consider the environment in which the arvicanthis comes from. In the sub-Saharan grasslands, average temperature can reach above 40° C as the day progresses, forcing the animal to rest during certain times, such as the "siesta-like" period of NREM activity seen between ZT5-9. Following this, activity slowly increases, maximizing around sunset and dusk, continuing after "lights-off" in a laboratory environment. It is likely at this time that the animal searches for food and shelter before sleeping during the night. Video recording also confirms that at certain times during both the light and dark period the animal is engaged in a waking state with restricted movement, confirmed with EEG and EMG. Further video comparison between animals showed that during the light and dark transitions most were engaged in foraging behavior, having buried their food in the preceding period. Furthermore, this bout of activity is repeated around light onset or sunrise/dawn, suggesting rather an ultradian cycle of 12-hours rather than a circadian period of 24-hours. Additionally, given the fact that this crepuscular behavior persists under constant darkness (**Fig. 6.5a**) and under an ultradian 1hL:1hD cycle (**Fig. 6.8b**), this is probably an evolutionary trait which is unable to be suppressed even in animals reared in a research setting with consistent lighting conditions. Moreover, these animals have only been bred for a few generations for experimental work in comparison to mice and rats which have long term adaptations to laboratory reproduction. Of note is the fact that animals nearer to the equator are exposed to relatively the same numbers of daylight across the year, making the 12hL:12hD cycle even more appropriate than for other rodents. If in fact this direct effect of light can be overridden in this animal at certain times of the day by a progressively suppressed mechanism, it asks the question as to whether in human beings a similar system exists underneath our forced daily activities. Finally, given the arvicanthis is as adapted to experimentation as it is, yet still retaining certain characteristics closer to what is likely observed in most rodents and other mammals in their natural environment, makes the model even more valuable. Finally, it would be of critical interest to study the circadian and crepuscular regulatory mechanisms under dawn and dusk experimental conditions as recent publications suggested in flies (Vanin, Bhutani et al. 2012) and another species, that a comprehensive re-examination of circadian behavior and its molecular readouts under

simulated natural conditions provide a more authentic interpretation of the regulatory mechanisms controlling the behavior.

The alerting effect of light and sleep promoting effect of darkness depend on time of day

When the animals are exposed to a single 1-hour light or dark pulse, reactivity occurs which is inverted to what is seen in a nocturnal rodent, however not consistently across all hours of the day. EEG analysis of the signal under the 1-hour light pulse showed an increase in alertness as measured by theta and gamma induction; however this induction was not sustained across the pulse as is normally seen in mice (see chapter 4). Mice also vary in their reactivity, though are more consistent across the day in general with melanopsin and rod/cones compensating for one another. The complexity of the arvicanthis retina may be responsible for waking up the animal, without actually being able to consistently activate the arousal system. Previously these animals were shown to react to very low luminosities, and the increased number of cones or ipRGCs in the retina may play a role (Boudard, Acar et al. 2011). Physiological studies on the retina of the arvicanthis have identified a highly complex light interpretation system unlike many nocturnal rodents. For example, Boudard et al. found highly light resistant structures in the retina which prevent the type of phototoxicity seen in nocturnal species, likely an adaptation for an active lifestyle in full sunlight (Boudard, Acar et al. 2011). Further research from the same group showed that this animal possesses ten times the number of cones as compared to other laboratory rodent species, sending visual as well as non-visual photic signals to the brain. This retinal structure difference suggests that these systems may not counterbalance as well, and as in mice during the subjective dark period, the effects might be melanopsin-dependent. However, the amplitude of wake reactivity to light pulses in arvicanthis is nearly identical to what is seen during a dark pulse in a mouse, leading us to conclude that this represents a significant regulatory mechanism. Furthermore, a sustained effect from light may also be present, yet without transgenic models it is difficult to address this question. An alternative to removal of melanopsin would be exposure to monochromatic light, preferentially stimulating the retinal substructures of the photodetection systems, allowing us to examine their isolated functionality. The fact that the pulse is given three hours after dark onset, during a time when sleep pressure is at its highest in these animals, may suppress the reactivity of the system to non-circadian light. The system seems to react more efficiently during the subjective dark period

Future experiments need to address these results and try to challenge the system more effectively, such as by increasing the length of the pulse to 2 or 3 hours, as analysis of the 1hL:1hD condition showed a promoting effect of light, though mostly consolidated to the second half of the pulses. Furthermore shifting the pulses further into the dark period away from the crepuscular activity zone around ZT12 persisting after light has been extinguished.

Criticism of the 1hL:1hD protocol usually concerns the fact that a light or dark pulse given during one hour will influence the reactivity of other periods during the cycle. During the 1hL:1hD cycle this is clearly underlined by the lack of reactivity under subjective light and a very clear and significant reactivity under subjective dark. One possible explanation is that the animal sleeps enough during the subjective light period due to the pulse activity, that it is able to awaken more easily at a time period where it is not expecting light. However, the reactivity also seems to show that dark pulses are promoting NREM sleep at this time as well. The direct effects of light and dark pulses on sleep have been recently revisited in mice (i.e. melatonin deficient animals which represent a main difference with arvicanthis). Melatonin, a hormone secreted by the pineal gland, is responsible for sleep promotion in most mammals, and can also powerfully synchronize the clock. According to previous studies on pineal secretion in arvicanthis, the peak of melatonin release is during the second half of the dark period starting at ZT2 (Garidou-Boof, Sicard et al. 2005). Melatonin production is suppressed by light, thus these pulses should be affecting its output, or at least tapering it to some extent. However, given the magnitude of the effects of the pulse during the first part of the dark period, it is unlikely that the alerting effect of light in arvicanthis depends only on melatonin suppression. Further studies in pinealectomized animals should clarify this point. Moreover, the amplitude of reactivity is the same in mice lacking melatonin and would be significantly different if this were a factor, though this is not the case.

In conclusion, the current study validates Arvicanthis ansorgei as a model for the translational approach in connecting findings from transgenic mouse models to human beings. The model represents a great compliment to others for improving our understanding on the neurobiology of sleep, as well as the switch between nocturnality/diurnality. Ultimately this will lead to a greater understanding of a variety of pathologies and sleep disorders seen in human beings.

6.5 - Materials and Methods

Animals

Arvicanthis ansorgei were obtained from our animal facility- the Chronobiotron, housed in the Institute for Cellular and Integrative Neurosciences CNRS UPR3212 in Strasbourg, France. Animals were raised under environmentally stable conditions [12-hour:12-hour light-dark (12hL:12hD); 25 ± 0.5° C, food and water ad libitum] and were managed according to the European Union guidelines for laboratory animal experimentation. All experimental sleep protocols were supervised by a veterinarian and approved by the appropriate committees.

Locomotor activity monitoring

Prior to electrode implantation, 14 male arvicanthis ansorgei were placed in individual running-wheel cages under 12hL:12hD conditions (DataportDP24, (VitalView, Minimitter). Analysis and production of the actograms was performed by Clocklab (Actimetrics) via Matlab.

Surgery

Surgery protocol for implantation consisted of anesthesia (intraperitoneal injection of pentobarbital- 68 mg/kg; protocol-approved), followed by implantation of electrodes, which were then secured using dental cement and sutured. Animals were implanted with two EEG on the dura, one reference, two EMG, and in a subset of rodents, two EOG electrodes and video recording to verify behavioral state in conjunction with EEG. Given the limited laboratory use of arvicanthis as compared to mice, the animals continue to retain certain savage tendencies such as increased activity and stress reaction to this environment. For this reason we designed a specific cable in order to prevent decoupling of the electrode chip as well as reinforcing it with INOX metal to prevent destruction by the animal. The increased weight of this system was offset by the size of the arvicanthis which averaged 150g at time of implantation. All sleep and light experiments were performed using male *arvicanthis ansorgei,* aged approximately 2 months at time of implantation. A minimum of 14 days was

given to recover from surgery and habituate to the baseline conditions before any protocol began. Animals were recorded under 48 hours 12hL:12hD) condition (white fluorescent lights, 150 lux, measured at the bottom of the cage) as a baseline measurement. Signals were recorded for analysis using commercially available hardware and software (Micromed France, SystemPLUS Evolution version1092). Other continuous sleep recordings were taken under several experimental conditions: (1) a 1hL:1hD) cycle for 24 hours, (2) 24 hour cycle of constant darkness, (3) 1-hour light pulse at zeitgeiber time ZT15, (4) 1-hour dark pulse at ZT3, (5) 6-hour sleep deprivation starting at ZT12. All experiments occurred on different days from one another and a minimum of 14 days under 12hL:12hD between each condition was used to re-habituate the animals to the control condition. All animals were recorded simultaneously.

EEG sleep scoring and power spectrum analysis

EEG and EMG signals were amplified, filtered, and analog-to-digital converted to 256Hz. The vigilance states for each 4-s epoch were then classified as waking, REM sleep, or NREM sleep, using visual inspection. As sleep and waking had previously not been categorized in these animals, initial scoring was verified in several animals using EEG/EMG/EOG in conjunction with video recording. Amounts spent in each vigilance state were calculated by averaging time spent either in 5min, and 1-, 12-, and 24-hour intervals. Video recording in several 4 animals was used to confirm concordance between behavior and EEG scoring (active wake vs. quiet during wake, cessation EMG activity during NREM, cessation of EMG activity coupled with EOG measured rapid-eye movements during REM sleep). In order to determine the qualitative aspects of these states, signals were modified using a Fast-Fourier Transform (FFT) to determine power spectra between 0 and 128 Hz (0.25Hz resolution) using a moving 4-s window. Power line artifacts disrupting the EEG were omitted with the frequencies between 49-51 Hz. For each vigilance state of the EEG, an average spectral profile was constructed using all 4-s epochs scored with the same state. Epochs which had visible signal artifacts were identified and excluded from power spectrum analysis. Epochs scored as wake showed high levels of desynchronized activity, which EEG power concentrated in the theta range (6-10 Hz), with reduced delta power (0.75-4 Hz), and active EMG indicating increased levels of movement. NREM sleep was identified according to highly synchronized slow oscillating delta waves, with a negligible presence of other

frequency bands, as well as a lacking any significant waveforms on the EMG, indicating stillness. REM sleep was categorized according to the presence of high peaks of theta in the absence of delta.

Under the 1hL:1hD schedule light-dark dependent changes were determined using sine-waves. For each animal, the 5-min values for the 6 dark-pulses given during the 12-hour subjective light period and the 6 dark-pulses given during the 12-hour subjective dark period were averaged. Sine-waves were then fitted using SigmaPlot (Systat v. 12) with amplitude, phase, y-offset as free parameters and period set to 2 hours.

Time-dependent changes in EEG power for specific frequency bands under NREM sleep, was performed for delta (0.75-4Hz), and during REM, theta (6-10Hz), and in wake, both theta (6-10Hz) and gamma (40-70Hz). EEG delta power during NREM sleep was normalized by expressing all values relative to the mean value of the period consistently showing the lowest period of homeostatic pressure, ZT20-23. The final hour was not included due to the crepuscularity of the animal. Changes in theta and gamma were normalized against ZT2-5, which represented the lowest power in these frequency ranges during both baseline and 1h:1h LD conditions. A heat map for the 1-hour light pulse was made by splitting the pulse into 13 time periods of overlapping 10-minute windows, normalized to identical times during the baseline period.

Anatomy (data not shown)

Prior to perfusion animals were sedated using high doses of pentobarbital and perfused with PBS pH 7.4 solution followed by paraformaldehyde transcardial fixation and dehydration for 48 hours in a 30% sucrose solution. Brains tissue was then cut at 18µm systematically from rostral to the caudal area. Immunostaining is underway in the laboratory using standard free-floating slice protocols routinely used in the lab (Tsai, Hannibal et al. 2009).

Photomicrographs are analyzed using a filtered microscope for detecting Cy5, Alexa 555. Images are taken using a non-confocal microscope (DMRXA2, Leica Microsystems) equipped with Metamorph v 2.1.39 (ref Olympus, Ballerup, Denmark) and light microscopy images from a Leica DC200 camera (Leica, Cambridge, UK). Image J is used to combine the images and Microsoft Publisher to assemble them into plates. References to various brain

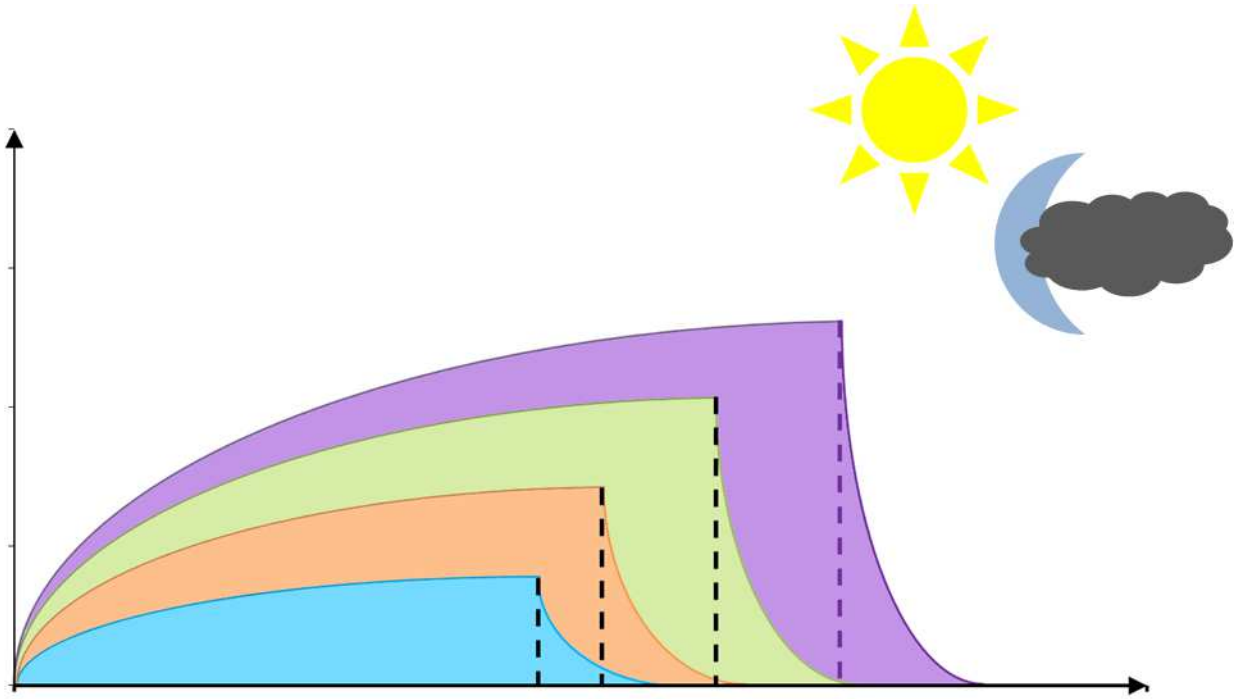
structures are made according to the Franklin and Paxinos atlas "rat brain in stereotaxic coordinates".

Statistical analysis

Statistical analysis was realized via Statistica (Statsoft v. 8) with graphic representations created either in SigmaPlot (Systat, v. 11) or Microsoft Excel (v. 2010). N-values differences were due to signal problems under certain light/dark regimes EEG spectrum analysis excluded certain animals with increased numbers which hindered accurate interpretation of the results, yet allowed for quantification of sleep and waking. Programs written in Visual Studio or Pascal were created by J. Hubbard to perform non-standard analyses.

6.6 - Perspectives

Here we have demonstrated for the first time that arvicanthis ansorgei is a valid model for studying the regulatory mechanisms of sleep and waking in a diurnal rodent. In addition, quantification of vigilance states and power spectrum analysis concluded the diurnality of the animal, though with a difference, in comparison to laboratory mice and rats, of a powerful crepuscular process overriding the circadian drive, as well the light dark influence. Furthermore, the acute photic regulation in this animal is similar to humans in terms of the alerting effect of light and sleep promotion of darkness. At this stage, we are in the process of determining whether the direct effects of light can alter the balance of the flip-flop neuronal switch responsible for the occurrence of sleep or waking with the final goal to propose the first neuronal mechanisms underlying the inversion of light direct effects between nocturnal and diurnal species. This would then represent a first step under the way of understanding the mystery of nocturnality and diurnality. The anatomy experiments are underway and should be completed within a few months. Thus, this article is in the stage of preparation for publication, yet we are still awaiting completion of anatomic analysis by another member of the research team, which will hopefully outline the brain regions involved in this switch. In the future, it remains to be seen as to whether pinealectomized animals, in conjunction with sleep recording, are necessary so as to evaluate the effect of melatonin suppression on reactivity to light and dark pulses at certain times of the day.



Chapter 7 - Does melanopsin-based photic regulation influence sleep homeostasis in nocturnal and diurnal rodents?

7.1 - Introduction

(Note: references in this chapter are given in the back of the manuscript)

Introduction and rationale

Light exerts pervasive effects on mammalian physiology and behavior, including sleep and alertness, yet its putative influence on sleep homeostasis remains unclear and is still under discussion. In 2009, our group reported that the peak of EEG delta activity reached after a 6 hour sleep deprivation was half decreased in mice lacking melanopsin(Tsai, Hannibal et al. 2009). This observation from a single sleep deprivation experiment and performed in a knockout mice from a mixed genetic background, implied the need for further investigation. However, this original finding raised the question as to whether a photopigment can act as a sleep homeostatic factor and thus whether melanopsin could be a biological link between light and sleep homeostasis. Therefore, we chose to revisit this possible influence of light. To answer these questions we performed various sleep deprivation experiments in melanopsin deficient mice of different genetic backgrounds and under various light and dark conditions. Additionally, because the effects of light in mice (i.e. nocturnal animals are inversed to humans), we also conducted the same protocol in a diurnal rodent model (a section of which is included in the previous chapter), arvicanthis ansorgei, that we previously validated as a model for sleep study. Ultimately, the aim is to examine whether light and/or sleep homeostasis might affect melanopsin expression, a part of the project which I participated in but is still underway by other members of the team. All mice used in the study were implanted specifically for this protocol. Due to time constraints arvicanthis were used which had previously underwent protocols as described in Chapter 6.

7.2 - Materials and Methods

Experimental light conditions

To determine the homeostatic response in both mice and arvicanthis, a comprehensive protocol involving sleep deprivations (SD) of varying lengths, under multiple light-dark conditions, was used. For mice, the first section of the protocol involved recordings under baseline conditions (12hL:12hD) and subsequently, a sequence of sleep deprivations planned in random order at light onset, of the following lengths: 90 minutes, 3-hour, 6-hour, and 9-hours, with each condition given several days apart and preceded by a recording of a new baseline day to control that sleep and wake returned to control values. Following this, 6-hour SD were performed after 72-hours under both constant light and constant darkness (the start of SD was shifted for 1 hour taking into account the three days of free running in DD or LL), as well as 6-hour SD at ZT0 and ZT12 under constant darkness. An identical protocol was implemented in arvicanthis, however all sleep deprivations were shifted 12-hours due to the animal's diurnality. An additional 6-hour sleep deprivation was performed at ZT0-6 in arvicanthis to examine the homeostatic response in regards to their crepuscular activity cycle (see below **Fig. 7.1**)

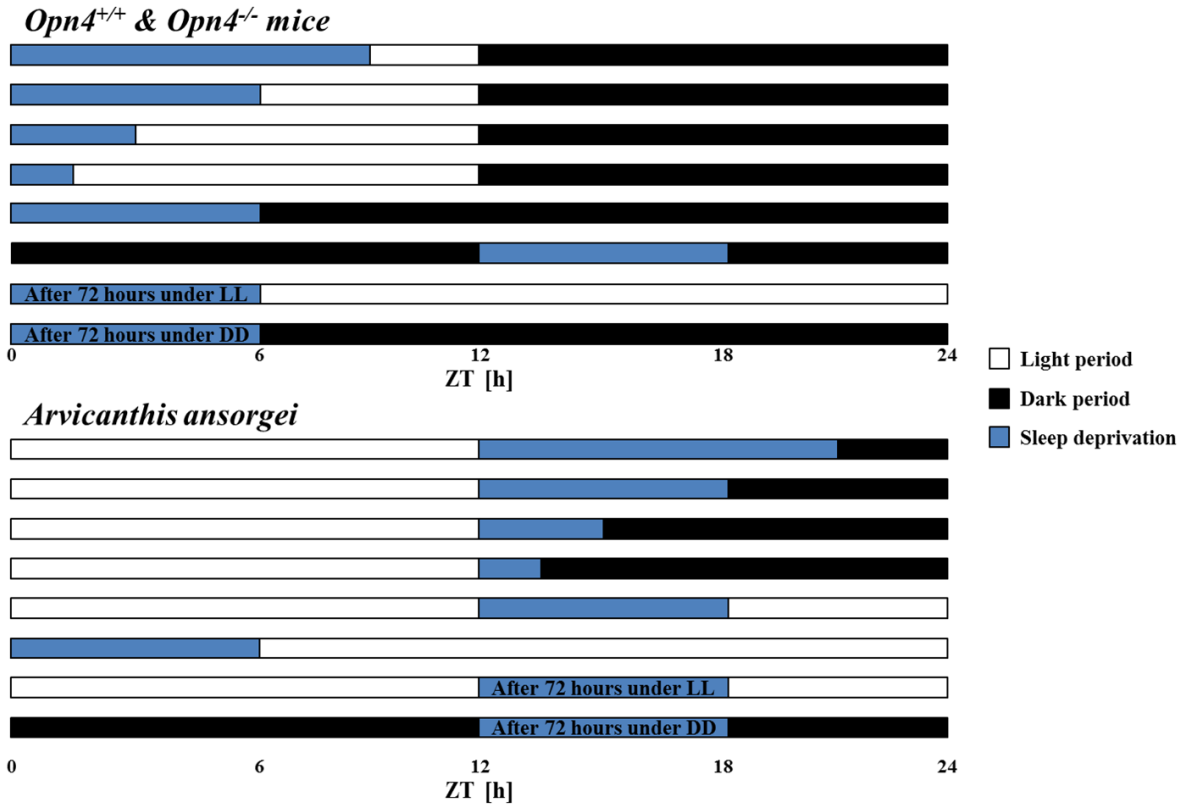


Figure 7.1: Sleep deprivation protocol for mice and arvicanthis

Animals

All sleep deprivation experiments were made using adult male animals aged approximately 2-months at onset. Opn4$^{+/+}$ and Opn4$^{-/-}$ from two different genetic backgrounds were used: mixed C57/Bl6&129P2 mice and backcrossed C57/Bl6. The Sudanian grass rat (arvicanthis ansorgei) a diurnal rodent available for experimentation at our facility, was also studied. The protocol was approved by the appropriate committees, conformed to local and international guidelines, and was supervised by the veterinarian in charge of the animal facility. Opn4 mice were originally obtained from Deltagen Laboratory, and bred in the Institute for Cellular and Integrative Neurosciences breeding facility at the University of Strasbourg where the backcross was also performed (10 generations). All animals were kept in stable conditions (12hL:12hD;, food and water ad libitum). Genotyping was performed with a standard PCR protocol (primers: Mel4: 5'–GCT CAC TAT ACC CTA GGC AC–3'; Mel2: 5'–GTC CAT GGC TAT GGC TGT CA–3'; TodoNeo1: 5'–CCG CTT TTC TGG

ATT CAT CGA C–3'from Integrated DNA Technologies, Inc.), previously described (Ruby, Brennan et al. 2002).

Each animal was implanted using a standard set of electrodes. EEG power spectrum was analyzed and quantified according to standard criteria (Franken, Malafosse et al. 1998).

EEG implantation

Prior to implantation, animals were anesthetized via intraperitoneal injection of pentobarbital (68 mg/kg; protocol-approved), implanted with two EEG, one reference, and two EMG electrodes. After two weeks of recovery from surgery and habituation to the recording conditions a baseline recording was performed during 48 under 12hL:12Dh (white fluorescent lights, 150 lux, measured at the bottom of the cage) using commercially available hardware and software (Micromed France, SystemPLUS Evolution version 1092). Animals were subsequently kept under standard LD conditions and subjected to sleep deprivations of varying lengths (see above), realized with gentle handling to prevent over-stressing. Each individual experiment occurred on differing days approximately one week apart. All animals were recorded simultaneously with their respective controls.

Delta power spectrum analysis

For the purposes of analysis, all EEG and EMG signals were amplified, filtered, and converted from analog-to-digital into 256Hz. Following this, a Discrete-Fourier Transform (DFT) was applied producing power spectra between 0 and 100 Hz (at 0.25Hz resolution) with a 4-s window. Vigilance states during each 4-s epoch were scored either as waking, REM sleep, or NREM sleep, via visual inspection of the EEG and EMG signals, from standardized criteria [3], without prior knowledge of either the genotype or condition. Those epochs which contained severe signal artifacts in the EEG, were removed and did not count for part of the spectral analysis. Additionally, frequencies between 49-51 Hz were taken out, as power-line interference artificially could modify the signal. Power spectrum analysis was focused specifically on the delta area of the spectrum (0.75-4 Hz) during all epochs classified as NREM. Values were normalized in relation to mean values during the last 4-hours (ZT8-12) of the light period (mice) or the ZT20-23 of the dark period (arvicanthis).

7.3 - Results

*All EEG recording conditions (i.e. the whole protocol described above) were completed in melanopsin KO mice of both genetic backgrounds and their controls, as well as in arvicanthis (Animals available for power spectrum analysis: C57/BL6 & 129P2 Opn4$^{+/+}$ n=6, Opn4$^{-/-}$ n=8; C57/BL6 backcross Opn4$^{+/+}$ n=5, Opn4$^{-/-}$ n=7; Arvicanthis ansorgei n=8). Given the length of time of the PhD, data analysis has not yet been completed and we present here preliminary results from the few experimental conditions that have already been investigated. One experimental result (**fig. 7.5a**) from the previous chapter is included here as well due to its compatibility with the mice protocol.*

The time course of EEG delta power under a 12hL:12hD cycle suggests an attenuation of the homeostatic response in the absence of melanopsin (C57/BL6 & 129P2 strain)

Mice were recorded for 48-hours consecutively under 12hL:12hD cycle and EEG delta power spectrum during NREM sleep bouts was analyzed. A pronounced and significant difference between *Opn4*$^{+/+}$ and *Opn4*$^{-/-}$ was observed across this light/dark condition, yet was most evident during periods of darkness (**Fig 7.2**), confirming previously published results (Tsai, Hannibal et al. 2009). As expected in wild type mice, EEG delta power was higher during the dark period due to the buildup of sleep pressure in relation to higher levels of activity at this time of the day. Delta activity was significantly reduced in melanopsin deficient mice, suggesting an attenuation of the homeostatic process in the absence of melanopsin.

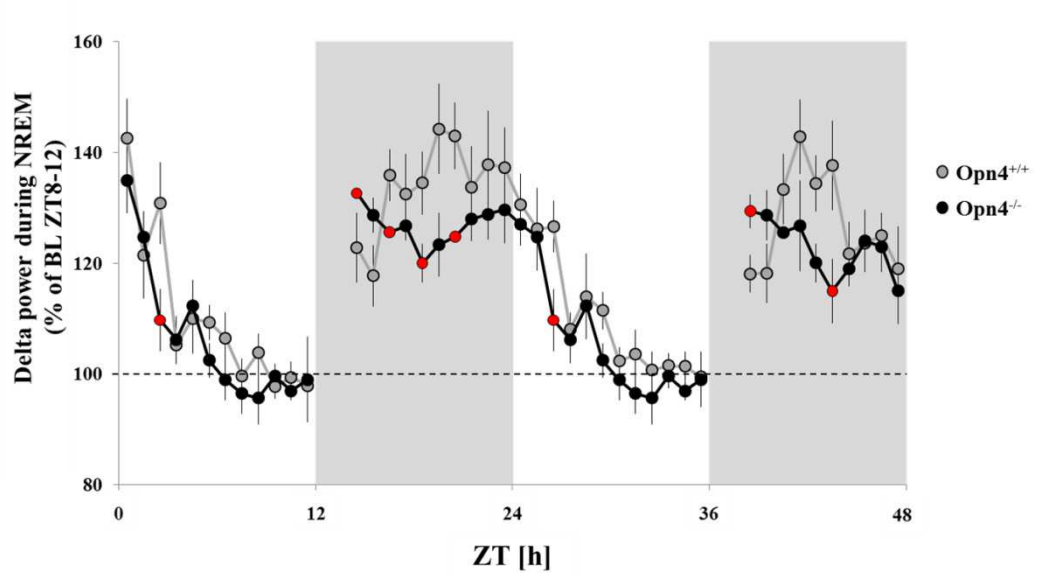


Figure 7.2: Time course of delta power during NREM sleep across 48-hours under a standard 12hL:12hD cycle.

During this condition clear genotype differences were observed though predominantly during the dark periods. Data represent delta power values ± s.e.m. A two-way ANOVA for between (Time course x genotype) showed significance ($p<0.05$). Red-filled circles denote time points found to be significant ($p<0.05$) after post-hoc tests. (*Opn4*$^{+/+}$, n=6; *Opn4*$^{-/-}$, n=8)

The buildup of EEG delta activity with time spent awake is altered in the absence of melanopsin in mice (C57/BL6 & 129ES strain)

To examine the effect of the lack of melanopsin on delta power as a function of time spent awake, sleep deprivation experiments of various lengths were used to elicit a dose response curve. The general agreement for SD in mice deemed necessary to illustrate a strong homeostatic response is 6-hours in length, beginning at ZT0 or light onset, the period of time with the highest pressure to sleep in mice. To generate the response curve, SD of 1.5-hours, 3-hours, 6 hours and 9-hours were applied. A clear genotype effect was seen at all levels of the SD protocol, with the difference between groups directly related to the increase in SD length (**Fig. 7.3a,b**).

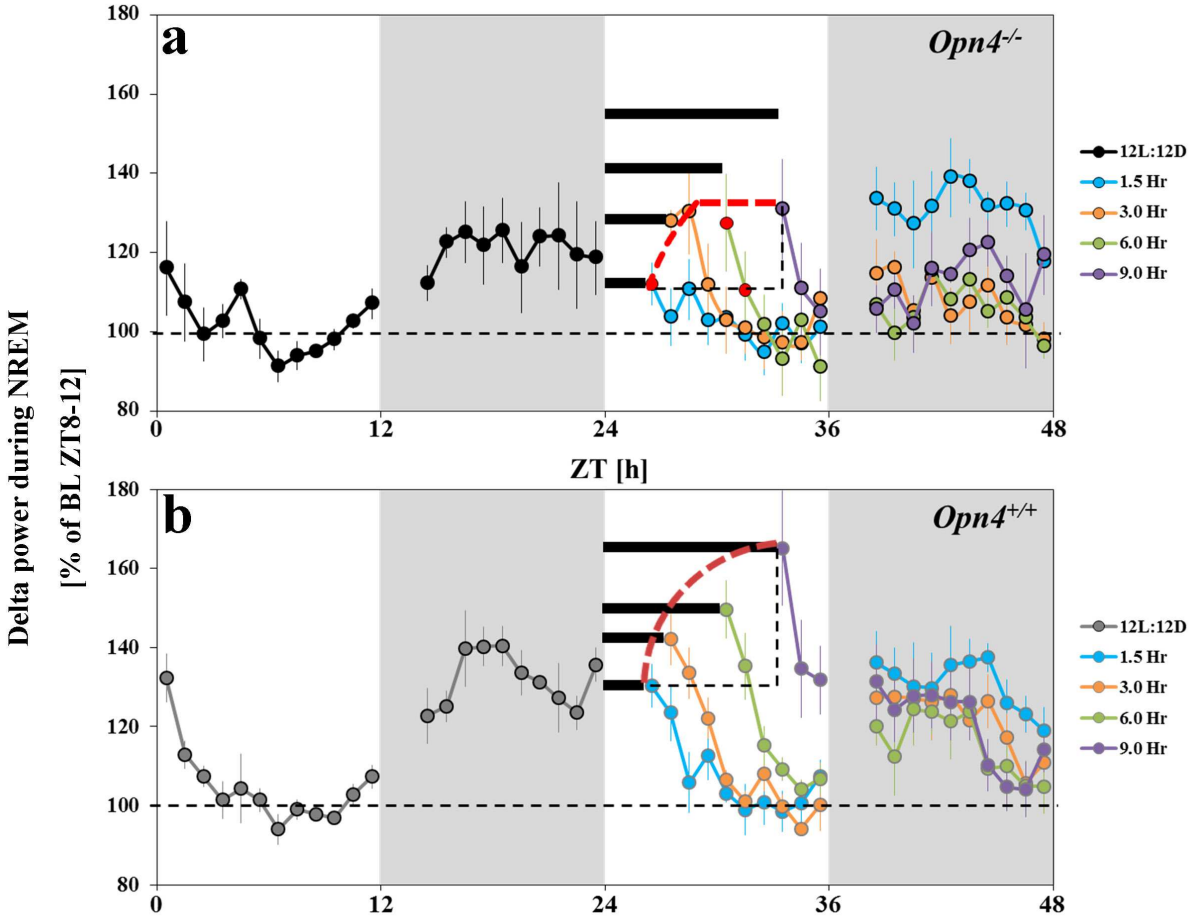


Figure 7.3: Time course of delta power in *Opn4*$^{+/+}$ and *Opn4*$^{-/-}$ mice during NREM sleep under sleep deprivations of various lengths and starting at ZT0.

Opn4$^{-/-}$ mice (**a**) showed smaller increases in delta power following SD as compared to *Opn4*$^{+/+}$ mice (**b**). In both groups, delta power increased with the length of the SD, though at a higher rate for *Opn4*$^{+/+}$. Following initial recovery there were not clear differences dependent on the length of deprivation for either group. Delta power is measured as a normalized value against the baseline period where sleep need is lowest, the end of the light phase (ZT8-12). Values represent mean ± s.e.m. Note only a single preceding baseline day is shown to more clearly illustrate the differences. (*Opn4*$^{+/+}$- 1.5 hr: n=6, 3.0 hr: n=4, 6.0 hr: n=6, 9.0 hr: n=5; *Opn4*$^{-/-}$- 1.5 hr: n=8, 3.0 hr: n=7, 6.0 hr: n=4, 9.0 hr: n=4) A Repeated measures ANOVA was performed between time x genotype (1.5 hr, time- $p<0.001$; 3.0 hr, time- $p<0.001$; 6.0 hr, time- $p<0.001$). One-way ANOVAs showed genotype significance ($p<0.05$) at certain time points (indicated by filled red circles)

Interestingly, delta power during recovery sleep in mice lacking melanopsin reached a plateau beyond 3-hours of time spent wake with similar levels of delta activity after 6-hour or 9-hour SD as compared with the 3-hour protocol (**Fig. 7.4**). This suggests a saturation of the system, and thus a severe alteration of the sleep homeostat. These noticeable and significant differences between groups imply a role for melanopsin in the regulation of sleep homeostasis.

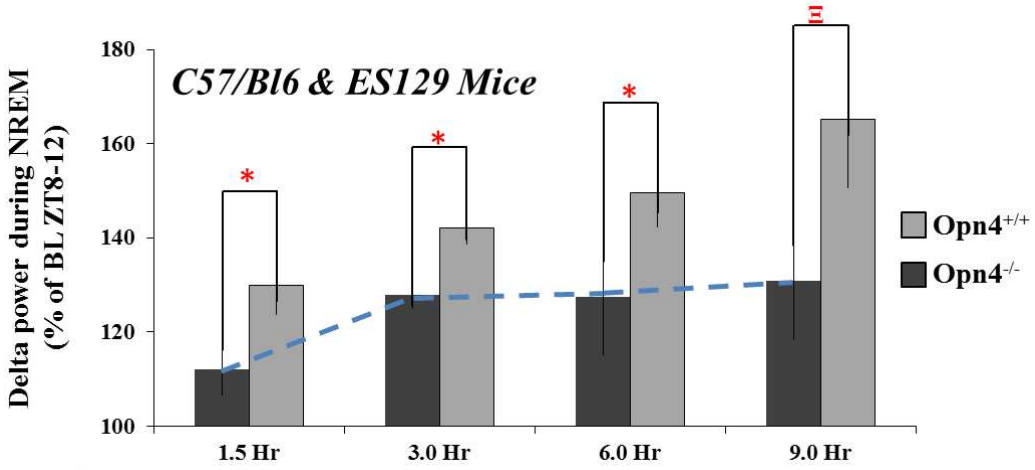


Figure 7.4: Level of delta power reached during recovery after sleep deprivation of various lengths in melanopsin KO mice.

Mean values for peak delta activity during the recovery period following SD identify significant differences, with Opn4$^{+/+}$ increasing substantially with the length of time spent awake. One-way ANOVA for genotype was performed for all experiments. Values represent mean ± s.e.m. Red-asterisk represents statistical significance ($p<0.05$) after post-hoc analysis, and red-xi symbol a statistical trend ($p<0.1$). (Opn4$^{+/+}$- 1.5 hr: n=6, 3.0 hr: n=4, 6.0 hr: n=6, 9.0 hr: n=5; Opn4$^{-/-}$- 1.5 hr: n=8, 3.0 hr: n=7, 6.0 hr: n=4, 9.0 hr: n=4)

Characterization of the buildup rate of sleep homeostatic pressure in a diurnal rodent, arvicanthis ansorgei

As a first step before examining the putative influence of light and dark on the sleep homeostatic response, we aimed to characterize the sleep homeostat in arvicanthis ansorgei, (i.e. to determine the kinetic of the buildup of sleep pressure as a function of time spent awake). As this animal is a diurnal rodent, the SD protocol was inverted as compared to mice,

with all conditions beginning at ZT12 or dark onset, the period of highest pressure to sleep in these animals. As expected, the delta power observed during recovery sleep increased with time spent awake under deprivation (**Fig. 7.5a**). Unfortunately due to a system malfunction, recordings during the 3-hour SD were unusable, though will be implemented in a future experiment. Clear differences between protocols were seen, with the highest levels of delta power during recovery sleep seen after 9-hours of SD (**Fig 7.5a**). As arvicanthis ansorgei are known to be active at both light and dark onset, and show significant periods of NREM activity during the light period (see chapter 5), a 6-hour SD was performed starting at ZT0 (**Fig. 7.5b**). Arvicanthis responded to the increased hours of wake, yet during sleep rebound the delta power was slightly lower than the corresponding SD at ZT12.

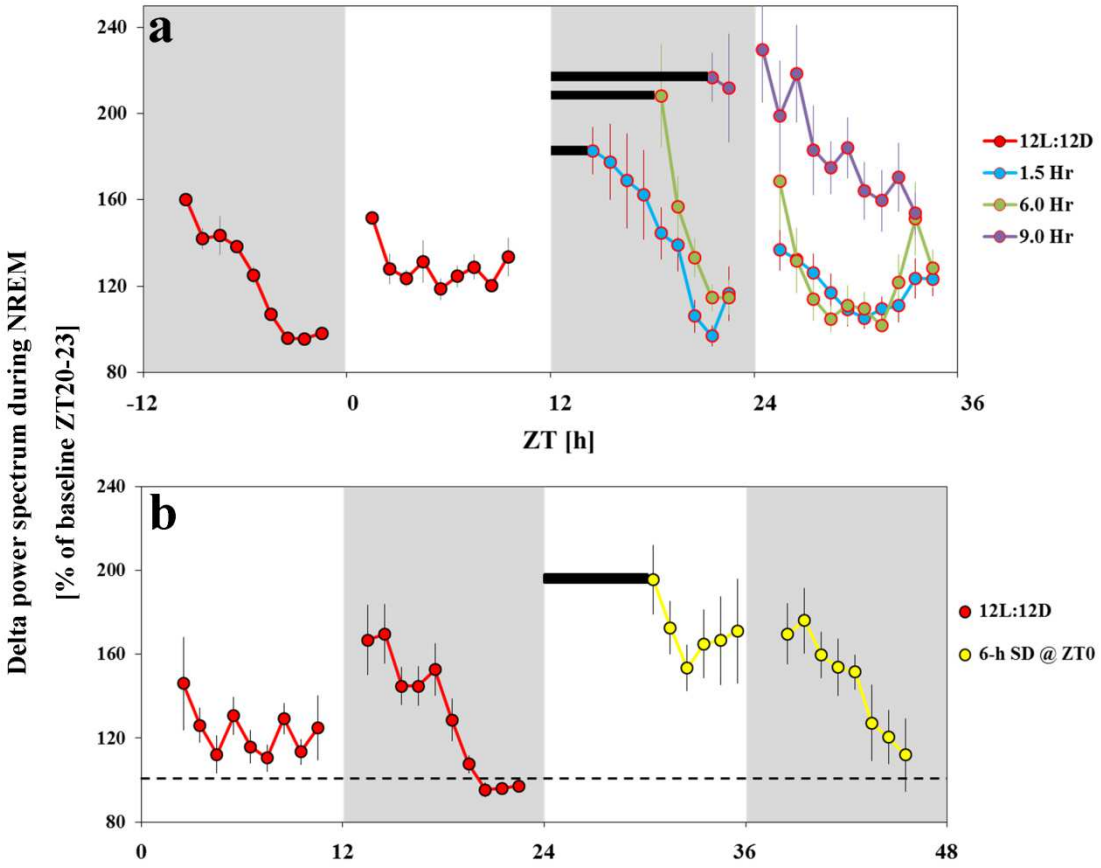


Figure 7.5: Arvicanthis ansorgei: Time course of delta power during NREM sleep following various lengths of sleep deprivation beginning at ZT12.

(**a**) Note the increase in delta power during recovery sleep as a function of the length of time spent awake. Additionally a 6-hour SD at ZT0 was performed (**b**) to examine sleep response during the active period. Delta power increased during recovery sleep, though at lower levels than a similar protocol at ZT12. Delta power is measured as a normalized value against the

baseline period where sleep need was determined to be lowest, the end of the dark cycle (ZT20-23). Values represent mean ± s.e.m. Note only a single preceding baseline day is shown to more clearly illustrate the differences. (BL: n=8; 1.5 hr: n=8, 6.0 hr @ ZT12: n=6, 9.0 hr: n=4; 6.0 hr @ ZT0: n=4)

An increase is seen in delta power during recovery as the time of sleep deprivation increases. When compared to mice the arvicanthis seems to have a slower buildup between the 6-hour and 9-hour sleep deprivations, as compared to mice (**Fig. 7.6**).

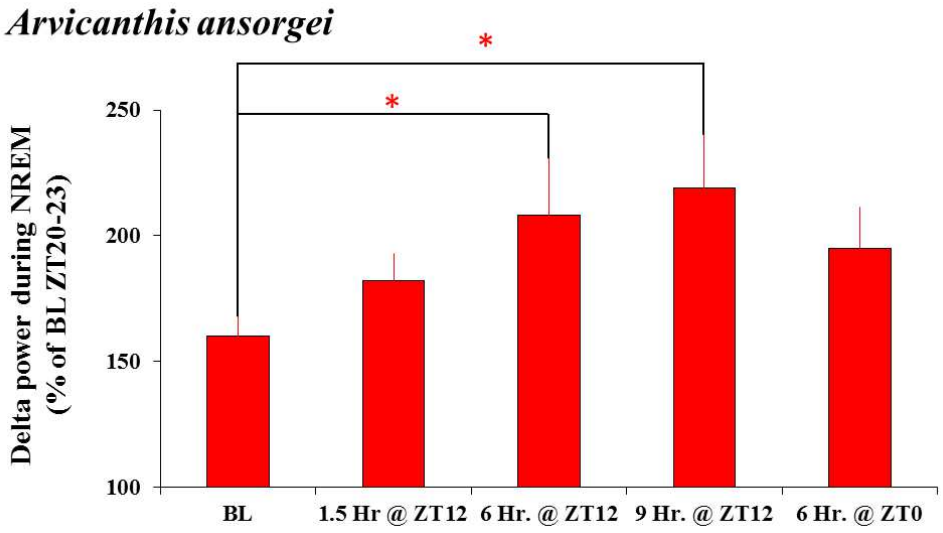


Figure 7.6: Level of delta power reached during recovery after sleep deprivation of various lengths in arvicanthis ansorgei.

Mean values for peak delta activity during baseline and the recovery period following SD identify differences, with a steady state increase depending on the length of the deprivation. Values represent mean ± s.e.m. One-way ANOVA for period was performed for all experiments. Red-asterisk represents statistical significance ($p<0.05$) after post-hoc analysis. (BL: n=8; 1.5 hr: n=8, 6.0 hr @ ZT12: n=6, 9.0 hr: n=4; 6.0 hr @ ZT0: n=4)

7.4 - Discussion

Context

Sleep homeostasis is a fundamental characteristic of almost all animals living on earth and especially humans. The kinetics of sleep homeostasis vary greatly among species, possibly as an evolutionary consequence to different environmental conditions, especially changes in the natural ambiance light and dark. The link between sleep homeostasis and light, if any, remains to be clarified. A surprising finding by Tsai et al., has suggested that light through melanopsin may influence the homeostatic process of sleep (Tsai, Hannibal et al. 2009). Here, we aimed to further explore this hypothesis by subjecting mice lacking melanopsin, as well as a diurnal rodent, arvicanthis ansorgei, to a variety of sleep deprivation (SD) under various lighting conditions.

At this stage of advancement of the analysis, we propose below to briefly discuss the preliminary results and perspectives.

The proof of concept that a photopigment, melanopsin, affects sleep homeostasis

Besides sleep deprivation, the reference condition to challenge the sleep homeostat, the analysis of the time course of EEG delta activity can give some indication of the homeostatic process. Under a 12hL:12hD cycle, melanopsin deficient mice display a one hour sleep loss. Despite this reduction in sleep time, delta power was dramatically decreased in $Opn4^{-/-}$ mice, mostly during the dark period, at a time when sleep pressure builds up due to the higher activity of the animal. This observation, in line with our previous analysis suggests an alteration of the homeostatic process that would affect the daily buildup of homeostatic sleep pressure (Tsai, Hannibal et al. 2009).

The realization of sleep deprivation of various lengths allows characterization of the time-course of the homeostatic process to definitively establish its relationship with Opn4. The buildup rate of EEG delta power peak after sleep deprivation increases as a function of SD lengths and decreases exponentially during recovery sleep, a time-course that nicely describes the homeostatic process. In mice lacking melanopsin, the delta power reached a plateau beyond 3-hours of time spent wake showing a saturation of the system, in essence a

severe alteration of the sleep homeostat. This might imply that the sleep homeostat would be set up to a different level in absence of melanopsin.

Finally, because these experiments have been conducted in KO mice of a mixed genetic background C57/BL6 & 129P2, one could argue that the background strain used to create the genetic modification is inappropriate for phenotypic analysis of the mutation. In order to control for this, we backcrossed the animals on a pure C57/BL6 background. Preliminary results (data not shown) suggest similar alterations of the sleep homeostat in $OPN4^{-/-}$ of different genetic backgrounds, strongly reinforcing this finding, as it is observed in a homogenous, as well as an enriched and diverse genetic group.

Quantification of the sleep homeostatic process in a diurnal rodent, arvicanthis ansorgei

The effects of light, either indirect through the phase shifting of circadian rhythms, or direct on the expression of sleep and waking, are inversed in mice that are night-active animals as compared to humans. Thus the need to study these effects in a diurnal model, for which arvicanthis ansorgei was used. This model has been extensively studied in the chronobiology field, and we previously validated it for sleep study (Chapter 6). To examine the influence of light and darkness on sleep homeostasis in this species, one must first characterize the homeostatic response. The buildup rate of EEG delta power with time spent awake, as well as the exponential decrease during subsequent recovery sleep, describes a nice time-course of the homeostatic process. In preliminary experiments the buildup of sleep pressure seems to be significantly higher as compared to mice, perhaps due to the animal's increased activity during waking periods. The inclusion of a diurnal animal into this protocol further confirms that sleep homeostasis is well conserved across multiple species. Previously, Dijk and Daan exposed a diurnal rodent, eustamicus sibericus to a 24-hour sleep deprivation, finding a 50% increase in the amount of power during the first 3-hours of sleep recovery (Dijk and Daan 1989; Dijk, Strijkstra et al. 1991), though spectrum analysis was broadened to include 1-10 Hz, thus not representative of "delta" power, and therefore not really describing the homeostatic response.

Sleep homeostatic response is altered under various light and dark conditions

In addition to the analysis shown in this article, several additional experimental light/dark conditions were used. Animals were placed in constant darkness for 24 hours to observe whether the effect of melanopsin is suppressed in the absence of light. In addition animals were given a 6-hour SD under constant darkness, as well as after 3 continuous days of constant darkness. Finally, animals were placed under constant light for 3 days and given a 6-hour SD, to observe the effects after an overexposure of light. Unfortunately logistic reasons inherent with a project this size inhibited completion of the analysis, though preliminary results are promising. Before publication this analysis will be realized to form a more complete story in addition to the data already presented.

Modulation of melanopsin RNA and protein expression across the day, and it's alteration under sleep deprivation

Under a 1hL:1hD cycle as shown in Tsai et al, and Hubbard et al (Chapter 4), the effects of melanopsin vary depending on the time of day. To more clearly understand why changes exist, regardless of a direct pulse of light or darkness, it is pertinent to examine the modulation of melanopsin across the day. Initial analysis of qPCR and RT-PCR experiments in both mice and arvicanthis (data not shown), ongoing from another member of the team (CM Gropp), has shown encouraging results, indicating a time-dependent change on expression which is altered when a sleep deprivation protocol is enforced. The final component will be to successfully adapt the western blot procedure to identify protein expression across the same periods, and determine the timeframe between RNA transcription and appearance of the melanopsin protein. Previously a western blot was attempted but with little success likely due to the minute quantity of melanopsin protein available for extraction from a murine retina, in conjunction with anti-bodies not specifically designed for this task.

Constant exposure to light and dark modulates the melanopsin system

Though not yet fully analyzed, one important question still unknown is the homeostatic response to mice in the absence of melanopsin following a sleep deprivation after several continuous days under constant days of light or darkness. Previous studies in rats by Hannibal et al. (Hannibal, Georg et al. 2005) on melanopsin showed that after several days of

darkness, overexpression of mRNA in the RGCs was evident, increasing with the number of days. Conversely prolonged exposure to light suppressed the melanopsin mRNA expression. It is therefore possible that the homeostatic process if related to this expression of melanopsin will similarly be affected if sleep deprivations are performed after these light-dark conditions.

Perspectives

This study represents the first comprehensive experimental protocol to examine the link between light and sleep homeostasis, utilizing not only a transgenic model, but a comparable diurnal rodent as well. After continued exploration on the remaining experimental conditions, in addition to melanopsin RNA and protein analysis, comprehension of this elusive regulatory mechanism will be closer to fruition. Furthermore, with the results seen in arvicanthis, this may serve as a valid translational model to human beings, allowing similar protocols to be implemented and studied in clinical settings. Sleep deprivation caused by insomnia and other sleep disorders is known to drastically affect mood, behavior, memory, and many other abilities essential to daily life. Understanding the mechanisms underlying the relationship of light to sleep homeostasis is a major step towards finding ways to treat dysfunctions.

Conclusions and Perspectives

The data presented in this dissertation represents strong evidence to support the existence of a third regulatory mechanism for sleep and alertness. The two-process model has been rigorously tested for years and established many of the concepts we currently know in the fields of chronobiology and sleep research. However, since the discovery of melanopsin and non-visual direct light effects on physiology, the model required revisiting. Along this axis the preceding research projects have therefore established several concepts:

(I) Reclassification of the suprachiasmatic nucleus as a relay system for approximately 50% of the melanopsin- and rod/cone-dependent non-circadian direct effects if light outside of its role as the central circadian pacemaker, to areas of the brain responsible for the regulation of sleep and waking.

(II) Impact of the sustained melanopsin-dependent direct effects of light across an habitual 12h:12h light-dark cycle, accounting for approximately 1/3 of the daily sleep and wake distribution under normal conditions.

(III) Efficacy of using a diurnal rodent, arvicanthis ansorgei to study the regulatory mechanisms of sleep, including the direct effects of light, and creating the first steps towards a translational model between classical laboratory rodents and basic human research for the study of sleep.

(IV) Establish that melanopsin affects the sleep homeostat to propose a first biological link between melanopsin, light, and sleep homeostasis, in both nocturnal and diurnal species.

The first research project identified a secondary non-circadian function of the SCN, previously alluded to, as a relay system for the direct effects of light on sleep and alertness. The prevailing consensus on the functionality for the SCN was that it serves as the master circadian pacemaker, responsible for timing peripheral clocks, and synchronizing around a 24-hour day. However, its physical location at the terminus of the retinohypothalamic tract makes it well-placed to receive non-visual direct light signals, transduced via rods and cones and melanopsin cells in the retinal ganglia. Indeed, removal of the clock removed half of the acute direct effects of light and darkness on sleep and waking, suggesting a relatively even functional distribution of retinal fibers extending both through and around the SCN.

More importantly, when the EEG was altered across the different mice groups under sustained exposure to light (normal 12hL:12hD conditions), leading to the conclusion that melanopsin-based direct photic input drives 1/3 of the daily distribution of sleep and waking. This observation calls into question years of chronobiology research that paid little consideration to the direct non-circadian effects of light, also termed “masking effects” given their ability to mask circadian rhythms. The daily partition of sleep and waking with light and dark was thought to be controlled mainly by the circadian clock, entrained by light. In fact, our findings establish, at least in nocturnal animals, that the Opn4-mediated direct effects of light represents a regulatory mechanism that can maintain a sleep-wake cycle with light and dark to keep the animal adapted to life on earth in the absence of a circadian drive. Lesioning of specific brain areas implies the possibility of damage to structures surrounding the SCN even through this point has been checked by functional anatomy. Fortunately transgenic mice possessing intact yet functionally disabled SCN tissue were available for comparison. Nearly identical results as compared to lesioned wild-type animals further validated our findings, each model controlling for the bias related to the other approach. Future experiments need to examine these sustained effects under different lighting conditions, such as using a dawn/dusk protocol, or increasing and decreasing the luminosity to mimic differing latitudes. However, the data should encourage revisiting the role of non-circadian effects of light in mammalian physiology.

The evolutionary switch between nocturnality and diurnality, in terms of the internal mechanisms which cause functionality to invert, remain misunderstood. Additionally the majority of basic rodent research is performed in animals which have inversed activity and sleep cycles to humans, presenting a fundamental issue for transfer applying these findings to human physiology. The second project sought to help elaborate on this problem through EEG recording in the *Arvicanthis ansorgei*, which allowed us to precisely examine the regulatory processes of sleep and wake in such an animal. Sleep EEG and the circadian and homeostatic regulation of sleep were characterized in arvicanthis ansorgei. The results confirmed the animal’s diurnal preference for waking, even if this proved less effective than in some of their murine counterparts, validating arvicanthis ansorgei as one of the first diurnal rodent models for the study of sleep. Moreover, a strong direct alerting effect of light, similar to humans and inversed to mice, was noted under several light/dark protocols. Further experiments by the team are underway, specifically seeking to identify brain structures which are activated by light, work previously performed in mice. These data will allow us to better understand the

neuronal switch responsible for the inversion of the direct effects of light in nocturnal and diurnal species. On a long-term perspective, arvicanthis ansorgei can be a useful model to further decipher the mysteries of nocturnality and diurnality.

Though the influence of light on the circadian process is well-known, the connection between light and sleep homeostasis remains to be clarified. Initial observations by our group, found a significant difference in sleep homeostasis between mice with and without melanopsin, a proof of concept that a photopigment can affect the sleep homeostat, though interpretation of this finding in the context of light remains to be established. In order to identify this, the sleep homeostat was challenged in mice lacking melanopsin through various sleep deprivation experiments with determination of the time course of EEG slow wave activity, the most reliable marker of sleep homeostasis. The homeostatic response, characterized by the increase in EEG delta as a function of time spent awake, was dramatically altered in the absence of melanopsin, and was independent of the genetic background. To put this finding in the context of the effects of light, these experimental conditions were also conducted during exposure to constant light and darkness over several days, though time constraints and the complicated nature of power spectrum analysis has postponed completion to a later date. Additionally, the protocol was performed in diurnal rodents with the final goal of deciphering how light and melanopsin might affect sleep homeostasis differently in nocturnal vs. diurnal rodents. Since melanopsin affects the homeostatic process, one could hypothesize that sleep homeostasis is influenced by melanopsin expression, a result confirmed by our team (at least at the mRNA level as at the protein level our experiments failed given the lack of efficient antibodies and low expression of the protein). Completion of this analysis will establish the role of light and melanopsin on sleep homeostasis, leading to future experiments in humans with possible applications to the study of sleep disorders related to neuropsychiatric diseases.

In essence this thesis work challenges, from multiple directions, the accepted 2-process model for the regulation of sleep and wake, proposing a third process which is independent yet also interrelated. These findings, taken together, suggest a more complex role of light which could affect sleep and waking in different ways: indirectly on the circadian drive, and directly on the expression of sleep and waking and through modulation the sleep homeostat. These results encourage a reevaluation on the role of light in mammalian physiology, and necessitate further validation of this model in humans, perhaps providing a new conceptual framework for understanding the disturbances associated to sleep disorders,

as well as the way we in our individual daily lives artificially expose ourselves to light and darkness.

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