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Influence non-circadienne de la lumière sur les comportements :

Identification des structures impliquées et application clinique

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INTRODUCTION

INTRODUCTION générale

I. Problématique générale

La lumière influence de nombreux paramètres physiologiques et comportementaux tels que la fréquence cardiaque (Scheer, Kalsbeek et al. 2003), la température corporelle (Scheer, Pirovano et al. 2005), l'humeur (McClung 2007), le niveau de performance cognitive (Hobson and Pace-Schott 2002) ou encore la qualité du sommeil et la vigilance diurne (Badia, Myers et al. 1991). En thérapeutique clinique, notamment dans les troubles de l'humeur, l'utilisation de la lumière est bien établie. Les mécanismes par lesquels la lumière influence les différents paramètres physiologiques et comportementaux restent cependant très mal connus. Ces effets sont soit indirects circadiens, liés à l'entrainement de l'horloge centrale, soit directs indépendants du système circadien.

Dans ce travail de thèse, je me suis intéressée aux effets directs, non-circadiens de la lumière. Les données récentes de la littérature soulignent l'importance physiologique de ces effets mais aussi la nécessité de comprendre les mécanismes sous-jacents qui restent très mal compris. Certains travaux suggèrent que ces effets résultent d'une modulation du système dopaminergique, cependant les voies neuronales qui contrôlent cette régulation photique directe sont mal caractérisées. Notre laboratoire ayant une valence fondamentale et clinique, c'est donc logiquement que j'ai développé une approche translationnelle expliquant l'organisation de cette thèse en trois parties.

Dans la première partie, j'ai utilisé des modèles de souris transgéniques pour préciser le rôle respectif des différents systèmes de photodétection impliqués et pour identifier des premières structures cérébrales de relai telles que, les « neurones inducteurs de sommeil » de l'aire préoptique ventrolatérale, et les noyaux suprachiasmatiques (NSC). Ainsi, nous avons montré que les NSC, au-delà de leur fonction d'horloge, influencent les états de vigilance en relayant les effets non-circadiens de la lumière. Par ailleurs, ces travaux permettent de souligner l'importance des effets directs de la lumière sur le sommeil et l'éveil.

La régulation photique directe est inversée entre espèces diurnes et nocturnes, comme l'illustre l'effet éveillant « alerting effect » de la lumière chez l'homme alors que

chez les espèces nocturnes cet effet éveillant résulte d'une exposition à l'obscurité. Pour pouvoir transférer à l'homme les données obtenues chez la souris, il est donc souhaitable d'avoir un modèle d'étude de rongeur diurne. Dans ce deuxième volet, j'ai donc validé comme modèle diurne d'étude du sommeil un rongeur diurne, *Arvicanthis ansorgei*, largement utilisé en chronobiologie et dans notre institut,. La caractérisation de la régulation du sommeil chez l'arvicanthis est en effet une étape préliminaire indispensable avant de pouvoir l'utiliser pour comprendre les mécanismes qui sous-tendent les effets inverses de la lumière observés en fonction de la nocturnalité ou diurnalité de l'espèce.

Enfin, chez l'homme, l'influence directe de la lumière, en particulier, « l'alerting effect » fait actuellement l'objet de nombreux travaux soulignant la diversité des effets observés, sur le sommeil, la vigilance, la performance et de nombreux autres paramètres. Chez le patient, la luminothérapie est utilisée en thérapeutique, en particulier pour traiter les troubles du rythme circadien ou les troubles de l'humeur. Les données récentes issues de la recherche fondamentale, ouvrent des perspectives intéressantes laissant envisager de nouvelles indications de la luminothérapie, notamment dans les troubles du sommeil et de l'éveil. Considérant que la régulation photique directe puisse moduler le fonctionnement dopaminergique, des premiers travaux ont cherché à évaluer l'intérêt de la luminothérapie dans des pathologies impliquant le système dopaminergique. C'est pour ces raisons que dans ce troisième volet, j'ai cherché à évaluer si la luminothérapie, via des effets directs, pouvait être un outil thérapeutique intéressant pour les symptômes non-moteurs de la maladie de Parkinson et pour les troubles du sommeil et de l'humeur dans le syndrome des jambes sans repos.

II. La lumière

La lumière exerce au niveau de l'œil deux types de fonctions, visuelle (aboutissant à la formation de l'image) et non visuelle (information sur l'intensité lumineuse). Les effets non-visuels de la lumière influencent de très nombreux paramètres physiologiques et comportementaux. Ainsi, la lumière supprime la synthèse de la mélatonine (Lewy, Wehr et al. 1980) et est le principal synchronisateur de l'horloge centrale (Czeisler, Shanahan et al. 1995; Wirz-Justice, Terman et al. 2004; Vandewalle, Balteau et al. 2006; Vandewalle, Schmidt et al. 2007).Elle influence la fréquence cardiaque (Scheer,

Kalsbeek et al. 2003), la température corporelle (Badia, Myers et al. 1991; Scheer, Pirovano et al. 2005), les fonctions cognitives (Vandewalle, Gais et al. 2007) ou encore la qualité du sommeil et la vigilance diurne (Badia, Myers et al. 1991).

La perception non-visuelle de la lumière par les cellules ganglionnaires à mélanopsine intrinsèquement photosensitives

Deux systèmes de photodétection, relativement indépendants l'un de l'autre, [mais avec des interactions complexes (Dacey, Liao et al. 2005; Dkhissi-Benyahya, Gronfier et al. 2007; Guler, Ecker et al. 2008)], interviennent dans la perception de la lumière : i) les cônes et les bâtonnets, des photorécepteurs du système visuel, répondent à des stimuli sensoriels appartenant à une large gamme d'intensités de lumière et de durée d'exposition. Une exposition parfois très brève, de l'ordre de quelques millisecondes, peut ainsi être détectée (Nelson and Takahashi 1991). ii) les cellules ganglionnaires à mélanopsine, intrinsèquement photosensibles (intrinsically photosensitive retinal ganglion cells: ipRGCs) (Freedman, Lucas et al. 1999; Provencio, Rollag et al. 2002).

Les ipRGCs jouent un rôle majeur dans les fonctions non visuelles de la lumière qui sont de deux types (Berson, Dunn et al. 2002), soit indirectes, via la resynchronisation de l'horloge centrale et l'entraînement du cycle veille/sommeil sur le cycle de lumière/obscurité (Reppert and Weaver 2002), soit directes agissant sur l'expression du sommeil et de la veille et qui sont encore mal comprises. Les effets indirects, circadiens de la lumière sont actuellement bien caractérisés. La lumière est le plus puissant synchroniseur de l'horloge centrale, localisée dans les noyaux suprachiasmatiques (NSC). De nombreuses variables physiologiques, hormonales, cognitives, la température corporelle, et en particulier le cycle veille/sommeil sont sous son contrôle (Reppert and Weaver 2002). Ces effets circadiens sont relayés principalement par les cellules à mélanopsine, via une projection monosynaptique distincte des voies visuelles depuis la rétine vers les NSC (Ruby, Brennan et al. 2002).

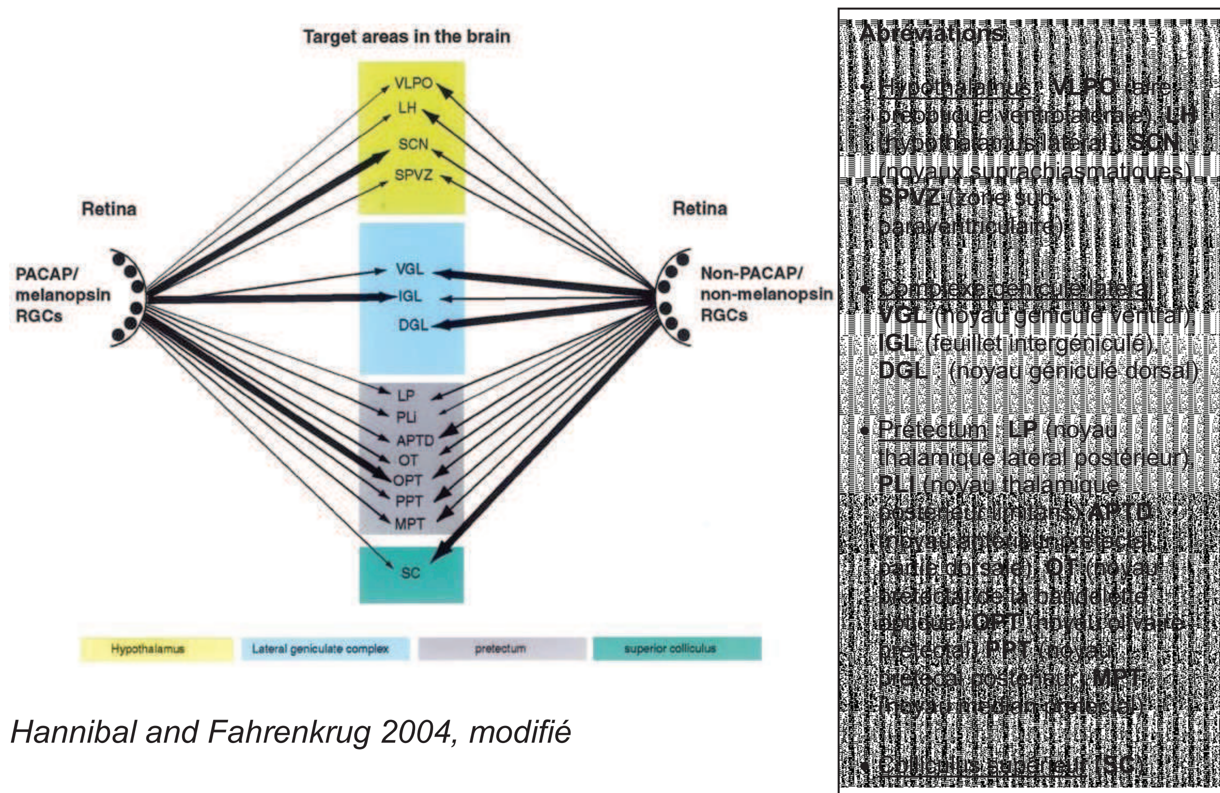
La mélanopsine n'est exprimée que dans environ 1-2% des cellules ganglionnaires de la rétine (Gooley, Lu et al. 2001; Hattar, Liao et al. 2002; Berson 2007). Alors que les bâtonnets sont très sensibles à des luminosités de faible irradiance, les cônes fonctionnent à des niveaux de lumière plus élevés. A l'opposé des photorécepteurs classiques, la mélanopsine est un photopigment rhabdomérique, qui possède la double fonction de photopigment sensoriel et de photoisomérase (Koyanagi, Kubokawa et al.

2005; Mure, Rieux et al. 2007). De ce fait, la mélanopsine résiste à l'épuisement du photopigment sous l'effet de la lumière (bleaching) et conserve la capacité de réponse photique à des niveaux d'irradiances élevés et à des durées prolongées (Zhang, Wong et al. 2008). Ce photopigment à type d'opsine / vitamine A est sensible à un spectre de lumière à λ_{max} autour de 460-480nmn et situé dans le spectre de la lumière bleue (Panda, Sato et al. 2002).

L'information lumineuse provenant des ipRGCs est transmise à de nombreuses structures cérébrales

Les ipRGCs dont les principaux neurotransmetteurs sont le PACAP (Pituitary adenylate cyclase-activating polypeptide) et le glutamate transmettent l'information lumineuse à de nombreuses structures du cerveau (Figure 1) (Hannibal and Fahrenkrug 2004; Hattar, Kumar et al. 2006).

L'entraînement de l'horloge biologique est influencé par les projections des ipRGCs au niveau des NSC (Ruby, Brennan et al. 2002) et des feuillets intergéniculés (Harrington 1997). Une autre cible est l'aire préoptique ventro-latérale (VLPO) qui contient des neurones galaninergiques « inducteurs de sommeil » et dont le rôle dans l'homéostasie du sommeil a été fortement suggéré (Sherin, Shiromani et al. 1996; Gvilia, Xu et al. 2006). Les projections sur l'hypothalamus antérieur peuvent expliquer les effets de la lumière sur la régulation de la température corporelle, ainsi que la modulation photique de sécrétions neuroendocrines impliquées dans la fonction reproductive (Moore and Danchenko 2002). Il en est de même pour les projections sur les noyaux supraoptiques (Cui, Jolley et al. 1997; Kostoglou-Athanassiou, Treacher et al. 1998). Les afférences des NSC vers la zone subparaventriculaire participent à l'influence circadienne et photique de l'activité locomotrice et du sommeil (Kramer, Yang et al. 2001; Moore and Danchenko 2002). Les projections au niveau du complexe géniculé latéral pourraient suggérer que les ipRGCs contribuent à une vision corticale, mais ceci reste très hypothétique. Le prétectum est fortement innervé par les ipRGCs, notamment le noyau olivaire contribuant ainsi à la régulation du réflexe photomoteur (Young and Lund 1994; Panda, Provencio et al. 2003). Au niveau du colliculus supérieur, les projections mélanopsinergiques ne respectent pas de franche organisation rétinotopique, suggérant plutôt un rôle modulateur des ipRGCs à ce niveau qu'un véritable rôle visuel (Barash, Melikyan et al. 1998).



Hannibal and Fahrenkrug 2004, modifié

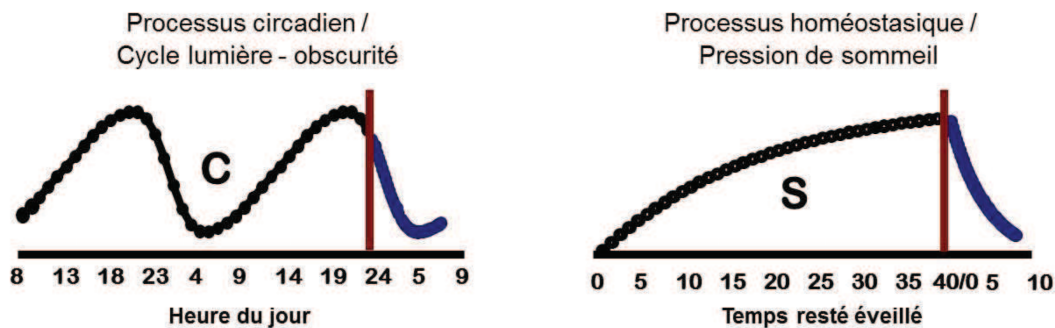
Figure 1 : Diagramme montrant les cibles de projection des cellules ganglionnaires à mélanopsine (PACAPergique et glutamatergique) ainsi que les cibles de projection des cellules ganglionnaires non mélanopsinergiques (non PACAPergiques). La densité des projections rétiniennes est indiquée par l'épaisseur des flèches. Les projections rétiniennes sont principalement controlatérales (pour des raisons de clarté les projections ipsilatérales ne sont pas représentées).

III. Le sommeil

Les troubles du sommeil affectent 15% à 30% de la population générale représentant un enjeu majeur de santé publique (Ohayon, Guilleminault et al. 2010). En l'absence de thérapeutiques suffisamment efficaces, il est nécessaire de mieux comprendre leur physiopathologie ce qui repose sur une meilleure connaissance des mécanismes de régulations du sommeil.

La régulation du sommeil conceptualisée sous la forme d'un modèle à deux processus : circadien et homéostasique

La régulation du sommeil dépend de deux mécanismes principaux : le processus circadien (C) et le processus homéostasique (S) (Figure 2) (Borbely 1982). La composante circadienne, définie par des cycles de veille et de sommeil d'une durée d'environ 24h, dépend de l'horloge centrale, endogène, située dans les noyaux suprachiasmatiques (NSC) (Reppert and Weaver 2002). Le processus homéostasique correspond à une accumulation 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 perdue étant récupérée lors du rebond de sommeil. Cette récupération est caractérisée par la mesure de la puissance spectrale delta de l'EEG (Franken, Dijk et al. 1991).



Borbely et al. 1982, modifié

Figure 2 : La régulation du sommeil à deux processus. Sous l'influence du processus circadien (C), la pression d'éveil augmente progressivement au cours de la journée en même temps que la pression de sommeil augmente sous l'influence du processus homéostasique (S). Au cours de la nuit, la pression d'éveil diminue sous l'influence du processus C et la pression de sommeil est évacuée au cours du sommeil via le processus S.

Les mécanismes neuronaux qui sous-tendent le processus S restent actuellement mal compris mais impliquent très probablement l'aire préoptique (Sherin, Shiromani et al. 1996). Différentes substances « somnogènes » telles l'adénosine, l'interleukine-1β, le facteur de nécrose tumorale TNF-α ou la prostaglandine D2 peuvent moduler le processus homéostasique du sommeil (Obal and Krueger 2003). Les deux processus C et S fonctionnent en parallèle afin de déterminer le niveau de pression de sommeil (Daan, Beersma et al. 1984).

Les rythmes biologiques, le processus circadien et les noyaux suprachiasmatiques

La rythmicité est une des propriétés fondamentales du vivant. De nombreux organismes, des plus simples aux plus complexes, présentent des rythmes biologiques. La période (intervalle de temps séparant le début de 2 phénomènes identiques) permet de classer les rythmes biologiques en 3 catégories : (i) les rythmes ultradiens d'une période très inférieure à 24 h (alternance sommeil lent et sommeil paradoxal), (ii) les rythmes infradiens d'une période très supérieure à 24 h (rythme d'hibernation) et (iii) les rythmes circadiens d'une période proche de 24 h, comme par exemple le cycle veille/sommeil. Les rythmes circadiens influencent de nombreuses variables comportementales, physiologiques et biochimiques incluant le cycle veille/sommeil (Pevet 2000). Ils sont endogènes, donc persistent en l'absence de facteurs environnementaux, comme le cycle lumière/obscurité, testé en condition d'obscurité constante. Cette rythmicité repose sur l'existence d'horloges et d'oscillateurs biologiques capables de synchroniser (c'est-à-dire de remettre à l'heure) un ensemble de fonctions à l'échelle de 24 h et d'établir une physiologie prédictive. L'horloge circadienne principale est localisée dans les noyaux suprachiasmatiques (NSC) de l'hypothalamus (Pevet, Pitrosky et al. 1996). L'activité des NSC est principalement modulée par des donneurs de temps externes (les « *Zeitgeber* »), le plus important étant le cycle journalier d'alternance lumière/obscurité. La lumière, perçue par la rétine, entraîne cette horloge par le biais du tractus rétinohypothalamique, une voie qui part des cellules ganglionnaires de la rétine jusqu'aux NSC. Ces derniers distribuent alors l'information vers différentes structures centrales ou périphériques dont les activités seront à leur tour synchronisées par l'intermédiaire de voies neuronales et neuroendocrines. La sortie principale de l'horloge est la mélatonine, une hormone synthétisée par la glande pinéale, et qui est supprimée par la lumière (Simonneaux and Ribelayga 2003). Sécrétée dans le sang, elle permet de synchroniser les horloges périphériques avec l'horloge centrale. Après administration pharmacologique, la mélatonine a un effet soporifique au niveau de la vigilance, mais est également capable de décaler la phase de l'horloge. La plupart des souris utilisées en laboratoire, contrairement à l'homme et au rongeur diurne *Arvicanthis ansorgei*, n'ont plus de mélatonine (Kasahara, Abe et al. 2010).

Chez le mammifère, la réception du signal lumineux a lieu exclusivement au niveau de la rétine [contrairement par exemple à la glande pinéale aviaire qui est

directement photosensible (Csernus 2006)]. Une modulation très complexe du signal lumineux a lieu au niveau de la rétine avant que cette information lumineuse, visuelle et non-visuelle, ne soit distribuée aux nombreuses structures cérébrales. Par ailleurs, les rétines de rongeurs sont structurellement et fonctionnellement différentes en fonction de la diurnalité ou nocturnalité de l'espèce (p.ex. 33% des photorécepteurs sont des cônes chez *arvicanthis ansorgei* versus 3% chez la souris) (Bobu, Craft et al. 2006). Ces considérations font de la rétine une cible privilégiée pour moduler le transfert de l'information lumineuse aux différentes structures cérébrales, et ceci de manière différente chez les espèces en fonction de leur diurnalité ou nocturnalité.

Les différents stades de sommeil et son architecture en cycles

On distingue trois stades de vigilance, l'éveil, le sommeil lent et le sommeil paradoxal (AASM Version 2). L'exploration du sommeil est réalisée par polysomnographie, permettant de reconnaître les différents stades de sommeil grâce aux enregistrements électroencéphalographique (EEG), électrooculographique (EOG) et électromyographique (EMG). Pendant l'éveil on observe une désynchronisation de l'activité électrique cérébrale avec des ondes de faible amplitude et ayant des fréquences mixtes, ainsi qu'un tonus musculaire élevé. **Chez l'homme** la veille calme est caractérisée par la présence d'ondes alpha (8-12Hz) qui apparaissent dans les régions postérieures à la fermeture palpébrale. Lors de l'endormissement, on observe l'apparition de mouvements oculaires lents et un ralentissement de l'activité électrique avec apparition d'ondes thêta (2-7Hz), ainsi que des pointes-vertex. Le sujet est alors entré en sommeil lent léger appelé stade 1. L'activité électrique continue à ralentir avec apparition des fuseaux de sommeil et des complexes K, des graphoéléments qui témoignent du sommeil lent léger stade 2. Les fréquences mixtes du sommeil stade 2 seront remplacées par des ondes lentes, delta (0,5-2 Hz) qui prédominent en sommeil lent profond, encore appelé stade 3. Le traitement du signal électrique du sommeil lent profond en appliquant une Transformation Rapide de Fourier (FFT : fast Fourier transform) permet de déterminer la puissance delta, marqueur de la pression homéostasique du sommeil. Le ralentissement électrique enregistré en sommeil lent résulte de l'activité des boucles de synchronisation thalamo-corticales. En fin de chaque cycle, l'activité électrique reprend un aspect désynchronisé, mais on note également l'apparition de pointes ponto-géniculées-occipitales et des mouvements oculaires

rapides, caractéristiques du sommeil paradoxal (SP), alors que le tonus musculaire est au plus bas.

Chez le rongeur, on ne fait habituellement pas de distinction entre les stades de sommeil lent (SWS : slow wave sleep) léger et lent profond. La veille calme correspond à une activité cérébrale désynchronisée sans prédominance d'activité alpha postérieure. On n'observe pas non plus les différents graphoéléments décrits chez l'homme : pointes-vertex, fuseaux de sommeil, complexes K ou pointes ponto-géniculées-occipitales. Le sommeil paradoxal (REM : rapid eye movement) est caractérisé par des rythmes thêta associés à une atonie musculaire et des mouvements oculaires rapides.

Chez l'homme, espèce diurne, on observe une organisation du sommeil consolidée sur la nuit, la période d'obscurité. Une sieste de durée variable peut être réalisée, le plus souvent vers le milieu de la période d'exposition lumineuse. Les souris et les rats, rongeurs nocturnes, présentent la majorité de leur sommeil pendant la journée, la période lumineuse. A l'opposé de l'homme, les rongeurs ont un sommeil qui est nettement moins consolidé et présentent de nombreux endormissements tout au long du nycthémère permettant d'évacuer au fur et à mesure leur pression de sommeil. Chez l'homme, on observe une organisation du sommeil en cycles qui se succèdent tout au long de la nuit avec une plus abondante activité en ondes lentes delta en début de nuit permettant d'évacuer la pression de sommeil cumulée tout au long de la phase d'éveil qui a précédé le sommeil. Cette activité delta décroit progressivement au détriment du sommeil paradoxal, particulièrement abondant en fin de nuit.

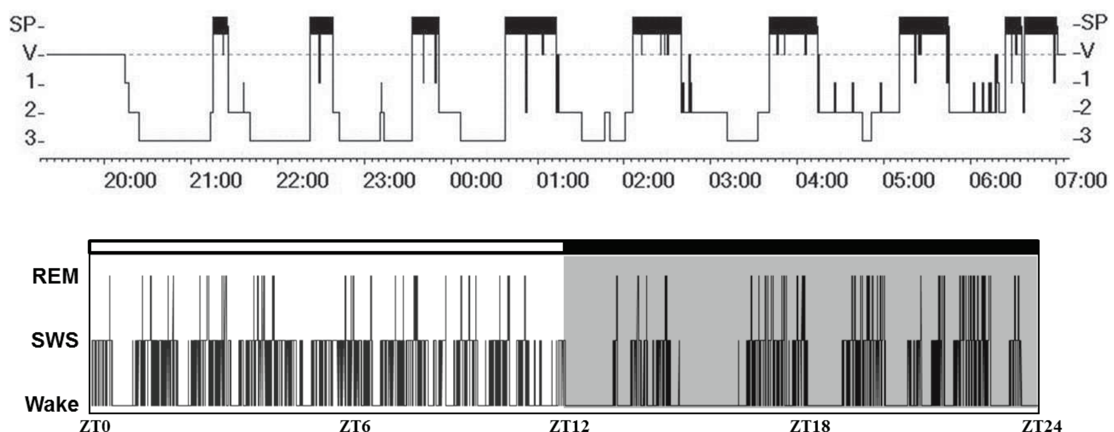


Figure 3 : Exemples d'un hypnogramme chez l'homme (cas d'un enfant), diurne (en haut) et chez la souris, nocturne (en bas). Alors que le rongeur répartit son temps de

sommeil sur tout le nycthémère avec une prédominance de veille la nuit, l'homme a un sommeil consolidé sur la période d'obscurité.

La zone subparaventriculaire est impliquée dans les processus de la diurnalité / nocturnalité, cependant les mécanismes précis, ainsi que les autres structures et réseaux neuronaux intervenant dans la diurnalité/nocturnalité expliquant cette réponse inversée de la veille et du sommeil à la lumière et à l'obscurité restent inconnus.

Le rongeur diurne, *Arvicanthis ansorgei*, est utilisé en chronobiologie pour l'étude de la diurnalité/nocturnalité

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, les effets circadiens et non circadiens de la lumière, ainsi que l'organisation circadienne de la veille et du sommeil de ces animaux sont inversés par rapport au rythme veille/sommeil et aux effets de la lumière observés chez l'homme.

La neurobiologie des rythmes est un des axes de recherche de l'Institut des Neurosciences Cellulaires et Intégratives (INCI). Le Chronobiotron, l'animalerie rattachée à l'Institut, est une plate-forme d'hébergement et d'exploration fonctionnelle unique en Europe, permettant d'étudier les rythmes circadiens et saisonniers des rongeurs. Depuis plus d'une quinzaine d'années, le Chronobiotron héberge un rongeur diurne pour les recherches sur les phénomènes de la diurnalité. Ce rongeur provenant de la région sub-saharienne (sud du Mali) appartient à la famille des Arvicanthis. *Arvicanthis ansorgei* est un muridé bien caractérisé sur le plan chronobiologique et s'est révélé être un modèle diurne pertinent en chronobiologie. De ce fait, *Arvicanthis ansorgei* semble être un candidat intéressant pour comprendre les effets directs de la lumière chez l'espèce diurne. Cependant, contrairement au rythme circadien d'activité locomotrice, le sommeil des rongeurs diurnes n'avait jamais été spécifiquement étudié. Caractériser le sommeil du rongeur diurne est une étape fondamentale avant d'extrapoler à l'homme (diurne) des données issues de la recherche chez le rongeur nocturne concernant les effets directs de la lumière sur la veille et le sommeil.

IV. La lumière en application clinique

La luminothérapie est devenue un outil important dans l'arsenal thérapeutique, que ce soit en pathologie du sommeil [insomnie (Campbell, Dawson et al. 1993; Guilleminault, Clerk et al. 1995; Murphy and Campbell 1996; Kirisoglu and Guilleminault 2004; Lack, Wright et al. 2005; Pallesen, Nordhus et al. 2005; Lack and Wright 2007; Friedman, Zeitzer et al. 2009), troubles du rythme circadien (Czeisler, Kronauer et al. 1989; Terman 2007)], en psychiatrie (Terman, Terman et al. 1989; Even, Schroder et al. 2008 ; Chellappa, Gordijn et al. 201) ou dans des indications nouvelles (Gruber, Grizenko et al. 2007; Daansen and Haffmans 2010). Chez le sujet sain, la lumière renforce la qualité de l'éveil (Chellappa, Gordijn et al. 2011).

Les effets thérapeutiques de l'exposition à une forte intensité lumineuse quotidienne (10000 lux pendant 30 minutes) se font via des mécanismes circadiens, bien explorés. Les troubles de l'humeur et plus particulièrement la dépression saisonnière sont des pathologies souvent associées à des perturbations du rythme circadien (Levitan 2007). Néanmoins, ces mécanismes circadiens, indirects de la lumière ne peuvent expliquer qu'une partie des effets de la luminothérapie sur les comportements, suggérant qu'ils sont aussi la conséquence d'effets directs. De plus, les substrats neuronaux sous-jacents à ces effets thérapeutiques, non-circadiens de la lumière, doivent être mieux identifiés. Dans ce travail de thèse, je m'intéresse à ces questions.

Outre l'implication de la mélanopsine dans ces effets thérapeutiques non visuels de la lumière, les noyaux suprachiasmatiques qui sont une cible majeure du tractus rétino-hypothalamique, pourraient être impliqués dans ces effets directs de la lumière, indépendamment de leur rôle d'horloge biologique.

V. Les interactions complexes entre la lumière et le système dopaminergique

Un effet bénéfique de la luminothérapie a été suggéré par deux études cliniques préliminaires dans la maladie de Parkinson, une pathologie en lien avec un déficit en dopamine (Paus, Schmitz-Hubsch et al. 2007; Willis and Turner 2007). Dans ces travaux,

l'administration de lumière entraînait non seulement une amélioration des symptômes moteurs caractéristiques de la maladie, mais également un bénéfice concernant les troubles non-moteurs qui sont fréquemment associés (Paus, Schmitz-Hubsch et al. 2007; Willis and Turner 2007).

Un certain nombre de données suggèrent l'existence d'interactions fortes et complexes entre les systèmes dopaminergique et mélanopsinergique. Celles-ci se font soit au niveau rétinien, où la dopamine est essentiellement exprimée par des cellules amacrines avec un vaste système de ramifications, soit au niveau du système nerveux central via leurs projections sur de nombreuses structures.

Au niveau de la rétine : la dopamine module le transfert de l'information lumineuse

Les deux principaux acteurs de la transmission lumineuse non-visuelle aux différentes structures cérébrales sont la mélanopsine contenue dans les ipRGCs, et la dopamine qui provient d'un sous-groupe de cellules amacrines, des neurones dopaminergiques situés dans la couche nucléaire interne de la rétine (Witkovsky 2004). La dopamine exerce différentes fonctions au niveau de la rétine et permet entre autres d'optimiser la vision et de réguler l'horloge périphérique autonome (Witkovsky 2004). Les ipRGCs et les cellules amacrines dopaminergiques possèdent des contacts étroits (Bloomfield and Dacheux 2001; Vugler, Redgrave et al. 2007). La libération de dopamine rétinienne suit un rythme circadien (sa sécrétion reste rythmée par un cycle d'environ 24 heures en obscurité constante) avec une sécrétion maximale pendant le jour subjectif et minimale au cours de la nuit subjective, une faible sécrétion de dopamine persiste pendant la nuit subjective (Doyle, McIvor et al. 2002). Par ailleurs, la réponse dopaminergique à une stimulation photique implique la mélanopsine, qui est capable de contrôler une dépolarisation soutenue dans un sous-groupe de neurones dopaminergiques (Zhang, Wong et al. 2008).

Au niveau du système nerveux central la lumière renforce le tonus dopaminergique

L'analyse des études de traçage ne fait pas apparaître de projections directes des ipRGCs vers les structures dopaminergiques cérébrales (Hannibal and Fahrenkrug 2004; Hattar, Kumar et al. 2006). Toutefois, les résultats de différentes études suggèrent que la lumière pourrait avoir un effet sur des structures dopaminergiques, tels le locus niger et

le striatum, via une projection indirecte depuis la rétine (Nieoullon, Cheramy et al. 1977; Chiodo, Antelman et al. 1980; Steinfels, Heym et al. 1983; Schwarz, Sontag et al. 1984; Strecker and Jacobs 1985).

L'application d'un stimulus lumineux, éveillant chez l'homme, peut moduler l'activité de neurones dopaminergiques de la substance noire (Chiodo, Antelman et al. 1980; Steinfels, Heym et al. 1983; Schwarz, Sontag et al. 1984). Cette réactivité est spécifique aux neurones dopaminergiques de la pars compacta sans entraîner d'effet au niveau des neurones non-dopaminergiques de la pars reticulata (Chiodo, Antelman et al. 1980; Schwarz, Sontag et al. 1984). Steinfels et al, ont caractérisé les neurones dopaminergiques de la pars compacta en fonction de différents stimuli répétés toutes les 2 secondes, comparant des flashs lumineux à de brèves stimulations auditives (Chiodo, Antelman et al. 1980; Steinfels, Heym et al. 1983; Schwarz, Sontag et al. 1984). Des enregistrements unicellulaires ont été réalisés chez le chat au cours des différents stades de sommeil. Aucune différence entre la veille calme, le sommeil lent et le sommeil profond n'a été enregistrée, mais un état basal plus actif en veille active a été constaté (Steinfels, Heym et al. 1983). Cette activation plus élevée en veille active était indépendante de l'activité locomotrice (Steinfels, Heym et al. 1983). Différents types de réponse ont été enregistrés suite à une exposition lumineuse suggérant différents sous-types de neurones (n'ayant pas de période d'inhibition post excitation, ayant une latence et une durée d'excitation légèrement plus longues, ayant une inhibition suite à une stimulation auditive, …) (Steinfels, Heym et al. 1983; Strecker and Jacobs 1985). L'application du stimulus acoustique n'entrainait pas la même réponse électrophysiologique que le stimulus lumineux, néanmoins les auteurs ne précisent pas la durée du flash visuel, possiblement de durée supérieure au stimulus acoustique (Steinfels, Heym et al. 1983). Les neurones stimulés par le flash lumineux et par le stimulus acoustique n'étaient pas toujours les mêmes (Steinfels, Heym et al. 1983; Strecker and Jacobs 1985). Ainsi, quelques neurones étaient stimulés par le flash visuel et inhibés par le click auditif, alors que d'autres cellules enregistrées répondaient seulement au stimulus lumineux, mais pas à l'application du stimulus acoustique (Steinfels, Heym et al. 1983).

Dans leur interprétation les auteurs parlent d'un stimulus visuel, sous-entendant qu'il passe par les voies visuelles. Néanmoins, une autre condition visuelle consistant dans l'exposition de l'expérimentateur dans le champ de vision du chat, entraînait des

réactions adverses avec inhibition de ces mêmes neurones (Steinfels, Heym et al. 1983). De manière globale les auteurs ont interprété ces résultats en relation avec un effet activateur des stimuli sur les neurones de la pars compacta de la substance noire, même si les réponses aux différentes stimulations pouvaient différer d'un neurone à l'autre. L'activité des neurones à dopamine de l'aire tegmentale ventrale varie en fonction de l'état de la vigilance avec une décharge en bouffées lors de l'éveil à la suite d'une consommation de nourriture sapide, ainsi qu'au cours du sommeil paradoxal (Dahan, Astier et al. 2007).

L'ensemble de ces conclusions restent cependant spéculatives car il est difficile de savoir si les effets observés correspondent à un effet direct de la lumière avec une activation du neurone dopaminergique de la substance noire, ou si la réponse neuronale observée est non spécifique, secondaire à une modulation des processus attentionnels. Ces expériences sont également limitées par l'exposition non soutenue très brève du stimulus lumineux et ne permettent pas de tester davantage le mode de décharge du neurone dopaminergique. Une décharge « phasique » en bouffée a été montrée comme étant plus efficace qu'une décharge « tonique » quant à la sécrétion de la dopamine dans la synapse striatale du rat (Floresco, West et al. 2003).

En plus de cette stimulation photique aiguë des neurones dopaminergiques nigrostriataux, une régulation circadienne des sécrétions de dopamine, de l'acide 3,4-Dihydroxyphénylacétique (DOPAC) et de l'acide homovanillique (HVA) mesurés par microdialyse est proposée. Au niveau du noyau accumbens, la rythmicité circadienne a été observée dans la condition alternant 12 heures de lumière et 12 heures d'obscurité, en condition d'obscurité constante pendant 24 heures et en condition de luminosité constante pendant 24 heures chez des rats non anesthésiés (Castaneda, de Prado et al. 2004). Au niveau du striatum, les données sont plus ambiguës avec perte de la rythmicité circadienne en condition de 24 heures d'obscurité constante, alors qu'elle restait présente en 24 heures de lumière constante (Castaneda, de Prado et al. 2004).

Finalement, et malgré les limitations inhérentes aux études citées, l'ensemble de ces données renforcent l'hypothèse que la sécrétion de dopamine au niveau du striatum puisse être influencée par la lumière.

VI. La maladie de Parkinson idiopathique

La maladie de Parkinson idiopathique (MPI) est une maladie neurodégénérative consécutive à des facteurs génétiques et environnementaux (Lees, Hardy et al. 2009). Sa prévalence est de 1 à 2/1000 en Europe, et son incidence est de 0,3 – 1/5000 habitants/ an. La prévalence augmente avec l'âge et le sex ratio homme/femme est de 1,4. La dégénérescence des neurones dopaminergiques de la substantia nigra rend compte des principaux **symptômes moteurs** de la maladie qui sont le tremblement de repos, l'akinésie et l'hypertonie plastique, mais n'explique pas tout le tableau clinique qui implique également d'autres mécanismes (Lees, Hardy et al. 2009). L'atteinte motrice est classiquement asymétrique avec une bilatéralisation des symptômes moteurs au cours de l'évolution de la maladie. Le tremblement de repos disparaît à l'action et correspond à un mouvement stéréotypé, répétitif, lent (4-6Hz). Il est majoré à l'émotion et peut prédominer au membre supérieur, classiquement décrit à type « d'émiettement », au membre inférieur ou plus rarement au niveau du menton. L'akinésie correspond à une réduction et à une lenteur d'initiation des gestes avec des mouvements rares, lents, de faible amplitude voire impossibles en cas de maladie évoluée. Elle se manifeste surtout pour des mouvements automatiques avec des difficultés à la marche et une mimique réduite. La rigidité ou hypertonie extra-pyramidale rencontrée dans la maladie de Parkinson, correspond à une résistance musculaire des membres qui cède par à-coups aux mouvements passifs imposés par un examinateur. Elle peut être douloureuse et source de déformations de mains ou du tronc.

La prise en charge pharmacologique des signes moteurs de la maladie repose essentiellement sur les médicaments dopaminergiques : L-dopa associée à un inhibiteur de la dopadécarboxylase périphérique, agonistes dopaminergiques, inhibiteur de la monoamine oxydase B (IMAO B), inhibiteurs de la COMT (cathécol O méthyl transférase) (Yamamoto and Schapira 2008). Tandis que pendant les premières années d'évolution ces médicaments dopaminergiques permettent souvent une amélioration fonctionnelle significative, l'évolution spontanée de la maladie nécessite une augmentation progressive des traitements dopaminergiques, eux-mêmes à l'origine de complications motrices : fluctuations d'efficacité (réapparition de plus en plus rapide des signes parkinsoniens), et dyskinésies biphasiques ou de pics de dose. Le passage brutal d'un état de dyskinésies invalidantes à un état d'akinésie parkinsonienne (effets

ON/OFF) devient alors extrêmement difficile à gérer. Bien qu'un traitement chirurgical (stimulation bilatérale à haute fréquence du noyau subthalamique) donne chez certains patients fluctuants des résultats intéressants (Benabid, Chabardes et al. 2009), les indications de ce traitement sont limitées et la procédure est associée à des risques non-négligeables. Finalement, les difficultés rencontrées pour équilibrer le traitement dopaminergique des patients sur la durée soulignent la nécessité de trouver des traitements adjuvants efficaces.

Avec le développement des traitements dopaminergiques, il est clairement apparu que la maladie de Parkinson n'est pas seulement une maladie de la motricité. Les symptômes non-moteurs sont importants à prendre en compte et à traiter, puisqu'ils exercent leurs propres effets délétères, y compris sur la symptomatologie motrice et le handicap (Poewe 2008). Les **symptômes non-moteurs** incluent de nombreux domaines, notamment des troubles du sommeil, des troubles de l'humeur ainsi que des troubles cognitifs. Longtemps négligés à tort, ces symptômes non-moteurs en particulier nécessitent d'autres alternatives thérapeutiques qu'un traitement dopaminergique, souvent peu efficace sur ces derniers (Arnulf, Leu et al. 2008; Park and Stacy 2009).

VII. Le syndrome des jambes sans repos

Le syndrome des jambes sans repos (SJSR) est une pathologie qui implique le système dopaminergique sans que le rôle de ce dernier ne soit bien compris (Allen, Picchietti et al. 2003). Il s'agit d'un trouble sensorimoteur, caractérisé par des sensations désagréables dans les jambes, dont la description a une importante variabilité d'un patient à l'autre (Karroum, Golmard et al. 2012). Les symptômes surviennent au repos avec un besoin impérieux de bouger les membres inférieurs, et le soulagement des troubles sensitifs par le mouvement volontaire, tous ces éléments étant caractéristiques de cette affection. Des mouvements périodiques nocturnes survenant à la veille ou au sommeil sont observés dans 80% des cas de SJSR, mouvements involontaires d'allure myoclonique touchant les membres inférieurs, et qui sont également atténués par le mouvement volontaire. L'évolution de la symptomatologie au cours du nycthémère est caractéristique, elle s'aggrave en soirée et en début de nuit (Allen, Picchietti et al. 2003).

Le diagnostic de cette affection est purement clinique, même si l'enregistrement vidéo-polysomnographique peut apporter des indices supplémentaires. Ainsi, on peut observer un patient qui présente à l'éveil une importante agitation prédominant aux membres inférieurs pouvant retarder son endormissement, ou être source d'éveils intrasommeil. Le sommeil est souvent fragmenté, lié ou non à des mouvements périodiques du sommeil.

Le SJSR constitue une entité assez hétérogène, autant sur le plan phénotypique qu'étiologique. Dans la cadre de mon activité de recherche clinique, j'ai pu décrire la variante du SJSR aux membres supérieurs [Annexes (Articles 5 et 6)] et des formes secondaires à un accident ischémique cérébral [Annexe (Articles 7 et 8)]. Les différences phénotypiques font probablement suite à des mécanismes physiopathologiques différents, encore mal connus dans le SJSR, ce qui rend difficile les études cliniques. D'importantes différences sont également observées dans les réponses thérapeutiques, pouvant peut-être s'expliquer par des mécanismes sous-jacents différents.

Deux avancées majeures ont été la mise en évidence de troubles du métabolisme du fer intracérébral (O'Keeffe, Gavin et al. 1994) et l'efficacité des produits à visée dopaminergiques en particulier des agonistes à faible dose (Hornyak, Scholz et al. 2014). Par ailleurs, on distingue dans le SJSR une forme idiopathique survenant souvent à un âge jeune et associée à une histoire familiale, alors que la forme secondaire survient habituellement plus tardivement. Dans ce cas le SJSR peut être associé à une neuropathie, à une insuffisance rénale, à une carence martiale, à une grossesse, à la prise de médicaments tels les neuroleptiques ou les antidépresseurs (tricycliques ou la famille des inhibiteurs de la recapture de la sérotonine). En outre, les analyses génétiques ont permis de mettre en évidence différents polymorphismes associés au SJSR (Trenkwalder, Hogl et al. 2009).

Cette pathologie est relativement fréquente, pouvant toucher entre 3,9 et 7,9 % de la population (Ohayon, Guilleminault et al. 2010). Si les formes peu sévères sont prédominantes, la prise en charge des formes sévères pose souvent problème. Les traitements peuvent être insuffisants, mal tolérés ou encore engendrer un syndrome d'augmentation avec recrudescence paradoxale des symptômes sous traitement (Garcia-Borreguero, Allen et al. 2007). Les conséquences de la maladie sont multiples : le sommeil est habituellement altéré, quantitativement et qualitativement, les répercussions diurnes entraînent souvent somnolence, troubles de l'humeur, asthénie,

altérations des fonctions exécutives et de la qualité de vie. Les troubles de l'humeur sont particulièrement difficiles à traiter, une majoration iatrogène du SJSR étant fréquemment observée avec la plupart des antidépresseurs (Picchietti and Winkelman 2005).

VIII. Hypothèse de travail / Objectifs

L'objectif de ce travail de thèse a été de mieux caractériser les effets non-circadiens de la lumière sur les comportements avec une approche translationnelle de l'animal à l'homme en passant par le rongeur diurne, permettant d'ouvrir des perspectives en application clinique à partir des données issues de la recherche fondamentale. Alors que les substrats neuronaux impliqués dans les effets directs de la lumière sur la veille et le sommeil chez le rongeur feront l'objet des première et deuxième parties de ce travail, la troisième partie porte sur l'application clinique de la lumière dans deux pathologies dopaminergiques, la maladie de Parkinson idiopathique et le syndrome des jambes sans repos.

Plus spécifiquement, les trois volets suivants sont abordés dans ma thèse:

Première partie :

Aspect fondamental: Identification des structures impliquées dans les effets non-circadiens directs de la lumière sur le sommeil et sur la veille chez le rongeur nocturne

i) rôle de la mélanopsine
ii) influence de la lumière sur les neurones galaninergiques de l'aire préoptique ventrolatérale
iii) rôle des noyaux suprachiasmatiques

Deuxième partie :

Approche translationnelle de l'animal à l'homme : Caractérisation du sommeil et de sa régulation dans un modèle de rongeur diurne, *Arvicanthis ansorgei*.

Troisième partie :

Aspect clinique : Application des effets non-circadiens directs (design expérimental permettant de contrôler l'absence d'effet sur la phase circadienne par l'application de la luminothérapie) de la lumière en pathologie dopaminergique

i) les effets directs de la lumière (utilisant une luminothérapie) sur les symptômes moteurs et non-moteurs de la maladie de Parkinson

ii) les effets directs de la lumière sur les symptômes sensori-moteurs du syndrome des jambes sans repos

Aspect clinique : Etude du système dopaminergique avec une approche en neuro-imagerie chez des patients atteints d'un SJSR dans les suites d'un accident ischémique cérébral dans le territoire lenticulo-strié

MATERIEL ET METHODES

Matériel et Méthodes

Les méthodes utilisées dans ce travail de thèse seront approfondies dans les rubriques respectives des différents articles.

Modèles animaux

Pour réaliser ce travail, nous avons utilisé les modèles suivants :

- **Souris sans mélanopsine (*Opn4*$^{-/-}$) (Ruby, Brennan et al. 2002)**
    - Melanopsin as a sleep modulator: circadian gating of the direct effects of light on sleep and altered sleep homeostasis in *Opn4*$^{-/-}$ mice. *Plos Biology 2009*. (page 60)
    - A model to predict how (melanopsin-dependent) lighting shapes the sleep-wake cycle. *En préparation*. (page 61)
    - Light irradiance positively influences sleep homeostasis through melanopsin-baseed phototransduction. *En préparation*. (Annexe; page 114)
    - Opn4-based dose-dependent direct mood improvement by light. *En préparation*. (Annexe ; page 116)
- **Souris sans pacemaker circadien** obtenues, soit par
    - lésion électrolytique des NSC (Easton, Meerlo et al. 2004)
        - A model to predict how (melanopsin-dependent) lighting shapes the sleep-wake cycle. *En préparation*. (page 61)
        - The sleep-wake dependent changes in clock gene expression and in EEG delta power do not depend on an intact SCN. *En préparation*. (Annexe; page 115)
    - invalidation de l'horloge par transgénèse (Synaptotagmin10 Cre/Cre Bmal1fl/-) (Husse, Zhou et al. 2011)
        - A model to predict how (melanopsin-dependent) lighting shapes the sleep-wake cycle. *En préparation*. (page 61)
- **Arvicanthis Ansorgei (Challet, Pitrosky et al. 2002).**
    - Arvicanthis ansorgei, a novel model for the study of sleep and waking in diurnal rodents. *Sleep, accepté*. (page 65)

Acquisition des données d'activité locomotrice (actimétrie)

L'activité locomotrice est enregistrée soit par :

- <u>une cage équipée d'une roue</u>

  - Arvicanthis ansorgei, a novel model for the study of sleep and waking in diurnal rodents. *Sleep, accepté*. (page 65)
  - The sleep-wake dependent changes in clock gene expression and in EEG delta power do not depend on an intact SCN. *En préparation*. (Annexe; page 115)

- <u>un capteur de détection infra-rouge de l'activité locomotrice</u>

  - A model to predict how (melanopsin-dependent) lighting shapes the sleep-wake cycle. *En préparation*. (page 61)
  - Light irradiance positively influences sleep homeostasis through melanopsin-baseed phototransduction. *En préparation*. (Annexe; page 114)
  - Opn4-based dose-dependent direct mood improvement by light. *En préparation*. (Annexe ; page 116)

Explorations du sommeil in vivo

chez l'animal

- Melanopsin as a sleep modulator: circadian gating of the direct effects of light on sleep and altered sleep homeostasis in *Opn4*$^{-/-}$ mice. *Plos Biology 2009*. (page 60)
- A model to predict how (melanopsin-dependent) lighting shapes the sleep-wake cycle. *En préparation*. (page 61)
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- The sleep-wake dependent changes in clock gene expression and in EEG delta power do not depend on an intact SCN. *En préparation*. (Annexe; page 115)

- **chez l’homme :**

  - Bedtime-related jerks in the upper limbs associated with restless arms syndrome. *Neurology*, accepté. (Annexe; page 110)

  - Characterization of periodic upper limb movement disorder in a patient with restless arms syndrome. *Movement disorders* 2012. (Annexe; page 109)

  - Restless legs syndrome as a first manifestation of a cerebral infarct. *Journal of Clinical Sleep Medicine*, 2014. (Annexe; page 111)

  - Brainstem stroke-related restless legs syndrome: frequency and anatomical considerations. *European Neurology*, accepté. (Annexe; page 112)

  - Hyperdopaminergism in lenticulostriate stroke-induced restless legs syndrome: a neuro imaging study. *En préparation*. (Annexe; page 113)

Conditions expérimentales

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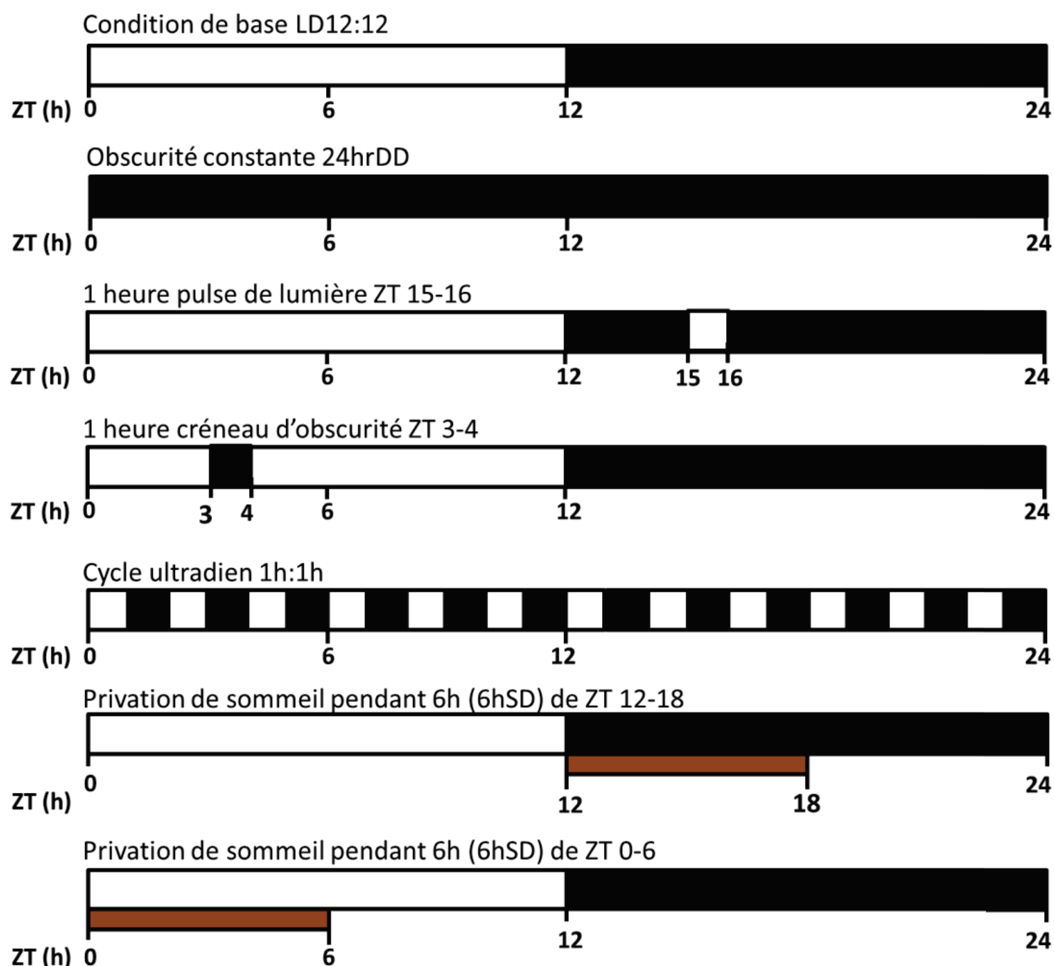

Figure 4 : Conditions expérimentales d'exposition lumineuse et de privation de sommeil.

ZT « *Zeitgeber time* » avec ZT0 qui correspond au début de la période lumineuse. Les barres blanches représentent les périodes lumineuses et les barres noires les périodes d'obscurité ; les barres brunes correspondent à la période de privation de sommeil. Les carrés blancs correspondent à des expositions d'une heure de lumière et les carrés noirs à des créneaux d'une heure d'obscurité. Le temps est exprimé en heure (h).

On parle de période lumineuse « subjective » lorsque l'animal est placé en condition expérimentale et que le moment de la période correspond à la période lumineuse habituelle. De même, la période d'obscurité « subjective » signifie le moment circadien de l'animal qui correspond à la période d'obscurité habituelle, alors que l'animal

en condition expérimentale se trouve par exemple en lumière constante ou exposé à des cycles ultradiens alternant des créneaux de lumière et d'obscurité.

Analyse des états de la vigilance (Bourgin, Fabre et al. 2007; American Academy of Sleep Medicine, 2005)

- **chez l'animal :**

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Anatomie

- Injection de la toxine cholérique, sous-unité B

    - A model to predict how (melanopsin-dependent) lighting shapes the sleep-wake cycle. *En préparation*. (page 61)

- Immunomarquages

    - Melanopsin as a sleep modulator: circadian gating of the direct effects of light on sleep and altered sleep homeostasis in *Opn4*$^{-/-}$ mice. *Plos Biology 2009*. (page 60)
    - A model to predict how (melanopsin-dependent) lighting shapes the sleep-wake cycle. *En préparation*. (page 61)
    - The sleep-wake dependent changes in clock gene expression and in EEG delta power do not depend on an intact SCN. *En préparation*. (Annexe; page 115)

Expériences cliniques

Les données cliniques concernant le syndrome des jambes sans repos ont été recueillies lors de la prise en charge habituelle des patients, et un avis favorable du Comité d'Ethique a été obtenu au préalable pour les résultats issus des expériences utilisant l'imagerie nucléaire.

- Bedtime-related jerks in the upper limbs associated with restless arms syndrome. *Neurology*, accepté. (Annexe; page 110)
- Characterization of periodic upper limb movement disorder in a patient with restless arms syndrome. *Movement disorders* 2012. (Annexe; page 109)
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RESULTATS

Résultats

1ère partie : ***« Identification des structures impliquées dans les effets directs de la lumière sur le sommeil chez la souris »***

Première partie: Introduction

Les structures et voies neuronales impliquées dans les effets circadiens de la lumière

Les effets circadiens de la lumière sur le cycle veille-sommeil sont bien connus et passent par des mécanismes de resynchronisation de notre horloge principale située dans les noyaux suprachiasmatiques (NSC). Le signal lumineux est détecté au niveau de la rétine par les photorécepteurs classiques, les cônes et les bâtonnets, mais c'est principalement la mélanopsine, un photopigment contenu dans un sous-groupe de cellules ganglionnaires de la rétine (ipRGS) qui détecte l'information non visuelle de la lumière. Par ailleurs, les ipRGC véhiculent également l'information lumineuse provenant des cônes et des bâtonnets (Goz, Studholme et al. 2008; Guler, Ecker et al. 2008; Hatori, Le et al. 2008). Les projections des ipRGCs se font par le tractus rétinohypothalamique, qui est PACAPergique (Pituitary adenylate cyclase-activating polypeptide) et glutamatergique, sur la partie rétinorécipiente des NSC. Dans l'organisation des NSC on distingue le centre (ou « core ») des NSC, partie rétinorécipiente, antérieure et ventrolatérale. Cette partie est particulièrement riche en VIP (Vasopressive Intestinal Peptide), alors que les neurones contenant le neuropeptide AVP (arginine vasopressine) forment la partie dorsomédiane des NSC, l'enveloppe (ou « shell ») (Moore, Speh et al. 2002). (Figure 5)

Cependant, les effets circadiens de la lumière ne dépendent pas uniquement de la mélanopsine. Ceci est illustré par un rythme circadien qui reste entraîné chez les souris ayant eu une invalidation du gène de la mélanopsine (Ruby, Brennan et al. 2002). En dehors des projections des ipRGC sur les NSC, des projections non-mélanopsinergiques / PACAPergiques permettent également de transmettre le signal lumineux sur les NSC (Hannibal and Fahrenkrug 2004). Il existe aussi des projections indirectes. Ainsi, le tractus géniculohypothalamique transmet aux NSC l'information lumineuse, notamment celle reçue par les feuillets intergéniculés (Morin 2013).

Au niveau des NSC, le signal lumineux permet de synchroniser l'horloge principale sur le rythme jour/nuit et cette information est distribuée au corps entier grâce à la mélatonine sécrétée par de la glande pinéale. Toutefois, la sécrétion de la mélatonine est également sous un contrôle photique direct. Chez le mammifère, [contrairement par

exemple à la glande pinéale aviaire qui est directement photosensible (Csernus 2006)], la réception du signal lumineux au niveau de la rétine aboutit à la suppression de la sécrétion de la mélatonine, cette dernière n'ayant lieu que dans le noir.

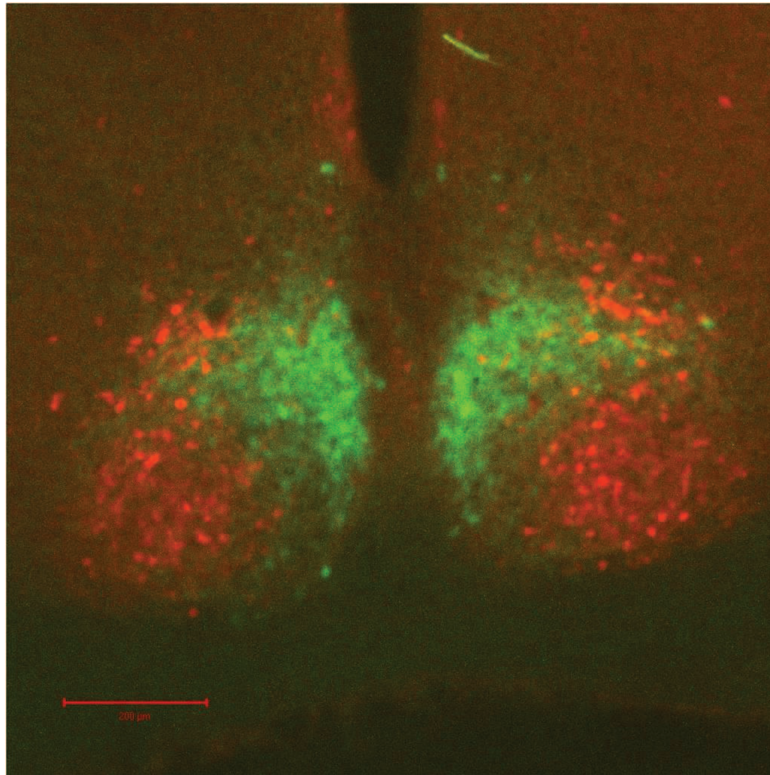

Figure 5. Vue globale des NSC. La stimulation lumineuse induit au niveau de la partie rétinorécipente l'expression de c-Fos (rouge), un marqueur d'activation neuronale. Les neurones vasopressinergiques, constituant l'enveloppe (ou « shell ») des NSC ont été marqués par immunofluorescence en vert.

Hypothèses concernant les structures et voies neuronales impliquées dans les effets non-circadiens, directs de la lumière

Les effets non-circadiens directs de la lumière restent beaucoup moins appréhendés. Les effets directs de la lumière ont une traduction comportementale rapide, contrairement aux effets circadiens qui nécessitent un délai d'environ 24 heures. La mélatonine, dont la sécrétion par la glande pinéale est supprimée suite à une exposition lumineuse, ne peut être un candidat majeur. Malgré l'absence de cette

hormone chez la plupart des souches de souris maintenues en laboratoire (Kasahara, Abe et al. 2010), les effets directs de la lumière persistent.

A ce jour, la traduction clinique des effets directs de la lumière la mieux étudiée est le réflexe photomoteur. La mélanopsine, de par ses projections au niveau du noyau olivaire du prétectum, en est un acteur principal. Son rôle dans la réponse pupillaire à la lumière est bien identifié avec une composante dépendante de la mélanopsine mesurable (Brown, Allen et al. 2013; Barrionuevo, Nicandro et al. 2014).

Néanmoins, les structures et voies neuronales impliquées dans les effets directs de la lumière sur le sommeil restent inconnues. Au niveau rétinien, l'implication de la mélanopsine, de par ses propriétés de photopigment intrinsèquement photosensible (Mure, Rieux et al. 2007; Zhang, Wong et al. 2008; Chen, Badea et al. 2011; Schmidt, Chen et al. 2011) et de par ses projections cérébrales multiples (Hannibal and Fahrenkrug 2004; Hattar, Kumar et al. 2006), semble un candidat évident.

Au niveau cérébral, deux structures semblent particulièrement intéressantes : l'aire préoptique ventrolatérale (VLPO) et les noyaux suprachiasmatiques (NSC).

Le rôle du VLPO dans la régulation sommeil est bien établi. Ce sont notamment les neurones galaninergiques et GABAergiques dits « inducteurs de sommeil » qui sont considérés comme un élément clef dans l'induction et l'homéostasie du sommeil (Gvilia, Xu et al. 2006). Ces neurones densément organisés en grappe (ou« cluster ») sont situés dans la portion la plus ventrale de l'aire préoptique et latéralement par rapport au chiasma optique (Figure 6). Ils sont activés en sommeil lent, et inhibés par la plupart des neurotransmetteurs impliqués dans l'éveil (Gallopin, Fort et al. 2000). Les neurones galaninergiques du VLPO étendu (ou « extended »), non regroupés et de localisation plus dorsale, sont actifs en sommeil paradoxal (Sherin, Shiromani et al. 1996; Lu, Greco et al. 2000). Une lésion bilatérale des VLPO entraîne une insomnie prolongée (Lu, Greco et al. 2000). Ces données évoquent que l'induction du sommeil ou de la veille résulte d'une interaction inhibitrice réciproque entre les structures de l'éveil et les neurones inducteurs du sommeil. De plus, il existe des projections directes des ipRGC sur le bord ventral du VLPO.

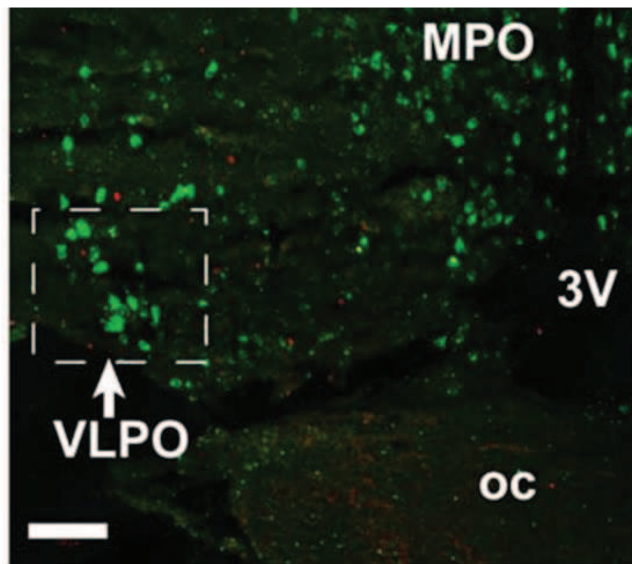

Figure 6. L’aire préoptique ventro-latérale (VLPO). Les neurones galaninergiques (vert) « inducteurs de sommeil » sont organisés en grappe (ou « cluster ») latéralement par rapport au chiasma optique (oc) et dans la partie la plus ventrale de l’hypothalamus antérieur (encadré). La barre d’échelle correspond à 100µm. 3V : troisième ventricule ; MPO : aire préoptique médiane.

Les NSC, cible principale du tractus rétino-hypothalamique, sont considérés seulement dans leur fonction d’hébergement de l’horloge biologique principale. Toutefois, il existe un réseau d’afférences et d’efférences particulièrement riche entre les NSC et les autres structures hypothalamiques, voire cérébrales (Morin 2013). Compte tenu de ces considérations, les NSC, en dehors de leur rôle d’horloge, pourraient également être une structure de relai dans les effets directs de la lumière sur le sommeil.

Première partie: Résultats

Les différents articles auxquels ce travail de thèse a abouti, ne sont pas rédigés pour se suivre selon la question neuroanatomique posée. Ainsi, dans un souci de clarté et de continuité des hypothèses de travail de cette thèse, mes principaux résultats sont repris dans cette section. Les articles qui s'y réfèrent sont insérés à la fin de ce chapitre.

La mélanopsine est impliquée dans les effets directs de la lumière sur le sommeil.

L'approche utilisée pour mieux identifier le rôle de la mélanopsine dans les effets directs de la lumière est l'étude du phénotype comportemental des souris transgéniques ayant eu une invalidation du gène de la mélanopsine (*Opn4*$^{-/-}$) et de les comparer au groupe de souris chez lesquels ce gêne est préservé, souris dites de type« sauvage » (*Opn4*$^{+/+}$). Leur sommeil est étudié en fonction de différentes conditions lumineuses.

<u>En l'absence de mélanopsine, les souris perdent une heure de sommeil pendant la période lumineuse en condition de base (12hL:12hD).</u>

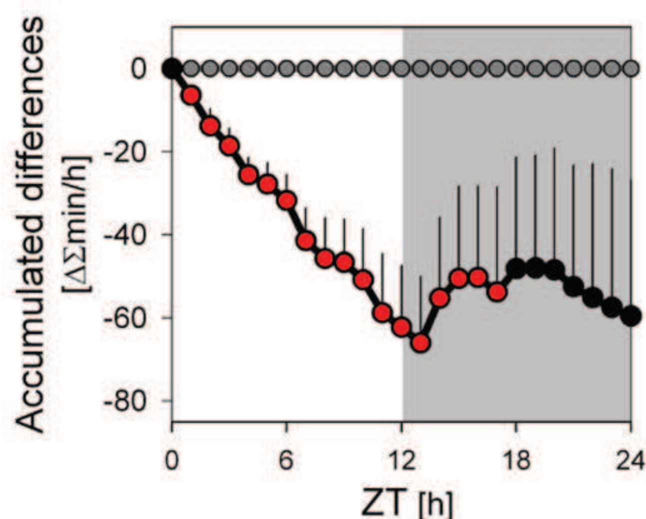

Figure 7. Différence cumulée en sommeil lent en condition 12hL : 12hD. Comparées aux souris *Opn4*$^{+/+}$, les souris *Opn4*$^{-/-}$ perdent de manière cumulée pendant la période lumineuse environ une heure de sommeil, alors qu'il n'y a pas de différence pendant la période d'obscurité .

En condition de base sous un régime simulant le jour et la nuit en alternant une période de luminosité de 12 heures suivie d'une période de 12 heures d'obscurité (12hL:12hD ; L= « light » et D= « dark »), on observe chez les souris *Opn4*$^{-/-}$ la perte

d'environ une heure de sommeil comparées aux souris *Opn4*$^{+/+}$. Cette perte de sommeil n'est observée que pendant la période lumineuse. En période d'obscurité, les deux types de souris ont un temps de sommeil non significativement différents, malgré que les souris *Opn4*$^{-/-}$ aient eu une heure d'éveil supplémentaire pendant la phase lumineuse qui précédait. Comme montré par des études antérieures, le rythme circadien de leur activité locomotrice n'avait pas été affecté (Ruby, Brennan et al. 2002). Ce résultat fait évoquer une implication de la mélanopsine soit au niveau de la régulation homéostasique du sommeil, soit au niveau d'une régulation photique directe du sommeil, soit en lien avec un pacemaker circadien moins puissant.

La régulation photique directe du sommeil est étudiée par la suite par deux conditions différentes. La première condition consiste en l'application d'une exposition lumineuse d'une heure pendant la période d'obscurité (1hLP : « light pulse » d'une heure). La deuxième condition, appelée ultradienne, consiste en l'alternance de 12 cycles d'une heure de lumière et d'une heure d'obscurité (LD1h:1h).

L'induction de sommeil par la lumière implique la mélanopsine

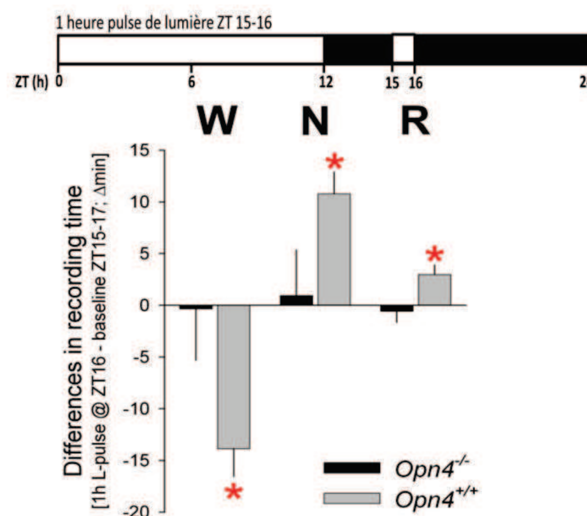

Figure 8. Effet direct d'un pulse de lumière d'une heure sur le sommeil. Induction de sommeil lent (N) au détriment de la veille (W) et avec un effet moindre sur le sommeil paradoxal (R) d'un pulse d'une heure de lumière (ZT 15 à ZT16) chez les souris *Opn4*$^{+/+}$, comparées aux souris *Opn4*$^{-/-}$ (p = 0.05; post hoc paired t-tests).

Chez les souris *Opn4*$^{+/+}$, l'induction du sommeil suite à l'application d'un pulse d'une heure de lumière en période d'obscurité est plus prononcée comparées aux souris *Opn4*$^{-/-}$ faisant évoquer une implication de la mélanopsine dans cette réponse photique.

<u>La régulation photique du sommeil implique la mélanopsine et dépend du moment circadien</u>

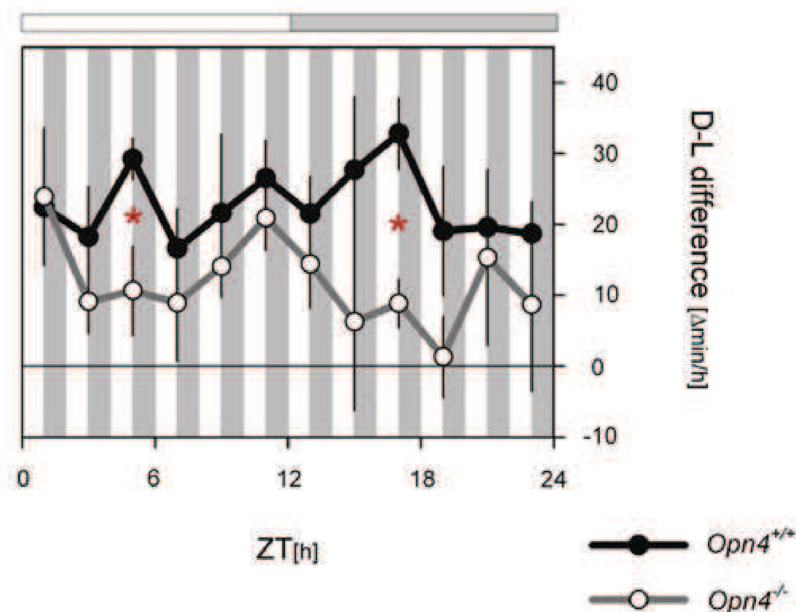

Figure 9. Le moment de la journée influence les effets directs de la lumière sur le sommeil. Illustration des différences entre les périodes de lumière et d'obscurité en condition ultradienne alternant des cycles d'une heure de lumière et d'une heure d'obscurité pendant 24 heures chez des souris *Opn4*$^{+/+}$ et *Opn4*$^{-/-}$. Les données représentent les variations concernant le temps passé éveillé (en minutes) entre les pulses d'obscurité et de lumière pour chaque cycle. Les barres verticales représentent l'erreur standard. Les analyses de la variance (ANOVA) montrent des différences entre pulse d'obscurité - lumière plus importante chez les souris *Opn4*$^{+/+}$ comparées aux souris *Opn4*$^{-/-}$ (p<0,05). L'astérisque montre des différences génotypiques significatives (p<0,05 ; post hoc *t*-tests).

La sécrétion de la mélanopsine ayant une forte modulation circadienne, une condition ultradienne a été appliquée alternant des cycles d'une heure de lumière et d'une heure d'obscurité tout au long de 24 heures. Cette condition permet de distinguer

l'importance du moment circadien dans la réponse photique au niveau du sommeil. Cette réponse dépend majoritairement de la mélanopsine pendant la période de l'obscurité subjective, alors que les cônes et les bâtonnets y contribuent largement pendant la période de lumière subjective,

L'absence de mélanopsine n'entraîne pas d'affaiblissement du pacemaker circadien

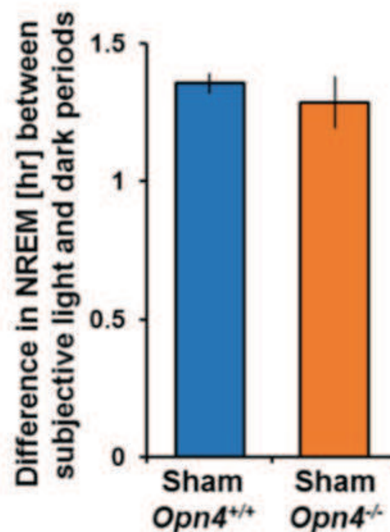

Figure 10. Absence d'influence circadienne de la mélanopsine sur le sommeil lent. La différence pour le sommeil lent entre les périodes lumineuse subjective et d'obscurité subjective en condition d'obscurité constante n'est pas différente significativement entre les deux génotypes.

Afin de tester si la perte de sommeil observée pendant la période lumineuse chez les souris *Opn4*$^{-/-}$ était en lien avec une régulation photique directe ou secondaire à un pacemaker circadien affaibli, les souris ont été enregistrées en condition d'obscurité constante. Les résultats montrent que la différence pour le sommeil lent entre la période lumineuse subjective et d'obscurité subjective en condition d'obscurité constante n'est pas différente entre les deux génotypes. La perte de sommeil lent en période lumineuse est donc en lien avec les effets directs de la lumière et non pas secondaire à un pacemaker circadien affaibli. Ce résultat est cohérent avec des données de la littérature ne mettant pas en évidence d'altération du rythme circadien de l'activité locomotrice (Ruby, Brennan et al. 2002).

La mélanopsine, en dehors de son rôle de photopigment est également impliquée dans l'homéostasie du sommeil

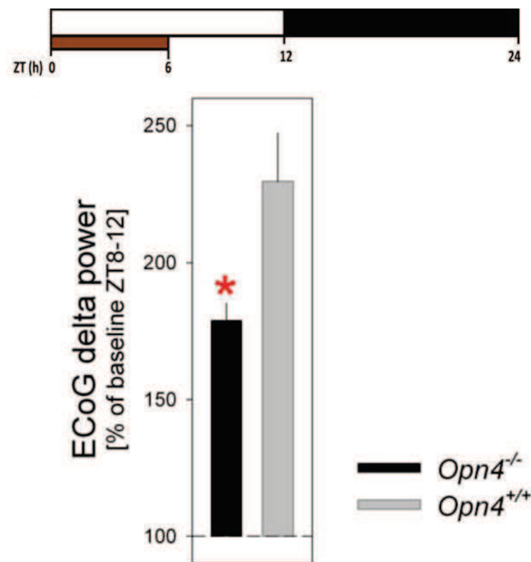

Figure 11. La mélanopsine est un facteur homéostasique du sommeil. La puisse spectrale delta mesurée lors du rebond de sommeil suivant une privation de sommeil de ZT0 à ZT6 (alors que leur pression de sommeil est déjà élevée suite à la période d'éveil nocturne qui a précédé) est significativement diminuée en l'absence de mélanopsine. Les valeurs moyennes (±SEM) sont exprimées en pourcentage par rapport aux valeurs obtenues la veille entre ZT8 et ZT12. Astérisque : différence génotypique (p= 0,05; post hoc t-tests).

Devant la perte d'environ une heure de sommeil pendant la période lumineuse en l'absence de mélanopsine, le processus homéostasique du sommeil a été testée par une condition de 6 heures de privation de sommeil. L'analyse de la puissance delta au cours du rebond de sommeil montre que les souris *Opn4*$^{-/-}$ maintiennent une pression homéostasique de sommeil plus faible en faveur d'un rôle de la mélanopsine en tant que facteur homéostasique de sommeil.

Ces différentes expériences montrent que la mélanopsine est impliquée dans les effets directs de la lumière sur le sommeil chez la souris. Elle n'intervient pas dans la régulation circadienne du cycle veille/sommeil, mais elle est à considérer comme un

facteur homésotasique du sommeil lorsque l'homéostat est défié par une privation de sommeil.

Les neurones galaninergiques « inducteurs de sommeil » sont activés par une stimulation photique avec implication de la mélanopsine

Dans un premier temps, une expérience de marquage de la protéine c-Fos, marqueur d'activation neuronale, a été réalisée au niveau des NSC chez des souris *Opn4*$^{+/+}$ et *Opn4*$^{-/-}$ afin de vérifier l'efficacité de notre application lumineuse d'une heure entre ZT 15 et ZT 16. Les résultats obtenus concordent avec ceux de Ruby et al. et montrent en l'absence de mélanopsine une réduction significative de c-Fos (en rouge) (Ruby, Brennan et al. 2002). Chez les souris de type sauvage, l'induction de c-Fos particulièrement marquée permet de valider notre condition expérimentale.

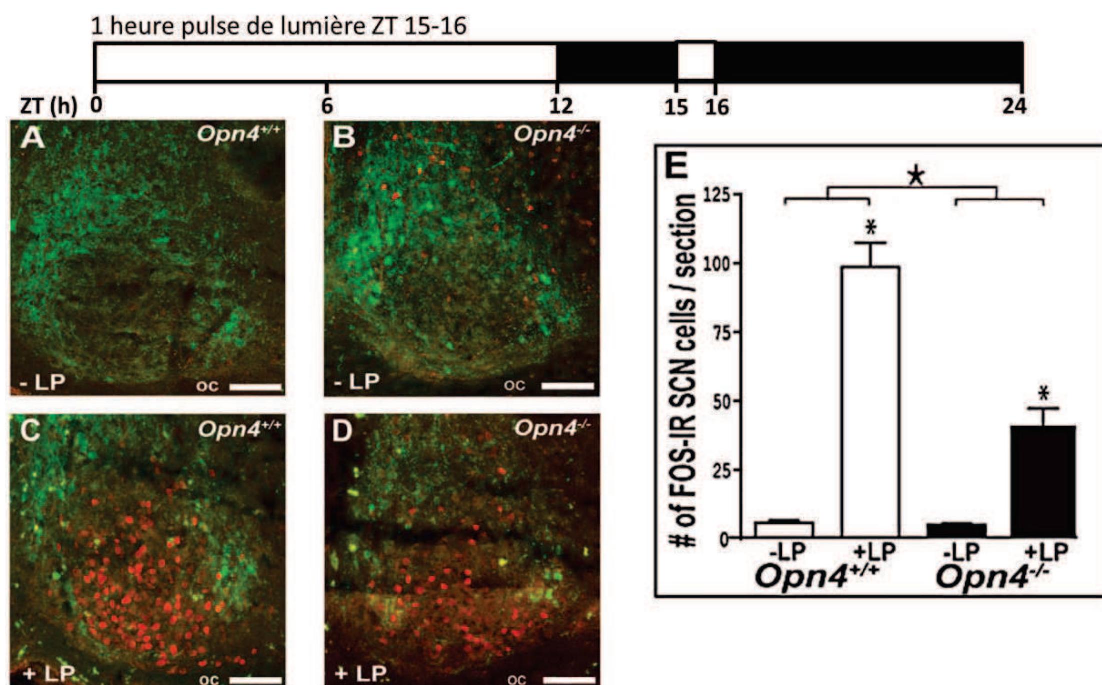

Figure 12 Activation photique des NSC. L'induction de c-Fos (rouge) en regard des NSC (AVP marqué en vert) est obtenue par l'application d'un pulse d'une heure de lumière entre ZT15 et ZT16 (C,D) avec absence d'activation neuronale en obscurité (A, B). Les souris déficientes en mélanopsine ont une réponse moindre (D, E) comparées aux souris de type sauvage. La quantification des neurones des NSC marqués à l'anticorps anti c-Fos (rouge) met en évidence une activation neuronale par la lumière,

diminuée en cas de déficience en mélanopsine (ANOVA à deux facteurs, light pulse: $p < 0,001$; astérisque (*) indique post hoc Fisher PLSD: p<,0.05), étoile : light pulse x genotype : $p<0,001$).

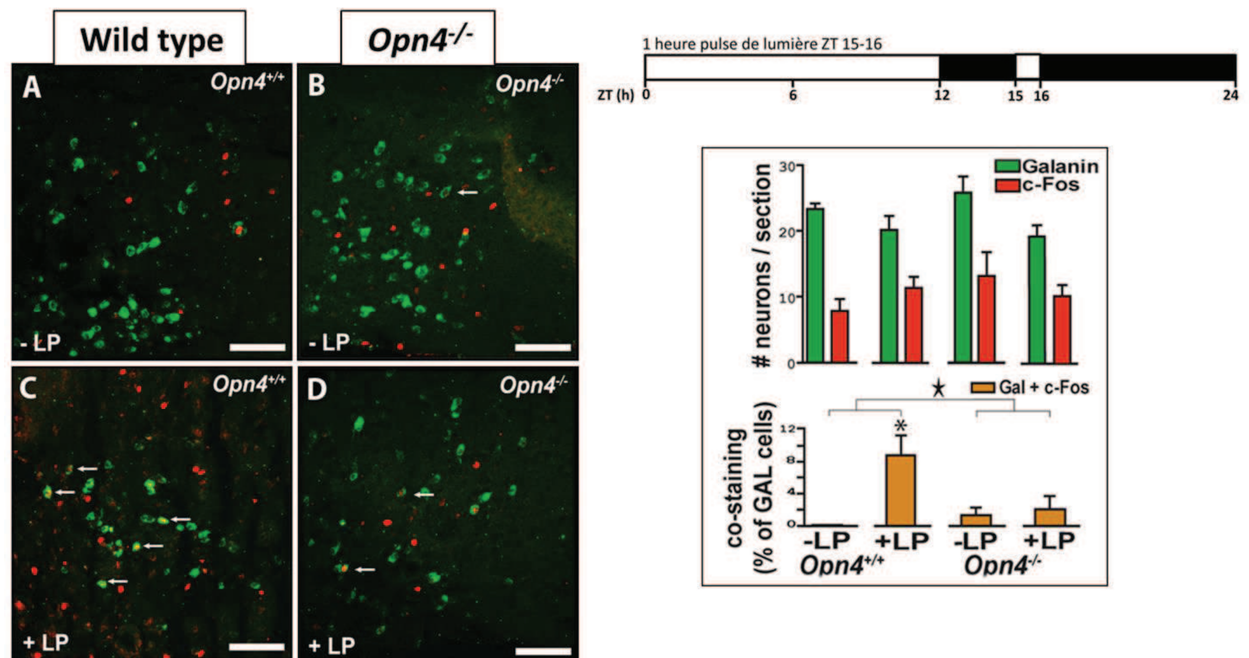

Figure 13. Activation photique des neurones « inducteurs de sommeil » du VLPO. L'application d'un pulse d'1heure de lumière [+LP (C,D); contrôle sans lumière : -LP (A, B)] entre ZT15 et ZT16 permet l'induction de c-Fos (rouge) au niveau du cluster des neurones galaninergiques (vert) du VLPO (C,D, histogrammes) avec une activation neuronale diminuée en cas de déficience en mélanopsine (D, histogrammes). La barre d'échelle indique 100µm. Les barres horizontales correspondent aux valeurs moyennes ± SEM. Une ANOVA à deux facteurs, light pulse : $p<0,05$; Light pulse x genotype : $p<0,05$ (étoile) ; astérisque indique post hoc Fisher PLSD : $p<0,05$) (B).

La quantification du comarquage de l'ARNm de la galanine (en vert) en hybridation *in situ* et de la protéine d'activation neuronale c-Fos (en rouge) en immunohistochimie met en évidence une augmentation de c-Fos dans les neurones galaninergiques « inducteurs de sommeil » à la suite de la stimulation photique. Cette augmentation est dépendante de la mélanopsine.

Les NSC, en dehors de leur fonction d'horloge, interviennent également comme structure de relai dans les effets directs de la lumière sur le sommeil.

Une double approche, lésionnelle et génétique, a été utilisée pour mieux identifier le rôle des NSC dans les effets directs de la lumière sur le sommeil. Chez ces deux modèles de souris, le pacemaker circadien n'était plus fonctionnel avec une arrythmie locomotrice mise en évidence par actimétrie.

Validation histologique du modèle expérimental

<u>Validation histologique de l'abolition du pacemaker circadien par lésions électrolytiques</u>

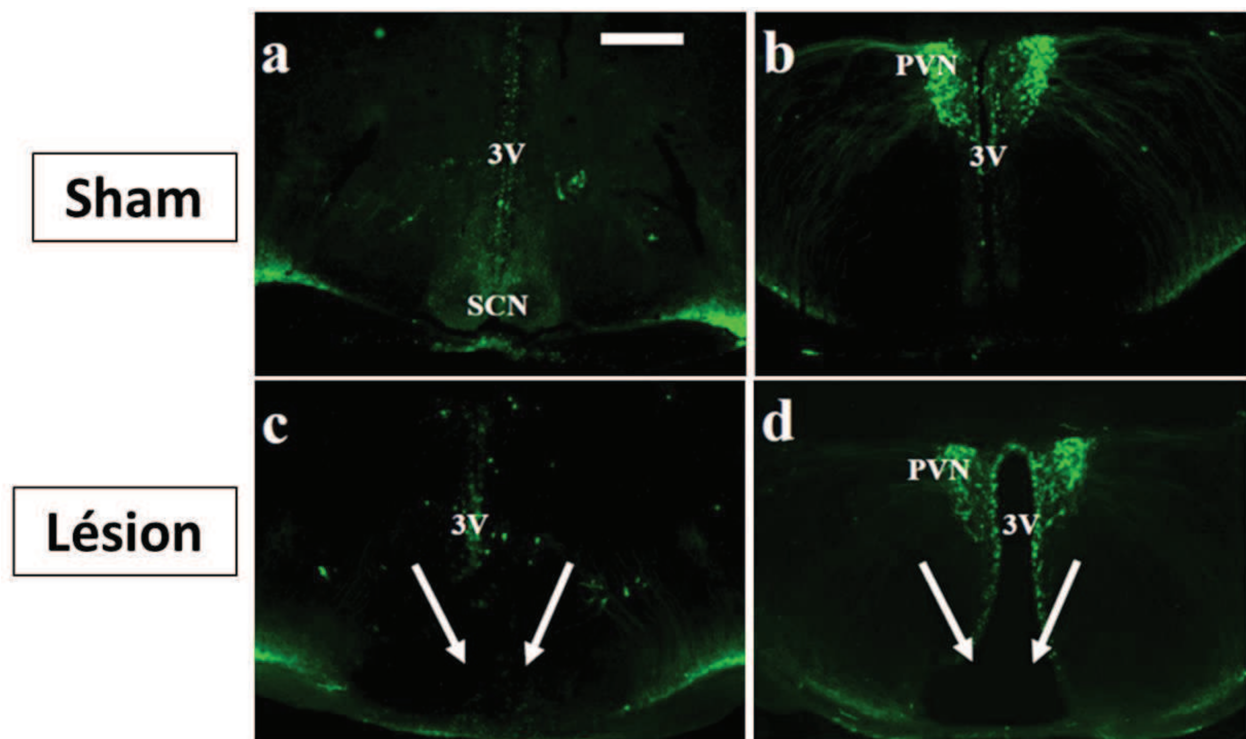

Figure 14. Les lésions sont complètes et limitées aux NSC. Sections coronales à hauteur des NSC dans leur partie moyenne (a, c) et postérieure (b, d) dans une souris « Sham » avec des NSC intacts (a,b) et une souris lésée (c,d) au niveau des NSC (flèches) sans endommager les structures adjacentes. La barre d'échelle indique 500µm ; PVN : noyaux paraventriculaires ; 3V : troisième ventricule.

Dans le modèle lésionnel, afin de vérifier l'efficacité des lésions, une analyse structurelle a été réalisée avec un marquage de l'AVP (en vert) ou en DAPI (non illustré). Les lésions sont complètes et restent limitées aux NSC, laissant les structures

adjacentes intactes, illustrées par les fibres des noyaux paraventriculaires projetant sur les noyaux supraoptiques, situés latéralement par rapport aux NSC.

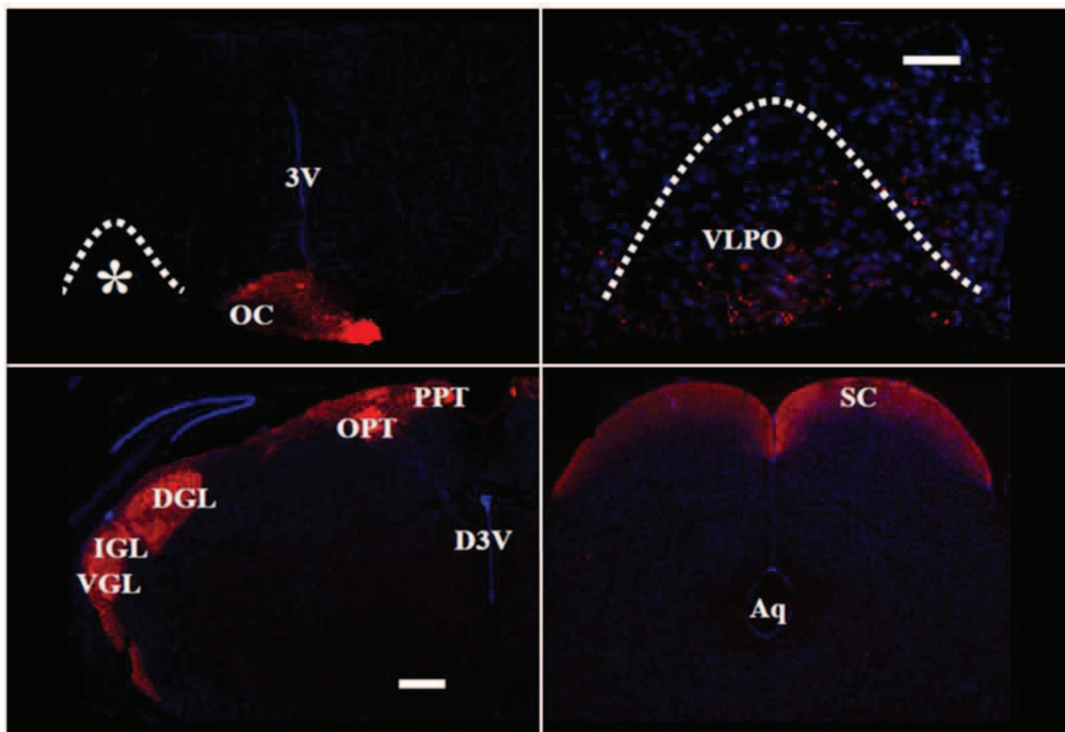

Figure 15. Les fibres provenant des ipRGC sont restées intacts. Coupes coronales d'un cerveau lésé de souris au niveau des NSC et injectée préalablement au sacrifice par la toxine du choléra (sous-unité B) pour tracer les fibres des ipRGC (en rouge) restées intactes. chiasma optique (oc) ; VLPO [correspond à un agrandissement de la zone marquée par un astérisque dans la photo en haut à gauche] ; noyaux du prétectum (OPT, PPT) et complexe géniculé (DGL, IGL, VGL) ; colliculus supérieurs (sc) ;3ième ventricule (3V), canal de l'aqueduc (Aq). La barre d'échelle représente 500µm et 20µm dans la zone agrandie (en haut à droite).

Dans le modèle lésionnel, afin de vérifier l'absence d'atteinte des fibres du tractus rétinohypothalamique à proximité des NSC lésés, les souris ont été injectées avec la sous-unité B de la toxine du choléra (rouge), un marqueur de traçage antérograde, et les cerveaux ont été analysés en postmortem. Chez les animaux lésés, le marquage du traceur est comparable à celui des animaux contrôles, non lésés (ou « sham ») avec des fibres bien présentes au niveau du chiasma optique, du VLPO, du complexe géniculé, du prétectum et du colliculus supérieur. En bleu, les noyaux cellulaires sont marqués par le DAPI.

Ces résultats témoignent que les lésions sont complètes et que les fibres en dehors des NSE sont restées intactes. Les effets observés en électrophysiologie peuvent donc être interprétés en lien avec la lésion et ne sont pas dus à une interruption des fibres ou à une lésion des structures avoisinantes.

Validation histologique et morphofonctionnelle de l'abolition du pacemaker par approche transgénique

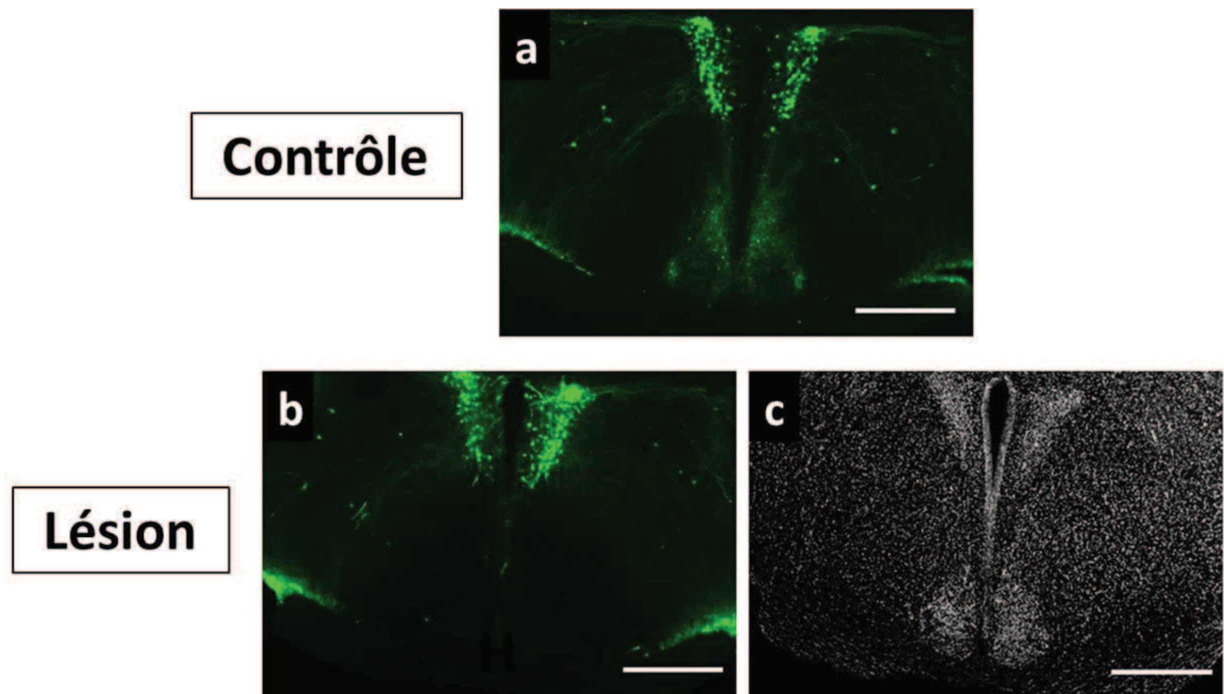

Figure 16. Les NSC des souris *Syn10$^{Cre/Cre}$Bmal1$^{fl/-}$* sont non fonctionnels et structurellement intacts. Absence d'expression de l'AVP (vert) dans les NSC chez les souris *Syn10$^{Cre/Cre}$Bmal1$^{fl/-}$*(b), alors qu'elle reste exprimée dans les structures avoisinantes (b) et chez la souris de contrôle (a). NSC structurellement intacts (DAPI, gris). La barre d'échelle représente 500µm (a, b, c).

Dans le modèle transgénique, afin de vérifier l'absence d'horloge fonctionnelle et des NSC structurellement intacts, un double marquage a été réalisé. L'AVP (vert) est absent au niveau des NSC chez la souris transgénique. Ce neuropeptide dont la régulation est sous le contrôle circadien reste bien exprimé dans les structures avoisinantes et chez les souris contrôles. Le marquage en DAPI (gris) permet de visualiser les NSC structurellement intacts chez les souris transgéniques.

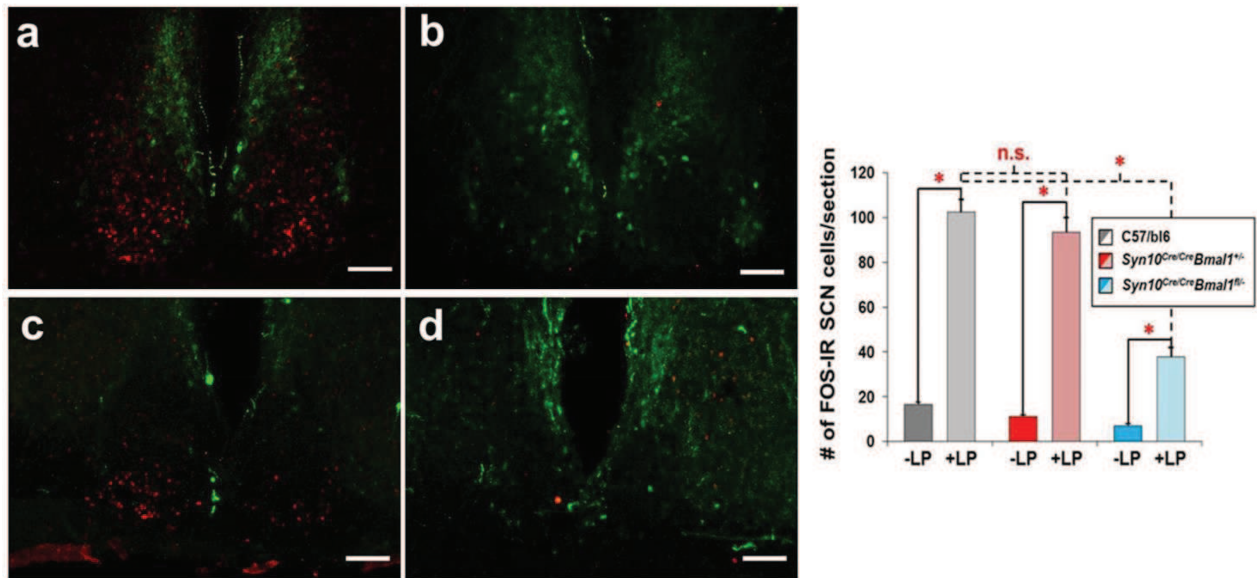

Figure 17. L'activation photique des NSC est diminuée chez les souris *Syn10Cre/CreBmal1fl/-*. Lors d'une stimulation par 1 heure de lumière entre ZT15 et ZT16 [+LP (a,c) ; -LP (contrôle)(b,d)] , on observe une moindre induction de c-Fos (rouge) chez les souris *Syn10Cre/CreBmal1fl/-*(c, histogrammes) comparées aux souris contrôles (a, histogrammes) dans les NSC (AVP, en vert). (histogrammes) ANOVA à deux facteurs; light pulse : $p<0.001$; genotype : $p<0.001$, (*) post-hoc t-test: $p<0.05$.

Dans le modèle transgénique, afin de vérifier la perception du signal lumineux, un pulse d'une heure de lumière a été appliqué entre ZT15 et ZT16. Les souris transgéniques gardent une induction de c-Fos suite à l'application lumineuse, mais de manière significativement réduite comparée à celle obtenue chez les souris contrôles. Les NSC, bien qu'ils aient perdu leur fonction de pacemaker circadien, gardent une faible activation à la lumière au niveau de la partie rétinorécipiente.

Ces différentes expériences nous permettent de conclure que les NSC des souris transgéniques sont structurellement intacts, mais non-fonctionnels concernant le pacemaker circadien et ne préservent que partiellement leur fonction de recevoir l'information lumineuse capable d'induire une activation neuronale dans la partie rétinorécipiente. Les résultats observés en électrophysiologie sont donc expliqués par la condition testée, et non pas par un biais du modèle animal utilisé.

Les NSC, au-delà de leur fonction de pacemaker circadien sont également une structure de relai dans les effets directs de la lumière sur le sommeil

En l'absence de pacemaker circadien, que ce soit par lésion électrolytique (SCNx *Opn* $4^{+/+}$) ou par transgenèse (*Syn10*$^{Cre/Cre}$*Bmal1*$^{fl/-}$), les souris montrent un comportement similaire en fonction des différentes conditions expérimentales. Ces deux modèles expérimentaux sont équivalents, malgré leurs approches différentes.

Persistance d'une réactivité aux effets directs de la lumière en l'absence de NSC fonctionnels

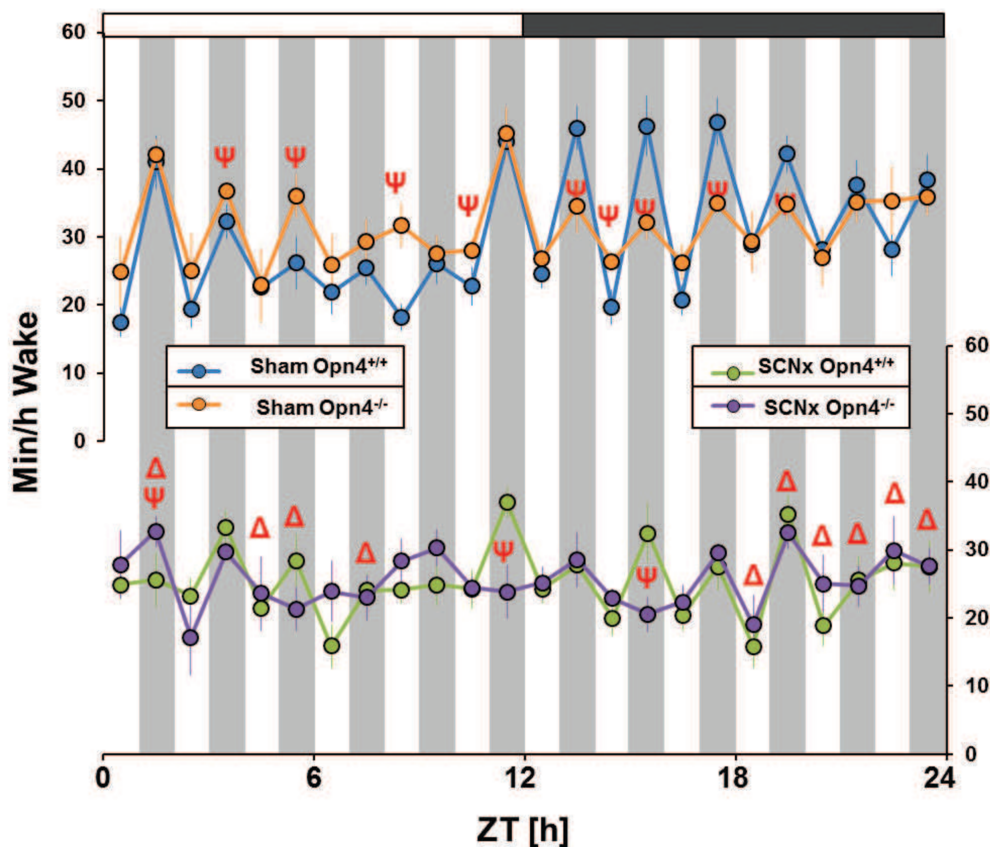

Figure 18. Les NSC contribuent à la régulation du sommeil par les effets directs de la lumière. Les valeurs moyennes de veille exprimées par heure pendant la condition ultradienne mettent en évidence chez les souris non lésées « Sham » (courbes bleue et jaune) une réactivité atténuée pendant la période d'obscurité subjective en cas de déficience en mélanopsine (courbe jaune). En cas de lésion des NSC (courbes verte et violette), il persiste une réactivité chez les souris *Opn* $4^{+/+}$ (courbe verte), alors qu'elle est presqu'abolie chez les souris *Opn* $4^{-/-}$, (courbe violette).

En condition de cycles ultradiens alternant une heure de lumière et une heure d'obscurité, les souris transgéniques (non illustrées) ont montré un comportement comparable à celui des souris lésées au niveau des NSC. Ce cycle qui vise à s'affranchir de la modulation circadienne du sommeil en réponse à la lumière, met en évidence chez les souris dépourvues de pacemaker circadien la persistance d'une réactivité à la lumière. Toutefois, cette dernière est moindre comparée aux souris de type sauvage et ne montre pas d'augmentation de la réactivité en période d'obscurité subjective.

Les NSC, en dehors de leur fonction d'horloge principale sont donc également une structure de relai pour les effets directs de la lumière sur l'induction du sommeil.

Les importances respectives des différentes composantes (mélanopsine, cônes et bâtonnets, NSC) intervenant dans les effets directs de la lumière sur le sommeil chez le rongeur nocturne.

<u>Persistance d'une alternance veille-sommeil calée sur le cycle lumière-obscurité chez des souris arythmiques</u>

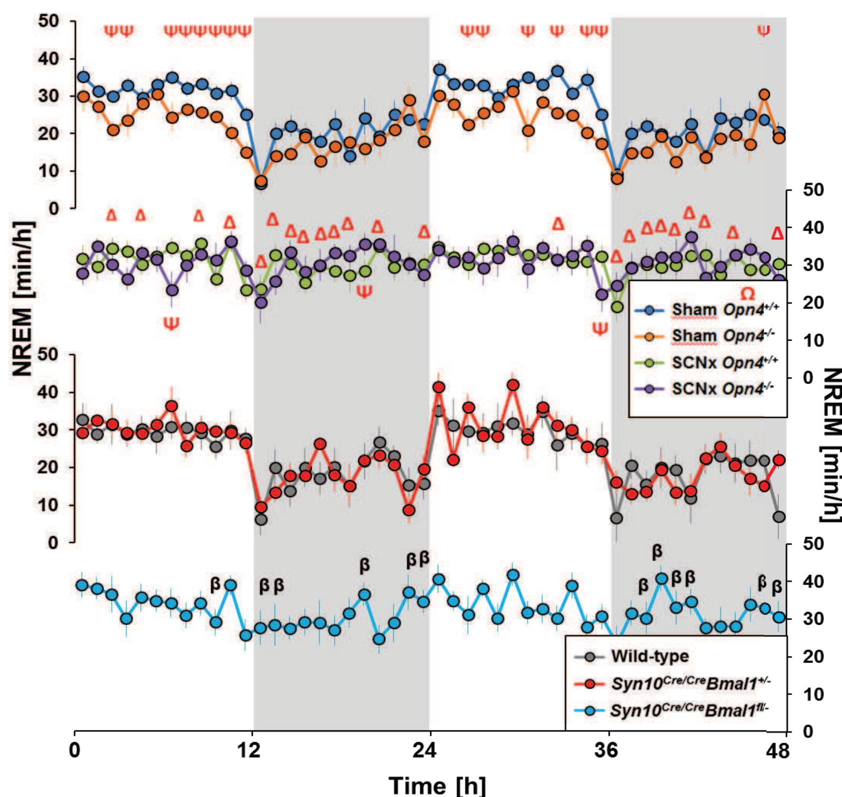

Figure 19. Les effets directs de la lumière sont capables de maintenir un cycle veille/sommeil en condition 12hL :12hD chez des souris arythmiques. Quantification du sommeil lent au cours de 48 heures chez des souris non lésées « Sham » des deux génotypes (courbes bleue et orange), souris lésées des NSC via lésion électrolytique des deux génotypes (courbes verte et violette), souris transgéniques ayant des NSC non fonctionnels (courbes rouge et grise) et souris de type sauvage (courbe bleue). En haut: Sham vs. SCN-lesioned mice (three-way ANOVA, *P*genotype=0.01; *P*genotype x time-course=0.01), SCN-lesioned (*P*SCN-condition<0.001). En bas: *Syn10Cre/CreBmal1fl/-* vs. controls (two-way ANOVA, *P*genotype=0.007). Les différences entre la veille et le sommeil sont abolies en cas d'absence de mélanopsine et de NSC (SCNx *Opn4−/−; P*SCN-condition x genotype=0.004)

En l'absence de pacemaker circadien, bien que l'animal soit arythmique sur le plan chronobiologique, et que son cycle veille/sommeil soit fortement perturbé comparé à celui des souris contrôles, il persiste une organisation de la veille et du sommeil en fonction de la lumière et de l'obscurité. Seules les souris déficientes en mélanopsine et lésées au niveau des NSC ne présentent plus de réactivité à la lumière.

<u>Importance des différentes composantes dans la régulation du cycle veille/sommeil</u>

Il existe une importante régulation photique aiguë sur le sommeil observée tout au long des cycles ultradiens alternant une heure de lumière et une heure d'obscurité. L'amplitude de la réactivité à la lumière dépend du modèle animal avec une moindre réponse chez les souris déficientes en mélanopsine et ayant été lésées au niveau des NSC comparées aux souris contrôles.

Cette réactivité du sommeil à la lumière n'est pas seulement aiguë, mais également soutenue. Ainsi, elle est présente tout au long de la période lumineuse en condition standard alternant 12 heures de lumière et 12 heures d'obscurité avec des amplitudes qui varient en fonction de la composante étudiée. Afin de déterminer l'importance de chaque composante dans l'amplitude de cette réponse du sommeil aux effets directs de la lumière, j'ai réalisé des analyses de soustraction à partir des différents résultats en condition 12hL :12hD et 24h :DD.

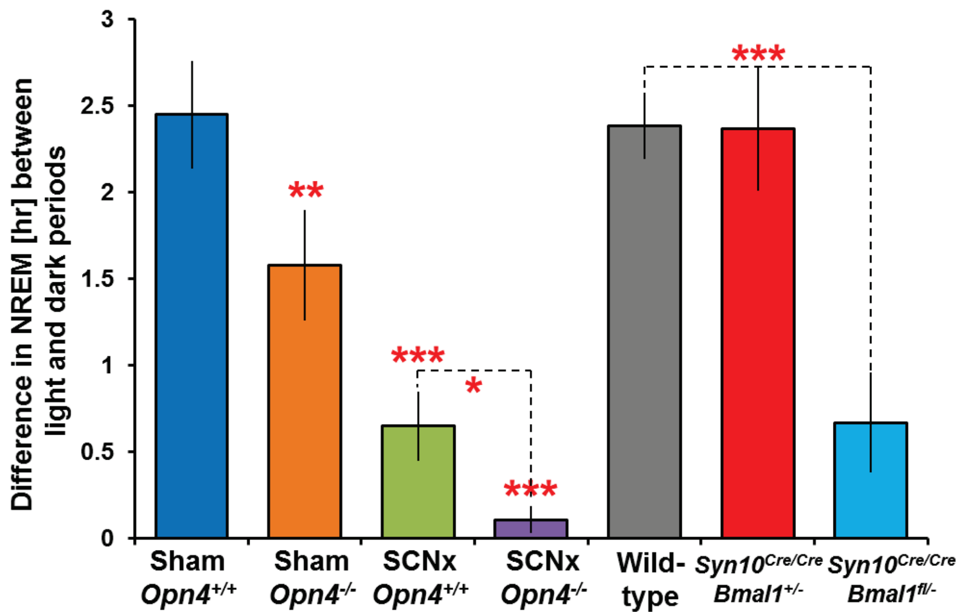

Figure 20. Expression soutenue des effets directs de la lumière sur le cycle veille/sommeil. Les différences de sommeil lent entre les périodes de lumière et d'obscurité en condition 12hL :12hD ne diffèrent pas entre les différents contrôles (Sham » *Opn 4*$^{+/+}$, Wild-type, *Syn10*$^{Cre/Cre}$*Bmal1*$^{+/-}$), mais sont significativement atténuées dans les autres conditions (ANOVA à deux facteurs, P*genotype*<0.0001), $P_{SCN\text{-}condition}$=0.002; ANOVA, P<0.001) et abolies en l'absence de mélanopsine et de NSC (absence de significativité avec zéro, one-sample signed rank test, *P*=0.22).

Le calcul de l'amplitude de cette réactivité est obtenu en soustrayant la quantité de sommeil lent en période d'obscurité de celle mesurée en période lumineuse. Chez les souris contrôles (« Sham » *Opn 4*$^{+/+}$, *Syn10*$^{Cre/Cre}$*Bmal1*$^{fl/-}$; (*Syn10*$^{Cre/Cre}$*Bmal1*$^{+/-}$), cette amplitude était d'environ 2,5 heures. Dans les autres groupes expérimentaux, cette amplitude était diminuée à des degrés différents en fonction de l'importance de la composante respective dans la phototransduction. La comparaison de ces amplitudes permet d'isoler et de quantifier les contributions de ces différents éléments dans l'organisation du cycle veille-sommeil. Dans la condition 12hL :12hD, l'importance des effets circadiens de la lumière sur le sommeil et la contribution des cônes et des bâtonnets passant par les NSC dans les effets directs de la lumière sur le sommeil n'ont pas pu être déterminées. Pour déterminer l'importance des effets circadiens de la lumière sur le sommeil, le calcul de l'amplitude des animaux avec et sans mélanopsine en condition d'obscurité constante a été réalisé (absence de différence significative – cf supra). Ainsi la perte en sommeil lent en condition 12hL :12hD chez les souris *Opn 4*$^{-/-}$,

fait suite à une perte des effets directs de lumière et n'est pas en lien avec un pacemaker circadien altéré.

<u>Le détail des calculs de soustraction</u>

Dans un souci de clarté, je détaille davantage les calculs mentionnés, de qui permet de disséquer l'importance de chaque composante de la phototransduction à partir des différents modèles et conditions expérimentales étudiés.

Les effets circadiens de la lumière sur le sommeil sont représentés par « CL » (Circadian light). Les effets non-circadiens directs de la lumière sur le sommeil sont désignés par « DL » (Direct light). Le terme « différences » est utilisé comme raccourci et signifie la différence en sommeil lent observée entre les périodes lumineuse et d'obscurité objectives ou subjectives pour un modèle de souris étudié soit en condition 12hL :12hD, soit en condition 24h :DD (cf Figure 9) .

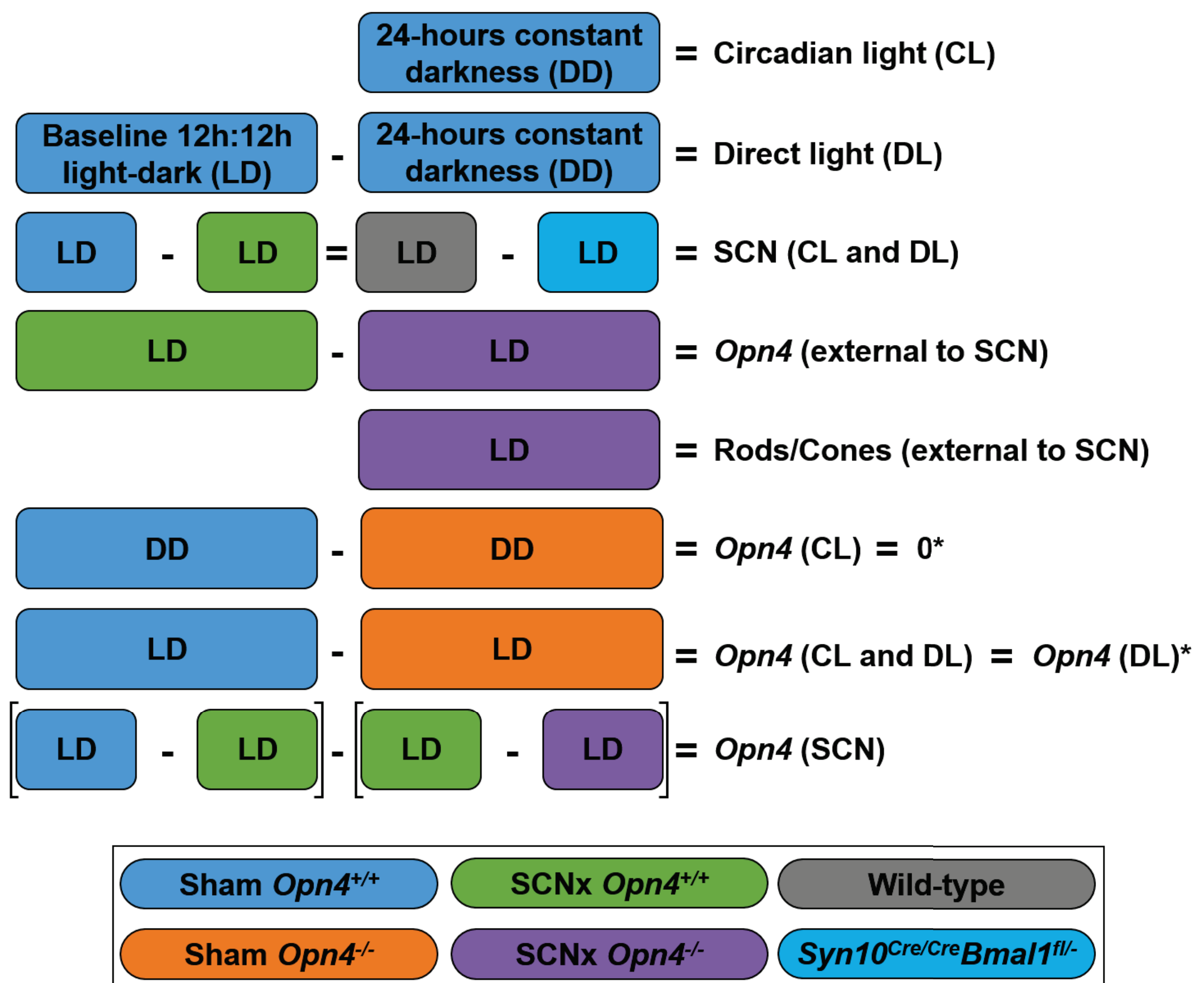

Figure 21. Modèle des calculs. Les calculs réalisés pour les soustractions permettant d'obtenir les différences pour le sommeil lent entre les périodes lumineuse et d'obscurité, objectives ou subjectives, en condition 12hL :12hD et 24h :DD selon la figure 20 ci-dessus

(a) La contribution de CL est obtenue à partir des différences obtenues en l'absence de lumière, c. à d. en condition d'obscurité constante auprès des souris « Sham » *Opn* $4^{+/+}$.
(b) La contribution totale de DL est représentée par la différence entre la condition 12hL :12hD et (a).
(c) Le pourcentage des effets passant par les NSC est obtenu à partir des différences entre les deux groupes de souris *Opn* $4^{+/+}$, avec et sans NSC (SCNx *Opn* $4^{+/+}$; Sham *Opn* $4^{+/+}$). Par ailleurs, le pourcentage obtenu à partir du modèle de souris ayant des NSC non fonctionnels transgéniques (*Syn10*$^{Cre/Cre}$*Bmal1*$^{+/-}$; *Syn10*$^{Cre/Cre}$*Bmal1*$^{fl/-}$) est identique.
(d) Le pourcentage de DL passant à l'extérieur des NSC est obtenu à partir de l'étude des différences entre les deux génotypes de souris ayant eu une lésion électrolytique des NSC (SCNx *Opn* $4^{+/+}$; SCNx *Opn* $4^{-/-}$).
(e) Le pourcentage de DL incriminé aux cônes et aux bâtonnets est obtenu à partir de la différence en condition 12hL :12hD chez les souris déficientes en mélanopsine et en NSC (SCNx *Opn* $4^{-/-}$)
(f) Aucune différence n'est observée entre les deux génotypes chez les souris ayant des NSC intacts (Sham *Opn* $4^{+/+}$; Sham *Opn* $4^{-/-}$) en l'absence de lumière, correspondant à CL.
(g) Les différences cumulées (CL et DL) en lien avec la mélanopsine résultent uniquement de DL (f) et ne sont pas sous influence circadienne.
(h) La différence observée entre les souris *Opn4*$^{+/+}$ avec et sans lésion des NSC (Sham *Opn* $4^{+/+}$ - SCNx *Opn* $4^{+/+}$) moins la différence observée entre les souris lésées au niveau des NSC des deux génotypes (SCNx *Opn* $4^{+/+}$ - SCNx *Opn* $4^{-/-}$) nous informe de la contribution de la mélanopsine passant par les NSC.

Schedule	Time Spent	Condition	*Opn4* Genotype	NREMS (h)
12h:12h LD	12-h light period	Sham	+/+	6.46±0.13
			-/-	4.99±0.45[a]
		SCNx	+/+	6.42±0.17[b]
			-/-	6.22±0.12[b]
	12-h dark period	Sham	+/+	4.00±0.23[c]
			-/-	3.44±0.25[c]
		SCNx	+/+	5.85±0.19[b,c]
			-/-	6.10±0.11[b]
	24 h	Sham	+/+	10.47±0.39
			-/-	8.43±0.66[a]
		SCNx	+/+	12.27±0.23[b]
			-/-	12.32±0.22[b]
24h DD	*Subjective* 12-h light period	Sham	+/+	4.67±0.33
			-/-	5.12±0.15
	Subjective 12-h dark period	Sham	+/+	3.72±0.45
			-/-	4.09±0.22
	24 h	Sham	+/+	8.39±0.69
			-/-	9.21±0.27

Table 1. Détail des quantités (en heures) de sommeil lent (NREM) enregistrées dans les différentes conditions expérimentales en fonction des génotypes.

Phototransduction des effets de la lumière sur le sommeil : Synthèse des résultats permettant de disséquer l'importance des différentes composantes

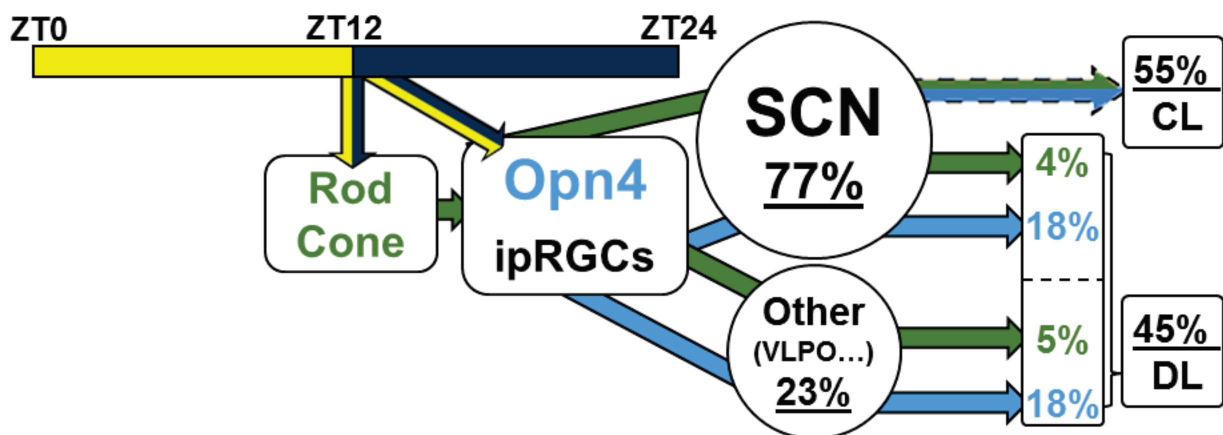

Figure 22. Synthèse des différentes composantes intervenant dans les effets directs de la lumière sur le cycle veille/sommeil. CL « Circadian light » : effets circadiens de la lumière ; DL « Direct light » : effets directs, non-circadiens de la lumière.

La synthèse des résultats obtenus à partir des calculs de soustraction entre les différentes conditions permet de mettre en évidence que 55% de l'amplitude du cycle veille/sommeil est expliqué par les effets circadiens de la lumière et 45% du cycle veille/sommeil est expliqué par les effets directs de la lumière. Ainsi les effets directs de la lumière expliquent pour plus d'un tiers (presque pour la moitié) l'amplitude du cycle veille/sommeil. Par ailleurs 22% des effets de la lumière passent par les NSC, qui sont également une structure de relai pour les effets de la lumière au-delà de leur rôle d'horloge. La mélanopsine explique pour 36% les effets directs de la lumière. Les 64% restants, non-mélanopsinergiques, sont présumés être sous le contrôle des cônes et des bâtonnets.

La démarche de ce premier volet de thèse était d'identifier les structures et voies neuronales impliquées dans les effets directs de la lumière. Il s'agit d'un travail qui est transversal sur les différents projets de l'équipe. Devant l'impossibilité de dissocier mes résultats issus des expériences anatomiques et impliquant les réseaux neuronaux, de ceux des autres membres de l'équipe, j'ai préféré inclure les articles auxquels j'ai contribué directement via mes données d'anatomie. Les publications auxquelles j'ai participé pour la réalisation de l'expérience (sans en être responsable), et/ou pour la réflexion et la rédaction de l'article sont annexées au manuscrit. Les résultats concernant les structures anatomiques et les voies neuronales sont répartis dans différentes publications, cependant leur synthèse et la démarche sous-jacente permettent de faire évoluer notre compréhension des « pathways » qu'utilise la lumière (et l'obscurité) dans ses effets directs sur le sommeil et la qualité de l'éveil.

Compte tenu du volume important des données présentées et afin d'éviter toute confusion, je tiens à préciser pour ces différentes publications ma part de contribution dans ces travaux qui sont ceux de notre équipe. Par ailleurs, mon travail de thèse a débuté à l'issu de mon master 2, lors de l'Année Recherche obtenue en 2008-2009. C'est dans ce cadre que j'ai pu bénéficier d'un séjour à Copenhague auprès du Dr Jens Hannibal, qui m'a initiée aux techniques d'anatomie et reste toujours d'un conseil précieux en cas de difficultés rencontrées dans les manipulations d'immunohistochimie ou d'hybridation *in situ*. La partie de ma thèse qui est en lien avec la publication *(Tsai, Hannibal, Hagiwara, Colas, Ruppert, Ruby, Heller, Franken, Bourgin: Melanopsin as a sleep modulator: circadian gating of the direct effects of light on sleep and altered sleep homeostasis in Opn4*$^{-/-}$ *mice. PLoS Biology, 2009)* a donc été réalisée à Strasbourg et à Copenhague à partir de cerveaux provenant des expériences débutées à Stanford par mon directeur de thèse.

Dans la suite des travaux de la première publication, ont démarré les expériences qui cherchent à mieux identifier le rôle des effets directs de la lumière et celui des NSC dans la régulation du cycle veille-sommeil. Les manipulations étaient souvent longues et incluaient un nombre de groupes d'animaux importants, ce qui explique la durée qu'ont pris la collecte et l'analyse des données tout au long de ma thèse. Ma contribution dans ce projet concerne le volet anatomique fonctionnel. Le Dr Jeffrey Hubbard (premier auteur) a réalisé les expériences concernant l'enregistrement des souris, le scorage et l'analyse des données EEG. De mon côté, j'ai participé à l'étude des données ayant permis d'établir le modèle décrivant l'impact des différentes structures intervenant dans la phototransduction, ainsi que dans la rédaction de l'article (Figure 5). J'ai également été impliquée dans le design de cette recherche, dans l'analyse globale des données et dans l'écriture de l'article. *(Hubbard, Ruppert, Frisk, Tsai, Robin-Choteau, Husse, Calvel, Eichele, Franken, Bourgin: A model to predict how (melanopsin-dependent) lighting shapes the sleep-wake cycle. En préparation)*.

Article 1: ***« Melanopsin as a sleep modulator: circadian gating of the direct effects of light on sleep and altered sleep homeostasis in Opn4$^{-/-}$ mice»***

Plos Biology, 2009

Article principal : pages 1-13

Matériel supplémentaire : 1-7

Melanopsin as a Sleep Modulator: Circadian Gating of the Direct Effects of Light on Sleep and Altered Sleep Homeostasis in *Opn4*$^{-/-}$ Mice

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Abstract

Light influences sleep and alertness either indirectly through a well-characterized circadian pathway or directly through yet poorly understood mechanisms. Melanopsin (Opn4) is a retinal photopigment crucial for conveying nonvisual light information to the brain. Through extensive characterization of sleep and the electrocorticogram (ECoG) in melanopsin-deficient (*Opn4*$^{-/-}$) mice under various light–dark (LD) schedules, we assessed the role of melanopsin in mediating the effects of light on sleep and ECoG activity. In control mice, a light pulse given during the habitual dark period readily induced sleep, whereas a dark pulse given during the habitual light period induced waking with pronounced theta (7–10 Hz) and gamma (40–70 Hz) activity, the ECoG correlates of alertness. In contrast, light failed to induce sleep in *Opn4*$^{-/-}$ mice, and the dark-pulse-induced increase in theta and gamma activity was delayed. A 24-h recording under a LD 1-h:1-h schedule revealed that the failure to respond to light in *Opn4*$^{-/-}$ mice was restricted to the subjective dark period. Light induced c-Fos immunoreactivity in the suprachiasmatic nuclei (SCN) and in sleep-active ventrolateral preoptic (VLPO) neurons was importantly reduced in *Opn4*$^{-/-}$ mice, implicating both sleep-regulatory structures in the melanopsin-mediated effects of light. In addition to these acute light effects, *Opn4*$^{-/-}$ mice slept 1 h less during the 12-h light period of a LD 12:12 schedule owing to a lengthening of waking bouts. Despite this reduction in sleep time, ECoG delta power, a marker of sleep need, was decreased in *Opn4*$^{-/-}$ mice for most of the (subjective) dark period. Delta power reached after a 6-h sleep deprivation was similarly reduced in *Opn4*$^{-/-}$ mice. In mice, melanopsin's contribution to the direct effects of light on sleep is limited to the dark or active period, suggesting that at this circadian phase, melanopsin compensates for circadian variations in the photo sensitivity of other light-encoding pathways such as rod and cones. Our study, furthermore, demonstrates that lack of melanopsin alters sleep homeostasis. These findings call for a reevaluation of the role of light on mammalian physiology and behavior.

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Abbreviations: ECoG, electrocorticogram; EEG, electroencephalogram; LD, light–dark; NREM, non-rapid eye movement; PLSD, protected least significant difference; REM, rapid eye movement; RGC, retinal ganglion cell; SCN, suprachiasmatic nucleus; VLPO, ventrolateral preoptic; ZT, Zeitgeber time.

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Introduction

Light exerts strong effects on human physiology and behavior, including entrainment of circadian rhythms [1–3], suppression of melatonin release [4], regulation of heart rate and body temperature [5], alertness [6], and cognition [7]. The effects of light on behavior have been classified either as indirect, shifting the phase of the circadian rhythm (photic entrainment), or as direct, affecting behavior in a circadian-independent fashion, such as occurs in light avoidance in nocturnal species (masking). Processing of photic information has been studied extensively in the context of circadian biology with an emphasis on the nonvisual effects of light [8–10] mediated by melanopsin, a photopigment involved in irradiance level detection [11–13]. In mammals, melanopsin is exclusively expressed in retinal ganglion cells (RGCs) [14] and retinal pigment epithelium [15]. Melanopsin plays a major role in the photic phase shifting of circadian rhythms [16,17]. The role of light in the circadian regulation of sleep and wakefulness and the pathways by which melanopsin-containing RGCs influence the circadian system, particularly the suprachiasmatic nuclei (SCN), are well documented [18,19]. In contrast, the noncircadian, direct effects of light on sleep and wakefulness as well as the pathways relaying these effects remain poorly understood. Direct effects of light are difficult to distinguish from visual and circadian influences. Such effects are mainly known to acutely promote alertness in day-active species and sleep in night-active species.

Author Summary

Light affects sleep in two ways: indirectly through the phase adjustment of circadian rhythms and directly through nonvisual mechanisms that are independent of the circadian system. The direct effects of light include the promotion of sleep in night-active animals and of alertness in diurnal species. We analyzed sleep and the electroencephalogram (EEG) under various light–dark regimens in mice lacking melanopsin (*Opn4*$^{-/-}$), a retinal photopigment crucial for conveying light-level information to the brain, to determine the role of melanopsin, as opposed to rod and cones, in mediating these direct effects of light. We show that melanopsin mediates the direct effects of light during the subjective dark period, whereas rods and cones contribute to these effects in the light period. Our finding that "sleep-active" (i.e., galanin-positive) neurons of the anterior hypothalamus are not activated by light in *Opn4*$^{-/-}$ mice suggests that these neurons are part of the circuitry whereby light promotes sleep. Also, the alerting effects of transitions into darkness were less pronounced in *Opn4*$^{-/-}$ mice judged on the reduced increase in EEG theta and gamma activity. Finally, and unexpectedly, the rate at which the need for sleep, quantified as EEG delta power, accumulated during wakefulness was found to be reduced in *Opn4*$^{-/-}$ mice both during baseline and sleep deprivation conditions, implicating a photopigment in the homeostatic regulation of sleep. We conclude that melanopsin contributes to the direct effects of light and darkness, and in interaction with circadian and homeostatic drive, determines the occurrence and quality of both sleep and waking. If confirmed in humans, our observations will have applications for the clinical use of light as well as for societal lighting conditions.

The purpose of this study was to take advantage of mice lacking melanopsin (*Opn4*$^{-/-}$) to provide an in-depth analysis of the direct effects of light on sleep, wakefulness, and the electrocorticogram (ECoG) and how these effects are modulated by time of day under various light–dark (LD) regimes. Two recent studies [20,21], performed concomitantly to ours, confirmed that in the absence of melanopsin a single light pulse, presented during the dark period, failed to induce sleep. This led to the conclusion that melanopsin mediates the direct effects of light on sleep [20,21]. By probing the effects of light on sleep across the 24-h day, we demonstrate here that the failure to respond to light in *Opn4*$^{-/-}$ mice was restricted to the dark period, implicating other light-encoding pathways in mediating the direct effects of light at other times of day. Another novel aspect of our study is the finding that melanopsin plays a role in shaping rhythmic ECoG activity. In particular, the amplitude of the sleep-wake–dependent changes in ECoG delta power, a marker of sleep need, was reduced in *Opn4*$^{-/-}$ mice compared to that of wild-type littermate controls. Finally, to gain insight into the pathways relaying these direct effects, we used c-Fos immunohistochemistry in SCN neurons and in galaninergic "sleep-active neurons" of the ventrolateral preoptic (VLPO) area: key circadian and sleep-promoting structures in the hypothalamus, respectively. The VLPO receives direct projections from melanopsin-containing RGCs [22–24] and thus represents a candidate brain area to mediate the direct effects of light on sleep.

Results

We compared the direct effects of light between mice carrying a targeted disruption of the melanopsin gene (*Opn4*$^{-/-}$; see [17] and Text S1 for details) and their wild-type, littermate controls (*Opn4*$^{+/+}$). We used various lighting schedules to evaluate the effect of light on sleep and the ECoG (for an overview, see Figure S1). Among these schedules were the standard baseline LD 12-h:12-h (12:12) schedule the mice were kept under, a single 1-h light and a single 1-h dark pulse given during the habitual 12-h dark and light periods, respectively, and 24 h under a LD 1-h:1-h (1:1) cycle.

Melanopsin Modulates the Direct Effects of Light on Sleep and the ECoG

In a first set of experiments, the acute effects of light and darkness on sleep and the ECoG were investigated by exposing mice of both genotypes to a single, 1-h pulse of light administered 3 h after the habitual LD transitions; i.e., Zeitgeber time (ZT)15–16 (with ZT0 referring to the time of light onset) and to a 1-h dark pulse given 3 h after light onset (i.e., ZT3–4). Mice were otherwise kept under LD 12:12 conditions, and at least 14 d separated any two recording conditions.

In *Opn4*$^{+/+}$ mice, the light pulse readily increased the amounts of both rapid eye movement (REM) and non-REM (NREM) sleep at the cost of wakefulness, whereas the same light pulse in *Opn4*$^{-/-}$ mice failed to affect time spent in either behavioral state for the duration of the light pulse (Figure 1A). This lack of a response demonstrates that, at least at this time of day (see LD 1:1 results below), melanopsin contributes significantly to the acute effect of light on sleep.

Light-to-dark transitions induce waking and alertness in nocturnal rodents. Accordingly, in *Opn4*$^{+/+}$ mice, a 1-h dark pulse induced an immediate increase in time spent awake (Figure 1B), and the ECoG during wakefulness showed a rapid and prolonged induction in ECoG theta (7–10 Hz) and gamma (40–70 Hz) activity (Figure 1C and 1D), the ECoG correlates of exploratory behavior and alertness in rodents [25,26]. The dark pulse also induced waking in *Opn4*$^{-/-}$ mice. Although their immediate response was somewhat delayed (compare genotypes for the increase in waking in the 5 min before and after dark onset in Figure 1B), the hourly amount of waking (and sleep) did not differ between genotypes (unpublished data). The changes in ECoG theta and gamma activity that follow the transition into darkness seemed, however, to be modulated by melanopsin because they were delayed by ca. 25 min (Figure 1C and 1D). Upon restoring the normal light condition 1 h later, *Opn4*$^{-/-}$ mice stayed awake longer, consistent with the light-pulse results (Figure 1B and 1A).

The Effects of Light and Dark Pulses on Sleep and Waking Vary with Time of Day

The single pulses of light and darkness probed their effects on sleep and waking at specific times of day only. To obtain a more complete account of the genotype differences of these interventions, we maintained mice under a short, 1-h LD cycle (LD 1:1) for a 24-h period. This lighting schedule was used first in the rat to investigate the direct effects of light on sleep across the circadian cycle [27]. Also under these conditions, 1-h periods of light induced sleep, and 1-h periods of darkness favored wakefulness. Irrespective of the effect of this ultradian LD cycle on sleep and wakefulness, a circadian modulation of the average levels of sleep and waking reached within the subjective 12-h light period and subjective 12-h dark period is preserved in wild-type mice (Figure 2A; Table 1). The protocol has the potential drawbacks that light given at any one hour might influence its effects during subsequent hours and that animals could entrain their ultradian sleep–wake organization to this schedule. We did not see evidence of the former effect as the relative effect of the LD alteration on

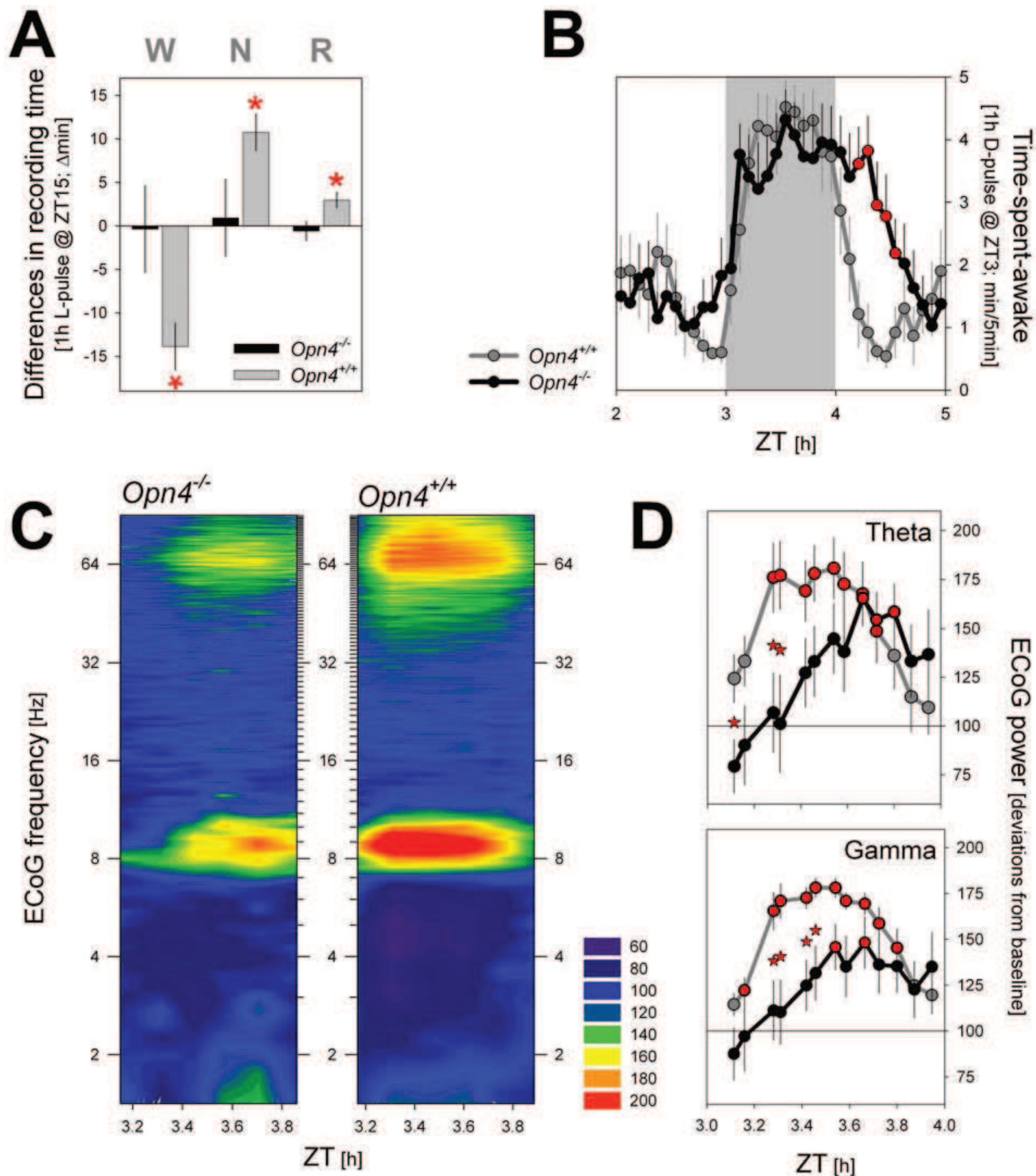

Figure 1. Direct effects of a 1-h light (L) and a 1-h dark (D) pulse on the sleep–wake distribution (A and B) and the waking ECoG (during the 1-h D pulse; panels [C and D]) under an LD 12:12 schedule. (A) The L pulse, administered during the habitual dark period (ZT 15–16), induced NREM (N) and REM (R) sleep at the expense of wakefulness (W) in *Opn4*$^{+/+}$ mice ($p<0.05$; post hoc paired *t*-tests). No response was observed in *Opn4*$^{-/-}$ mice. Values during the 1-h L pulse were compared to baseline values obtained in a 3-h window centered around the time of the pulse (ZT 14–17). (B) Time course of time spent awake across the 1-h D pulse, administered during the habitual light period (ZT3–4). Waking values (waking minutes/5-min intervals) over 3 h (1-h light before and 1-h light after D pulse) and centered on the D pulse (ZT3–4; grey area). Note the delayed response in *Opn4*$^{-/-}$ mice after the D pulse when light is applied again. Melanopsin-deficient mice stay awake and return to sleep only after 35 min. Red asterisks (*) denote 5-min intervals with significant genotype effects ($p<0.05$; post hoc *t*-tests). (C) Heat map of the time course of spectral changes in the waking ECoG during the 1-h D pulse. ECoG power density was expressed as percentage of individual waking ECoG spectra obtained during the baseline light period (=100%). Warmer colors denote relative increases, colder colors, decreases versus baseline ECoG activity. Relative spectral profiles were calculated over 10 min of waking at 5-min increments (i.e., 13 spectra/hour). (D) as in (C) but summarized for the theta (7–10 Hz) and gamma (40–70 Hz) frequency bands. Note the smaller and delayed activation in *Opn4*$^{-/-}$ mice. Asterisks indicate significant genotype differences ($p<0.05$; post hoc *t*-tests); red-filled circles indicate significant differences from baseline ($p<0.05$; post hoc paired *t*-tests). Values represent means±SEM ($n=6$ and 7, for *Opn4*$^{+/+}$ and *Opn4*$^{-/-}$, respectively).
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time spent awake did not vary with time of day in wild-type mice (Figure 2A–2C), but after the initial three cycles (i.e., after ZT6), wild-type mice seemed capable of anticipating dark onset, because increases in wakefulness started preceding it (Figures 2B and S2).

Visual inspection of the hourly values of wakefulness reached under the LD 1:1 protocol suggests that *Opn4*$^{-/-}$ mice have a reduced response to light in the (subjective) dark period (Figure 2A); i.e., the time at which we and others [20,21] administered the single 1-h light pulse (see above). This reduction was, however, not observed at other times of day, and the LD-induced changes in time spent awake did not differ from wild-type mice during the subjective light period (Figure 2A–2C). The capacity of the 1-h LD cycles to alter the sleep–wake distribution was further analyzed by aligning the subsequent 1:1-h LD cycles (Figures 2B and S2). This time course analysis revealed that, as was observed after the single dark-pulse experiment (Figure 1B), *Opn4*$^{-/-}$ mice were again slower in initiating sleep after light onset (Figure 2B). Analyses of variance confirmed that the time course of the LD-induced changes in wakefulness and NREM sleep varied significantly according to genotype and time of day (i.e., 12-h subjective light period versus 12-h subjective dark period; Figure 2B). Changes in the time course observed in Figure 2B were summarized by estimating the amplitude of sine waves of best fit for individual mice (Figure 2C; see Text S1 for details). This analysis also confirmed that genotype affected the light-induced changes in wakefulness only during the subjective dark period (Figure 2C). Especially between ZT15–21, the increase of wakefulness during the 1-h dark intervals compared to the 1-h mean value in the preceding light intervals was no longer significant in *Opn4*$^{-/-}$ mice (+4.2±4.1 min; $p = 0.38$ and +26.2±5.3 min; $p = 0.038$; for *Opn4*$^{-/-}$ and *Opn4*$^{+/+}$ mice, respectively; paired *t*-tests), and this increase differed between genotypes (one-way ANOVA; $p = 0.020$). It thus seems that only during this time melanopsin contributed significantly to the direct effects of light on wake and sleep duration.

Light-Induced c-Fos Expression in SCN Neurons and Galaninergic Neurons of the VLPO

As expected from our previous work and that of others [16,17], a 1-h light pulse administered during the dark period induced c-Fos immunoreactivity in the SCN in both genotypes (two-way-ANOVA, light-pulse effect: $p<0.001$, followed by post hoc Fisher protected least significant difference [PLSD]: $p<0,05$); however, the induction was half that observed in *Opn4*$^{+/+}$ mice (two-way ANOVA, light×genotype interaction: $p<0,001$; light-pulse effect:

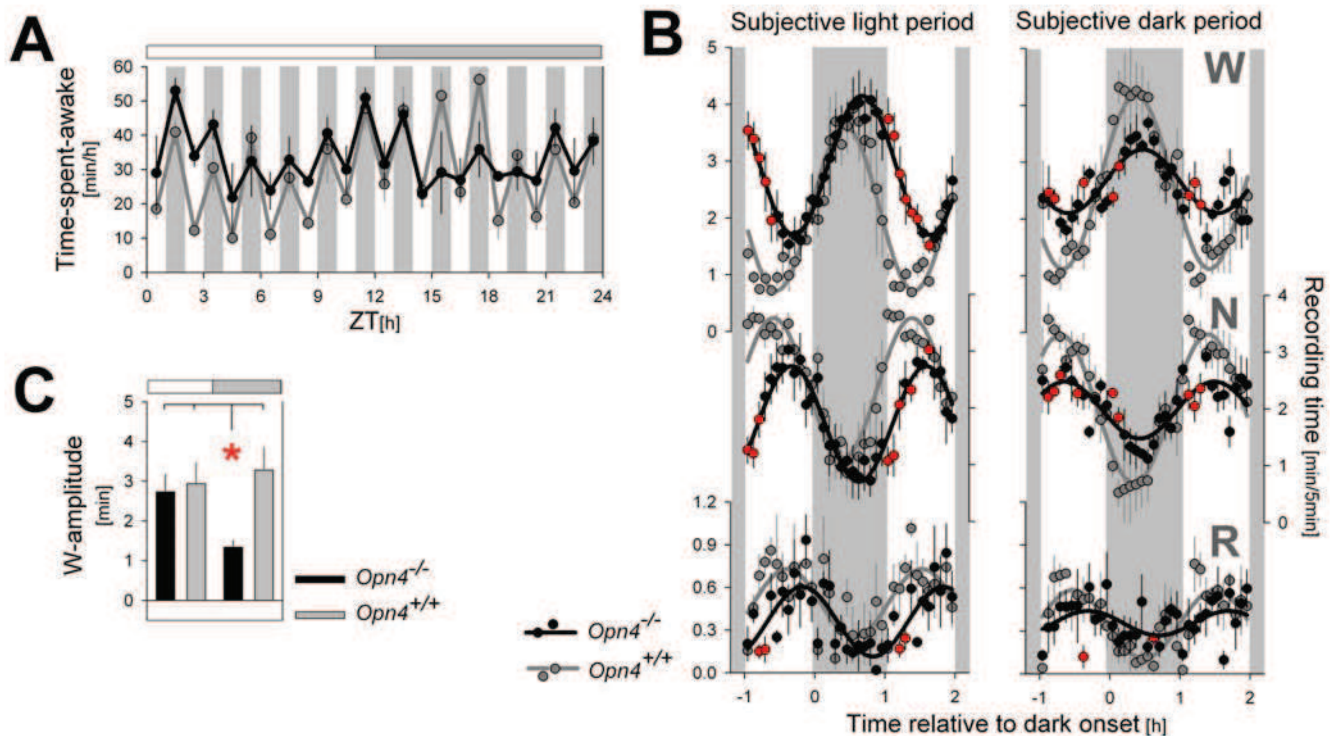

Figure 2. Direct effects of 1-h light (L) and 1-h dark (D) pulses on the sleep–wake distribution under a LD 1:1 schedule. (A) Hourly mean values of wakefulness during the LD 1:1 cycle. Hourly L pulses suppressed wakefulness, but this effect seemed to vary with time of day in *Opn4*$^{-/-}$ mice and was especially small between ZT15 and ZT21. Grey horizontal bars mark the subjective dark period (i.e., the 12-h dark period of the preceding days under LD 12:12). (B) Average time course of the LD-induced changes in wakefulness (W; upper), NREM sleep (N; middle), and REM sleep (R; lower panels) during the 12-h subjective light (left) and subjective dark (right) period. Values represent means (±SEM) over 5-min intervals in the hour preceding, during, and following the six 1-h dark pulses in the subjective light and dark periods, respectively. 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 W and N, time course was significantly affected by time of day and genotype (interactions: genotype×time of day: W and N: $p<0.0001$; R: $p = 0.017$; genotype×time course: W and N: $p<0.0001$; R: $p = 0.14$; time of day×time course: W: $p = 0.019$; N: $p = 0.048$; R: $p = 0.18$). Red filled circles denote 5-min intervals with significant genotype effects ($p<0.05$; post hoc *t*-tests). Black (*Opn4*$^{-/-}$) and grey (*Opn4*$^{+/+}$) sine waves represent best fits to the data points. (C) Changes in the time course observed in (B) were summarized and quantified by determining the amplitude of sine waves of best fit for each individual mouse (see Text S1 for details). The thus estimated amplitude of the LD-induced changes in wakefulness was affected by time of day (two-way ANOVA interaction genotype×time of day $p = 0.033$) and was significantly smaller in *Opn4*$^{-/-}$ mice during the subjective dark period compared to the subjective light period and compared to the values obtained in wild-type mice in both conditions (red asterisk; post hoc *t*-tests; $p<0.05$).
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Table 1. Time spent asleep and awake under the LD 12:12 and LD 1:1 schedules.

Schedule	Time spent	*Opn4* Genotype	Waking (h)	NREMS (h)	REMS (min)
LD12:12	12-h light period	$-/-$	4.80±0.21[a]	5.92±0.18[a]	77.1±2.6
		$+/+$	3.74±0.17	6.95±0.17	77.8±11.1
	12-h dark period	$-/-$	6.59±0.20[b]	4.56±0.18[b]	50.8±2.3[b]
		$+/+$	6.79±0.37[b]	4.51±0.33[b]	41.9±6.5[b]
	LD difference	$-/-$	1.80±0.17[a]	1.36±0.14[a]	26.3±3.2
		$+/+$	3.04±0.34	2.44±0.29	35.9±6.6
	24 h	$-/-$	11.39±0.37	10.48±0.33	127.9±3.6
		$+/+$	10.53±0.47	11.47±0.44	119.7±16.9
LD1:1	*Subjective* 12-h light period	$-/-$	6.96±0.34[a]	4.19±0.29[a]	50.8±3.8[a]
		$+/+$	5.15±0.48	5.66±0.40	71.3±5.1
	Subjective 12-h dark period	$-/-$	6.44±0.26	4.71±0.21	51.2±4.7
		$+/+$	6.48±0.18[b]	4.69±0.16	49.9±1.7[b]
	24 h	$-/-$	13.40±0.36[a]	8.9±0.34	101.9±3.4[a]
		$+/+$	11.63±0.65	10.35±0.56	121.2±6.7
	12 1-h light periods	$-/-$	5.51±0.29[a]	5.47±0.23[a]	61.3±5.2[a]
		$+/+$	3.53±0.36	7.13±0.29	80.5±4.2
	12 1-h dark periods	$-/-$	7.89±0.25[b]	3.44±0.23[b]	40.6±3.6[b]
		$+/+$	8.10±0.87[b]	3.22±0.76[b]	40.7±7.1[b]

Under both schedules, *Opn4*$^{-/-}$ mice displayed significantly less NREM sleep (NREMS; and were awake more) than *Opn4*$^{+/+}$ mice during the (subjective) 12-h light (L) periods. This resulted in a significantly decreased amplitude of the circadian distribution (LD difference) of NREMS time (and waking) in *Opn4*$^{-/-}$ mice. Under the LD 1:1 schedule, *Opn4*$^{-/-}$ mice slept less compared to *Opn4*$^{+/+}$ mice during all 12 1-h L pulses of the entire 24-h d. Also under the LD1:1 schedule, the circadian distribution of sleep and waking observed under the LD 12:12 condition is maintained only in *Opn4*$^{+/+}$ mice (subjective 12-h L period, ZT0–12, compared to the. subjective 12-h dark (D) period, ZT12–24).

[a]Indicates significant genotype differences.

[b]The difference between (subjective) 12-h D and L periods for each lighting schedule and genotype ($p<0.05$; post hoc *t*-test). Values represent means±SEM (LD 12:12 $n=9$/genotype; LD 1:1 $n=3$ and 4, for *Opn4*$^{+/+}$ and *Opn4*$^{-/-}$, respectively).

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Opn4$^{+/+}$ vs. *Opn4*$^{-/-}$, post hoc Fisher PLSD, $p<0{,}05$) (Figure 3). This reduction in c-Fos immunoreactivity has been functionally linked to the reduced ability to phase shift circadian rhythms in these mice. This does not, however, rule out the possibility that the reduced activation of SCN neurons could also contribute to the reduced direct effects of light on sleep and waking described here.

We further determined whether melanopsin conveys light information to the VLPO, in particular to the galaninergic sleep-active neurons, using double staining for c-FOS protein (immunohistochemistry) and for galanin mRNA (in situ hybridization). Whereas overall c-Fos immunoreactivity in the VLPO area was not significantly affected by the light pulse, in *Opn4*$^{+/+}$ mice, the percentage of galanin-containing (GAL) neurons coexpressing c-Fos did significantly increase compared to the control condition (i.e., darkness) (Figure 4). In contrast, in *Opn4*$^{-/-}$ mice, GAL c-Fos-costained neurons were present at low levels in both lighting schedules (2%), and no induction of c-Fos in GAL neurons was observed, suggesting that at least at this time of day, melanopsin-containing RGCs contribute significantly to the effects of light on the activity of these sleep-active VLPO neurons (two-way ANOVA, light-pulse effect: $p<0.05$, light×genotype interaction: $p<0{,}05$; light-pulse effect: *Opn4*$^{+/+}$: $p<0{,}05$; *Opn4*$^{-/-}$: $p=0{,}23$, post hoc Fisher PLSD).

Sleep and Quantitative ECoG Analyses under Standard Light–Dark Conditions

The mouse is a nocturnal species that avoids light of higher intensity. *Opn4*$^{-/-}$ mice are no exception and, similar to their littermate wild-type controls (*Opn4*$^{+/+}$), mostly sleep during the light period and are awake during the dark period of the LD 12:12 cycle (Figure 5A; Table 1). Nevertheless, *Opn4*$^{-/-}$ mice lose ca. 1 h of NREM sleep per day relative to *Opn4*$^{+/+}$ mice. This marked loss of sleep occurred during the 12-h light period exclusively (Figure 5B) and resulted in an attenuation of the diurnal distribution of sleep and waking (Table 1). This genotype difference could be due to a reduced capacity of light to induce sleep or to suppress wakefulness, an interpretation underscored by the results of the light- and dark-pulse experiments (Figure 1A and 1B) and the LD 1:1 experiment (Figure 2A). Indeed, for both genotypes, the levels of sleep reached during the 1-h light intervals during the subjective light period of the LD 1:1 schedule were similar to those reached during the light period of the LD 12:12 schedule; i.e., *Opn4*$^{-/-}$ mice slept less when light was present during the (subjective) light period of both the LD 1:1 and LD 12:12 (Table 1). Moreover, the sleep loss observed in *Opn4*$^{-/-}$ mice was associated with an overall deficit in ECoG delta power during NREM sleep (see below).

The wakefulness present during the light period is organized in more or less regularly occurring bouts lasting on average about 20–25 min [28] (Figure S1; Table S1). To examine in more detail the mechanism underlying the differences in sleep time during the light period, we analyzed these spontaneous waking bouts. In *Opn4*$^{+/+}$ mice, after 20 min of sustained wakefulness, waking values reverted quickly to the low values characteristic of the light period (Figure 5C). In contrast, in *Opn4*$^{-/-}$ mice, these waking bouts lasted on average 11 min longer (Table S1), and values remained above wild-type levels for 30 min (Figure 5C). The similarity between these results and the results observed after the single dark pulse (Figure 1B)

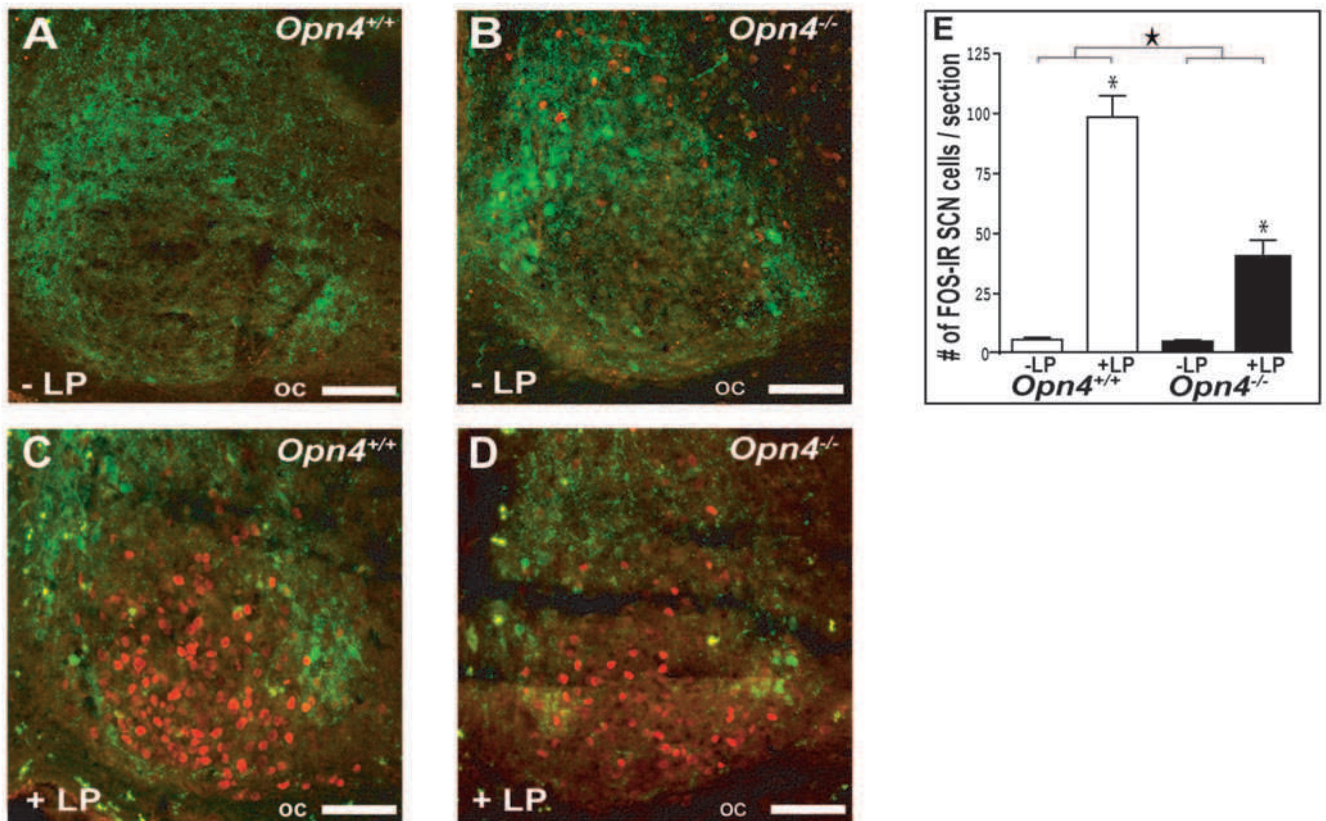

Figure 3. Effects of a 1-h light (L) pulse on c-Fos immunoreactivity in the SCN. (A–D) Effects of the L pulse administered during the habitual dark period (ZT15–16) on c-Fos immunoreactivity in the SCN of *Opn4*$^{+/+}$ and *Opn4*$^{-/-}$ mice. Few c-Fos immunoreactive cells (labeled red) are found in the dorsomedial (arginine-vasopressin [AVP]-containing) SCN (labeled green) in control (no L pulse) animals (A and B). Light induced c-Fos in the retino-recipient zone of the SCN in *Opn4*$^{+/+}$ (C) and to a lesser extend in *Opn4*$^{-/-}$ mice (D). oc; optic chiasm. Scale bars in (A–D) indicate 75 µm. (E) Number of c-Fos immunoreactive neurons in the SCN. The L pulse induced c-Fos in the retino-recipient part of the SCN in both genotypes (two-way ANOVA, light pulse effect: $p<0{,}001$; an asterisk (*) indicates post hoc Fisher PLSD: $p<0.05$), but this light induced c-Fos immunoreactivity is significantly reduced in *Opn4*$^{-/-}$ mice (two-way ANOVA, the star indicates light pulse×genotype interaction: $p<0{,}001$).
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suggest that the reduced capacity of light to suppress wakefulness and to induce sleep in *Opn4*$^{-/-}$ mice also contributed to an overall reduction in sleep time during the light period.

Quantitative analysis of the ECoG during the three behavioral states revealed an increase in ECoG power density in the theta and gamma frequency bands in *Opn4*$^{-/-}$ mice compared to in *Opn4*$^{+/+}$ mice (Figure 6). These differences were present both during the light and dark periods of LD 12:12 (unpublished data). Such ECoG changes during NREM sleep are typically associated with reduced sleep quality [29], whereas during wakefulness, ECoG activity in these bands is associated with exploratory behavior, alertness, and cognition [25,26,30], and is thus markedly increased during waking compared to NREM sleep [31]. Genotype effects on theta activity could be analyzed in more detail in REM sleep because theta oscillations are especially prevalent and regular during this state [32]. Theta peak power during both REM sleep and wakefulness was nearly doubled in *Opn4*$^{-/-}$ mice compared to controls (Figure 6; Table S2). The frequency of the theta oscillation during REM sleep was faster in *Opn4*$^{-/-}$ mice compared to *Opn4*$^{+/+}$ mice during the light period, but not during the dark period. In mice, theta frequency during REM sleep is usually slower in the light compared to the dark period [32] (Table S2). In *Opn4*$^{-/-}$ mice, no evidence for such dark–light slowing was, however, found (Table S2), suggesting that the melanopsin pathway can directly or indirectly modulate hippocampal activity.

Altered Homeostatic Regulation of Sleep in *Opn4*$^{-/-}$ Mice

Curtailing sleep time usually results in an increased need for sleep. It is therefore surprising that in *Opn4*$^{-/-}$ mice, levels of ECoG delta power during NREM sleep, a reliable correlate of sleep need, were generally lower while less time was spent in NREM sleep compared to *Opn4*$^{+/+}$ mice. This reduction in delta power was especially pronounced in the dark periods of the LD 12:12 schedule (Figure 7A). The same difference was observed during the subjective dark period under the LD 1:1 schedule (Figure 7B) and thus did not depend on the presence or absence of light. Moreover, this reduction was not associated with an increased fragmentation of sleep (Figure S3) that could have interfered with the expression of delta oscillations.

The unexpected reduction of delta oscillations observed in melanopsin-deficient mice might result from alterations in the properties of the sleep homeostat. We tested the possibility that the build-up of a pressure for sleep when animals are awake occurs at a slower rate in a sleep deprivation experiment. Mice of both genotypes were kept awake by gentle handling for 6 h starting at light onset (ZT0–6). The level of delta power reached during NREM sleep immediately following the sleep deprivation was significantly lower in *Opn4*$^{-/-}$ mice as compared to wild-type animals (Figure 7C), suggesting that in the absence of melanopsin, the dynamics of the sleep homeostat are altered.

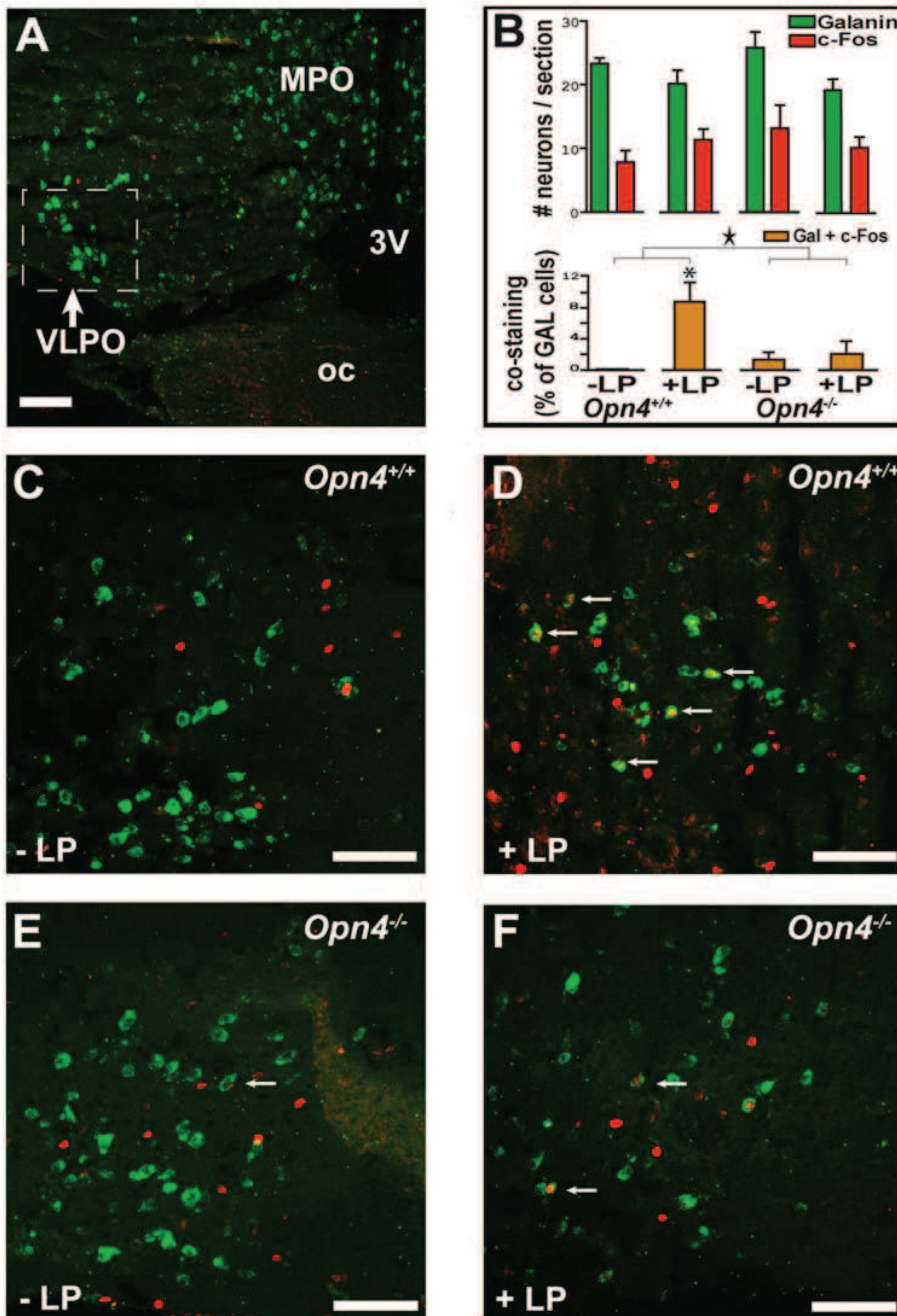

Figure 4. Effects of a 1-h light (L) pulse on c-Fos immunoreactivity in the VLPO. (A) ''Sleep-active neurons'' of the VLPO identified by ISH contain galanin mRNA (labeled green). (B) Top: histograms represent the number of VLPO neurons expressing galanin mRNA or c-FOS protein per section (mean±SEM). Bottom: VLPO costained (galanin+c-Fos) neurons expressed as a percentage of the total number of galanin mRNA-positive neurons (mean±SEM). A two-way ANOVA (with factors light pulse and genotype) revealed that the L pulse induced an increase in VLPO costained cells in *Opn4*$^{+/+}$, but not in *Opn4*$^{-/-}$ mice (Two-way ANOVA, L-pulse effect: $p<0,05$; L pulse×genotype interaction: a star indicates $p<0.05$; the asterisk [*] indicates post hoc Fisher PLSD: $p<0.05$). The light-induced immunoreactivity in VLPO in wild-type animals is specific to galaninergic neurons and of a large magnitude; however, the proportion of c-Fos-stained galanin neurons is low (e.g., *Opn4*$^{+/+}$: +L pulse: 9% of total number of galanin mRNA-containing cells). (C) In the absence of a L pulse (control condition at ZT16), c-Fos immunoreactivity was found in nongalanin mRNA-containing neurons in the VLPO of *Opn4*$^{+/+}$. (D) The L-pulse-induced c-Fos expression (labeled red) in some of the galanin mRNA-positive (green) neurons of the VLPO in *Opn4*$^{+/+}$ (indicated by arrows). (E and F) In *Opn4*$^{-/-}$ mice, the same low number of galanin mRNA-positive neurons express c-Fos in both conditions, without (E) or with (F) light pulse. 3v, third ventricle; LP, L pulse administered during the habitual dark period (ZT15–16); MPO, medial preoptic area; oc, optic chiasm. Scale bars in (A) and (C–F) indicate 100 µm.
doi:10.1371/journal.pbio.1000125.g004

Discussion

Melanopsin-containing RGCs modulate a broad range of physiological responses to light, ranging from pupil constriction to circadian phase shifting. Here, we demonstrate that melanopsin-containing RGCs can also contribute to the acute light induction of sleep. Previously, it was assumed that only the rod–cone system was involved in these direct effects of light through conscious alerting. In humans, a role for melanopsin in the direct effects of light has already been suggested [33,34] because the sensitivity of sleep–wake to acute light exposure is at a maximum at short wavelengths (460–480 nm), corresponding to melanopsin's peak light sensitivity at 480 nm. The application of blue light in normal subjects has been reported to activate multiple brain areas within seconds [35], and the observed responses to 1-h light or dark pulses quantified here in mice occur quickly as well (within minutes).

Circadian Gating of the Direct Effects of Light

A main finding of our study is the result of the LD 1:1 experiment that revealed that time of day modulated the acute effects of light and dark only in *Opn4*$^{-/-}$ mice. During a 7-h period of the subjective dark period, *Opn4*$^{-/-}$ mice did not respond to the ongoing LD alterations with respect to sleep–wake induction. During this time, we and others [20,21] applied the single 1-h light pulse that has led to the conclusion that melanopsin mediates the direct effects of light. Our data show that at other times of day, melanopsin is not necessary for mediating these acute light effects on sleep duration.

Because light perception in *Opn4*$^{-/-}$ mice depends solely on rod–cone photoreception, it can be argued that the inability to respond to light at specific times of day results from circadian changes in rod–cone sensitivity. In intact mice, no time-of-day effect was observed in the response to the LD 1:1 schedule, suggesting that melanopsin compensates for circadian changes in rod–cone sensitivity. Such interaction between the two photosensitive systems could take place in melanopsin-containing RCGs since these cells integrate rod–cone input important for mediating nonvisual light information to the brain [10]. Circadian modulation of retinal output has been described, and for example, variations in rod–cone electrical coupling were shown to lead to higher rod–cone photosensitivity during the dark period [36]. In contrast, another study showed higher cone sensitivity during the

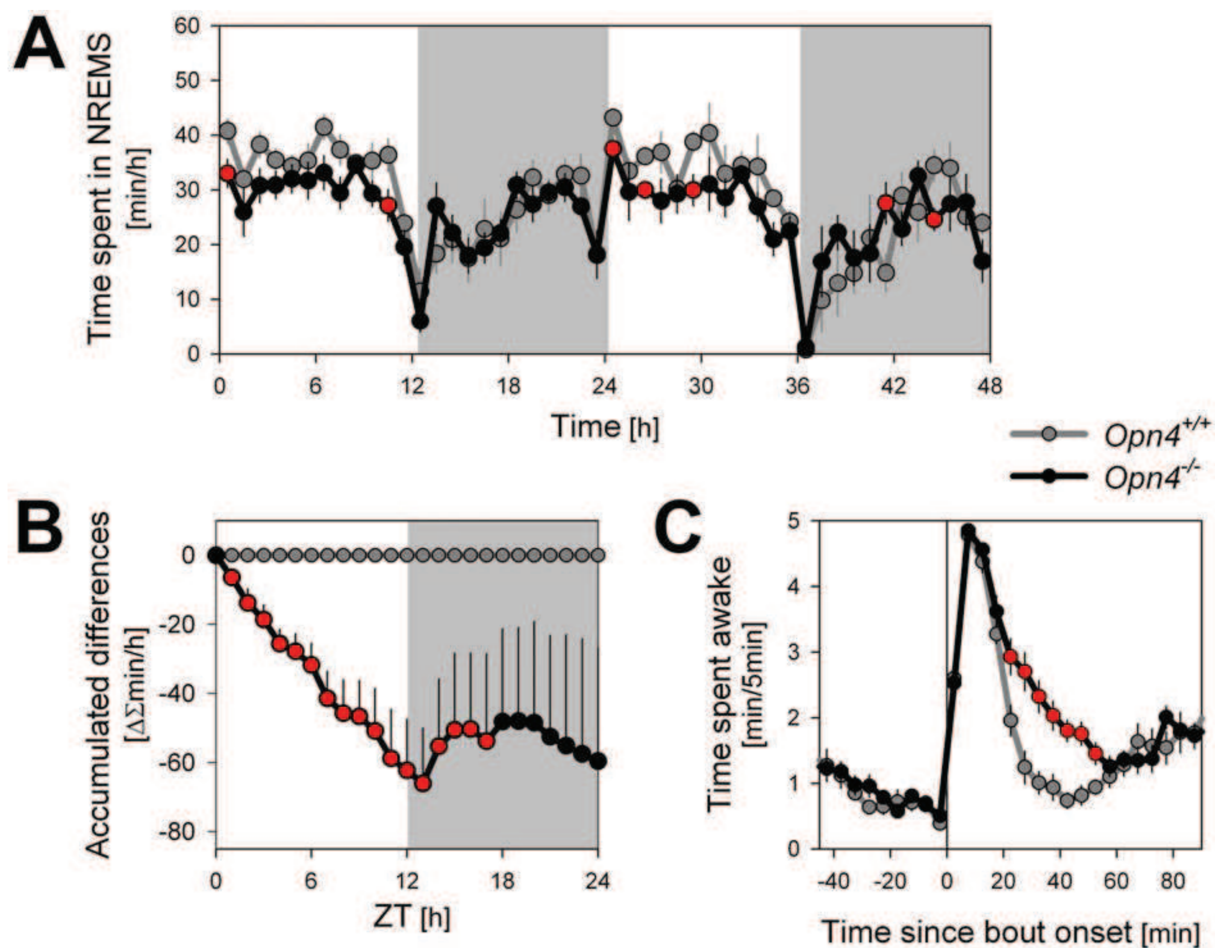

Figure 5. Time course of NREM sleep time and wake distribution under a standard LD 12:12 schedule. (A) Despite a similar time course, NREM sleep levels attained during the 12-h light period were generally lower in *Opn4*$^{-/-}$ mice. (B) Dynamics of the accumulated differences demonstrate that *Opn4*$^{-/-}$ mice lose 1 h of NREM sleep per day, a loss incurred during the light period exclusively (average of the two baseline days; see also Table 1). (C) Sustained waking bouts in *Opn4*$^{-/-}$ are longer than in wild-type animals. See Table S1 for details on the definition of a sustained bout. Red-filled circles denote significant genotype differences ($p<0.05$; post hoc *t*-tests). Values represent mean±SEM (both days $n=9$/genotype; for some mice the first or second day could not be included; *Opn4*$^{+/+}$: day 1 $n=7$, day 2 $n=5$; *Opn4*$^{-/-}$ day 1 $n=9$, day 2 $n=6$).
doi:10.1371/journal.pbio.1000125.g005

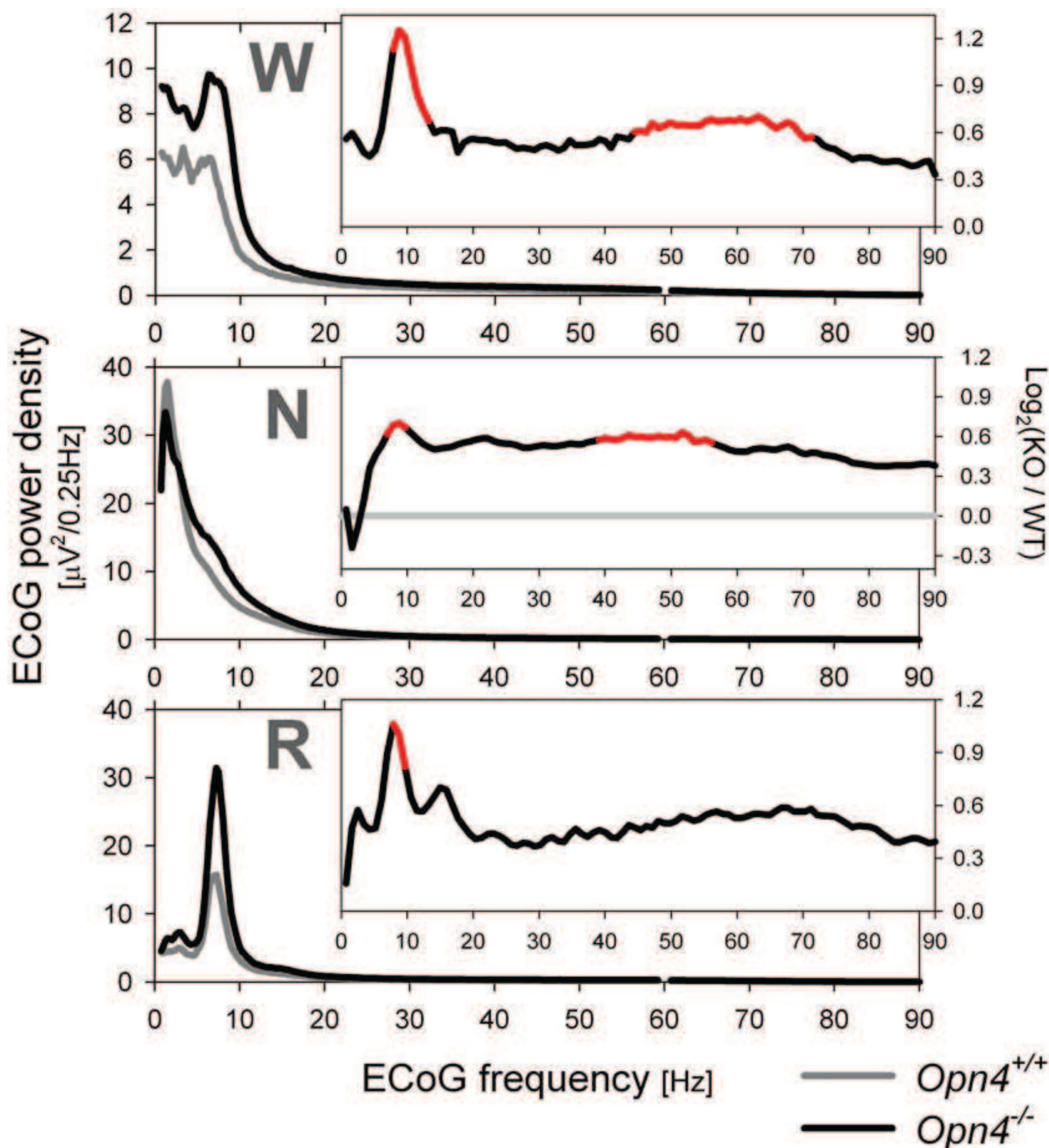

Figure 6. Average ECoG spectral profiles for each behavioral state recorded during 2 d under standard LD 12:12 conditions. Power density in the theta and gamma frequency ranges was generally higher and in the delta frequencies lower (during NREM sleep) in *Opn4*$^{-/-}$ mice. Insets: relative spectral changes quantified as $\log_2$ of the ratio between *Opn4*$^{-/-}$ (KO) and *Opn4*$^{+/+}$ (WT) power density values (e.g., 1 signifies a two-fold increase). Line segments in red denote significant genotype differences ($p<0.05$; post hoc *t*-tests; $n=9$/genotype). N, NREM sleep; R, REM sleep; W, wakfulness.
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(subjective) light period, a circadian difference that required the presence of melanopsin [37]. It is unknown whether such effects exist also for the nonvisual, acute effects of light on sleep. Melanopsin protein levels are also known to vary across the day due to both circadian- and light-dependent influences reaching their highest levels during the light period and lowest during the dark period [38]. Assuming that differences in protein levels translate into differences in (blue) light sensitivity further complicates the issue and calls for further investigation.

We want to emphasize here that the above-described effects concern the acute effects of light observed at the transitions between lighting conditions. We presented evidence that melanopsin also affects several aspects of sleep during the sustained 12-h light period of the LD 12:12 schedule, including the spectral composition of the ECoG, the frequency of theta oscillations during REM sleep, and waking bout duration. The genotype differences in the ECoG activation during the 1-h dark pulse was also observed during this period of the day.

Neuronal Pathways Relaying the Direct Effects of Light on Sleep

Although circadian gating already occurs at the level of the retina [36,39,40], the 50% reduction in the activation of SCN neuronal activity after a light pulse in *Opn4*$^{-/-}$ mice may also be relevant for the circadian variation of the direct effects of light in these mice. As SCN functionality is conserved in the absence of melanopsin [9], a potential role of the SCN in also mediating the acute, noncircadian effects of light could explain the time-of-day-

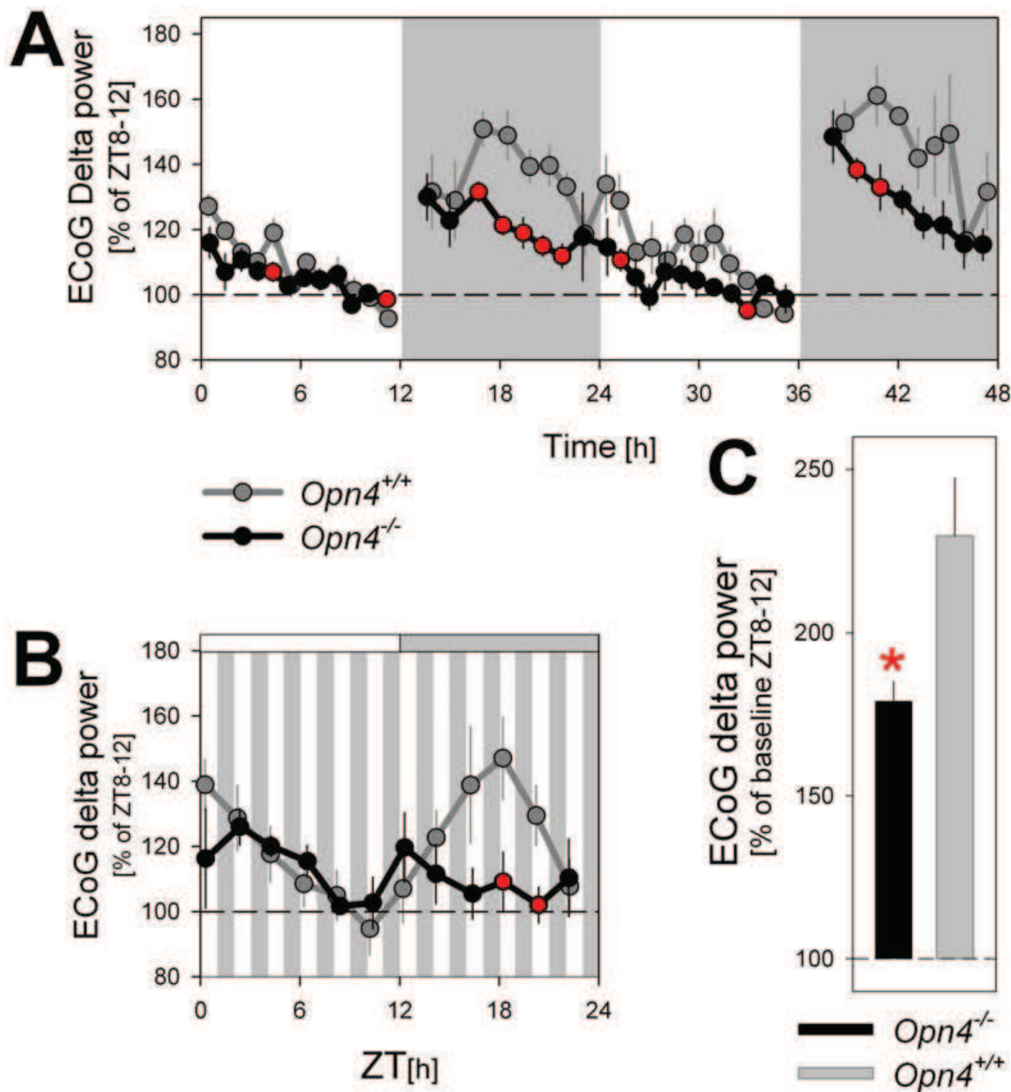

Figure 7. Time course of ECoG delta power (1–4 Hz) during NREM sleep under a standard LD 12:12 cycle, a LD 1:1 cycle, and during recovery sleep after sleep deprivation. (A and B) Time course analyses of ECoG delta power revealed that both under LD 12:12 (A) and LD 1:1 (B) schedules values were generally lower in *Opn4*$^{-/-}$ mice during the (subjective) dark periods. (C) Level of delta power reached after 6 h of sleep deprivation (starting at light onset; ZT0–6) was lower in *Opn4*$^{-/-}$ mice. Mean values ($\pm$SEM) were expressed as percentage of level reached during ZT8–12 of a preceding baseline day. Red filled circles and asterisk denote significant genotype differences ($p<0.05$; post hoc *t*-tests; (A) $n=9$/genotype; (B) $n=4/3$ for *Opn4*$^{-/-}$ and *Opn4*$^{+/+}$ mice, respectively; (C) $n=4$/genotype).
doi:10.1371/journal.pbio.1000125.g007

dependent variation in light sensitivity. The SCN is well placed to relay such activation since it influences key areas for sleep–wake control, such as the VLPO.

Besides the SCN, other anatomical targets of melanopsin-containing RGCs have been identified [22,24,41]. Among the various projection areas, the VLPO is a good candidate for relaying light information to sleep–wake neuronal networks. The VLPO has been implicated in the induction of NREM sleep [42], and galanin- and GABA-releasing neurons within the VLPO have been functionally characterized as a cluster of sleep-active neurons. These sleep-active neurons are thought to actively promote sleep by inhibiting the ascending arousal systems [43,44]. A regional analysis of *c-Fos* expression within the VLPO area was conducted by Lupi et al., 2008, by real time PCR from punched tissues [21]. This analysis revealed a 2-fold increase in *c-Fos* following a 1-h light pulse in wild-type mice that was absent in *Opn4*$^{-/-}$ mice. Using a different method (i.e., counting the number of c-FOS immunoreactive cells in the VLPO), we found little evidence for a light-induced general activation of the VLPO area. Only the costaining of galanin and c-Fos used in our study allows for an evaluation of transcriptional changes specific to those VLPO neurons that could mediate the sleep-promoting effects of light. Here, we provide the first evidence that light indeed activates galanin-containing cells in the VLPO. This light-induced c-Fos immunoreactivity was absent in *Opn4*$^{-/-}$ mice. This anatomical result is consistent with the complete absence of sleep–wake changes during the 1-h light pulse at this time of day, suggesting that the VLPO might play a role in mediating these effects of light on sleep. Our findings thus suggest that light directly impinges on VLPO sleep-active neurons thereby shifting the balance of the reciprocal inhibitory interaction towards sleep promotion and arousal inhibition.

Melanopsin Modulates the Induction of the ECoG Correlates of Alertness and Cognition

The rapid induction of ECoG activity in both the theta and gamma frequency bands during the dark pulse in *Opn*$^{+/+}$ mice is consistent with the alerting effects of light-to-dark transitions in a nocturnal species. Theta oscillations accompany exploratory behavior [45] and are also involved in long-term potentiation (LTP) and learning [46–48]. Gamma activity accompanies a wide variety of cognitive processes, including perceptual processing, attention, arousal, object recognition, and in humans, language perception [49]. As in the present study, gamma activity is often associated with the presence of theta oscillation in rodents [50]. The downstream events initiated by melanopsin have been suggested to affect LTP as well as performance in learning and memory tasks [51]. In humans, nonvisual responses related to alertness and cognition are associated with changes in regional brain activity detected by positron emission tomography (PET) or functional magnetic resonance imaging (fMRI) [52,53]. Application of blue light during a working memory task induces specific brain activity changes within a time frame of seconds [7].

A Role for Melanopsin in Sleep Homeostasis?

ECoG delta power is widely used to reflect a sleep homeostatic process because its level monotonically increases with wake duration and decreases during NREM sleep [54]. The reduction of ECoG delta power in *Opn4*$^{-/-}$ mice was pronounced and present during both the LD 12:12 and LD 1:1 schedules. This reduction was all the more surprising because NREM sleep time was reduced in these mice, and reduced sleep time is usually associated with increased delta power. The unexpected reduction of delta oscillations in melanopsin-deficient mice might result from alterations in the properties of the sleep homeostat. One possibility is that NREM sleep may have been more efficient in reducing sleep need in *Opn4*$^{-/-}$ mice. However, by comparing the ECoG spectral profiles during NREMS, evidence to the contrary was found; the increase in theta and gamma activity combined with reduction of delta activity in *Opn4*$^{-/-}$ mice indicate that NREM sleep seems less profound and, if anything, likely to be less efficient in reducing sleep need and ECoG delta power [55]. The other possibility is that the buildup of a pressure for sleep when animals are awake occurs at a slower rate. We confirmed this possibility in a sleep-deprivation experiment; the level of delta power reached during NREM sleep immediately following the sleep deprivation was significantly lower in *Opn4*$^{-/-}$ mice as compared to wild-type animals. In fact, delta power levels reached after sleep deprivation in *Opn4*$^{-/-}$ mice were as low as the lowest levels observed in a panel of six inbred strains of mice [56]. This reduced compensatory response to sleep loss suggests that indeed the need for sleep increases at a slower rate in *Opn4*$^{-/-}$ mice. The experiment also demonstrates that the reduced delta power is not specific to lighting condition or time of day.

Although a role for melanopsin in sleep homeostasis is unexpected and not easily reconciled with current hypotheses on sleep homeostasis, the modulation of VLPO sleep-active neurons by melanopsin-containing RGCs could hint to a possible mechanism. Sleep-active neurons in the VLPO are thought to be a neuronal substrate of sleep homeostasis because more than 50% of galanin-containing neurons express c-Fos during recovery sleep after a sleep deprivation [57]. A final consideration that applies to all studies using noninducible loss-of-function mutants is that the altered relationship between the sleep–wake distribution and ECoG delta power reflects a developmental effect.

Conclusions

Our results provide evidence that melanopsin-containing RGCs contribute to the noncircadian, nonvisual direct effects of light on sleep and the ECoG correlates of alertness and cognition. Melanopsin's contribution to the acute affects of light on sleep duration was however limited to a ca. 7-h time window. Our findings suggest that the acute photic sleep promotion stems, at least partly, from a stimulation of VLPO sleep-active neurons, which in return, would lead to an inhibition of the arousal systems. Apart from these direct effects of light observed at the transitions between lighting conditions, the daily loss of NREM sleep over the 12-h light period in *Opn4*$^{-/-}$ mice provides evidence that melanopsin also modulates the expression of sleep under sustained periods of light exposure. If confirmed in humans, our observations concerning the time-dependent effects of melanopsin's contribution to the acute effects of (blue) light as well as the effects of sustained light exposure will have applications for the clinical use of light therapy as well as for 24-h patterns in luminance [58]. Finally, the discovery that the homeostatic regulation of sleep need can be affected by a photopigment is intriguing and represents a novel concept in the field of sleep regulation.

Materials and Methods

Animals

All experiments were performed on adult male *Opn4*$^{-/-}$ mice and wild-type littermates (as controls), and carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals as well as local veterinary office and use committees at Stanford University (for details and genotyping, see Text S1 and Ruby et al. (2002) [17]).

ECoG Recordings and Analyses

The methods concerning the recording and analysis of the ECoG in mice are described in detail in Text S1 and elsewhere [32,59]. Briefly, ECoG and electromyogram (EMG) recordings from mice implanted with a classical set of electrodes, were collected (using commercial hardware, EMBLA, and software, Somnologica-3) for the following conditions: (1) two continuous baseline days under LD 12:12 cycle; (2) a 1-h light pulse (white fluorescent tubes, Philips F32T8/TL741 Hi-Vision 40 W, 4,100 K [Philips Lighting], broad spectrum, mainly 400–800 nm) administered 3 h after dark onset (ZT 15), (3) 1-h dark pulse administered 3 h after light onset (ZT 3), (4) a 1:1 LD cycle for 24 h, and (5) 6-h sleep deprivation starting at light onset. The behavior in each 4-s epoch was classified as waking, REM sleep, or NREM sleep based on the ECoG and EMG signals according to standard criteria [32]. The ECoG signal (analog-to-digital converted) was subjected to discrete Fourier transform (DFT) yielding power spectra at 0.25-Hz resolution. For each state, an ECoG spectral profile (0 to 90 Hz) was constructed by averaging all 4-s epochs scored as that state.

In Situ Hybridization (ISH) and Immunohistochemistry (IHC)

Galanin ISH and c-Fos immunostaining on frozen sections with or without (control condition) a prior 1-h light exposure was carried out as described previously [60,61] and are detailed in Text S1. Staining of GAL mRNA was chosen instead of staining the protein because it has been shown in mice that cell body staining of galanin-containing neurons by IHC is only reliable in colchicine-treated animals. In *Opn4*$^{+/+}$ and *Opn4*$^{-/-}$ mice ($n = 6$ of each genotype), identification of c-Fos immunoreactive cells and

sacrifice of animals occurred at the conclusion of a 1-h light pulse as well as without a light pulse as a negative control. Sleep was recorded in these animals, several weeks before, under a light pulse identical to the light pulse administered the day of the perfusion to confirm that their response to light was similar to those of the whole group. Before ISH series of 18-µm-thick sections were pretreated by an antigen retrieval procedure [62]. The mouse galanin probe (National Center for Biotechnology Information [NCBI] BC044055, covering a 716-base sequence of the GAL prepro-mRNA) was used in a dilution of 1:500. The ISH protocol preceding the immunohistochemical protocol was identical to the procedure described previously [60,61]. GAL mRNA was visualized using a horse radish peroxidase (POD)-labeled sheep-anti-digoxiginin antibody (Roche 1207733, diluted 1:200), and Alexa-tyramide 488 (Molecular Probe; diluted 1:100). Hereafter, sections were incubated with a rabbit anti-c-Fos antiserum (c-Fos antibody dilution: 1:500; code no: 9412, [23] and visualized by Alexa568-conjugated goat anti-rabbit antibody (Molecular Probe; diluted 1:1,000). Counting of GAL- and c-Fos-expressing neurons in the VLPO and SCN was conducted using a confocal microscope (Zeiss LSM 510; Brock and Michelsen) equipped with appropriate filter settings for detecting Alexa488 and Alexa568 was used. The quantification method is detailed in Text S1.

Statistical Analysis

Differences in sleep amounts and quantitative EEG variables were determined by single- or multiple-way ANOVAs, followed by post hoc *t*-tests if 5% significance levels were reached. The differences in number of c-Fos, Gal; or c-Fos+Gal neurons were assessed by two-way (light and genotype conditions) ANOVA, followed by post hoc Fisher PLSD.

Supporting Information

Figure S1 Overview of wakefulness expressed per 5-min intervals in one *Opn4*$^{-/-}$ (left) and one wild-type (right) animal. Shown are the various LD regimens used, including two consecutive days of baseline (top), 1-h dark pulse administered at ZT3 and 1-h light pulse administered at ZT15 (middle) and a 24-h d under a 1-h:1-h LD cycle (bottom). A minimum of 10 d was allowed between each experimental condition. Recordings started 1 d prior to each condition to verify that sleep–wake amounts and architecture returned to baseline values.
Found at: doi:10.1371/journal.pbio.1000067.s001 (1.08 MB TIF)

Figure S2 Heat map of the light (L) and dark (D) and time-of-day-dependent changes in time spent awake under the LD 1:1 schedule (see Figure 2B). Waking values (waking minutes/5-min intervals; warmer colors correspond to more waking/5 min) over 3 h were aligned according to the onset (0 h; grey horizontal bars) of the 1-h dark periods. Only in *Opn4*$^{-/-}$ mice does the capacity of the light and dark pulses to shape the sleep–wake distribution vary with time of day. This is especially clear between ZT15 and ZT21 during the subjective dark period (ZT12–24; grey vertical bars). Note that values depicted between time 1 and 2 at one ZT corresponds to the values between −1 and 0 of the subsequent ZT interval. Also note that *Opn4*$^{+/+}$ mice learn to anticipate dark-period onset as the day progresses.
Found at: doi:10.1371/journal.pbio.1000067.s002 (4.02 MB TIF)

Figure S3 Relative frequency distribution of episode duration of NREM sleep (N), REM sleep (R), and waking (W) episodes under standard LD 12:12 conditions. Vertical bars represent the number of episodes (mean±standard error of the mean [SEM]) expressed per hour of time spent in each state for nine categories of episode duration. For none of the behavioral states did genotype affect the distribution.
Found at: doi:10.1371/journal.pbio.1000067.s003 (0.55 MB TIF)

Table S1 Duration and number of sustained waking bouts during baseline recordings under a LD 12:12 schedule. Sustained waking bouts in *Opn4*$^{-/-}$ are longer than in wild-type animals (*Opn4*$^{+/+}$) during the 12-h light period ($p<0.05$; post hoc *t*-tests). See Text S1 below for selection criteria of sustained waking bouts. All values represent means±1 SEM (both days $n=9$/genotype; for some mice the first or second day could not be included; *Opn4*$^{+/+}$: day 1 $n=7$, day 2 $n=5$; *Opn4*$^{-/-}$ day 1 $n=9$, day 2 $n=6$).
Found at: doi:10.1371/journal.pbio.1000067.s004 (0.03 MB DOC)

Table S2 ECoG theta activity during REMS differed between genotypes. ECoG power density at peak frequency was higher both in absolute and relative terms (not shown, but see Figure 6) in *Opn4*$^{-/-}$ mice. In the light period, theta oscillated at a higher frequency in *Opn4*$^{-/-}$ mice, reaching values normally attained during the dark period. As a result, the normal LD difference in theta peak frequency was absent in *Opn4*$^{-/-}$ mice. An asterisk (*) indicates significant genotype differences; a section mark (§) indicates significant LD differences ($p<0.03$; post hoc *t*-test). Values represent mean±SEM (light period: $n=8$ and 9; dark period: $n=7$ and 7, for *Opn4*$^{-/-}$ and *Opn4*$^{+/+}$, respectively).
Found at: doi:10.1371/journal.pbio.1000067.s005 (0.03 MB DOC)

Text S1 Supplemental experimental procedures: detailed procedures.
Found at: doi:10.1371/journal.pbio.1000067.s006 (0.04 MB DOC)

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Author Contributions

The author(s) have made the following declarations about their contributions: Responsible for supervision and data collection: PB. Developed the study protocol: JWT, JH, GH, DC, ER, NFR, HCH, PF, PB. Performed anatomical experiments: JH, ER. Performed ECoG recordings and scoring: JT, GH, DC. Analyzed sleep and ECoG: PF. Drafted and prepared the final manuscript: HCH, PF, PB. Reviewed, revised, and approved the final paper: JWT, JH, GH, DC, ER NFR, HCH, PF, PB.

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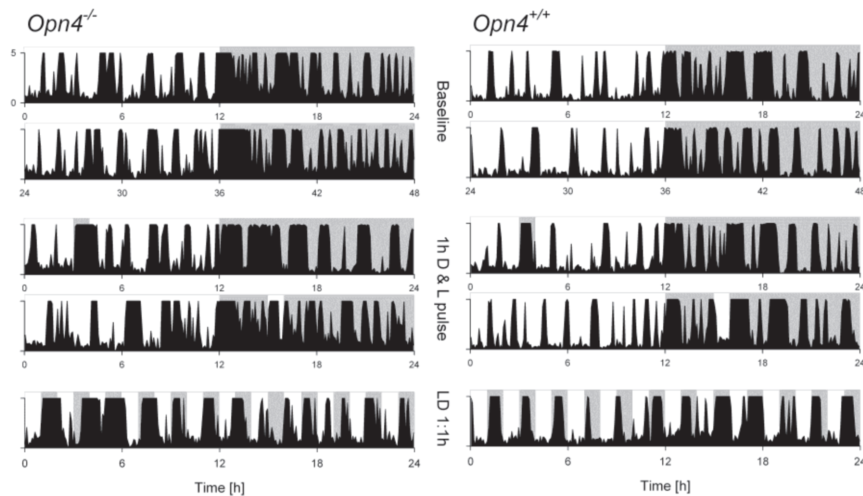
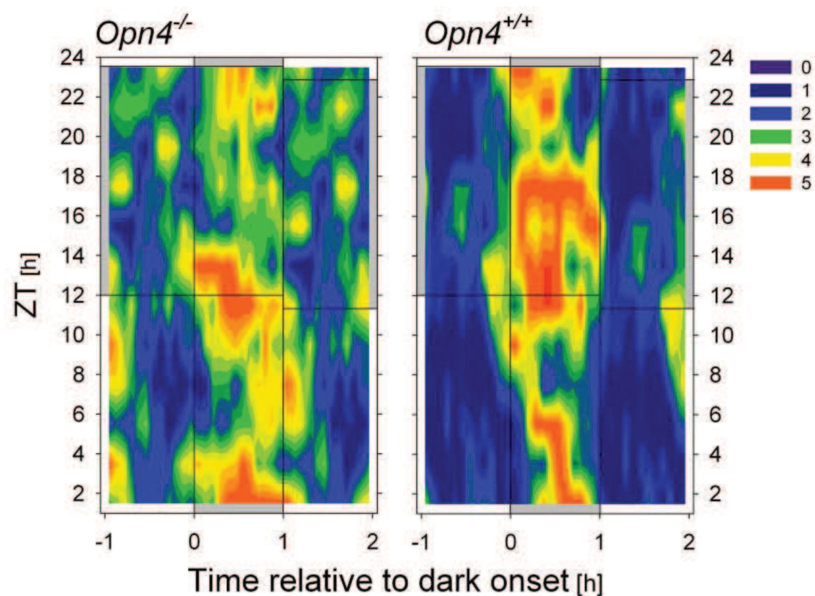
SUPPORTING INFORMATION

Figure S1



Overview of wakefulness expressed per 5-min intervals in one *Opn4*$^{-/-}$ (left) and one wild-type (right) animal. Shown are the various LD regimens used, including two consecutive days of baseline (top), 1-h dark pulse administered at ZT3 and 1-h light pulse administered at ZT15 (middle) and a 24-h d under a 1-h:1-h LD cycle (bottom). A minimum of 10 d was allowed between each experimental condition. Recordings started 1 d prior to each condition to verify that sleep–wake amounts and architecture returned to baseline values.

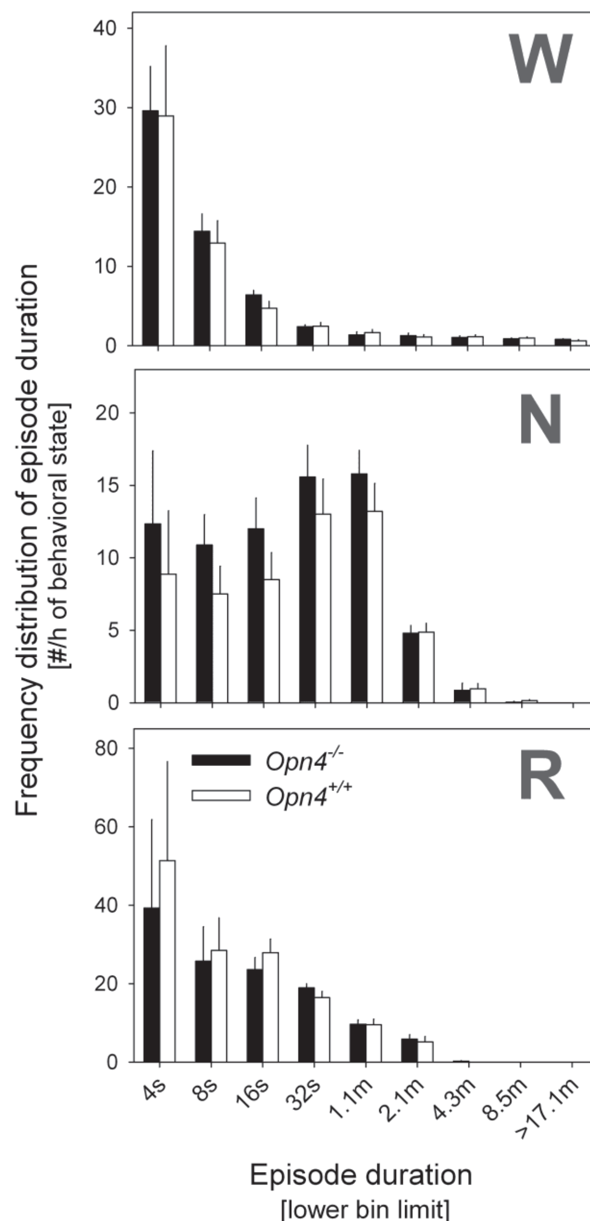
Figure S2



Heat map of the light (L) and dark (D) and time-of-day-dependent changes in time spent awake under the LD 1:1 schedule (see Figure 2B). Waking values (waking minutes/5-min intervals; warmer colors correspond to more waking/5 min) over 3 h were aligned according

to the onset (0 h; grey horizontal bars) of the 1-h dark periods. Only in *Opn4*$^{-/-}$ mice does the capacity of the light and dark pulses to shape the sleep–wake distribution vary with time of day. This is especially clear between ZT15 and ZT21 during the subjective dark period (ZT12–24; grey vertical bars). Note that values depicted between time 1 and 2 at one ZT corresponds to the values between −1 and 0 of the subsequent ZT interval. Also note that *Opn4*$^{+/+}$ mice learn to anticipate dark-period onset as the day progresses.

Figure S3



Relative frequency distribution of episode duration of NREM sleep (N), REM sleep (R), and waking (W) episodes under standard LD 12:12 conditions. Vertical bars represent the number of episodes (mean±standard error of the mean [SEM]) expressed per hour of time

spent in each state for nine categories of episode duration. For none of the behavioral states did genotype affect the distribution.

Table S1

Duration and number of sustained waking bouts during baseline recordings under a LD 12:12 schedule. Sustained waking bouts in *Opn4*$^{-/-}$ are longer than in wild-type animals (*Opn4*$^{+/+}$) during the 12-h light period (p<0.05; post hoc t-tests). See Text S1 below for selection criteria of sustained waking bouts. All values represent means±1 SEM (both days n=9/genotype; for some mice the first or second day could not be included; *Opn4*$^{+/+}$: day 1 n=7, day 2 n=5; *Opn4*$^{-/-}$ day 1 n=9, day 2 n=6).

	12h L-period		12h D-period	
	number	length [min]	number	length [min]
Opn4$^{-/-}$	6.7 ± 0.3	34.3 ± 2.2	6.2 ± 0.4	66.6 ± 4.6
Opn4$^{+/+}$	7.2 ± 0.5	23.3 ± 1.0	6.0 ± 0.5	77.9 ± 9.4
P	*0.42*	*0.0003*	*0.73*	*0.30*

Table S2

ECoG theta activity during REMS differed between genotypes. ECoG power density at peak frequency was higher both in absolute and relative terms (not shown, but see Figure 6) in *Opn4*$^{-/-}$ mice. In the light period, theta oscillated at a higher frequency in *Opn4*$^{-/-}$ mice, reaching values normally attained during the dark period. As a result, the normal LD difference in theta peak frequency was absent in *Opn4*$^{-/-}$ mice. An asterisk (*) indicates significant genotype differences; a section mark (§) indicates significant LD differences (p<0.03; post hoc t-test). Values represent mean±SEM (light period: n=8 and 9; dark period: n=7 and 7, for *Opn4*$^{-/-}$ and *Opn4*$^{+/+}$, respectively).

	Opn4 genotype	12h Light	12h Dark
Peak frequency [Hz]	−/− +/+	7.28 ± 0.07* 6.93 ± 0.10	7.30 ± 0.10 7.34 ± 0.07§
Peak power [μV^2/0.25]	−/− +/+	31.9 ± 4.5* 14.6 ± 3.2	36.3 ± 4.5* 13.7 ± 4.3

Supplemental Experimental Procedures: Detailed Procedures

Animals:

The animals, originally obtained from Deltagen Laboratory, were reared under control environmental conditions (12h:12h light-dark; 25 ± 0.3° C, food and water ad libitum) and handled in agreement with the ethical rules for experimentation on laboratory animals in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The experimental protocols were approved by the local veterinary office and use committees at Stanford University. Genotype was validated by 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.) as described previously [1].

ECoG Recordings and Analyses:

Surgical preparation occurred under anesthesia delivered intraperitoneally with Nembutal (68 mg/kg; Stanford University IRB-approved). Adult male *Opn4*$^{-/-}$ (n=10) and *Opn4*$^{+/+}$ (n=10) mice were implanted with two ECoG and two EMG electrodes for sleep recordings at 10-12 weeks of age. Mice were allowed at least 14 days of recovery from surgery and habituation to experimental conditions before the experiments. Two undisturbed baseline days (48h) were recorded using commercial hardware (EMBLA™) and software (Somnologica-3™) under LD12:12h conditions (white fluorescent lights, 100lux, measured at the bottom of the cage). Continuous sleep recordings were also performed under the following conditions: (i) a 1 hour white-light pulse of (200 lux;) administered 3 hours after dark onset (ZT 15), (ii) 1-hour dark pulse administered 3 hours after light onset (ZT 3), and (iii) a 1-1 LD cycle for 24 hours. In a subset of mice a 6h sleep deprivation was performed at the end of all experiments. Sleep deprivation was performed by gentle handling [2] starting at light onset (ZT0-6). These experiments took place on separate days with at least 14 days in 12-12 LD between each experiment. The day of recording preceding the experimental condition was used to confirm that sleep-wake amounts and architecture had returned to baseline conditions. Both genotypes were recorded at the same time.

ECoG and EMG signals were amplified, filtered, and analog-to-digital converted at 2000Hz, subsequently down-sampled and stored at 200Hz. The ECoG signal was subjected to Discrete-Fourier Transform (DFT) yielding power spectra between 0 and 90 Hz (0.25Hz

resolution) using a 4-s window. The behavior in each of these 4-s epochs was classified as waking, rapid-eye-movement (REM) sleep, or non-REM (NREM) sleep by visual inspection of the EEG and EMG signals without knowledge of the recording and genotype condition according to standard criteria [3]. Four-second epochs containing ECoG artifacts were marked so they could be excluded from ECoG spectral analyses. Genotype differences in sleep amount were calculated by averaging time spent in each state over 5min, and 1-, 12-, and 24h intervals. The frequency distribution of episode duration of NREMS, REMS, and waking was calculated as described previously [2]. In addition, the sustained waking bouts were defined by using a moving average (10-min window, 4-s increments) according to Franken et al. (2006) [4]. Ten-minute windows in which waking prevailed (>75%) were deemed "awake". Awake windows separated by <20 min were merged into a single waking bout.

To determine the amplitude of the light-dark dependent changes in wakefulness under the LD1:1 schedule, sine-waves were fitted to the individual 5min values for wakefulness obtained in the 1h before, during, and after the 1h dark periods. Within subjects, the 5min values for the 6 dark-pulses given during the 12h subjective light period and the 6 dark-pulses given during the 12h subjective dark period were averaged. Sine-waves were fitted to the average time courses using SAS (SAS Institute Inc, Cary, NC; Proc NLIN) with amplitude, phase, y-offset as free parameters and period set to 2 hours (see Fig.2B). Thus obtained individual amplitudes for the subjective light and dark period were further analyzed using a 2-way ANOVA with factor genotype and repeated measures for factor light or dark period.

For each state an ECoG spectral profile was constructed by averaging all 4-s epochs scored as that state. The frequency range 59-61 Hz was omitted due to power-line artifacts in some of the recordings. Time dependent changes in ECoG power in specific frequency bands was performed for delta (1-4Hz) in NREM sleep, and theta (6-10Hz) and gamma (40-70Hz) during wakefulness. ECoG delta power during NREMS was normalized by expressing all values relative to the mean value obtained in the last 4h of the (subjective) light period. Determination of theta peak frequency and theta peak power in the REMS ECoG was performed according to Franken et al. (1998)[3].

In Situ Hybridization (ISH) and Immunohistochemistry (IHC)

In *Opn4*$^{+/+}$ and *Opn4*$^{-/-}$ mice (n=6/genotype), identification of c-Fos immunoreactive cells and sacrifice of animals occurred at the conclusion of a 1h light pulse and without light pulse as a negative control. Sleep was recorded in these animals, several weeks before, under a light

pulse identical to the light pulse administered the day of the perfusion to confirm that their response to light was similar to those of the whole group. At the end of the 1h light pulse (ZT16), mice were deeply anesthetized with CO_2 and perfused with heparin/NaCl followed by transcardial fixation for 15 min with 4% paraformaldehyde in PBS, pH 7.4 for in situ hybridization and combined immunohistochemistry. After dehydration in 30% sucrose for 48h the brains were frozen and cut in a freezing microtom as 18μm thick sections in series of four containing the VLPO and the SCN, respectively.

Before ISH all sections were pretreated by an antigen retrieval procedure as described by the manufacturer (DAKO TechMate 500/1000, Copenhagen, Denmark) using antigen retrieval buffer (DAKO ChemMate, code No. S 203120) in distilled water. The mouse galanin (GAL) probe used was obtained from NCBI (BC044055, cDNA clone MGC:54666, base 1 to 716 of the GAL prepro-mRNA inserted into a pCMV-SPORT6). Antisense and sense probes were labelled with digoxiginin using T7 and SP6 polymerase and used in a dilution of 1:500. The ISH protocol preceding the immunohistochemical protocol were identical to the procedure described previously [5,6]. GAL mRNA was visualized using a horse radish peroxidase (POD)-labelled sheep-anti-digoxiginin antibody (Roche 1207733, diluted 1:200), and Alexa-tyramide 488 (Molecular Probe diluted 1:100). Hereafter sections were washed and incubated over night with a well-characterized rabbit anti-c-FOS antiserum (c-Fos antibody dilution: 1:500; (code #9412) raised against amino acids 4-17 of the human/rat proteins [7]; kindly donated by Dr. Philip J. Larsen, The Panum Institute, Copenhagen, Denmark; see [8] and visualized by Alexa568 conjugated goat anti-rabbit antibody (Molecular Probe, diluted 1:1000). Hybridization with the GAL sense probe gave no specific labeling (data not shown).

To investigate the number of GAL expressing neurons in the VLPO also expressing c-Fos immunoreactivity a confocal microscope (Zeiss LSM 510, Brock and Michelsen, Birkerød, Denmark) equipped with appropriate filter settings for detecting Alexa488 and Alexa568 was used. The number of GAL expressing neurons in the VLPO cluster was counted by an experimenter blind to experimental conditions, in an area of 225μm x 225μm at a level corresponding to bregma -0.1mm [9] (Figure 2). Two brain sections from each animal representing the VLPO area were analyzed and cells containing GAL, c-Fos and GAL+c-Fos was manually determine using a grid on each image.

Sections from each animal containing the SCN were used as control of the light stimulation paradigm. SCN was stained for arginin-vasopressin (AVP antibody was raised in

guinea pig (code no.: GHC 8103, Peninsula Laboratories, San Carlos, CA, USA, dilutes 1:1000) and co-stained for c-Fos which is strongly induced in the SCN after light stimulation at night [10]. C-Fos was visualized as described above and AVP as described previously [11]. The number of c-Fos immunoreactive neurons in the SCN was counted the same way as above. Elimination of the primary antisera eliminates all specific staining.

Supplemental References:

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Article 2: ***« A model to predict how (melanopsin-dependent) lighting shapes the sleep-wake cycle»***

Soumission prévue février 2015

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Figures : pages 9-14

Références : pages 15-16

Table supplémentaire : page 17-18

Figures supplémentaires : pages 19-21

Texte supplémentaire : pages 22-28

1. TITLE PAGE. *Original paper*

Title: A model to predict how (melanopsin-dependent) lighting shapes the sleep-wake cycle

Short title: Light shapes the sleep-wake cycle

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Conflict of interest: The authors have no conflict of interest to declare.

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Tables and Figures: 3 Figures, 2 supplemental tables, 4 supplemental figures, extended materiel and methods section

Keywords: direct effects of light, sleep regulation, direct photic light, melanopsin, mice, Synaptotagmin10-Cre

Word count: Main text = 2443; Introductory paragraph = 292; References =22

2. MAIN MANUSCRIPT:

Introductory Paragraph:

Evolutionary conservation suggests that timing physiology and behavior to Earth's light/dark cycle is critical to the survival of most species. This rhythmic expression is driven by the circadian process generated by the primary clock located in the suprachiasmatic nucleus (SCN)[1]. Light entrains the circadian pacemaker, synchronizing the sleep-wake cycle to the time of day (CL: circadian effect of light). Light also exerts a direct clock-independent effect (DL: direct effect of light), which has been the subject of growing interest since the discovery of melanopsin (Opn4), a photopigment crucial for conveying non-image forming light to the brain[2,3].

As CL and DL, are difficult to disentangle, it remains unknown whether and to which degree DL shapes the sleep-wake cycle[4-8]. Here we quantified for the first time, the contribution of each component by studying EEG in SCN- and melanopsin-disabled mice. Sleep reactivity to an ultradian hourly light/dark cycle (LD1:1) and to a standard 12-hour light/12-hour dark regimen (LD12:12) was diminished in clock- or melanopsin-disabled mice, and abolished in animals lacking both. Our results suggest that CL shapes about half of the 24-hour sleep-wake cycle, with DL being responsible for the other half, mediated mostly by Opn4-based phototransduction. Furthermore, our findings show that the SCN is not only a clock but also mediates close to 50% of DL, and is critical for the alerting effects of darkness in nocturnal mice. We portray our results in a model integrating the respective contributions of the main phototransduction pathways regulating the natural sleep-wake cycle, which, contrary to previous belief, attributes a critical role to DL. The model was successfully applied to the LD1:1, suggesting that this rationale could be used to predict the shape of the sleep-wake cycle in the perturbing lighting conditions commonly experienced in modern society.

Main text:

In modern society, we have come to rely less and less on Earth's natural light/dark cycle. As a result, we increasingly see alterations in sleep-wake architecture leading to circadian disorders, insomnia, daytime somnolence, mood alteration, and poorer cognitive performance[9,10]. This points to the need for a better understanding of the photic regulation of physiology and behavior. Non-visual rod/cone- and Opn4-based photic information is signaled to the brain through a subset of retinal ganglion cells rendered intrinsically photosensitive through Opn4 expression[11,12]. These cells project to the SCN, entraining the clock, but also to other structures directly controlling vigilance states such as the sleep-promoting neurons of the ventrolateral preoptic area (VLPO)[11,12].

Here we have dissected the photic regulation of sleep by studying EEG in SCN- and melanopsin-disabled mice under various light/dark regimens in order to determine the respective contribution of each component (CL vs. DL, melanopsin vs. rods/cone based photoreception, SCN vs. non-SCN brain relays). SCN and clock disablement was achieved through: 1) lesioning of the SCN (SCNx), and 2) a novel transgenic mouse model (*Syn10$^{Cre/Cre}$Bmal$^{fl/-}$*) characterized by a functional invalidation of the clock with a structurally intact SCN[13]. The use of both models controls for lesion-induced structural damages as well as for the limitations associated with transgenesis. Additionally, the contribution from melanopsin and rods/cones was distinguished using SCNx mice lacking Opn4 (*Opn4$^{-/-}$*)[14].

Both models were arrhythmic regarding sleep and locomotor activity under constant darkness (DD), confirming the efficacy of clock disablement. In addition, as lesioning of the SCN generates structural damages, we verified the complete conservation of retinal projections to non-SCN structures using the anterograde fiber tracer, cholera toxin subunitB (CtB) (Fig. 1a). Moreover, immunostaining with arginine vasopressin (AVP), a marker of SCN output[15], and 4',6-diamidino-2-phenylindole (DAPI), a marker of cell nuclei, confirmed the complete destruction of the SCN while sparing surrounding areas (Fig. 1a).

To assess SCN functioning in *Syn10$^{Cre/Cre}$Bmal$^{fl/-}$* mice, we quantified c-Fos, a marker of neuronal activation, in response to a 1-hour light pulse administered during the dark period (ZT15-16) when light-induced c-Fos immunoreactivity and phase delay of circadian rhythms in locomotor activity are maximal. *Syn10$^{Cre/Cre}$Bmal$^{fl/-}$* mice showed a severely diminished light-induced c-Fos expression compared to their controls (*Syn10$^{Cre/Cre}$Bmal$^{fl/-}$* and WT), indicating the lack of reactivity of SCN neurons to light (Fig. 1b). Moreover,

immunoreactivity of AVP was absent in the DAPI-stained SCN cells of the *Syn10$^{Cre/Cre}$Bmal$^{fl/-}$* mice, despite a normal distribution in surrounding areas, further confirming SCN-disablement (Fig.1b)[15].

Acute administration of light is known to promote sleep in mice and other nocturnal species whereas dark pulses promote wake[6]. However the temporal organization and componential breakdown (SCN vs. other structures and Opn4 vs. rod/cone mediation) of the transmission of these acute effects of light and dark (D) remain to be clarified. To examine the acute influence of DL and its interaction across the day with C, we subjected all experimental groups to an ultradian 1-hour light : 1-hour dark (LD1:1) cycle applied continuously over 24 hours (Fig. 2a). All control groups (wild-type; *Syn10$^{Cre/Cre}$Bmal1$^{+/-}$*, Sham-lesioned *Opn4$^{+/+}$*), showed a response correlating with previous literature (Fig 2; Supplementary Fig. 1, Supplementary Table 1, 2)[8]. A comparison of the reactivity to LD1:1 among the experimental groups allowed us to extrapolate the influence of each of the above named components. The greater reactivity to photic stimulus in controls compared to SCNx and *Syn10$^{Cre/Cre}$Bmal$^{fl/-}$* demonstrates that the SCN plays a critical role in mediating DL, especially during the subjective dark period (ZT12-24, i.e. the 12-hour dark phase of the preceding days) (Fig. 2b). Furthermore, the time-course analysis of EEG theta and gamma, correlates of exploratory behavior and alertness[16-18], indicate that the SCN is a crucial mediator of the alerting effects of darkness in a nocturnal mouse (Fig. 2c). The comparison of results obtained from Opn4 transgenic mice and controls indicate that both rods/cones and Opn4 relay DL, with an Opn4 dominance during the subjective dark and rod/cone dominance during the subjective light, a likely explanation for the greater effect of DL during the subjective light period. This is further supported by the 24-hour cyclic expression of melanopsin, suggesting that the circadian process modulates D throughout the day[19,20].

In humans, light is known to acutely promote alertness, waking EEG, and cognition, with the highest efficiency being blue light centered around 460-480nm, within the spectral response peak of melanopsin[10]. Here, in mice under LD1:1, the wake promoting effect of darkness was associated with a rapid and prolonged induction of EEG theta (7–10 Hz) and gamma (40–70 Hz) activity (Fig. 2c, Supplementary Fig. 3) over the course of all 1h-dark pulses. This alerting effect of darkness, which was observed at all times of the day, was attenuated though not abolished in melanopsin-disabled mice, suggesting that it is mostly mediated by Opn4 but also to some extent by rod-cone-based photodetection. In addition, we studied the dark-associated theta and gamma activities in SCNx and *Syn10$^{Cre/Cre}$Bmal$^{fl/-}$* mice,

which produced some remarkable observations. These EEG markers of alertness were dramatically decreased in both SCN-abolished models, both during the dark pulses of LD1:1 (Fig. 2c, Supplementary Fig. 3) as well as in the 12h-dark period of LD12:12 (Supplementary Fig. 4). This finding indicates that the alerting effect of darkness in nocturnal animals is mostly signaled by the SCN, a structure known to influence, albeit indirectly, activity in the hippocampus, a center for cognition and theta/gamma rhythm generation[21]. Thus, we can speculate that Opn4-signaling and the SCN signifies an essential neuronal substrate of the alerting effect of light in humans, with this pathway representing a framework for the further understanding of the powerful influence of light on arousal systems and cognition.

Under a standard 12-hour light/12-hour dark condition (LD12:12), mice express a cyclic sleep-wake pattern with a 24-hour period and an amplitude representing the difference in sleep/wake amounts between the light and dark phases. It has generally been assumed that a primary clock is necessary for maintaining this cycle, with light cues playing a role in adjusting the clock to match Earth's light/dark cycle. The homeostatic process in this condition does not affect the overall period or amplitude of the sleep-wake cycle as the mice are not sleep deprived, housed in single cages, with food and water provided ad libitum. Thus the animals are allowed to follow their natural napping pattern throughout the protocol, and therefore do not build up a homeostatic sleep need over a longer period. As a mouse naps frequently, this parallels, in humans, the multiple nap protocol in which the homeostatic process averages to zero[22]. Given the significance of the aforementioned acute photic regulation of sleep observed at all times of the day, we examined whether DL is sustained over each 12-hour lighting period to uphold a 24-hour organization of the sleep-wake cycle. To do this, we analyzed the amplitude of the cycle under LD12:12 to determine whether DL, even in the absence of a circadian system, is capable of maintaining a significant sleep/wake amplitude (Fig. 3c).

The amplitude of the sleep-wake cycle in the Sham-lesioned *Opn4*$^{+/+}$ mice was about 2.5 hours (Fig. 3c). Amplitudes of the experimental groups were decreased, with degree of reduction determined by the removal of phototransductive components. Comparison of the amplitudes among the experimental groups allowed us to isolate and quantify the contribution of all but two of the components to the sleep-wake cycle, as this method does not give us the respective contributions of DL mediated by rod/cone phototransduction via the SCN and CL. Therefore, we calculated the amplitude of the sleep-wake cycle of WT and *Opn4*$^{-/-}$ mice obtained under DD, i.e. a condition lacking DL (Fig. 3b). This isolates CL, the circadian drive

entrained by the LD12:12 condition of the preceding days. CL was similar in both genotypes, indicating that it is not significantly affected by the loss of Opn4. This is coherent with previous observations based on actimetry that in the absence of Opn4-based phototransduction, entrainment of CL is largely compensated by rods and cones[14]. Thus, we conclude that the curtailed magnitude of the sleep-wake cycle in *Opn4*$^{-/-}$ mice under LD12:12 results from a loss of DL and is not due to altered circadian output.

Our results can be summarized in a model illustrating the contribution of each component in the determination of the sleep-wake cycle, compiling to CL and DL (Fig. 3d). According to our model, DL is responsible for about 45% of the amplitude of the sleep-wake cycle, meaning that rather than just a “masker” of CL, DL plays a significant, and likely even equivalent role to CL in the shaping of the daily sleep-wake pattern. The calculations shown here are based on values of NREM, but the same distribution between CL and DL is observed in wake and REM (Supplementary Table 1, 2). Furthermore, we have also broken down the roles which Opn4 and rods/cones play in transmitting the direct effects of light, and found that Opn4-based phototransduction is responsible for the majority of DL, with rods/cones playing a minor role. These results corresponded with the distribution obtained with a second mode of calculation based on the isolation of CL from DL in melanopsin-disabled animals and their controls, reinforcing the accuracy of our data (Supplementary Table 1, 2). In addition to the finding that DL is responsible for about half of the shaping of the sleep-wake pattern, we find, through experiments with SCN-invalidated animals and controls, that the SCN is responsible for mediating about half of DL. This means that the SCN, in addition to modulating CL, has an auxiliary function in the formation of the sleep-wake pattern. This finding was also confirmed with nearly identical results obtained through two independent approaches: the use of both SCNx and *Syn10*$^{Cre/Cre}$*Bmal*$^{fl/-}$, which control for each other.

If the sleep-wake cycle is represented as in our model (Fig. 3d), two wave-like curves resulting from DL and CL added together, one can imagine that a lack of temporal coordination between the two would result in a decreased overall amplitude of the sleep-wake cycle. This possibly corresponds to disturbances observed in jet-lag or seasonal changes in photoperiod and suggests that our model can predict the amplitude of the sleep-wake cycle in these and other altered lighting conditions. Here we test the predictive power of our model by anticipating and verifying the amplitude of the sleep-wake cycle in the LD1:1 condition. In this condition, the 1-hour light/dark pulses result in acute changes in vigilance state, but as there are 24 pulses, these acute changes based on lighting condition should average to about

zero. Thus, by taking the difference between the average sleep amounts in the subjective light and dark, we get the amplitude of a sleep-wake cycle lacking DL (Supplementary Table 1, 2). According to our model, this amplitude of CL should be about 55% of the amplitude of the sleep-wake cycle under standard conditions in wild-type animals. Our calculations indicate that the amplitude of the sleep-wake cycle in the LD1:1 condition, and thus the amplitude resulting solely from CL, is 50% and 52% of the amplitude observed in the LD12:12 condition of the two wild-type control groups. Remarkably, this is within 3-5 percentage points of the expected value, even though the LD1:1 is an extreme lighting condition far from anything observed in a natural scenario.

There are, however, certain limitations to the model as the shaping of the sleep-wake cycle cannot be as simple as adding two components together. Our model does not take into account the kinetics of the sleep-wake cycle, as DL interacts with the circadian system and varies across the 24-hour day as seen in the LD1:1 condition described above. Moreover, in the LD12:12, we consider DL as the compilation of an acute effect, coming with the change in lighting (acute DL) and a 12-hour sustained effect (sustained DL). This is highlighted in the LD1:1 condition, where there are 12 light/dark pulses, and thus acute DL is overrepresented. Thus, changes in vigilance state observed with each 1-hour light/dark pulse is greater than the 45% of the sleep-wake cycle expected from our model.

Our findings challenge the current understanding of the photic regulation of the natural sleep-wake cycle. Here we reveal that CL determines only about half of this regulation, with DL, half of which actually is relayed by the SCN, being as critical a mechanism. In fact, DL is even capable of compensating for the absence of a clock-driven influence to help maintain a sleep-wake cycle timed with the external light/dark cycle. This is a natural temporal coordination essential for proper behavior and body function, but can also be used to generate a pseudo-clock to treat circadian sleep rhythm disorders. Our model integrating the respective contributions of the main phototransduction pathways regulating the natural sleep-wake cycle and could provide an explanation for the physiological and behavioral disturbances induced by exposure to the aberrant light observed in our modern society as well as predict the shape of the sleep-wake cycle under varying lighting conditions. Concretely, it could be useful for improving our management of circadian sleep rhythm disorders including jet-lag, night work associated disturbances, and seasonal perturbations commonly observed at extreme latitudes and should also be considered in the development of optimal societal lighting.

Methods Summary:

Animals: All experiments were performed on young adult male mice using 1) *Opn4*$^{-/-}$ and wild-type littermate controls[8] and 2) *Syn10*$^{Cre/Cre}$*Bmal1*$^{fl/-}$, animals rendered arrhythmic through a conditional deletion of the clock gene *Bmal1* in the SCN using a Syt10Cre driver[13]. The *Syn10*$^{Cre/Cre}$*Bmal1*$^{fl/-}$ animals were compared to *Syn10*$^{Cre/Cre}$*Bmal1*$^{+/-}$ to control for the "floxed" *Bmal1* allele as well as to WT mice. To control for both the Cre driver and the heterozygosity of *Bmal1*, we verified that *Syn10*$^{Cre/Cre}$*Bmal1*$^{+/-}$ and WT mice displayed similar EEG and sleep-wake patterns. The experiments were supervised by a veterinarian and approved by the ethical committees for animal research at the University of Strasbourg and CNRS.

Surgery: All mice were implanted with a classical set of electrodes for EEG/EMG recordings. Electrolytic lesions of the SCN (SCNx) under stereotactic conditions were also performed in *Opn4*$^{(-/-)}$ and their controls (the electrode was lowered into brain without the application of electrical current in Sham-lesioned animals).

Recordings: General activity was monitored under DD to confirm the loss of circadian rhythmicity in SCNx and *Syn10*$^{Cre/Cre}$*Bmal1*$^{fl/-}$ animals. Sleep and the EEG were analyzed under 3 different lighting schedules LD12:12 cycle, LD1:1 cycle applied for 24-hours and under DD for a subgroup of animals.

Anatomy: To control for SCN lesion size and conservation of retino-cerebral projections, immunohistochemistry was used in SCNx and Sham-lesioned animals to stain AVP, DAPI and CtB which was previously injected into the posterior chamber of the eyes. To evaluate SCN reactivity to light in *Syn10*$^{Cre/Cre}$*Bmal*$^{fl/-}$ and their controls, c-Fos expression was quantified in the SCN with AVP and DAPI co-staining following a 1-hour light pulse administered at ZT15 (no light pulse for controls).

Full Methods and any associated references are available in the online version of the paper.

3. FIGURES

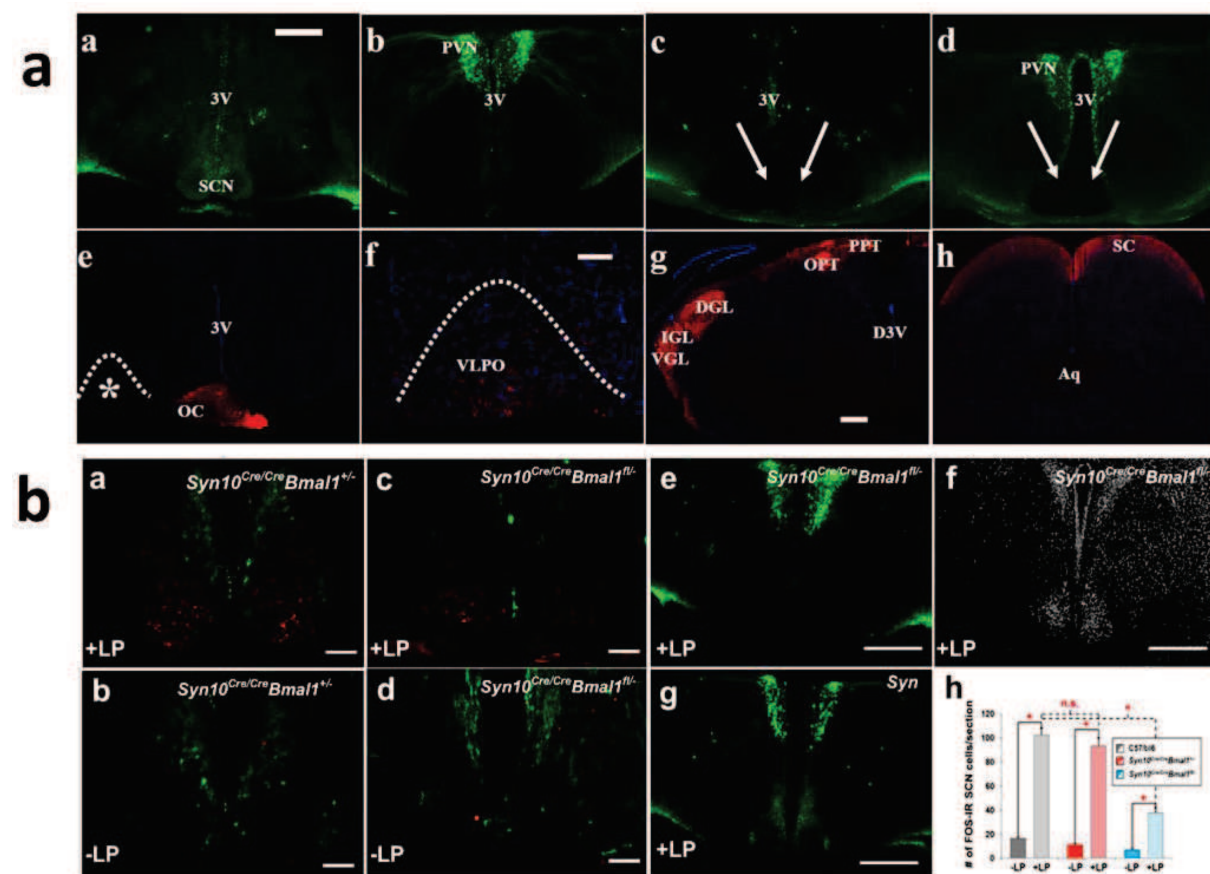

Figure 1:

(a) 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-lesioned mouse (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.

(b) Lack of SCN-mediated photic signaling in Syn10Cre/CreBmal1fl/- mice

(a-d): Effects of a 1-hour light pulse (LP) administered during the dark period (ZT15-16) (a,c) on c-Fos immunoreactivity in the SCN. Similar light-induced c-Fos immnoreactivity (red) in the retino-recipient zone of the SCN in Syn10$^{Cre/Cre}$Bma11$^{+/-}$ (a) and WT (data not shown). The number of Fos-positive SCN neurons is dramatically reduced in Syn10$^{Cre/Cre}$Bmal1$^{fl/-}$ mice (c) and in control condition (no light-pulse, b,d). AVP immunoreactivity (a-e, g, green) in the SCN is preserved in Syn10$^{Cre/Cre}$Bma11$^{+/-}$ (a,b,g, comparable to WT, data not shown), but not in Syn10$^{Cre/Cre}$Bmal1$^{fl/-}$ mice in which AVP staining remains unaltered in adjacent regions (c-e) and whereas the SCN remains structurally intact as shown by DAPI-staining (grey) (f). Scale bars: 100µm in a-d; 500µm in e-g. (h) Number of c-Fos immunoreactive SCN neurons after a 1-hour LP. (Two-way ANOVA; $P_{light\ pulse}$=<0.001; $P_{genotype}$=<0.001, post hoc t-test: *P<0.05). No differences between both controls (*Syn10$^{Cre/Cre}$Bmal1$^{fl/-}$*, WT). Sham Opn4$^{+/+}$ n=5; Sham Opn4$^{-/-}$ n=5; SCNx *Opn4$^{+/+}$* n=5; SCNx *Opn4$^{-/-}$* n=5; Wild-type n=6; *Syn10$^{Cre/Cre}$Bmal1$^{+/-}$* n=8; *Syn10$^{Cre/Cre}$Bmal1$^{fl/-}$* n=6.

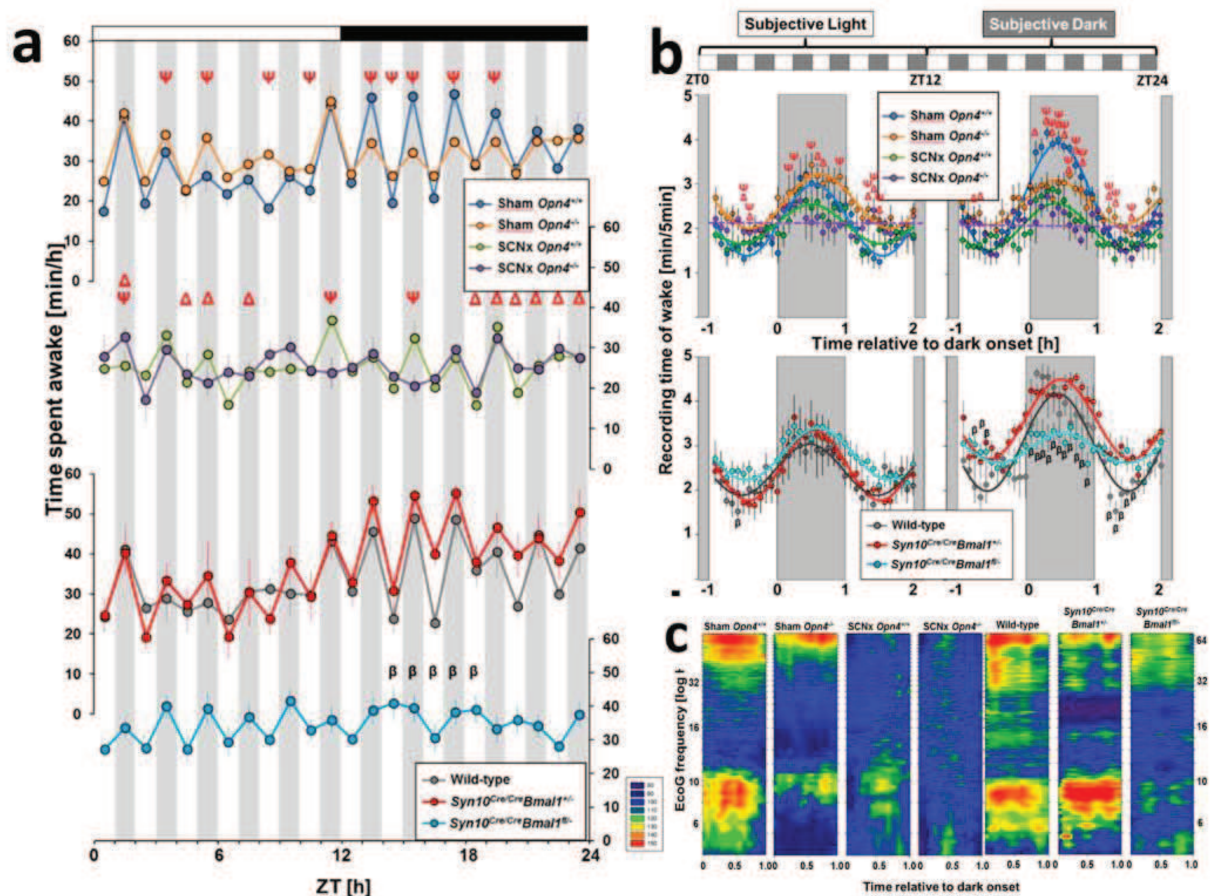

Figure 2: The SCN contributes to the direct photic regulation of sleep and waking signaled by melanopsin- and rod/cone-based photodetection

(**a**) Experimental protocol: LD1:1 pulses repeated over 24-hours. Average time-course of changes in wake (W) during the 12-h subjective light (left) and subjective dark (right) period. Values represent means (±SEM) over 5-min intervals in the hour preceding, during, and following the six 1-h dark pulses. In controls (Sham-lesioned *Opn4*$^{+/+}$, *Syn10*$^{Cre/Cre}$*Bmal*$^{+/-}$ and Wild-type) light pulses promoted N (Supplementary Table 1, 2) whereas dark pulses increased W; an effect that was greater during the subjective dark period (top: four-way ANOVA, $P_{\text{time-course}}$<0.0001, bottom: three-way ANOVA, $P_{\text{time-course}}$=0.003). Reactivity was significantly attenuated in the absence of melanopsin (P_{genotype}=0.003) or of a functional SCN (SCNx *Opn4*$^{+/+}$: $P_{\text{SCN-condition}}$=0.004; *Syn10*$^{Cre/Cre}$*Bmal1*$^{fl/-}$: P_{genotype}=0.004) and abolished in mice lacking both (SCNx *Opn4*$^{-/-}$, $P_{\text{genotype x SCN-condition}}$=0.04). Red *Psi* (top) and *beta* (bottom) symbols indicate significant genotype differences and *delta* (top) SCN-condition differences (*P*< 0.05; post hoc *t*-tests). Corresponding sine waves represent best fits to the data points. For representation of the hourly time course, see Supplemental Fig. 1. (**b**) NREM differences

between the two surrounding light pulses and inclusive dark pulse were quantified and averaged for all 1-hour pulses across the 24-hour period, representing the amplitude of reactivity. The photic regulation of sleep is attenuated by about half in animals lacking melanopsin (two-way ANOVA, $P_{genotype}$=0.004) or a functional SCN (SCNx *Opn4*$^{+/+}$, $P_{SCN\text{-}condition}$=0.009; *Syn10*$^{Cre/Cre}$*Bmal1*$^{fl/-}$, ANOVA, *P*<0.019) and flattened in the absence of both (not significantly different from zero, one-sample signed rank test, *P*=0.48). (post-hoc *t*-tests, *******P*<0.01, ****P*<0.001). Sham *Opn4*$^{+/+}$ n=9; Sham *Opn4*$^{-/-}$ n=7; SCNx *Opn4*$^{+/+}$ n=10; SCNx *Opn4*$^{-/-}$ n=8; Wild-type n=7; *Syn10*$^{Cre/Cre}$*Bmal*$^{+/-}$ n=6; *Syn10*$^{Cre/Cre}$*Bmal1*$^{fl/-}$ n=8.

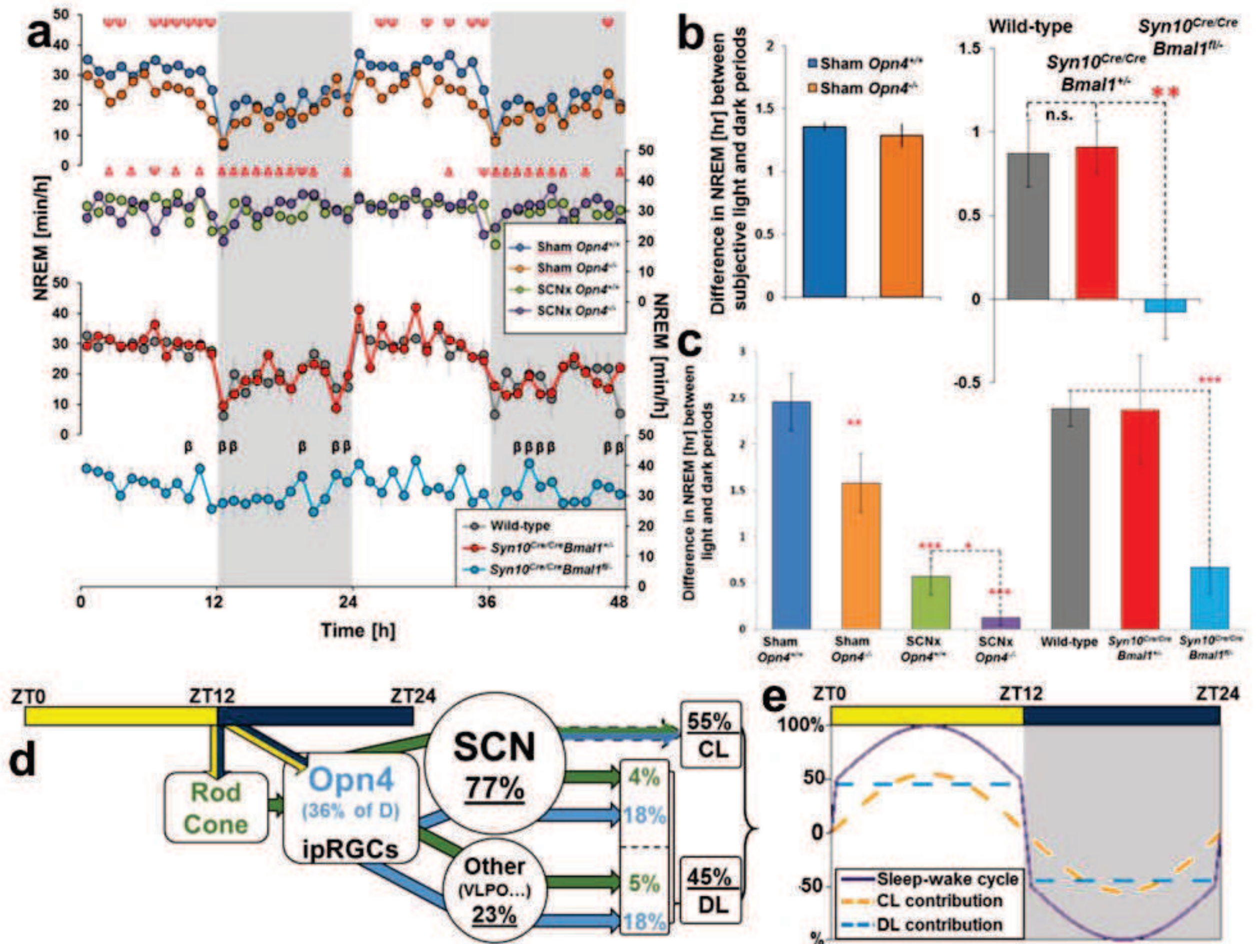

Figure 3: Melanopsin-based direct photic regulation is sufficient to maintain a sleep-wake cycle synchronized with light and dark phases

(**a**) Top: *Opn4*$^{-/-}$ displayed lower NREM during light period (three-way ANOVA, $P_{genotype}$=0.01; $P_{genotype\ x\ time\text{-}course}$=0.01). SCN-lesioned ($P_{SCN\text{-}condition}$<0.001) and *Syn10*$^{Cre/Cre}$*Bmal1*$^{fl/-}$ (two-way ANOVA, $P_{genotype}$=0.007) displayed higher NREM during dark period, resulting in a flattened 24-hour sleep-wake cycle. This cycle is suppressed in absence of both, melanopsin and SCN ($P_{SCN\text{-}condition\ x\ genotype}$=0.004). (**b**) In *Opn4*$^{-/-}$ decreased NREM during the light period results from a loss of melanopsin-dependent direct effects of light and not from weakened circadian drive, as confirmed by DD experiments showing comparable NREM reduction in both genotypes (two-way ANOVA, $P_{light\ condition\ x\ genotype}$=0.005. (**c**) Sleep rhythmicity under 24-hours of DD, expressed as the L-D difference in NREM between subjective light and subjective dark periods, is abolished in *Syn10*$^{Cre/Cre}$*Bmal1*$^{fl/-}$ but not in either control groups ($P_{genotype}$ = 0.005). (**d**) Magnitude of the sleep-wake cycle (expressed as in Fig. 1a) is decreased in absence of melanopsin (two-way ANOVA, $P_{genotype}$<0.001), reduced

in the same proportions in both SCN-disabled models (SCNx *Opn4*$^{+/+}$, $P_{\text{SCN-condition}}$=0.002; *Syn10*$^{Cre/Cre}$*Bmal1*$^{fl/-}$, ANOVA, *P*<0.001) and abolished in absence of both (not significantly different from zero, one-sample signed rank test, *P*=0.22. (**e**) Schema representing the respective contribution of the different pathways by which the daily light-dark cycle shapes the 24-hour temporal organization of sleep and waking. Calculations based upon inter-group differences according to data in d (for detailed calculations see extended materials). The Opn4-mediated direct phototransduction accounts for one third of the sleep-wake cycle, independently of the clock influence. Mean ± SEM. Sham *Opn4*$^{+/+}$ n=9; Sham *Opn4*$^{-/-}$ n=7; SCNx *Opn4*$^{+/+}$ n=10; SCNx *Opn4*$^{-/-}$n=8; Wild-type n=7; *Syn10*$^{Cre/Cre}$*Bmal*$^{+/-}$ n=7; *Syn10*$^{Cre/Cre}$*Bmal1*$^{fl/-}$ n=10. ***P* < 0.01, ****P* < 0.001, post-hoc *t*-tests.

4.

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5. SUPPLEMENTARY DATA

Supplementary Table 1: Time spent asleep and awake under the LD 12:12 and LD 1:1 schedules in SCN lesioned Opn4-/- mice and their controls

Schedule	Time Spent	Condition	*Opn4* Genotype	Waking (h)	NREMS (h)	REMS (min)
12h:12h LD	12-h light period	Sham	+/+	4.06±0.08	6.69±0.13	74.8±3.9
			-/-	5.48±0.48[a]	5.23±0.45[a]	77.8±3.1
		SCNx	+/+	4.51±0.21[b]	6.42±0.17[b]	64.1±2.4[b]
			-/-	4.92±0.14[b]	6.15±0.12[b]	55.7±4.1[b]
	12-h dark period	Sham	+/+	7.41±0.26[c]	3.93±0.23[c]	39.3±3.8[c]
			-/-	7.80±0.29[c]	3.52±0.25[c]	40.9±3.7[c]
		SCNx	+/+	5.23±0.23[b,c]	5.75±0.19[b,c]	54.0±3.1[b,c]
			-/-	4.78±0.28[b]	6.26±0.11[b]	56.1±5.4[b]
	24 h	Sham	+/+	11.47±0.37	10.62±0.39	114.1±7.7
			-/-	13.27±0.70[a]	8.75±0.66[a]	118.7±5.3
		SCNx	+/+	9.74±0.31[b]	12.18±0.23[b]	118.1±4.5
			-/-	9.70±0.28[b]	12.41±0.22[b]	111.8±8.7
1h :1h LD	*Subjective* 12-h light period	Sham	+/+	5.30±0.19	5.73±0.22	59.0±7.6
			-/-	6.25±0.44[a]	4.79±0.33[a]	56.7±9.0
		SCNx	+/+	5.12±0.15	6.03±0.14	50.8±2.6
			-/-	5.10±0.18[b]	5.87±0.17[b]	52.4±2.2
	Subjective 12-h dark period	Sham	-/-	6.79±0.23[c]	4.50±0.20[c]	39.6±7.6[c]
			+/+	6.31±0.31	5.06±0.43	37.7±8.6[c]
		SCNx	-/-	5.06±0.16[b]	6.11±0.15[b]	50.0±1.5
			+/+	5.13±0.22[b]	6.03±0.23[b]	50.1±2.6
	24 h	Sham	+/+	12.08±0.31	10.23±0.32	98.6±14.8
			-/-	12.56±0.65	9.86±0.49[a]	94.4±17.4
		SCNx	+/+	10.18±0.25[b]	12.14±0.25	100.8±3.2
			-/-	10.23±0.31[b]	11.91±0.25	102.6±3.3
	12 1-h Light periods	Sham	+/+	4.54±0.16	6.41±0.18	62.3±8.8
			-/-	5.49±0.38[a]	5.55±0.38[a]	57.4±12.2
		SCNx	+/+	4.36±0.22[c]	6.73±0.22[c]	55.2±2.7
			-/-	4.83±0.18	6.29±0.16	53.0±4.7
	12 1-h Dark periods	Sham	+/+	7.54±0.24[c]	3.82±0.22[c]	38.6±5.6[c]
			-/-	7.07±0.20[c]	4.31±0.19[c]	37.0±12.1[c]
		SCNx	+/+	5.82±0.26[b,c]	5.42±0.24[b,c]	45.6±2.7
			-/-	5.41±0.36[b]	5.78±0.33[b]	48.6±2.7

12h:12h LD - *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

24hr 2-way ANOVA genotype x SCN: genotype W p=0.04, N p=0.04; SCN p<0.0001

1h:1h LD - *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

24hr 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±SEM

Supplementary Table 2: Time spent asleep and awake under the LD 12:12 and LD 1:1 schedules in *Syn10*$^{Cre/Cre}$*Bmal1*$^{fl/-}$ and their controls

Schedule	Time Spent	Condition	*Bmal1* Genotype	Waking (h)	NREMS (h)	REMS (min)
12h:12h LD	12-h light period	Wild-type	+/+	5.30±0.14	5.94±0.08	45.9±7.0
			+/-	5.11±0.62	6.12±0.60	45.6±9.3
		SCN-disabled	fl/-	4.38±0.37	6.83±0.33	47.5±3.8
	12-h dark period	Wild-type	+/+	8.18±0.28	3.55±0.24	14.4±2.8
			+/-	8.05±0.19	3.75±0.22	12.3±3.7
		SCN-disabled	fl/-	5.42±0.30[a]	6.15±0.28[a]	21.3±3.0[a]
	24 h	Wild-type	+/+	13.48±0.39	9.63±0.30	63.5±9.3
			+/-	13.16±0.65	9.87±0.99	58.0±10.7
		SCN-disabled	fl/-	9.81±0.59[a]	12.98±0.54[a]	68.8±4.3
1h:1h LD	*Subjective* 12-h light period	Wild-type	+/+	5.90±0.48	5.45±0.47	38.7±6.1
			+/-	6.08±0.64	5.17±0.55	45.0±6.3
		SCN-disabled	fl/-	6.70±0.18[a]	4.30±0.17[a]	49.2±8.4
	Subjective 12-h dark period	Wild-type	+/+	7.44±0.42	4.20±0.41	20.9±3.3
			+/-	8.73±0.23	2.92±0.20	20.9±3.5
		SCN-disabled	fl/-	7.11±0.23[a]	4.30±0.19[a]	35.8±4.2[a]
	24 h	Wild-type	+/+	13.3±0.88	9.66±0.83	59.6±9.1
			+/-	14.8±0.62	8.09±0.57	65.9±8.6
		SCN-disabled	fl/-	13.8±0.35	8.78±0.31	85.0±11.6
	12 1-h Light periods	Wild-type	+/+	5.11±0.25	6.28±0.21	36.6±4.6
			+/-	6.06±0.41	5.25±0.39	41.2±4.3
		SCN-disabled	fl/-	6.32±0.32[a]	4.88±0.28[a]	48.2±6.1
	12 1-h Dark periods	Wild-type	+/+	8.23±0.67	3.38±0.65	22.9±5.2
			+/-	8.76±0.35	2.84±0.29	24.6±5.0
		SCN-disabled	fl/-	7.49±0.26	3.90±0.19	36.8±6.0

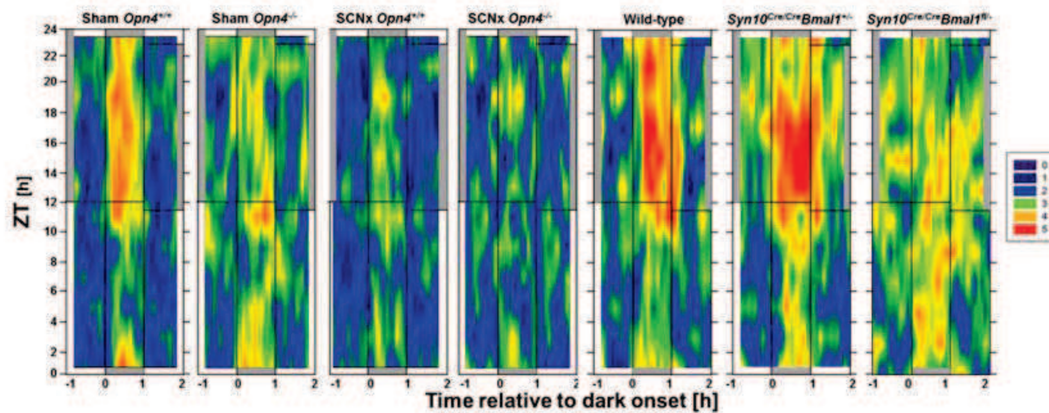
12h:12h LD- Light vs Dark Repeated measures ANOVA between Genotype x Light/Dark: Genotype x LIGHT W P=0.007, N P=0.007; Genotype W P<0.0001, N P=0.007; LIGHT W P=0.005, N P<0.001, R P<0.001

24hr One-way ANOVA genotype: genotype W P=0.005, N P=0.007,

1h:1h LD- ***Light vs Dark*** 3-way ANOVA genotype x TIME: genotype x TIME W P=0.021, N P =0.014; TIME W P <0.0001, N P <0.0001, R P <0.0001

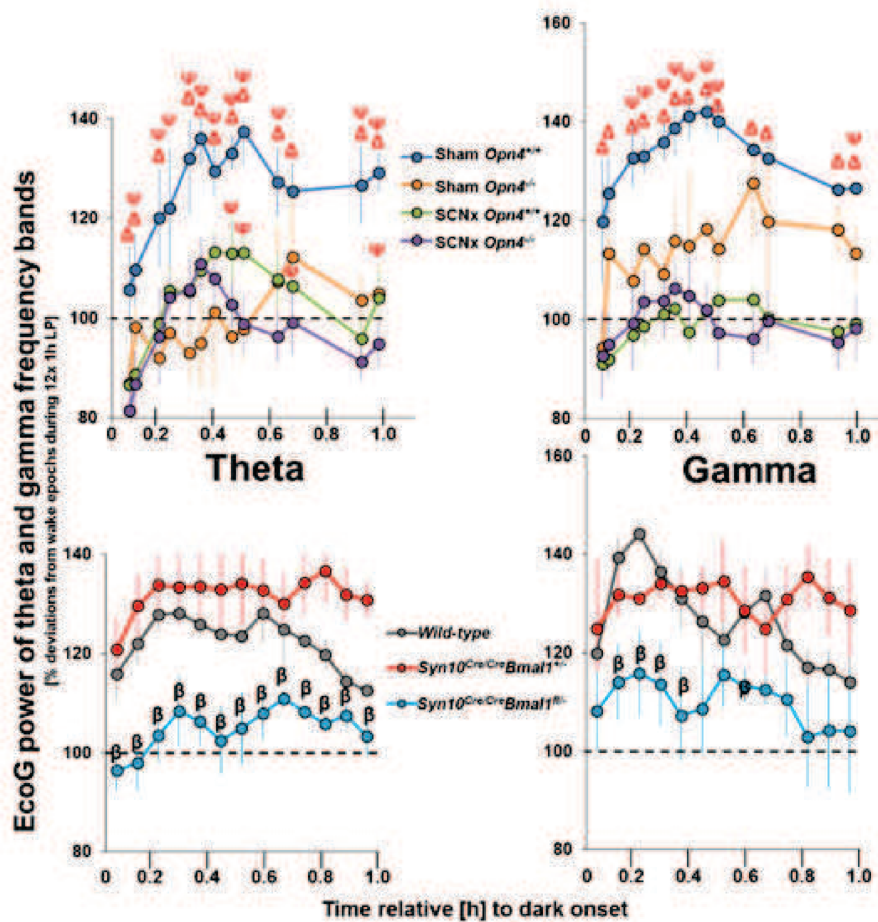
12x L vs D pulses 2-way ANOVA genotype x TIME: genotype x TIME W P =0.03, N P =0.02; TIME W P <0.0001, N P <0.0001, R P =0.003

(**a**) Indicates significant genotype 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±SEM



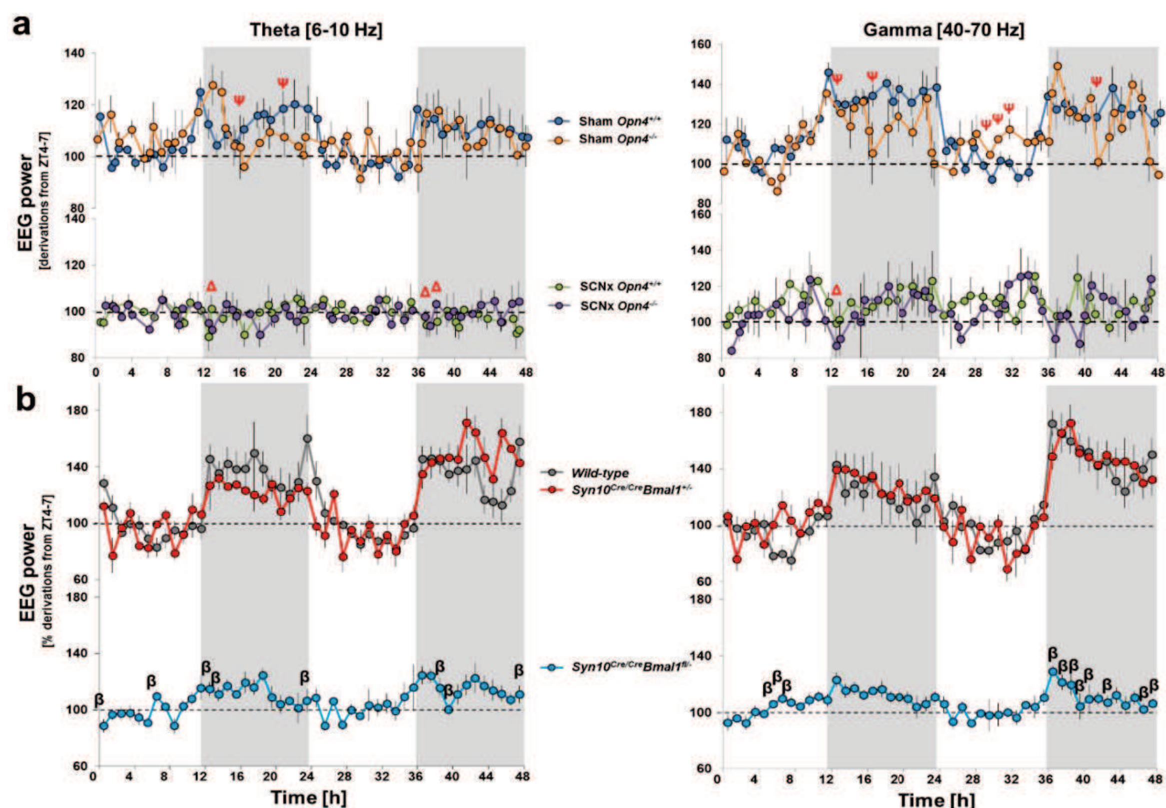
Supplementary Figure 1: Time-course of wake during LD1:1

Waking values expressed as minutes/5 minute bout. In the absence of melanopsin, sham 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. *Syn10*$^{Cre/Cre}$*Bmal1*$^{fl/-}$ show strong arrhythmicity which is not seen in their controls (wild-type and *Syn10*$^{Cre/Cre}$*Bmal1*$^{+/-}$). x-axis numbers denote the time before and after the dark pulse (centered).



Supplementary Figure 2: The alerting effect of darkness is primarily signaled by the SCN

Quantification of theta (7–10 Hz) and gamma (40–70 Hz) power spectra, in the waking ECoG during all averaged 12 1-hour dark pulses across the 24-hours of the LD1:1. Note the significant increase in theta and gamma power spectra in all control groups. This dark-induced activation is delayed and attenuated in *Opn4*$^{-/-}$ and abolished in the absence of SCN. (Top) 3-way ANOVA: Theta: $P_{\text{SCN-condition}}$=0.003, $P_{\text{time-course}}$ =0.0005; Gamma: $P_{\text{SCN-condition}}$<0.001, $P_{\text{time-course}}$<0.001). (bottom) In *Syn10*$^{Cre/Cre}$*Bmal1*$^{fl/-}$ and their controls, 2-way repeated measures ANOVA: Theta: $P_{\text{time-course}}$=0.021, P_{genotype}=0.006; Gamma: $P_{\text{time-course x genotype}}$=0.044, $P_{\text{time-course}}$ <0.001, P_{genotype} =0.007). Red *psi* (top) and *beta* (bottom) symbols indicate significant genotype differences and *delta* SCN-condition differences (*P*<0.05; post-hoc *t*-tests). Dark-associated theta/gamma activities are also suppressed under LD12:12 (see Supplementary Fig. 3) Sham *Opn4*$^{+/+}$ n=5; Sham *Opn4*$^{-/-}$ n=6; SCNx *Opn4*$^{+/+}$ n=5; SCNx *Opn4*$^{-/-}$ n=5; Wild-type n=5; *Syn10*$^{Cre/Cre}$*Bmal1*$^{+/-}$ n=5; *Syn10*$^{Cre/Cre}$*Bmal1*$^{fl/-}$ n=6.



Supplementary Figure 3: Theta and Gamma per hour of wake under LD12:12 in SCN-lesioned *Opn4*$^{-/-}$, *Syn10*$^{Cre/Cre}$*Bmal1*$^{fl/-}$ mice and their controls

(**a**) 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 and gamma (across the 48-hours of LD12:12. 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*) (**b**) *Syn10*$^{Cre/Cre}$*Bmal1*$^{fl/-}$ mice and their controls. One way ANOVA between controls and *Syn10*$^{Cre/Cre}$*Bmal1*$^{fl/-}$ were significant ($P < 0.05$) of changes in theta (**a**) and gamma (**b**) during certain hours across 48-hours of 12h:12h LD. Post-hoc significance is represented by beta symbols. For day 1 and 2 (*wild-type n=6; Syn10*$^{Cre/Cre}$*Bmal1*$^{+/-}$ *n=5; Syn10*$^{Cre/Cre}$*Bmal1*$^{fl/-}$ *n=8*).

6. EXTENDED MATERIALS & METHODS

Animals

All experiments were performed on adult male *Opn4*$^{-/-}$ mice and wild-type littermates (as controls), and 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[1]. After completion of the protocol, the anterograde tracer cholera toxin subunitB (CtB) was injected under anesthesia into the posterior chamber of the eye. After sacrifice and perfusion of the animals three days later, 4',6-diamidino-2-phenylindole (DAPI) and immunostaining to arginine vasopressin (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 (LD12:12; 23 ± 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.[2].

A complimentary group of transgenic mice were used to verify the effects in the 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*[3]. 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 regarding the suprachiasmatic nucleus, before electrode implantation as previously described[4]. 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;[5]). 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 ketamine 80mg/kg and xylazine 7mg/kg. Male *Opn4*$^{-/-}$ (n=16) , *Opn4*$^{+/+}$ (n=19), *Syn10*$^{Cre/Cre}$*Bmal1*$^{fl/-}$ (n=10), *Syn10*$^{Cre/Cre}$*Bmal1*$^{+/-}$ (n=7), and *wild-type* (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 LD12:12 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 LD1:1 cycle for 24 hours, (2) 24-hour cycle of constant darkness (DD) under red light. All experiments occurred on different days from one another and a minimum of 14 days under LD12:12 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 LD1:1 schedule were determined using sine-waves, fitted to the individual 5-min values for wakefulness obtained in the 1-hour before, during, and after the 1-hour dark periods. For each mouse, 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 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-second 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 Model Figure 3d

Under a standard LD12:12 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 LD12:12 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 as 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 2, (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), time course was significantly affected by SCN condition (W: P=0.004) and genotype (W: P=0.003) (interactions: genotype x SCN condition: W: P=0.04; genotype x time course: W: P=0.0005; SCN condition x time course: W: P<0.0001). Red *Psi* denote significant genotype differences, red *Delta* 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.

For figure 3, (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 *Syn10*$^{Cre/Cre}$*Bmal1*$^{fl/-}$ 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. (e) . 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 wild-type ; 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[6]. 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].

Supplementary References

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Deuxième Partie : Introduction

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. Le premier axe de cette thèse s'est intéressé aux structures neuronales impliquées chez la souris dans les effets hypnotiques observés suite à une exposition lumineuse. A l'inverse, l'application d'un créneau d'obscurité améliore la qualité de l'éveil du rongeur nocturne. Pourtant, les effets circadiens et non circadiens de la lumière ainsi que l'organisation circadienne de la veille et du sommeil de ces animaux sont inversés par rapport au rythme veille/sommeil et aux effets de la lumière et de l'obscurité chez l'homme.

Les mécanismes neuronaux sous-tendant ces effets inverses de la lumière et de l'obscurité observés chez les espèces nocturnes comparées aux diurnes, restent incompris. Dans une approche de recherche translationnelle de l'animal à l'homme, il devient primordial, notamment pour l'étude des effets directs de la lumière sur le comportement (régulation du sommeil, qualité de l'éveil, humeur…) de mieux comprendre les mécanismes impliqués dans ce switch des effets directs de la lumière et de l'obscurité entre espèces diurnes et nocturnes.

Le sommeil des rongeurs diurnes n'avait jamais été spécifiquement étudié, contrairement au rythme circadien de leur activité locomotrice. L'*Arvicanthis ansorgei* est un modèle animal diurne bien établi, comme le montrent les nombreuses études chronobiologiques. Ce muridé a été introduit au sein de notre laboratoire (Chronobiotron - Institut des Neurosciences Cellulaires et Intégratives) en mars 1998 par le Dr Paul PEVET à partir de 10 animaux mâles et 15 femelles, capturés au sud du Mali (Samaya: 12° 33' 14.8'' N/8° 03' 1.3'' W). Les animaux reproducteurs pour le maintien de la colonie sont systématiquement enregistrés par actimétrie de roue afin de ne sélectionner que des animaux ayant un chronotype clairement diurne. Néanmoins, il conviendrait encore de montrer que ce rongeur diurne constitue un bon modèle dans cette approche translationnelle.

Ainsi, ce deuxième axe qui s'intéresse à la caractérisation et aux mécanismes de régulation du sommeil chez ce rongeur diurne, constitue une première étape dans

l'approche translationnelle. Cette étape est préliminaire et indispensable aux étapes suivantes.

A l'heure actuelle, il reste hypothétique à savoir si les effets inverses de la lumière sur le sommeil observés chez le nocturne comparé au diurne sont sous l'influence d'un mécanisme unique de switch de la diurnalité/nocturnalité ou si chaque comportement est sous le contrôle d'un mécanisme différent. L'extrapolation des données issues de la recherche chez le rongeur nocturne à l'espèce diurne demande une meilleure compréhension des substrats neuronaux qui sous-tendent la diurnalité/nocturnalité. Ceci fait l'objet d'une recherche actuellement en cours.

Les expériences en lien avec la publication suivante dans laquelle je suis premier co-auteur avec le Dr Jeffrey Hubbard *(Ruppert, Hubbard, Calvel, Robin-Choteau, Gropp, Allemann, Sage-Ciocca, Reibel, Bourgin: Arvicanthis ansorgei, a novel model for the study of sleep and waking in diurnal rodents. Accepté, Sleep)* ont été réalisées majoritairement en 2011. L'analyse des données et l'interprétation des résultats, ainsi que l'écriture de l'article ont été plus longs et ont été poursuivis tout au long de ma thèse.

Article 3: ***«Arvicanthis ansorgei, a novel model for the study of sleep and waking in diurnal rodents»***

Sleep

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1. Title page. ***Original paper*** **SLEEP -**

Title: ***Arvicanthis ansorgei*****, a novel model for the study of sleep and waking in diurnal rodents**

Short title: Sleep EEG characterization of diurnal *Arvicanthis ansorgei*

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ABSTRACT

Study Objectives: Sleep neurobiology studies use nocturnal species, mainly rats and mice. However, as their daily sleep/wake organization is inverted as compared to humans, a diurnal model for sleep studies is needed. To fill this gap, we phenotyped sleep and waking in *Arvicanthis ansorgei,* a diurnal rodent widely used for the study of circadian rhythms.

Design: video-Electroencephalogram (EEG), EMG and EOG recordings.

Setting: Rodent sleep laboratory

Participants: 14 male *Arvicanthis ansorgei*, aged 3 months

Interventions: 12hL:12hD baseline condition, 24-hour constant darkness, 6-hour sleep deprivation

Measurements and Results: Wake, NREM, and REM showed similar electrophysiological characteristics as nocturnal rodents. On average, animals spent 12.9h±0.4 awake per 24-hour cycle, of which 6.88±0.3 was during the light period. NREM sleep accounted for 9.63h±0.4, which of 5.13h±0.2 during dark period, and REM sleep for 89.9min±6.7, which of 52.8±4.4 during dark period. The time-course of sleep and waking across the 12hL:12hD was overall inverted to that observed in rats or mice, though with larger amounts of crepuscular activity at light and dark transitions. A dominant crepuscular regulation of sleep and waking persisted under constant darkness, showing the lack of strong circadian drive in the absence of clock reinforcement by external cues such as a running wheel. Conservation of the homeostatic regulation was confirmed with the observation of higher delta power following sustained waking periods and a 6-hour sleep deprivation, with subsequent decrease during recovery sleep.

Conclusions: *Arvicanthis ansorgei* is a valid diurnal rodent model for studying the regulatory mechanisms of sleep and so represents a valuable tool for further understanding of the nocturnality/diurnality switch.

Keywords: *Arvicanthis ansorgei*, diurnality, sleep regulation, sleep deprivation, sleep homeostasis, circadian rhythm, direct effects of light, crepuscular, rodent, nocturnality

INTRODUCTION

The majority of laboratory sleep research today focuses on nocturnal rodents, primarily mice due to the transgenic tools available, including optogenetics. Drosophila and zebrafish, two other powerful genetic models, have been used for sleep studies, yet restrictions such as the lower complexity of neuronal networks limit their interest for research on mammalian physiology and behavior. To date, sleep has been characterized in a large range of species. Historically, sleep research was conducted on disparate animals such as cats or even rabbits, though now is all but abandoned (experiments and colonies expensive to maintain, lack of genetic and biological tools). Though mice and rat research have provided major insights into sleep neurobiology, these animals are nocturnal, underlying the need for a diurnal rodent model.

To our knowledge, a chipmunk *Eutamias sibiricus* and a ground squirrel *Citellus spp*, are the only truly diurnal rodents in which sleep has been studied using EEG recordings.[1,2] Sleep and wake distribution across the 24-hour day of the chipmunk showed that they sleep for about one quarter of the 12-hour light period and three quarters of the 12-hour dark period.[1]Additionally, a significant increase in the level of EEG delta power, a marker of sleep need, was seen following a longer 24-hour sleep deprivation.[1] Though these initial results were promising, further experimentation was stopped, probably due to the isolated nature of this research, conducted on a poorly characterized model, as well as the lack of laboratory colonies leading to difficulties in performing additional studies. Sleep regulation has also been studied in *Octodon degus,* a dual-phasing rodent showing crepuscular timed episodes of sustained waking evocative of a bimodal crepuscular modulation of arousal.[3,4] Furthermore, selective REM sleep deprivation resulted in consistent REM sleep rebound only after nocturnal deprivation, which suggested a unimodal promotion of nocturnal REM sleep. Inevitably, the *Octodon degus* exhibits no strong preference for sleep during the light or dark phase, and thus further sleep studies were not conducted in this species.

In the field of chronobiology, the diurnal muridae of the Arvicanthis family (*A. ansorgei* and *A. niloticus*) have proven to be useful models. Originally, more than one hundred *Arvicanthis ansorgei* were screened for daily patterns of wheel-running activity in our laboratory.[5] The colony was then extended and maintained for fifteen years, with recurrent import of new animals to reinforce the genetic diversity of the species. Most of the animals expressed a clear diurnal pattern of locomotor activity.[6,7] Subsequent studies analyzed the circadian expression of clock genes (*Per1, Per2, Cry2, Bmal1*)[8] and clock-related

neuropeptides (AVP, VIP and GRP)[9] in the suprachiasmatic nucleus (SCN) where the primary clock is located. The circadian rhythmic expression of these clock genes and neuropeptides was roughly similar to what was observed in nocturnal rodents.[8,9] Melatonin and its rate-limiting enzyme, arylalkylamine N-acetyltransferase (AA-NAT) are expressed in *Arvicanthis ansorgei* during the night or dark phase, similarly to the distribution observed in human.[10,11] This underlies, not only a parallel to humans for translational research, but also the possibility to study the melatoninergic regulation of sleep, whereas most laboratory mice strains used in research do not express melatonin (i.e. C57BL6/J, 129/Sv).[12] Moreover, the phase response curve to light pulses in *Arvicanthis ansorgei* is comparable to both nocturnal and other diurnal species,[8] whereas the circadian responses to dark pulses differ from those of nocturnal rodents.[13] Resetting of the circadian clock through the use of hypocaloric feeding in *Arvicanthis ansorgei* results in phase delays of the SCN pacemaker, contrary to nocturnal rodents.[14] Additionally, the proportion of the retinal photoreceptors, rods, cones and melanopsin, in the retina of the *Arvicanthis ansorgei*, and subsequently their phototransduction systems which mediate the non-visual effects of light, are closer to what is observed in humans.[15,16] These data taken together have confirmed the relevance of *Arvicanthis ansorgei* as a diurnal model, yet it remains essential to phenotype sleep and to characterize sleep regulatory mechanisms in this species.

In the current study we recorded sleep for the first time in *Arvicanthis ansorgei* and sought to address whether this species is a valid diurnal model for studying the regulatory mechanisms of sleep and waking.

METHODS

Animals, Housing Conditions, and Sleep Deprivation

Arvicanthis ansorgei were obtained from our animal facility- the Chronobiotron CNRS UMS 3415, housed at the Institute for Cellular and Integrative Neurosciences, France. Animals were raised under environmentally stable conditions [12-hour:12-hour light-dark schedule (12hL:12hD); 150 lux; 23 ± 0.5° C; food and water ad libitum] and were maintained according to the European Union guidelines for laboratory animal experimentation. LD and DL-transitions switched instantly, thus not mimicking natural twilight. Sleep deprivation was performed by gentle handling as described previously.[17] All experimental sleep protocols were supervised by a veterinarian and approved by the ethical committees for animal research at the University of Strasbourg and CNRS.

Wheel-running activity monitoring

Prior to electrode implantation, running-wheel actimetry (DataportDP24) was performed under standard 12hL:12hD conditions using appropriate software (VitalView, Minimitter). Analysis and production of the actograms were performed using Clocklab (Actimetrics) following data transformation via Matlab. 14 male *Arvicanthis ansorgei* were included in the study, all of which showed a clearly diurnal profile of wheel running activity (Figure 1A).

Surgery and experiments

Surgery protocol for electrodes implantation was performed according to methods previously described[17] with minor changes to adapt to the *Arvicanthis* species. The electrodes were implanted under deep anesthesia (intraperitoneal injection of ketamine 80mg/kg and xylazine 7mg/kg), then soldered to a connector and cemented to the skull, before the skin was sutured. Animals were implanted with two EEG on the dura using the same coordinates as in the rat (frontal: 2.0 mm lateral to midline, 2.0 mm anterior to bregma; parietal 2.0 mm lateral to midline, 2.0 mm anterior to lambda),[18] one reference electrode, two EMG inserted into the neck muscles along the back of the skull and, in a subset of rodents (n=4), two EOG electrodes placed next to the orbital socket to record eye movements observed during waking and especially during REM sleep. Video was also recorded in these animals to verify the behavioral state in conjunction with EEG. Given the increased activity of *Arvicanthis,* compared to laboratory mice or rats, we designed a specific cable in order to prevent decoupling of the electrode chip as well as we reinforced it with INOX metal to prevent destruction by the animal. The cable material, however, was flexible and relatively light and the increased weight of this system was offset by the size of the *Arvicanthis ansorgei* which averaged 150g at time of implantation. All experiments were performed using male *Arvicanthis ansorgei,* aged approximately 3 months at time of implantation. A minimum of 14 days was given to recover from surgery and to habituate to the baseline conditions before any protocol began. Signals were recorded for analysis using commercially available hardware and software (Micromed France, SystemPLUS Evolution version 1092). Sleep recordings were performed under several experimental conditions: (1) baseline 12hL:12hD cycle, (2) 24-hour of constant darkness (24h D:D), (3) 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 conditions. 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, NREM sleep, or REM sleep, using visual inspection without knowledge of the recording condition. The scoring was performed according to criteria similar to those classically used for other rodents: The EEG during REM was characterized by a regular, low-amplitude theta (6-10 Hz) rhythm and a low EMG. During NREM, the EEG amplitude was larger and dominated by both delta (0.75–4 Hz) and theta-frequency components and the EMG was low. Wakefulness was characterized by a higher and variable EMG and a low-amplitude EEG with both slower (delta during drowsiness) and faster (theta during exploratory behavior) components. Furthermore, 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 infrared video recording (n=4). Video recording was made using a commercially available night-vision camera (Sony HDR-CX550VE) mounted on a tripod to confirm concordance between behavior and EEG scoring (wake, cessation of EMG activity during NREM, cessation of EMG activity coupled with EOG measured rapid-eye movements during REM sleep). The EEG signal was subjected to a rectangular Discrete-Fourier Transform (DFT) with a 50% overlap, yielding power spectra between 0 and 128Hz (0.25Hz resolution) using a 4-second window and a 50Hz filter to remove power-line artifacts. All four-second epochs containing other signal artifacts were marked so they could be excluded from EEG spectral analyses. Amounts spent in each vigilance state were calculated by averaging time spent either in 5min, 1-, 12-, and 24-hour intervals. Moreover, an average spectral profile was constructed using all 4-s epochs scored with the same vigilance state. The absolute spectral power of the delta band frequencies were calculated (0.75-4 Hz) and normalized to the period during baseline (12hL:12hD) with the lowest average value corresponding to the last four hours of darkness (ZT20-24). Previously, this type of analysis has been performed in mice and rats, however their lowest delta power is seen at the end of the light period (ZT8-12), consistent with their inherent nocturnality. Only continuous NREM periods of greater than 1 minute were included in the analysis. To sleep deprive the animals, a starting time was chosen consistent with the animal's chronobiological diurnality (ZT12-18), opposite to previous experiments performed in nocturnal laboratory rodentss.[17] Delta power analysis following this 6-hour NREM sleep deprivation was performed in the same manner. 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).

Statistical analysis

Statistical analysis was realized using Statistica (Statsoft v. 8) with graphic representations created either in SigmaPlot (Systat, v. 11) or Microsoft Excel (v. 2010). To measure time-dependent changes in vigilance states amounts or bout lengths and power spectra of EEG, either one-way or repeated measures ANOVA were used. Specific tests are listed in each figure legend. To determine post-hoc significance Protected Least Significant Difference (PLSD) were performed unless otherwise specified.

RESULTS

In chronobiology, locomotor activity is the most widely used method for identifying the daily rhythm of the biological clock in order to determine whether the studied animals are diurnal or nocturnal, however the characterization of their sleep-wake regulation was missing. In the present study all activity recordings confirmed a diurnal profile using wheel running actimetry (Figure 1A, 1B).

Polysomnographic Characterization of Sleep and Waking in *Arvicanthis Ansorgei*

Initial scoring was verified in four animals using EEG /EMG/EOG in conjunction with video recording (Supplemental Video 1-3; for details, see methods above). Epochs scored as wake (W) showed highly desynchronized EEG and high muscle activity on the EMG, as well as non-rhythmic eye movements on the EOG (Figure 2A, Supplementary Video 1). The EEG spectral profile showed higher power within the theta (6-10 Hz), and delta range (0.75-4 Hz), this latter being also observed in nocturnal rodents during wake (Figure 2B). NREM sleep (N) was identified according to highly synchronized slow oscillating delta waves and lower activity of higher frequency bands, associated with low muscle tone, consistent with a resting state. Additionally no eye movements were observed (Figure 2A). The EEG Power spectrum was dominated by delta frequencies (0.75-4 Hz), characteristic EEG rhythm of NREM sleep (Figure 2B, Supplementary Video 2). REM or rapid-eye-movement sleep was characterized by highly desynchronized EEG activity, coupled with complete muscle atonia, and rapid eye movements every several seconds (Figure 2C,-Supplementary Video 3). Note the presence of high peaks of theta-waves in the absence of delta associated to muscle atonia and an EEG characteristics of this state (Figure 2C). These vigilance states were additionally verified using

video monitoring of a subset of animals (n=4). During wake states animals were visibly active and commonly engaged in food burying. This activity increased during light-to-dark and dark-to-light transitions (Supplementary Video 1). Under NREM sleep animals remained immobile and in a similar position to that classically observed in laboratory rodents (Supplementary Video 2). Finally when animals were engaged in REM sleep the video confirmed the lack of movements and muscular tone (Supplementary Video 3).

Distribution of Sleep and Wakefulness during the 12L:12D Baseline

Analysis of two consecutive days under a standard 12hL:12hD cycle showed that wake accounted on average for 58% (7.01h ± 0.3) during the light period compared to 50% (5.99h ± 0.3) in the dark phase (Figure 4A, 4C, Supplemental Table 1). There was a significant difference of time spent in NREM [37% (4.42h ± 0.3) vs. 43% (5.13h ± 0.2)] or REM [4.7% (34.8min ± 4.5) vs. 7.3% (52.8min ± 4.4)] between the light and dark periods. Baseline day 1 and 2 were not significantly different (Supplemental Table 1). Across the 24-hour period, wake and NREM sleep are relatively mirrored to one another (Figure 4A). Moreover, the length of wake and sleep bouts was not different between light and dark phases (Figure 3C). Finally, under a standard 12hL:12hD condition the 24-hour sleep and wake distribution, represented as illustrated by a hypnogram was overall inverted to that observed in rats or mice (Figure 3A). This is further confirmed by the 24hrs distribution of NREM sleep expressed per 5 minutes bouts (Figure 3B).

Circadian, Direct Photic, Crepuscular and Homeostatic Components Regulate Sleep and Waking

Circadian and direct photic non-circadian regulation of sleep and waking in Arvicanthis ansorgei

We analyzed sleep under a 24-hour constant darkness condition to determine whether the 24-hour distribution of sleep and waking observed under a standard 12hL:12hD cycle was regulated by a clock-driven mechanism and/or due to the direct influence of light on sleep.[17,20] The results confirm that both sleep regulatory mechanisms play a role. Under constant darkness, sleep and waking display an overall daily distribution relatively similar to that of baseline 12hL:12hD confirming the clock-driven circadian regulation of the sleep-wake cycle (Figure 4A, 4B). The difference in sleep amounts was quantified between the light and dark phase under 12hL:12hD, which represents the amplitude of the daily sleep-wake cycle. When comparing this light/dark difference to the difference in sleep amounts between the

corresponding subjective periods under 24hD:D (ZT 0-12, subjective light period and ZT12-24, subjective dark period), we observed that the amplitude of the sleep-wake cycle is attenuated in constant darkness, due to the lack of wake promotion by light (Figure 4C). Indeed, in constant darkness we observed during the subjective light period, higher amounts of shorter wake bouts as compared to the standard 12hL:12hD condition, suggesting a light-dependent consolidation of wakefulness (Figure 3C). The longest wake bout lasted on average 57.1 (±8.5) minutes during the 12hL:12hD condition, whereas it was of 27.2 (±2.7) minutes during 24-h darkness (One-way ANOVA Light condition: $p=0.007$). Conversely, during the subjective dark period, we observed under constant darkness lower amounts of shorter NREM sleep bouts as compared to the standard 12hL:12hD condition. Finally, the frequency distribution of REM sleep bouts did not show any particular differences between the 12hL:12hD and 24hD:D conditions.

When the average length of sleep bouts are calculated a significant difference is seen for wake bouts under 12hL:12hD condition (light: 9.11± 0.65 min vs. dark: 7.24± 0.44 min) (Supplemental Table 2). Under constant darkness there is a significant difference in the duration of episodes between subjective light and dark periods for both wake (sub. light: 5.04± 0.18 min, sub. dark: 7.84 min± 0.89) and NREM (sub. light: 2.28± 0.18 min, sub. dark: 3.25± 0.15 min) bout lengths. Interestingly, there is a significant increase in average wake bout length during the light period under 12hL:12hD (9.11 ± 0.65 min) vs. subjective light under 24h D:D (5.04 ± 0.18 min), whereas no significant change is found during dark and subjective dark periods (Supplemental Table 2). Average NREM bout length is increased during the subjective dark period under 24h D:D (3.25 ± 0.15 min), as compared to the dark period under 12hL:12hD (2.72 ± 0.14 min) (Figure 3B, Supplemental Table 2).

Crepuscular regulation of sleep and waking

Abrupt changes in wake and sleep are observed in Arvicanthis at L-D-L transitions with two sustained periods of wake at light and dark onsets, referred to as crepuscular (Figure 4A, Figure 5A, 5B).[4] This bimodal profile persists under constant darkness (Figure 4B, 4C, Figure 5A, 5B**)** with a maximum of waking amounts two hours immediately before and following the light or dark onset (Figure 5B). Additionally, EEG power spectrum analysis at L-D-L transitions showed highly increased EEG theta and gamma activities (Figure 5C), EEG correlates of cognition and exploratory behavior in rodents.[21,22] Thus, this bimodal expression of waking corresponds to a cyclic pattern with a period of 12 hours. Therefore, we then applied sine-wave calculations to the results obtained during baseline under 12hL:12hD and

24-hour constant darkness. The time course of each vigilance state fits a sine wave curve of 12 hours periodicity without significant differences between both lighting conditions (Figure 5A). These data suggest that *Arvicanthis ansorgei* has a 12-hour periodic expression of sleep and waking, resulting from a prevailing crepuscular regulatory mechanism.

Process S is conserved in Arvicanthis ansorgei

EEG delta activity is the most reliable marker of the buildup of homeostatic sleep need with time spent awake. Sleep deprivation represents the best approach to challenge the sleep homeostat.[23,24] Analysis of baseline days revealed an increase in delta power following activity periods corresponding to the animal's crepuscular peaks of activity at light and dark transitions (Figure 6A). The EEG delta activity exponentially decreased during subsequent NREM rebound following these activity peaks, describing the classical time-course of sleep homeostasis (Figure 6A, 6B).[23,24] This is consistent with the increases seen in theta markers for alertness, observed during the activity peaks preceding these bouts of NREM sleep (Figure 5C). 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 nocturnal rodents are usually performed at light onset (ZT0).[17,23] Following sleep deprivation, animals showed a significant increase in the EEG delta power peak consistent with standard sleep deprivation protocols showing augmented sleep pressure (Figure 6A, 6C). Sleep rebound was similar to that seen in mice under a similar length deprivation, exponentially decreasing within 4-hours to the baseline levels observed before sleep deprivation.[17]

DISCUSSION

Here we characterized sleep and waking in *Arvicanthis ansorgei.* Our findings reveal that sleep and waking are mainly regulated by a crepuscular component with a bimodal waking distribution with light/dark/light transitions. The main sleep regulatory mechanisms classically described in mammals are conserved in *Arvicanthis Ansorgei*, a species that has been characterized as diurnal from extensive research in the field of chronobiology. This suggests that *Arvicanthis ansorgei* may represent a useful model for future sleep research.,, especially from the perspective of deciphering the mystery of the neuroanatomical switch between diurnality and nocturnality.

Why is *Arvicanthis ansorgei* a model of interest for sleep research?

Our comprehension of the neurobiology of sleep and wakefulness results mainly from studies performed in nocturnal rodents, mice and rats, whose sleep-wake cycle is inverted in comparison to humans. This underlines the importance for the development and characterization of a diurnal model that could satisfy the requirements for the study of sleep neurobiology. Animal research on circadian rhythms has been largely conducted on rats and mice, facing the same challenge. The field of chronobiology addresses this issue through the extensive study for more than fifteen years of the rodent Arvicanthis. Two subspecies of this African grass rat have been used for investigations, the Nile *Arvicanthis niloticus* and *Arvicanthis ansorgei* from Mali. Interestingly, the *ansorgei* subtype has been maintained under laboratory conditions for over fifteen years, which turned the animal behavior from wild to a behavior closer to laboratory conditions.[45,25] Finally, Arvicanthis proved to be an appropriate model for laboratory experiments and the species has been widely utilized for the study of circadian rhythms. As a result and as described in the introduction, the species has already been well-characterized and specific biological tools have been created, in addition to the development of transgenic models and other genetic tools, which is underway. However, as of this writing, sleep has never been phenotyped in Arvicanthis, although a few sleep recordings were performed in different rodent species. 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.[19,26] 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.[19,26] Moreover, the main processes known to regulate sleep and waking are conserved in *Arvicanthis ansorgei* and differences with other rodent species are discussed below.

Arvicanthis ansorgei is a Diurnal Rodent Whose Sleep is regulated by Light and Circadian, Crepuscular and Homeostatic Processes

Arvicanthis ansorgei display a lack of strong circadian drive in the absence of clock reinforcement by external cues such as a running wheel or photic stimulation

In mammals, the distribution of sleep over the 24-h cycle is regulated by a circadian process generated by an endogenous clock located in the suprachiasmatic nuclei.[28] In

Arvicanthis ansorgei the sleep-wake distribution under a standard 12hL:12hD condition shows a weak, but significant, diurnal circadian rhythmicity. This circadian distribution of sleep and wakefulness is clock-driven, as it is also observed under 24h-hour of constant darkness. Yet, some differences are observed between the 12hL:12hD and 24hD:D conditions, more likely due to a sustained direct effect of light promoting wakefulness in diurnal species. This is supported by what is observed when light is removed using a constant darkness condition, as average wake bout length change only under light and subjective light periods, but not during the dark period under 12hL:12hD vs. subjective dark under 24h D:D. The direct influence of light, through melanopsin-based phototransduction, plays a major role in sleep regulation in mice, as it did here in *Arvicanthis ansorgei*. Therefore, further experiments are needed especially to understand by which mechanisms the direct photic regulation of sleep switches between diurnal and nocturnal animals.

Crepuscular activity overrides the circadian drive

Under standard 12hL:12hD cycles the animals display peaks of wakefulness at L-D-L transitions which are conserved, albeit slightly altered, under constant darkness. These consolidated periods of waking are sustained, beginning about two hours before light and dark onset, and ending about two hours after light and dark offset. Certain animal species are incredibly active at light and dark onset, referred to as crepuscular.[3,4] The crepuscular regulation of waking observed in *Arvicanthis ansorgei* is far more pronounced than what is normally seen in laboratory rats and mice, and its persistence under constant darkness implies crepuscularity as a major sleep-wake regulatory component. This 12-h bimodal rhythm is also found for core body temperature in the absence of a running-wheel[7] and in corticosterone release with peak values close to L-D and D-L transitions.[30] The wheel-running locomotor activity recordings show, however, a clear diurnal pattern of locomotor activity. (Figure 1A, 1B). This indicates that the species can engage its daily sleep-wake distribution from a bimodal crepuscular pattern in the absence of external cues to a clear diurnal pattern of sleep-wake rhythmicity in presence of circadian Zeitgebers such as a running wheel. For *Arvicanthis ansorgei*, several reasons might explain this crepuscular wake behavior. Primarily one must consider the environment in which the *Arvicanthis ansorgei* 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 of the

day, 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, the repetition of this activity around light and dark onsets suggests an ultradian cycle of 12-hours rather than a circadian period of 24-hours. Finally, it would be of interest to study the circadian and crepuscular regulatory mechanisms under experimental conditions mimicking dawn and dusk, as suggested in flies.[30]

In humans, a bimodal organization of sleep and wakefulness can be observed under specific conditions. In the case of a Mediterranean climate or in the Sahel, people adapt to the afternoon heat by taking a prolonged siesta. In shift workers, a scheduled siesta during the day often compensates for sleep loss. Celebrating the Matins splits the night of cloistered monks and nuns with a biphasic core body temperature profile.31 However, it remains speculative as to whether the crepuscular regulation of the sleep-wake cycle in Arvicanthis ansorgei shares any similarities with the bimodal organization of sleep and wakefulness sometimes observed in human populations under specific conditions.

The homeostatic regulation of sleep in Arvicanthis ansorgei

Arvicanthis ansorgei is an active animal compared to laboratory rats, and increased activity is known to augment homeostatic pressure.[32,33] The time-course of delta activity following the two periods of high activity suggests that sleep pressure builds up faster in *Arvicanthis ansorgei* than in mice or rats, yet this remains to be further established. A 6-hour sleep deprivation started at dark onset challenged the sleep homeostat with a significant increase in the amount of delta power at sleep rebound. As compared to delta peaks after crepuscular wake bouts under baseline, the delta peak obtained at recovery sleep was relatively low. Given that there are two peaks of activity, it may be pertinent to perform a sleep deprivation at crepuscular activity onset. A more complete analysis of all increases in EEG delta activity in relation with periods of waking needs to be examined for further integrated understanding of the interaction between the circadian, the crepuscular, and the homeostatic components.

The Sleep-Wake Rhythm has a Weaker Circadian Organization as Compared to Wheel-Running Activity

In *Arvicanthis ansorgei*, the amplitude of the sleep-wake circadian rhythm is weak as compared to that of wheel-running locomotor activity. Locomotor activity is known to reinforce circadian rhythmicity and, indeed, in our experiments the diurnal pattern of locomotor activity in *Arvicanthis ansorgei* is largely strengthened in presence of a running wheel. Therefore, further experiments are needed to explore whether the Arvicanthis diurnal sleep-wake rhythm might be solidified in the presence of external cues such as a running-wheel.[3,4] The influence of locomotor activity on the circadian system can even be more pronounced in certain species such as the diurnal unstriped Nile Grass rat. Indeed, in the related species *Arvicanthis niloticus* have been shown to switch partially or totally their locomotor profile from a diurnal to a nocturnal pattern when a wheel is available.[3,5] However, this observation was not reproduced in *Arvicanthis ansorgei* as no significant changes were noticed after recording animals using infrared motion detectors in the absence of a wheel,[5] except for a few animals displaying a behavioral switch from “predominantly diurnal” to “predominantly nocturnal”.[7] This behavioral variety is probably due to different constitutive traits of the species, even though *Arvicanthis niloticus* and *Arvicanthis ansorgei* share common characteristics.

In conclusion, this is the first characterization of sleep and waking, as well as of their regulatory mechanisms in the rodent *Arvicanthis ansorgei.* This animal’s diurnal sleep-wake rhythm is strongly overridden by a crepuscular regulatory process. It remains speculative whether this crepuscular pattern in Arvicanthis has similarities with the bimodal organization of sleep and wakefulness described in humans under specific conditions. A stronger synchronization of the diurnal sleep-wake profile might be obtained through exposure of the animals to external cues such as light-dark condition as well as reward properties of wheel-running. Our findings suggest that *Arvicanthis ansorgei* may represent a powerful model for further research aiming at deciphering the mystery of the diurnality/nocturnality switch. This represents an important step for translational research from nocturnal animals to humans.

ACKNOWLEDGMENTS/

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3. Figures:

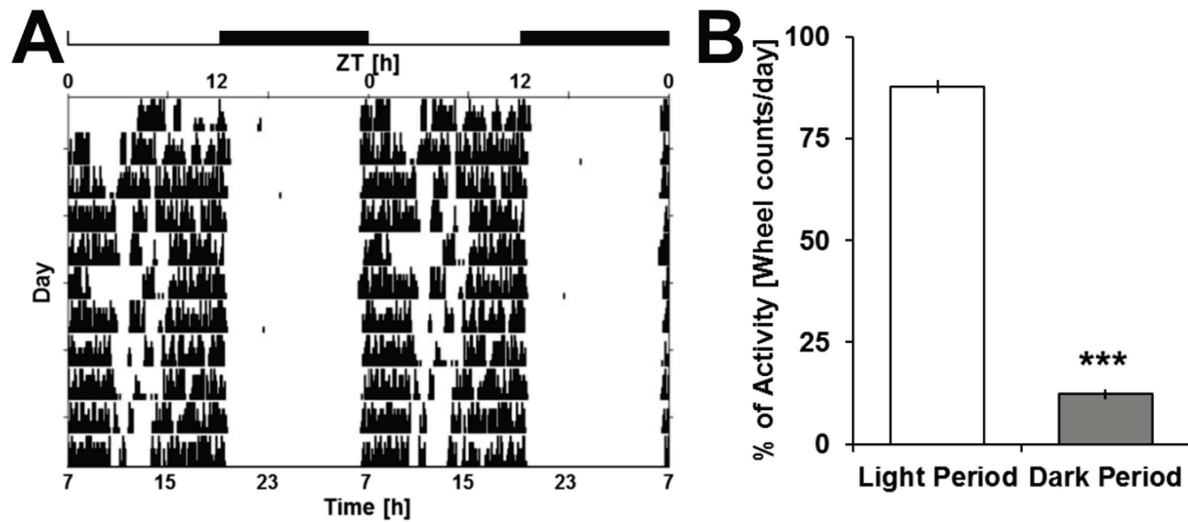

Figure 1: Daily wheel-running activity under a standard 12hL:12hD cycle

(A) Actimetry sample 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 ($p<0.001$). Asterisk represents Student t-test significance ($p<0.00001$).

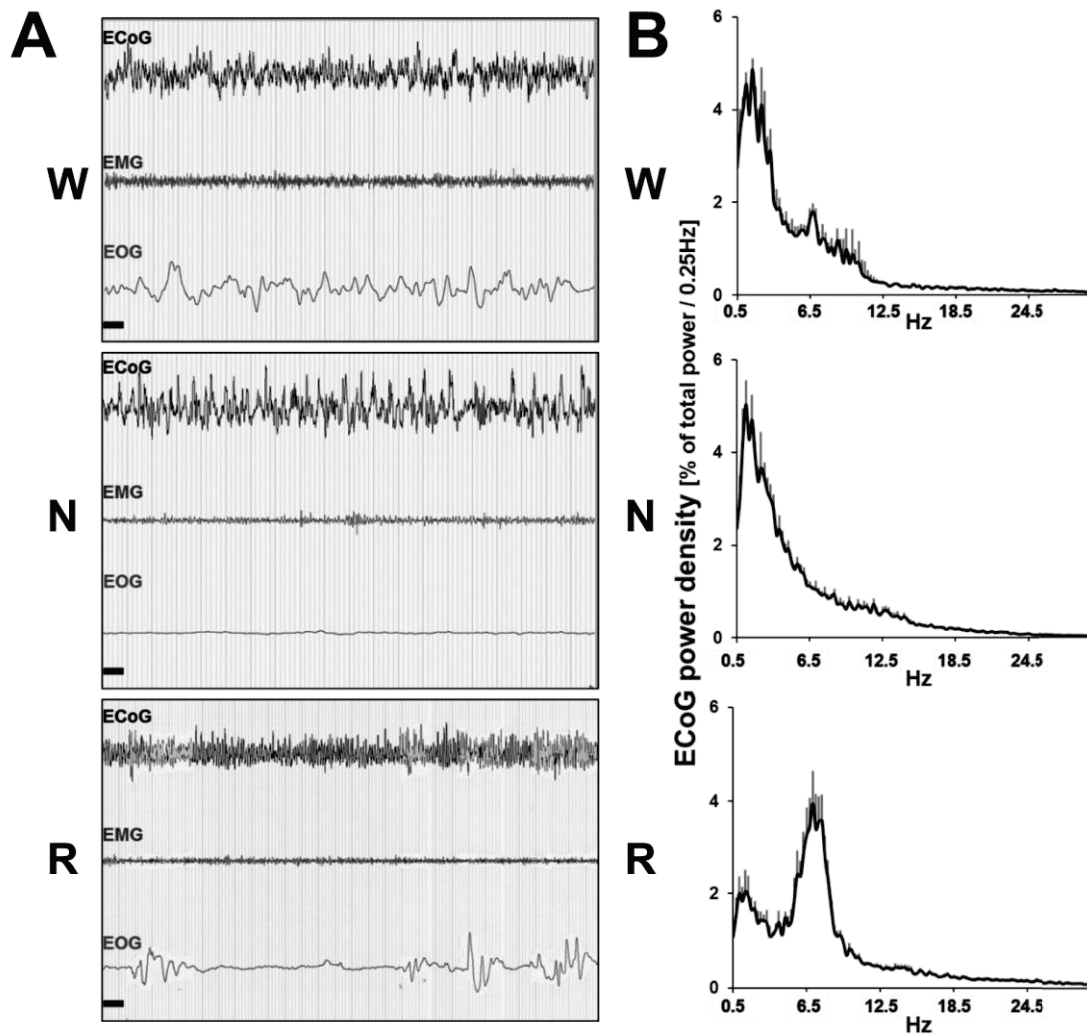

Figure 2: Samples of polygraphic recordings and EEG **power spectrum profile in W, NREM and REM under baseline condition.** Samples of EEG, EMG and EOG recordings obtained during baseline 12L:12D condition: **(A)** During waking a desynchronized EEG pattern occurs in parallel with high levels of EOG and EMG activities, **(B)** Power spectrum analysis of all waking epochs during baseline. Peaks are noticed in both the delta (0.5-4 Hz) and theta (6-10 Hz) ranges. **(C)** Recordings during NREM show synchronized slow-wave activity oscillations in the EEG, with a near complete suppression of EMG and EOG activity. **(D)** Spectral profile is dominated by delta frequencies. **(E)** During REM sleep a desynchronized EEG pattern emerges with a total flattening of the EMG, reflecting the atonic state of the animal. Rapid eye movements are indicated by EOG activity. **(F)** Power spectrum profile is dominated by theta activity. Graph represents peak relative frequencies of total power between 0.5-25 Hz. Horizontal black bars: 1 second, window represents 20 seconds of recording.

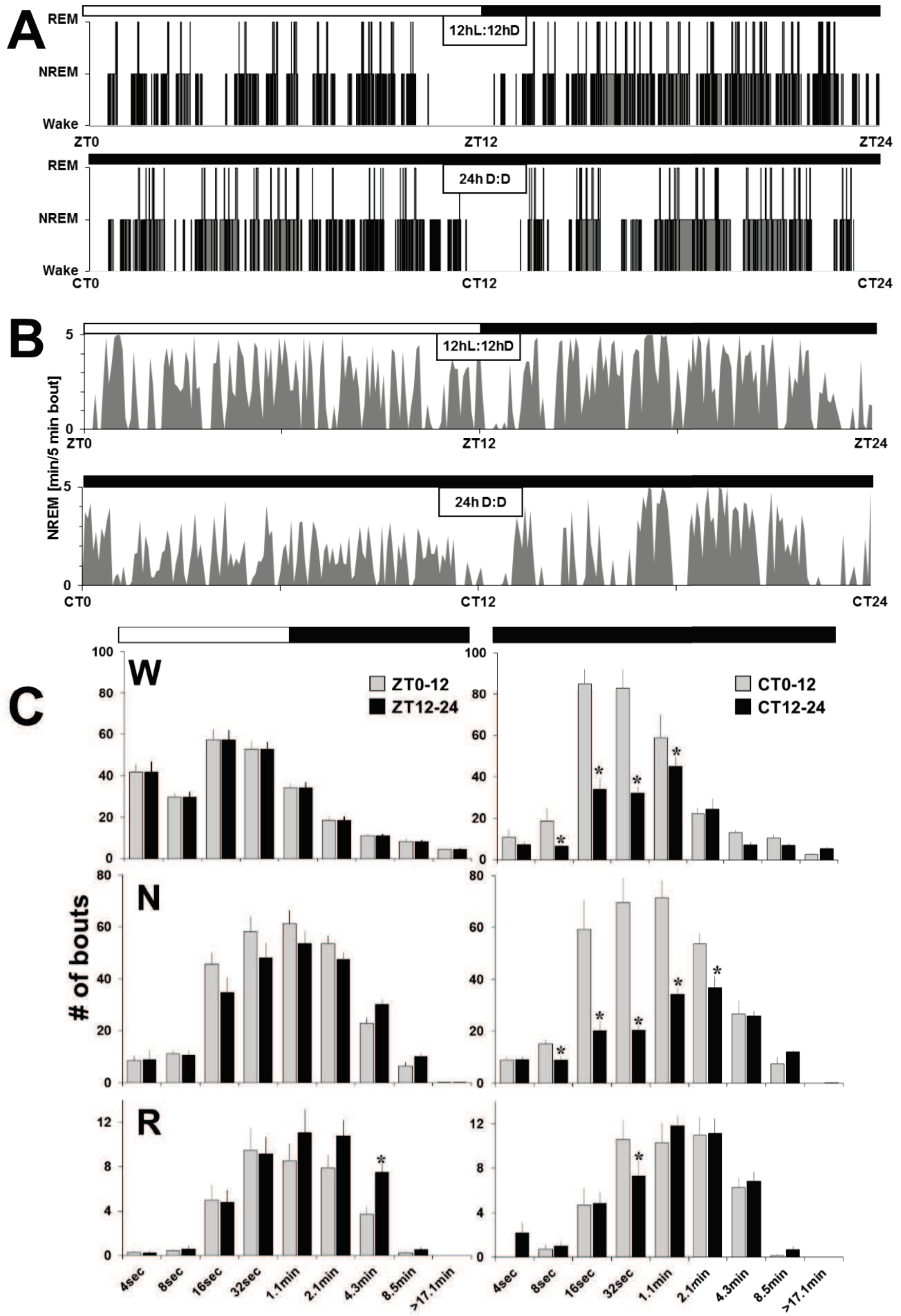
A
REM
NREM
Wake
12hL:12hD
ZT0
ZT12
ZT24
24h D:D
CT0
CT12
CT24
B
NREM [min/5 min bout]
C
W
N
R
ZT0-12
ZT12-24
CT0-12
CT12-24
# of bouts
4sec
8sec
16sec
32sec
1.1min
2.1min
4.3min
8.5min
>17.1min

Figure 3: 24- hours distribution of sleep and waking under 12hL:12hD and 24h D:D. Examples of a hypnogram based on 4-second epoch scoring **(A)** and of NREM sleep per 5 minute bouts **(B)** in two different *Arvicanthis ansorgei* across 12hL:12hD and 24h D:D conditions. Frequency distribution of W, NREM and REM episode lengths **(C)** during 12L:12D vs. 24h D:D. Vertical bars represent the number of episodes (mean ± SEM) expressed as time spent during light or dark periods (left) or subjective light or dark periods (right), in each state by episode duration. Two-way ANOVAs were performed to examine bout length differences as a function of light/dark period as well as lighting condition (12hL:12hD vs. 24h D:D). Significance was seen at differing points. $p_{Light\ condition\ x\ L\ vs.\ D}<0.05$ (W- 16s, 17.1m; N- 16s, 32s, 1.1m; R- 4s). $p_{Light\ condition}<0.05$ (W- 4s, 8s, 1.1m; R- 4s). $p_{L\ vs.\ D}<0.05$ (W- 8s, 16s, 32s, 4.3m, 8.5m; N- 8s, 16s, 32s, 1.1m, 8.5m; R- 4s, 4.3m). Asterisks represent significance between subjective light and dark phases (one-way ANOVAs, post-hoc t-tests p<0.05).

Figure 4: Time course of sleep and waking under 12L:12D and 24h D:D. (A) Vigilance states are represented as the amount of minutes per hour across 48-hours of the 12L:12D cycle **(A)** or 24h D:D **(B). (C)** Difference between the light and dark periods of total amounts of W, NREM and REM sleep during the 24-hour 12L:12D baseline condition (left). **(C)** Difference of vigilance states between the subjective light and dark periods under 24h D:D (right). All values are expressed as mean ± s.e.m. A two-way ANOVA for time-course and light condition showed significance for any vigilance states between baseline and constant darkness (black points- one-way ANOVA for light condition, post-hoc t-test, p<0.05). Asterisks denote significant differences (p<0.05).

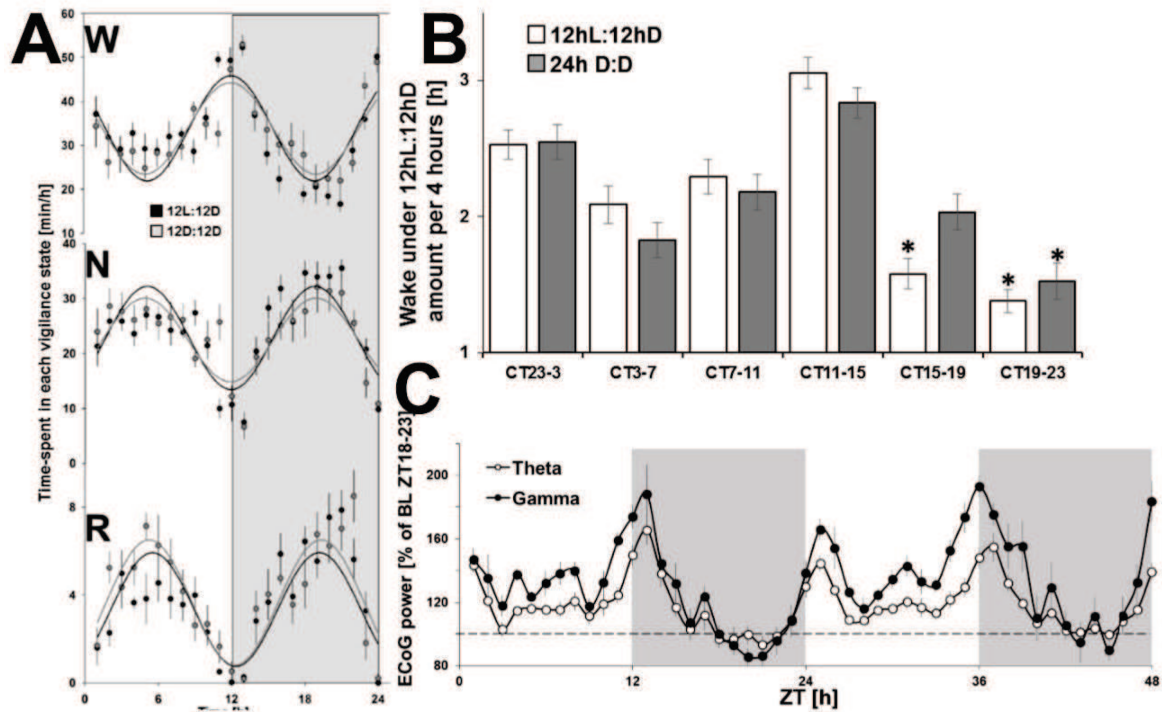

Figure 5: Crepuscular regulation of sleep and waking. (A) Sine-waves were calculated for each vigilance state to determine their mathematical profile during 12L:12D (grey) and 24h D:D (black). No significant difference was observed between the two conditions. **(B)** Amounts of wake per periods of 4 hours centered on the (subjective – grey bar) D-L-D transitions show predominant wake activity at (subjective – grey bar) crepuscular time points and an increased wake during the (subjective – grey bar) light period outside of the (subjective – grey bar) crepuscular zone, as compared to the dark (subjective – grey bar) period outside the (subjective – grey bar) crepuscular zone. Asterisks denote significant differences ($p<0.05$) by *t*-test, between the different 4-hour periods. All values are expressed as mean ± s.e.m. **(C)** Time course of EEG theta and gamma power spectrum during waking epochs expressed per hour across the two baseline days.

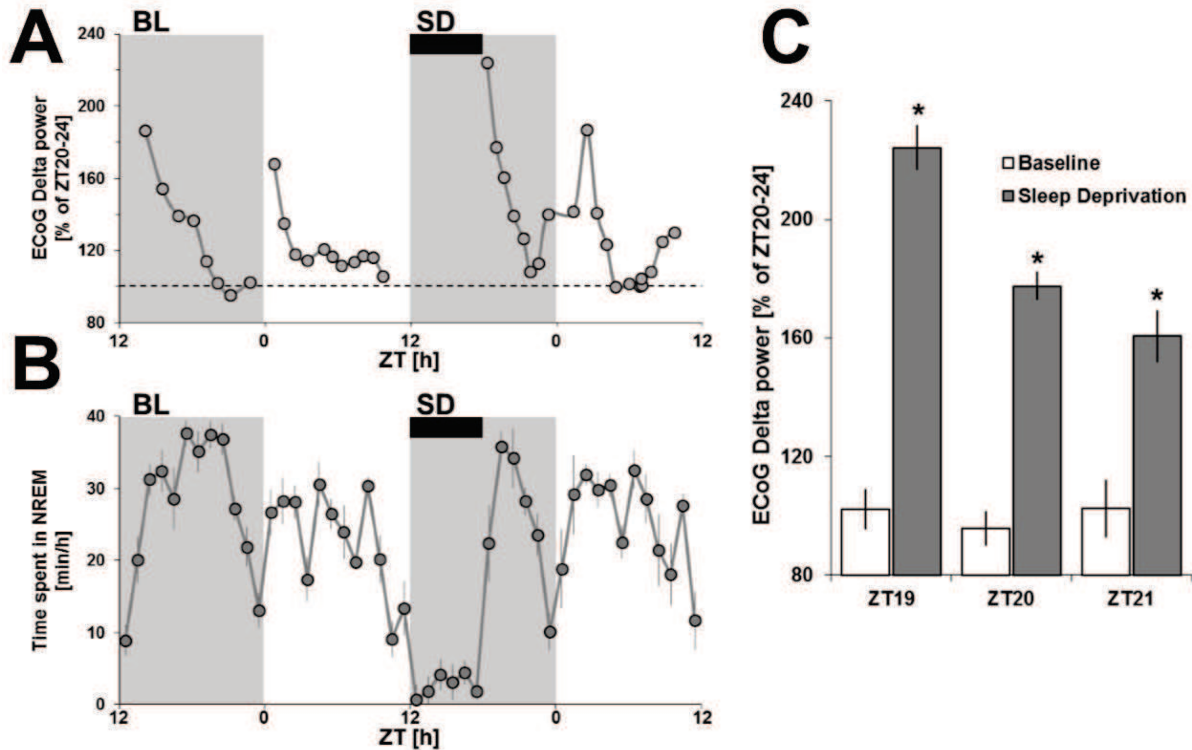

Figure 6: NREM sleep and EEG **delta power under sleep deprivation at ZT12. (A)** EEG delta power expressed as a percentage of ZT20-ZT24 during baseline (ZT20-ZT24 was determined to consistently be the period with the lowest sleep pressure). The 6-hour sleep deprivation is displayed as well as the preceding baseline day. **(B)** NREM sleep amounts under baseline conditions are at the maximum level at ZT 18. The rebound of NREM following the 6-hour SD occurs at ZT 18, which more likely explains why NREM amounts following a 6-hour SD stay within the baseline range. However, the peak of EEG delta power reached after sleep deprivation, a more reliable marker of the homeostatic process, is increased compared to baseline values. **(C)** Histograms representing EEG delta power during the three first recovery hours after the sleep deprivation as compared to the same baseline ZT (n=8). A repeated measures ANOVA for baseline vs. sleep deprivation and time-course showed significance. Asterisks denote significant differences following post-hoc PLSD ($p<0.05$).

Supplemental Table 1 – Daily amounts of wake (h), NREM sleep (h) and REM sleep (min) in 12hL:12hD cycle and 24 D:D cycles.

Schedule	Time spent	W (h)	N (h)	R (min)	R/N (%)
12hL:12hD Day 1	*12-h light period*	7.24 ± 0.3	4.24 ± 0.3	31.5 ± 5.4	12.4 ± 1.9
	12-h dark period	5.83 ± 0.3	5.27 ± 0.3	54.1 ± 5.6	17.1 ± 2.0
	24-hour Total	13.1 ± 0.4	9.51 ± 0.4	85.5 ± 7.2	15.0 ± 1.7
12hL:12hD Day 2	*12-h light period*	6.78 ± 0.4	4.59 ± 0.3	38.0 ± 3.9	13.8 ± 1.1
	12-h dark period	6.15 ± 0.3	4.99 ± 0.3	51.5 ± 4.4	17.2 ± 1.6
	24-hour Total	12.9 ± 0.6	9.58 ± 0.5	89.5 ± 6.4	15.6 ± 1.2
24h D:D	*Sub. 12-h light period*	6.34 ± 0.4[a]	4.87 ± 0.4	47.0 ± 3.3[a]	17.2 ± 2.3
	Sub. 12-h dark period	6.60 ± 0.1[a]	4.54 ± 0.1	50.9 ± 4.4	18.9 ± 1.9
	24-hour Total	13.0 ± 0.5	9.41 ± 0.5	97.9 ± 6.7	17.9 ± 1.9

Values are expressed as mean ± s.e.m. ***a*** denotes significant differences between baseline day 1/2 and 24h D:D ($p<0.05$) by *t*-test. Baseline day 1 and 2 were not significantly different.

Supplemental Table 2 – Average bout length in wake (min), NREM (min), and REM sleep (min) in 12hL:12hD cycle and 24 D:D cycles.

Schedule	Time spent	Wake (min)	NREM (min)	REM (min)
12hL:12hD	*12-h light period*	9.11 ± 0.65[a,b]	2.45 ± 0.13	1.94 ± 0.06
	12-h dark period	7.24 ± 0.44[a]	2.72 ± 0.14[b]	2.19 ± 0.07
	24-hour period	8.29 ± 0.49[b]	2.63 ± 0.11	2.08 ± 0.07
24h D:D	*Sub. 12-h light period*	5.04 ± 0.18[a,b]	2.28 ± 0.18[a]	2.17 ± 0.09
	Sub. 12-h dark period	7.84 ± 0.89[a]	3.25 ± 0.15[a,b]	2.11 ± 0.10
	24-hour period	6.44 ± 0.41[b]	2.76 ± 0.15	2.14 ± 0.07

Values are expressed as mean ± s.e.m. Two-way ANOVA for 12-hour periods (schedule x time spent) showed significance for wake (schedule- $p=0.01$; schedule x time spent- $p= 0.0001$) and NREM (time spent- $p=0.002$; schedule x time spent- $p= 0.03$) bout lengths. For 24-hour periods a one-way ANOVA (schedule) showed significance only for wake bout length ($p=0.03$). ***a*** denotes significant differences between light and dark periods, ***b*** denotes significant differences between schedules ($p<0.05$, post-hoc Fisher PLSD).

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Résultats

3ème partie : ***« Les effets directs de la lumière en application clinique : La luminothérapie en pathologie dopaminergique »***

Troisième partie : **Introduction**

Les effets directs de la lumière chez l'homme

Le rôle crucial des effets directs de la lumière a été caractérisé chez le rongeur dans la première partie de ce travail de thèse. L'implication de la mélanopsine a été bien identifiée, que ce soit au niveau des effets directs de la lumière sur le cycle veille/sommeil ou sur les comportements anhédonique, « depression-like » et anxieux. Outre la mélanopsine, les NSC jouent également un rôle important dans la phototransduction des effets non-circadiens de la lumière. En pathologie, la luminothérapie est utilisée principalement dans les troubles de l'humeur (Even, Schroder et al. 2008), notamment dans la dépression saisonnière (Terman, Terman et al. 1989) via des mécanismes circadiens bien explorés (Wirz-Justice 2003). Néanmoins, ces mécanismes circadiens, indirects de la lumière ne permettent d'expliquer qu'une partie des effets de la luminothérapie sur les comportements. Ceux- ci devraient aussi être la conséquence d'effets directs. On ne connaît que peu des effets directs de la lumière chez l'homme, ni des mécanismes impliqués.

La caractérisation de ces effets reste limitée devant l'absence de techniques bien validées permettant d'analyser chez l'homme l'implication des différentes composantes identifiées chez l'animal. Une autre difficulté dans l'application chez l'homme de ces études sur les effets directs de la lumière en pathologie du cycle veille/sommeil ou de l'humeur concerne le manque de connaissances sur les substrats biologiques de ces pathologies, ainsi que l'importante hétérogénéité entre patients. Les indications actuelles de la luminothérapie (hors troubles du rythme circadien) concernent les troubles de l'humeur (Chellappa, Gordijn et al. 2011), l'insomnie (Friedman, Zeitzer et al. 2009), la maladie de Parkinson (Paus, Schmitz-Hubsch et al. 2007; Willis and Turner 2007) et le trouble de déficit d'attention avec ou sans hyperactivité (Gruber, Grizenko et al. 2007). Un substrat neuranatomique commun à ses différentes pathologies et pouvant être impliqué dans les effets directs de la lumière est le système dopaminergique (cf Introduction « Les interactions complexes entre la lumière et le système dopaminergique »).

La luminothérapie dans la maladie de Parkinson : Données de la littérature

Deux études cliniques préliminaires suggèrent un effet bénéfique de la luminothérapie dans la maladie de Parkinson, pathologie où on peut observer une fluctuation parfois importante des symptômes au cours du nycthémère (Paus, Schmitz-Hubsch et al. 2007; Willis and Turner 2007). Dans ces travaux, l'administration de lumière entraînait une amélioration des symptômes moteurs de la maladie de Parkinson (Paus, Schmitz-Hubsch et al. 2007; Willis and Turner 2007), une diminution de la posologie des traitements dopaminergiques utilisés pour équilibrer les patients (Willis and Turner 2007), ainsi qu'une amélioration de l'humeur (Paus, Schmitz-Hubsch et al. 2007; Willis and Turner 2007), du sommeil (Paus, Schmitz-Hubsch et al. 2007; Willis and Turner 2007), de l'appétit et de l'autonomie motrice.

Paus et al. ont administré la luminothérapie le matin selon les mêmes modalités qu'elle est utilisée dans la dépression saisonnière, alors que Willlis et al. ont appliqué la lumière à moindre intensité sur une durée plus longue avant le coucher recherchant un effet de suppression de la mélatonine (Paus, Schmitz-Hubsch et al. 2007; Willis and Turner 2007). L'application de la luminothérapie le matin entraîne une avance de phase, alors que celle du soir retarde la phase circadienne. Compte tenu du design expérimental de ces deux études, il est difficile d'affirmer qu'il s'agisse i) d'effets directs de la lumière ii) d'effets circadiens passant soit par une meilleure synchronisation des rythmes circadiens soit par un décalage de la phase circadienne connus pour être perturbés dans la maladie de Parkinson (Bordet, Devos et al. 2003; Lin, Du et al. 2014) iii) d'effets directs en lien avec la suppression par la lumière de la mélatonine ayant un effet négatif sur le tonus dopaminergique (Willis and Armstrong 1999) et dont des taux résiduels ont été observés en début de journée chez le patient atteint de maladie de Parkinson (Lin, Du et al. 2014). Cette dernière hypothèse est à considérer toutefois avec précaution, il existe des données contradictoires. Des indications de thérapie par mélatonine ont été proposées au cours de la maladie de Parkinson lors du trouble du comportement en sommeil paradoxal (Kunz and Mahlberg 2010), d'insomnie (Dowling, Mastick et al. 2005; Medeiros, Carvalhedo de Bruin et al. 2007) ou à titre de neuroprotection (Capitelli, Sereniki et al. 2008; Ma, Shaw et al. 2009; Srinivasan, Cardinali et al. 2011). Cette discordance pourrait s'expliquer par un bais méthodologique dans les études chez l'animal, les rats ayant été testés pendant la période de lumière subjective en condition de lumière constante, c. à d. à un moment où les souris contrôles dorment probablement

davantage (Willis and Armstrong 1999). De plus, il a été suggéré (chez le primate non humain) qu'en l'absence de dopamine et en condition de lumière constante, les animaux deviennent arythmiques sur le plan locomoteur (Fifel, Vezoli et al. 2014). La lumière, en dehors d'un effet suppresseur sur la sécrétion de la mélatonine, exerce de multiples fonctions dont il faut tenir compte.

La luminothérapie dans le syndrome des jambes sans repos (SJSR): Données de la littérature

La littérature sur l'utilisation de la luminothérapie dans le SJSR est particulièrement pauvre.

La seule étude publiée (Whittom, Dumont et al. 2010) est basée sur une hypothèse d'un effet aggravant de la mélatonine sur le SJSR. Cette hypothèse est issue des résultats d'une étude réalisée en condition de routine constante modifiée, et qui mettait en évidence que l'exacerbation vespérale du SJSR était précédée de l'augmentation de la sécrétion de la mélatonine (Michaud, Dumont et al. 2004). Afin de tester cette hypothèse, le groupe de J. Monplaisir a étudié dans des conditions contrôlées les effets d'une administration de mélatonine exogène (3 mg à 19h00) et ceux d'une luminothérapie (3000 lux de 19h00 à minuit) (Whittom, Dumont et al. 2010). Il s'agissait d'une administration aiguë, ne permettant pas au système circadien d'interagir lors de leur paradigme expérimental. La mélatonine exogène a majoré l'index des mouvements périodiques au test d'immobilisation suggéré, la luminothérapie a légèrement amélioré les symptômes sensitifs du SJSR lors de ce même test.

Hypothèse de travail

Le système dopaminergique est une cible potentiellement impliquée dans les effets directs de la lumière sur les comportements chez l'homme. Dans la maladie de Parkinson, pathologie en lien avec une déficience acquise en dopamine, on observe des symptômes moteurs, mais également des symptômes non-moteurs, dont les troubles du cycle veille/sommeil et de l'humeur. Les résultats des deux études préliminaires sur l'indication de la luminothérapie dans la maladie de Parkinson sont encourageants, mais ils nécessitent une validation par des études de type preuve de concept. Des troubles du cycle veille/sommeil et de l'humeur sont également plus fréquents chez les patients atteints d'un syndrome des jambes sans repos, une autre pathologie dans laquelle le système dopaminergique joue un rôle majeur.

Afin de mieux analyser les effets directs de la lumière sur les comportements chez l'homme, nous avons étudié l'impact de la luminothérapie dans ces deux pathologies impliquant la dopamine. Nous allons nous intéresser particulièrement aux effets de la lumière sur le cycle veille/sommeil et sur l'humeur. Pour éviter tout décalage de la phase circadienne, et étudier les effets non-circadiens directs, le moment de l'application de la lumière a été calculé individuellement pour chaque patient.

Concernant la maladie de Parkinson, l'étude est multicentrique et contrôlée contre placebo. Elle bénéficie d'un financement de type PHRC National. Ce protocole de recherche clinique est actuellement en cours dans notre service et les résultats concernant les premiers patients ne sont pas encore accessibles. Une première analyse (nécessitant une levée de l'aveugle des patients ayant terminé le protocole) n'est prévue qu'après inclusion de la première moitié des patients. De ce fait, dans la section des résultats, je me limiterai à la description du protocole expérimental de cette étude.

Concernant l'étude de la luminothérapie dans le syndrome des jambes sans repos, l'analyse des résultats est actuellement en cours. Cette étude a été réalisée par mes collègues les Dr Ulker KILIC-HUCK et Mme Christelle MEYER. Mon implication concernait la mise au point du test d'immobilisation suggéré dans notre service. J'ai participé au recrutement des patients, de même qu'aux analyses des résultats.

Troisième partie : **Résultats / Perspectives**

La luminothérapie dans la maladie de Parkinson : Effet sur les symptômes moteurs, le sommeil, les rythmes circadiens et l'humeur

Introduction et justification scientifique de l'étude

Dans la maladie de Parkinson il existe une dégénérescence acquise des neurones dopaminergiques à l'origine de symptômes moteurs (tremblements, akinésie, rigidité) et non-moteurs, dont des troubles du cycle veille/sommeil et de l'humeur (cf Introduction VI. « Maladie de Parkinson »). Malgré une grande efficacité des traitements pharmacologiques à base de dopamine et de la chirurgie par stimulation profonde des noyaux sous-thalamiques, nombreux sont les patients qui éprouvent de grandes difficultés pour équilibrer la symptomatologie. Il existe donc une nécessité de trouver des traitements adjuvants efficaces autant sur le plan moteur que non moteur. La luminothérapie pourrait représenter une telle approche thérapeutique innovante, ce d'autant plus qu'elle est habituellement bien tolérée et ne présente pas de réelles interactions médicamenteuses.

Il existe des interactions complexes entre le système dopaminergique et la lumière. Celles-ci peuvent avoir lieu au niveau de la rétine, notamment par le biais du contrôle de la sécrétion de la mélanopsine par la dopamine. La rétine étant atteinte dans la maladie de Parkinson, les symptômes observés peuvent être favorisés par un tonus dopaminergique diminué au niveau de l'œil. Des interactions entre la lumière et le système dopaminergique ont également été mis en évidence au niveau cérébral (cf Introduction). Ainsi, une activation des neurones dopaminergiques a été mise en évidence au niveau de la substance noire réticulée à la suite d'un exposition lumineuse.

Cette étude a également une dimension mécanistique permettant d'évaluer les effets directs de la lumière. Pour cela, le moment de l'application de la luminothérapie est calculé pour chaque patient afin d'éviter un décalage de la phase de son rythme circadien. Par ailleurs, des mesures circadiennes permettent de vérifier l'absence d'effet circadien de la luminothérapie (agenda de sommeil, actimétrie, profil de mélatonine, DMLO).

Objectifs et résultats attendus

L'objectif principal de cette étude est d'évaluer en comparaison à un groupe placébo (lampe inactive), l'effet de 3 semaines de traitement par luminothérapie sur la sévérité des symptômes moteurs de la maladie de Parkinson.

Les bénéfices attendus au niveau des symptômes non-moteurs font partie des objectifs secondaires : la qualité du sommeil, la somnolence diurne, le renforcement des rythmes circadiens, l'humeur et l'apathie

Par ailleurs, cette étude a une dimension exploratoire. A l'heure actuelle, nous ne disposons pas d'outil bien validé pour explorer le système mélanopsinergique. La mélanopsine étant fortement impliquée dans la régulation du réflexe photomoteur, différentes équipes ont mis en place des pupillomètres qui cherchent à distinguer dans la réponse photomotrice, la composante spécifique de la mélanopsine. Cette analyse sera réalisée à Strasbourg avec l'aide du système mis en place par un centre collaborateur à Amsterdam sous la responsabilité du Pr E. Van SOMEREN . A Lyon, les patients seront testés par le matériel mis au point par l'équipe lyonnaise (Mure, Rieux et al. 2007). En parallèle, une imagerie par tomographie par cohérence optique (OCT) permettra de corréler l'effet de la luminothérapie avec des indicateurs structuraux (OCT) et fonctionnels (pupillométrie) rétiniens. De plus, en effectuant des mesures chronobiologiques incluant l'actimétrie, la température centrale, le rythme de mélatonine urinaire et le DMLO ou « Dim light melatonin onset » (amplitude et phase du rythme circadien), l'objectif est d'évaluer si l'effet de la luminothérapie sur les symptômes moteurs et non-moteurs est direct non-circadien et/ou circadien (action sur l'amplitude et/ou la phase de l'oscillateur endogène).

Plan expérimental de la recherche

Dans ce projet d'étude planifiée sur une durée de 3 ans, nos hypothèses seront testées via une étude interventionnelle randomisée placébo-contrôlée en insu sur une période de 4 semaines. Cette étude est multicentrique, réalisée aux CHU de Strasbourg et Lyon. Le centre de Besançon n'est pas encore ouvert.

Après une évaluation initiale en condition « baseline » sur une semaine, les patients recevront l'un des deux traitements suivants: 5 000 lux 'bright light' (lumière

blanche ; traitement actif) ou 70 lux de ‘dim light’ (lumière rouge ; placébo), administré bi-quotidiennement 60 minutes au domicile sur trois semaines. L’évaluation finale après traitement sera conduite pendant la troisième semaine de traitement. Les horaires d’administration du traitement sont calculés individuellement pour chacun des patients en fonction de son rythme circadien sur la base des mesures chronobiologiques (Morningness/Eveningness questionnaire, actimétrie, agenda de sommeil et enregistrement de température centrale). L’actimétrie, incluant une mesure de la luminosité ambiante (luxmètre), sera poursuivie pendant la période intégrale de l’étude (4 semaines), permettant ainsi de vérifier la « compliance » au traitement par luminothérapie aux horaires individuellement programmés pour chacun des patients (semaine 2-4), un point clef dans le succès du traitement.

Il est prévu d’inclure 75 patients, en anticipant 5 sorties d’étude, afin d’avoir 35 paires de patients ayant eu le protocole complet. Le traitement, soit la luminothérapie active soit la lumière placebo, sera attribué de façon randomisée aux membres de chaque paire. Les deux groupes de patients seront appariés en termes d’âge, de sexe, d’équivalent de traitement dopaminergique.

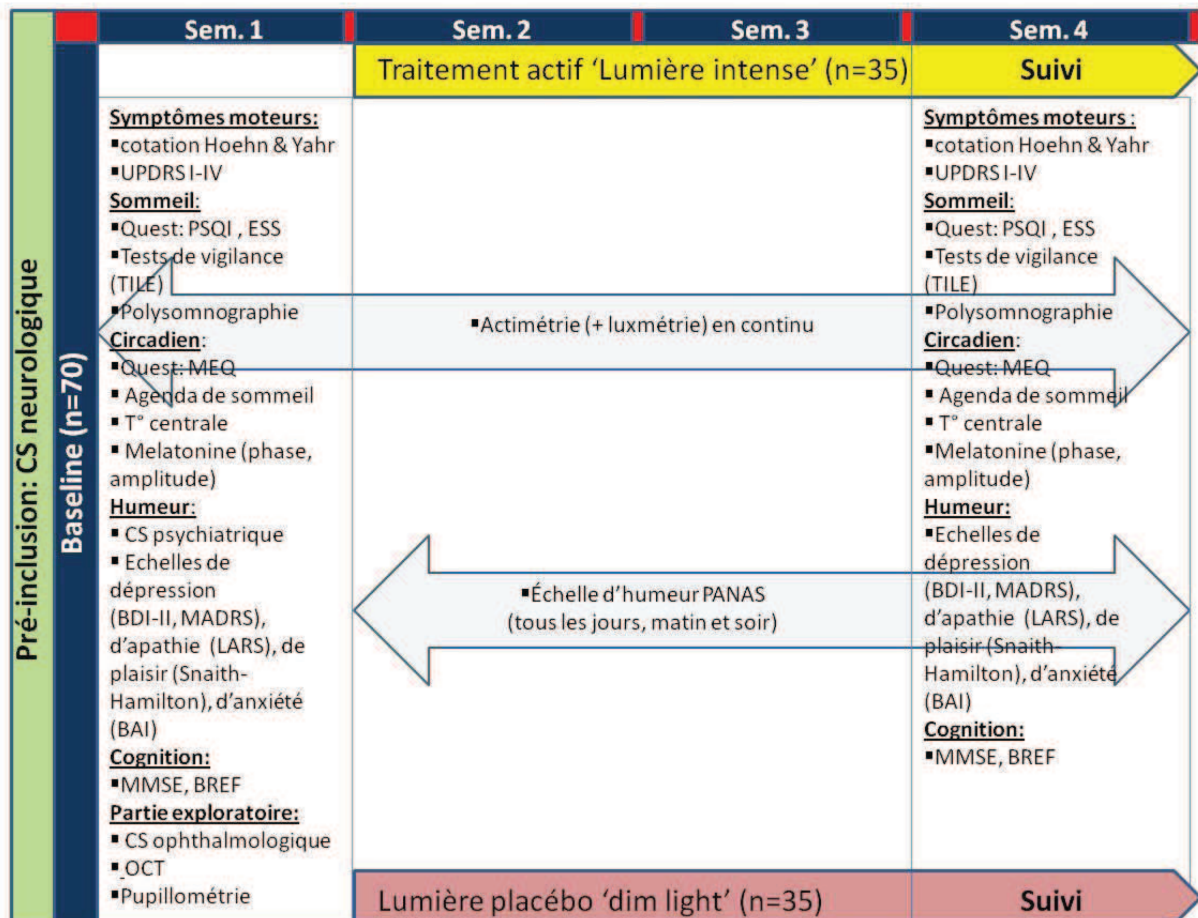

Figure 23. Résumé de la méthodologie de l'étude. Les mesures de température centrale et actimétrique sont effectuées sur une semaine avant traitement (sem 1) et à la fin du traitement (sem 4). L'actimétrie est conservée tout au long du protocole pour contrôler le suivi du traitement. Les autres mesures sont effectuées à la fin des semaines 1 (baseline) et 4 (fin de la période de 3 semaines de traitement).

Critères d'inclusion et d'exclusion

Critères d'inclusion : Sujet âgé entre 45 et 65 ans, atteint de maladie de Parkinson idiopathique peu ou modérément sévère (score < 4 sur l'échelle de Hoehn & Yahr) évoluant entre 4 et 10 ans.

Critères d'exclusion : Présence d'un syndrome démentiel (score MMS <24) ou d'une dépression sévère (BDI-II: score global < 30 ou score à l'item 9 < 2). Patient n'ayant pas une médication stable pendant le mois précédent l'inclusion et pendant les 4 semaines de durée de l'étude. Pathologie intercurrente sévère ou trouble respiratoire au cours du sommeil sévère. Présence de pathologies ophtalmologiques (cataracte avec indication opératoire; glaucome ; rétinopathies sévères)

Etat Actuel de l'Etude

L'étude est actuellement en cours. Pour l'instant, moins d'un quart des patients ont été inclus. Les premières analyses seront réalisées dès que la moitié des inclusions aura été faite.

La Luminothérapie peut-elle améliorer le Syndrome des Jambes sans Repos ? Une étude pilote contrôlée contre placébo

Introduction et justification scientifique de l'étude

Dans le syndrome des jambes sans repos, il n'existe pas de franc argument en faveur d'une dégénérescence au niveau des neurones dopaminergiques. Ce serait plutôt une sécrétion inappropriée de dopamine qui favoriserait les impatiences (Annexe x). Il s'agit d'une symptomatologie à type d'inconfort dans les jambes avec besoin de bouger. L'activité motrice fait céder (au moins transitoirement) les impatiences. Le SJSR évolue avec une rythmicité vespérale. Même si l'utilisation des agonistes dopaminergique dans le traitement du SJSR constitue une avancée majeure, de nombreux patients ne sont pas suffisamment soulagés par ce type de traitement ou ne le tolèrent pas. D'autres traitements existent avec toutefois souvent soit des problèmes de tolérance, soit une efficacité insuffisante. Il existe donc une nécessité de trouver des traitements alternatifs ou adjuvants. La luminothérapie pourrait représenter une telle approche thérapeutique innovante, ce d'autant plus qu'elle est habituellement bien tolérée et ne présente pas de réelles interactions médicamenteuses.

Il existe des interactions complexes entre le système dopaminergique et la lumière, déjà précisés dans ci-dessus (cf paragraphe sur le parkinson ; Introduction).

Objectifs et résultats attendus

L'objectif global de l'étude a été d'analyser l'effet de la luminothérapie active contre une condition placebo sur la sévérité du SJSR, tout en contrôlant les effets confondant sur le sommeil, le rythme circadien et l'humeur. L'hypothèse principale était celle d'un effet bénéfique de la luminothérapie sur la sévérité subjective et objective du SJSR avec amélioration des scores au questionnaire IRLS et le niveau d'inconfort au test d'immobilisation suggéré.

Les hypothèses secondaires concernent un bénéfice de la luminothérapie sur la qualité (subjective et objective) du sommeil, un renforcement du rythme circadien et une amélioration de l'humeur.

Plan expérimental de la recherche

Pour remplir les objectifs de cette étude pilote, un design d'étude interventionnelle contrôlée contre placébo a été utilisé. 24 patients ont été inclus et appariés en deux groupes, l'un recevant un traitement par luminothérapie active (10,000 lux lumière blanche), l'autre étant exposé à une lumière placébo (70 lux lumière rouge). La lumière a été administrée le soir, avant l'émergence des symptômes du SJSR, selon un horaire individuellement programmé en fonction de la phase circadienne de chaque patient afin de ne pas décaler la phase circadienne. L'évaluation des sujets a été effectuée pendant une semaine avant traitement, puis après 3 semaines de traitement, la durée totale de l'étude se déroulant sur 4 semaines.

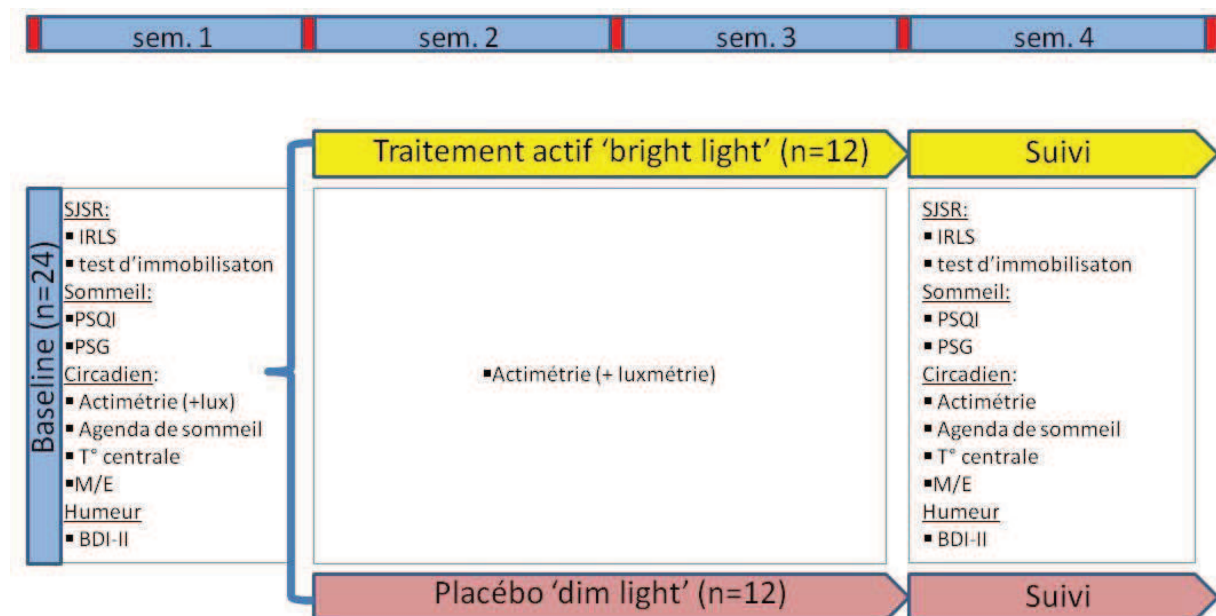

Figure 24. Résumé de la méthodologie de l'étude. Les mesures de température centrale et actimétrique sont effectuées sur une semaine avant traitement (sem 1) et à la fin du traitement (sem 4). L'actimétrie est conservée tout au long du protocole pour contrôler le suivi du traitement. Les autres mesures sont effectuées à la fin des semaines 1 (baseline) et 4 (fin de la période de 3 semaines de traitement).

Critères d'inclusion et d'exclusion

Critères d'inclusion : Sujet âgé entre 18 et 65 ans, atteint d'un syndrome des jambes sans repos de sévérité modérée à très sévére (IRLS>11).

Critères d'exclusion : Présence d'un syndrome des jambes sans repos secondaire ou de troubles de sommeil non liés au SJSR. Patient n'ayant pas une médication stable pendant le mois précédent l'inclusion et pendant les 4 semaines de durée de l'étude. Pathologie intercurrente sévère. Présence de pathologies ophtalmologiques (cataracte avec indication opératoire; glaucome ; rétinopathies sévères)

Etat actuel des analyses

Il n'a pas été observé d'amélioration subjective ou objective concernant la sévérité du syndrome des jambes sans repos ou des mouvements périodiques nocturnes (questionnaire de sévérité IRLS, test d'immobilisation suggéré, mouvements périodiques nocturnes à la polysomnographie).

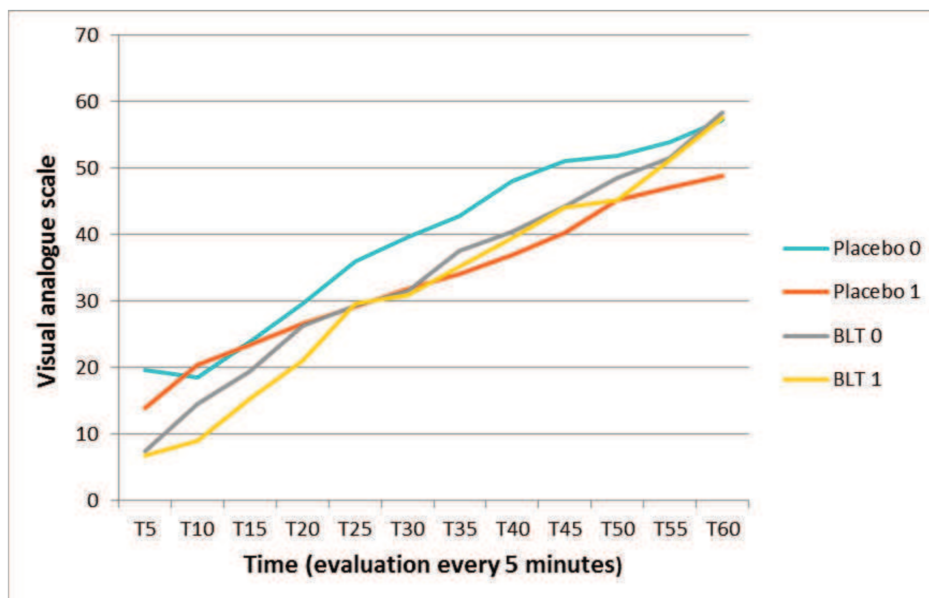

Figure 25. Effet de la luminothérapie sur la sévérité du SJSR. Absence d'amélioration de la sévérité du SJSR évaluée par un test d'immobilisation suggéré avant la mise en place de la luminothérapie (lampe active : courbe grise; lampe inactive : courbe bleue) et après 3 semaines d'utilisation de la luminothérapie (lampe active : courbe jaune ; lampe inactive : courbe rouge).

En revanche, il a été observé une amélioration significative au niveau des paramètres du sommeil, tels la durée du sommeil et le taux du sommeil paradoxal. Cette amélioration a également été mise en évidence, toutefois sans significativité statistique, au niveau de données enregistrées par actimétrie et par agenda du sommeil avec une tendance à un sommeil de plus longue durée avec moins de réveils intra sommeil (Figure 26).

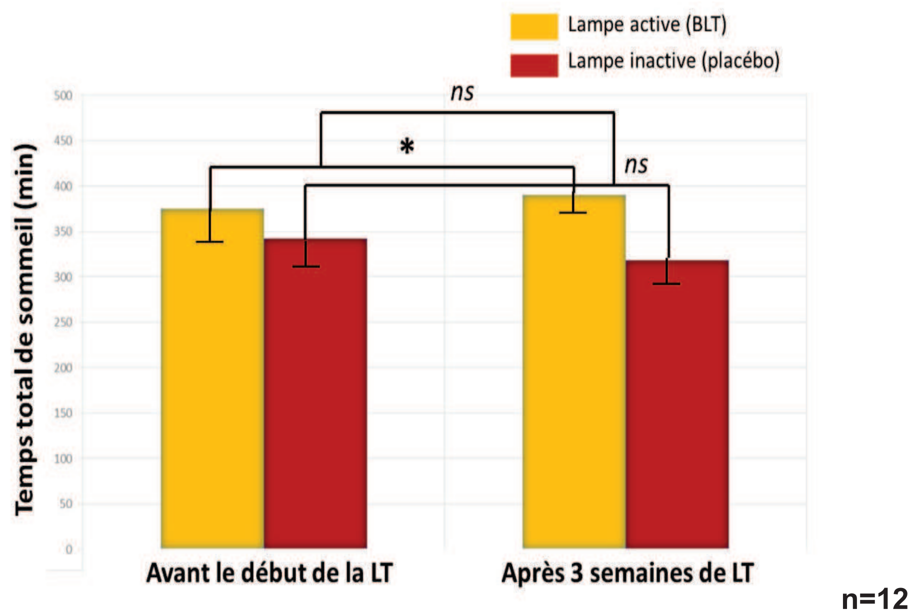

Figure 26. Amélioration du sommeil par la luminothérapie. L'exposition à 3 semaines de luminothérapie permet d'allonger significativement le sommeil comparé à la première évaluation, mais absence d'allongement significatif dans la condition placébo et lorsqu'on compare les deux groupes. (*) : Amélioration significative ; *ns* : non significatif ; méthode bayésienne.

De plus, on observe une amélioration de l'humeur à l'échelle de dépression de Beck chez les patients ayant eu un score élevé à l'inclusion. Compte tenu du faible nombre, une analyse statistique n'a pas été possible. L'effet thérapeutique de la lumière sur l'humeur étant bien connu, ces données permettent de valider la condition expérimentale utilisée.

Par ailleurs, aucun décalage de la phase circadienne n'a été observé après trois semaines d'utilisation de la lampe.

Interprétation

Devant l'absence de décalage de phase induit par la luminothérapie, les améliorations du sommeil et de l'humeur observées résultent donc des effets non-circadiens directs de la lumière. Ces résultats suggèrent que de la présence de troubles du sommeil secondaires à un SJSR pourraient constituer une indication à la luminothérapie. De même, la présence d'un trouble de l'humeur, comorbidité particulièrement fréquente chez les patients atteints d'un SJSR, pourrait être améliorée par la luminothérapie. Ceci est une indication d'autant plus précieuse chez cette population de patients, que les antidépresseurs habituels (inhibiteurs de la recapture de la sérontonine, tricycliques) aggravent souvent les impatiences.

L'absence d'effet de la lumière sur la sévérité du SJSR est à l'encontre de l'hypothèse de départ postulant que la luminothérapie pouvait renforcer le tonus dopaminergique chez le patient atteint d'un SJSR et par ce biais améliorer la symptomatologie.

Il est à considérer que cette étude pilote de type « preuve de concept » n'a inclus que 24 patients et nécessite la réalisation d'études complémentaires à plus large échelle. De même il sera intéressant d'étudier les effets de la luminothérapie plus spécifiquement dans des groupes de patients avec une plainte concernant la qualité de leur sommeil et / ou présentant un trouble de l'humeur.

Implication du système dopaminergique dans le syndrome des jambes sans repos post accident vasculaire cérébral des ganglions de la base

Le traitement par agonistes dopaminergiques est efficace à petites doses, mais l'augmentation de la posologie expose au risque de syndrome d'augmentation avec exacerbation des impatiences. Les données de la littérature évoquent plutôt un hyperdopaminergisme (Wetter 2013), hypothèse renforcée par nos données récentes auprès d'une série de patients ayant développé un SJSR post accident ischémique dans le territoire lenticulo-strié, explorés en imagerie métabolique (Article 4)

La collecte des données concernant les quatre patients décrits dans le suivant article a débuté en début d'année 2013. Le dernier patient inclus dans cette étude a eu son imagerie au cours de l'année 2014. Le recrutement des patients a eu lieu via ma consultation au centre des troubles du sommeil en collaboration avec mes confrères du service de neurovasculaire. L'analyse des données, leur interprétation et l'écriture de l'article ont été réalisées au fur et à mesure de la collecte des données.

Article 4 : ***« Hyperdopaminergism in lenticulostriate stroke-induced RLS: a neuro imaging study»***

En préparation

Article: pages 1-8

Figure : pages 9-10

Références : pages 11-13

(1) Title page. ***Article (Smaller scope studies)***

1 Article title: ***Hyperdopaminergism in lenticulostriate stroke-related restless legs syndrome: an imaging study***

2 Word count: Main text = 1131 words; Abstract = 235 words; Title: 94 characters; references: 10; figures: 2.

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6 Search terms:[249] restless legs syndrome; [122] PET; [123] SPECT; [313] basal ganglia, [2] All cerebrovascular disease / Stroke

(2) Page 2

7 Authors' Roles:

ER, MB, IJN and PB did the conception and the design of the study; ER, MB, IJN, LT, AH, LH, UKH were involved in the acquisition of data; ER, MB, IJN, LT, AH, LH, UKH, VW and PB did the analysis and interpretation of the data; ER, MB and JH did the drafting of the submitted material, PB did a critical revision of the submitted material.

8 Financial Disclosure / Conflict of interest concerning the research related to the manuscript: This study did not benefit from financial support / The authors have no conflict of interest or financial disclosures to declare.

9 Funding sources for the study: This study did not benefit from financial support

(3) Abstract:

Objectives: Restless legs syndrome (RLS) is a common disorder whose pathophysiology involves a poorly understood dopaminergic dysregulation. Stroke-related RLS primarily involves the basal ganglia, the paramedian pons and the thalamus. To further investigate the dopaminergic system in patients with stroke-related RLS, we performed isotopic explorations of both glucose consumption and nigrostriatal dopamine terminal functioning.

Methods: Four patients presenting with RLS symptomatic of lenticulostriate infarct, either de novo RLS or exacerbation of RLS existing prior to stroke were included. Structural imaging was performed using brain MRI and the stroke-induced metabolic modifications were assessed by ^{18}F-FDG PET. Dopamine reuptake via DAT was explored using ^{123}I-FP-CIT SPECT. PET with ^{18}F-F-DOPA evaluated the functional integrity of the presynaptic dopaminergic synthesis.

Results: The body of the caudate nucleus was the only structure damaged in all patients using MRI. ^{18}F-FDG PET showed a hypometabolism in the infarction area, the ipsilateral thalamus and the contralateral cerebellum. Three patients displayed decreased dopamine transporter binding in the ipsilateral putamen, with one patient having normal levels. Moreover ^{18}F-F-DOPA uptake was increased in the ipsilateral putamen of all patients.

Conclusions: The present findings suggest an ipsilateral striatal synaptic hyperdopaminergism as a compensatory mechanism in response to the stroke-induced neuronal loss. This mechanism might participate to RLS pathogenesis and should encourage further research on dopaminergic dysregulation associated with RLS. The study of well-defined lesions associated with RLS may contribute to comprehending the pathophysiological mechanisms underlying this condition.

(4) Main manuscript

Introduction:

Restless legs syndrome (RLS) is a common sensory-motor disorder characterized by an urge to move limbs due to unpleasant or uncomfortable feelings in the lower limbs. Symptoms occur at rest, are relieved by motor activity, and are worsened in the evening or at night.[1] Severe RLS has not only a strong impact on sleep and quality of life, but also on mortality.[2] pointing for the need to better understand its pathophysiology. Dramatic benefits from dopamine agonists strongly suggest involvement of dopaminergic system.[3] Regarding the type of dopamine dysregulation in RLS some controversies persist. In primary RLS, important differences were seen between studies where dopamine transporter density in the striatum was found to be normal, increased or decreased. F-DOPA PET studies showed either no abnormalities or in some cases a slight but significant decrease in presynaptic nigrostriatal dopaminergic projections among RLS patients, as compared to controls.[4] However, if dopaminergic dysregulation underlying pathology is highly suggested, it remains to be characterized.

The study of focal lesions associated with RLS is another approach to investigate RLS pathogenesis. Only a few cases of stroke-related RLS have been reported and lesion localizations were overall common suggestive of functional anatomy underlying stroke-related RLS.[5,6,7] Thus, by examining small well-defined lesions associated with the pathology more closely using both structural and metabolic explorations with appropriate radiotracers, dissection of individual circuits potentially accounting for its emergence may be possible. We performed isotopic explorations of both glucose consumption and the dopaminergic pathways, in four individuals with stroke-related RLS involving the basal ganglia.

Methods

We prospectively included all patients referred to our sleep disorders center from December 2012 to December 2013, who presented with stroke-related RLS after infarction of the basal ganglia. Patients were then assessed for severity using the Restless Legs score International Scale (IRLS), and were included based on whether sufficient criteria were met[1], after which

they were given a polysomnographic examination. A total of four patients were included. All patients underwent a standard brain MRI during the initial phase of their stroke. The images were then analyzed with the help of an experienced neuroanatomist (LT) to specify the injured anatomical structures for each patient. To analyze the neural and brain dopamine metabolism, the patients received two PET with ^{18}F-FDG (2-deoxy-2-[fluorine-18]fluoro- D-glucose) and ^{18}F-F-DOPA (3,4-dihydroxy-6-^{18}F-fluoro-l-phenylalanine), and a SPECT with ^{123}I-FP-CIT ([123]I-2beta-carbometoxy-3beta-[4-iodophenyl]-N-[3-fluoropropyl]nortropane). Dopamimetics were discontinued at least 7 days prior to isotopic explorations. All these examinations were performed in the department of nuclear medicine and interpreted by the same operator (IJN).

This study was approved by the Ethics Committee of the Faculty of Medicine, Strasbourg. Patient's consent was obtained for all four patients.

Results

Clinical history:

All patients presented infarct in the deep territory of the middle cerebral artery (MCA), right-sided for patients N°1, 2 and 4 and left-sided for patient N°3 (Figure 1). Exacerbation of RLS existing prior to the stroke, appeared either abruptly on the second day (patient N°2), or 3 weeks (patient N°3) after. In two cases where RLS was not present before, it appeared on either day 8 (patient N°1) or day 4 (patient N°4) following stroke with an independent evolution from initial stroke symptoms. Symptoms were overall similar to those described in idiopathic RLS. Treatment with low doses of pramipexole (0.09-0.18 mg daily) was immediately efficient in all patients. Metabolic brain imaging was performed at six or eight weeks (patients N°1 and 2), and three or nine months (patients N°3 and 4), after stroke.

Structural and functional imaging:

The body of the caudate nucleus was the only brain structure that was lesioned in all patients as illustrated by MRI in Flair (N°1, 3, 4) or diffusion (N°2) sequences (Figure 1). In addition to a pronounced hypometabolism in the infarction area shown by ^{18}F-FDG PET (Figure 1) the four patients displayed an hypometabolism in the ipsilateral thalamus (Figure 1) and in the

contralateral cerebellum (Figure 2), these latter being structurally intact according to the MRI (Figure 2).

The ^{18}F-F-DOPA PET showed in all patients an increased expression of dopamine precursor in the putamen, ipsilaterally to the infarct (Figure 1). In three patients, striatal DAT availability was decreased ipsilaterally to the lesion, as assessed by SPECT with ^{123}I-FP-CIT whereas it was normal in patient N°4 (Figure 1).

Discussion

Here, we report four patients with RLS symptomatic of lenticulostriate infarction. We found that corpus of caudate nucleus was the common lesioned structure but more strikingly, isotopic imagings suggest hyperdopaminergic state of ipsilateral putamen in all patients.

RLS symptomatic of focal lesions give opportunity to identify neural pathways underlying pathogenesis. In our patients ^{18}F-FDG PET shows thalamic and cerebellar diaschisis. Both, the thalamus and the cerebellum may be involved in the pathophysiology of RLS.[5] Furthermore, stroke-related RLS after thalamic infarct have been reported,[5,6,7] and the involvement of pontocerebellar fibers has been suggested in the pathophysiology of the disease.[6] However, it should be emphasized that thalamic and cerebellar diaschisis is a non-specific well established finding after middle cerebral artery infarction.

More interestingly, we had opportunity to study how a focal lesion affects ipsilateral dopamine functioning in comparison to the other hemisphere, with the hypothesis that ipsilateral dopamine changes would be responsible for occurrence of RLS symptoms. Thus, healthy controls are not needed for this comparison between hemispheres. Stroke-related RLS is a rare comorbidity, and likely predisposing factors are required. This raises the question whether dopamine changes induced by a focal lesion are similar to the dopamine dysregulation responsible for the idiopathic form of RLS.

We found hyperdopaminergism in comparison to the non-lesioned side. The hypothesis of an increase in synaptic dopamine in subjects with primary RLS is also supported by a recent isotopic study on the striatal dopamine-2 (D2) receptor binding potentials.[8] The same group found that dopamine transporter was decreased in sriatum of idiopathic RLS patients.[9] Further evidence of a hyperdopaminergic state in RLS has emerged showing that dopamine

metabolites (3-ortho-methyldopa and homovanillic acid) in the CSF of these patients were increased, as well as post-mortem tyrosine hydroxylase in the substantia nigra.[10] In reference to the hypothesis of a hyperdopaminergic tone, efficacy with low doses of dopamimetics in RLS could be explained by stimulation of inhibitory D2-like autoreceptors.

The present findings suggest an ipsilateral striatal synaptic hyperdopaminergism as a compensatory mechanism in response to the stroke-induced neuronal loss. This mechanism might participate to RLS pathogenesis and should encourage further research on dopaminergic dysregulation associated with RLS.

(5) Acknowledgment:

We thank the sleep technicians who helped with scoring the polysomnography.

(6) Authors' Roles:

ER, MB, IJN and PB did the conception and the design of the study; ER, MB, IJN, LT, AH, LH, UKH were involved in the acquisition of data; ER, MB, IJN, LT, AH, LH, UKH, VW and PB did the analysis and interpretation of the data; ER, MB and JH did the drafting of the submitted material, PB did a critical revision of the submitted material.

(7) Financial Disclosures of all authors (for the preceding 12 months): None

(8)

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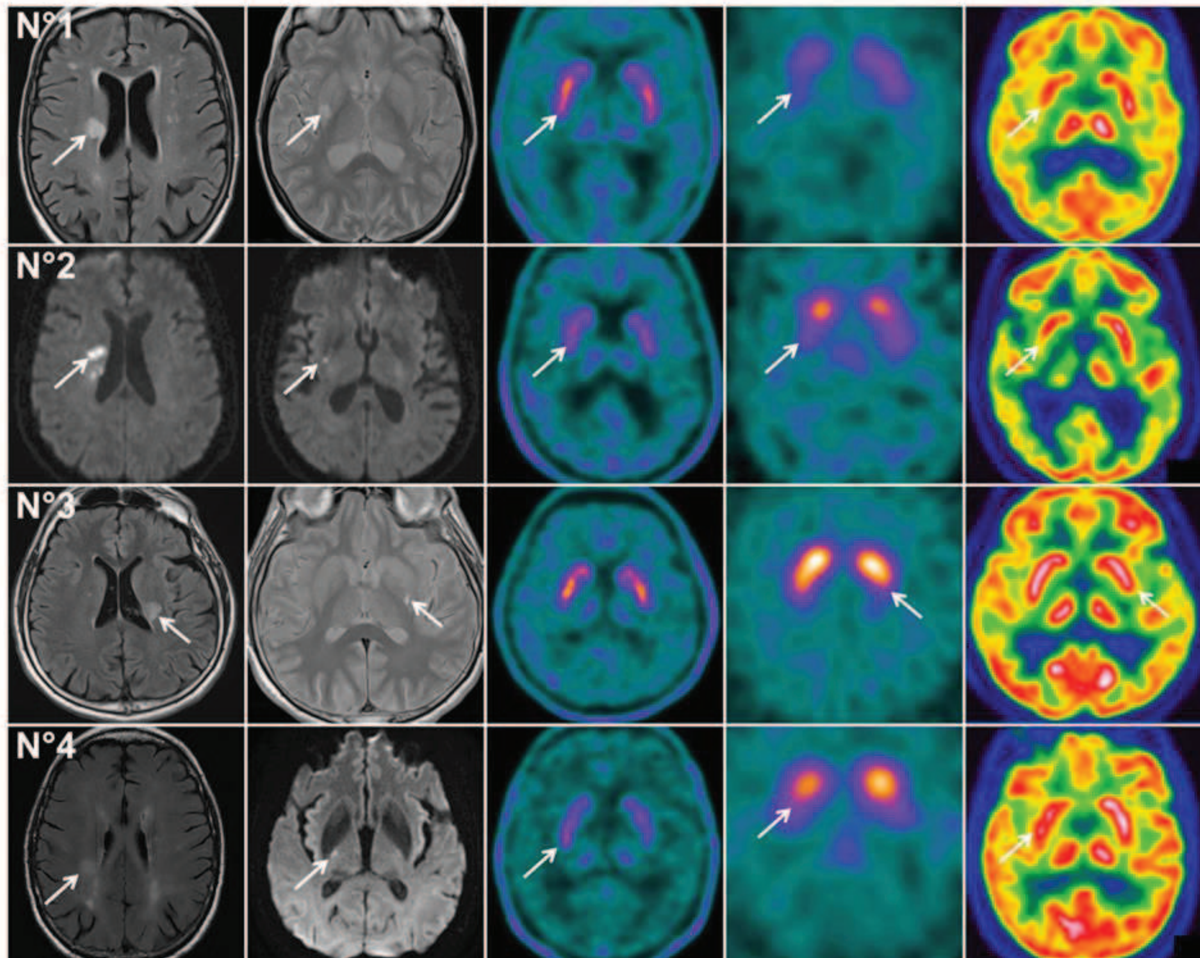
(9). Figures

Figure 1

Structural and functional brain imaging of the dopaminergic system

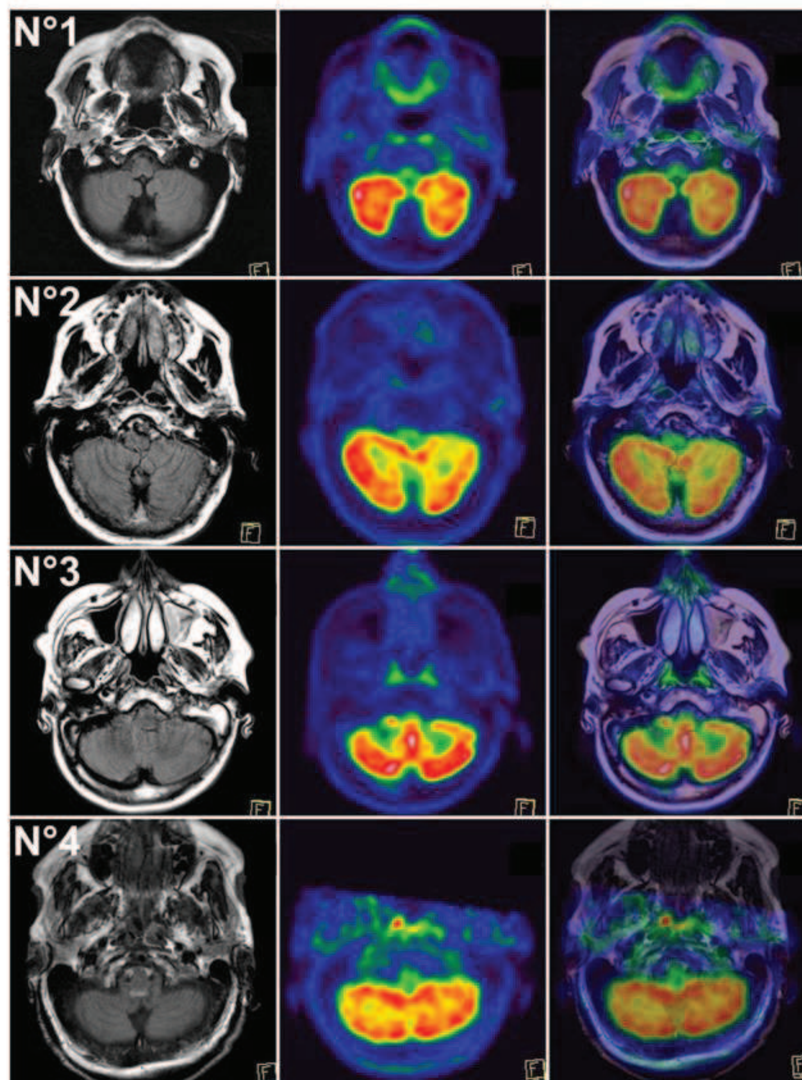


Legend to Figure 1.

Arrows: caudate nucleus (body)(1st column); lenticular nucleus using (N°1, 3) proton density weighted or (N°2, 3) diffusion sequences (2nd column); increased dopamine precursor using PET with ^{18}F-F-DOPA (3rd column) and decreased dopamine transporter using SPECT with ^{123}I-FP-CIT in putamen (4th column); hypometabolism in infarction area using PET with PET with ^{18}F-FDG (5th columns).

Figure 2

Structural and functional brain imaging of the cerebellum



Legend to Figure 2.

From left to right: transversal sections of structurally intact cerebellum in Flair sequences (1st column) and hypometabolism using PET with 18F-FDG in cerebellum contralateral to stroke (2nd column) or fusioned MRI / 18F-FDG PET imaging (3rd column).

DISCUSSION

Discussion

Le rôle crucial des effets directs de la lumière sur le cycle veille/sommeil

Selon le modèle classique, la régulation du sommeil est décrite comme étant sous l'influence de deux processus : circadien et homéostasique (Borbely 1982). La pression homéostasique du sommeil en condition standard de laboratoire chez des souris maintenues en cycle alternant 12 heures de lumière et 12 heures d'obscurité avec accès à la nourriture *ad libidum* est faible. L'animal n'ayant aucune contrainte externe se trouve en condition de routine constante lui permettant d'évacuer la pression de sommeil au fur et à mesure de son accumulation au cours du temps passé en état d'éveil.

Les résultats obtenus auprès des souris transgéniques ont permis de mettre en évidence un rôle crucial des effets directs de la lumière sur le cycle veille/sommeil. Par ailleurs, ils impliquent fortement la mélanopsine, notamment pendant la période d'obscurité subjective. En période lumineuse subjective les cônes et les bâtonnets jouent également un rôle important. Enfin, les NSC sont une structure de relai pour les effets directs de la lumière, au-delà de leur fonction d'horloge biologique principale.

Dans les effets de la lumière sur le sommeil, la phototransduction passe par les ipRGC (soit via la mélanopsine, soit en relayant l'information provenant des cônes et des bâtonnets), mais également directement via les cônes et les bâtonnets. Par la suite, le signal lumineux passe à 77% par les NSC dont 55% font intervenir le processus circadien et 22% sont liés à des effets directs de la lumière. En plus, 45% du sommeil sont expliqués par des effets directs de la lumière et pour lesquels 36% impliquent la mélanopsine.

Les effets directs de la lumière constituent donc un mécanisme majeur de régulation du cycle veille/sommeil en condition standard alternant 12 heures de lumière et 12 heures d'obscurité. Dans ces conditions, les effets directs de la lumière expliquent pour presque la moitié (45%) le cycle veille-sommeil, le restant étant expliqué par les effets circadiens de la lumière (55%).

Ces différents résultats nous permettent de conclure que les effets directs de la lumière sont un mécanisme majeur de régulation du sommeil. Ainsi, l'évolution du

modèle classique à deux processus vers un modèle plus complet à trois processus, élargi aux effets directs de la lumière, est proposé.

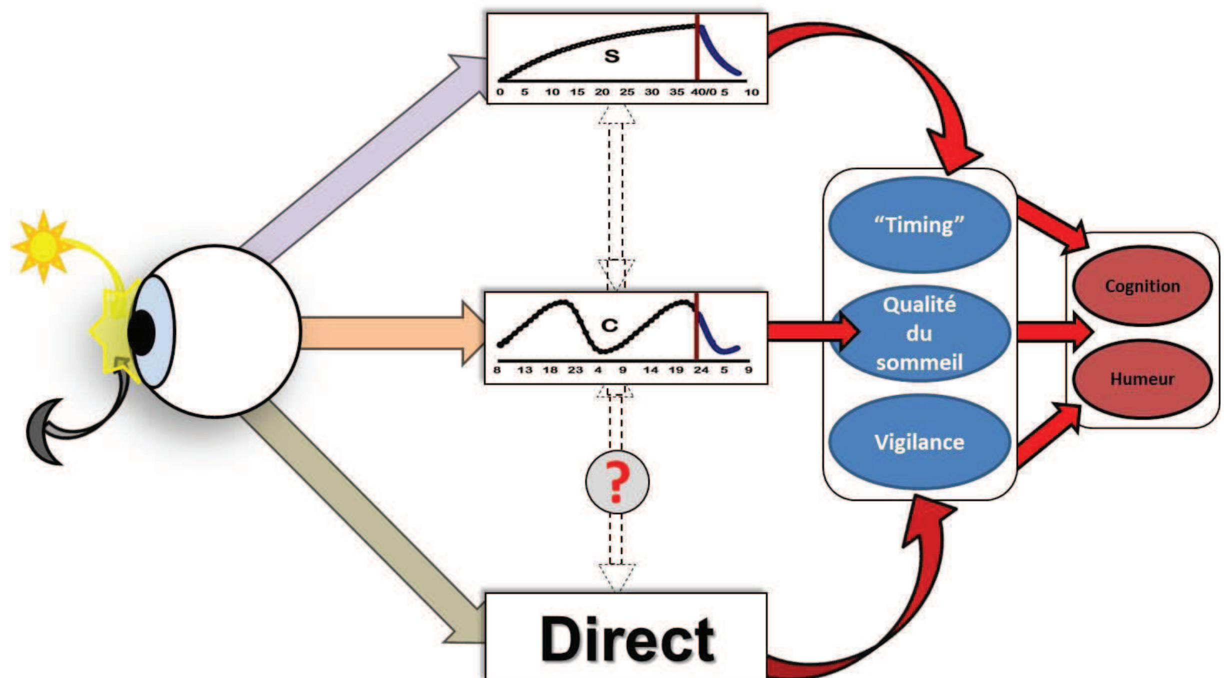


Figure 27 : La régulation du sommeil conceptualisée sous la forme d'un modèle à trois processus.

Les interactions complexes entre les différents processus de régulation du sommeil

Il existe une interaction importante entre les effets directs de la lumière et le processus homéostasique du sommeil. La mélanopsine, outre son rôle de photopigment est également un facteur homéostasique du sommeil. Les souris ayant une invalidation du gène de la mélanopsine ont une pression homéostasique du sommeil altérée, que ce soit en condition habituelle ou après une privation de sommeil.

Le rôle de la mélanopsine dans l'interaction entre les effets directs non circadiens de la lumière et le processus homéostasique du sommeil a été précisé davantage. Lors des expériences supplémentaires, des souris des deux génotypes (*Opn4*$^{+/+}$, *Opn4*$^{-/-}$) ont été enregistrées pour le sommeil dans des conditions de luminosité différentes (<10 lux ; 150 lux ; 600 lux) avec mise au défi de leur homéostat par des privations de 6 heures de sommeil. Les résultats (Annexe 1) montrent que la mélanopsine impacte le processus

homéostasique du sommeil. À l'inverse, il reste inconnu si la sécrétion de la mélanopsine est influencée par l'homéostasie du sommeil. Des expériences sont actuellement en cours dans notre équipe afin de répondre à cette question. Selon les données électrophysiologiques, la mélanopsine n'influence pas la régulation circadienne du sommeil. La sécrétion de la mélanopsine est contrôlée par le processus circadien, en plus d'une régulation photique

Dans ce jeux d'interactions complexes, l'implication des NSC dans l'homéostasie du sommeil a été étudiée en réalisant des expériences de privations de sommeil chez des souris lésées (complètement ou partiellement) au niveau des NSC avec analyse électrophysiologique du sommeil et études moléculaires de différents gènes impliqués dans les processus circadiens et homéostasiques du sommeil (Annexe 2). Les résultats mettent en évidence, que les NSC sont également impliqués dans le processus homéostasique du sommeil, en plus de leur rôle dans le processus circadien et dans les effets directs de la lumière sur le sommeil.

Les structures impliquées dans les effets directs de la lumière sur le cycle veille/sommeil

La démarche de ma thèse était d'identifier les structures anatomiques et les voies neuronales impliquées dans les effets directs de la lumière. Ces derniers dépendent majoritairement de la mélanopsine, mais les cônes et les bâtonnets sont également impliqués. Les NSC, en dehors de leur rôle d'horloge sont également une structure clef relayant les effets directs de la lumière aux réseaux neuronaux contrôlant les états de la vigilance. L'induction du sommeil se fait par le biais d'une activation des neurones « inducteurs de sommeil » au niveau du VLPO.

Les effets directs de la lumière sont inverses chez les espèces nocturnes comparées aux diurnes en induisant le sommeil chez les uns et en rendant les autres plus alertes. Chez le rongeur nocturne, l'induction du sommeil par la lumière est influencée par la mélanopsine et passe par le biais d'une activation des neurones « inducteurs de sommeil » du VLPO. Le cycle veille/sommeil résulte d'une inhibition réciproque entre les centres de régulation du sommeil, notamment le VLPO et les systèmes monoaminergiques de l'éveil (cf introduction).

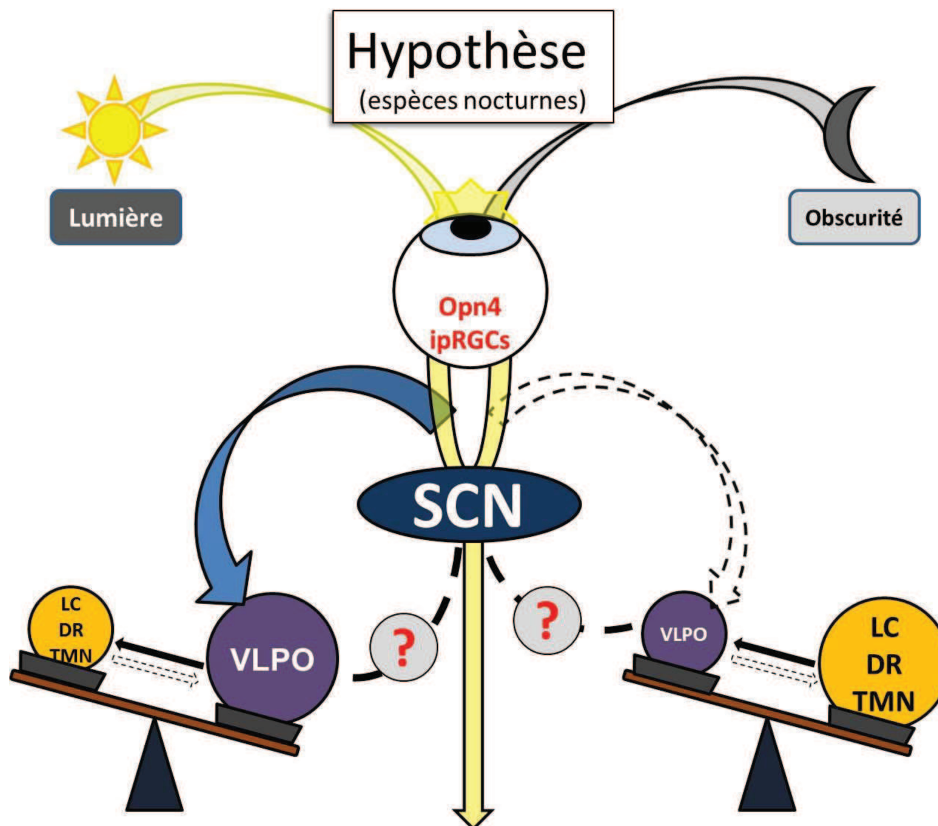


Figure 28 : Hypothèses concernant les effets différentiels de la lumière et de l'obscurité en fonction de la diurnalité ou nocturnalité de l'espèce.

LC : locus coeruleus (noradrénergique) ; DR : noyau du raphé dorsal (sérotoninergique) ; TMN : noyaux tubéromamillaires (histaminergiques).

Le rôle des effets directs de la lumière sur l'humeur

Outre son implication dans la régulation du cycle veille/sommeil, la lumière influence de nombreux autres comportements dont l'humeur. Le sommeil et l'humeur sont des comportements ayant de très fortes interactions fonctionnelles. La majorité des sujets insomniaques présentent un trouble de l'humeur. A l'inverse, la plupart des patients souffrant de dépression ont également des perturbations de l'initiation et du maintien du sommeil.

L'humeur, comme le sommeil, est sous le contrôle de processus circadien et homéostasique puisqu'elle est fortement influencée par une interaction complexe et non-additive de la phase circadienne et du temps passé éveillé. Alors qu'une privation

chronique de sommeil est globalement dépressogène, la privation aiguë de sommeil est utilisée en psychiatrie pour ses effets antidépresseurs.

L'étude comportementale chez des souris des deux génotypes (*Opn4*$^{+/+}$, *Opn4*$^{-/-}$) en fonction de différentes conditions de luminosité (<10 lux ; 150 lux ; 600 lux) a mis en évidence un effet bénéfique d'une forte luminosité et une apparition en cas de faible luminosité d'un phénotype anhédonique et « depression-like » (Annexe 3). Ses résultats ont été obtenus en dehors de toute modification de la photopériode ou du rythme circadien. Les rongeurs nocturnes ayant globalement un comportement de photophobie, on aurait pu s'attendre à un effet plutôt dépressogène et anxiogène de la lumière. De même, les effets directs de la lumière sur le sommeil sont inverses en fonction de la diurnalité/nocturnalité. Or, il n'était pas établi antérieurement si cette inversion existait également au niveau de l'humeur et de l'anxiété.

L'état « depression-like » observé chez les souris en condition de faible luminosité a été réversible à la suite de l'administration de fluoxétine, un inhibiteur de la récapture de la sérotonine. Devant les interactions entre le sommeil et l'humeur, les effets antidépresseurs d'une privation de sommeil ont été étudiés. Cependant, aucune réversibilité du trouble de l'humeur induit n'a été obtenue suite à l'application de la privation de sommeil (résultats de l'équipe). Il reste débattu à quel point la modulation de l'humeur par les effets directs de la lumière observée dans notre condition expérimentale est indépendante ou passe par un effet direct de la lumière sur le sommeil. Une interaction dans le sens inverse, c.à d. une amélioration du sommeil par le biais d'un effet direct de la lumière sur l'humeur est également envisageable.

Ces résultats sont particulièrement importants. Ils permettent, d'une part, de clarifier le rôle des effets directs de la lumière sur l'humeur au regard des données de la littérature, en particulier du groupe de Hattar et collaborateurs, qui suggère que les effets directs de la lumière détériorent la qualité de l'humeur lorsque celle-ci est administrée de manière «aberrante » (LeGates, Altimus et al. 2012; LeGates, Fernandez et al. 2014). D'autre part, ils permettent d'envisager que la luminothérapie puisse exercer un effet antidépresseur dans différents sous-types de troubles de l'humeur via des effets directs non-circadiens.

Le système dopaminergique constitue-t-il une cible pour les effets directs de la lumière ?

Le traitement par la luminothérapie est bien validé dans la dépression, surtout dans la dépression saisonnière. Les études qui cherchent à élargir ses indications sont nombreuses comme par exemple au trouble de déficit d'attention avec ou sans hyperactivité (TDAH) (Gruber, Grizenko et al. 2007), au trouble du comportement alimentaire (Daansen and Haffmans 2010) ou encore à la maladie de Parkinson (Rutten, Vriend et al. 2012). Il s'agit de pathologies qui peuvent avoir en commun une certaine implication du système dopaminergique. Ce neurotransmetteur intervient dans la modulation d'un nombre important de comportements et il est également une cible du signal lumineux non-visuel (cf introduction).

Le renforcement du tonus dopaminergique peut avoir lieu à deux niveaux, soit dans la rétine, soit dans les structures cérébrales. Dans la maladie de Parkinson, on observe une dégénérescence des neurones dopaminergiques de manière globale, y compris au niveau de la rétine (Harnois and Di Paolo 1990). Toutefois, l'atteinte rétinienne semble nettement moins marquée comparée à celle qui existe au niveau des locus niger. Même s'il est bien établi que le tableau neurologique observé est en lien avec une atteinte des ganglions de la base, il reste possible qu'une réduction de la modulation du signal lumineux par le système dopaminergique au niveau de la rétine soit également impliquée dans les symptômes non-visuels de la maladie de Parkinson. Il n'existe pas de données quant à une atteinte dopaminergique rétinienne dans les autres maladies où ce neurotransmetteur est impliqué comme le syndrome des jambes sans repos (SJSR), la dépression ou encore la schizophrénie.

Dans la troisième partie, l'étude des effets directs de la lumière sur les comportements a été ciblée auprès de populations de patients présentant soit une maladie de Parkinson, soit un syndrome des jambes sans repos.

L'étude concernant les effets directs de la lumière chez des patients atteints de maladie de Parkinson étant encore en cours, il est trop tôt pour se prononcer sur d'éventuels résultats. Devant l'absence d'outil de mesure et de groupe contrôle idéal, il ne sera toutefois pas possible par cette approche clinique de préciser clairement l'implication du système dopaminergique dans ces effets directs de la lumière.

L'hypothèse formulée est celle d'une amélioration des symptômes moteurs et non-moteurs secondaire à un renforcement du tonus dopaminergique par la lumière.

Quant au SJSR, l'utilisation de la luminothérapie permettait d'améliorer le sommeil des patients via des effets non-circadiens et indépendamment de la sévérité de leurs impatiences qui reste inchangée. Les difficultés majeures de la recherche dans le SJSR concernent l'évolution spontanément capricieuse de la symptomatologie et l'importante hétérogénéité du tableau clinique (cf introduction). Compte tenu d'un effectif réduit de cette étude pilote (N=24 patients) et d'une importante variabilité intra- et intersujet des variables mesurées, il est nécessaire de confirmer ces résultats auprès d'une plus grande population.

L'absence d'amélioration de la sévérité des impatiences est un argument plutôt en défaveur d'une implication du système dopaminergique dans les effets directs de la lumière. Cependant, le rôle de la dopamine dans cette pathologie n'étant pas clair (cf introduction), il est trop tôt pour en tirer des conclusions.

Des recherches supplémentaires sur les bénéfices de la luminothérapie dans les différentes pathologies sont nécessaires pour approfondir nos connaissances sur son efficacité et les mécanismes impliquées. La luminothérapie pourrait constituer une thérapeutique innovante et les avancées permettront peut-être d'ouvrir des pistes sur le (ou les ?) système(s) neuronal (aux) impliqué(s) dans les effets directs de la lumière sur les différents comportements.

La diurnalité et la régulation crépusculaire du cycle veille/sommeil

Le sommeil fait partie des fonctions vitales de l'organisme. La plupart des études sur la régulation du sommeil utilisent des souris et des rats, rongeurs nocturnes et qui ont des réponses inversées concernant la lumière comparés à l'homme, diurne. Dans la deuxième partie, nous avons caractérisé le sommeil chez *Arvicanthis ansorgei*, un rongeur diurne. Disposer d'un modèle de rongeur diurne est une étape importante dans l'approche translationnelle de l'animal à l'homme pour l'étude des effets directs de la lumière sur les comportements, dont le sommeil et la veille.

Le sommeil chez *Arvicanthis ansorgei*, celui des rongeurs nocturnes et celui de l'homme sont globalement sous les mêmes mécanismes de régulation. Cependant, il

existe des différences assez importantes dans l'organisation du cycle veille/sommeil, certaines composantes de la régulation pouvant prédominer en fonction de l'espèce.

Chez l'homme, le sommeil est consolidé sur la période nocturne avec une composante circadienne très importante. Il est possible que ce profil particulièrement diurne soit favorisé par une organisation sociale qui exige un maintien soutenu de l'éveil tout au long de la période lumineuse, permettant une importante augmentation de la pression homéostasique du sommeil. Dans certaines populations spécifiques, on observe l'apparition d'un profil bimodal. Ainsi, la réalisation d'une sieste prolongée l'après-midi est habituelle dans la population méditerranéenne et les régions de fortes chaleurs. Les travailleurs de nuit font souvent des siestes avant et après la prise de poste. Enfin, les moines et les sœurs qui assistent à l'office des Matines ont un lever nocturne prolongé avec une organisation bimodale de leur rythme veille/sommeil, mais qui concerne également d'autres rythmes biologiques comme la température corporelle (Arnulf et al.).

Le rythme veille/sommeil chez *Arvicanthis ansorgei* montre une régulation crépusculaire prédominante. Ce rythme crépusculaire (il persiste en condition d'obscurité constante) est à l'origine d'une organisation bimodale du cycle veille/sommeil avec une période se rapprochant plus d'un cycle de 12 que de 24 heures. Ce caractère bimodal semble plus marqué en l'absence de certains *Zeitgebers* tel une cage équipée d'une roue. La composante crépusculaire peut également être plus marquée chez certaines espèces de rongeurs nocturnes. Les mécanismes sous-jacents à cette régulation crépusculaire restent inconnus. On discute actuellement si l'homme garde une tendance à la régulation crépusculaire, le moment le plus propice au sommeil se situant au milieu de la période d'obscurité et la qualité de l'éveil étant au plus bas en postprandial vers le milieu de la période lumineuse. Il est envisageable que la composante crépusculaire de la régulation du cycle veille/sommeil ait été perdue au cours du développement de l'homme, compte tenu du fonctionnement de notre société. Un rythme bimodal peut (encore ?) être retrouvé dans des populations spécifiques et leur permettant une meilleure adaptation de l'homme à des conditions écologiques particulières, telles des fortes chaleurs.

Concernant la régulation homéostasique du sommeil, en l'absence de privation de sommeil imposée, le rongeur a tendance à céder à la pression homéostasique du

sommeil au fur et à mesure qu'elle augmente. Ceci résulte en des périodes de veille et de sommeil peu consolidées en condition de laboratoire.

Chez les animaux explorés en laboratoire, on ne connaît que peu de choses sur leur comportement veille/sommeil en milieu de vie habituel. Une étude réalisée chez le Paresseux Tridactyle De Bolivie (*Bradypus variegatus*) a mis en évidence un temps de sommeil très nettement diminué en milieu naturel (9.63h) comparé à des conditions de laboratoire (~16h) (Rattenborg, Voirin et al. 2008). Cet allongement du temps de sommeil en condition de laboratoire peut faire suite à l'absence des stimulations auxquelles l'animal est habituellement exposé dans son environnement: l'activité physique soutenue, la recherche de nourriture, le danger d'un prédateur, la défense de leur territoire, la recherche de partenaires etc. Le déconditionnement provoqué par la captivité en cage du rongeur avec accès à la nourriture *ad libidum*, lui permet de se laisser aller à la sieste dès que la pression de sommeil augmente. On observe chez l'homme, qu'un temps passé allongé au-delà du besoin de sommeil, est source de difficultés du maintien du sommeil. L'allongement du temps passé au lit ne permet pas l'accumulation de pression homéostasique suffisante pour permettre la consolidation du sommeil. De même, en cas d'insomnie avec des difficultés de maintien du sommeil, la mesure consistant à imposer au patient une restriction du temps de sommeil fait partie des techniques utilisées en thérapeutique. En parallèle, on demande aux patients de renforcer les *Zeitgebers*. L'animal maintenu en laboratoire standard se trouve en condition de routine constante, ni son rythme circadien, ni sa pression homéostasique de sommeil ne sont renforcés.

Les souris habituellement enregistrées diffèrent d'*Arvicanthis ansorgei* par l'absence de mélatonine, cette dernière étant déficiente chez la plupart des souris de laboratoire (Kasahara, Abe et al. 2010). L'importance de la mélatonine dans la régulation du sommeil et dans les effets directs de la lumière sur le cycle veille/sommeil chez ce muridé diurne nécessite d'être explorée par des recherches supplémentaires. La régulation de la mélatonine est très similaire chez *Arvicanthis ansorgei* comparée au rat avec un pic de la sécrétion de la mélatonine survenant 9 heures après le début de la période lumineuse versus 7 heures pour le rat Wistar (Garidou, Gauer et al. 2002).

Malgré leur organisation du sommeil et de la veille différente au cours du nycthémère, le rongeur reste un modèle intéressant et très utilisé pour étudier le

sommeil. *Arvicanthis ansorgei* est un modèle de rongeur diurne qui diffère du rongeur nocturne par une composante crépusculaire particulièrement marquée. Néanmoins, ce modèle diurne reste valide. Des données de notre équipe montrent chez ce muridé qu'une exposition lumineuse en période d'obscurité subjective permet d'augmenter des corrélats électrophysiologiques de la qualité de l'éveil. Ainsi, *Arvicanthis ansorgei* est un modèle prometteur pour étudier les effets directs de la lumière sur la veille et le sommeil et pour mieux comprendre le switch de la nocturnalité/diurnalité.

Quelques perspectives futures pour avancer dans notre compréhension sur les structures et systèmes impliquées dans les effets directs de la lumière

Les mécanismes sous-jacents à ces effets inverses en fonction de la diurnalité/nocturnalité restent à être mieux précisés. En cas d'un interrupteur (ou « switch ») global, il serait tentant de le situer au niveau de la rétine. Une modulation très complexe du signal lumineux a lieu au niveau de la rétine avant que l'information lumineuse, visuelle et non-visuelle, ne soit distribuée aux nombreuses structures cérébrales. Par ailleurs, les rétines de rongeurs sont structurellement et fonctionnellement différentes en fonction de la diurnalité ou nocturnalité de l'espèce (Bobu, Craft et al. 2006). Ces considérations font de la rétine une cible privilégiée pour moduler le transfert de l'information lumineuse aux différentes structures cérébrales.

Toutefois, en aval de la rétine, que ce soit au niveau des NSC ou au niveau de la sécrétion de la mélatonine par la glande pinéale, on n'observe que peu de différences entre les rongeurs diurnes et nocturnes (Challet, Pitrosky et al. 2002; Caldelas, Poirel et al. 2003; Dardente, Menet et al. 2004; Cuesta, Clesse et al. 2009). De même, on peut imaginer que les effets directs de la lumière n'aient pas d'effet inverse sur tous les comportements. Ainsi l'humeur est améliorée par les effets non-circadiens de la lumière, indépendamment de la diurnalité/nocturnalité de l'espèce étudiée (souris nocturne et homme diurne). On pourrait alors s'imaginer que le « switch » ne soit pas global. Ainsi, il est possible qu'il existe un interneurone inhibiteur, interposé entre le signal lumineux et la structure cible, permettant de moduler la réponse dans un sens ou dans l'autre en fonction du profil diurne ou nocturne de l'espèce et du comportement étudié. Cette possibilité laisse davantage de flexibilité au système permettant d'intégrer également d'autres paramètres extérieurs (température, stress en lien avec un prédateur,

accessibilité à la nourriture…) et de dissocier au besoin des comportements associés en condition classique comme l'horloge alimentaire et l'activité locomotrice.

Une première approche permettant de fournir des éléments supplémentaires concerne les effets de la lumière et de l'obscurité sur le VLPO chez l'arvicanthis, rongeur diurne. Cette étude est actuellement en cours. D'autres études envisagées concernent l'analyse des structures monoaminergiques de l'éveil en fonction des conditions lumineuses, chez la souris, rongeur nocturne et chez l'arvicanthis, rongeur diurne.

Les modèles animaux permettront de mieux caractériser le rôle du système dopaminergique dans les effets directs de la lumière. Ainsi, dans la continuité des données issues de la recherche clinique, il serait intéressant d'étudier chez des souris transgéniques (*Opn4*$^{+/+}$ et *Opn4*$^{-/-}$) une induction d'un marqueur d'activation neuronale, par exemple c-Fos, au niveau de la substance noire, particulièrement touchée dans la maladie de Parkinson ou dans l'aire A11, zone impliquée dans le SJSR. Une approche comportementale pourrait être réalisée auprès des modèles de rongeur pour la maladie de Parkinson [induction toxique de la maladie par le MPTP (Sundstrom, Fredriksson et al. 1990)] ou pour le SJSR [modèle avec carence martiale chronique et lésion de l'aire A11 (Qu, Le et al. 2007)].

Concernant les retentissements sur l'humeur de l'ambiance lumineuse, la nature des structures anatomiques et voies neuronales impliquées reste hypothétique. Le noyau du raphé dorsal, structure sérotoninergique et à moindre degré dopaminergique, est un candidat intéressant. Cette structure qui intervient dans l'humeur, est une cible des projections des ipRGC et projette également sur les NSC (Morin 2013).

Concomitamment avec notre article *(Tsai, Hannibal, Hagiwara, Colas, Ruppert, Ruby, Heller, Franken, Bourgin: Melanopsin as a sleep modulator: circadian gating of the direct effects of light on sleep and altered sleep homeostasis in Opn4-/- mice. PLoS Biology, 2009)*, deux autres équipes ont publié leurs données concernant les effets directs mélanopsine-dépendants sur le rythme veille/sommeil (Altimus, Guler et al. 2008) ou sur le rythme activité locomotrice/repos (Lupi, Oster et al. 2008) avec des résultats similaires. Parallèlement à l'avancement des expériences concernant l'article *(Hubbard, Ruppert, Frisk, Tsai, Robin-Choteau, Husse, Calvel, Eichele, Franken, Bourgin: A model to predict how (melanopsin-dependent) lighting shapes the sleep-wake cycle.)*, dans un travail de revue de la littérature, je me suis intéressée aux réseaux neuronaux impliqués dans ces effets directs de la lumière qui constituent un important mécanisme de régulation du sommeil chez le rongeur nocturne et améliorent chez l'homme la qualité de l'éveil *(Hubbard, Ruppert, Gropp, Bourgin: Non-circadian direct effects of light on sleep and alertness: lessons from transgenic mouse models. Sleep Medicine Reviews, 2013)*. Cette analyse et les données précisant le rôle des NSC dans les effets directs de la lumière m'a permis de proposer un modèle pour expliquer les voies neuronales impliquées par la lumière et par l'obscurité pour influencer la balance de l'éveil et du sommeil (ou « flip/flop switch » Saper, Scammel et al. 2005).

Article 5***: «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. Taking these data into account we therefore propose a novel model of sleep regulation based on three 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.

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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

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Abbreviations

5-HT	serotonin
ADTA	ablated diphtheria toxin-A
ANOVA	analysis of variance
CNGA3	cyclic nucleotide-gated channel alpha 3
EEG	electroencephalogram
fMRI	functional magnetic resonance imaging
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
OPN	olivary pretectal nucleus
Opn4	melanopsin photopigment
Opn4	opsin-4 (melanopsin)
OPT	olivary pretectum
PER3	period circadian protein homolog 3
PET	positron emission tomography
rd/rd cl	rodless/coneless
REM	rapid eye movement
RGC	retinal ganglion cell
SC	superior colliculus
SCN	suprachiasmatic nuclei
SPVZ	subparaventricular zone
VLPO	ventrolateral preoptic nucleus
WT	wild type
ZT	zeitgeber time

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-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 regard 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 raise 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

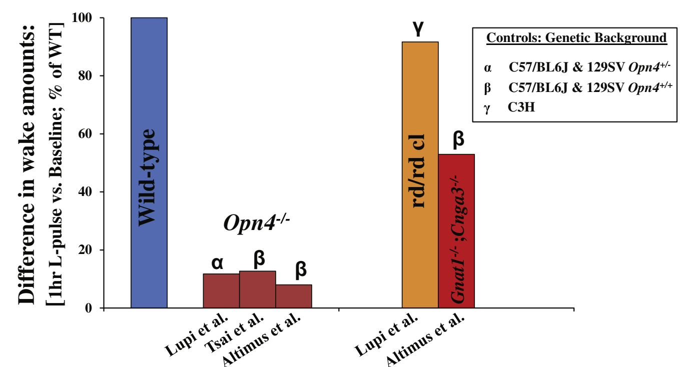

Fig. 1. Differences between studies in wake amounts during a 1-h light pulse administered at the same zeitgeber time (ZT) (early part of the dark phase). Effect of a 1-h light pulse administered at ZT14, ZT15, or ZT16 on wake suppression in different transgenic mouse models (maintained under 12-h:12-h 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 wild-type (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.

intensities and spectral compositions and administered the light pulse at approximately the same zeitgeber 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, 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 had 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 effective 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 is that it 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 no 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 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.

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.

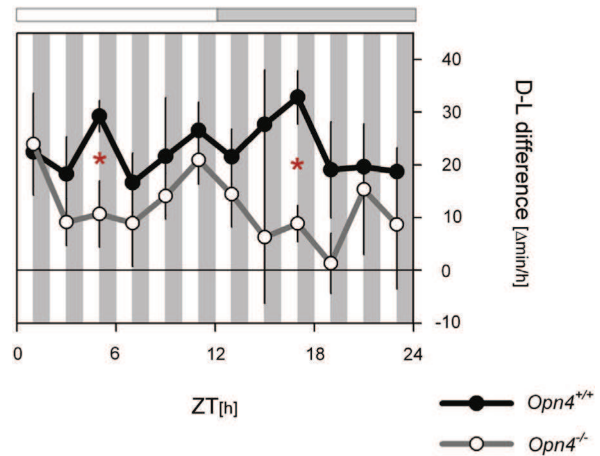

Fig. 2. Time of day influence on the direct photic input to sleep and waking. Dark-light difference in *Opn4*$^{-/-}$ and *Opn4*$^{+/+}$ under a 1 hL:1 hD cycle for 24-h. Data represent the change in wake amounts (min) between 1 h dark and 1 h light pulses for each cycle across the 24-h of the 1 hL:1 hD ultradian cycle. Vertical lines represent s.e.m. Data were extrapolated from Tsai et al.[30] Analysis of variance (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).

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 a similar type of analysis in these other transgenic models may clearly illustrate the underlying 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 these 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 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 sleep 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 period circadian protein homolog 3 (*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 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 sub-paraventricular 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 functional magnetic resonance imaging (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 positron emission tomography (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.

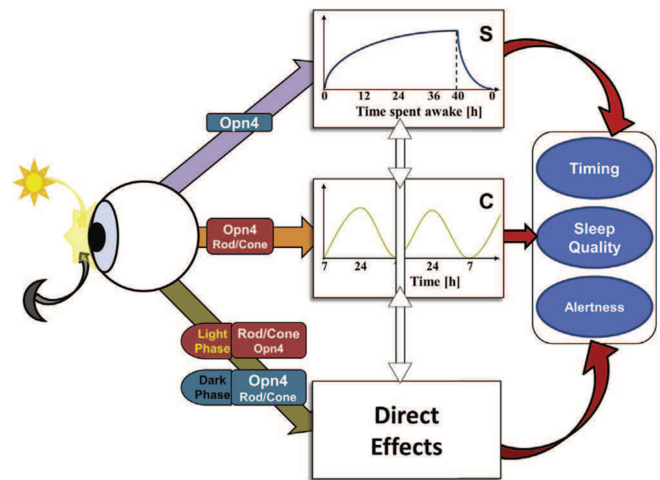

Fig. 4. Schema proposing a three-process model for sleep and wake regulation. Light and darkness exert a more complex role through a direct acute and sustained influence on the expression of sleep and waking as well as indirectly through the entrainment of the circadian timing system. Additionally, the 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.

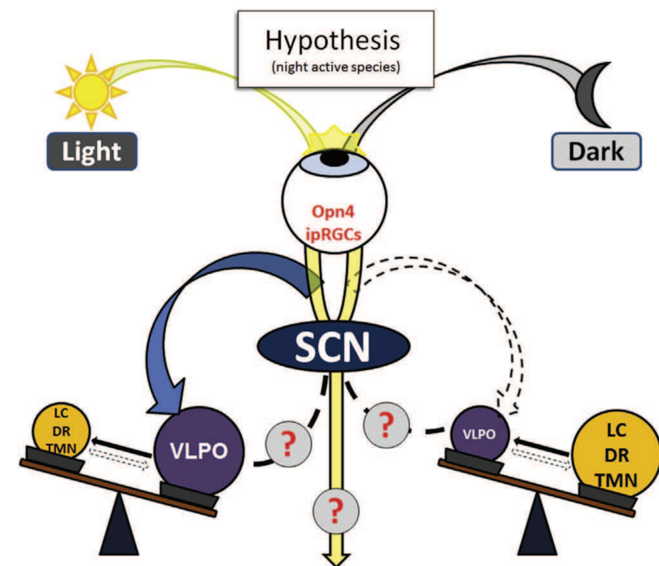

Fig. 3. A model in which the direct effects of light and darkness impinge on the balance of the flip-flop switch to promote sleep or waking. Here, the model is described for nocturnal rodents, with the influence of light and darkness on the flip-flop switch being reversed in diurnal species. The flip-flop 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); SCN: suprachiasmatic nucleus; TMN: tuberomammillary nucleus (histamine).

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 two-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

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).

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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CONCLUSION

Conclusion

La lumière exerce des effets directs sur de nombreux comportements et les données chez les rongeurs mettent en évidence que ces effets dépendent de la mélanopsine. Dans ce travail de thèse, je me suis particulièrement intéressée aux structures et systèmes neuronaux impliqués dans ces effets directs de la lumière.

Ces résultats ont permis d'identifier l'importance de la mélanopsine dans les effets directs de la lumière sur le cycle veille/sommeil et sur l'humeur. Par ailleurs, les NSC au-delà de leur fonction d'horloge biologique principale, jouent également un rôle crucial dans les effets non-circadiens directs de la lumière sur le sommeil. En plus, l'induction du sommeil par les effets directs de la lumière se fait par le biais d'une activation des neurones galaninergiques « inducteurs de sommeil » au niveau du VLPO.

L'identification des voies neuronales impliquées chez l'homme est plus délicate. Nous ne disposons pas de techniques qui soient bien validées pour explorer les différentes composantes mises en évidence au cours des expériences chez l'animal. De plus, les effets directs de la lumière sur le sommeil sont inverses en fonction de la nocturnalité/diurnalité. Pour s'affranchir ce cette problématique, nous avons caractérisé le cycle veille/sommeil chez *Arvicanthis ansorgei.* Ce rongeur diurne est un modèle intéressant dans l'étude de la nocturnalité/diurnalité,

La dopamine est un candidat intéressant pour intervenir dans les effets directs de la lumière. Ce neurotransmetteur intervient dans la modulation d'un nombre important de comportements et il est possiblement impliqué dans différentes pathologies qui semblent sensibles à la lumière. Par ailleurs, sa sécrétion peut être favorisée par une stimulation photique (rétinienne ou cérébrale).

Une meilleure compréhension des effets directs de la lumière et des mécanismes de régulation du sommeil permet de donner un cadre conceptuel pour avancer dans la compréhension de la pathogénèse des maladies du sommeil, mais également d'avancer dans la compréhension de certains troubles neuropsychiatriques. L'humeur, l'anxiété ou encore la cognition ont des interactions fortes avec le sommeil et constituent également des cibles du signal lumineux. La caractérisation des effets directs de la lumière permet de redéfinir les indications de la luminothérapie qui sont actuellement basées sur les effets circadiens de la lumière.

Des études cliniques avec des protocoles chronobiologiques sont prévus avec l'ouverture de la structure CIRCSom (CNRS / CHRU Strasbourg) fin 2014. Ces protocoles permettent de distinguer les processus C et S de la régulation du sommeil, ainsi que les effets directs de la lumière. Un premier projet permettant de caractériser chez le sujet sain les trois processus de régulation du sommeil circadien, homéostasique et effets directs de la lumière, est prévu. En perspective, leur rôle en pathologie du sommeil et en neuropsychiatrie sera analysé.

Enfin, la lumière artificielle est omniprésente. Alors qu'une exposition appropriée à la lumière est bénéfique pour les différents paramètres physiologiques et comportements, une exposition inappropriée peut être délétère. Mieux comprendre les effets directs de la lumière permettra une meilleure utilisation de l'éclairage artificiel, que ce soit au domicile, au lieu de travail ou dans des conditions particulières tels le travail posté ou le « jet lag ».

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ANNEXES

Article 6: *« Characterization of periodic upper limb movement disorder in a patient with restless arms syndrome»*

Movement Disorders, 2012

Article: pages 1-2

Characterization of Periodic Upper Limb Movement Disorder in a Patient With Restless Arms Syndrome

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ABSTRACT

Background: Restless legs syndrome (RLS) and the frequently associated periodic limb movements (PLM) are common neurological disorders whose pathophysiology remains elusive. We report on the case of a 40-year-old patient presenting with severe restlessness in the upper limbs, a poorly known variant of RLS.
Case Report: Video-polysomnography was performed because of the associated poor sleep quality and daytime sleepiness evocative of PLM. An electromyogram of the extensor carpi radialis muscle was added. Remarkably, our patient had movements of repeated extension of the small finger that contrasted with the extension of the hallux, characteristic for PLM. Pramipexol was an effective treatment relieving the patient's upper limbs of discomfort and ameliorating her restless sleep.
Conclusions: Involvement of the upper limbs in RLS is relatively common, but restlessness may be located on the upper limbs solely. One should be aware of the upper limb variant, and that treatment by dopaminergic agonists proves to be very efficient. ©2012 *Movement* Disorder Society
Key Words: periodic limb movement; restless arms syndrome; restlessness of upper limbs; restless limbs disorder; periodic arm movements

Additional Supporting Information may be found in the online version of this article.
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Restless legs syndrome (RLS) and the frequently associated periodic limb movements (PLM) are highly prevalent neurological disorders whose pathophysiology remains elusive.[1] We report on the case of a patient presenting with restlessness in the upper limbs, a poorly known variant of RLS.[2–4] Remarkably, our patient had movements of extension of the small finger that, in a way, mimic periodic movements of the legs, but also contrast with it because PLM are characterized by the extension of the hallux, and not of the little toe.

Case Report

A 40-year-old woman complained of major upper limb discomfort and poor sleep quality that had appeared 5 years earlier. She suffered from pins and needles in her arms, predominantly distal, causing a severe discomfort with an urge to move her upper limbs, which gave her relief. These symptoms appeared at rest and evolved with a striking circadian rhythmicity, fulfilling the criteria for restless "arms" syndrome. Symptoms remained isolated to the upper limbs for 3.5 years before involving the lower limbs, causing minor discomfort in the legs. Iron supplementation and dopaminergic agonist treatment (pramipexol: 0.27 mg/day) relieved the patient's upper limb discomfort and the associated insomnia, which consisted of delayed sleep onset and nocturnal awakenings. Because she continued complaining of restless sleep and daytime sleepiness, arousal-associated PLM were suspected.

The patient's medical history revealed a status of focal epilepsy with periventricular lesions resulting from perinatal complications. Epilepsy was well controlled with low doses of oxcarbazepine (600 mg/day). On examination, she presented a mild right hemiparesis.

Three nights of video-polysomnography (VPSG) were conducted to characterize the patient's sleep architecture and record limb movements: 1 night with the patient already treated with dopaminergic agonist; 1 night after treatment withdrawal; and, last, 1 night

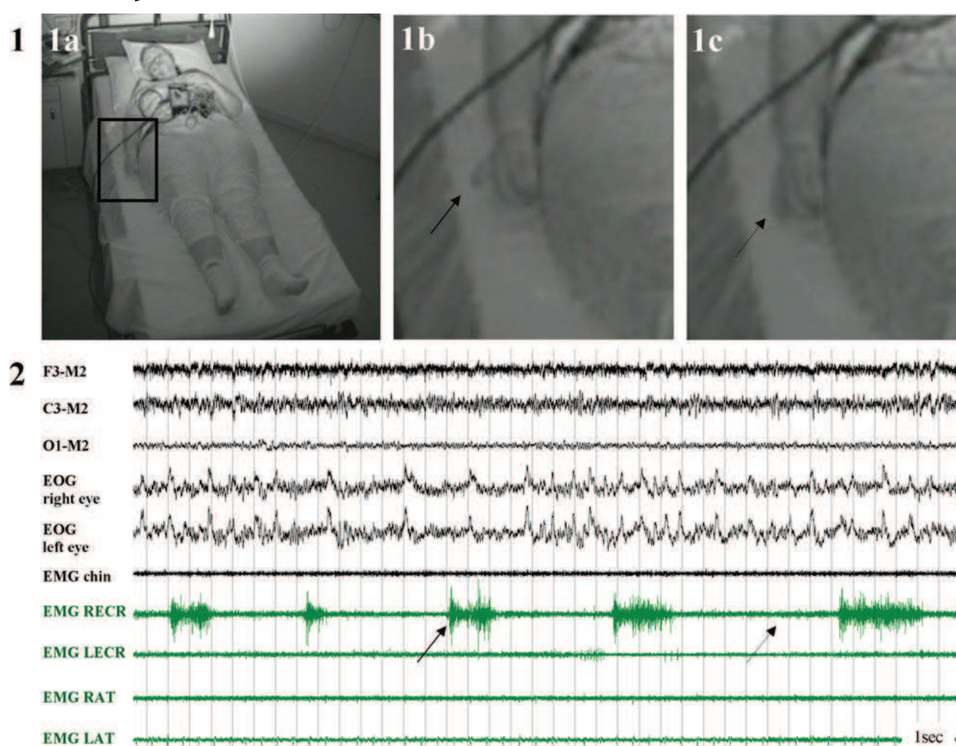

FIG. 1. Pictures illustrating the periodic upper limb movements are taken directly from the video capture during PSG. Inserts are an enlargement of the original video of the whole body, explaining the low resolution. F3-M1, C3-M1, and O1-M1, left frontal, central, and occipital electroencephalogram derivations; EOG, electrooculogram; RECR and LECR, right and left extensor carpi radialis; RAT and LAT, right and left anterior tibialis. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

after reintroduction of the dopaminergic agonist compound. The patient had a clear benefit from pramipexol, then a major recrudescence of restlessness in both arms subsequent to pramipexol withdrawal, and was relieved again when the molecule was administrated at 12 p.m. on the following day. VPSG, as well as an electromyogram (EMG) on the extensor carpi radialis (ECR) muscle were performed to record periodic movements of the upper limbs.[5] Surprisingly, a repeated extension of the small finger was observed, especially during pramipexol withdrawal (Fig. 1A, insert 1b,c; or see Video). The upper limb movements could occur unilaterally (right or left), bilaterally, or extend to the lower limbs and were observed during periods of wakefulness and non-REM sleep. EMG activity of the ECR (Fig. 1, part 2; or see Video) fulfils the American Academy of Sleep Medicine criteria for PLM disorder, [6] apart from for their localization.

Discussion and Conclusions

Involvement of upper limbs in RLS, although poorly known, is relatively frequent.[7] We report on a case that confirms that RLS can be limited only to the arms. Remarkably, a repeated extension of the small finger, mimicking the typical extension of the hallux, characteristic of PLM, was noticed. One should be aware of the upper limb variant of this syndrome, and that treatment by dopaminergic agonists proves to be very efficient.

Legends to the Video

Video 1. Video montage illustrates periodic upper limb movements and is taken directly from the video capture during PSG. Insert shown is an enlargement of the original video of the whole body, explaining the low resolution. EEG recording: F, frontal; C, central; P, parietal; T, temporal; O, occipital; uneven numbers, left. EOGD G2 and EOGG G2: electrooculogram. Ment+Ment−: chin EMG. JbG+JbG−; JbD+JbD−: left and right anterior tibialis EMG. DelD+DelD−; DelG+DelG−: right and left deltoid muscle EMG. DphD+DphD−; DphG+DphG−: right and left extensor carpi radialis EMG.

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Article 7: ***« Bedtime-related jerks in the upper limbs associated with restless arms syndrome»***

Neurology, accepté février 2015

Article principal : pages 1-2

Matériel supplémentaire (non soumis) : pages 3-5

VIDEO NEUROIMAGES

Bedtime-related jerks in the upper limbs associated with restless arms syndrome

A 73-year-old man complained at bedtime of "electric shock" sensations, corresponding to myoclonic-like jerks, observed solely in both arms, causing severe insomnia. These involuntary movements appeared at rest, and were accompanied by an urge to move that relieved symptoms (video on the *Neurology*® Web site at Neurology.org). To date, few observations have been reported on arm restlessness and periodic movements of the upper limbs.[1,2] This variant shares common features with restless legs syndrome and periodic limb movement disorder, such as therapeutic response to dopaminergic agonists. Clinicians should be aware of restlessness of the upper limbs, which likely remains underdiagnosed and requires appropriate therapeutic management.

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Author contributions: E.R. and P.B. conceived of the design of the case report. E.R., C.T., N.C., and U.K.-H. were involved in the acquisition of data. E.R., N.C., and U.K.-H. performed the analysis and interpretation of the data. E.R. and U.K.-H. wrote the submitted manuscript. C.T. and P.B. performed a critical revision of the submitted material.

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Article 8: ***« Restless legs syndrome as a first manifestation of a cerebral infarct»***

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NEW RESEARCH

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CASE REPORTS

Restless Legs Syndrome as a First Manifestation of a Cerebral Infarct

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The onset of restless legs syndrome (RLS) is usually progressive and the neural substrates underlying its pathophysiology remain to be identified. Here we report on a patient presenting with acute-onset RLS that was symptomatic of a right anteromedial pontine infarction. This case is exceptional because RLS appeared several hours before the occurrence of a regressive dysarthria clumsy-hand syndrome. Additionally, millimetric MRI sections showed that the structures possibly involved in RLS pathogenesis were the corticospinal tract, the pontine nuclei, and the pontocerebellar fibers. Although this is uncommon, clinicians should be aware that RLS characterized by a sudden onset can be a clinical manifestation related to stroke.

Keywords: pontine stroke, pontine anteromedial infarction, restless legs syndrome, RLS, periodic limb movements, RLS pathophysiology

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Restless legs syndrome (RLS) is a common neurological disorder whose symptoms develop progressively.[1] The neural substrates underlying RLS pathophysiology remain to be identified.[1] Here, we report an exceptional case of a patient who presented with acute onset RLS as the first clinical manifestation of a right anteromedial pontine infarction. Additionally, we analyzed, using millimetric MRI sections, the structures affected by the lesion.

REPORT OF CASE

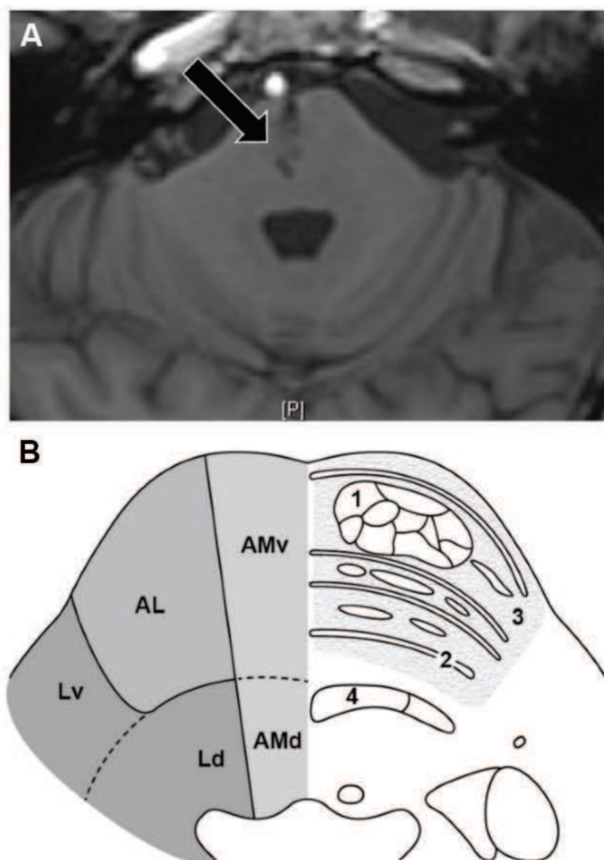
A 64-year-old patient presented a severe RLS characterized by sudden onset. He complained, for the first time ever, of an urge to move the legs with paresthesia, worsened during rest and as he lay down. The symptoms were relieved by mobilization, fulfilled the criteria of the International study group for diagnosis of RLS, and caused unusual and severe sleep-onset insomnia. The following morning the patient presented additionally with dysarthria-clumsy hand syndrome. He was a former smoker and was previously diagnosed with hypertension. Upon admission, MRI scans revealed a recent right anteromedial pontine infarction.

Investigations were otherwise unremarkable, and the stroke was presumed to be atherothrombotic. Treatment consisted of aspirin, atorvastatin and nebivolol. Ten days after RLS onset, the neurological examination was normal, yet RLS persisted and was evaluated as severe (24/40) using the International Restless Legs Syndrome Severity Scale. Biological assessment of the patient's body iron status was within the normal range, with ferritin at 118 µg/L (N: 58-319), transferrin saturation at 0.44 (N: 0.20-0.40), transferrin at 2.00 (1.73-2.77), and TIBC at 46 (N: 45-90). Polysomnography was performed and scored manually according to the AASM 2007 criteria. Sleep was highly fragmented (arousal index: 35.62/h), mainly due to a high index of periodic leg movements during sleep ([PLMS] total PLMS index: 95.8/h; right PLMS: 54.3; left PLMs: 54.3; PLMS associated with arousal: 31.26/h). Pramipexole was introduced, and improvement of RLS and sleep quality was prompt and substantial. After 3 months, RLS improved from severe to mild, based on the IRLS severity scale (8/40), and total PLMS index decreased to 4.51/h. After 16 months, the patient continued responding positively to a pramipexole dosage of 0.27 mg.

DISCUSSION

We describe the case of a patient presenting with RLS and presumably PLMSs whose onset was coincident with a classic lacunar stroke syndrome (i.e., clumsy-hand dysarthria syndrome). The neural substrate and the generator(s) responsible for RLS and PLMS remain unknown. Stroke-related RLS might emerge from interrupted fibers of presumably descending "inhibitory" pathways causing a supraspinal disinhibition. How can lesions of the paramedian pons be responsible for RLS occurrence? Using MRI with millimetric sections, we analyzed, in reference to the Duvernoy brain atlas, the structures that might be involved. Firstly, the reticular formation located in the tegmentum was spared, in contrast to previously reported

Figure 1



(A) Infarction of the ventral part of the pontine anteromedial territory on T1 MRI sequence lateral section (arrow). **(B)** Section of the middle part of the pons. (1) Corticospinal tract split into small fasciculi (2) Pontocerebellar fibers (3) Pontine nuclei (4) Medial lemniscus. AMv, Anteromedial pontine arterial territory (ventral part); AMd, Anteromedial pontine arterial territory (dorsal part); AL, Anterolateral pontine arterial territory; Lv, Lateral pontine arterial territory (ventral part); Ld, Lateral pontine arterial territory.

brainstem stroke-related RLS.[2] Lesioning was observed in the corticospinal tract, the pontine nuclei, and the pontocerebellar fibers (**Figure 1**). The involvement of the latter is especially interesting because these fibers project to the contralateral cerebrocerebellum. A specific pontocerebellar circuitry, which may imply opioid receptors in the human cerebellum,[3] has been suggested in the pathophysiology of RLS.[4] Functional MRI studies conducted during RLS symptoms and periodic leg movements show an activation of several structures, including bilateral cerebellum.[5-7] This neural network may be affected in the present case. Finally dopaminergic agonists have a therapeutic effect, and the dopaminergic neurons of the A11 area are thought to be implicated in RLS pathogenesis.[8] In our patient, it remains unclear whether the diencephalospinal tract conveying projections from the A11 neurons to the dorsal horn of the spinal cord was damaged by the infarction.[8] Additionally, the occurrence of RLS related to stroke may also require predisposing genetic risk factors.[9] Given the improvement of neuroimaging techniques including spatial resolution, a systematic study of small lesions associated with RLS may contribute to improving our understanding of the pathophysiological mechanisms underlying this condition.

Although this is uncommon, clinicians should be aware that sudden onset RLS can be a clinical manifestation revealing a brainstem stroke.

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Article 9: *« Brainstem stroke-related restless legs syndrome: frequency and anatomical considerations»*

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Brainstem Stroke-Related Restless Legs Syndrome: Frequency and Anatomical Considerations

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Key Words
Restless legs syndrome · Pontine stroke · RLS pathophysiology · Stroke-induced RLS · Periodic limb movements · Pontine anteromedial infarction

Abstract
Background: Given the discordant results of studies that have reported cases of RLS associated with brainstem stroke and the absence of RLS in large series describing the clinical spectrum of brainstem infarctions, we decided to assess RLS in all patients admitted for brainstem stroke. ***Methods:*** All patients who were consecutively referred to the Strasbourg stroke unit for brainstem infarction were prospectively evaluated for RLS. The different parameters analyzed were the topography of the ischemic lesions (magnetic resonance imaging), the different symptoms (sensory, motor, cerebellar, cranial nerves and dysarthria) and the NIH stroke scale. Statistical analyses used the Bayesian paradigm. ***Results:*** Thirty patients have been included, and RLS was observed in three patients (10%). Two patients suffered from an exacerbation of symptoms anterior to the stroke, and the other patient a de novo, but transient, RLS. Patients with stroke-induced sensory symptoms have a higher risk to develop brainstem stroke-related RLS as compared to patients without sensory symptoms. ***Conclusion:*** The results suggest that RLS should be systematically screened in patients affected with brainstem stroke, especially in the case of stroke-induced sensory symptoms. Clinicians should be aware of this association, especially as efficient treatments are available and allow improving the management of patients affected with stroke.
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Introduction

Restless legs syndrome (RLS) is a common disorder characterized by an urge to move limbs due to unpleasant or uncomfortable feelings in the legs. Symptoms occur at rest, are relieved by motor activity and are worsened in the evening or at night [1]. The prevalence is higher in women than in men and ranged from 3.9 to 14.3% in community-based surveys [2]. When the frequency/severity is taken into account, the prevalence ranged from 2.2 to 7.9%, and when differential diagnosis is applied, the prevalence estimates are between 1.9 and 4.6% [2]. The pathophysiology of RLS remains poorly understood [3]. Two major findings concern the impaired metabolism of intracerebral iron [4] and the therapeutic effect of dopaminergic agonists [5]. One distinguishes an idiopathic form with early-onset and frequent family history from a late-onset secondary form. The latter is commonly associated with neuropathy, renal failure, pregnancy or medication such as neuroleptics and antidepressants (tricyclic or SSRI) [3]. Cases of secondary RLS related to stroke have been reported [6], but the frequency of this association remains unclear. Considering brainstem stroke, only a few cases have been reported [7–9], contrasting with 17.4% of brainstem-stroke induced RLS in the series of Lee et al. [10]. Given the discordant results of these observations, and the absence of RLS in a large series describing the clinical spectrum of brainstem infarctions [11–13], we decided to prospectively investigate for RLS all patients admitted for brainstem stroke. The objective of this study is both, to establish the frequency of RLS associated with brainstem stroke, and to identify brainstem infarction territories as well as clinical signs that might be predictive of RLS.

Methods

All patients consecutively referred to the stroke unit for brainstem infarction over a year, between January 31st 2012 and January 31st 2013, were assessed for RLS symptoms, according to the International Restless Legs Syndrome Study Group [1], within 48 h after stroke onset. When IRLS criteria were met, the patient was additionally evaluated by a sleep specialist.

Infarction territory was determined using MRI, including diffusion, T2*, FLAIR and TOF sequences and involved arterial territories were defined according to the human brain atlas of arterial territories of the brainstem and the cerebellum [14].

The different parameters analyzed were the topography of the ischemic lesions, the different symptoms (sensory, motor, cerebellar, cranial nerves, dysarthria) and the NIH stroke scale.

Statistics have been carried out using the Bayesian paradigm. Results of the quantitative variables are expressed as mean ± standard error of the mean (SEM) and results of the qualitative variables are expressed as proportions and credibility intervals. Comparisons of proportions were made with logistic regressions using uninformative priors for betas (N(0,10)) and comparisons of means were made with linear regressions using uninformative priors for betas (N(0,10)). All the analyses were made with R 3.0.2 and WinBUGS software.

Results

During the study period, 30 patients were admitted for brainstem infarction. Data on demographics of the population and infarction localization are shown in table 1. The mean age of the population was 62.8 ± 3.01 with 60% [95% credibility interval (CrI) 42.19–75.45%] male patients (18/30). Nine patients presented with an infarction of the medulla (30%) [95% CrI 16.68–48.04%], seventeen patients presented with an infarction of the pons (57%) [95% CrI 39.08–72.68%] and four patients with an infarction of the midbrain (13%) [95% CrI 5.45–29.83%] (fig. 1).

Three patients (10%) [95% CrI 3.63–25.75%] met the RLS criteria. Two patients had exacerbation of preexisting RLS (Patients 14 and 22) and one patient had symptoms that regressed within three days and did not need medication (Patient 26). All patients complained of typical RLS with symptoms in both legs evaluated as moderate or severe. No patient had previously diagnosed, but unchanged RLS symptoms.

The implicated territories in all three patients that presented with RLS after brainstem stroke were right-sided and consisted of anteromedial pontine infarction (Patient 26), of anteromedial and anterolateral pontine infarction (Patient 14) and of anteromedial medullary infarction (Patient 22) (fig. 2).

Brainstem stroke-related RLS was 17-fold higher when the patient had poststroke-sensory symptoms, as compared to patients without sensory symptoms. The credibility interval was at 95% comprised between 1.389 and 330.773. The probability that sensory symptoms (independent of RLS) influence RLS was of 98.8%. Conversely, the presence of dysarthria, motor or cerebellar symptoms or dysfunction of cranial nerve did not increase the risk for stroke-associated RLS. Neither the topography of the ischemic lesion nor the NIH stroke scale were associated with a higher risk for brainstem stroke-related RLS.

Patient 14

Patient 14 described a very mild and intermittent form of RLS that had appeared about 5 years earlier. She had been asymptomatic for at least six months. Symptoms

Table 1. Characteristics and clinical symptoms of the patients admitted for brainstem infarction

N°	Sex	Age	Localization of infarction	NIH	S	M	C	CN	D	RLS
1	F	81	L AM + AL pontine	9	No	Yes	No	No	Yes	No
2	M	61	R AM pontine	10	No	Yes	Yes	No	Yes	No
3	M	71	L P medullary	9	Yes	No	Yes	Yes	Yes	No
4	M	69	R Lat + P medullary	3	No	No	No	Yes	Yes	No
5	M	85	R AM pontine	4	No	Yes	No	No	Yes	No
6	M	37	R AM midbrain	2	No	No	No	Yes	No	No
7	F	59	R AM + AL + Lat and L AM + AL pontine	13	Yes	Yes	No	No	Yes	No
8	F	78	R AM + AL pontine	7	No	Yes	Yes	No	Yes	No
9	F	75	L AM pontine (tegmentum)	1	No	No	Yes	Yes	No	No
10	M	52	R AM + AL pontine	5	No	Yes	No	No	Yes	No
11	M	48	R P medullary	3	Yes	Yes	No	No	Yes	No
12	M	60	L AL pontine	2	No	Yes	No	No	Yes	No
13	F	29	L AM pontine	3	Yes	Yes	Yes	Yes	No	No
14	**F**	**58**	**R AM + AL pontine**	**6**	**Yes**	**Yes**	**Yes**	**No**	**Yes**	**Yes***
15	M	50	R Lat medullary	0	No	No	No	No	No	No
16	M	66	L AM midbrain	5	No	No	No	Yes	Yes	No
17	F	90	R Lat pontine	6	No	No	Yes	No	Yes	No
18	F	52	R AM pontine	7	No	No	Yes	Yes	Yes	No
19	M	76	R AM pontine	2	No	Yes	No	No	Yes	No
20	M	57	R AM midbrain	1	No	No	No	Yes	No	No
21	M	26	L P medullary	0	No	No	No	No	No	No
22	**M**	**59**	**R AM medullary**	**1**	**Yes**	**No**	**No**	**No**	**No**	**Yes***
23	M	79	L Lat + P medullary	4	No	No	Yes	No	No	No
24	F	50	L AL + Lat + P medullary	5	Yes	No	Yes	Yes	Yes	Non
25	M	64	L AM pontine (tegmentum)	4	Yes	No	No	No	No	No
26	**M**	**49**	**R AM pontine**	**5**	**Yes**	**Yes**	**Yes**	**No**	**Yes**	**Yes****
27	F	77	L AM pontine + R Lat pontine	15	No	Yes	Yes	No	No	No
28	F	86	L AM pontine	2	No	Yes	No	No	No	No
29	F	85	R AM pontine (tegmentum)	3	No	No	Yes	Yes	No	No
30	M	51	L AM midbrain	11	Yes	Yes	No	Yes	Yes	No

S = Sensory; M = motor; C = cerebellar; D = dysarthria; CN = cranial nerves; L = left; R = right; AM = anteromedial; AL = anterolateral; Lat = lateral; P = posterior. * Exacerbation of previous symptoms. ** Transient symptoms.

had reappeared in the evening following stroke and were described as very severe when compared to the earlier symptoms severity. The IRLS-scale, validated to evaluate symptoms severity for time periods of a week, was used after four days with a score of 27/40, corresponding to severe RLS [15]. Upon admission, the NIH score was at 6 and neurological examination showed dysarthria – clumsy hand syndrome, a left central facial paresis, mild paresis and hypoesthesia of the left limbs as well as instability with the Romberg test. MRI showed a right anteromedial and anterolateral pontine infarction (fig. 2). Video-polysomnography was performed and PLMS index was evaluated at 40.0/h (PLMS associated with arousal: 6.67/h; PLMS not associated with arousal: 19.20/h; PLMS associated with a respiratory event: 14.13/h). The patient also had a moderate obstructive sleep apnea syndrome (Apnea-Hypopnea Index: 21.3/h). As the patient had never previously undergone polysomnography, we do not know if PLMS were already present before stroke. Pramipexole was efficient and the follow-up IRLS score under treatment was moderate (12/40).

Patient 22

Patient 22 complained of an RLS that had worsened after an anteromedial medullary infarction (fig. 2), and was evaluated as moderate at IRLS (16/40). At neurological examination he presented with a transient paresis and a persistent hypoesthesia of the left limbs. He had an anxiety disorder and was chronically treated with benzodiazepine, which had possibly a beneficial effect on the RLS.

PROOF

Fig. 1. Diffusion sequences of brain MRI realized upon admission in all of the 30 patients included in the study. Arrows point to the infarction area (axial views).

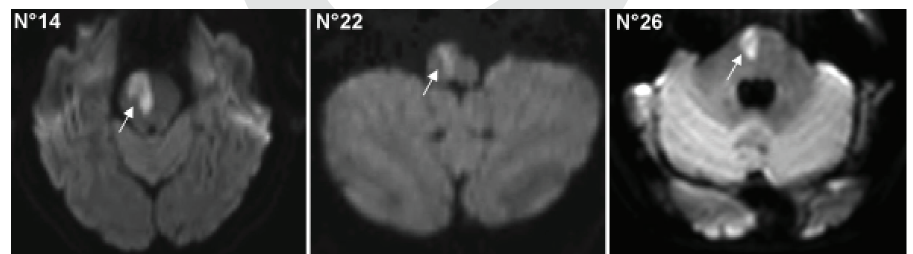

Fig. 2. Diffusion sequences of brain MRI realized upon admission in the three patients that presented stroke-induced RLS. Arrows point to the infarction area (axial views).

Patient 26

Patient 26 had a right anteromedial pontine infarction (fig. 2) revealed by dysarthria, regressive paresthesia of the left side, paresis of the left limbs and instability. The NIH score at admission was at 5. He complained of a transient RLS during the three first nights following stroke.

Discussion

In this prospective cohort, the frequency of RLS in patients with brainstem infarction was 10%, either de novo, or as a clear exacerbation of earlier present symptoms. This is slightly less than previously reported by Lee et al. (4/23; 17.4%), but confirms that the association between the two disorders is not exceptional [10]. Similarly, Kim et al. reported two cases of pontine infarction associated with periodic limb movements during sleep [7]. The frequency of brainstem stroke-related RLS reported in our study and by Lee et al. [10] are discordant with the absence of RLS reported in a large series describing the clinical spectrum of brainstem infarctions [11–13]. Different hypotheses could be proposed to explain these discordances. First, the overall relatively high prevalence of RLS could mask the diagnosis of stroke-related RLS [2]. Additionally, the presence of RLS complaints might be mistaken for poststroke-sensory symptoms as our results show that RLS develops more likely in patients with sensory symptoms. Finally, since RLS is a purely clinical diagnosis, it might be difficult to assess the symptoms in patients having stroke-induced communication problems.

The presence of poststroke-sensory symptoms increases the risk for brainstem stroke-related RLS in our monocentric case series. Whether specific arterial territories responsible for ischemic damages augment this risk as well remains elusive. Strikingly, in the present cohort, as well as in the study by Lee et al., all patients with RLS had an anteromedial pontine infarction except one who had an anteromedial medullary stroke [10]. Given the improvement of neuroimaging techniques including spatial resolution and functional neuroimaging of the dopaminergic system, a systematic study of small lesions associated with RLS may contribute to improving our understanding of the pathophysiological mechanisms underlying this condition.

The pathophysiology of RLS needs to be further clarified, but it is likely, that predisposing genetic factors are required to develop brainstem stroke-related RLS [16]. This could explain the poststroke exacerbation of idiopathic RLS in our patients.

Conclusion

Our series has limitations and, given the limited number of patients with RLS after brainstem stroke, a multicentric study with a larger cohort and an adequate control group is needed to further characterize the relation between brainstem stroke and RLS. However, to our knowledge, this is the largest cohort studying the frequency of RLS symptoms in patients with brainstem stroke. These results that came forth from our routine clinical practice indicate that an RLS complaint after brainstem stroke is not exceptional. We suggest that RLS be systematically screened in patients affected with brainstem stroke, especially in the case of stroke-associated sensory symptoms and of anteromedial pontine or medullary infarction. Clinicians should be aware of this association, especially as efficient treatments are available and allow improving the management of patients affected with stroke.

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PROOF

Article 10: ***« Light irradiance positively influences sleep homeostasis through melanopsin-based phototransduction»***

En préparation

Article principal : pages 1-11

Table and Figures : pages 12-18

Références : pages 19-21

1. Title page. ***Original paper***

Title: ***Light irradiance positively influences sleep homeostasis through melanopsin-based phototransduction***

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2. ABSTRACT

Study Objectives: Light influences sleep and waking through circadian and direct effects, yet its putative influence on sleep homeostasis remains unclear. We previously reported that melanopsin (*Opn4*), a retinal photopigment, affects sleep homeostasis. Here we sought to determine whether light irradiance influences the sleep homeostatic process in melanopsin KO mice.

Design: Backcross male *Opn4*$^{-/-}$ mice and their littermate controls were exposed to three different light intensities (< 10, 150, or 600lux, n=6-8/group) for 7 days (exposure duration based on a previous preliminary study). Sleep was recorded under baseline conditions and during a 6-hour sleep deprivation by gentle handling and starting at ZT12. EEG power spectrum was performed, specifically to quantify EEG delta power, a reliable marker of sleep need.

Setting: Rodent sleep laboratory

Participants: 36 male mice (18 *Opn4*$^{-/-}$, 18 controls) aged 3 months, 25-30g

Interventions: Sleep deprivation and variable light intensities

Measurements and Results: In wild-type the level ($p< 0.05$) delta power reached after sleep deprivation increased as a function of light intensity, with the highest under 600 lux (240% of baseline), vs. 150 lux (170%) and < 10 lux (141%).. Comparatively, mice lacking melanopsin displayed lower level of EEG delta activity following sleep deprivation. Moreover, in the absence of melanopsin, especially at <10 et 150 lux. However, the delta power peak observed after sleep deprivation increased with light, but only at higher irradiance (600 lux), suggesting for these light intensities the existence of a compensatory mechanism mediated by cones-based photoreception.

Conclusions: These preliminary results indicate a positive relationship between light intensity and the increase in sleep-need with time-spent-awake, an effect mediated primarily through melanopsin-based phototransduction, yet the rods and cones might also play a role at higher irradiance.

Keywords: Melanopsin, sleep homeostasis, non-visual light, phototransduction, rod-cone, EEG delta power, sleep need, knockout mice, NREM sleep, sleep deprivation.

3. MAIN TEXT

INTRODUCTION

Light plays an essential role in the homeostasis of biological functions and survival of species living on earth. Light exerts pervasive effects on physiology and behavior, including phase shifting of circadian rhythms, sleep, alertness, leraning and memory but also heart rate The retina is a crucial part of the eye involved in the translation and transmission of both visual and non-visual light information to different brain structures. Non-visual light is transmitted through a subset of ganglion cells located in the retina which are inherently photosensitive, in contrast to the two traditional photoreceptors, rods and cones. These phototransductive cells contain a transmembrane protein called melanopsin, which has been highlighted and studied for a little over a decade (Provencio et al 2000, Lucas et al. 2003 Hattar et al., 2003, Panda et al., 2002, Ruby et al. 2002). The primary circadian clock, the suprachiasmatic nucleus (SCN) serves as the main target of this non-visual light. As information passes from the retina, through the retinohypothalamic tract, light entrains this clock. Additionally, other non-visual information is relayed to further brain structures. In human beings and other mammals, these cells are involved in a variety of non-visual functions such as photoentrainement, phase shifting, suppression of melatonin secretion, the pupillary reflex, and the activation of locomotion, some of which are termed the direct, non-circadian effects of light (Lockley et al. 2006 Cajochen et al., 2007). Several teams working in mice models, including ours, have demonstrated the essential role of the non-circadian melanopsin-mediated direct effects of light on the sleep-wake cycle, motor activity, the pupillary reflex, alertness, and mood (Tsai et al., 2009 Lupi et al. 2008 Altimus et al. 2008, Hubbard et al., 2013, and Legates. al 2012).

In the current study we characterized the sleep-wake cycle in a mouse model lacking melanopsin (*Opn4*), in order to highlight the direct, non-circadian effects of light, especially when applied during the first part of the night. Furthermore, we aimed to study the impact of these effects on the sleep-wake cycle and motor activity, as light intensity was varied during the illuminated period. The finding of a negative consequence of light when presented at non-standard hours, is indeed alters certain biological functions such as sleep, memory, alertness, mood, driven by shift-work or jetlag with a sleep debt accumulated in transmeridian travelers

(Boivin 2000, Boivin and James, 2005, Chang et al., 2013, Bedrosian et al. 2013). Insofar as there is evidence that a neurobiological mechanism exists, depressive conditions as seasonal affective disorder (SAD), cannot be explained purely by circadian alterations, as sleep and depression have a strong functional interaction (Wirz Justice et al. 1993, Cajoren et al. 2000, Savelyev et al. 2012). Thus, it is useful to construct experimental protocols where the quality of light (intensity or wavelength) is varied, without interfering with the time or duration of application. Here we propose a study on a mouse model lacking melanopsin (*Opn4*$^{-/-}$), previously characterized for sleep and the non-circadian direct effects of light via melanopsinergic phototransduction. (Tsai et al. 2009)

MATERIALS AND METHODS

Animals

The animal cohort consisted of 36 males, C57BL/6 backcrossed (>F10), *Opn4*$^{-/-}$ mice and their wild-type (*Opn4*$^{+/+}$) controls, maintained in our animal facility the Chronobiotron (UMS 3415) housed at the Institute for Cellular and Integrative Neurosciences, Strasbourg, France. All mice were separated at 4 weeks and placed in individual cages for social isolation for a minimum of three weeks. Animals were raised under environmentally stable conditions [12-hour: 12-hour light-dark (12hL:12hD); 150 lux; 25 ± 0.5° C; food and water ad libitum] and 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 ethic committees at the University of Strasbourg, and CNRS.

Light-controlled experimental cabinet:

During the variable light exposure phase of our paradigm, where sleep and locomotor activity recordings were made, mice were placed in a ventilated two-compartment cabinet controlling moisture, temperature and lighting conditions. Programming of the light and dark cycle was controlled by a clock, and operated on a pulse method wherein the hour of illumination and extinction is defined, as well as the number of pulses (from 1 to 6 corresponding to the respective intensities of 600, 50, 150, 300, 450, 600) for the desired

phase of lighting for each day. Each compartment of the cabinet has the capacity to accommodate up to 10 cages of type 2 and 4 cages of type 2L.

Genotyping:

Genotyping to verify the genetic background was performed using a standard PCR described previously in (Ruby, et al. 2002). Primers used were:

Forward (Mel4E3) TCA-TCA-ACC-TCG-CAG-TCA-GC

Forward (Todo Neo) CCG-CTT-TTC-TGG-ATT-CAT-CGA-C

Reverse (Mel2E4) CAA-AGA-CAG-CCC-CGC-AGA-AG

The fragment corresponding to the allele for *Opn4*$^{+/+}$ is 289 bp and the mutant allele at 919 bp.

EEG implantation

Mice were anesthetized using a combination of Ketamine/xyalzine (80g/kg, 7g/kg; protocol-approved). Following anesthesia, mice were implanted with two EEG, one reference EEG, and two EMG electrodes placed subcutaneously in the cervico-auricularis muscles of the neck, in order to record vigilance states (Wake, NREM sleep, and REM sleep). Mice were given at least 14 days to recover from surgery and habituate to the baseline control conditions before any further experiments were carried out. Following surgery, visual inspections were made daily to ensure adequate recovery. Verification of physiologic and behavioral parameters was realized by weighing each animal, observing changes in water level, the healing of the tissue surrounding surgery site, and the movement of the animal in the cage. Mice were recorded continuously throughout several light conditions: (1) <10, (2) 150 lux, (3) 600 lux, all carried out using the controlled light cabinet (described previously). Different mice were used for each condition in order to eliminate any mood or behavior effects consecutive to light changes. 72 hours following surgery animals were connected to their recording systems for data acquisition. Sleep was recorded using using commercially available hardware and software (Micromed France, SystemPLUS Evolution version 1092).

Sleep deprivation

After 7 days of exposition to a specific light condition, each mouse underwent a standard NREM sleep deprivation protocol through means of "gentle handling" in order to remove as much bias as possible. This method was previously described in Franken, et al. 1998. Sleep deprivations began at ZT0, the time at which the light is switched on in the cabinet, corresponding to the habitual rest period for the mouse. Following each sleep deprivation mice underwent a sucrose preference test for anhedonia behavior.

Actimetry recording and analysis

In addition to EEG/EMG recordings of brain activity, movement was measured using an infrared camera system throughout the course of the experiment. Each camera was mounted facing downwards into the cage and was interrupted so as not to record movement from surrounding animals. Actimetry was recorded using the CAMS system, creating files of 5 minute activity bouts. Analysis was performed using commercially available software, ClockLab. Results were categorized in terms of 5-min, 12-hour, and 24-hour bouts.

EEG scoring and power spectrum analysis:

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.

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).

RESULTS

Opn4$^{-/-}$ behaved differently under various vigilance states depending on the lighting condition they were exposed to. Under a standard 150 lux condition, mice lacking melanopsin slept approximately one-hour less during the light period as compared to their wild-type counterparts, a NREM loss which was not recovered during the dark period (table 1). This finding was consistent with previous experiments using these mice (Tsai et al. 2009). Interestingly this observation was not seen to the same degree in either the low-light (14-minute difference) or high-light (30-minute difference), conditions. Differences during the dark period under low and standard light conditions were negligible, though KO mice slept one hour more under high intensity light. The time-course of NREM under standard lighting confirmed that differences seen between genotypes were relatively confined to the light period (Figure 1-middle). Under low-light no genotype differences were noticeable (Figure 1-top), which was also the case under high-intensity light (Figure 1-bottom). Interestingly, the overall amount of NREM sleep was lower under this condition.

After exposure to 7 consecutive days of 12hL:12hD, all mice were subjected to a 6-hour sleep deprivation beginning at ZT0 (light onset). Mice of both genotypes under 10 and 150 lux conditions showed similar time-course during the SD recovery period, as during the dark periods of the days preceding it. However, following sleep deprivation under high intensity light, mice continued to sleep levels higher than typical sleep under dark periods. In order to determine the quality of sleep following the sleep deprivation, the EEG was analyzed for power spectrum and specifically delta power, a marker for sleep need. Delta power during baseline days under 10 lux was relatively similar between genotypes, with only a few time-points showing significant differences (Figure 2- top). Following sleep deprivation, WT mice showed a significantly higher peak as compared to KO (Figure 3). During recovery animals displayed similar patterns, however, whereas WT mice built-up sleep pressure again during the dark period following the sleep deprivation, KO mice failed to do so. Under 150 lux, mice behaved nearly identically to previously published data under standard laboratory conditions (Figure 2- middle, Tsai et al). Interestingly, while WT mice continued to increase in delta power following deprivation, KO mice were unable to rise much above those in low-light conditions (Figure 3). Finally, in high-intensity conditions WT mice reached an even higher peak than under standard light, though unexpectedly, KO mice did as well (Figure 2- bottom, figure 3).

To examine the alertness level of these mice under baseline and to determine the existence of a genotype effect, theta and gamma, markers of rodent cognition and exploratory behavior, were analyzed during waking. In dim light, mice lacking melanopsin seemed to show higher levels of alertness during the dark period as compared to their WT controls, though followed similar time-courses (Figure 4- top). Under standard lighting no genotype differences were seen (Figure 4- middle). This was true as well for the high-intensity condition, however animals showed a significantly higher level of alertness during the dark phase, followed by a sharp decline during the light phase (Figure 4- bottom).

Finally, REM sleep was analyzed during the 12hL:12hD period for all animals, under all conditions. Under dim and standard light conditions, a time of day significance was seen (light vs dark period; Table 1), yet not a genotype difference. This was not the case under 600 lux though, as WT mice showed almost identical amounts of REM under light and dark periods. KO mice behaved similar under all conditions. The length of the first REM bout during the light period was calculated though no significant genotype or light condition changes were observed (Figure 5-bottom). However, when the start of the first REM bout was calculated a genotype significance was seen in dim and high light, as well as light condition differences for KO mice (Figure 5-top).

DISCUSSION

The genotype differences observed under 150 lux in this study were similar to our previous studies with these animals (Tsai, et al. 2009 Hubbard et al. 2013). *Opn4*$^{-/-}$ mice slept one hour less during the light period as compared to *Opn4*$^{+/+}$. Conversely, during exposure to the high intensity condition (600 lux) our results highlight two important elements. The first concerns wild-type animals which unlike their *Opn4*$^{-/-}$ counterparts remain awake for an extra hour during the dark period at the expense of NREM. Secondly the duration of REM during the subjective day is nearly twice as long as compared to both the "dim" and "standard" light conditions, regardless of genotype, and remains the same in *Opn4*$^{-/-}$ mice. Specifically, *Opn4*$^{+/+}$ mice show an even distribution across day and night, with a reduction of 50% as compared to wild-type mice exposed to the "standard" condition. This seems to suggest that the impact of the light and genotype is negligible under dim and standard conditions, yet highly involved in REM sleep distribution under elevated irradiance,

implying a direct, melanopsin-dependent, non-circadian regulation. In rodents, alternating cycles of NREM-REM are known to be ultradian, and lasting for several minutes at a time. According to the theory put forth by Bennington and Heller, the regulation of REM sleep is homeostatic in nature, not unlike NREM, wherein the pressure for REM operates as a function of NREM duration, and not the length of time spent awake (Benington and Heller 1994). More precisely, the intervals between a given REM bout is a function of the time spent in NREM. However, this NREM does not need to be consolidated. Thus, the homeostatic regulation of REM, represents an independently controlled mechanism, external to sleep-wake circadian, or NREM homeostatic regulations (Datta and MacLean 2007, Shea et al. 2008). These results are also similar to other studies we have performed in these mice (data not yet published), where a loss of total REM sleep was seen during the light phase in *Opn4*$^{+/+}$ SCN-lesioned as compared to sham controls, displaying a distribution akin to their arrhythmic sleep across the light/dark cycle. In summary, under the 600 lux condition, if we consider the time-course and total amount of time in each vigilance state, we find that *Opn4*$^{+/+}$ mice are awake 4 hours more, sleep almost 4.5 hours less, and lose 28 minutes of REM during the light period, when viewed against baseline values under the "standard" condtion. Given these results, we must consider that the principle regulatory mechanism for REM may be non-circadian melansopin-dependent phototransduction. In contrast to depressed human beings, which show increased latency and shortened duration for their first REM episode, in mice this ultradian NREM-REM repartition across the sleep/wake cycle is reinforced by the presence of bimodal increases in activity during light-to-dark and dark-to-light transitions. Thus these REM sleep markers of depression are not applicable in a nocturnal mouse model.

As previously stated, sleep deprivations and delta power analysis of NREM sleep homeostasis, were performed in both genotypes under each light condition. We observed that the increase in light intensity in relation to delta power following sleep deprivation, constitutes a dose-response curve. Under 600 lux, delta power is higher, reflecting an increased need to sleep, likely due to increased activity bouts, or at least greater ranges of movement which fatigue the animal more quickly. Previously we found a direct relationship between melanopsin and the homeostatic regulation of sleep, under baseline conditions as well as following sleep deprivation (Tsai et al. 2009). However, our current data suggest that this direct non-circadian effect functions outside of the presence of melanopsin at higher light intensities. This is evident given that although differences in delta power levels exist between

wild-type and knock-out animals, *Opn4*$^{-/-}$ mice still show higher delta power values as compared to the other light conditions. This may be due to an effect observed in a behavioral study using these same mice and light conditions (Calvel et al. 2014 under review), in which cooperation between cones and melanopsinergic cells in the retina, interact at higher light intensities (Lall et al., 2010, Morin and Sudholme 2011 Gooley et al., 2012).

The impact of total sleep deprivation on delta power and the duration of NREM during the recovery period varies depending on the light conditions and genotype. One of the key elements is the absence of a homeostatic modulation in dim light after sleep deprivation, where delta power is approximately 160% (versus 250% in the "high" condition). Furthermore, the following sleep deprivation in the "high" condition, delta power does not exponentially decrease as in other conditions, but maintains itself beyond the light phase. This seems to confirm that the quality of the sleep homeostat in this condition is related to the increase in NREM sleep during the subsequent dark phase. This data is particularly interesting in that these non-circadian direct effects of light via melanopsinergic phototransduction, directly alter the sleep homeostat.

Consecutive to this study, our team conducted experiments using the same mouse model, applied the same light paradigm, and studied the locomotor activity as a means to examine what conditions might lead to the development of a depressive phenotype. By comparing these results from those described above in the context of sleep, we can identify that higher irradiance lighting given during the day, is beneficial to the quality of sleep, mood, and promotes the behavioral drive for activity during the night, at least in nocturnal mice, and that these direct effects of light involve melanopsinergic phototransduction. Currently, lighting systems in animal research facilities are maintained at a standard lower than gave during the “high intensity” condition, a requirement due to the fact that mice are considered photophobic. However, given these findings on sleep homeostasis, mood, and locomotor activity, laboratory mice may actually benefit from an increase in light intensity during the day period, at least in rooms where mice are kept prior to experimentation. From this point of view, the mouse, a nocturnal mammal, and humans which are diurnal, the differences seem much slighter than previously thought, with respect to light. There is still an inverse relationship between light and dark in regards to the direct effects on sleep and wake in nocturnal vs. diurnal animals (Borbely 1976, Benca et al. 1998, Miller et al. 1998, Hubbard et al. 2013). Although, these aberrant and harmful effects on sleep homeostasis during either night or day are the result of light applications outside the appropriate times (e.g. 1-hour light

pulse during the dark period) (Campbell et al. 1995, Lupi et al. 2008, Tsaï et al. 2009, Legates et al. 2012). In contrast to protocols used by other teams, our bright light paradigm modulates only the intensity of the light period without changing habitual timing of light and dark onset. The strength of this protocol is that it allows us to highlight a direct effect of light on mood and cognition in terms of the functionality of these melanopsin cells, through modulation of the photic intensity without alteration of the application of light (Legates et al., 2012). Conversely, it likely imperative that attention be paid to the physical parameters of the light source in terms of duration, intensity or wavelength, as this seems essential for a healthy maintenance of sleep homeostasis, irrespective of whether the animal is diurnal or nocturnal. The current study underline an important need for understanding more completely the hypotheses related to both circadian rhythm and mood disorders, and ned to take into account the important role that the non-circadian, melanopsinergic phototransduction, plays.

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5. TABLES

Condition	Period	Genotype	W (h)	N (h)	R (min)	Total Sleep (h)
<10 lux	12-hours L	*Opn4*$^{+/+}$	$5.70 \pm 0.2^{b,c}$	$5.38 \pm 0.2^{b,c}$	54.6 ± 3.4	$6.30 \pm 0.2^{b,c}$
		Opn4$^{-/-}$	$5.68 \pm 0.4^{b,c}$	$5.15 \pm 0.3^{b,c}$	69.5 ± 7.2	$6.31 \pm 0.2^{b,c}$
	12-hours D	*Opn4*$^{+/+}$	8.26 ± 0.3	3.39 ± 0.3	$20.8 \pm 1.6^{a,b}$	3.74 ± 0.3
		Opn4$^{-/-}$	8.23 ± 0.4	3.33 ± 0.3	26.0 ± 3.2	3.77 ± 0.4
	24-hours	*Opn4*$^{+/+}$	14.0 ± 0.4	8.78 ± 0.4	75.4 ± 3.8^{a}	10.0 ± 0.4^{c}
		Opn4$^{-/-}$	13.9 ± 0.1	8.50 ± 0.1	95.5 ± 1.2	10.1 ± 0.4^{c}
	L-D Difference	*Opn4*$^{+/+}$	$-2.55 \pm 0.4^{a,c}$	1.99 ± 0.4^{a}	$33.7 \pm 3.5^{a,c}$	2.55 ± 0.3^{a}
		Opn4$^{-/-}$	-2.54 ± 0.5	1.82 ± 0.5	$43.6 \pm 6.6^{b,c}$	2.54 ± 0.1^{b}
150 lux	12-hours L	*Opn4*$^{+/+}$	$4.73 \pm 0.1^{a,d}$	$5.97 \pm 0.6^{a,d}$	78.0 ± 7.7^{d}	7.27 ± 0.7^{a}
		Opn4$^{-/-}$	5.77 ± 0.3	4.84 ± 0.2	83.1 ± 6.9	6.23 ± 0.3
	12-hours D	*Opn4*$^{+/+}$	7.46 ± 0.1^{d}	3.86 ± 0.2^{d}	40.8 ± 3.6^{d}	4.54 ± 0.1^{d}
		Opn4$^{-/-}$	7.80 ± 0.1	3.44 ± 0.1	46.0 ± 0.3	4.20 ± 0.1^{d}
	24-hours	*Opn4*$^{+/+}$	12.2 ± 0.9	9.83 ± 0.8^{a}	118.8 ± 4.0	$11.8 \pm 0.8^{a,d}$
		Opn4$^{-/-}$	13.6 ± 0.3	8.28 ± 0.2	129.2 ± 7.2	10.4 ± 0.3^{d}
	L-D Difference	*Opn4*$^{+/+}$	-2.72 ± 0.1^{a}	$2.10 \pm 0.4^{a,d}$	37.2 ± 11.3^{a}	$2.72 \pm 0.6^{a,d}$
		Opn4$^{-/-}$	-2.02 ± 0.3	1.41 ± 0.2	37.1 ± 6.7	2.02 ± 0.3
600 lux	12-hours L	*Opn4*$^{+/+}$	6.74 ± 0.2	4.23 ± 0.2^{a}	50.8 ± 7.7	5.08 ± 0.2^{a}
		Opn4$^{-/-}$	6.80 ± 0.1	4.73 ± 0.1	58.5 ± 9.5	5.71 ± 0.1
	12-hours D	*Opn4*$^{+/+}$	9.86 ± 0.1^{a}	1.89 ± 0.6^{a}	47.8 ± 11^{a}	2.68 ± 0.4^{a}
		Opn4$^{-/-}$	8.82 ± 0.1	2.96 ± 0.1	29.9 ± 5.8	3.45 ± 0.1
	24-hours	*Opn4*$^{+/+}$	16.6 ± 0.3^{a}	6.12 ± 0.3^{a}	98.6 ± 12	7.76 ± 0.3^{a}
		Opn4$^{-/-}$	15.6 ± 0.1	7.70 ± 0.2	88.4 ± 2.1	8.56 ± 0.1
	L-D Difference	*Opn4*$^{+/+}$	-3.11 ± 0.2^{a}	2.34 ± 0.1^{a}	2.95 ± 5.1^{a}	2.39 ± 0.1
		Opn4$^{-/-}$	-2.01 ± 0.1	1.78 ± 0.1	28.6 ± 0.8	2.26 ± 0.2

Table 1: Time-spent under each vigilance state during baseline 12hL:12hD in various luminance conditions

Two-way ANOVA Genotype x Light condition:

SubL: W- $P_{Light\ condition}$=0.003; N- $P_{Light\ condition}$ =0.001; TS- $P_{Light\ condition}$ =0.004

SubD: R- $P_{Light\ condition}$=0.004

24-hr: N- $P_{Light\ condition}$=0.04

LD-Diff: W- $P_{Light\ condition}$=0.008; N- $P_{Light\ condition}$ =0.02; R- $P_{Light\ condition}$ =0.008; TS- $P_{Light\ condition}$ =0.007

Post-hoc significance:

[a] Significant genotype differences

[b] Significant differences between 10 and 150 lux

[c] Significant differences between 10 and 600 lux

[d] Significant differences between 150 and 600 lux

6. FIGURES

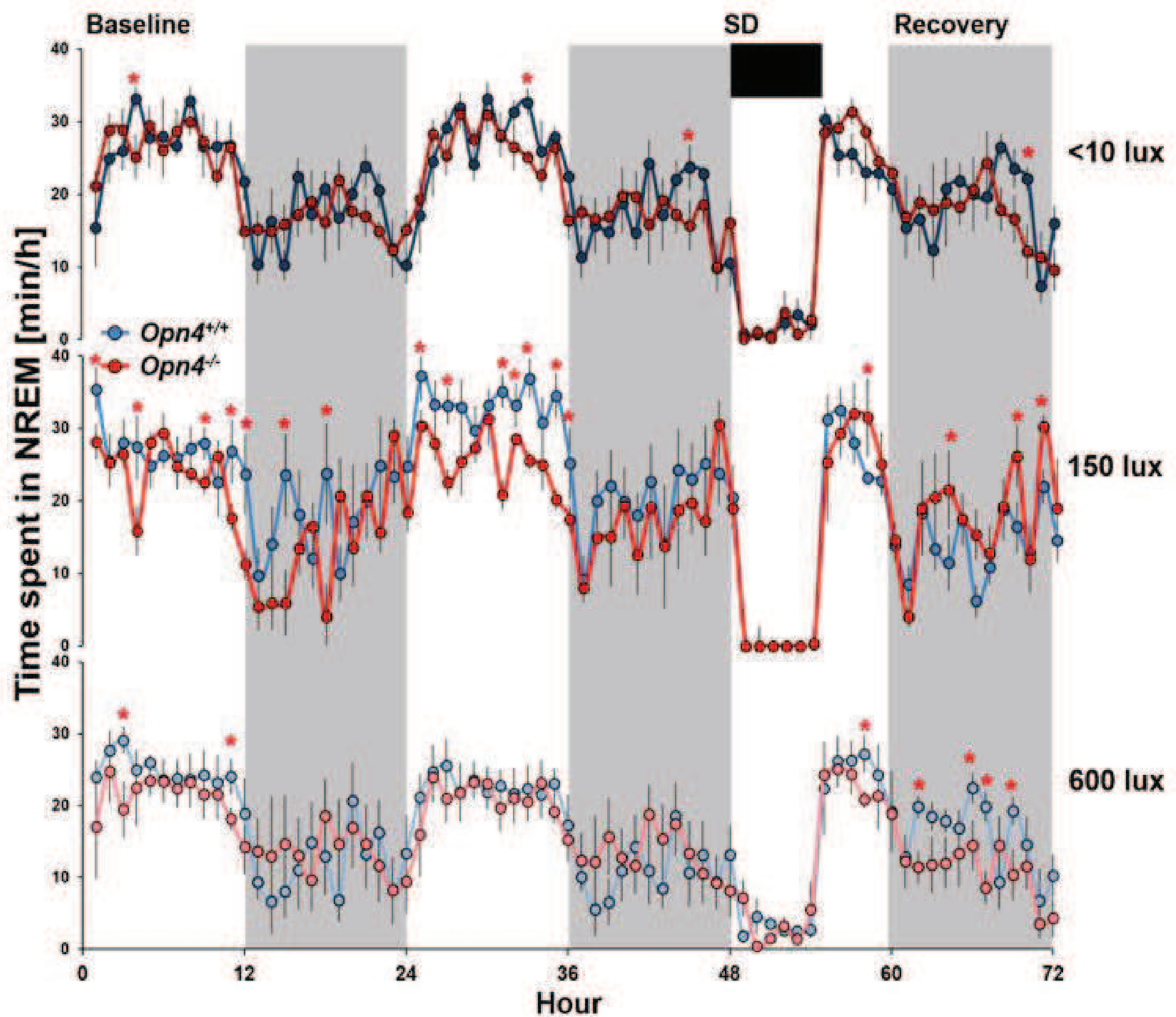

Figure 1: Time-course of NREM sleep during 48 hours of Baseline (12hL:12hD) followed by a 6-hour sleep deprivation (ZT0-6).

All values represent average ± SEM. Red asterisks represent genotype differences observed following post-hoc testing. [<10 lux: *Opn4*$^{+/+}$n=5, *Opn4*$^{-/-}$ n=6; 150 lux: *Opn4*$^{+/+}$n=6, *Opn4*$^{-/-}$ n=6; 600 lux: *Opn4*$^{+/+}$n=5, *Opn4*$^{-/-}$ n=5]

Baseline time-course [0-48]:

Repeated-Measures ANOVA Light Condition x Genotype x Time-course-

$P_{light\text{-}condition}$=0.04; $P_{time\text{-}course}$<0.00001; $P_{time\text{-}course\ x\ light\text{-}condition}$<0.00001; $P_{time\text{-}course\ x\ genotype}$=0.02; $P_{time\text{-}course\ x\ light\text{-}condition\ x\ genotype}$=0.001;

Post-hoc (p<0.05): <10 vs. 600 *Opn4*$^{-/-}$; <10 vs. 600 *Opn4*$^{+/+}$; 150 vs. 600 *Opn4*$^{+/+}$; *Opn4*$^{+/+}$ <10 vs. *Opn4*$^{-/-}$ 600; *Opn4*$^{+/+}$ 150 vs. *Opn4*$^{-/-}$ 600

Following Sleep Deprivation [49-72]:

Repeated-Measures ANOVA Light Condition x Genotype x Time-course-

$P_{light\text{-}condition}=0.07$; $P_{time\text{-}course}<0.00001$; $P_{time\text{-}course\ x\ light\text{-}condition}<0.00001$

Post-hoc ($p<0.05$): <10 vs. 600 *Opn4*$^{-/-}$; <10 vs. 600 *Opn4*$^{+/+}$; 150 vs. 600 *Opn4*$^{-/-}$; *Opn4*$^{+/+}$ <10 vs. *Opn4*$^{-/-}$ 600;

Overall time-course [0-72]:

Repeated-Measures ANOVA Light Condition x Genotype x Time-course-

$P_{light\text{-}condition}=0.02$; $P_{time\text{-}course}<0.00001$; $P_{time\text{-}course\ x\ light\text{-}condition}<0.00001$; $P_{time\text{-}course\ x\ light\text{-}condition\ x\ genotype}=0.001$;

Post-hoc ($p<0.05$): <10 vs. 600 *Opn4*$^{-/-}$; <10 vs. 600 *Opn4*$^{+/+}$; 150 vs. 600 *Opn4*$^{+/+}$; *Opn4*$^{+/+}$ 150 vs. *Opn4*$^{-/-}$ 600

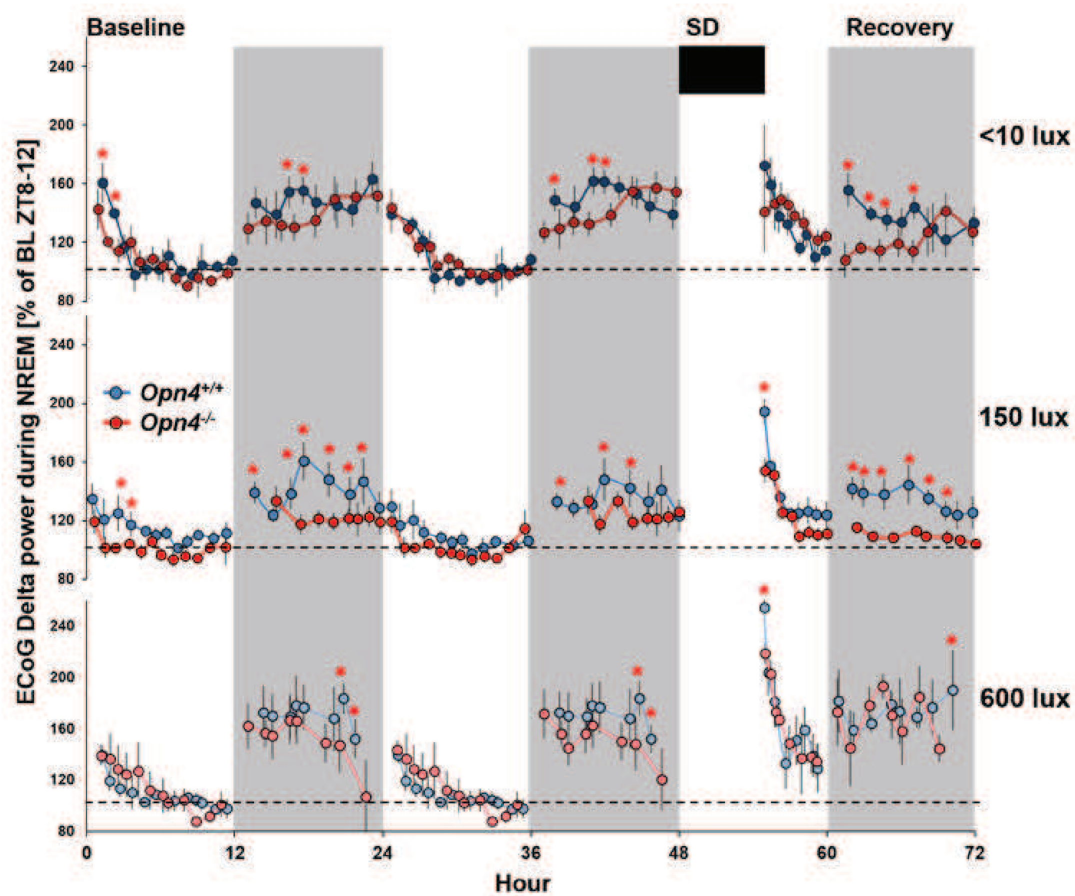

Figure 2: ECoG delta power time-course under 2 days of baseline (12hL:12hD), followed by a 6-hour sleep deprivation at light onset (ZT0-6).

All values represent mean delta power normalized to baseline ± SEM. Red asterisks represent genotype differences observed following post-hoc testing. [<10 lux: *Opn4*$^{+/+}$n=5, *Opn4*$^{-/-}$ n=6; 150 lux: *Opn4*$^{+/+}$n=6, *Opn4*$^{-/-}$ n=6; 600 lux: *Opn4*$^{+/+}$n=5, *Opn4*$^{-/-}$ n=5]

Baseline time-course [0-48]:

Repeated-Measures ANOVA Light Condition x Genotype x Time-course-

$P_{time\text{-}course}$<0.00001; $P_{time\text{-}course\ x\ genotype}$=0.003;

Following sleep deprivation [55-72]:

Repeated-Measures ANOVA Light Condition x Genotype x Time-course-

$P_{time\text{-}course}$<0.00001

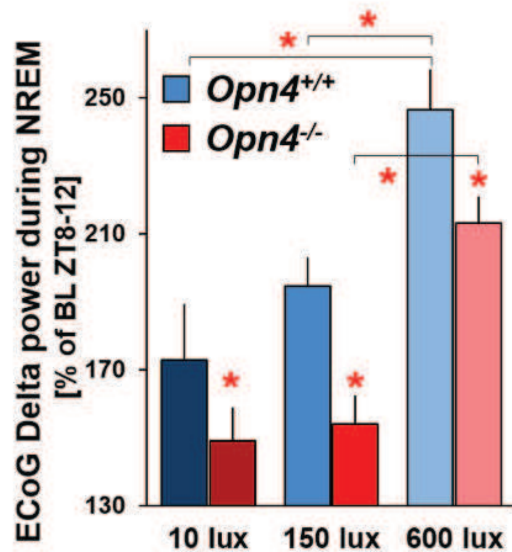

Figure 3: ECoG delta power peak of NREM following a 6-hour sleep deprivation.

Values represent mean delta power (0.5-4 Hz) during NREM sleep expressed as a percentage of the lowest mean delta power during baseline (ZT8-12). Error bars represent SEM. Red asterisks represent genotype and/or light condition differences observed following post-hoc testing. [<10 lux: *Opn4+/+*n=5, *Opn4$^{-/-}$* n=4; 150 lux: *Opn4+/+*n=5, *Opn4$^{-/-}$* n=6; 600 lux: *Opn4+/+*n=4, *Opn4$^{-/-}$* n=4]

2-way ANOVA for factors light condition x genotype: $P_{light\text{-}condition}<0.0001$; $P_{genotype}=0.0008$

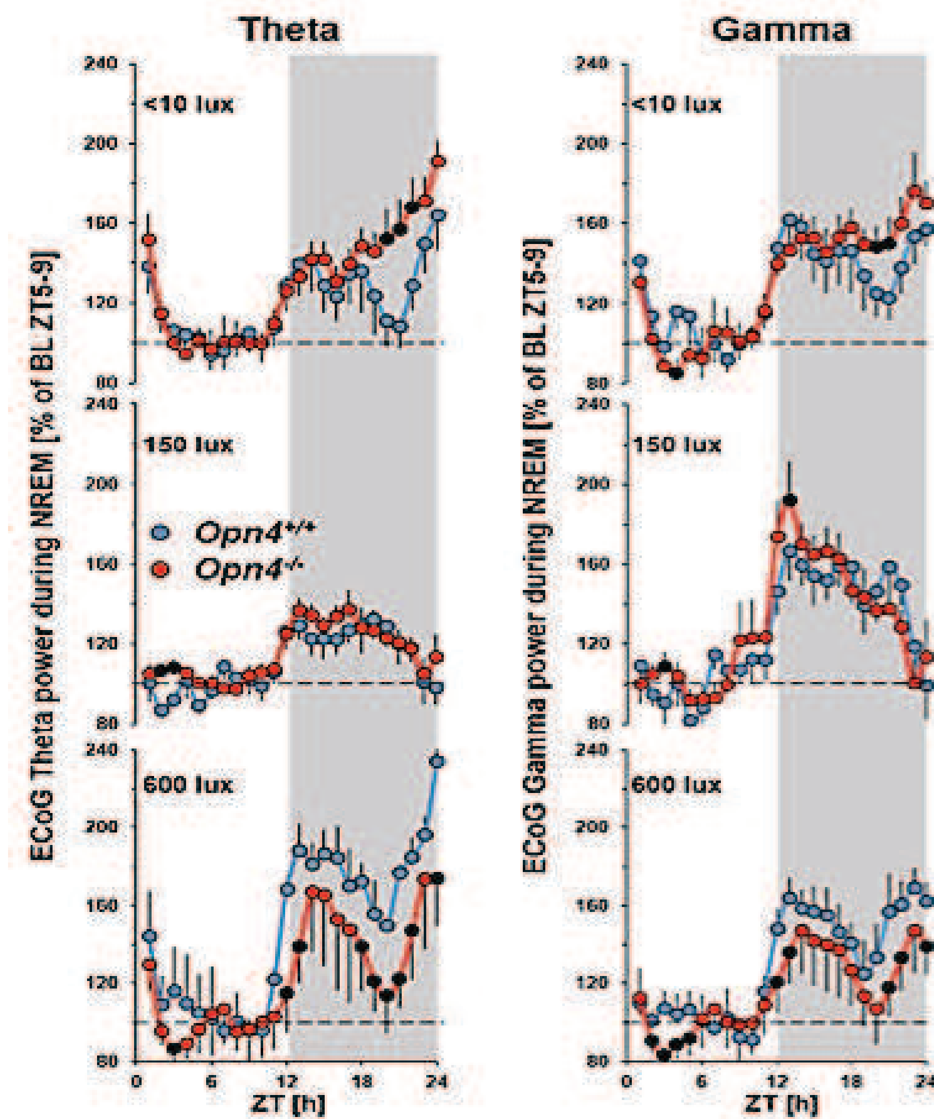

Figure 4: Time-course of relative theta and gamma power during wake across the baseline period under different light intensities

Theta baseline time-course [0-24]:

Repeated-Measures ANOVA Light Condition x Genotype x Time-course-

$P_{Light\ Condition}$=0.01; $P_{time\text{-}course}$<0.00001; $P_{time\text{-}course\ x\ Light\ Condition}$<0.00001; $P_{time\text{-}course\ x\ Light\ Condition\ x\ Genotype}$=0.01

Post-hoc Light Condition x Genotype-

WT: 150 lux vs 600 p=0.04

KO: 150 lux vs 600 p=0.01

Theta baseline L vs D [ZT0-12 vs 13-24]:

Repeated-Measures ANOVA Light Condition x Genotype x Light/Dark x Time-course-

$P_{Light\ condition}$=0.001; $P_{Light\ vs\ Dark}$=0.01; $P_{time\text{-}course}$<0.00001; $P_{time\text{-}course\ x\ Light\ Condition}$<0.00001; $P_{time\text{-}course\ x\ Light\ vs.\ Dark}$<0.00001; $P_{time\text{-}course\ x\ Light\ Condition\ x\ Light\ vs\ Dark}$<0.00001

Gamma baseline time-course [0-24]:

Repeated-Measures ANOVA Light Condition x Genotype x Time-course-

$P_{time\text{-}course\ x\ Light\ Condition}<0.00001$

Post-hoc Light Condition x Genotype-

10 lux: WT vs KO p=0.07

Gamma baseline L vs D [ZT0-12 vs 13-24]:

Repeated-Measures ANOVA Light Condition x Genotype x Light/Dark x Time-course-

$P_{Genotype}=0.03$; $P_{Light\ vs\ Dark}<0.00001$; $P_{time\text{-}course}<0.00001$; $P_{time\text{-}course\ x\ Light\ Condition}=0.002$; $P_{time\text{-}course\ x\ Light\ vs.\ Dark}<0.00001$; $P_{time\text{-}course\ x\ Light\ Condition\ x\ Light\ vs\ Dark}<0.00001$

Article 11: ***« The sleep-wake dependent changes in clock gene expression and in EEG delta power do not depend on an intact SCN »***

En préparation

Article: pages 1-15

Figures : pages 16-19

Figures et table supplémentaires : pages 20-23

Références : pages 24-25

1. Title page. ***Original paper***

Title: The sleep-wake dependent changes in clock gene expression and in EEG delta power do not depend on an intact SCN

Short title: The role of the SCN in the sleep homeostasis

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Tables and Figures: 4 Figures, 4 supplemental figures, 1 supplemental table

Keywords: sleep regulation, homeostatic sleep process, circadian sleep process, direct effects of light, suprachiasmatic nucleus, SCN lesions, clock genes, Homer 1a

Word count: Main text = 4842; Abstract = 240; References =29

2. MAIN MANUSCRIPT:

ABSTRACT

Study Objectives: Considerable cross-talks between the homeostatic and the circadian processes have been observed in the regulation of sleep. Additionally, recent data showed that the direct effects of light are a further major mechanism in shaping the daily sleep/wake cycle with the suprachiasmatic nucleus (SCN) mediating direct effects of light beyond its crucial role in the circadian process. We investigated the contribution of the SCN in the sleep/wake distribution and in the sleep homeostasis through both, a behavioral and a molecular approach.

Design: Electrocorticogram (ECoG), qPCR and ISH data were obtained for baseline and recovery from sleep deprivation in all groups of animals.

Setting: Mouse sleep laboratory.

Participants: Male mice.

Interventions: Sham, complete and partial lesions of the SCN, 6-hrs sleep deprivation starting at ZT0/CT0 under 12:12 LD condition and under darkness.

Measurements and Results: Contrarily to locomotor activity, the integrity of the SCN is essential for a robust daily sleep/wake distribution. In partial SCN-lesioned mice an uncoupling of NREM sleep from locomotor activity was observed and did not depend on *Per2* clock gene. The sleep homeostasis process is regulated differently under a habitual 12:12 LD condition with the SCN having a major influence, and as challenged by a SD condition where the role of the SCN seems inexistent.

Conclusions: Under standard conditions, the SCN strongly influence the sleep homeostasis. At least on a molecular level, this interaction does not imply the direct effects of light, but the circadian process.

Keywords: sleep regulation, homeostatic sleep process, circadian sleep process, direct effects of light, suprachiasmatic nucleus, SCN lesions, clock genes, Homer 1a

INTRODUCTION

In the sleep regulation study, two processes are classically considered: a homeostatic and a circadian process (Borbely 1982; Daan, Beersma et al. 1984). The homeostatic process tracks sleep need and can be analyzed by observing changes in the electrocorticogram (ECoG) derived variable delta power that can quantify the delta oscillation (1-4 Hz) characteristic of the non rapid-eye-movement (NREM) sleep. The circadian process gives time-context to most physiological processes including the sleep/wake cycle. In mammals, the suprachiasmatic nucleus (SCN) is located in the hypothalamus and is able to generate self-sustained oscillations (Klein, Schaad et al. 1992). The SCN ensures the proper entrainment of internal rhythms to the daily light/dark cycle and thus, the distribution of sleep over the 24-hour day is strongly determined by the circadian process (Reppert and Weaver 2002). Studies in animals rendered arrhythmic by lesioning the SCN have shown that the homeostatic regulation of sleep is not affected and thus does not depend on intact circadian rhythms (Trachsel, Edgar et al. 1992; Easton, Meerlo et al. 2004; Larkin, Yokogawa et al. 2004). Thus, these two processes develop independently although their interaction determines the timing, the duration and the quality of sleep and of wakefulness (Trachsel, Edgar et al. 1992; Dijk and Czeisler 1995; Easton, Meerlo et al. 2004; Larkin, Yokogawa et al. 2004).

However, more recent observations indicate some “cross talk” between the two processes. Sleep deprivation, for instance, is able to phase shift the circadian clock (Antle and Mistlberger 2000) and firing rates of SCN neurons decreased during sleep (Deboer, Vansteensel et al. 2003). Furthermore, mice with genetic targeted disruptions of the core clock genes not only show an altered circadian phenotype but also the homeostatic regulation of sleep seems affected (Shaw and Franken 2003; Franken, Dudley et al. 2006). In our previous papers, we also demonstrated that *Per1*, *Per2* and *Dbp* clock genes were affected in the cerebral cortex by sleep deprivation (SD) supporting that at least at the molecular level, there is a relation between clock genes and sleep homeostasis (Franken, Thomason et al. 2007; Maret, Dorsaz et al. 2007; Franken and Dijk 2009; Curie, Mongrain et al. 2013). Thus it became crucial to understand the role of the SCN in the sleep homeostasis.

In this paper, we investigated the contribution of the SCN in the sleep/wake distribution and in the sleep homeostasis through both, a behavioral and a molecular approach. We measured the homeostatic component of sleep in baseline and also after a 6-hrs SD (between ZT0-6) by following the electrocorticogram (ECoG), in three groups of mice: total SCN-lesioned mice that were arrhythmic, partial SCN-lesioned mice that were still

rhythmic and sham control mice. We quantified the slow-wave activity during NREM sleep and analyzed in these groups of mice the EEG delta power, a measure thought to only index homeostatic sleep need with circadian factors having negligible influence (Franken, Dijk et al. 1991; Franken and Dijk 2009). The time course of delta power predicted on the sleep-wake distribution was also simulated using the same model parameters determined previously (Franken, Dudley et al. 2006). In that case, our simulation was able to predict the empirical levels of EEG delta power reached in baseline and after the ZT0-6 SD in all the three groups of mice.

Finally, we analyzed using qPCR and *in situ* hybridization (HIS) the levels of the clock genes *period- (Per)1*, *Per2*, and of the clock-controlled gene *D-site albumin-promotor binding protein* (*Dbp)* in SCN-lesioned (SCNx) arrhythmic mice in baseline condition and after a 6hrs-SD (ZT0-6 SD). Our results demonstrated that SD-induced changes were decreased for *Per1* and *Per2*, and increased for *Dbp*, in the forebrain of arrhythmic mice immediately at the end of SD, supporting a role for the SCN in the sleep homeostasis. Light did not influence *Per1*, *Per2* and *Dbp* gene expression as shown under constant darkness.

Taken together, our data reveal that the integrity of the SCN is necessary for a normal distribution of the sleep/wake cycle and that at the molecular level, clock genes are strongly implicated in the sleep homeostasis.

METHODS

Animals and housing conditions

All animals were kept under a 12h-light/12h-dark cycle (12:12 LD; lights on at 9 A.M., 110lux) and were singly housed with food and water available *ad libitum*. We use Zeitgeber time (ZT) to indicate time-of-day with ZT0 (or ZT24) marking light onset and ZT12 dark onset. Cages were placed in sound attenuated and temperature controlled recording rooms (25°C). In all experiments, animals were sleep deprived for 6h (6hr-SD) by what is referred to as ‘gentle handling‘ which consists of displacing bedding or introducing novel objects in the cage as soon as the animal attempted to sleep (Franken, Dijk et al. 1991). Touching the animal was done only when it no longer responded to other stimuli.

C57BL6/J mice (B6) were purchased from Jackson Laboratory (Bar Habor, ME) except for the B6 mice used in the EEG experiment which were purchased from Charles River (Lyon, France). All experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals as well as local veterinary office and use committees at Stanford University. All EEG experiments were approved by the Ethical Committee of the State of Vaud Veterinary Office, Switzerland.

Suprachiasmatic nuclei (SCN) lesions

Bilateral lesions of the two SCNs were performed stereotaxically (Kopf Instruments, 963LS, Miami Lakes, Florida, USA) under ketamine/xylazine aneasthesia (intraperitoneal injection, 75- and 10mg/kg, at a volume of 8ml/kg) and using radiofrequency lesions aimed at the SCN according to published protocols (Easton, Meerlo et al. 2004). Briefly, two electrodes (0.3mm in diameter) were 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; (Franklin and Paxinos 2007)] corresponding to the two SCN nuclei. Locomotor activity was recorded during the whole experiment and effectiveness of lesions was assessed by periodogram analysis of locomotor activity (ClockLab, Actimetrics, Wilmette, IL, USA) (Supplemental Figure 1) and SCN lesions were verified histologically by performing Nissl staining on coronal brain sections (Supplemental Figure 2, Figure 3C, 3F).

Locomotor activity

Locomotor activity of male mice (age 2 months) was recorded under a 12h:12h LD cycle (12h-light/12h-dark, lights on at 9 A.M., 110lux). Then lesions were done on the two SCN and locomotor activity of mice was recorded under 12h:12h LD, followed by complete darkness (DD) and then under 12h:12h LD to verify the circadian organization of overt behavior under Zeitgeber free conditions. Activity was quantified using passive infra-red (PIR) sensors (Visonic SPY 4/RTE-A). ClockLab software was used both for data acquisition and analyses. Presence or absence of significant circadian rhythmicity in locomotor activity was evaluated using a chi-square periodogram analyses ($P<0.01$).

Histochemical procedures and Nissl Staining

After following activity of mice, anatomical site of lesion was verified with Cresyl violet staining of coronal brain sections. Briefly, mice were anaesthetized by

ketamine/xylazine (intraperitoneal injection, 75- and 10mg/kg, at a volume of 8ml/kg) and subjected to intracardiac perfusion with PBS-heparin (2units/mL) and with 4% paraformaldehyde in PBS, pH7.4. Brains were carefully removed and incubated in 10% and 20% of sucrose for 1h each and in 30% of sucrose in PBS for 48h at 4°C, and then embedded in OCT compound at -20°C until cryostat sectioning. Coronal brain sections (20 µm) were washed in PBS and then fixed again in PFA 4% at 4°C for 10 minutes. Brain sections were then incubated for 10-15 minutes in Cresyl violet (Sigma-Aldrich®) and washed in distilled water. Then brain slides were successively immerged in ethanol 95%, ethanol 100%, and three xylene baths before to be coverslipped in Eukitt medium (Fluka). Chemical staining from whole brain coronal views was finally acquired using a stereomicroscope MZ16FA (Leica).

EEG/EMG recording and analysis

EEG/EMG surgeries were performed according to the methods previously described (Franken, Malafosse et al. 1998) with minor changes. Briefly, EEG and EMG electrodes were implanted under deep ketamine/xylazine anesthesia (intraperitoneal injection, 75- and 10mg/kg, at a volume of 8ml/kg). Two gold-plated screws (diameter 1.1mm) served as EEG electrodes and were screwed through the skull over the right cerebral hemisphere (frontal: 1.7mm lateral to midline, 1.5mm anterior to bregma; parietal 1.7mm lateral to midline, 1.0mm anterior to lambda). Four additional screws were used as anchor screws. Two semi-rigid gold wires were used for EMG electrodes and were inserted into the neck musculature along the back of the skull. The four electrodes were soldered to a connector and cemented to the skull. After recovery from surgery (4-7 days) mice were connected to a swivel contact through recording leads to which they could habituate for 7 days prior to the experiment.

B6 male mice, 3 months old, (9 Control (CTRL), 9 Sleep-deprived (SD); total n=18) were recorded for 72 continuous hours of which the first 48h served as baseline followed by 6h of SD initiated at ZT0 (ZT0 light onset) and recovery. EEG and EMG signals were amplified, filtered, and analogy-to-digital converted (256Hz). The behavioral states wakefulness (W), rapid eye movement sleep (REM), and non-REM sleep (NREM) were visually assigned for consecutive 4-second epochs as described previously (Franken, Malafosse et al. 1998). EEG signals were subjected to a discrete Fourier transform (DFT) to determine EEG power density in the delta frequency range (i.e., delta power, 1-4Hz) for 4-second epochs scored as NREM. Differences in absolute levels of delta power among individuals were accounted for by expressing it as a percentage of the mean delta power over the last 4h of the two baseline light

periods (ZT8-12) when sleep pressure is at its lowest. Delta power was averaged for 12 intervals to which an equal number of NREM epochs contributed (i.e., percentiles) during the 12h-light periods, for 6 intervals during the 12h-dark periods, and for 8 intervals during the 6h immediately following SD (recovery). Choice of the number of percentiles per recording segment depended on the amount of NREM present.

Expected levels of delta power were calculated using previously published parameter estimates (Franken, Dudley et al. 2006). In these simulations the time course of delta power is predicted solely on the sleep-wake distribution by assuming that a homeostatic need for sleep ('Process S') increases according to a saturating exponential function when the animal is awake (or in REM) according to $S_{t+1} = UA - (UA - S_t)*e^{-dt/\tau i}$, and decreases exponentially when animals are in NREM according to $S_{t+1} = LA + (S_t - LA)*e^{-dt/\tau d}$; where S_{t+1} and S_t are consecutive values of Process S (time resolution of iteration dt=4s) which varies between an upper (UA=282%) and lower (LA=55%) asymptote with time constant τ_i (=7.9h) and τ_d (=1.9h) for the increase and decrease, respectively [for details see (Franken, Chollet et al. 2001); parameter estimates taken from (Franken, Dudley et al. 2006)].

RNA extraction and Quantitative PCR Analyses:

For gene expression level, two different and separate experiments have been performed from different sets of mice groups.

Experiment 1: Sleep deprivation effect in clock gene expression changes in SCN-lesioned mice:

Sleep deprivation was performed between ZT0 and ZT6. Sleep-deprived mice (n=8) were killed at ZT6 with their non sleep-deprived control mice (n=8). Total RNA was isolated from left frontal cerebral cortex at ZT6 (Supplemental Figure 3). Then quantification of the RNA level was done by qPCR Taqman analyses. *Homer1a, Dbp, Per2*, and *Per1* genes were quantified and normalized using *GusB, Rps9 and Tbp* as housekeeping genes.

Experiment 2: *Light effect on the sleep deprivation in clock gene expression changes*:

Sleep deprivation was performed between ZT0 and ZT6 with lights off. Sleep-deprived mice (n=6) and their control (n=6) were killed at ZT6 and total RNA from the whole forebrain was extracted (Supplemental Figure 3). Quantification of the RNA level was done

by qPCR Taqman analyses. *Dbp*, *Per1* and *Per2* genes were then quantified and normalized using *Eef1a1, Rps9 and Tbp* genes.

In both experiments, after 6h of SD, B6 male mice were killed by cervical dislocation together with their non-SD controls. Brains were rapidly removed and frozen at -80°C. The brain tissues were then used for quantitative PCR (Taqman) assessment of the steady state expression levels of genes of interest (Supplemental Table 1).

Briefly, RNA from the left frontal cortex (piriform and dorsal cortex) (Experiment1) or from the whole forebrain (Experiment2) was isolated and purified with the RNeasy Lipid Tissue Midi kit (Qiagen, Hombrechtikon, Switzerland) and DNase-treated. RNA quantity was assessed with a NanoDrop ND-1000 spectrophotometer (ThermoScientific, Wilmington, DE, USA) and RNA quality was controlled on Agilent 2100 bioanalyzer chips (Agilent Technologies, Basel). For cDNA synthesis, 1mg of total RNA was reverse-transcribed using random hexamers and Superscript III reverse transcriptase (Invitrogen, Basel), according to standard procedures. The cDNA equivalent of 20ng of total RNA was PCR-amplified in an ABI PRISM 7700 detection system (Applied Biosystems, Switzerland). Three technical replicates were analyzed. Forward, reverse primers and probes sequences were chosen from the coding regions of the genes of interest and were determined using Primer Express version 1.0 software (Applied Biosystems, Switzerland). To confirm specificity of the nucleotide sequences chosen for the primers and probes and the absence of DNA polymorphisms, BLASTN searches were conducted against the dbEST and nonredundant set of GenBank, EMBL and DDBJ databases. Primers (Invitrogen) and probes (Eurogentec, Seraing, Belgium) sequences used are listed in Supplemental Table 1. Three technical replicates were analyzed. Gene expression level was calculated using the modified ddCt method from qBase (Hellemans, Mortier et al. 2007) and normalized relative to three housekeeping genes selected with gNorm software (Vandesompele, De Preter et al. 2002).

***In Situ* Hybridization** (*To be completed)*

A complementary approach using *in situ* hybridization (IHS) was used to analyze the clock genes *Per1* and P*er2* expression levels in animals exposed to a 6-hrs SD from ZT0-6 (same conditions as "Experiment 1"). Brains were perfused and prepared as explained above (section "histochemical procedures") for ISH in the dorsal and piriform cortex of the mid/fore-brain in SCN-lesioned and Sham animals (Supplemental Figure 3).

Statistical Analysis

To assess the effects of sleep deprivation (SD vs. control) in the absence of light or in the absence of SCN, *t-test* analysis was performed. Significant effects for the t-tests were obtained and statistical significance was set to P=0.05 and results were reported as mean ± SEM. SigmaPlot 11 (Systat Software Inc., Chicago, Il, USA) was used for non-linear fitting of Gaussian and exponential saturating functions.

RESULTS

Mice are nocturnal animals and thus are mainly active during the night and especially the first part of the night (ZT12-ZT18) and are essentially asleep during the day (ZT0-ZT12). When mice are placed under constant darkness, they start to free-run (Supplemental Figure 1A). At the opposite, total SCN-lesioned mice (referred to as “SCNx arrhythmic”) that have no traces of positive Nissl-stained cells in the SCN (Supplemental Figure 2C) become arrhythmic confirming the primordial role of the SCN in generating rhythmic locomotor activity (Supplemental Figure 1C). Between these two extremes, we also noticed that partial SCN-lesioned mice (referred to as “SCNx rhythmic”) which had only an absence of a part of the SCN (Supplemental Figure 2B) kept rhythmic locomotor activity (Supplemental Figure 1B). This means that even if there are few neurons in the SCN, this is enough for generating rhythmic circadian locomotor activity, although the activity of these SCNx rhythmic animals seems less high than in sham animals but overall, these animals are still rhythmic under LD cycle and free run under complete darkness (Supplemental Figure 1B).

The absence of the SCN influences the level of the EEG delta power in the sleep/wake distribution.

During two baseline days (48 hours), the typical sleep/wake distribution of Sham mice was observed with a clear preference for sleep during the light periods (ZT0-12), lowest levels in the first half of the dark periods (ZT12-18), and a transient increase in sleep time in the second half of the dark period (ZT18-24) (Figure 1B). The distribution of sleep gave rise to the equally typical dynamics of EEG delta power reaching highest levels during sleep immediately following the initial period of sustained wakefulness in the dark period, followed

by a gradual decline in function of the presence of NREM sleep, and reaching lowest levels in the last 4h of the light periods correlating with previous literature (Figure 1C) (Tsai, Hannibal et al. 2009).

Contrarily to rhythmic locomotor activity, the daily sleep/wake distribution of SCNx rhythmic animals was comparable to what was observed in the SCNx arrhythmic mice (Figure 1B). In both groups, SCNx arrhythmic and SCNx rhythmic mice, the sleep-wake distribution during the 48 hours of baseline was considerably attenuated and their NREM sleep level was higher during the night period, as compared to the Sham mice (Figure 1A,B).

Further, when considering delta power under baseline light/dark cycle in SCNx rhythmic animals, its diurnal variation was of similarly decreased amplitude as compared to the arrhythmic SCNx mice, and contrasted with Sham mice exhibiting strong diurnal variation in NREM sleep delta power (Figure 1C). In SCNx mice distribution of NREM sleep and of delta power, remained slightly higher as compared to the dark phase (Figure 1B, C). In Sham animals, NREM and ECoG delta power was strongly decreased at the transition of the light-dark cycle, and to a lesser extend increased at the dark-light transition. These changes persisted in the SCNx mice although the reactivity was largely attenuated in case of SCN lesions (Figure 1B, C). However, SD raised EEG delta power above the highest levels reached during baseline in all the three considered groups (Figure 1C) and there was no significant difference between the three groups in absolute values.

Interestingly, when we compared total SCN-lesioned and partial SCN-lesioned mice, although, partial SCN-lesioned mice have still rhythmic locomotor activity under LD, their NREM sleep was similar as compared to SCNx arrhythmic mice (Figure 1A, B). These findings suggest that the integrity of the SCN is necessary for a normal distribution of NREM sleep over the day.

The SD-induced variations in clock gene expression are reduced in SCN-lesioned mice.

In control conditions, except for *Per2*, (Figure 2D, 3D, 3E) we did not observe any statistical differences at ZT6 between SCN-lesioned mice which were arrhythmic (SCNx arrhythmic) and Sham mice (statistical tests using a *t*-test and a *P*-value <0,05) (Figure 2). The difference observed in *Per2* can be explained at least in part by the model we described previously (Curie, Mongrain et al. 2013).

After 6hrs-SD (ZT0-6), Sham mice had a significant increase in *Per1* (Figure 2C, 3B), *Per2* (Figure 2D, 3D, 3E), and *Homer1a* (Figure 2A) (fold change of 1,38; 1,83 and 2 for

Per1, *Per2* and *Homer1a* respectively) and a significant decrease for *Dbp* (Figure 2B) gene expression (fold change of 0,72 for *Dbp*), correlating with previous literature (Franken, Thomason et al. 2007; Curie, Mongrain et al. 2013). This observed change was still significant in SCNx arrhythmic mice (*t-test* and a *P*-value <0,05) for *Per1* (Figure 2C, 3B) , *Dbp* (Figure 2B) and *Homer1a* (Figure 2A) (fold change of 1,15; 1,53 and 2,23 for *Per1*, *Dbp* and *Homer1a* respectively). SD-induced change was significant for *Per2* in both the piriform (PC) and the dorsal cortex (DC) (Figure 3D, 3E) as measured by ISH, but not in the frontal forebrain using qPCR (Figure 2D). The fold-change observed in arrhythmic mice (SCNx arrhy) was slighly reduced for *Per1*, *Per2*, and not modified in the case of *Homer1a*. However, this change was largely increased in the case of *Dbp* (fold change of 0,72 in Sham mice versus 1,53 in SCNx arrhythmic mice). At the opposite, *Homer1a* transcript level was always significantly increased after 6hrs-SD independently of the lesion of the SCN (Figure 2A) and although this increase is slightly higher in the Sham as compared to the arrhythmic mice (SCNx arrhy) (fold change of 2,23 in Sham mice as compared to 2 in SCNx arrhythmic mice) (Figure 2A). Following 6hrs-SD, SCNx rhythmic mice behaved similarly as Sham mice for clock and clock-controlled genes (Figure 2B, C, D), whereas SD-induced *Homer 1a* gene expression was as strongly altered as in SCNx arrhythmic animals (Figure 2A). Regarding *Per1*, no significant SD-induced augmentation was observed in the piriform cortex (Figure 3A).

Furthermore, *Per1* and *Per2* mRNA levels in the SCN did not show any significant variations after the 6-hrs SD as compared to the control condition in intact mice (Supplemental Figure 4).

The influence of lighting condition was estimated by performing SD under constant darkness.

We analyzed clock genes in a darkness condition to determine whether the SD-induced gene expression observed under a standard 12:12 LD cycle was regulated by a clock-driven mechanism and/or due to the direct influence of light on sleep (Tsai, Hannibal et al. 2009) (Hubbard, Ruppert et al. submitted). A 6-hrs SD was performed in intact mice starting from Circadian Time (CT) 0 to CT6 in darkness (without switching lights on at CT0) for study of *Per1, Per2* and *Dbp* gene expression levels (Figure 5). As a result, we still observed a significant increase for *Per1* and *Per2* and a significant decrease for *Dbp* (*t-test*, *P*-value <0,0001) (Figure 5). Following 6-hrs SD, there were no differences in expression levels for *Per1* and *Per2* in animals under normal light/dark cycle as compared to the darkness

condition (Figure 5, 2C, 2D). However, the decrease of the *Dbp* expression level was increased in the absence of light (reduction of 1,5 fold time under darkness compared to 0,72 fold time in intact animals under L/D (Figure 5, Figure 2B), even if we have to take into account that both experiments were done with two different and separate groups of mice. This suggests that except for *Dbp*, the absence of light does not modify the effect of the SD in the *Per1*, *Per2* gene expression level in the forebrain.

All these results support the idea that the expression of the *Per2* circadian gene integrates both time-of-day (circadian) and sleep-wake dependent (homeostatic) influences compared to *Homer1a* which is mainly activity (or waking) induced.

DISCUSSION

Recent data showed that, contrary to what was commonly assumed circadian factors might contribute to the homeostatic dynamics and expression of the EEG delta power (Curie, Mongrain et al. 2013). Further, the SCN, beyond their role as a central biological clock play a crucial role in the direct effects of light mediating melanopsin-dependent and rod/cone-dependant non-visual light information to structures regulating sleep and alertness (Hubbard, Ruppert et al. submitted). We investigated the role of the SCN in the sleep homeostasis process by a behavioral and a molecular approach using SCN-lesioned mice.

The integrity of the SCN is essential for the daily sleep/wake distribution

Surprisingly, our results showed that in partial SCN-lesioned mice the circadian rhythm of rest/locomotor activity was preserved, while the circadian process of NREM regulation was no more functional. Regarding sleep, partial SCN-lesioned mice behaved similarly as total SCN-lesioned arrhythmic mice, contrary to their locomotor activity, meaning that although the activity is rhythmic, the integrity of the SCN is necessary for having normal distribution of NREM sleep over the day. This was further underlined by SD-induced *Homer 1a* expression level which was similarly decreased in partial and total SCN-lesioned mice as compared to Sham animals. This was not the case for both clock and clock-controlled genes, where SCN rhythmic mice behaved overall similarly as Sham mice following SD.

Reactivity to light-dark-light transitions under 12:12 LD baseline condition were preserved, albeit reduced in both total and partial SCN-lesioned mice. They also kept a slighly higher expression of NREM during the light period, due to the direct effects of light still shaping a daily sleep/wake cycle as predicted by our previously published model (Hubbard, Ruppert et al., submitted). These data suggest that following partial SCN lesions, we observe the circadian uncoupling of NREM sleep from locomotor activity. Under 12:12 LD baseline condition, the SCN are necessary to keep a strong daily variation of the sleep homeostasis as shown by the nearly abolished variation of ECoG delta power in SCNx mice. On the opposite, the sleep homeostasis system as challenged through a 6-hour sleep deprivation condition was completely normal in the absence of the SCN. These results suggest that the SCN is involved in the interaction between processes C and S, both together determine the timing, the duration and the quality of sleep and wakefulness. On the other hand, as previously shown, the SCN are not necessary for the sleep homeostasis process challenged through sleep deprivation (Trachsel, Edgar et al. 1992; Easton, Meerlo et al. 2004), a condition that probably implies different survival mechanisms when occurring in the wild. Thus, the sleep homeostasis process is regulated differently under a habitual 12:12 LD condition with the SCN having a major influence and under a SD condition where the role of the SCN seems inexistent. Whether these effects are due to the SCN's crucial function in the circadian process (Reppert and Weaver 2002), or to its role in mediating the direct effects of light to structures involved in the sleep/wake regulation (Hubbard et al. submitted), remains unclear.

The SD-induced variations in expression levels of clock and clock-controlled genes are reduced in SCN-lesioned mice.

In previous studies, we determined the role of circadian genes (also called clock genes) in the sleep homeostatic process of sleep (Wisor, O'Hara et al. 2002; Franken, Dudley et al. 2006; Franken, Thomason et al. 2007, Curie, Mongrain et al. 2013). In this study, our aim was to assess how the SD interacts with the expression of core clock genes such as *Per1* and *Per2* and the clock-controlled transcription factor *D-site albumin promoter binding protein* (*Dbp*) in the absence of the SCN. We also used as a control the activity-induced transcript *Homer1a* that we previously identified as a molecular marker of sleep need (Maret, Dorsaz et al. 2007). In intact animals, a SD-induced increase in expression levels of *Per1* and *Per2* genes was observed in the cortex, but not in the SCN. Previous data already demonstrated the considerable cross-talk between the circadian and homeostatic process, and that *Per2* gene

expression from whole brain responds to both sleep loss and time of day (Curie, Mongrain et al. 2013). The mathematical model previously created to explain how sleep can affect *Per2* gene expression is here reinforced by our results (Curie, Mongrain et al. 2013). Our data further confirm that *Per2* is a well-positioned molecule to track time spent awake and asleep (Franken and Dijk 2009; Curie, Mongrain et al. 2013).

Interestingly, contrarily to SCNx arrhythmic mice, steady state expression level of *Per2* was not affected in partial SCN-lesioned rhythmic animals. These data suggest that *Per2* is not implicated in the loss of circadian rhythms in NREM sleep in the presence of a partial SCN lesion.

The influence of lighting condition was estimated by performing SD under constant darkness.

Our experiments, as well as previously reported SD studies were performed under standard 12:12 LD conditions (Curie, Mongrain et al. 2013). Recent findings underline the importance of the non-circadian direct effects of light on sleep and alertness (Altimus, Guler et al. 2008; Lupi, Oster et al. 2008; Tsai, Hannibal et al. 2009; Hubbard, Ruppert et al. 2013; Hubbard, Ruppert et al. submitted). Non-visual light, through melanopsin-based and rod/cone-driven direct effects, is a crucial mechanism for the regulation of the sleep/wake cycle (Hubbard, Ruppert et al. submitted). Furthermore, the retinal photopigment melanopsin is strongly implicated in the sleep homeostasis process, as in its absence animals have significantly lower levels of sleep pressure, both under baseline and following a SD condition (Altimus, Guler et al. 2008; Tsai, Hannibal et al. 2009). Light transmission outside of the SCN remained most probably intact in our mice, as histological staining showed a very limited lesion on the SCN, sparing the surrounding areas which of the optic chiasm. Given the major influence of the direct effects of light on sleep, we tested whether the observed influence of the SCN was due either to its role as major circadian process or to its role as a relay system of the direct effects of light. SD-induced *Per1* and *Per2* gene expression levels remained unchanged when sleep deprivation was performed under darkness, without switching lights on at ZT0. These results illustrate that the influence of the SCN on the sleep homeostasis, at least on a molecular level, does not imply the direct effects of light, but the circadian process.

CONCLUSION

The integrity of the SCN is essential for the considerable cross-talks observed between the circadian and the homeostatic process resulting in a robust daily sleep/wake distribution. Contrarily, partial SCN-lesioned animals continued to show rhythmic locomotor activity so that an uncoupling of NREM sleep from locomotor activity was observed. The *Per2* clock gene integrates both circadian and homeostatic influences, but this well positioned molecule to track time spent awake and asleep, is not implicated in the loss of circadian rhythms in NREM sleep, in partial SCN-lesioned mice. The sleep homeostasis process is regulated differently under a habitual 12:12 LD condition with the SCN having a major influence, and as challenged by a SD condition where the role of the SCN seems inexistent. Further, we demonstrated that the influence of the SCN on the sleep homeostasis, at least on a molecular level, does not imply the direct effects of light, but the circadian process.

ACKNOWLEDGMENTS/
The authors thank xxx for financial support.

3. FIGURES

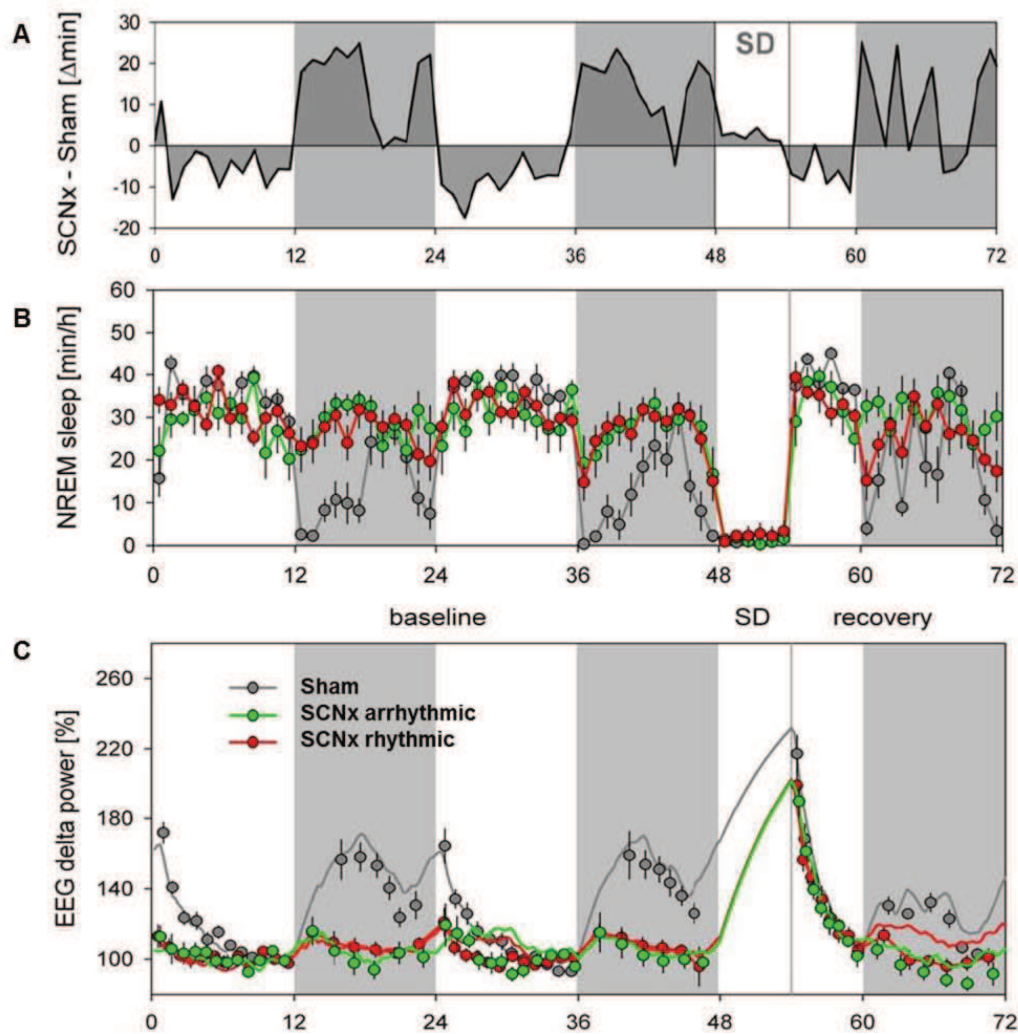


Figure 1: Effects of sleep deprivation on NREM and on ECoG delta power in SCN-lesioned mice. *(to be completed for statistics)*

Recordings during 48 hours baseline and after a 6h-sleep deprivation (ZT0-6) followed by recovery sleep in 12:12 LD cycle for Sham, SCNx rhythmic and SCNx arrhythmic mice. Grey areas delineate the dark periods. (**A**) Differences in times spent in NREM (min/recording hour) between SCNx mice (arrhythmic + rhythmic) and Sham mice. (**B**) Times spent in NREM (min/recording hour) during baseline, 6-hrs SD (ZT0-6) and recovery sleep for sham mice (grey circles), SCNx arrhythmic mice (green circles) and SCNx rhythmic mice (red circles). (**C**) Time course of NREM ECoG delta power during baseline, 6-hrs SD (ZT0-6) and recovery sleep for sham mice (grey circles), SCNx arrhythmic mice (green circles) and SCNx rhythmic mice (red circles). Simulation of homeostatic process S is shown with lines for Sham mice (grey line), SCNx arrhythmic mice (green line) and SCNx rhythmic mice (red line). (**B**) and (**C**) Values are expressed as mean (± SEM).

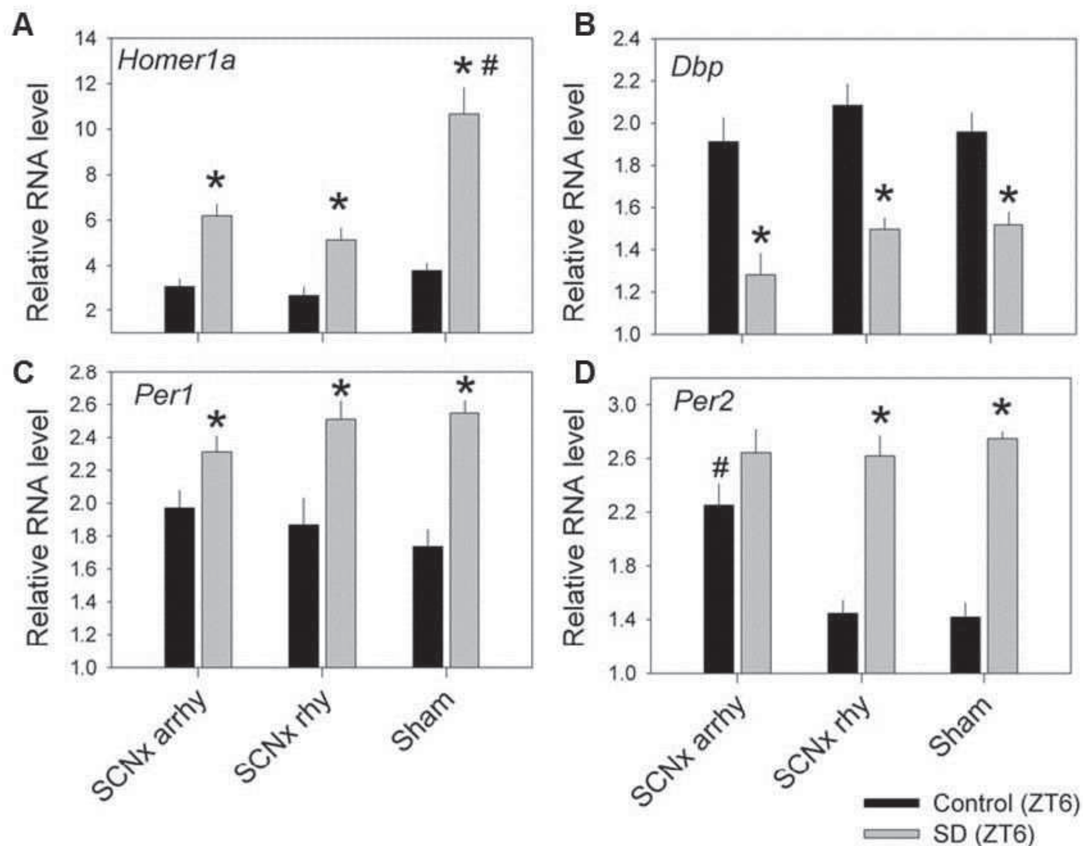
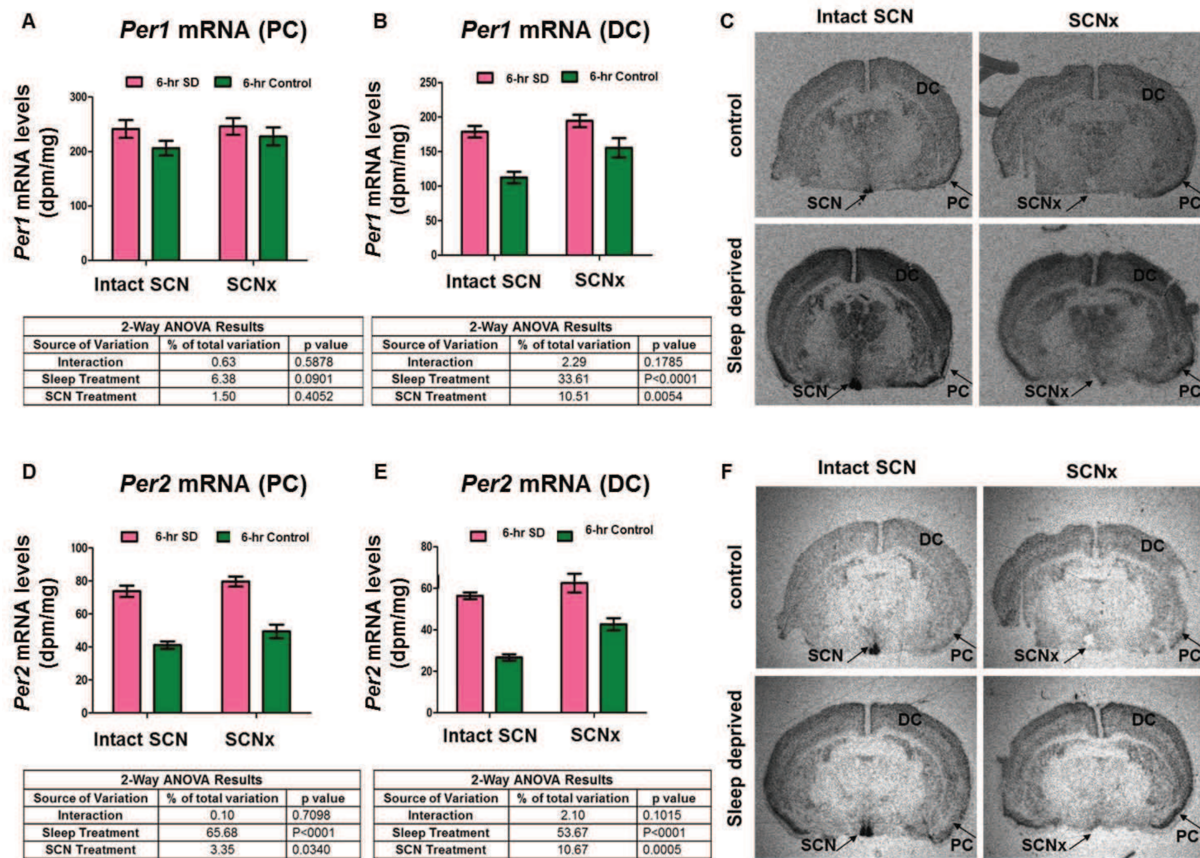


Figure 2: Effects of sleep deprivation and SCN lesion on forebrain mRNA levels of *Per1*, *Per2*, *Dbp* and *Homer1a* as measured by qPCR in the three groups of mice. *(to be completed for statistics)*

Gene Expression levels of (**A**) *Homer1a*, (**B**) *Dbp*, (**C**) *Per1* and (**D**) *Per2* for B6 mice in 12:12 LD cycle after a 6hrs-SD started at light-onset (ZT0-6) (grey columns) and their controls (black columns) for SCN-lesioned arrhythmic mice (SCNx arrhy), SCN-lesioned rhythmic mice (SCNx rhy) and intact mice (Sham).

Asterisks (*) indicate significant differences (with *P* value <0.05) between controls and sleep-deprived mice within the same group; either SCNx arrhy, SCNx rhy or Sham mice, at the same ZT time point. The symbol (#) indicates significant differences (with *P* value <0.05) between control and control or sleep-deprived and sleep-deprived mice between both groups of mice SCNx arrhy and Sham mice.



2-Way ANOVA Results		
Source of Variation	% of total variation	p value
Interaction	0.63	0.5878
Sleep Treatment	6.38	0.0901
SCN Treatment	1.50	0.4052

2-Way ANOVA Results		
Source of Variation	% of total variation	p value
Interaction	2.29	0.1785
Sleep Treatment	33.61	P<0.0001
SCN Treatment	10.51	0.0054

2-Way ANOVA Results		
Source of Variation	% of total variation	p value
Interaction	0.10	0.7098
Sleep Treatment	65.68	P<0001
SCN Treatment	3.35	0.0340

2-Way ANOVA Results		
Source of Variation	% of total variation	p value
Interaction	2.10	0.1015
Sleep Treatment	53.67	P<0001
SCN Treatment	10.67	0.0005

Figure 3: Effects of 6-hrs SD and SCN lesion on mRNA levels of *Per1* and *Per2* in the dorsal and piriform cortex as measured by *in situ* hybridization.

Gene Expression levels of *Per1* (top) and *Per2* (bottom) in (**A, C** for *Per1*; **D, F** for *Per2*) the dorsal and (**B, C** for *Per1* and **E, F** for *Per2*) piriform cortex in B6 mice under 12:12 LD after a 6hrs-SD started at light-onset (ZT0-6) (rose columns) and their controls (green columns) for Sham animals with intact SCN and SCN-lesioned arrhythmic mice (SCNx) (**C** for *Per1*, **D** for *Per2*) as quantified from coronal brain sections at the level of the mid SCN area.

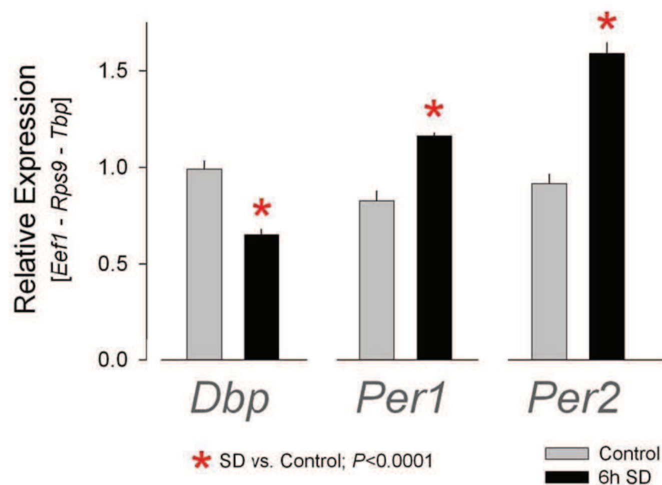
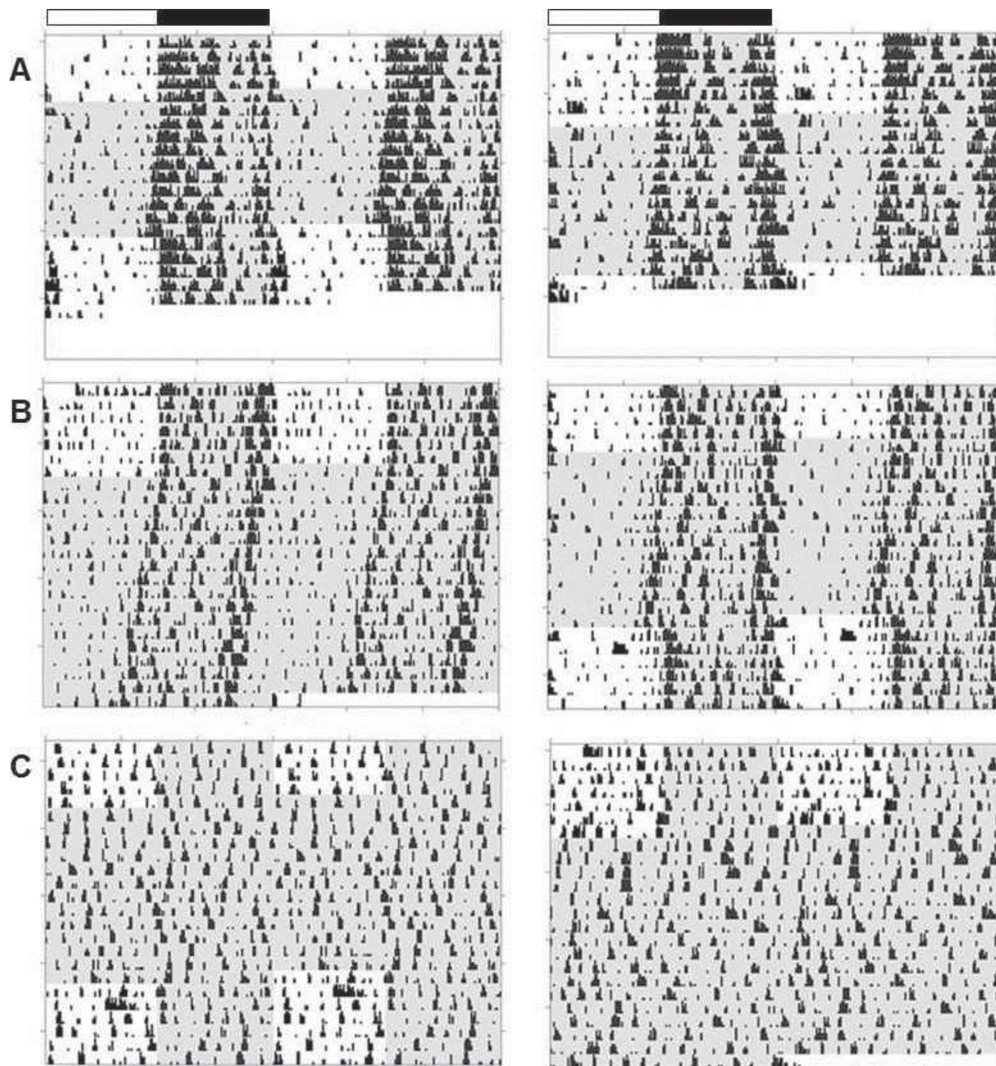


Figure 4: Effects of sleep deprivation performed in darkness on forebrain mRNA levels of *Per1*, *Per2* and *Dbp* as measured by qPCR in intact mice.

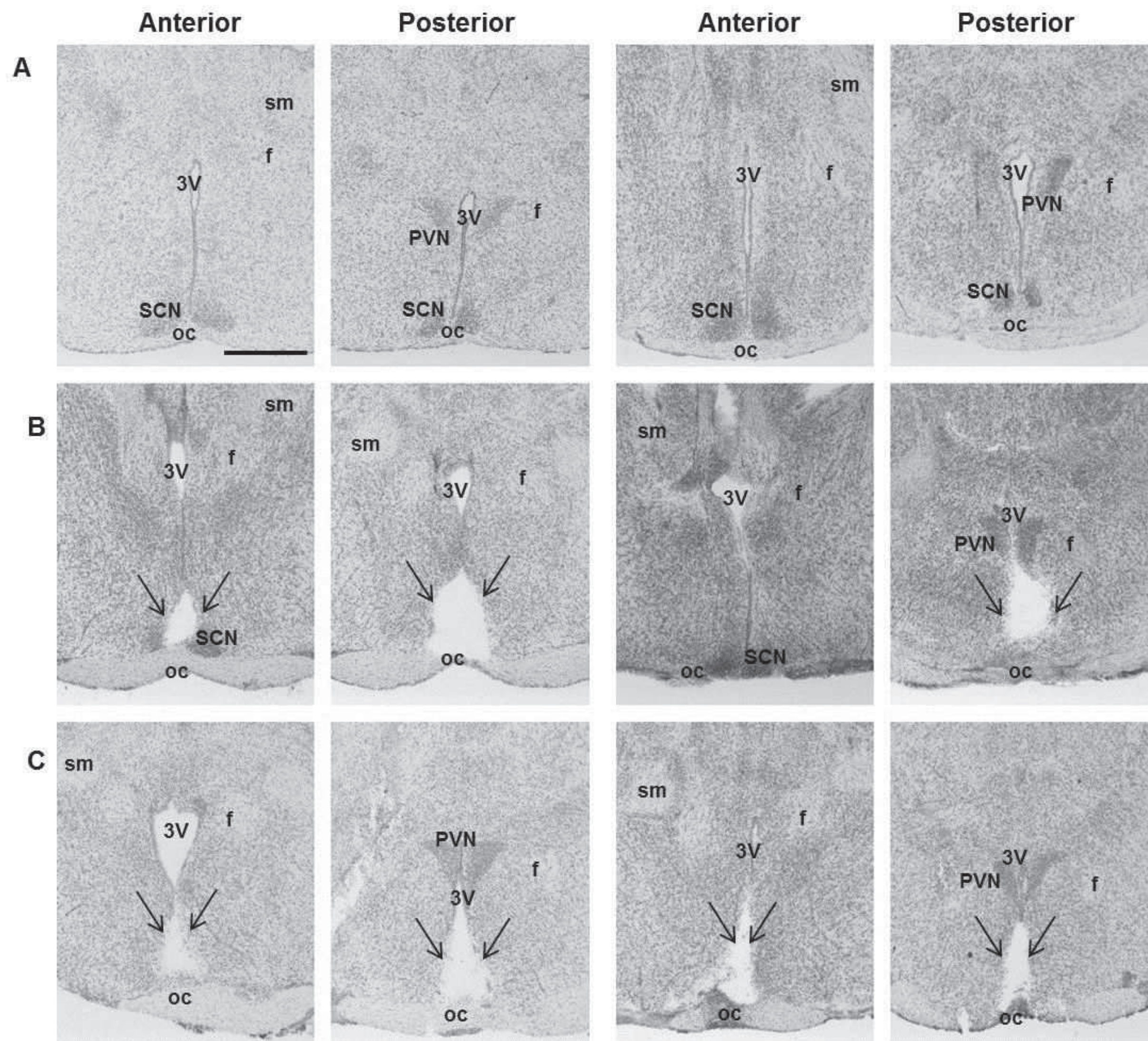
Gene Expression levels of *Dbp, Per1* and *Per2* for B6 mice were analyzed after a 6hrs-SD performed in darkness from Circadian Time (CT) 0 to CT6 (lights were not switched on at CT0) (black columns) and their controls (grey columns) for intact mice. Asterisks (*) indicate significant differences (with *P* value <0.0001) between controls and sleep deprived mice.

4. SUPPLEMENTARY FIGURES



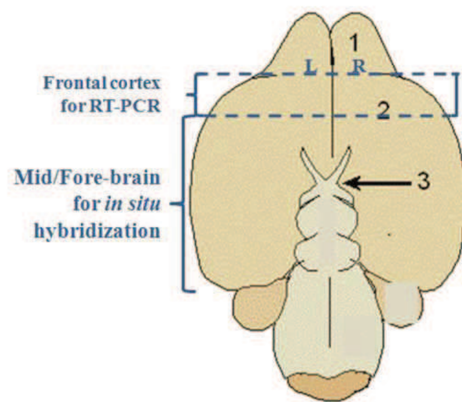
Supplemental Figure 1: Daily infra-red measured locomotor activity under a standard 12h:12h LD cycle and constant darkness (24 DD)

Actimetry samples from two different animals of each condition showing double-plotted locomotor activity centered at ZT0 (9 A.M.). Grey areas delineate the dark periods. Animals were under standard 12h:12h LD cycle (top of each actogram), under 24 DD (middle) and put back again to 12h:12h LD for several animals (bottom). (**A**) Sham animals were light-entrained under 12:12LD and free-ran under 24 DD. (**B**) Mice with a partial lesion of the SCN behaved similarly as (**A**) Sham animals, whereas (**C**) mice with a total lesion of the SCN showed a very week cycle under 12:12 LD, but were arrhythmic under 24 DD. When put back again to 12:12 LD cycle, (**B**) mice with a partial lesion of the SCN were able to entrain to the light dark cycle (right), as did (**A**) the Sham mice (left).



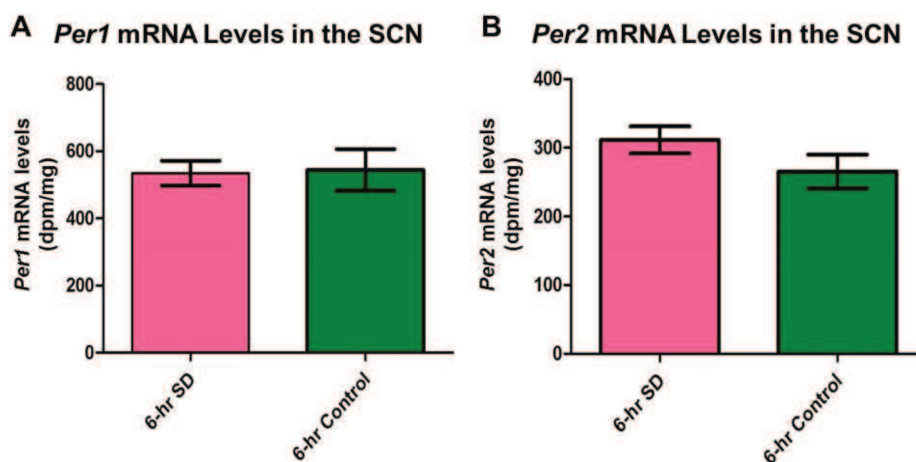
Supplemental Figure 2: Histological control of the quality of Sham or SCN-lesions.

Samples from two different animals of Nissl stained coronal sections centered on the anterior (ant) and posterior (post) SCN area for (**A**) Sham animals, (**B**) mice with a partial lesion of the SCN and (**C**) mice with a total lesion of the SCN. SCN = suprachiasmatic nucleus, oc = optic chiasm, 3V = third ventricule, PVN = paraventricular nucleus, f = fornix, sm = stria medullaris. Arrows are centered on SCN lesions (total or partial). Scale bar (valid for all sections): 500µm. *Bregma* -0.34mm (C left ant; B left ant); *Bregma* -0.46mm (A left ant; A right ant; B right ant; C right ant); *Bregma* -0,58mm (C right post); *Bregma* -70mm (A left post; A right post; B left post; B right post); *Bregma* -82mm (B right post)



Supplemental Figure 3:

Anatomical schema illustrating (**1**) frontal cortex used for RT-PCR (**2**) mid/forebrain used for *in situ* hybridization and (**3**) sections centered around the SCN for histological control of the SCN lesion, either total or partial, and the intact SCN in Sham animals.



Supplemental Figure 4: Effects of sleep deprivation on mRNA levels of *Per1* and *Per2* in the SCN measured by *in situ* hybridization.

Gene Expression levels of *Per1* (**A**) and *Per2* (**B**) in the SCN of B6 mice under 12:12 LD after a 6hrs-SD started at light-onset (ZT0-6) (rose columns) and their controls (green columns) for Sham animals with intact SCN.

SupplementaL Table 1: Primer and probe sequences used for real-time qPCR.

Gene Symbol	Direction	Sequence 5' to 3'	Accession #
Per1	fwd	ACCAGCGTGTCATGATGACATAC	NM_011065
	rev	CTCTCCCGGTCTTGCTTCAG	
	probe	CCGTCCAGGGATGCAGCCTCT	
Per2	fwd	ATGCTCGCCATCCACA AGA	NM_011066
	rev	GCGGAATCGAATGGGAGAAT	
	probe	ATCCTACAGG CCGGTGGACAGCC	
Dbp	fwd	CGTGGAGGTGCTTAATGACCTTT	NM_016974
	rev	CATGGCCTGGAATGCTTG A	
	probe	AACCTGATCCCGCTGATCTCGCC	
Homer1a	fwd	GCATTGCCA TTTCCACATAGG	NM_011982
	rev	ATGAACTTCCATATTTATCCACCTTACTT	
	probe	ACACATTCAATTCAGCAATCATGA	
GusB	fwd	ACGGGATTGTGGTCATCGA	NM_010368
	rev	TGACTCGTTGCCAAAACTCTGA	
	probe	AGTGTCCCGGTGTGGGCATTG TG	
Tbp	fwd	TTGACCTAAAGACCATTGCACT TC	NM_013684
	rev	TTCTCATGATGACTGCAGCAAA	
	probe	TGCAAGAAATG CTGAATATAATCCCAAGCG	
Rps9	fwd	GACCAGGAGCTAAAGTTGATTGGA	NM_029767
	rev	TCTTGGCCAG GGTAAACTTGA	
	probe	AAACCTCACGTTTGTTCCGGAGTCCATACT	

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Article 12 : *« Opn4-based dose-dependent direct mood improvement by light»*

En préparation

Article: pages 1-6

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1. Title page. *Original paper (brief communication)*

Title: Opn4-based dose-dependent direct mood improvement by light

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This work was performed at the Institute of Cellular and Integrative Neurosciences, Strasbourg, France

Conflict of interest: The authors have no conflict of interest to declare.

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Tables and Figures: 1 Figure

Word count: Main text = 618; References = 10

Keywords: mood, anxiety, depresssion, seasonal affective disorder, melanospin, direct effects of light, fluoxetine, mice

2. Main manuscript:

Within the last several decades our societies progressed further with the advent of new technologies necessitating work and social activities outside of hours naturally determined by the solar light-dark cycle. These changes have led to an increased use of artificial light as a compensatory mechanism for a variety of activities. Alterations in light exposure and intensity have been shown to contribute to mood alterations, yet the conditions under which this occurs and the mechanisms involved remain to be clarified[1]. Specifically, there are indications that lower intensity light may be linked to an increase in mood dysfunctions, such as Seasonal Affective Disorder (SAD)[2]. Non-visual light has a pervasive influence on many aspects of physiology and behavior, and a majority of this information is transmitted via intrinsically photosensitive retinal ganglion cells (ipRGCs) to brain areas responsible for sleep and wake, as well as mood regulation[3-5]. In humans, light is known to acutely promote alertness, waking EEG, and cognition, with the highest efficiency being blue light centered around 460-480nm, within the spectral response peak of melanopsin[6]. The phase-angle between the circadian pacemaker and delayed sleep-wake cycle is potentially depressogenic and corresponds to a mechanism previously described as the "internal coincidence hypothesis"[7,8] providing a rationale for the application of light therapy and chronotherapeutics to treat mood disorders. In the current study, we sought to elucidate the relationship between light intensity, mood alteration, and anti-depressive treatment in wild-type mice and a genetic knockout lacking melanopsin (*Opn4*$^{-/-}$), in order to determine the manner in which this system functions.

We subjected wild-type and *Opn4*$^{-/-}$ mice to seven days of various light intensities (10, 150, 600 lux), investigating the effect on depression-like behavior using the forced swim test (FST), tail suspension test (TST), as well as sucrose anhedonia. The lighting schedule respected their habitual light-dark cycle (12h:12h LD) in order to avoid changes in either circadian phase or sleep integrity[9].

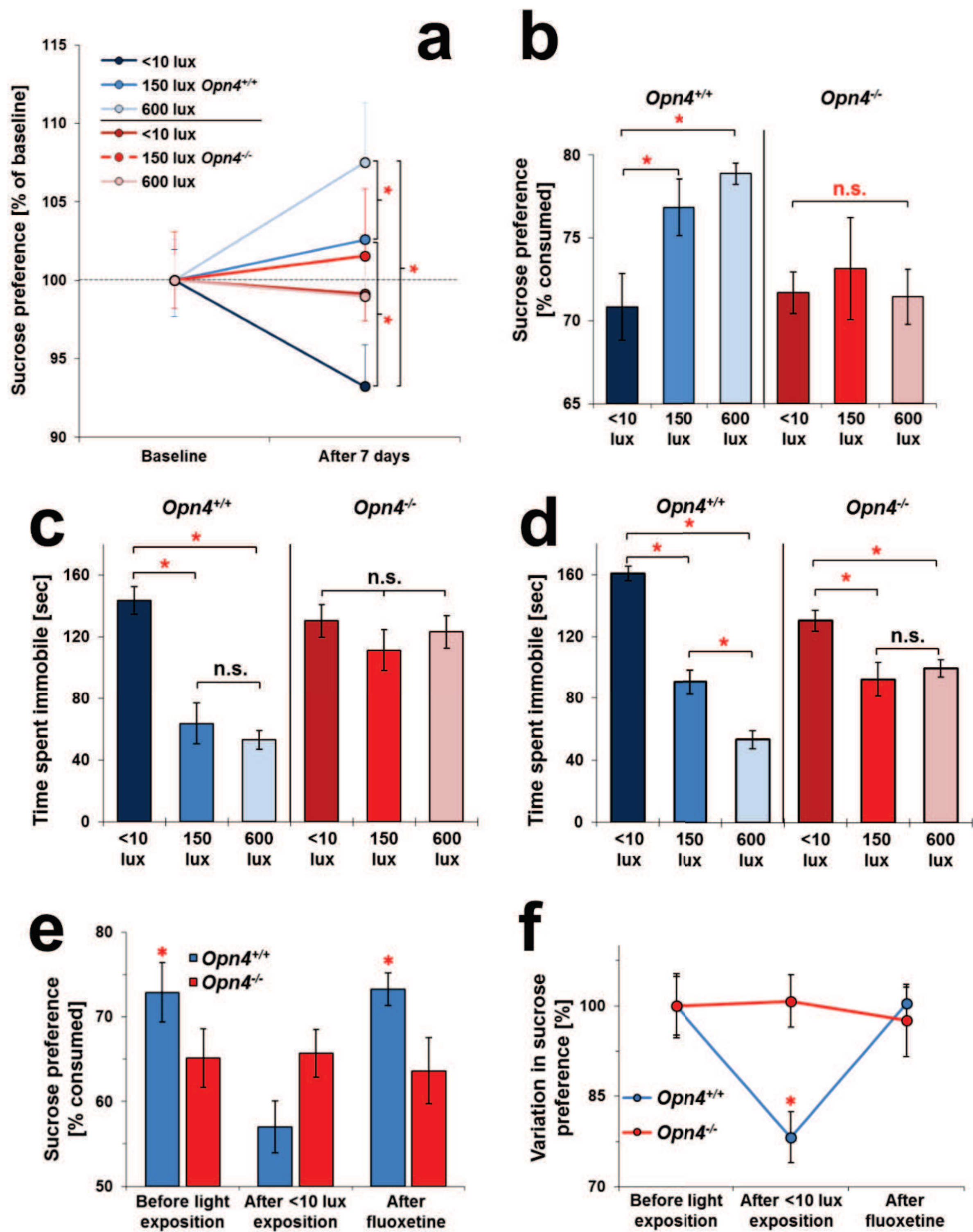
Light irradiance was significantly correlated with mood in a dose-dependent manner, modulating sucrose anhedonia (Fig. 1a,b) and behavioral despair (Fig 1c,d). Wild-type (*Opn4*$^{+/+}$) animals exposed to low levels of light showed less sucrose preference and spent significantly more time immobile during the FST and TST than mice housed under habitual (150 lux) or higher (600 lux) light conditions, implying a change in depression-like behavior. In comparison, *Opn4*$^{+/+}$ mice exposed to higher luminance increased their sucrose preference

and displayed less immobility time (FST, TST), indicative of mood improvement. In the absence of melanopsin this effect of light was abolished (sucrose anhedonia, Fig. 1a,b) or severely attenuated (behavioral despair, Fig. 1c,d). Additionally, the increased immobility time at <10 lux in *Opn4*$^{-/-}$ mice may indicate a possible role for rods and cones at lower irradiance levels. In order to examine whether the depressogenic effects of limited light (<10 lux) were reversible, we administered a commonly prescribed anti-depressant (fluoxetine). Mice lacking melanopsin were not depressed by the light condition and thus showed no reaction to anti-depressant treatment (Fig. 1e,f). However, *Opn4*$^{+/+}$ mice were not only significantly depressed by the weak luminosity as compared to baseline, but recovered completely after drug administration, to normal levels (Fig. 1e,f).

Our results demonstrate that light, independent of circadian rhythms or changes in sleep, positively influences depression-like behaviors such as despair, resignation (FST, TST), and sucrose anhedonia, an effect mediated by melanopsin-based photoreception. A positive relationship between mood improvement and the level of illumination exists via transmission through melanopsin-containing ipRGCs, and that a low amount of light can elicit a depressive effect. Furthermore, our findings may explain why light therapy is effective for treating winter depression independently of its effect on the circadian system[10]. The clarification of the nature of direct photic influence is critical given the ubiquitous use of artificial lighting in our society.

Full Methods and any associated references are available in the online version of the paper.

3. FIGURE:



4. LEGEND TO FIGURE:

Figure 1: Light directly improves anhedonia and despair behavior via melanopsin-expressing cells and low-light depression can be reversed using anti-depressant pharmaceutical therapy

(**a**) Sucrose preference between baseline and after 7 days of differential light conditions, in wild-type but not *Opn4*$^{-/-}$. Two-way ANOVA (with factors genotype and light); light: p=0.015, genotype x light: p=0.014. No post-hoc significance was found between *Opn4*$^{-/-}$ groups. (**b**) Difference between consumption of sucrose after 7 days of differential light conditions, expressed as a percentage. One-way ANOVA for genotype: p=0.034; one-way ANOVA for light showed significance for *Opn4*$^{+/+}$ (p=0.002), but not for *Opn4*$^{-/-}$. (n: *Opn4*$^{+/+}$ - <10=26, 150=12, 600=21; *Opn4*$^{-/-}$- <10=22, 150=17, 600=21) (**c**) Immobility time in seconds during FST. A significant dose-dependent decrease is observed in *Opn4*$^{+/+}$ mice, not seen in *Opn4*$^{-/-}$, except between the lowest and highest luminosity conditions. Two-way ANOVA (with factors genotype and light): genotype: p<0.0001, light: p<0.0001, genotype x light: p<0.0001. (n: *Opn4*$^{+/+}$- <10=26, 150=17, 600=19; *Opn4*$^{-/-}$- <10=37, 150=12, 600=20) (**d**) Immobility time in seconds during TST. Results are similar as in (**c**). Two-way ANOVA (with factors genotype and light): genotype: p=0.03, light: p<0.0001, genotype x light: p<0.0001. (n: *Opn4*$^{+/+}$- <10=37, 150=17, 600=19; *Opn4*$^{-/-}$- <10=37, 150=12, 600=20). (**e**) Sucrose preference changes between baseline, after 7 days of exposure to low light (<10 lux), and following anti-depressant treatment with fluoxetine. Mice showed a melanopsin-dependent decrease in sucrose preference following a short exposure to low luminosity, which was increased to its original levels following 3 days of treatment by anti-depressant (fluoxetine). A repeated measures ANOVA (with factors genotype and time) showed significance; genotype x time: p=0.02. Post-hoc signifance was found between the low-light exposition protocol, and the other two periods, for the *Opn4*$^{+/+}$mice. No post-hoc significance was found between *Opn4*$^{-/-}$ periods. (**f**) Variation in between baseline, after 7 days of exposure to low light (<10 lux), and following anti-depressant treatment with fluoxetine, expressed as a percentage. A repeated measures ANOVA (with factors genotype and time) showed significance; genotype x time: p=0.01. Post-hoc signifance was found between the low-light exposition protocol, and the other two periods, for the *Opn4*$^{+/+}$mice. For all graphs, error bars indicate standard error; ANOVAs were followed by post hoc-tests for intergroup comparisons with * p<0.05, n.s.: non significant.

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6. EXTENDED DATA:

Methods:

Experiments were performed on C57/Bl6 backcrossed adult male (F10) *Opn4*$^{-/-}$ mice and wild-type littermates (as controls), aged 2 to 6 months and housed under a standard 12h:12h light-dark cycle (for details and genotyping, see Tsai et al.[1]). The protocol was approved by the local veterinary office and use committees and pursuant to French National Law (License 67-32) and to the rules of the European Committee Council Directive of November 24, 1986 (86/609/EEC). After a period of social isolation (21 days), the animals were exposed for 7 days (based on preliminary data) to the experimental lighting condition (<10 lux, 150 lux or 600 lux) before behavioral evaluation. All tests were performed during the habitual dark period at the same ZT (14 to 20) under dim red light (<5 lux) to control for light irradiance at the time of measurement[2]. Behavioral tests included the sucrose preference test (anhedonia), the forced swim test and tail suspension test (despair, resignation) and were performed at least 2 days apart from one another. Subchronic treatment by Fluoxetine (10mg/kg/j) was administered under darkness for 3 days following 7 days of exposure to dim light (<10 lux)[3,4].

Supplementary References:

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UNIVERSITÉ DE STRASBOURG

Elisabeth RUPPERT

Influence non-circadienne de la lumière sur les comportements

Résumé

La lumière influence fortement la physiologie et le comportement en exerçant des effets non-visuels de deux types : i) indirects, via la resynchronisation de l'horloge centrale qui est située dans les noyaux suprachiasmatiques (NSC), ii) directs, indépendants du processus circadien, via des mécanismes encore mal compris. Nos travaux chez la souris ont montré que l'influence directe de la lumière constitue un mécanisme majeur de régulation du sommeil, de l'éveil et de l'humeur, au même titre que le processus circadien. Ces effets sont majoritairement médiés par la mélanopsine, un photopigment exprimé dans la rétine, et relayés au niveau cérébral par différentes structures comme les NSCs et le VLPO. Ainsi, le rôle des NSCs ne doit pas être interprété qu'au travers de leur fonction d'horloge. Ensuite, dans une perspective de recherche translationnelle de l'animal à l'homme, nous avons validé *Arvicanthis ansorgei*, comme modèle d'étude du sommeil afin de pouvoir interpréter nos résultats chez un rongeur diurne. Enfin, de nombreuses données suggérant que les effets directs de la lumière modulent l'activité du système dopaminergique, nous avons évalué l'intérêt de la luminothérapie dans des pathologies dopaminergiques (maladie de Parkinson, syndrome des jambes sans repos, troubles de l'humeur). Ces avancées ouvrent de nombreuses perspectives pour une meilleure utilisation de la lumière dans notre société ainsi qu'en pathologie. Mots clefs : effets directs de la lumière, mélanopsine, modèle de régulation du sommeil à trois processus, noyaux suprachiasmatiques, syndrome des jambes sans repos, *Arvicanthis ansorgei*

Résumé en anglais

Light influences physiology and behavior through both types of non-image-forming effects: i) indirect, synchronizing the circadian master clock located in the suprachiasmatic nucleus (SCN), ii) direct effects, independent from the circadian process though mechanisms poorly understood. Our studies in mice demonstrate that the direct influence of light constitutes a key mechanism of regulation for sleep, alertness and mood and is as important as the circadian process. The direct effects of light are mainly mediated through melanopsin, a retinal photopigment that projects to the different structures of the brain such as the SCN and the VLPO. The SCN, beyond their role as circadian clock are also a relay system for the direct effects of light. Further, we validated *Arvicanthis ansorgei* as a diurnal model for the study of sleep regulatory mechanisms. This is an important step in the translational approach from animal research to applications in humans. Various data suggest that the direct effects of light interact with the dopaminergic system. In the last part of this thesis, we evaluated the indication of bright light therapy in dopaminergic pathologies (Parkinson disease, restless legs syndrome, mood disorders). These advances open up new perspectives for possible applications of light therapy and may help improving societal lightening conditions Key terms: direct effects of light, melanopsin, three process model of sleep regulatory mechanism, suprachiasmatic nucleus, restless legs syndrome, *Arvicanthis ansorgei.*